IV. MATERIALS AND METHODS

1. Host: *Piper betle* L. (Bengali vernacular name 'Pan') is an important chewing crop which is cultivated throughout India. This crop has several cultivars. But among several cultivars five cultivars were taken for present investigation, these are - Mitha, Sanchi, Dhal Bangla, Kali Bangla and Mitha-Bangla. This crop has been found to be infected by different types of diseases in West Bengal viz., wilt, leaf spots, leaf rot, root rot, stem rot. But this crop has been found to be heavily infected by leaf spot disease causing pathogen viz., *Fusarium scirpi*. This disease causes heavy economic losses to the farmers every year. The seeds (setts i.e. five nodes vines) of *Piper betle* (cultivars Mitha, Sanchi, Dhal-Bangla, Kali-Bangla and Mitha-Bangla) have been collected from Tamluk, Midnapur, West Bengal and they are cultivated for experiment at Tamluk, Midnapur, West Bengal.

2. Fungi: During random collection of different types of leaf spot diseases of *Piper betle* from different localities in West Bengal, several pathogens were isolated from diseased parts in Potato dextrose agar slants. The cultures were purified by simple monosporic culture techniques and stock cultures were maintained on Potato dextrose agar slants at 10°C by regular subculturing at the interval of 30 days. The identity of the
The pathogen was confirmed from Identification Service of Commonwealth Mycological Institute, Ferry Lane, Kew, Richmond, Surrey TW9 3AP, England and culture has been deposited there. The pathogen, *Fusarium scirpi* Lamb & Pautr. (IMI-244823) causing leaf spot disease of *Piper betle* was collected from Bankura, West Bengal.

3. **Medium used**: The following media were used during the present investigation.

### Potato Dextrose Agar Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peeled Potato (Decoction)</td>
<td>400 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
<td>25 g</td>
</tr>
<tr>
<td>Distilled Water upto</td>
<td>1000 g</td>
</tr>
</tbody>
</table>

### Richard's Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium sulphate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Zinc Sulphate</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Ferric Chloride</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>upto 1000 ml</td>
</tr>
</tbody>
</table>
4. **Buffers**: For maintaining the required pH, the following buffers were used during the experimental period.

   a) 0.1 M Acetate buffer - (pH - 5.3)
   b) 0.1 M Sodium phosphate buffer - (pH - 7.1)
   c) 0.2 M Sodium phosphate buffer - (pH - 7.0)
   d) Sodium citrate phosphate buffer - (pH - 4.1)

5. **Chemicals**: All chemicals and reagents used during the course of the present investigation were of analytical grade. Chemicals were manufactured by M/s Sunkist Growers Inc., U.S.A., Hercules Powder Co., U.S.A., Sigma Chemical Co., U.S.A., E. Merck, Germany and Sarabhai Merck, India.

6. **Methods of Cultivation in the field**: Cultivation of betel plant is highly specialised and is carried out with intensive care regarding preparation of land, mode of propagation, after care in manuring & irrigation. The best soil for betel cultivation is clayey loam. When the soil was light and loamy, suitable manuring and addition of tank silt preceded cultivation. For the covering of cultivated land i.e. for the purpose to make a hedge, bamboos were used (i.e. boroj), while the thatch was made up of bamboos or other materials like locally available grass Sclerostachya fusca (Roxb.) A. camus ('Khari' - local name). The thatch was so made as to facilitate entry of less sunshine and rainfall.
For the preparation of land, at first land was ploughed and harrowed to obtain a fine tilth. The soil also was sterilized by spraying 2% formaldehyde and then the soil was allowed to evaporate the formaldehyde in sun. The betel plants were grown on somewhat elevated beds, alternating with strips of narrow trenches, where water is kept flowing or stored in pits. The plants are planted on 'chur' (local name) which is raised by the addition of soil during first plantation and pai (local name) remain blank for the retention of water. So, 'Chur' and 'Pai' are arranged alternately in separate lines. Betel plants are climbers and need support for their growth. Here non-living wooden plants are used as supporters e.g. - Corchorus sp., Sclerostachya fusca (Roxb.) A.Camus and certain grasses like Imperata cylindrica (Linn.) P. Beauv for binding. Betel plant is propagated only vegetatively by cutting from healthy plants at least two years old. Each cutting contained 5 nodes and was planted in such a manner that 2 nodes were buried in the soil and 3 nodes were above the soil. On the 'chur' the cuttings were planted in the month of July. Immediately after planting the setts of cutting were covered by straws and were given required amount of water. The setts established in about 3 weeks time and first leaf was put forth in about a month. After 30 to 40 days the covered straws were opened and were supported by non-living supporting plants and they were tied by 'Zun' (local name of locally available grass Imperata cylindrica (Linn.) P. Beauv) to support the vine at intervals of 20 to 25 cms apart. The setts produced roots and established themselves firmly. When the
plants reached a height of about 2 m, they were often 'lowered' (local name is 'Ula') by a process in which the plants are again tied from their first 5-6 nodes areas and the remaining portion was coiled and buried in the soil (i.e. local name is 'Kharapota'), leaving free only a few nodes at the growing end of the plant. The lowering of plants was done once in the 1st year and 4 times per year in the next consecutive years. 'Ula' or lowering is practised 4 times per year in the different seasons and the names are to be given according to the name of the season -

