MATERIALS & METHODS
MATERIALS AND METHODS

The present investigation was undertaken to study the epidemiology and management of leaf spot and fruit rot of chillies incited by Alternaria alternata. The materials used and methods followed are described below.

1. General
1.1 Cleaning and sterilization of apparatus

The glasswares used during the course of investigation are of Corning and Borosil make. Prior to use, each glass are was cleaned with chromic acid solution (sulphuric acid 300ml, potassium dichromate 80g and distilled water 400ml) followed by thorough washing with detergent powder and finally rinsed with normal tap water as per need. The dried glasswares were sterilized in an electric hot air oven at 180°C for 120 minutes. The media were sterilized in an autoclave at 1.05 kg cm² (15 lb per square inch) for 15 minutes, whereas soil at 1.05kg cm for two and a half hours. Plastic pots were sterilized with 0.1% Mercuric chloride solution, followed by thorough washing with sterilized water. Surface sterilization of chilli plant parts and chilli seeds was done by dipping them in 0.1% mercuric chloride solution for 60 to 90 seconds, followed by three regular changes of sterile distilled water. Sterilization of earthen pots was done by dipping in 5% copper sulphate solution, followed by thorough washing with sterile water.

The inoculation needles, forceps and biological needles were sterilized by dipping them in alcohol and heating over a flame.

The Petridishes containing the medium were stored for 24 hours inside the inoculation chamber prior to use to avoid the possibility of
contaminations. Inoculated plates were exposed to 40w Philips day light tubes for 6 hours every alternate days. Virulence of the isolated *Alternaria alternata* was tested every alternate month by inoculating healthy chilli fruits by pin prick method and incubated at 25° C.

1.2. Media

The ingredients of the media used during the course of investigation are given below.

1.2.1. Non-synthetic media

(i) **Potato dextrose agar medium (PDA)**

- Peeled and sliced potato: 200g
- Dextrose (anhydrous): 20g
- Agar-agar: 20g
- Distilled water: 1000ml

(ii) **Rice meal agar (RMA)**

- Rice meal: 20g
- Agar-agar: 20g
- Distilled water: 1000ml

(iii) **Corn meal agar (CMA)**

- Corn meal agar: 20g
- Distilled water: 1000ml

(iv) **Soil extract agar**

- Soil extract agar: 100ml
- Glucose: 1g
- Dipotassium phosphate: 0.5g
- Agar-agar: 20g
- Sterile water: 900ml

Soil extract was prepared by steaming 1000g of garden soil in tap water in an autoclave at 1.05kg/cm² for two and a half hours. A Small
quantity of calcium carbonate was added to it and soil suspension was filtered through a whatman 42 double filter paper. The turbid filtrate was then poured into a flask and sterilized and made 900 ml by adding sterile water.

(v) **Chilli seed extract agar (CSE)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Chilli seed extract</td>
<td>200g</td>
</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Agar agar</td>
<td>20g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
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</table>

Two hundred gram chilli seed was ground and boiled, then the extract was passed through muslin cloth.

(vi) **Oat meal agar (OMA)**

Fifty gram oat meal was boiled in 300ml water till quantity of water remained half and volume was made upto 1000ml and 20g agar-agar was added.

(vii) **Soil-maize medim (SMM)**

<table>
<thead>
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<tr>
<td>Soil + Sand (1:1)</td>
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<tr>
<td>Ground maize</td>
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<tr>
<td>Distilled water</td>
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1.2.2. **Synthetic media**

(viii) **Asthana and Hawker's medium**

<table>
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<tbody>
<tr>
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<td>Potassium nitrate</td>
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<tr>
<td>Potassium dihydrogen phosphate</td>
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<td>Magnesium sulphate</td>
<td>0.75g</td>
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<tr>
<td>Agar-agar</td>
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(ix) **Czapek's agar**

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<tbody>
<tr>
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<td>Sucrose</td>
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<tr>
<td>Sodium nitrate</td>
<td>2g</td>
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<tr>
<td>Potassium chloride</td>
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<tr>
<td>Ferrous sulphate</td>
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<tr>
<td>Agar-agar</td>
<td>20g</td>
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(x) **Richard's agar medium**

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<tbody>
<tr>
<td>Potassium nitrate</td>
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<tr>
<td>Potassium dihydrogen orthophosphate</td>
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<td>Magnesium sulphate</td>
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(xi) **Martin's medium**

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<tbody>
<tr>
<td>Dextrose</td>
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<tr>
<td>Peptone</td>
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<tr>
<td>Potassium dihydrogen phosphate</td>
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<td>Magnesium sulphate</td>
<td>0.5g</td>
</tr>
<tr>
<td>Agar-agar</td>
<td>20g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
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1.3. **Incubation chamber**

To provide optimum conditions for the germination of seeds and growth of mycoflora associated with chilli seeds, petriplates containing three moiste blotters and 25 seeds plated on them were incubated in a wooden growth chamber during the course of study. The dimensions of the chamber were length 3m, width 0.75m and height 0.9m. Two sets of Philips 40w day light tubes were provided in the chamber horizontally at a height of 40 cm. Alternate cycles of 12 hr light and 12 hr dark periods
were maintained. One of the sides of the chamber was provided with a water cooler to maintain the temperature at 28\(^0\)C and to minimise the losses of water from blotters and medium in the petridishes.

