APPENDICES
APPENDIX - 1

Media composition:

**Nutrient Agar Medium**

<table>
<thead>
<tr>
<th>Component</th>
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<tr>
<td>Bacto Peptone</td>
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<tr>
<td>Beef extract</td>
<td>3.0</td>
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<tr>
<td>Sodium Chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>Distilled water</td>
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<tr>
<td>pH</td>
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**Nutrient Broth Medium**

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<tr>
<td>Bacto Peptone</td>
<td>5.0</td>
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<tr>
<td>Beef extract</td>
<td>3.0</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>Distilled water</td>
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**Yeast Dextrose Calcium carbonate Agar Medium (YDCA)**

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<td>Agar</td>
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**Basal Medium**

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<td>K$_2$HPO$_4$</td>
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<tr>
<td>MgSO$_4$.7H$_2$O</td>
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<td>NaCl</td>
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<td>Yeast extract</td>
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<tr>
<td>Sucrose</td>
<td>1% (as carbon source)</td>
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<tr>
<td>Casein hydrolysate</td>
<td>0.1% (as nitrogen source)</td>
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APPENDIX - II

A. BIOCHEMICAL CHARACTERISTICS OF X. a. pv. vesicatoria.

a. Gram staining: (Anjea, 1996)

Thin smears of bacteria on glass slides were air dried, heated and covered with crystal violet stain for 30 seconds. The slide was washed with distilled water for a few seconds and the smear was stained with iodine solution for 30 seconds. The iodine was then washed off with 95% ethyl alcohol (95 ml ethyl alcohol made up to 100 ml with double distilled water). The slides were washed with double distilled water and drained. Safranin stain was applied to the smear for 30 seconds and then washed with distilled water. This slide was observed for the appearance of colour under a microscope and the color of the smear was noted.

b. Catalase test: (Kado, 1980)

About one drop of the culture was placed on a clear glass slide, to which one drop of H₂O₂ was added and kept under observation for appearance of air bubbles for few minutes.

c. Starch Hydrolysis Test. (Kado, 1980)

The culture was streaked on the surface of ABP Agar medium containing (100 ml, Auline blue - 60 mg; soluble starch - 40 g, glucose - 0.05 g, yeast extract-0.67 g; agar - 1.2 g) and incubated at 28°C. The plate was observed for the appearance of a clear unstained zone around the bacterial growth indication of starch hydrolysis in contrast with the blue black colour of the surrounding agar.
d. **Gelatin - Liquefaction Test**: (Iswaran, 1980)

The Potato Dextrose medium (g/l: peeled potatoes - 200, Dextrose - 200, Agar - 200) with 2% gelatin in Petri plates were spot inoculated with the Xav5 isolate and incubated for 48 hr at 28°C. These plates were flooded with mercuric chloride solution (15g HgCl₂, 20ml HCl, 100ml double distilled water). Hydrolyzed gelatin appears as a clear zone around the bacterial growth.

e. **Test for Tolerance to NaCl**: (Kado, 1980)

Concentrations of 10%, 15%, 20% or 50% NaCl were added to 10 ml of PDA medium and made into slants. The slants were inoculated with the Xav5 isolate, incubated for 48 hr and observed for growth of the bacterium.

f. **H₂S Production from Cysteine**: (Iswaran, 1980)

Slants containing PDA medium supplemented with 2% peptone and 0.05% cysteine were inoculated with the Xav5 isolate and incubated for 48 hr at 28°C. Dried Whatman No 1 filter paper discs soaked in a 10% solution of saturated lead acetate were introduced aseptically into slants and incubated for 24 hr. If H₂S is evolved, the lead acetate paper will blacken within 1 to 24 h.

g. **Acid Production Test**: (Iswaran, 1980)

Triple sugar iron agar medium (g/l: peptone - 20, Lactose - 10, Sucrose - 10; Glucose - 10; NaCl - 10, Ferrous ammonium sulphate - 0.2, Sodium thiosulphate - 0.2; Phenol red - 0.025; agar - 13 g) slants were inoculated and observed for gas production after 48 hours of incubation at 25°C.
h. **Phenylalanine deaminase enzyme activity** (Kado, 1980)

Phenylalanine agar medium (g/l: D, L - phenylalanine - 2.0; yeast extract - 3.0, NaCl - 5.0, Na₂HPO₄ - 1.0, Agar - 1.2) slants were inoculated, incubated for 24 hr; after which 4 or 5 drops of ferric chloride reagent (FeCl₃ - 12.0 g; Conc HCl - 2.5 ml, double distilled water - 100 ml) were added directly to the culture. The tube was rotated gently to loosen the bacterial growth and observed for colour appearance.

i. **Hydrolysis of Urea** (Iswaran, 1980)

Potato Dextrose agar medium containing 2.0 % urea and phenol red indicator was inoculated with bacteria and incubated at 37°C for 4 hr or over night and observed for the colour change.

j. **Test for Ammonia Production**: (Iswaran, 1980)

To the bacterial culture grown in 0.225 % peptone water for 5 days at 37°C or at optimal temperature, 1 ml of Nessler's reagent was added and observed for the appearance of colour.

k. **Urea Test**: (Iswaran, 1980)

The slants containing urea agar (containing per liter, casein hydrolysate-1.0 g; NaCl-5.0 g, KH₂PO₄-2.0 g; glucose-1.0 g; urea-20 g (sterilized separately by filtration and then added to cooled autoclaved medium), phenol red-6.0 ml of a 0.2 % solution; pH - 6.8) was streaked with a 48 hr culture of Xav5 up to the top.
\( \frac{2}{3} \) of the slant with the bottom \( \frac{1}{3} \) kept as a colour control. The slants were incubated at 27°C and examined after 4-24 h. A urease positive reaction is indicated by a bright pink colour.

1. **Test for Nitrile** (Iswaran, 1980)

The culture was grown in tubes containing nitrate agar medium (g/l sucrose-10.0, KNO\(_3\)-5.0, yeast extract-1.0, K\(_2\)HPO\(_4\)-2.0; MgSO\(_4\) 7H\(_2\)O-0.1, agar-15.0) for 48 h. The slants were flooded with 2 ml of nitrite reagent (1 ml of reagent A and 1 ml of reagent B as below) Appearance of a red colour indicates a positive test. To confirm the presence of nitrate in a negative reaction, a bit of zinc dust is added to the medium and then the nitrite reagent Zinc reduces any remaining nitrate in the medium to nitrite.