(i) Asari kundal - in the month of June, (ii) Bhadra kundal - in the month of August, (iii) Kartika kundal - in the month of October, (iv) Tatha kundal - in the month of March. When the Chur was filled by algal dipositions then it was covered by newly formed fine tilth of soil which is known as 'Pas' (local name). As the plants are perennial, they survive year after year.

7. Method of inoculation in the experimental field of betelvine:

At 4-months of plant growth, healthy plants were inoculated with the virulent strains of Fusarium scirpi in separate plants in the following way. The fungus was grown in large number of 250 ml conical flasks containing Potato - dextrose agar medium. After 10 days of inoculation the fungus sporulated profusely. Conidial suspension in sterile distilled water was prepared after 15 days
of growth by gently shaking and followed by filtering through a layer of muslin to get rid of mycelial fragments and media. The suspension was then centrifuged at 5,000 g for 15 minutes. Conidial suspension was prepared by addition of sterile distilled water. The concentration of conidia in suspension was adjusted to $8 \times 10^6/\text{ml}$. This conidial suspension was used as inoculum.

The sand was also sterilized by 2% formaldehyde and pH was maintained at 7.0 (Neutral).

First the leaves of the plants were rubbed by sterilized sand particles and then the formerly prepared conidial suspension was sprayed over the rubbed areas of the leaves by Ajitomizer (sprayer) and then the leaves were covered with moist polythene bags. Simultaneously some rubbed leaves covered with moist polythene bags were also kept as control just like inoculated ones. Different sets of plants both normal and inoculated were harvested after 2 days, 5 days, 7 days, 10 days and 15 days after inoculation. Each type of harvested leaves were then washed with distilled water and were kept in different polythene bags and were stored at 4°C for biochemical studies.

8. **Temperature**: In all the cases, plants were grown at 26° - 30°C which is optimum for the growth of the host plant as well as for infection.
9. **Relative humidity**: The plants were grown at a relative humidity of 80-85% (per cent) excepting the periods after inoculation when 90-95% relative humidity was maintained.

10. **Determination of dry weight**: An amount of fresh sample was weighed and dried overnight in an oven at 60°C, cooled, desiccated and fixed weight recorded till no variation was found.

11. **Biochemical Methods**:

   (a) **Method for estimation of Calcium**:

   Calcium was estimated following the method of Vogel (1961).

   **Preparation of sample**: 5 g of fresh material (leaves) was crushed, diluted with dist. water and volume was made upto 50 ml with dist. water. This prepared solution was used for calcium estimation.
Reagents:

i) NH$_4$OH solution

ii) NH$_4$Cl

iii) Solochrome dark blue indicator

iv) 0.01 (M) EDTA (Ethylene-diamine-tetra acetic acid)

Procedure: 5 ml of calcium solution was taken in a conical flask and 2 ml of buffer solution (pH 10, made up by mixing 142 ml of NH$_4$OH with 17 g of NH$_4$Cl) was added to it. Then 4-5 drops of indicator solochrome dark blue (4 g in 100 ml methanol) solution was also added to it. The mixture turned red. The whole mixture was then titrated with 0.01 (M) EDTA solution until the colour changed from red to pure blue. Disappearance of the last tint of reddish colour indicated the end point. By repeating this process several readings were taken.

Given data -

1 ml of 0.01 (M) EDTA complexes with 0.4008 mg of calcium
(b) **Estimation of Nitrogen:**

Total, soluble and insoluble nitrogen of healthy and infected plants were estimated by the colorimetric method of Polin and Wu (1919) and Vogel (1961).

1) **Estimation of Total nitrogen:** Fresh healthy and infected leaves were dried in a hot air oven at 50°C for 24 hours and then the leaves were powdered in a Wiley mill and passed through 60-screen mesh. The powdered materials were then stored in a stoppered bottles within a desiccator and used in the experimental studies.

