Chapter 4

MATERIALS AND METHODS

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CHAPTER IV

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An ample supply of food is a prerequisite to human health as well as to the social and economic growth of our society. But about thirty percent of the food we grow is lost due to diseases caused by pathogens and about thirty four percent of population in developing countries suffers from malnutrition. The pathogens not only caused quantitative losses but also affect the quality of the produce.

The main thrust of cultivators has been towards application of pesticides for combating the disease menace, because of convenience and easy availability of chemicals. But with their indiscriminate and excessive use on diverse crops, many complex and diversified problems have been realised, such development of resistance to pesticides in pathogens, resurgence of secondary pathogens, disturbances to natural ecosystem, besides environmental pollution etc. It has, therefore, become necessary to search an alternative for plant pathogen management technology that are ecofriendly, biosafe, economically viable and socially acceptable, to combat the menace of pathogens for all cropping systems.

The efforts have been made towards the evaluation of some management tactics to manage the problem of pathogens effectively. Several workers have explored the utility of various botanical as one of the potential alternatives.
The aqueous extracts of botanical like *Ipomea* and *Azadirachta indica* (leaves and seeds) have been found promising. But still the information on microbial properties of various botanicals is lacking.

In view of the above present investigation entitled “Studies on microbicidal properties of some plants for management of plant pathogens”, so as to reduce the pesticidal consumption and to formulate effective, ecofriendly, and economical alternatives for management strategies against various plant pathogens.

### 4.1 Data collection and screening of Plants:

- The information regarding the availability of cultivated medicinal plants in and around the Akola was collected from District Forest Office, as well as from Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

- The collected data was appropriately processed with respect to local names, percentage of total plant community in Forest jurisdiction under cultvate sector, and total percentage of plantation etc. The processed data was presented in the form of Table No. 1 and graphically represented in Figure No1.

- To avoid the constraints in availability of the medicinal plants to farmers in Akola, only those plants which are most frequently available i.e. above 25% in the Akola region and were selected for the present studies and presented in Table No. 2.
4.2 **Preparation of the Plant Extracts**

- Plants (n=10) were used in the present study. Plant materials such as fresh leaves and roots were collected and transported to the research laboratory.
- The plant parts were further subjected for extraction using solvent like aqueous and acetone adopting the standard methods suggested by Shekhawat *et al.* (1971).
- All the plant extracts were then preserved at 10°C till further use.

4.2.1 **Preparation of Aqueous Extracts**

- The plant materials viz. fresh leaves were separately collected, washed in tap water and then crushed in mortal and pestle by adding small quantity of distilled water.
- The crushed material was then homogenized by using sterilized distilled water (95:5w/v).
- The homogenized suspension of plant materials were filtered through muslin cloth.
- The marc remaining on the muslin cloth is squeezed into the beaker thoroughly and the suspension is used for reextraction.
- Finally the filtrate obtained was further used as plant extract of 100% concentration.
- The extract of various concentration viz. 25%, 50%, and 75% were further prepared by raising the volume with sterilized distilled water in appropriate quantity of plant extracts.
4.2.2. Preparation of Acetone Extracts :-

- The selected plant leaves were taken and were cut into pieces and dehydrated at room temperature by evaporation.
- The dehydrated pieces were then powdered by grinding and passing through hundred mesh sieves.
- The powder thus obtained was preserved in air tight glass bottle.
- Acetone extraction was done by simple crude method suggested by Bambode and Shukla, 1973.
- Powder (10g, w/w) was suspended in 80% acetone and refluxed for about an hour at 60°C on water bath.
- The acetone was evaporated at room temperature followed by addition of 10 ml distilled water to get stock solution. All the extracts were filtered through muslin cloth and further the filtrate was used as acetone extract.
- The extracts of various concentrations such as 25%, 50%, and 75% were prepared by diluting with sterilized distilled water, whereas stock solution was considered as 100%.

4.3 Isolation and Identification of plant pathogens :-

- The frequently encountered diseased plants in common crops of Akola i.e. cotton (*Gossypium hirsutum* L.), *mung* (*Vigna radiata* L. Wilczek) and Tomato (*Lycopersicon esculentum* Mill) were screened during the cultivation period.
• The infected plants were excavated from locally cultivated farms and transported to the laboratory in sterilized containers.

• The infected plants were surface sterilized using (1:10,000) solution of mercuric chloride, followed by sterilized distilled water.