1.4 Meteorological data

Data on rainfall, relative humidity, maximum and minimum temperature were recorded from the meteorological observatory at Department of Meteorology IGFRI, Jhansi.

1.5 Field Experiments

Observations on the field experiments were recorded from IGFRI, Jhansi till 2005.

METHODS

1. Survey

To study the distribution and occurrence of chilli at regular interval survey were conducted at 20 locations around Banda during 2002-03 and 16 locations of Jhansi during 2003 and 2004. One square meter (1x1m) was thrown randomly at 10 points in a field and total infected plants within the square were counted to calculate the disease incidence. The average of 10 observations was calculated as below:

\[ \text{DI} = (\text{TIP} \times 100) / \text{TPO} \]

Where,

DI = Disease incidence i.e. per cent plant exhibiting symptoms.
TIP = Total number of infected plants.
TPO = Total number of plants observed

Infected plants and diseased plant parts were collected in envelops and brought to the laboratory and observations were recorded on the locality, variety, crop stage, soil type, crop pattern and disease incidence.
2. **Collection of chilli seed samples and diseased parts**

During survey, 20 seed samples from each place were collected representing the specific locality. Seed samples of 19 varieties/lines were also obtained from the Scientist (Horticulture), Deptt. of Horticulture, C.S.A. University of Agri. & Tech., Kanpur. Eleven seed samples were collected from I.V.R.I., Varanasi. Overall 50 seed samples of chilli were selected and categories into two group:

(i) Seed samples variety-wise.

(ii) Seed samples locality wise.

Seed samples were numbered and stored in paper envelops at $4^\circ$C to avoid deterioration.

3. **Symptomatology**

Symptoms produced by *Alternaria alternata* on chilli seeds, seedlings, leaves and fruits were studied.

3.1. **Seeds**

Chilli fruits (Jawhar Mirch 218 and Pusa Jwala) naturally infected with *Alternaria alternata* were collected from fields and graded into no infection, slight infection (1-10%), moderate infection (10-25%) and severe infection (26% and above). The fruits were kept in refrigerator ($4^\circ$C) for two months. Seeds were collected separately and were used for study. Seeds were stored in wax paper envelops at room temperature.

3.2. **Seedlings**

Symptoms produced on chilli seedlings were studied under artificial conditions by raising seedlings in soil infested with *Alternaria alternata*, while proving pathogenicity and under natural conditions where seedlings were grown on seed bed.
3.2.1. *In Vitro*

Soil maize medium was prepared 100g in each 250 ml conical flask infested with *Alternaria alternata* and incubated at 25°C for 30 days. Culture of one flask was mixed with 1Kg sterile soil. Alluminium trays (51 x 34 x 1cm) were filled with such infested soil. Suitable control i.e. trays with sterile soil (uninfected) were maintained. Surface sterilized chilli seeds of Pusa Jwala were sown in the trays. One hundred seeds were sown in each row. The trays were irrigated every day after sowing (150ml/tray). After 10 days of sowing, the seedlings were covered with perforated polythene bags for 3 days. The symptoms on the trays were examined upto 30 days.

3.2.2. *In vivo*

Seed beds identified to be naturally infested during survey were used for the study. Surface sterilized seeds were sown and symptoms were observed at different growth stages and isolations were made from the plant parts.

3.3. Leaves

The leaf spot disease frequently occurred on chillies every year in the vicinity of Banda and Janshi region. Diseased leaves from different localities/varieties were collected and studied for the association of the fungus.

3.4 Fruits

Naturally infected fruits of different varieties and stages were collected and symptoms incited by *Alternaria alternata* were studied and isolations were made.
4. Isolation, purification, identification and maintenance of *Alternaria*.

On fruits and leaves exhibiting typical symptoms of the disease were collected from different locations and brought to the laboratory for isolation. The diseased plant parts were cut into small pieces and surface sterilization with 0.1 per cent mercuric chloride solution for 30 seconds, followed by three washing and then small disease parts put on 20 ml solidified PDA. The Petridishes were incubated at 25 ± 1°C. Isolations were made from surface sterilized plant parts. The fungus was subcultured, purified with fungal hyphal tip method and single spore method (Exekiel, 1930). The identification of the isolated fungus was done on the basis of morphological characters (Subramanian, 1971; Ellis, 1971).