**Reagents:**

i) Nessler's Reagent

ii) Conc. $H_2SO_4$

iii) $H_2O_2$

**Procedure:** 20 mg of plant materials together with 2 ml of conc. $H_2SO_4$ digested in a digestion flask inside a fume chamber till a homogenous black solution appeared. The flask
was then cooled and 1 ml of hydrogen peroxide ($H_2O_2$) was then added to the flask and again was heated until the mixture became colourless. The volume was finally made up to 10 ml.

0.5 ml of the digested solution was then taken in an Erlenmeyer's flask. Then 5 ml of Nessler's reagent was added to the mixture and was diluted up to 25 ml. Colorimetric reading was then taken using blue filter (420 m\textmu) after 20 minutes.

ii) Method for estimation of soluble nitrogen:

Preparation of sample: 1 g of fresh leaves were crushed in a clean mortar with chemically pure sand. The extract was then made up to 50 cc by adding distilled water and was centrifuged at 5000 g for 10 minutes. 19 ml of supernatant was taken and to it 1 cc of 50% TCA was added and kept for half an hour for precipitation of all the colloidal substances (Proteins etc.). It was then centrifuged for 15 minutes at 5000 g and supernatant was decanted off and was taken in a beaker and was used for soluble nitrogen estimation.
Reagents:

i) 50% TCA soln. (Trichloroacetic acid)

ii) Nessler's Reagent

iii) Conc. H₂SO₄

iv) H₂O₂

Procedure: Then 4 ml of the prepared solution was taken in a digestion flask and 2 ml of Conc. H₂SO₄ was added to it and was digested inside the fume chamber till a homogenous dark brown colour appeared. The flask was then cooled and 1 ml H₂O₂ was added to it and again was heated until the mixture became colourless. The volume was finally made up to 10 ml.

0.5 ml of digested solution was then taken in an Erlenmeyer's flask and to it 5 ml of Nessler's reagent was added and was diluted up to 10 ml. Colorimetric reading was then taken using blue filter (420 mν) after 10 mins.

iii) Estimation of insoluble Nitrogen: Insoluble nitrogen was estimated in the following way:-

Concentration of insoluble nitrogen = Concentration of total nitrogen - Concentration of soluble nitrogen.
(c) **Method of Protein Estimation**: 

Total protein was estimated from insoluble Nitrogen by the following formula:

\[
\text{Total protein} = 6.25 \times \text{Insoluble Nitrogen}.
\]

(d) **Method for estimation of pectic substances**: 

The method for estimation of pectic substances was followed according to McComb and McCready (1952) and McCready and McComb (1952).

**Preparation of samples**: Fresh healthy and infected leaves were dried in a hot air oven at 60°C for 24 hrs. and then the dried plant materials were crushed for producing powder in a clean mortar with pestle. Then this powder was stored in a stoppered bottle in a desiccator and used for experimental purpose.

**Reagents**:

1. 0.01 (N) HCl
2. 0.05 (N) NaOH
3. Ethyl alcohol (purified)

100 ml of 95% reagent grade ethyl alcohol was refluxed with 4 g of zinc dust and 4 ml of 1:1 sulphuric acid for 24 hrs. Then distilled alcohol was redistilled.
iv) Carbazole Reagent

0.15 g of reagent grade carbazole (recrystallized from toluene) was dissolved with stirring in 100 ml of purified ethyl alcohol.

v) H₂SO₄ reagent grade concentrated

vi) Galacturonic acid, monohydrate, reagent grade. The purity was checked by titration.

Procedure: 500 mg of dried material was boiled with dilute HCl (0.01 N) at pH 3 for 60 minutes. The extracted pectic substances were deesterified by treatment with 0.05 (N) NaOH solution at 25°C to 30°C, neutralised, filtered and diluted to 125 ml.

2 ml aliquotes from the above were taken into Pyrex tube (25 x 200 mm) and 12 ml of conc. H₂SO₄ (cold) was added to each of the tubes. The contents were shaken and cooled in ice bath. Then the contents of the tubes were heated for 10 minutes in a boiling water bath. After boiling, the tubes were again cooled, 1 ml of alcoholic solution of carbazole reagent was added. The temperature of the mixture was kept below 30°C, otherwise mixture would char and give erroneous results. The tubes were well shaken and allowed to stand at room temperature for 30 minutes. The
(e) **Extraction and separation of phenolic substances**

Phenolic substances (F-I, F-II, and F-III) were estimated by the method of Kuc' (1964).