**Reagent A** 8 g sulfanilic acid was first dissolved in 287 ml of glacial acetic acid and then diluted with boiled distilled water to 1000 ml

**Reagent B** 5 g alpha-naphthyl amine dissolved in 290 ml of glacial acetic acid was diluted with boiled distilled water to 1000 ml

2. **Arginine Dihydrolase test** (Kado, 1980)

The bacteria were stab inoculated in arginine agar medium (containing per litre peptone - 5.0 g, beef extract - 5.0 g; bromocresol purple - 0.1 g, pyridoxol - 5.0 g; glucose - 0.5 g, L-arginine-monochloride - 10 g; agar - 8.0 g) in screw cap vials. The media were covered with sterile mineral oil and incubated for 2 to 3 days. Appearance of a bright magenta colour indicates the positive response of the bacterium.
n. **Test for poly β-hydroxy butyric acid** (Kado, 1980)

Heat fixed smears of the bacterial suspension were prepared and flooded with sudan black solution (0.3 g in 70 % ethanol) and allowed to air dry for 24 hr. The slides were cleared with xylene and counter stained with 5% safranin solution. After 2 or 3 minutes, the slides were washed with water and observed under a microscope for black staining of cells as a positive reaction.

o. **Utilization of carbon source**: (Iswaran, 1980)

925 E minimal medium enriched with yeast extract (g/l: (carbon source arabinose, dextrose, mannose, maltose, lactose, xylose, glucose, sucrose-5.0), K₂HPO₄ - 3.0, KH₂PO₄ - 1.0, NH₄Cl - 1.0; MgSO₄.7H₂O - 0.3; yeast extract - 0.25, agar - 15.0, bromocresol purple - 1.0) containing blanks were inoculated with a 48 hr culture of Xav5 and incubated at 28°C for 48 hr after covering one set with sterile mineral oil and leaving one set uncovered. Observations were made after 48 hours.

p. **Nicotinic acid as carbon source**: (Kado, 1980)

The medium containing (g/l: nicotinic acid - 10.0; peptone - 2.0; NaCl-5.0, K₂HPO₄-0.3, 1% bromo thymol blue-3.0 ml; agar-4.0; pH 6.8) was poured into test tubes and sterilized. The test tubes were kept in vertical position and allowed to solidify. These were stab inoculated with a 48 hr old bacterial colony and observed for a green to deep blue colour change of the medium.
q. Utilization of the carbon source (Iswaran, 1980)

i. 2-Ketogluconate:

Test tubes containing 10 ml of 92% Broth (containing per litre potassium glutonate - 5.0 g; K₂HPO₄ - 3.0 g; NaH₂PO₄·12H₂O - 1.0 g; NH₄Cl - 1.0 g; MgSO₄·7H₂O - 0.5 g) were inoculated with a loopful of culture and incubated on a rotary shaker at 28°C. One ml aliquots were withdrawn 2, 7 and 15 days after incubation. These aliquots were tested by adding 10 ml of Benedict's solution (17.3 g sodium citrate and 19 g Na₂CO₃ dissolved in 80 ml of hot water) 1.8 g CuSO₄·5H₂O dissolved in 10 ml water and added to the citrate carbonate solution by stirring and made up to 100 ml with distilled water. The tubes were heated for 10 minutes at 100°C. Reduction of copper to a reddish to yellowish colour indicates a positive reaction.

ii. Succinate:

Tubes containing a light green coloured succinate medium (g/l. sodium succinate - 10.0, peptone - 2.0, NaCl - 5.0, bromo thymol blue-3 ml, agar - 4.0, pH 6.8) was inoculated and observed daily for the appearance of a deep blue colour indicating utilization of succinate.

iii. Tartrate:

Test tubes containing 10 ml of the light green coloured tartarate medium (g/l: sodium tartarate - 10.0; peptone - 2.0; NaCl - 5.0; K₂HPO₄ - 0.3; bromo thymol blue- 3 ml of 1% solution; agar - 4.0; pH 6.8) was inoculated and incubated for 48 hr. The medium was observed daily for formation of a deep blue colour which indicates tartrate utilization.
iv. Malonate:

Test tubes containing malonate medium (g/l: sodium malonate - 10.0; peptone - 2.0, NaCl - 5.0, K₂HPO₄ - 0.3; agar - 4.0; bromo thymol blue; 3 ml of 1% solution, pH - 6.8) were inoculated and observed daily for a colour change of the medium.

v. Propionate:

The procedure followed was same as for malonate except sodium malonate was replaced by 10.0 g of sodium propionate in the medium.

vi. Citrate:

Slants containing the light greenish yellow citrate medium (g/l: sodium citrate - 5.0, K₂HPO₄ - 3.0, NH₄H₂PO₄ - 1.0; MgSO₄.7H₂O - 0.2, NaCl - 5.0, bromo thymol blue - 0.08 and agar 20.0) inoculated and incubated at 28°C were observed daily for a blue colour which indicates a positive reaction.

vii. Tobacco Hypersensitivity test:

The Xav5 isolate was grown on nutrient agar medium slants for 48 hr before preparing a bacterial inoculum suspension adjusted to 1.0 O.D. using sterile distilled water. The bacterial suspension in test tubes were kept in a water bath for 30 to 45 minutes at 90-95°C. At this temperature, the bacterium was killed. These heat killed bacterial cells were used for the hypersensitive study.
To study the effect of HR, the bacterial suspensions were pre-inoculated. The bacterial suspension (living cells of Xav5 compared with dead cells of Xav5) were infiltrated into intercellular spaces of tobacco leaf with the help of a sterile (No 25) hypodermic needle. Mature leaves were more suitable for injection and the injection was made near the lateral veins of the leaf (lower surface) where the tissue was thicker. The carefully injected suspension spread over a larger surface, usually surrounded by a bigger vein. These areas were marked and labelled. After sometime, the infiltrated water evaporated from the intercellular spaces and the leaf regained its original appearance. The tobacco plant was exposed to light for 24 hr continuously before the challenge inoculation with the pathogen. This was accomplished by infiltrating the Xav5 suspension into the intra-venous areas, where the markings were drawn. Care was taken not to spread the bacterial suspension beyond the markings. A control without prior inoculation was maintained. The plants were kept for observation.

