CHAPTER-3:
MATERIALS AND METHODS
Materials and Methods:

1. Survey & screening of diseased leaves of selected crops from different fields
2. Isolation of fungal pathogens
3. Pathogenicity test
4. Growth and cultural characteristics on the basis of
   A. Selection of basal media
   B. Sugar biomass ratio
5. Biochemical analysis of isolated pathogens of
   A. Protein (by Lowry’s method)
   B. Chromatographic analysis of
      a. Amino acids
      b. Organic acids
      c. Sugars
6. Nutritional analysis
   A. Mineral composition
   B. Trace elements
7. Enzymological studies
   A. Extracellular enzymes
      a. Cellulases (Cx)
      b. Pectolytic-Polymethylgalacturonase (PMG)
         Pectinmethylsterase (PME)
   B. Intra cellular enzymes
      a. Acid phosphatase
      b. Alkaline phosphatase
8. Disease protection (in-vivo, in-vitro)
   A. Antibiotics- Grisofulvin, Teramycin, Norflaxacin
   B. Fungicides- Blitox,Carbendazim+ Mancojeb, Antracol
   C. Angiospermic sources- Ziziphus jujuba L., Eugenia jambolana L.,
      Carica papaya L., Psidium guajava L.
Survey

A periodical survey was conducted for the collection of infected leaves of leguminous crops during September 2012 to March 2014. Ten legume crops 5 each from Kharif and Rabi crops were selected for isolation viz.

Kharif crops:

1. *Cajanus cajan* L. (Arhar)
2. *Dolichos lab-lab* L. (Sem)
3. *Glycine max* L. (Soyabean)
4. *Vigna radiata* L. (Mung)
5. *Vigna mungo* L. (Urid)

Rabi crops:

1. *Lathyrus sativus* L. (Teora)
2. *Pisum arvense* L. (Chhota batra)
3. *Pisum sativum* L. (Matar)
4. *Lens culinaris* L. (Massoor)
5. *Trigonella foenum-graecum* L. (Methi)

The samples were collected from five different locations of Raipur district. The selected sites were --

1. Indira Gandhi Krishi Vishwavidyalaya, Raipur
2. Village-Jora
3. Village-Bhatagaon
4. 2 Kitchen Farm houses of Kushalpur

The kharif and rabi crops were cultivated in 4 hectare area in Indira Gandhi Krishi Vishvavidyalaya, Raipur and 1 acre each in Jora and Bhatagaon and about half acre area in Farmhouse and Kitchen garden in Kushalpur area.

Collection

Infected leaves with chlorotic or necrotic spots were collected from the different locations and brought to the laboratory in sterilized polythene bags for the isolation and
identification of the causal organisms. Degree of infection and percentage disease intensity of each crop was calculated on the basis of number of infected leaves out of ten leaves of per ten plants per crop field. Effect and impact of climatic conditions on disease incidence were analysed from the meteorological data (2012-2014) obtained from Indira Gandhi Krishi Vishvavidyalaya, Raipur (C.G).

Collection of leaf samples of kharif and rabi crops done during later harvesting period.

**Screening**

The infected leaves were screened out on the basis of symptoms. The disease initiated with chlorotic resulting in necrotic, blight, leaf spots and shot holes. Symptoms varied as brown, reddish brown, black, hard black dot, leaf blight, necrotic and chlorotic spots.

**Isolation**

- Potato Dextrose Agar (PDA) medium was used as the substrate (250.0 g potato, 20.0 g Dextrose, 20.0 g Agar, 1000ml distilled water, pH 4.5) for isolation of pathogens.
- Infected portion of leaves were cut by means of sterilized razor in small pieces and dipped in 0.01% mercuric chloride solution for 30 seconds.
- The diseased pieces were then successively washed thrice in sterilized distilled water and placed on pre-poured petridishes containing PDA medium.
- The plates were inoculated at room temperature at 28°C ± 2°C for seven days.
- The entire operations were carried out under aseptic conditions. The organisms thus obtained were repeatedly subcultured in order to get pure cultures.
- Pure cultures were maintained on PDA slants for further studies.