5. Pathogenicity Test

Pathogenicity of the isolated *Alternaria alternata* was tested by three ways; (i) seed infestation, (ii) soil infestation, and (iii) fruit inoculation.

5.1. Standardization of time required for maximum conidial production

The standardization of duration required for maximum conidial production of *Alternaria alternata* was done prior to pathogenicity test. The fungus was grown on PDA in petridishes for 6, 8, 10, 12, 14, 16 and 18 days at 25°C. The Petridishes were inoculated with a 5mm culture disc. After completion of incubation periods, the observations on conidial production were recorded.

A 5mm culture disc was transferred to the test tube containing 10 ml of sterile water. The suspension was prepared by shaking the tube gently. The number of conidia per ml of suspension was determined with the
help of a haemocytometer. The incubation period required for the maximum conidial production was followed for multiplication of the inoculum.

5.2. Seed infestation technique

5.2.1. Eight day old culture of *Alternaria alternata* grown on potato dextrose agar medium was used. Seed samples free from natural infection of *A. alternata* were used in the test. The seeds were pretreated with 0.1% mercuric chloride solution for 60 seconds, washed thoroughly with sterile water and soaked in water for 120 minutes. Soaked seeds were rolled in actively grown culture of the test fungus. Ten such infested seeds were placed on moist blotters in petridishes. One hundred seeds were used. Seed without fungal infestation severed as control. Petridishes containing these seeds were incubated in the chamber and were observed for the development of disease symptoms after 7 days.

5.2.2. Seed infestation method was tried in another way, where the seed coat of pretreated chilli seeds was injured with the help of sterilized needle and then rolled on the actively grown culture of *A. alternata*. Seeds were placed on moist blotters as in 5.2.1. Seeds injured but without infestation were kept simultaneously as control. Observations on the disease development were made after seven days of incubation.

5.3. Soil-infestation method

Inoculum of *Alternaria alternata* was grown in 250 ml Erlenmeyer conical flasks each containing 100ml potato sucrose broth. After sterilization of the medium, 5mm disc of seven day old culture was transferred in each flask and incubated at room temperature 25 ± 1°C for 15 days. After incubation, mycelial mat was removed and washed thoroughly in sterile water to remove the traces of culture filtrate and mixed in sterile soil at the rate of the fungus of one flask per 200g. The
infested soil was thoroughly mixed and was filled in plastic pots (10 cm diameter). The fungus was allowed to grow for 10 days at room temperature. Ten surface sterilized seeds with no natural infection of *Alternaria alternata* were sown in each pot. Un-infested sterilized soil served as control. Observations were recorded on the development of the disease in seeds and seedlings. Isolations were made from the infected seeds and seedlings on PDA medium for the associated fungi.

5.4. **Fruit and leaf inoculation method**

Four methods were used to test the pathogenicity of the fungus on fruits (ripe and turning red stage) and leaves under laboratory and field conditions on intact and detached parts. Spore suspension was prepared by adding 10ml sterile water in 8 day old culture grown in test tube. The inoculated leaves and fruits were covered with belljar and humidity was maintained with the help of moist cotton placed inside the belljar. In field, inoculated fruits and leaves were covered with polythene bags with moist cotton.

5.4.1. **Pin prick method**

Ten pin heads (entomological needles) were mounted on cork covering 1 sq. cm area and five pricks were given on each intact and detached leaf and fruits prior to spray of spore suspension of the fungus.

5.4.2. **Spore suspension spray method**

Spore suspension of *Alternaria alternata* was sprayed with the help of atomizer on dorsal and ventral side of chilli leaves and in case of fruits entire surface was covered by spraying the spore suspension.

5.4.3. **Carborandum rub method**

Spore suspension spray method was modified by making injury with the help of carborandum and the spore suspension was sprayed with the help of an atomizer on detached and intact leaves and fruits.
5.4.4. Tooth prick method

Tooth prick with small bits of mycelial mat was inserted in the furits and observations were recorded on the symptom development.

The inoculated leaves and fruits were kept in sterile clean petridishes under laboratory conditions and provided with 12hr dark and 12 hr light periods at 25 ± 1°C. Humidity was maintained with the help of a wet-cotton piece placed with petridishes containing inoculated leaves/fruits under belljars. Under field conditions, temperature ranged from 20-30°C while humidity was 40-60%.