s. *Tyrosinase Test:*

The medium containing 0.12% K$_2$HPO$_4$, 0.08% KH$_2$PO$_4$, 0.02% MgSO$_4$·7H$_2$O, 0.2% sucrose and 0.3% tyrosine in 150 ml flasks was seeded with the Xav5 isolate and kept on a rotary shaker. Flasks were observed for four days for pigment.
B. PHYSIOLOGICAL STUDIES OF THE PATHOGEN

i. Electrolyte leakage conductivity:

About 100 mg of fresh bacterial mass grown on nutrient agar was suspended in 100 ml of distilled water and 25 ml transferred to a 100 ml beaker. This bacterial suspension was used as a stock from which dilutions of $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{8}$ were made separately in 100 ml beakers. The approximate number of bacterial cells in each of these dilutions was calculated based on the number of bacterial cells per gram fresh weight of the bacterial mass. Beakers containing the suspensions were kept constantly shaking on a rotary shaker at 28°C. Conductivity measurements of the bacterial suspensions were recorded at four different intervals of 15, 30, 60 and 120 minutes with a Philips model PR 9500 conductivity bridge using a conductivity cell with 1.46 cell constant. The temperature correction factor was calculated as $1 + (T - 25) \times 0.02 = \chi$, where $T$ is the temperature at which readings were taken (28°C) and the Temperature Correction factor = $\chi \times$ cell constant = 1.548. The conductance multiplied by the temperature correction factor gives the conversion of conductivity values into micromhos and is given as

\[
\text{Conductivity in micromhos} = \frac{1}{R} \times \text{temperature correction factor} \times 10^6
\]

ii. Protease enzyme activity:

This was estimated in both the bacterial mass and culture filtrate
a. In bacterial mass and culture filtrate:

Two loopfuls of 48 hr old bacterial growth was transferred to modified Hussain Kelman's medium. After 72 hours, cell free crude enzyme solutions were obtained by centrifugation at 10,000×g for 30 minutes. The enzyme was extracted and assayed from the bacterial cells and supernatant according to the method of Kunimichi Nanto et al. (1979).

The residue obtained from 25 ml of Hussain Kelman's medium was homogenized with 2 ml of ice cold 0.1M phosphate buffer (pH 6.5), 2 ml of 1% vitamin free casein solution in sodium phosphate buffer (pH 6.5) was added, and the mixture was incubated for two hours at 37°C before adding 1 ml of cold TCA followed by centrifugation at 500 rpm. Proteolytic activity of the supernatant was measured by after reaction with Folin-phenol reagent taking the absorption values at 750 nm against a zero time blank (Lowry et al., 1951).

The supernatant from Hussain Kelman's medium was dialysed for 24 hr against distilled water in a cold room and 2 ml of the supernatant was assayed by the same procedure described above.
APPENDIX - III

Physiological and biochemical analysis of the host-pathogen complex:

Photosynthetic Pigments:

Chlorophylls and carotenoid pigments were estimated in both healthy and infected leaves of both susceptible (LCA-304) and resistant (Pusa jwala) varieties of chillies at five different stages of disease development i.e., at 2, 6, 10, 20 and 30 days after inoculation.

a Total chlorophylls:

Chlorophyll content was estimated according to the method of Ammon (1949).

One gram of the leaf samples was washed with distilled water, cut into small bits and ground in a mortar using 80% (v/v) acetone-AR. The paste was centrifuged at 3000 rpm for 15 min and the supernatant collected. This was repeated until the residue appeared colourless. The absorbance of the supernatant was measured in a Bausch and Lomb spectroscope-20 photoelectric colorimeter at 645 nm and 663 nm. The chlorophyll content was determined as follows:

Total chlorophyll (mg/ml) = (20.2 X O.D.645) + (8.02 X O.D.663)

chlorophyll a (mg/ml) = (12.7 X O.D.663) - (2.69 X O.D.645)

chlorophyll b (mg/ml) = (22.9 X O.D.663) - (4.68 X O.D.645)

The chlorophyll content was expressed as mg chlorophyll per gram fresh weight of leaf tissue.
b. **Total Carotenoids:**

Total carotenoids were determined as per the method of Jensen and Jensen (1971) where about one gram of freshly harvested leaf material was washed thoroughly with distilled water, cut into small bits and macerated with a portion of 80% (v/v) acetone-AR in a mortar. The extract was centrifuged and the residue reextracted until no more pigment was observed. All the supernatants were pooled and the acetone-AR was removed in vacuo. The residue after complete removal of acetone was dissolved in a small volume of petroleum ether (40°C-60°C) and the same volume of 10% methanolic potassium hydroxide solution was added. This mixture was allowed to stand at room temperature for two hours before being transferred to a separatory funnel. The reaction was stopped by the addition of 5% aqueous sodium chloride for the removal of methanol. The salt facilitates the separation of phases and prevents the formation of emulsion. The ether extract was dried over anhydrous sodium sulphate and the petroleum ether was removed in vacuo. This residue was dissolved in acetone and the absorbance was measured in a Schimadzu UV-240 photoelectric spectrophotometer at 445 nm, being the midpoint of the main absorption maximum of the extract. Total carotenoids present in the samples was calculated as follows.

\[
C = \frac{D \times V \times F}{10 \times 2500},
\]

Where \(C\) = Total carotenoids in mg

\(D\) = O.D. at 445 nm

\(V\) = Total volume in ml

\(F\) = Dilution factor for the sample measured

2500 = Average coefficient for carotenoids
APPENDIX - IV

Carbohydrate fractions:

Extraction of carbohydrates was accomplished by the method of Mahadevan et al. (1963) where about one gm of the leaf sample was transferred to about 10 ml of boiling 80% ethanol (v/v)-AR grade and reflux extracted for five minutes on a hot water bath. The cooled mixture was homogenized in a mortar and the material was transferred to a small quantity of fresh boiling 80% ethanol and re-extracted for five minutes. All the extracts were pooled and centrifuged. The supernatant was concentrated on a boiling water bath to 5 ml.

a. Reducing sugars:

Estimation of reducing sugars was determined by Nelson-Somogy's (Nelson, 1944) method.