**Reagents:**

1. Methanol
2. 5% NaHCO₃
3. Diethyl Ether
4. HCl
5. 10 (N) H₂SO₄
6. NaCl

**Procedure:** 2 g of fresh leaves were extracted with adequate amount of methanol. Methanol was removed under reduced pressure and the volume was made up to 10 cc with 5% NaHCO₃ solution. The aqueous layer was then extracted thrice with equal volume of diethyl ether. Ether was evaporated to dryness which is the free phenols (Fraction-I).

The aqueous layer was acidified to pH 3.0 with HCl and then extracted thrice with equal volume of ether. Ether was evaporated to dryness which is the phenolic acids (Fraction - II).

The aqueous layer was hydrolised with equal volume of 10 (N) H₂SO₄ by refluxing for 1 hr. The hydrolised substances were

intensity of the colour was measured in Beckman Spectrophotometer DU at 520 mµ.
saturated with NaCl which was then extracted similarly with ether. Ether was evaporated to dryness which is the bound phenolic substances (Fraction - III).

The weights of these F-I, F-II and F-III were taken by Electrical balance.

(f) Enzyme assay Methods

Preparation of sample: Fungus was grown on Richard's medium in conical flasks. The inoculated flasks were incubated at 27° ± 0.05°C for 10 days. The mycelium was harvested by centrifugation at 5000 g and filtered through Whatman No. 42 with the aid of Buchner funnel and partial vacuum.

The filtrate was placed in cellophane dialysis tube and dialysed in a large volume of deionised distilled water for 24 hrs. at 20°C. The dialysed filtrate was used for enzyme analyses. Five g of infected and non-infected host materials were properly collected and homogenized in 20 ml of 0.25 M NaCl solution. The extraction, centrifugation and dialyses procedures, however, were the same as used in preparation of culture filtrate.

Procedure:

1) Pectin methyl esterase (PME) activity was measured by Bateman and Beer (1965) by continuous titration at pH 5.0 of
reaction mixture consisting of 20 ml of 1.2% pectin, 2.0 ml of 0.125 N NaCl and 3.0 ml of enzyme preparation or culture filtrate. Activity was expressed as ml of 0.02 N NaOH necessary to maintain a pH of 5.0 for 2 hrs.

2) The Endopolygalacturonase activity was measured at 30°C by the method of Bell et al. (1955). 2 ml of the enzyme preparations were added to 4 ml of 1.2% Pectin buffered at pH 4.5 with citric acid - NaOH (0.05 M). Viscosity readings were taken after reaction time of 10, 20 and 40 minutes. Data are based on the relative enzymatic activities expressed as 1000/t ml filtrate or extract where 't' equal minutes required for viscosity to the preparation to be reduced 50% or enzyme activity units per mg protein.

3) The Phenoloxidase and Peroxidase activities were measured by the method of Maxwell and Bateman (1967).

**Preparation of sample:** Enzyme extracts were prepared by grinding the sample in 0.1 M sodium phosphate buffer at pH 7.1 (2 ml/g fresh weight) in a clean mortar in ice cold condition. These tissues were filtered through Whatman No. 42 filter paper with the aid of Buchner funnel and partial vacuum under cold condition. The filtrates were centrifuged at 15,000 g for 20 minutes at 6°C. The supernatant fluid was used for the enzymic assay.
Procedure: Phenoloxidase and peroxidase enzyme assays were carried out in a Spectronic 20 Colorimeter at 27±0.05°C. When the compounds were transferred into a colorimeter tube, it was rapidly inverted several times and placed in the colorimeter. The needle was adjusted to nil point and readings were taken every 30 seconds for 5 minutes.

To determine the phenoloxidase activity, the reaction mixture contained 2.0 ml enzyme extract, 1.0 ml 0.2 M sodium phosphate buffer at pH 7.0 and 1.0×10⁻³ M catechol brought to a final volume of 6.0 ml with distilled water. The activity of phenol-oxidase was expressed as the change in absorbance/μl of extract per minute of 495 μM.

To determine the peroxidase activity, the reaction mixture contained 2.0 ml of extract diluted 1 : 1000 with distilled water, 1.0 ml sodium citrate phosphate buffer at pH 4.1, 0.4 ml 0.1% H₂O₂ and 1.0 ml of 0.1 M P-phenylene diamine brought to a final volume of 6.0 ml with distilled water, pH 5.8. The activity was expressed as the change in absorbance/0.001 ml extract per minute at 485 μM.