6. Detection of *Alternaria alternata* and other mycoflora associated with chilli seeds:


All the collected seed samples of chilli from different localities and varieties were tested by Standard Blotter Method (ISTA, 1985) for the mycoflora associated with chilli seeds. Three circular pieces of blotter papers of the size of petridish were cut and dipped in sterilized water, excess water was drained off and placed in each sterilized petridish. Twenty-five chilli seeds were placed in each petridish with the help of sterile forceps under sterile conditions in the inoculation chamber (16 seeds in outer circle, eight in the inner and one in the centre) so as to allow equal distance between seeds. The plated petridishes were kept for incubation in the growth chamber. Two hundred petridishes treated and untreated with 0.1% mercuric chloride for 30 seconds and then washed in three changes of sterile water. Petridishes were examined on the eighth day of incubation. Fungi were observed and identified by making slides and on the basis of colony and habit characters under stereoscopic binocular.
6.2. Naked eye examination

Seed samples of four selected seed samples were examined by naked eye and were classified into following groups:

- A = Bold apparently healthy seeds
- B = Deformed shrunked and brown seeds

The classified seeds were incubated on moist blotter as in Standard Blotter Method (ISTA, 1985) without any pretreatment.

7. Effectiveness of various methods in the detection of *Alternaria alternata* and other important fungi associated with chilli seeds:

The following methods were tested for the association of *Alternaria alternata* with four selected seed samples of chilli obtained from Jhansi, Hamirpur, Mahoba and Banda exhibiting the maximum natural infection of the fungus. Two hundred seeds of each sample were tested by each of the following methods:

(i) Standard Blotter Method (ISTA, 1985)
(ii) Standard Agar-plate Method (Modified Ulster method Muskett and Colhoun, 1948)
(iii) 2, 4-D Method (Neergaard, 1973)
(iv) Deep Freezing Blotter Method (Limonard, 1968)

7.1. Standard Blotter Method

Standard Blotter Method (ISTA, 1985) was used for the detection of mycoflora associated with four selected seed samples of chillies. Surface sterilized and unsterilized seeds were used. On the eight day of incubation the seeds were examined for the association of fungal flora. The details of the method is given in 6.1.
7.2. **Standard Agar plate Method**

In this method, potato dextrose agar medium was used, instead of previously used malt extract. In each sterilized petridish, ten pretreated seeds were placed with the help of sterilised forceps on 20 ml solidified PDA medium at equal distance, nine seeds in outer ring and one seed in the centre. Observations on the associated fungi were recorded on the fifth day of incubation with the help of making slides of growing fangi.

7.3. **2, 4-D Method**

7.3.1. **Chilli seed soaking method**

Sterilized petridishes were prepared as in Standard Blotter Method. The seeds were dipped in 2000 ppm of solution of 2, 4-D (Sodium salt of 2, 4-dichlorophenoxy acetic acid) in muslin cloth for 15 minutes, then 25 seeds were plated on moist blotter in each petridish and incubated for seven days in the incubation chamber. Observations were recorded on the associated mycoflora.

7.3.2. **Blotter dip method**

Three circular pieces of blotter, as used in Standard Blotter Method, were dipped in 2000 ppm solution of 2, 4-D and after dripping off the extra solution, these were placed in each sterile petridish. Twenty-five seeds were plated on the moist blotters. The seeds were examined for the presence of fungi associated after incubation period of seven days. In this method, sterile water was replaced by 2, 4-D solution to dip the blotters.

7.4. **Deep freezing blotter method**

The procedure for preparing petridishes (with moist Blotters and 25 seeds) was similar to the Standard Blotter Method. After 24 hr of incubation, the petridish containing seeds were transferred to the deep freezer at -7°C in complete darkness. Petridishes were taken out after 24
hr an kept again in the chamber for incubation. On the seventh day of incubation, the seeds were examined for the associated fungi.

8. Use of modified methods for the detection of *Alternatia alternata* associated with chilli seeds
8.1. Modification of Standard Blotter Method

Modification of Standard Blotter Method was tried by changing the initial hydrogen ion concentration (pH) of water used for wetting the blotters. Three levels of pH i.e. 6, 7 and 8 were adjusted with the help of 0.1 N NaOH and 0.1 NHCl.

8.2. Modification of Standard agar plate Method

Modification of agar plate method was tried by incorporating chilli seed extract in potato sucrose agar medium. Rest of the method was the same to that of agar plate method. Observations on the fungi associated with chilli seeds were recorded on fifth day of incubation.

8.3. Modification of Deep-freeze blotter method

Modification of deep freeze blotter method was tried in which the blotters were soaked in solution of antibiotics instead of plain sterile water. The blotters were dipped in 200 ppm solution of streptomyacin. Water without addition of antibiotics served as control. The seeds exhibiting the presence of *A. alternata* were recorded after seventh day of incubation.