To one ml of an ethanolic leaf tissue extract, 1 ml of a mixture of reagent A (25 g of anhydrous sodium carbonate, 25 g of Rochelle salt, 20 g of Na₂CO₃, 200 mg of anhydrous sodium sulphate were dissolved in 800 ml distilled water and the solution was made up to one liter with distilled water) and reagent B (15 g of CuSO₄ dissolved in 100 ml of distilled water and to this one or two drops of concentrated H₂SO₄ was added) in 25:1 proportions was added and heated for 20 min in a boiling water bath and then cooled in tap water. One ml of ascorbomolybdate colour reagent (25 g of ammonium molybdate, 2 ml of concentrated
H₂SO₄ and 3 g of sodium arsenate in 25 ml distilled water was added to 450 ml of distilled water and the mixture was kept in a glass stoppered brown bottle to prevent oxidation in an incubator at 37°C for 48 hr) was added and the solution was thoroughly mixed and diluted to 25 ml with distilled water. The blue colour intensity was read at 497 nm in a Schimadzu spectrophotometer and the reducing sugar content was calculated with a standard graph plotted with known equivalents of glucose.

b. **Non reducing sugars**:

Non reducing sugars present in the ethanol extract were first hydrolyzed to reducing sugars (Inman 1962) by evaporating one ml of ethanol extract in a test tube to dryness on a water bath. To this residue, 1ml of distilled water and 1 ml of 1 N sulphuric acid were added and the mixture was hydrolyzed by heating at 49°C for 30 min. The resulting solution was neutralized with 1N NaOH using methylred indicator. The reagent blanks were prepared with 1 ml of distilled water instead of ethanol extract. The quantity of non-reducing sugars was calculated by subtracting the reducing sugar content from total sugars and expressed in terms of glucose equivalents.

c. **Starch**:

The residue remaining after ethanolic extraction of the leaf material was used for estimation of starch content according to the method of Mc Cready et al., (1950) by solubilizing it in 52% perchloric acid for 30 min, filtering, and making it
up to 100 ml with distilled water. One ml of this extract was diluted to 5 ml with distilled water in a test tube and 10 ml of freshly prepared anthrone reagent (200 mg of anthrone was dissolved in 100 ml of 95% cold sulphuric acid) was added under cold conditions. The tube and its contents were heated for exactly 7.5 min at 100°C in a boiling water bath. The solution was cooled rapidly to room temperature and the colour intensity was measured at 630 nm using a Bausch and Lomb spectronic-20 photoelectric colorimeter. The starch content was measured by reading the value on a standard graph for glucose and multiplying the glucose equivalents present in the sample by 0.9.


APPENDIX - V

Nitrogen fractions:

Total nitrogen and soluble protein nitrogen fractions were estimated in both 48 hr old bacterial culture and in leaves of healthy and infected chilli plants.

a. Total nitrogen: was estimated according to the method of Markham (1942)

One gram of fresh material was taken in a 25 ml micro Kjeldahl flask to which about 100 mg of catalyst (1g CuSO₄+5g K₂SO₄+1g SeO₂) was added to aid digestion. Three ml of fuming concentrated analytical grade Sulphuric acid and 1 ml of Hydrogen peroxide were also added and the sample was allowed to digest on a hot plate until a clear colourless solution was obtained. The final volume of the solution was made up to 25 ml with distilled water. Five ml aliquots of the digest were transferred to a distillation unit and 10 ml of 40% sodium hydroxide was added just prior to distilling for 20 minutes. The ammonia liberated was absorbed into a burette acid - indicator mixture in a volumetric flask. The completion of distillation was recognized by the change in pH of the indicator solution in the receiver from pink to green. After complete distillation, the indicator solution was titrated against 1N Hydrochloric acid until the pink colour reappeared. The amount of nitrogen present in the sample was calculated as 1ml of 1N HCl = 0.14 mg of nitrogen and the results were expressed as mg per gram fresh weight.
Boric acid indicator mixture:

16 g of boric acid and 200 ml of absolute alcohol were mixed and added to the indicator solution consisting of 0.033 mg bromoresol green and 0.666 mg of methyl red in 100 ml of absolute alcohol. This indicator mixture was made up to one litre with distilled water and the pH was adjusted to 5.0.

b. Protein nitrogen:

Protein nitrogen was estimated by the method of Thimann and Loss (1957) as follows.

About one gram of fresh material was macerated with 10 ml of 10% TCA at 4°C and centrifuged at 2000xg for 30 minutes. After decanting the supernatant, the precipitate was washed with 5 ml of 5% TCA. Again 5 ml of 5% TCA was added to the washed precipitate and the mixture was incubated for 30 min at 80°C to remove nucleic acids. The precipitate obtained after centrifugation was analysed by the microkjeldahl method as mentioned above for total nitrogen.

c. Soluble Nitrogen:

Soluble nitrogen can be estimated by subtracting protein nitrogen from total nitrogen. The results were expressed as mg nitrogen per gram fresh weight of the sample.
APPENDIX - VI

Lipid fractions

a. Total Lipids:

Total lipids were extracted from leaf samples of both resistant and susceptible chilli varieties by the method of Hoppe and Hertefuss (1974).