The % disease intensity was recorded after 7 days and was calculated using the following formula:

\[
\text{% Disease intensity} = \frac{\text{Total number of infected leaves}}{\text{Total number of leaves}} \times 100
\]
Pathogenicity Test

Pathogenicity has been tested and proved by infection experiments. In case of disease caused by parasitic organisms, Koch’s “rules of proof”, postulates must be satisfied to determine the relationship of a particular organism to the disease under investigation. These postulates are:

1. The organisms question in must be found constantly associated with a particular symptom.
2. It must be isolated and studied in pure culture.
3. The organisms grown in pure culture must be inoculated into a healthy plant to produce the particular disease. The symptoms grown in pure culture and inoculated plant should be the same as the symptoms first observed.
4. The organisms must be re-isolated from the inoculated plant and compared with the first culture to and should be the same as the original culture.

Pathogenicity test was conducted using attached leaf method to ensure Koch’s postulate, and was confirmed by attached leaf method under greenhouse conditions. (Chature et al. 2014).

- The leaves were pin pricked using sterilized needle and cultures were inoculated on the respective leaves in triplicates under aseptic conditions.
- The fifteen days old seedlings were inoculated with spore suspension.
- Before inoculation of plant leaves were injured with sterilized needle for easy infection.
- All pots were kept in moist chamber for 24 hours for the development of disease symptoms.
- Observations of disease symptoms were recorded up to 8-15 days after seedling inoculation.
- One control pot was maintained as without inoculated sterilized water.
Mineral Analysis

Macro elements (Nair, 1994; Sharma, 2011)
- Nitrogen (N)
- Phosphorus (P)
- Potassium (K)

Micro elements (Nair, 1994)
- Iron (Fe)
- Zinc (Zn)
- Copper (Cu)
- Mangnese (Mn)

Total nitrogen

Total nitrogen in plant samples was determined by the Kjeldahl method. The method involved:

1. Digestion of the samples to convert the nitrogenous compound to ammonium (NH₄)
2. Determination of ammonium (NH₄) form in the digest through distillation.

Requirements

1. Concentrated H₂SO₄ (98%)
2. Salt and catalyst mixture: A mixture of K₂SO₄+CuSO₄ in the ratio of 10:1
3. Digestion unit with digestion tubes
4. Standard sulphamic acid (0.1N)
5. 40% NaOH (0.1 MO/L)
6. Boric acid + Mixed indicator (Bromocresol green and methyl red)

Procedure

- 0.5 gram leaf sample of each crop was taken in digestion tube and 10 ml of concentrated H₂SO₄ was added.
- 1 gram of salt mixture was added to each digestion tube in order to raise the boiling temperature of the digestion mixture and to shorten the digestion time.
- After pre-digestion, the samples were digested at 200°C. The digest was cooled to room temperature.
For distillation automated version of Kjeldahl distillation was used.
The digestion tubes were directly fitted in Kjeldahl apparatus.
In a conical flask, 10 ml of 2% boric acid solution was taken containing mixed
indicator to which the condenser outlet of the distillation unit was dipped.
The distillate was than titrated against 0.1N sulphamic acid.

Phosphorus (P)

Determination of phosphorus was done by spectrophotometric method. Wet
method was used for digestion using di-acid (nitric acid and perchloric acid), (Sharma
2011; Mali et al. 2009)

Reagents

Preparation of vandamolybdate

Ammonium molybdate–ammonium vandate in HNO₃: 22.5g (NH₄)₆ MO₇O₂₄·4H₂O
was dissolved in 400 ml diatilled water.

1. 1.25 g ammonium vanadate was dissolved in 300 ml boiling distilled water.
2. Vandate solution was then added to the molybdate solution and cooled to room
temperature than 250 ml of concentrated HNO₃was added and diluted to 1 litre.