• The diseased part especially from foliar region were then removed with the help of sterile knife and further used for isolation of pathogen.

• The infected tissues were then homogenized and separately inoculated into Nutrient broth and Potato Dextrose Broth, further incubated at 37°C for 24 hours at room temperature for four days respectively.

• The enriched cultures were then used for isolation of pathogens on solid media.

• The enriched broths were further inoculated on Nutrient Agar and Potato Dextrose Agar respectively.

  The nutrient Agar plates were maintained at 37°C for 24 hrs, whereas Potato Dextrose Agar plates were incubated at room temperature for 4 days.

• Further identifications of the plant pathogens were done on the basis of colony characters, conventional, cultural and biochemical characters followed by comparing with available standard literature (Breed et al., 1957; Singh et al., 1991).

• All the isolated pure cultures were maintained at respective temperatures followed by sub-culturing after every 2-3 weeks on specific medias.
4.4 *In-vitro* studies on antimicrobial properties of plant extracts :-

The studies on antimicrobial activities of plant extracts against isolated plant pathogens were conducted adopting the method suggested by Nene, (1977) and Horsfall (1956).

Disc diffusion method for bacterial pathogens, and Poisoned food technique for fungal isolates were adopted and elaborated as follows. The plant extracts of the concentrations 100%, 75%, 50% and 25% were considered in the present study. All these operations were carried out under aseptic condition in Laminar air flow.

**Disc Diffusion Method :-**

- Approximately the disc of six mm diameter was prepared using Whatmann Filter paper No. 42 with the help of punching machine.
- All the discs were sterilized by autoclaving followed by drying in oven.
- The seeded plates of test pathogens were prepared by incorporating 2 ml of cell suspension (10⁹/ml) in sensitivity test medium before solidification.
- The culture was shaken thoroughly for uniform mixing in the medium and poured in sterile petri plates at the rate of 20 ml per plate.
- The sterilized filter paper discs were soaked in respective plant extracts up to saturation.
- The saturated plates were finally placed at the centre of seeded plates.
• All the plates were kept at refrigeration temperature for 10-15 minutes and further incubated at 37°C for 24 - 48 hours.

• Each treatment was replicated thrice with control having disc dipped in sterilized distilled water and placed on seeded plates.

• Followed by incubation, all the plates were examined for the presence of zone of inhibition.

• The size of zones were measured and recorded in mm.

• The presence of clear area around the disc was considered as zone of inhibition.

**Poisoned food technique Method:**

• The principle involved in this method is to poison the nutrient medium with a fungi-toxicant and the growth response for the test fungi was studied by observing the mycelial inhibition.

• In 250 ml of conical flask 100 ml of sterilized molten Potato Dextrose Agar was prepared.

• To this medium, requisite quantities of plant extracts were added, so as to get the desired concentrations.

• About 20 ml of melted, poisoned Potato Dextrose Agar was poured in sterilized petri-plates and allow to solidify.

• All the plates were then inoculated with test fungal cultures by transferring the pure culture (inoculum disc) with the help of sterilized cork borer.
• The surface of inoculum disc was kept in inverted position on the surface of plates.

• The control plates were maintained, where the inoculum disc were grown under same condition on Potato Dextrose Agar without plant extract.

• Three plates were inoculated for each fungi for every kind of extract.

• All the plates were incubated at room temperature of 27°C ± 2 for 7 days.

• In observations regarding growth resistance, the colony diameter were recorded and compared with the control.

• The percent inhibition of the growth was calculated using following formula (Vincent, 1947)

\[
I = 100 \times \frac{(C - T)}{C}
\]

Where, 
\(I\) = % inhibition of growth.
\(C\) = Growth of fungus in control (mm)
\(T\) = Growth of fungus in treatment (mm)

4.5 Field Trials

Akola has Cotton (Gossypium hirsutum L.), Mung (Vigna radiata L. Wilczek) and Tomato (Lycopersicon esculentum Mill.) as the major crops. However, during the field trials, studies on microbicidal activities of leaves extracts of Azadirachta indica L. for management of foliar diseases in Mung were conducted during the period 2001-2004. The details of materials used and methods adopted during the course of present investigations are given as follows.
4.5.1 Experimental Site :-

The experiment was conducted on local vertisol soils. The cropping pattern followed by local farmers on the experimental site of preceding three years are presented as follows:

<table>
<thead>
<tr>
<th>Year</th>
<th>Crop cultivated and harvested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998 – 1999</td>
<td><em>Mung</em></td>
</tr>
<tr>
<td>1999 – 2000</td>
<td><em>Cotton</em></td>
</tr>
<tr>
<td>2000 – 2001</td>
<td><em>Mung</em></td>
</tr>
<tr>
<td>2001 – 2002</td>
<td><em>Mung</em> (Present investigation)</td>
</tr>
<tr>
<td>2003 – 2004</td>
<td><em>Mung</em> (Present investigation)</td>
</tr>
</tbody>
</table>

4.5.2 Metrological Data :-

Akola is situated in Sub-tropical zone at the latitude of 22.42°N and longitude of 77.02 E at an altitude of 307.42 m above mean sea level. The weekly metrological data on some important weather parameters recorded at the agrometro observatory of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during the course of investigations.

4.5.3 a. Experimental Details :-

The present investigation was laid out in Randomised Block Design on vertisol soils with 4 replications and 5 treatments.
Details of field experiment at glance

1. Name of Crop - *Mung Vigna radiata* L. Wilczek variety
2. Experiment design - R.B.D. Randomized Block Design
3. No. to treatments - 5
4. No. of replications - 4
5. No. of plots - 20
6. Size of plot - 2 mm x 2 mm
7. Location - Vivara local farm, Survey No.151/2.
8. Season - Kharif
9. No. of plants/plot - 10

The infected plants were screened and counted at 15 days interval on the basis of following indicative symptoms, on mung crop.

1) Leaf Spots - *Pseudomonas aeruginosa* infection (Kale, 1999)

2) Leaf blight - *Rhizoctonia bototicola* infection (Sakuja, 1975)

4.5.3. b. **Induction of plant infections :-**

- The pure culture of isolated pathogens i.e. *Rhizoctonia bototicola* and *Pseudomonas aeruginosa* were enriched using Potato Dextrose Agar and *Pseudomonas* media respectively.
The mycodial suspension of *Rhizoctonia botanicola* was prepared by mixing the mycelium into sterilized distilled water with 1:10 w/v ratio, whereas, bacterial suspensions were prepared by 1:5 v/v ratio using sterile distilled water and considered as an infective dose.

Both the suspensions were further used for induction of infection in test crop.

The infective dose was spread on each plant routinely 5 days prior to each treatment of plant extract.

The infected plants were screened and counted.

4.5.4. Details of experimental treatments :-

The quantity of plant extract required per plot was calculated as per treatment and spread with a sprayer in respective plots. The spraying as per treatment was done at 7, 14, 28, 42, and 56 DAS. The details of experimental treatment are as follows.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Control (untreated)</td>
</tr>
<tr>
<td>T2</td>
<td>25% concentration of plant extract @ 2 liter/hectare.</td>
</tr>
<tr>
<td>T3</td>
<td>50% concentration of plant extract @ 2 liter/hectare.</td>
</tr>
<tr>
<td>T4</td>
<td>75% concentration of plant extract @ 2 liter/hectare.</td>
</tr>
<tr>
<td>T5</td>
<td>100% concentration of plant extract @ 2 liter/hectare.</td>
</tr>
</tbody>
</table>
4.5.5 Sampling technique :-

For recording various observations viz. germination percentage, Plant Disease Intensity (P.D.I.) and yield performance, among all the treatments, the plots were randomly selected in all replications. The observations were made as follows

a) Germination percentage was calculated after 15 DAS.

b) Plant Disease Intensity was calculated after 15\textsuperscript{th}, 30\textsuperscript{th}, 45\textsuperscript{th}, and 60\textsuperscript{th} DAS.

c) Yield performance was calculated after harvesting (60 DAS)

4.5.6 Statistical Analysis :-

The data recorded were subjected to statistical analysis as per procedure given by Panse and Sukhatme, (1978) using software on personal computer. The critical difference (C.D.) was worked out at 5 percent level of significance for comparison of individual treatment.
Photo Plate No. 1: Medicinal Plants used under study

**Plant No. 1:**
Azadirachta indica L.

**Plant No. 2:**
Chlorophytum borivilianum

**Plant No. 3:**
Acacia arabica

**Plant No. 4:**
Withania somnifera
Plant No. 5: 
*Annona squamosa*

Plant No. 6: 
*Datura innoxia*

Plant No. 7: 
*Bauhinia mosa*

Plant No. 8: 
*Ocimum canum*
Plant No. 9:
*Annona reticulata*

Plant No. 10:
*Zizyphus jujuba*