One gram of leaf sample was homogenized in 22 ml of boiling solvent mixture consisting of 6 ml chloroform, 12 ml methanol and 4 ml water (1:2:0.8 v/v/v), filtered and reextracted with 14 ml methanol in a mortar. The residue was washed with 20 ml chloroform. All the extracts were combined in a separating funnel and 18 ml of water was added to make the ratio of chloroform-methanol-water to 2:2:1:3 (v/v/v). After removing the lipid layer, the water layer was extracted three times with 20 ml chloroform. These layers were evaporated to dryness at 43-45°C in a rotary evaporator. Two ml of benzene was added to the residue and evaporated under nitrogen. The dried lipid residue was quantitatively dissolved in chloroform into a previously weighed bottle and evaporated to dryness under nitrogen. The bottle was kept in a vacuum desiccator over KOH under reduced pressure for several hours and again weighed. The weight of total lipids present in one gram leaf samples was calculated by taking the difference in the above weights and recorded as mg/g fresh weight. The lipid residues were dissolved in chloroform in 5 to 10 ml volumetric flasks and stored in a deep freeze for further studies.
b. **Total Glycolipids**

Estimation of total glycolipids was done by the Phenol-sulphuric acid method of Dubois et al., as described by Roughan and Bate (1968).

An aliquot of the lipid extract was placed in a test tube, evaporated to dryness and 0.9 ml methanol and 0.1 ml 5 N NaOH were added. A marble was placed over the tube and the mixture heated for an hour at 45-50°C in a water bath. The hydrolysate was evaporated to dryness under compressed air, 1 ml of 2N H₂SO₄ was added to the residue. A marble was placed on the tube and it was again hydrolysed for 2½ hours on a boiling water bath. After hydrolysis, 1 ml chloroform was added to partition pigments. To an aliquot from the aqueous phase, 0.5 ml water and 0.5 ml 4% phenol were added and shaken well. Four ml of concentrated sulphuric acid was added and the contents of the tubes were thoroughly mixed and allowed to stand at room temperature for 15 min before reading the color intensity at 480 nm in a Schimadzu spectronic spectrophotometer. Galactose standards containing 10 to 20 µg/tube were run simultaneously; each time and the concentration of glycolipids was expressed as mg/gram fresh weight of the material.

c. **Total Phospholipids:**

To determine the phosphorus content in phospholipids, Bartlett's (1959) procedure with a slight modification was followed.
Lipid samples containing 1 to 10 µg of phosphorus were placed in a test tube marked in 10 ml and the organic solvent was removed by passing compressed air. One ml of 60% perchloric acid was added to each tube and the samples were digested at 170-180°C in a heating block until the samples were clear. After digestion, 4.5 ml of 0.44% ammonium molybdate (4.4 g of ammonium molybdate added to 14 ml concentrated sulphuric acid made to one litre) followed by 0.2 ml of 1-amino, 2-naphthol, 4-sulphonic acid (0.5 g 1-amino, 2-naphthol, 4-sulphonic acid, 6 g sodium sulphite, 30 g sodium bisulphite dissolved in 250 ml distilled water) were added to each test tube. The contents were mixed thoroughly, heated in a boiling water bath for 10 minutes, cooled and the volume was adjusted to 10 ml with glass distilled water. Colour intensity was measured in a Schimadzu spectronic spectrophotometer at 660 nm wave length. Aliquots of KH₂PO₄ solution containing 2 µg of phosphorus/ml were used as a standard and simultaneous blanks were maintained. Total lipid content was calculated by multiplying the lipid phosphorus by 25 and expressing it as µmoles of phosphorus per gram fresh weight of the material.
A comparative analysis of the molecular weight of polypeptides of *Capsicum frutescens* leaves was investigated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE), inorder to study the gene products (proteins) expressed in pathogen infected and healthy leaf samples of susceptible (LCA – 304) plants

**a. Experimental samples:**

- 45 day old healthy leaves from the susceptible variety, LCA-304
- 10 day old XavS infected leaves from the susceptible variety, LCA-304 of 45 day old chilli plants

**Extraction of buffer soluble proteins:**

Five grams of freshly harvested leaf sample were washed thoroughly with tap water and blotted to dryness between filter paper folds. The leaf bits were homogenized at 4°C (0.1 M Tris-HCl, pH 8.3 containing 0.5 M sucrose and 0.5% 2-mercaptoethanol) in a mortar at the rate of 2 ml/g tissue. The homogenate was squeezed through muslin cloth and centrifuged at 10,000 rpm for ten minutes. The soluble proteins of the supernatant were precipitated with an equal volume of 20% Trichloro Acetic acid (TCA) for two hours at 4°C. The TCA precipitate was collected by centrifugation at 10,000 rpm for ten minutes. The pellet was washed twice with 5% TCA and thrice with ice cold solvent ether. The final protein pellet
was dried under vacuum and solubilized in a minimal known volume of 10% SDS
Insoluble material was recovered by centrifugation at 8000 rpm for 10 minutes and
the soluble proteins in the supernatant were collected, stored at 4°C until used and
the protein content was estimated according to Lowry et al., (1951)

a. **Quantitative Estimation of Proteins:**

To a 50 ml protein sample, 5 ml of freshly prepared alkaline copper
sulphate (by mixing 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide)
10 ml of 0.5% hydrated copper sulphate (CuSO₄·5H₂O) and 1% sodium
potassium tartarate mixing well and allowing the solution to stand for 10 minutes
Before adding 0.5 ml of folin phenol reagent (diluted in a 1:1 ratio with distilled
water before use) with thorough mixing) After thirty minutes, the blue colour
intensity was read at 660 nm in a Shimadzu UV-VIS spectrophotometer The
amount of protein was calculated by using a bovine serum albumin standard curve

b. **SDS Poly Acrylamide Gel Electrophoresis (SDS-PAGE):**

a. **Stock Reagent Solutions:**

Stock solutions for SDS-PAGE were prepared according to Laemmli

1. **Acrylamide Solution Bisacrylamide (30:0 8)**

Acrylamide-30.0 g, N,N-methylene bisacrylamide-0.8g were dissolved in
60ml distilled water, made up to 100 ml with distilled water, filtered and
stored at 4°C in the dark in an amber coloured bottle
Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

Tris-HCl Buffer (18.15 g) was dissolved in 80 ml distilled water, the pH was adjusted to 8.8 with 1N HCl and made up to 100 ml with distilled water. The buffer was filtered and stored at 4°C.

10% SDS

Sodium Dodecyl Sulphate (SDS) (10.0 g) was dissolved in 80 ml of distilled water, with gentle stirring, filtered, made up to 100 ml and stored at room temperature.