Procedure

1. Acid mixture was prepared by mixing two parts HNO₃ with one part HClO₄ .
2. 0.5 g leaf sample was taken into 50 ml digestion tube.
3. 5 ml of acid mixture was added to it.
4. Tubes were placed in block digester.
5. Samples were then heated at 60°C for 15 minutes until reaction was completed.
6. Temperature was raised to 120°C and samples were digested for 75 minutes until
clear sample was obtained.
7. Tubes were then removed from block digester.
8. After cooling the solutions were filtrated through Whatman’s No. 1 filter paper
and volume of the filtrate was make up to 100 ml using double distilled water.
9. 10 ml of the above sample was transferred in 50 ml volumetric flask and 10ml of vanadium molybdate reagent (yellow reagent) was added and dilute upto 100 ml with distilled with distilled water and absorbance was taken at 420 nm by spectrophotometer (Systronics company).

**Potassium (K)**

*Mali et al. 2009*

Determination of potassium was done by flame photometric method.

**Procedure**

1. Acid mixture was prepared by adding two parts HNO₃ with one part HClO₄.
2. 0.5 g of each leaf sample was taken into 50 ml digestion tube.
3. 5 ml of acid mixture was added to it.
4. Sample was heated at 60°C for 15 minutes until reaction was completed on a block digester.
5. Temperature was raised to 120°C and sample was digested for 75 minutes until clear sample was obtained.
6. Tubes were then removed from block digester for cooling.
7. After cooling the solutions were filtrated through Whatman’s No. 1 filter paper and volume of the filtrate was make up to 100 ml using double distilled water.
8. 5 ml aliquot of the above sample was transferred into 25 ml volumetric flask and make up 25 ml then absorbance was taken 548 nm in flame photometer (Systronics).

**Estimation of Micro Elements (Trace Elements)**

- Iron (Fe)
- Zinc (Zn)
- Copper (Cu)
- Manganese (Mn)

Estimation of iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) in the selected host crops was done by using the following method.
Iron (Fe), Zinc (Zn), Copper (Cu) and Manganese (Mn) estimation was done by spectrophotometric method. Leaf samples were digested by wet method using di-acid mixture, HNO₃ and HClO₄.

**Estimation of Iron (Fe)**

**Procedure**

1. 0.5 gm of each leaf sample was placed in a digestion tube and 11 ml of di-acid mixture (9 ml HNO₃, 2 ml HCl) was added and kept overnight at room temperature for pre-digestion.
2. Digestion was carried out at temperature 180°C in a microwave digestion chamber (CEM-MARS equipment) for 45 minutes.
3. Completion of digestion was confirmed when the liquid turned colourless.
4. After cooling, the solution was filtered through Whatman’s filter paper number 1 and volume of the filtrate was make up to 50 ml using double distilled water.
5. The leaf sample thus prepared were used for the analysis of iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn).

**Estimation of Iron (Fe)**

1. Aliquot of the above solution was taken for determination of iron and read at 248 nm in atomic absorption spectrophotometer (ECIL Company).

**Estimation of Zinc (Zn)**

1. Each digested leaf sample was tested for the presence of zinc (Zn) at taken 213 nm in atomic absorption spectrophotometer (ECIL Company).

**Estimation of Copper (Cu)**

1. For the estimation of copper (Cu) the digested solution of each sample was tested at 213 nm in atomic absorption spectrophotometer (ECIL Company).

**Estimation of Manganese (Mn)**

1. Aliquot of the above solution was taken for determination of manganese (Mn) then absorbance was recorded at 279 nm in atomic absorption spectrophotometer.
Selection of Basal Media

To find out the suitable basal medium for the optimum growth, five culture media were used viz.

- Richard’s Broth (RB)
- Potato Dextrose Broth (PDB)
- Asthana Hawker’s Broth (AHB)
- Sabouraud’s Broth (SB)
- Czapex Dox Broth (CDB)

Richard’s Broth (RB)

- KNO3 – 10.00 g
- KH$_2$PO$_4$ – 5.00 g
- MgSO$_4$.7H$_2$O – 2.50 g
- Sucrose – 50.00 g
- Distilled water – 1000 ml
- pH – 4.5

Potato Dextrose Broth (PDB)

- Potato – 250.0 g
- Dextrose – 20.00 g
- Distilled water – 1000 ml
- pH – 4.5

Asthana Hawker’s Broth (AHB)