9. Factors influencing growth and sporulation of *Alternaria alternata*
9.1. Effect on growth
9.1.1. Effect of media

In all ten media were used to find the influence on the growth and sporulation of *A. alternata* isolated from infected chilli fruits. The media used were Potato-dextrose agar (PDA), Rice-meal-agar (RMA), Corn-meal-agar (CMA), Soil-extract-agar (SEA), Chilli seed extract agar (CSE), Oatmeal-agar (OMA), Czapek's agar (CA), Asthana and Hawker's agar (AHA), Richard's agar (RA) and Martin's agar (MA).

Media were prepared and sterilized in an autoclave and 20ml lukewarm medium was poured into each sterilized petridish. 7mm discs from 8 day old culture of *A. alternata* were cut, one disc was placed in the centre of each petridish and incubated at 25°C. Colony growth was measured and cultural characters were recorded after 5th and 8th day of incubation. For determination of spore production, ten mm disc was cut with the help of cork borer and suspended in 5ml of water in a test tube. the disc was macerated and the suspension was used for counting the spores per microscopic field. Further desired calculations were made.

The procedure of testing was the same as the Standard Blotter method (ISTA, 1985). Observations on the association of *Alternaria alternata* were recorded on the eighth bay of incubation.

9.1.2. Effect of temperature

Petridishes containing PDA and inoculated with seven mm culture disc of *A. alternata* were incubated at 20, 25, 30 and 35°C. Observations were recorded on the colony character and sporulation.

9.1.3. Effect of hydrogen ion concentrations (pH)

Eight pH levels (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) were used to study the influence of pH on growth and sporulation of *A. alternata* on PDA. Seven mm disc was cut from 8 day old culture and placed in the centre of petridish. Petridishes were incubated at 25°C. Colony characters
were studied. Conidial pH was adjusted with the help of 0.1 NHCl and 0.1 N NaOH before sterilization.

9.2. Effect on spore germination

Cavity slide method was employed for studying the spore germination of *A. alternata* isolated from chilli fruits of variety Pusa Jwala. Conidial suspension was made in sterile water in culture tube and standardized to about 15 conidia under low power of compound microscope. One ml of the conidial suspension was placed in each of the three cavities of each slide. Four slides were kept for each treatment.

The slides were kept in moist chamber prepared by using petridishes provided with moist blotter and glass rods. The data on germination of spores were recorded.

9.2.1. Effect of temperature

With a view to see the effect of temperature on spore germination, the cavity slides with sores were kept in the Petridishes at 15, 22, 25, 27 and 30°C. The data on spore germination were recorded.

9.2.2. Effect of relative humidity

Four humidity levels (90, 75, 50 and 25) were used in the experiment. The humidity levels were maintained by using saturated solutions kept in the desicators.

- 90% Distilled water
- 70% NaCl + KCl equivalent parts
- 50% (NH₄NO₃ + KNO₃ equivalent parts)
- 40% KNO₂

The cavity slides were kept in the sterile desicators in which the different humidity levels were maintained. The spores were kept dry and placed at different relative humidities.
9.2.3. Effect of exogenous supply of suger

In order to find the effect of exogenous supply of carbohydrate, various concentrations of sucrose ranging 0.01, 0.25, 0.5, 1.0 and 1.5% were used. Distilled water, without sucrose served as control. The observations were recorded on spore germination upto 5 hours.

9.2.4. Effect of seed-exudates

Five gram of seeds of chilli of Pusa Jwala were soaked in 20 ml distilled water in 250ml Erlenmeyer conical flask and kept for germination at room temperature. After 5 days, the water was decanted and passed through sterile unit of Seitz filter. The filtrate was tested for its effect on germination of spores of *A. alternata*. The spore suspension was prepared in the filtrate. One drop of the suspension was placed on a cavity slide with the help of sterile pippette. After 5 hours, the final germination was recorded.

10. Transmission of *Alternaria alternata*

Transmission of the fungus on the host from one season to another season was studied.

10.1. Seed to plant

10.1.1. Role of seed-borne infection in causing diseases

Role of *A. alternata* in causing seed and seedling diseases was determined by the method recommended by Khare *et al.* (1977). In this method, 10ml hot plain water agar of one per cent strength was poured in each of the 100 rimless test tubes of 30ml capacity. Mouth of test tubes was closed with slightly loose cotton plugs and autoclaved. After solidification of the medium, one seed of sample No. 27 was placed in each tube with the help of sterile forcep on water agar. The tubes were plugged again and kept in growth chamber for incubation. The plugs of
the tubes were removed when seedlings reached the top. Observations were recorded on the seed infection and seedling infection due to *A. alternata* after 12 days.