Sample buffer (Laemmli buffer) consisting of 0.5 M Tris-HCl (pH 6.8) (1.25 ml), glycerol (1.00 ml), 10% SDS (2.00 ml), 2-mercaptoethanol (0.50 ml) and 0.05% bromophenol blue (W/V) (0.25 ml) were made up to 10 ml with distilled water and stored at room temperature.

The electrode (tank) buffer consisting of Tris-HCl (1.80 g), glycine (8.62 g) and SDS (0.60 g) This was dissolved in 800 ml distilled water and adjusted to pH 8.3 with 1N HCl. The solution was made up to 1000 ml and stored at room temperature.

10% Ammonium Persulphate (APS) was prepared by dissolving 100 mg ammonium persulphate prepared freshly just before use in 10 ml distilled water.

The staining solution (100 ml) consisted of 100 mg Coomassie brilliant blue R₂₅₀ (Bio-Rad) in 50 ml methanol, 10 ml Glacial acetic acid and 40 ml distilled water. The solution was filtered and stored in an airtight bottle.

The destaining solution (100 ml) consisted of 20 ml methanol in 70 ml Glacial acetic acid and 100 ml distilled water.

The gel storage solution was prepared by dissolving 10 ml glacial acetic acid in 90 ml distilled water.
<table>
<thead>
<tr>
<th>STOCK REAGENTS</th>
<th>RESOLVING GEL (12%)</th>
<th>STACKING GEL (6%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>2.4 ml</td>
<td>0.60 ml</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>1.5 ml (pH 6.8)</td>
<td>0.75 ml (pH 6.8)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.0 ml</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>600 μl</td>
<td>300 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>600 μl</td>
<td>300 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>60 μl</td>
<td>30 μl</td>
</tr>
<tr>
<td>Total</td>
<td>6.026 ml</td>
<td>3.013 ml</td>
</tr>
</tbody>
</table>

b. *Poly Acrylamide Gel preparation:*

Leaf soluble proteins were analyzed by SDS-slab gel electrophoresis (Studier, 1973; Ames, 1974)

A 1.5 mm thick gel was cast in the gel mold consisting of two glass plates, spacers and well forming comb. The glass plates were clamped to the electrophoresis tank with the help of back fold clips and the edges sealed with 3% molten agar. The gap between the inner plate and the lucite support was filled by a cushion pad and clamped tightly. The resolving gel solution was prepared and poured into the mold by gravity. The flow rate was maintained at approximately
160 ml per minute. Distilled water was overlaid on the resolving gel to obtain a
flat gel surface after gelling. After complete polymerization of the resolving gel,
the water layer was decanted carefully without disturbing the surface of the
resolving gel. The slot former (comb) was inserted to form 1 cm long spacer gels
on the resolving gel. The stacking gel solution was prepared freshly and poured
without air bubbles. The stacking gel was allowed to polymerize at room
temperature for one hour. After polymerization, the comb was removed and the
ells were washed with distilled water. The bottom spacer also was removed. The
lower and upper reservoirs were filled with electrode buffer without any air
bubbles.

e. Sample application and Electrophoresis:

After protein estimation, the samples were made to equal concentration
with Laemmli sample buffer. Samples were digested in boiling water bath for two
minutes and cooled in an ice bath for few seconds. Samples were spun at 10,000g
for 10 minutes to remove the undigested particles. Samples were loaded into gel
slots (wells) by means of micropipettes. An initial current of 50 volts was passed
until the samples entered the stacking gel and then the current was increased to
150 volts till the end (15 hr) at room temperature under constant voltage
conditions with Briviga Powerpack. The run was terminated when the
bromophenol blue migration front was one cm above the bottom of the gel or
when tracking dye just crossed the bottom of the gel.
The Bio-Rad marker proteins, with their molecular weights in parentheses used were α-lactalbumin (14,200 Da), soyabean trypsin inhibitor (20,100 Da), carbonic anhydrase (29,000 Da); glyceraldehyde 3-phosphate dehydrogenase (36,000 Da) albumin egg (45,000 Da) and bovine albumin (66,000 Da). The proteins were dissolved in single strength sample buffer at a concentration each of 1 mg/ml. The proteins determined in healthy as well as infected samples were calculated with the help of a log graph (No 1) plotted for \( R_f \) values (i.e., relative motility) on the ‘X’-axis and the molecular weights of the standard marker proteins on the ‘Y’-axis. The \( R_f \) is expressed as

\[
R_f = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}
\]

Staining and destaining:

After completion of the run, the gel mold was carefully dismantled, immersed in a glass tray (corning) containing staining solution, and then shaken gently on a rotary shaker for at least three hours before soaking in destaining solution until the background was clear.
APPENDIX - VIII

Total free amino acids

Free amino acids were analyzed from leaves of control and inoculated plants at five different stages of disease development. The total free amino acids present in the leaf samples were extracted according to the method of Das Chowdary et al., (1967)

Extraction:

About 500 mg of freshly harvested healthy and infected samples were extracted with boiling 80% ethanol by refluxing for 30 min on a boiling water bath. The extracts were decanted and residues were reextracted several times in hot 80% ethanol. The pooled extracts were filtered, centrifuged and the supernatant evaporated to dryness was redissolved in 1 ml of 80% ethanol and stored in tightly stoppered vials at 0°C

Estimation:

To 0.1 ml of ethanolic extract in a test tube, 1 ml of ninhydrin reagent (20 g of ninhydrin dissolved in 500 ml of methyl cellosolve was added to 0.8 g stannous chloride dissolved in 500 ml of 0.2 m citrate buffer (pH 5.0)) and made up to 2 ml with double distilled water. This was boiled in a boiling water bath for 20 min and 5 ml of the diluent was added. The contents were thoroughly mixed and incubated for 15 min. The purple colour obtained was read against a reagent blank in a Bausch and Lomb spectronic 20 photoelectric colorimeter at 570 nm using the reagent blank with 0.1 ml of 80% ethanol. A standard curve was prepared using known concentrations of leucine.
APPENDIX - IX

Nucleic Acids:

Nucleic acids (DNA and RNA) were extracted and estimated according to the method of Nieman and Poulsen (1963) from both healthy and infected leaf tissues at five stages of disease development.