- Glucose – 5.00 g
- KNO3 – 3.50 g
- KH$_2$PO$_4$ – 1.75 g
- MgSO$_4$.7H$_2$O – 0.75 g
- Distilled water – 1000 ml
- pH – 4.5
**Saubaroud’s Broth (SB)**

- Peptone: 10.00 g
- Dextrose: 40.00 g
- Distilled water: 1000 ml
- pH: 4.5

**Czapek-Dox Broth (CDB)**

- NaNO₃: 2.00 g
- KH₂PO₄: 1.00 g
- MgSO₄·7H₂O: 0.50 g
- KCl: 0.50 g
- FeSO₄·7H₂O: 10.00 g
- Sucrose: 30.00 g
- Distilled water: 1000 ml
- pH: 4.5

Optimum growth was recorded in Richard’s Broth (RB) media and was selected for enzymological studies and *in-vitro* control studies.

**Biochemical Characteristics of the Fungal Pathogens**

**Sugar utilization**

Sugar utilization was done with Dreywood’s Anthrone reagent (Morris, 1948; Rao *et al.* 2014). The isolates were cultured in Asthana Hawker’s medium (Glucose-5.00gm, KNO₃-3.50 gm, KH₂PO₄-1.75gm, MgSO₄·7H₂O 0.75, Distilled water 1000 ml, pH-4.5).

**Process of inoculation**

- Each of the culture medium was prepared in one litre and 25 ml of Asthana Hawker poured in 100ml borosil flask and sterilized at 121°C for 20 minutes.
- The selected pathogenic isolates were inoculated in triplicates and inoculated at 28°C ± 2°C for seven days.
- Mycelial mat was removed by filtering the broth through Whatman filter paper number 1.
• Dry weight of mycelium was recorded after subtracting the weight of filter paper from total weight of mycelium and filter paper.

• pH of aliquot was taken by pH meter and then total volume of filtrate was measured.

• The biomass was taken in triplicate after seven days of incubation.

Procedure

• After 7 days of growth, 2 ml of culture filtrate was transferred to the test tube, kept on ice bath and 8 ml of anthrone reagent (0.2% in conc. H₂SO₄) was added to it. Test tubes were then kept in boiling water bath for 15 minutes.

• A golden green colour was developed.

• Absorbance was noted at 620 nm.

• The blank samples contained distilled water and reagent only was used to test the 100% transmission.

• Standard calibration curve of glucose solution (concentration 0.1% to 0.9%) was plotted.

• Concentration of glucose of the filtrate was obtained from the curve. The biomass and pH changes were also recorded.

Protein Test

Protein estimation was done by Lowry’s method (Lowry’s 1951; Gupta 2010).

A. Preparation of solution-

1. 10%Trichloroacetic Acid (TCA)
2. Ethanol: Ether in 3:1 ratio
3. 1 N NaOH

• The pathogens were cultured in Richard’s Broth medium for protein estimation for 7 days.
• From the mycelial extract which was taken as a protein source, 1 ml aliquot was pipetted into a centrifuge tube to which 1 ml of 10% TCA was added to precipitate the protein.

• The mixture was allowed to stand for 15 minutes on the ice bath and centrifuged at 10000 rpm.

• The supernatant was filtered twice.

• The pellet was washed with ethanol-ether mixture, centrifuged and supernatant was discarded.

• The resulting pellet was dissolved in 1 N NaOH. This sample was used for protein estimation.

B. Method

• 1 ml of aliquot was taken in a test tube and 5 ml of freshly prepared alkaline CuSO₄ solution was added to it.

• After 5 minutes, 0.5 ml of Folin- Ciocalteu’s phenol reagent was added to it.

• The solution was shaken immediately and optical density (OD) of the colour developed was read on spectrophotometer at 750 nm after 15 minutes.

• The optical density was read against a blank of 1 ml 1N NaOH which was processed as the test sample.

• Standard graph was prepared by using different concentrations of Bovin albumin serum and total protein content of the test sample was calculated with reference to the standard graph.