10.1.2. Effect of seed-borne *A. alternata* was also determined by sowing the seeds in sterilized sand filled in sterilized plastic pots of 15 cm diameter. Observations were recorded on the infection on seed and seedlings. In each pot, 10 surface sterilized seeds were sown and observations were recorded after 20 days. The pots were provided alternate cycles of dark and light periods of 12hr. The infected seeds and seedlings were examined under microscope and isolations were made on PSA medium for the association of *A. alternata*.

10.1.3. **Location of *A. alternata* inside the chilli seeds (site of infection)**

10.1.3.1. **Seed component plating**

The method recommended by Sinha ans Khare (1977) was adopted to locate the site of infection.

10.1.3.2. Location of *A. alternata* was confirmed by histopathological studies after cutting the graded section of leaf and stem of chilli plant. Following steps were adopted for histopathological studies.

10.1.3.3. **Fixation of samples**

Samples infected due to the fungus were collected, cut into 5x5 and 5x2 mm with the help of a sharp knife and fixed in FAA solution (Formalin 40%-5ml, Acetic acid glacial-5ml and Ethyl alcohol 50%-90ml).

10.1.3.4. **Dehydration**

Fixed samples were washed thoroughly to remove the traces of FAA and then passed through the following series for one hour in each solution:
25% alcohol 50% alcohol 70% alcohol
90% alcohol absolute alcohol

10.1.3.5. Infiltration

Samples kept in absolute alcohol were passed through alcohol: xylene series for 1 hour in each solution.
25% xylene, 50% xylene, 70% xylene, pure xylene

10.1.3.6. Embedding

Parafin wax 58-60°C (E. Merck, Bombay was used for making blocks.

10.1.3.7. Sectioning

Prepared wax blocks containing infected samples were mounted on rider and sectioning was done by rotatory microtome (Spencer, USA). Transverse sections were cut of 15-25μ thickness. Cleam microslides were coated with Haupt's adhesive and ribbons were placed on it. Warming of these slides was done after flooding them with 4% Formalin solution for 3 minutes.

10.1.3.8. Staining

One set of slides were processed for mounting directly and another set of slides were passed through xylene Alcohol series and then alcohol: distilled water series (backward series) for 2 minutes in each series. Sections were stained in 0.5% safrarin aqueous solution for 5 minutes. Stained sections ware again passed through forward series (2 minutes in each) upto xylene series.

10.1.3.9. Mounting

Slides taken out from xylene pure were immediately mounted with 2 or 3 drops of Canda Balsam. Rectangular cover slip was placed before taking microscopic observations, the slides were kept in over (60°C) for overnight.
10.2. Plant to seed

10.2.1. Fruit Inoculation Technique

Fruits of different age of Pusa Jwala were inoculated with 3ml of spore suspension of *A. alternata* of 8 day old culture through hypodermic syringe (Needle No. 22) and covered with polythene bags containing wet cotton to provide moisture/humidity for 6 days, under field conditions. Sterile water was injected in fruits served as control. At maturity, seeds of such fruits were collected and plated on PDA medium after surface sterilization. Similarly, seeds of naturally infected fruits were collected and tested for association of *A. alternata* by blotter method as well as PDA method.

10.2.2. Infected plant parts like twig, stem, fruits and leaves were collected and observed for the association of *A. alternata* under microscope and isolations were made.

10.2.3. Naturally infected stem, twig and fruits were collected, initially checked under microscope for the association of the fungus and stored in paper envelops under room temperature for periodical observations and isolations were made for the associated fungus.

10.2.4. Naturally linfected twigs, stem and fruits were mixed and cut into small portions and buried in the field soil placed in the pots. Isolations from the small bits of those buried plant debris were made on PDA. Observations were recorded on the survival and viability of the fungus.

11. Influence of toxin produced by *Alternaria alternata* on the seed germination and root-shoot length
11.1. Preparation of culture filtrate

A bio-assay method based on the inhibition of root shoot elongation was planned (Das and Shrivastava, 1969). Toxicity was determined in terms of inhibition of seed germination and root/shoot elongation on chilli seedlings over check. Richard's medium was used as the basal medium and pretreated healthy chilli seeds having no natural infection of \textit{A. alternata} were used. In each 150ml Erlenmeyer flask, 50ml of the medium was taken and autoclaved. Five mm disc of culture was placed in each flask. Flasks were prepared for six incubation periods 5, 10, 15, 20, 25 and 30 days. Flasks were incubated at room temperature $28 \pm 1^\circ$C. After the incubation period the medium was filtered through muslin cloth, cotton pad followed by Seitz filter, to obtain a bacteria free culture filtrate. The crude culture filtrate (CF) was considered as 100%. Twenty-five pretreated seeds were placed in sterile petridishes containing 5, 10, 15, 20, 25 and 30 day old culture filtrate. The culture filtrate exhibiting maximum inhibition of chilli seed germination was further tested at different dilutions. Seeds were soaked in cultur filtrate for 3 hours and plated on blotter. Seed soaked in sterile water for the same period served as control.