Extraction

About 250 mg of freshly harvested leaf material was extracted with cold methanol, centrifuged and the residue repeatedly washed with cold methanol to remove pigments and alcohol soluble phosphorous. To the residue, 5% ice cold trichloro acetic acid (TCA) was added, centrifuged and the resulting residue was washed twice with 90% ethanol and ether (ether:ether (2:1) w/v). For each extraction, the solvent was allowed to boil for 20 seconds. The residue was carefully dried, powdered and used for further extraction of RNA and DNA.

1. Estimation of RNA:

The dried residue was suspended in 5 ml of 0.3 N sodium hydroxide and held at 30°C for 18 hours. The sediment was centrifuged and washed with 5 ml of 0.3 N sodium hydroxide. Both the wash and extract were combined, made to 10 ml with 0.3 N NaOH, acidified to pH 1.0 with 15% PCA, held at 4°C for 40 mm and then centrifuged. The supernatant obtained contained the RNA fraction.
The DNA (protein) sediment was resuspended with 2 ml water followed by the addition of 1N Per chloric acid (PCA). The suspension, held at 4°C for 20 min, was centrifuged and the supernatant added to the RNA fractions before bringing the final RNA fraction up to 20 ml with distilled water.

ii. Estimation of DNA:

The DNA present in the sediment was suspended in 3 ml of 0.5 N PCA, heated at 70°C for 15 min, centrifuged at 4000 rpm at 2°C and the wash added to the DNA fraction. The final DNA extract was made up to 5 ml with 0.5 N PCA and the DNA extract was measured using 0.5 N PCA as the blank. O.D values were measured at 260 nm using a Schumadzu spectrophotometer and using sperm DNA and yeast RNA respectively as standards.
APPENDIX - X

Total phenols:

The quantitative determination of total phenols was estimated according to the method of Swain and Hallis (1959) where about one gram of freshly harvested leaf samples were boiled in 5 ml of 2 N HCl on a water bath for 20 min and filtered. The residue obtained was ground in 5 ml of 2 N HCl, filtered, the filtrates were pooled and then centrifuged at 3000 rpm for 15 minutes. The supernatant was extracted twice with 20 ml of diethyl ether. Both diethyl ether extracts were pooled, evaporated and the residue was dissolved in 1 ml of 95% ethanol and diluted to 7 ml with distilled water. To the mixture, 0.5 ml fohin-phenol reagent was added, shaken thoroughly and held for three min, before adding 1.0 ml of saturated sodium carbonate solution and making up to 10 ml with distilled water. The blue colour intensity developed after one hour was measured at 725 nm in a Shimadzu UV-240 spectrophotometer using coumarin as the standard solution.
APPENDIX - XI

Estimation of enzymes:

The enzymes like protease, RNase, catalase, peroxidase, polyphenol oxidase, β-amylase, phenylalanine ammonia-lyase, glutamic dehydrogenase and ascorbic acid oxidase were determined quantitatively in both healthy and infected leaves of resistant and susceptible chulli plants

a. Protease activity

Protease activity was estimated by the method of Kunihiko Naito et al., (1979) where one gram of leaf sample was homogenised with 5 ml of ice cold 0.1M phosphate buffer (pH 6.5). The homogenate was centrifuged at 11,500 g for 30 min and the supernatant was assayed. Proteolytic activity was determined as the capacity to hydrolyze casein. In the assay, 1 ml of enzyme extract was incubated with 2 ml of 1% vitamin free casein solution in sodium phosphate buffer (pH 6.5) and incubated at 37°C for 2 hr. Then, 1 ml of cold TCA was added and the mixture was centrifuged. Proteolytic activity was measured by absorbance at 750 nm of the TCA soluble fraction (supernatant) after reacting with Folin-phenol reagent against a zero time blank according to the method of Lowry et al., (1951). Enzyme activity was measured in arbitrary units as one enzyme unit, defined as an increase of one A750 unit per hour under the assay condition.
RNase activity in the leaf samples were determined quantitatively according to the method of Bagi and Farkas (1967) where one gram of sample was homogenized in a prechilled mortar at 2-4°C with distilled water. The homogenate was squeezed through a four layered muslin cloth and the extracts were centrifuged at 3000 rpm for 15 minutes in an MSE refrigerated centrifuge. The supernatant thus obtained was used to assay for RNase activity.

The assay system containing 0.5 ml enzyme extract, 2 ml of 7.5 mg/ml yeast RNA and 0.5 ml 0.1M acetate buffer (pH 5.6) was incubated at 37°C in a water bath for 30 min, terminated by adding 0.5 ml of Mc Fadyen's reagent (0.25% Urankyl acetate in 2.5% TCA), shaken well and set overnight at 4°C. The mixture was centrifuged and the supernatant diluted (1/10) with distilled water. O.D values were measured against a zero time control at 260 nm with a Schimadzu UV-240 spectronic spectrophotometer. Activity was measured in arbitrary units/ml as

$$A_{260} = \frac{(\text{ml assay solution} + \text{ml precipitating agent}) \times \text{Dilution factor}}{\text{ml enzyme solution assayed} \times X \text{ time in minutes}}$$

c. Catalase activity

Catalase enzyme activity was measured according to the method of Gopalachari (1963) where two hundred mg of leaf material was ground with 20 ml of cold phosphate buffer (pH 7.0) and the homogenate was filtered through pyrex glass wool and then made up to 25 ml. The reaction mixture (2 ml enzyme extract
and 1.0 ml of 0.045M H₂O₂ was incubated for 5 min and the reaction stopped by adding 1.0 ml of 12% sulphuric acid. The mixture was titrated immediately against 0.05 N KMnO₄ and the end point was denoted by the first appearance of a pink colour, which lasted for 30 seconds. The blank was run simultaneously substituting the enzyme extract by an equal volume of buffer. Enzyme activity was expressed as mg of H₂O₂ destroyed in 15 minutes per gram weight of the tissue

\[ C = \frac{25/2 \times 0.85 \times V/W}{W} \]

\[ V = \text{Difference in titre value between control and treated} \]

\[ W = \text{Weight of the sample in grams} \]

\[ 0.85 \mu g \ H₂O₂ = 1.0 \ mg \ KMnO₄ \]

d. Peroxidase activity:

Peroxidase activity was estimated according to the method of Manczaruk, Kar and Dhahanandhu (1976) by grinding the leaf sample with 0.1 M phosphate buffer (pH 7.0) in a pre-chilled mortar and centrifuging the homogenate at 15000 g at 4°C for 30 min. The aliquot obtained was used as the source of the enzyme.