Chromatographic Analysis

1. Amino acids
2. Organic acids and
3. Sugars

Quantitative analysis was done in all isolates using ascending paper chromatography.
Procedure

- The pure culture of the isolates were inoculated in Asthana Hawker’s medium and incubated at 28°C ± 2°C for 7 days.
- After 7 days, the culture filtrates were collected and concentrated to about its half volume at 60°C.
- The aliquots of cultures were used for chromatographic analysis of amino acids, organic acids and sugars.
- Aliquots of the cultures were plotted with 30 µl micropipette and dried at room temperature.
- Whatman paper No.1 was used as absorbent.

Amino acids (Kohli, 2004; Rao et al. 2014)

The solvent system used was n-butanol: acetic acid: water, in 4:1:5 v/v, solvent system and ninhydrin (0.2% in acetone) was used as the detecting reagent.

Organic acids (Nair, 1994)

- The chromatogram was developed in n-butanol: acetic acid: water, 4:1:5 v/v as solvent system and 01% aqueous bromocresol green as the detecting reagent.

Sugars (Rawte, 2011)

The solvent system was run in n-butanol:acetic acid:water, in 12:3:5 v/v as solvent system and aniline-phthalate reagent (0.6gm phthalic acid+930 mg aniline +100mlwater saturated n-butanol) as detecting reagent.

The separation of standard samples of amino acids, organic acids and sugars were also carried out by ascending paper chromatography. The unknown samples obtained from the respective culture filtrates were plotted and their Rf values were calculated by the formula-

\[ Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}} \]

Enzymological Studies

In-vitro production of extracellular and intracellular enzymes were determined in selected isolates.
A. Extracellular enzymes

Cellulolytic: Cellulase (Cx)

Pectolytic: Polymethyl galactoronase (PMG)

Pectinmethyl esterase (PME)

Cellulase (Cx)

Libration of sugar, as glucose, was measured by DNS method (Gascoigne and Gascoigne, 1969, Ahamed et al. 2008)

Reaction mixture:

- Enzyme preparation - 1.0 ml
- 0.55% carboxy methyl cellulose solution (pH 5.5) - 3.5 ml
- DNS reagents - 1.0 ml

Procedure

- The reaction mixture consisting of 1 ml of enzyme sample and 1 ml of 0.55% carboxy methyl cellulose (CMC) in sodium citrate buffer (pH 5.5) was taken in a test tube and incubated for one hour.
- 1 ml of aliquot from each test-tube was taken out in another tube to which 1 ml of Dinitrosalicylic acid (DNS) reagent was added.
- This mixture was heated on boiling water bath for 5 minutes and cooled under tap water to room temperature.
- Distilled water was then added to make up the volume up to 10 ml.
- The absorption of the sample was measured at 540 nm.
- The amount of reducing sugar liberated was obtained from standard curve, obtained by plotting absorbance against concentration of standard aqueous solutions of D-glucose.

One unit of cellulase enzyme activity is defined as the amount of enzyme required to liberate reducing sugar equivalent to 10 µg of glucose.
Polymethyl galacturonase (PMG)

The Polymethyl galacturonase (PMG) enzyme were estimated according to the method of methods and procedures described (Hussian, 1958; Hancock et al. 1964 and Bateman, 1963; Chaurasia et al. 2014)

Enzyme assay was made viscometrically. The reaction mixture contained

- 1.2% citrus pectin solution (pH 5.5) - 3.5 ml
- McIlvaines buffer (pH 5.5) - 1.5 ml
- Distilled water - 1.5ml
- Enzyme preparation - 1.5ml

McIlvaines buffer Composition

- Na₂HPO₄·H₂O 0.2M (35.61g in 1 litter distilled water)
- Citric acid 0.1M (21.01g in 1 litter distilled water)

Procedure

- The enzyme mixture was placed in the viscometer and the time of efflux was recorded immediately as at 0 hours at 30°C in each case.
- Subsequently the reading of the efflux time was noted after incubation of 30, 60, 90, & 120 minutes.
- Boiled culture filtrates served as control. Percentage loss in viscosity was calculated.