The culture filtrate dilutions were prepared with sterile water. The observations on seed germination, root shoot length were recorded after 72hr. Thermal inactivation period was also determined.

11.2. Dilutions of culture filtrate

Various dilutions viz. 1:1, 1:2, 1:3, 1:4 and 1:5 of the culture filtrate of 15 day old were made with sterile distilled water. Three blotters were soaked and placed in each petridish. Seeds of Pusa Jwala were used after surface sterilization with mercuric chloride (1:1000) solution, followed by three washings of distilled sterile water to remove the traces
of mercuric chloride. Twenty-five seeds were plated in petridish as in Stabard Blotter Method (ISTA, 1985). Germination was recorded after 13 days of incubation. Blotters soaked in sterile Richard's medium corresponding dilutions with sterile water served as control following the method adopted by Meehan and Murphy (1947).

11.3. Determination of absorption time of toxic metabolite by chilli seeds

In order to know the time taken for absorption of toxic metabolite by seeds, the pertreated seeds of Pusa Jwala were soaked for 1, 2, 3, 6, 9 and 12 hr in culture filtrate of *A. alternata*. Immediately after soaking, the seeds were washed with distilled water three times so as to remove the excess of filtrate present on the surface of seeds. Twentyfive treated seeds were placed in each petridish containing three moist blotters. The petridishes were incubated for 7 days in growth chamber. Seeds soaked in Richard's medium for 1, 2, 3, 6, 9 and 12 hours as their respective control. Inhibition in germination indicated the presence of toxic metabolite in the seeds.

11.4. Influence of temperatures on the activity of toxic metabolite

Culture of *A. alternata* was grown on Richard's medium for 15 days at room temperature. Culture filtrate was sterilized by filtering it through Sietz filter, and diluted with distilled water (1:1). Fifty ml of this culture filtrate was transferred to 250ml Erlenmeyer flask. These flasks were placed in water bath at 30, 40, 50, 60, 70, 80 and 90°C for 10 minutes. After cooling, the volume of each flask was made upto 50ml (where required) with distilled water. Three blotters were soaked with such solution and placed in petridishes. Twenty-five seeds were placed in each petridish and incubated for 7 days in growth chamber. Reduction of
inhibition in chilli seed germination indicated the degradation of toxic metabolite. Four replications were kept.

12. Factors affecting disease development

12.1. Under field conditions

Observations were recorded on the development of fruit rot and twig infection and relation between temperature and humidity was worked out at Janshi and Banda conditions.

12.2. Under laboratory conditions

12.2.1. Influence of temperature and relative humidity on the disease development was determined under laboratory conditions. Fruits of Pusa Jwala and Jawahar Mirch 218 were brought to the laboratory and incubated with pin prick method. The inoculated fruits were exposed to 10, 15, 20, 25, 30 and 35°C and 50, 60, 70, 80, 90 and 100% relative humidity levels. Observations were recorded on the development of fruit rot at 10 and 15 days after inoculation. In each treatment 100 fruits were inoculated.

12.2.2. Influence of stage and various age of fruits on infection was determined by collecting the fruits of Pusa Jwala and Jawahar Mirch 218. Inoculation was done by pin prick method. The fruits were kept at 25°C. Observations were recorded after 15 days. Fruits of eight stages were collected. Green, green yellow (dominating green) I, green yellow (yellow dominating) II, complete yellow, yellow red (yellow dominating) I, yellow red (red dominating) II, complete red and full ripe red fruits of almost equal size and shaps were inoculated.
13. Estimation of capsaicin content in chilli fruits

Capsaicin content of naturally infected chilli fruits and healthy fruits was estimated by thin layer chromatography (TLC) method.

13.1. Preparation of TLC plates

Silicagel (30g) containing calcium sulphate as binder was mixed with 60ml of double distilled water. The slurry was poured into a TLC spreader adjusted to a thickness of 150m and on 6 glass plates of 20x20 cm. The plates were dried in an electric oven at 100-105°C for 30 minutes and stored in a desiccator.

13.1.1. Preparation of sample

Chilli fruits with natural infection of *A. alternata* were collected, oven dried and powdered. Dry powder of chilli (20 g) was used. Apparently helathy fruits were selected for control centrifuged for 5 minutes and the optical density of the remaining mixture was read on a spectrophotometer at 720mm.

13.1.2. Preparation of standard graph

To determine the amount of capsaicin, the spot from standard graph on concentration of pure vanillin against optical density was multiplied by a factor 2 to correct the difference in the molecular weight of capsaicin and vanillin. Different volume of a standard aqueous solution of vanillin reacted with Folin-Denis reagent and optical density was determined. A standard garph was drawn with the weight of vanillin against optical density.