The assay mixture for peroxidase contained 2 ml 0.1M phosphate buffer (pH 7.0), 1 ml 0.01 M pyrogallol, 1 ml 0.005 M H₂O₂ and 1 ml enzyme extract. The reaction was stopped by adding 1 ml of 2.5 N sulphuric acid after 5 minutes, incubated at 25°C and the amount of peroxidase formed was estimated by measuring absorbance at 420 nm. Enzyme activity was recorded in terms of absorbancy units.
e. Polyphenol Oxidase:

The method followed to estimate polyphenol oxidase activity was same as that for peroxidase except that the reaction mixture contained no $\text{H}_2\text{O}_2$. Absorbance was measured at 420 nm.

f. $\beta$-amylase:

$\beta$-amylase activity was estimated according to the method of Bernfeld (1955) where an aqueous extract of the material was prepared by grinding one gram of leaf tissue in 10 ml chilled water. The extract was filtered and the filtrate was made up to 10 ml. A one ml sample of the extract was incubated for three minutes at 20°C with 1 ml of starch solution (1 g of starch dissolved in 100 ml of 0.016 M acetic buffer, pH 4.8). The enzyme reaction was then interrupted by the addition of 2 ml of 3,5-dinitrosalicylic acid reagent (1 g of 3, 5-DNS in 20 ml of 2 N sodium hydroxide and 50 ml distilled water; 30 g of Rochelle salt was added and the mixture made up to 100 ml with distilled water). The tube was heated for 5 min in a boiling water bath and cooled in running tap water. After adding 25 ml water, the optical density of the solution containing a brown reduction product was determined photometrically at 540 nm. A blank was maintained without adding the enzyme. A calibration curve established with maltose was used to convert the data into mg of maltose. Amylase activity was measured in terms of mg of maltose liberated in 3 min at 20°C by 1 ml of the enzyme solution.
g. *Phenylalanine ammonia lyase:*

Activity of PAL-ase was determined according to the method of Brehn et al., (1968) with slight modification as follows:

One gram of freshly harvested material was ground in a chilled mortar with 10 ml of ice cold 0.1M borate buffer, pH 8.8 at 4-5°C, in a cold room. The homogenate was filtered through several layers of muslin cloth. The extract was then centrifuged in the cold at 10000 g for 30 min. The supernatant was made up to 15 ml and used to measure PAL-ase activity.

The reaction mixture (2 ml of enzyme extract, 1 ml of 0.05M L-Phenylalanine and 2 ml of 0.1M Borate buffer pH 8.8) was incubated for 1 hr in a test tube at 40°C and the reaction was stopped by adding 0.1 ml of 5 N HCl. A zero time control prepared at the same time was assayed. The acidified reaction mixture was measured at 268 nm in a Hilger and Watts spectrophotometer. The values, in terms of cinnamic acid formed, were calculated from a standard curve prepared from known amounts of cinnamic acid.

h. *Glutamic Dehydrogenase Activity:*

GDH activity was determined by the procedure of Smith and Waygood (1963) where one gram of freshly harvested leaf material was ground at 0°C in a chilled mortar with 10.0 ml of ice cold phosphate buffer, pH 8.0, with 0.01M cysteine (1.2 w/v) and filtered. The filtrate was centrifuged at 20000 g for 30 min at 0°C. The supernatant was made up to 20 ml and used as a source of enzyme.
The enzyme was assayed at $21 \pm 1^\circ\text{C}$ following the oxidation of NADH at 340 nm in a spectrophotometer using α-ketoglutarate and ammonia as substrates. To the reaction mixture (0.2 ml of NADH (0.2 μ moles), 0.5 ml (NH₄)₂SO₄ (750 μ moles), 1.9 ml 0.1 M phosphate buffer (pH 8.0) and 0.2 ml enzyme extract), 0.2 ml α-ketoglutarate (40 μ moles) was added. Optical density readings were made at 1 minute intervals and the specific activity was calculated as

$$\text{EV/mg protein}$$

Where

$$E = \text{Extinction decrement during a six minute period.}$$

$$V = \text{Volume of the reaction mixture}$$

1. **Ascorbic acid Oxidase:**

Five hundred mg of fresh leaf material was ground at 0°C in a chilled glass mortar with 20 ml of ice cold phosphate citrate buffer, pH 5.0. The homogenate was filtered through pyrex glass wool and made up to 25 ml with the same buffer and used for the enzyme assay. The reaction mixture (4.0 ml of enzyme assay, 2.5 ml of 2% ascorbic acid reagent) in 25 ml conical flasks was incubated for 30 min and the reaction was stopped by the addition of 1 ml of 1% TCA. For the control, 1 ml of TCA was added to the reaction mixture containing 4 ml of ascorbic acid reagent without any time lag. The total volume in each of the flask was made up to 10 ml. One ml of this solution was titrated against 0.001 N 2,6-DCP (30 mg of
2,6-dichlorophenol dissolved in 100 ml of distilled water to which two or three drops of 0.1N NaOH was added) until a permanent pink colour was attained. Ascorbic acid oxidase activity was measured for 30 min and recorded per gram fresh weight of tissue. The specific enzyme activity was determined as

\[ A = \frac{V}{W} \times 0.008 \times 10 \times 25/4 \]

Where \( A \) = ascorbic acid oxidase activity expressed as mg of ascorbic acid per gram fresh weight of plant tissue.

\( V \) = difference in titre values between the control and treatment in ml

\( W \) = fresh weight in gram of plant material

0.008 mg ascorbic acid = 1 ml of 2,6-DCP.