\[
\text{\% loss in viscosity} = \frac{\text{ET}_0 - \text{ET}_t}{\text{ET}_0 - \text{ET}_w} \times 100
\]

where

- ET₀= efflux time at 0 hours
- ETₜ= efflux time at time t
- ETₗ= efflux time of control

Pectin Methyl Esterase (PME)

The PME was carried out by the continuous titration method of Hancock et al 1964, Kumar et al (2007).
**Procedure**

- A solution of 1.2% citrus pectin (pH 5.5) was the enzyme substrate, 4.5ml of filtrate was added to 30 ml of the substrate and pH was immediately recorded as at 0 hours.

- After reaction times of 30, 60, 90, and 120 minutes, the pH change of the reaction mixture was recorded.

- The enzyme substrate mixture was then back titrated with 0.02 N NaOH and the amount of 0.02 N NaOH required to bring back the pH of 0 hour was recorded.

- Boiled culture filtrate was taken as control.

The relative enzyme activity represented the reciprocal of 1000 and was calculated by the formula:

\[
\text{REA at time } t = \frac{0.02 \text{ N NaOH in ml}}{4.5} \times 100
\]

**Intracellular enzyme**

The intracellular enzymes were

1. Acid phosphatase (ACP)
2. Alkaline phosphatase (AKP)

**Reagents for determination of Acid Phosphatase**

- Glycine–NaOH buffer (0.05M, PH5.5)
- NaOH solution (0.085 N)
- MgCl₂ solution (10.5 mM)
- \(p\)-nitrophenyl phosphate (35mM)
- Standard solution of \(p\)-nitrophenol (100 mM)

**Reagents for determination of Alkaline Phosphatase**

- Glycine –NaOH buffer (0.05M, pH10.5)
- NaOH solution (0.085 N)
- MgCl₂ solution (10.5 mM)
- P-nitrophenyl phosphate (35 mM)
- Standard solution of p nitrophenol (100 mM)
Determination of Acid & Alkaline Phosphatases

All the operations for preparation of the tissue extract were carried out in cold at 0-4°C.

Procedure

- 1g of the mycelium was taken and grinded into chilled pestle in mortar in presence of 10 ml of glycine – NaOH buffer (in case of acid phosphatases 0.05M, pH 5.5 was maintained and for alkaline phosphatases 0.05M, pH 10.5 was maintained).
- A small amount of acid washed river sand was used as an abrasive to facilitate complete breakage of the cell.
- The homogenate was centrifuged in a refrigerated centrifuge at 10,000 rpm for 20 minutes.
- Supernatant was decanted and used for enzyme preparation.
- Nine test tubes were taken and 3 ml of glycine–NaOH buffer, 0.1 ml MgCl₂ and 0.3 ml of above enzyme preparation were added into each of them.
- These tubes were then transferred to a water bath maintained at 37°C. After 3 minutes 0.1ml p-nitrophynyl phosphate was added in seven of the above tubes to start the reaction.
- Exactly after 30, 60, 90, 120 minutes 9.5 ml of 0.085 N NaOH solution was added to each tube to stop the reaction.
- In the eighth tube NaOH was added followed by 0.1ml of p-nitrophynyl phosphate which represented as 0 minutes control. In the ninth tube, instead of p-nitrophynyle phosphate, 0.1 ml of 0.05 M glycine–NaOH buffer (pH 5.5) was added and was served as the reagent blank.
- The spectrophotometer was adjusted to 410 nm and absorbance for reagent blank and other samples were recorded.
- The result was calibrated with standard curve.
**In-vitro Control Measures**

Three different control agents i.e. fungicides, antibiotics, angiospermic sources were used for *in-vitro* control of the pathogens. The poisoned food technique was followed for broth and semi solid media (Nene and Thapalyal, 1965 and Meena *et al* 2014). Potato Dextrose Agar (PDA) medium was used for plate method and Richard’s broth medium to analyse the morphological variations in growth as well as biomass in treated and control samples. The pH of both media was maintained at 4.5.

1. **Antibiotics**
   
   Three antibiotics viz: Grisofulvin, Teramycin, Norfloxacin, 0.01%, 0.02% and 0.03% aqueous concentration were selected to test out their effectivily against all the isolates.

2. **Fungicides**
   
   Three fungicides viz. Blitox, combination of Carbendazim + Mancozeb, Antracol (SAF) (concentrations 0.01%, 0.02%, and 0.03% each) to evaluate their disease protection capacity against the isolates isolates.

3. **Angiospermic sources**
   
   Four angiospermic sources viz. *Carica papaya*, *Ziziphus jujuba*, *Eugenia jambolana*, *Psidium guajawa* with three different concentrations 0.01%, 0.02%, and 0.03% each) were tested to control the growth of all the isolates.

**Growth on Semisolid Media**

- To study the antifungal activities of different control agents, antibiotics viz. Norfloxin, Teramycin and Grisofulvin (0.01%, 0.02%, 0.03% concentration of each), fungicides viz. Biltox, Carbendazim + Mancozeb, Antrocol (SAF) (0.01%, 0.02%, 0.03% concentration of each) were added to Potato Dextrose Agar (PDA) media.

- The medium was shaken for uniform mixing of extracts. 20 ml of poisoned medium was poured into each of sterilized petriplates.

- Plates were inoculated at 28°C ± 2°C for seven days and radial growth was measured.
Control was also maintained for the growth of the pathogens on Potato Dextrose Agar (PDA) medium without treatment.

The radial size of the colony was measured after seven days of incubation and percentage growth inhibition was calculated by the following formula.

\[
\text{% Growth Inhibition} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100
\]

Preparation of Extract

- Excess water was removed before grinding them to a paste. The extract was prepared in 100 ml distilled water and filtered through muslin cloth.
  - From this homogenet 0.01%, 0.02% and 0.03% aqueous solution of each angiospermic solution were prepared from the each stock solution.
  - 1 ml of each concentration was added in each flask to 24 ml of sterilized Richard’s broth in triplicates.
- The medium was thoroughly shaken for uniform mixing of the extract.
- 25ml of this media was poured in to conical flask and selected pathogenic isolates were inoculated in triplicates and incubated at 28°C ± 2°C for seven days.
- Biomass was recorded after seven days of incubation and percentage inhibition was calculated.

Richard broth medium without culture was used as control.

*in-vivo control* (*Jagtap et al. 2013 and Meena et al. 2014*)

Antibiotics

- Three antibiotics viz grisofulvin,teramycin, norfloxacinwere selected to study the *in vivo* control of the disease on the plants 0.01%,0.02%,0.03% aqueous solution of these antibiotics were prepared.
Fungicides

- Three fungicides blitox, Carbendazim+ Mancojeb, Antracol with 0.01%, 0.02%, 0.03% concentrations were selected in vivo control of the disease.

Angiospermic sources

- Four Angiospermic sources viz., *Zyzyphus jujuba* L., *Eugenia jambolana* L., *Carica papaya* L., *Psidium guajava* L. three aqueous concentrations of 0.01%, 0.02%, 0.03, of were used in in- vivo control of disease.

Rabi & Kharif Crops

- Field experiments were carried out in field at Raipur city during 2015-2016.
- Five plants each from kharif and, rabi total ten legume crops, were selected and cropped them in pots under moderate temperature and high relative humidity.
- 15 days older plants were then inoculated with pathogen and covered them polythene bags to maintain high relative humidity. Disease symptoms constantly observed after 8-15 days of inoculation.
- Solution of of 0.01%, 0.02%, 0.03%. Concentrations of antibiotics, fungicides, and angiospermic sources were prepared in distilled water (Aqueous).
- The leaves were sprayed with these solutions separately.
- Data of seedling mortality, percent disease leaf area covered by leaf spots were recorded twice in 15 days interval.
- Percentage disease intensity in each plant was calculated five from leaves from main branch.
- One pot/replication was maintained as unsprayed control.
- \% Disease incidence was calculated by the formula.

\[
\% \text{ Disease incidence} = \frac{\text{Average number of infected plants}}{\text{Average number of total plants}} \times 100
\]