Percentage of capsaicin in oleoresin was calculated as :

\[ \frac{(A \times 100)}{(10^6 \times 10 \times 0.01)} \times 100 \]

where,

\[ A = \text{Weight of capsaicin (Vanillin} x^2\text{)} \text{ in the spot} \]
14. Estimation of carotene

In a flask, two gram of dry powder of red fruit was placed and 50ml saturated n-butyl alcohol was added by a pipette and filtered through what man filter paper No. 42.

The optical density of filtrate was measured at 435.8 nm in spectrophotometer. The content of carotene was calculated and expressed in ppm.

\[
C = \frac{(5 \times \text{absorbance})}{(b \times K)}
\]

where,

\[
C = \text{Pigment for carotene}
\]

\[
b = \text{Cell thickness}
\]

\[
K = \text{Constant 0.16632 absorbativity (mg/lit.) for carotene at 435.8 nm in water saturated n-butyl alcohol.}
\]

15. Ascorbic acid estimation

One gram of fresh fruit was taken, sample was blended with 3% metaphosphoric acid (HPO₃) and volume was made upto 10ml with metaphosphoric acid dilution and filtered. One ml of aliquot was taken and titrated with dye solution till the change in colour from pink to colourless was there for 15 seconds. Ascorbic acid content was calculated as:

\[
\frac{(\text{Titration reading} \times 25 \times 100)}{\left(\text{Wt. of sample} \times 2.4 \times \text{Wt. of extract}\right)} \times 100
\]

16. Influence of A. alternata on growth parameters

Effect of fungal infection on various growth parameters of chilli plant was determined in four commonly available and grown varieties to know the extent of losses caused by A. alternata. Observations were recorded on the crop grown at IGFRI, Jhansi.
Observations on the number of fruits per plant, fruit length, fruit girth, fresh weight, plant height and yield were recorded in healthy and naturally infected chilli plants.

16.1. Chilli plant height

Height of normal healthy and diseased plants was measured from base to the top of the main stem with the help of a thread and then on the meter scale. The observations were recorded on 25 randomly selected healthy and diseased plants at 130 and 160 days.

16.2. Number of fruits per plant

Since the harvesting of chilli is irregular, the number of fruits per plant was calculated by adding the total number of fruits after four pickings of selected plants and dividing the summation with four.

16.3. Fruit length and girth

The length and girth of the diseased and healthy fruits was calculated by a thread from the joint of the calyx to the apex and then measuring it on a scale. Data were recorded on 25 fruits of each variety. Similarly, the girth was recorded.

16.4. Fresh weight

Weight of 25 healthy and diseased fruits was recorded.

17. Management of the disease:
17.1. Host resistance

Twenty-one varieties/lines of chilli were tested against fruit rot and twig infection due to A. alternata under natural conditions of IGFRI, Jhansi. Twenty plants of each variety were selected randomly, tagged and disease incidence was recorded on leaves, fruits and stem. Following grading was done:
<table>
<thead>
<tr>
<th>Numerical value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy</td>
</tr>
<tr>
<td>1</td>
<td>Leaf/fruit/twig Covered up to 5%</td>
</tr>
<tr>
<td>2</td>
<td>Leaf/fruit/twig Covered up to 6-25%</td>
</tr>
<tr>
<td>3</td>
<td>Leaf/fruit/twig Covered up to 26-50%</td>
</tr>
<tr>
<td>4</td>
<td>Leaf/fruit/twig Covered up to 51-100%</td>
</tr>
</tbody>
</table>

The disease index was calculated by using the following formula:

\[
\text{Disease Index} = \left( \frac{\text{Sum of numerical values}}{\text{Total No. of observations}} \right) \times \left( \frac{100}{4} \right)
\]

<table>
<thead>
<tr>
<th>Disease Index</th>
<th>Disease Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Disease Free (I)</td>
</tr>
<tr>
<td>1-5</td>
<td>Resistant (R)</td>
</tr>
<tr>
<td>6-25</td>
<td>Moderately Resistant (MR)</td>
</tr>
<tr>
<td>26-50</td>
<td>Susceptible (S)</td>
</tr>
<tr>
<td>51-100</td>
<td>Highly Susceptible (HS)</td>
</tr>
</tbody>
</table>

17.2. Evaluation of fungicides under laboratory conditions

Following fungicides in different concentrations were tried against *A. alternata*:

<table>
<thead>
<tr>
<th>SL.N.</th>
<th>Fungicide</th>
<th>Chemical Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Indofil M-45</td>
<td>Zinc ions+manganese ethylenebisdithio-carbamate</td>
</tr>
<tr>
<td>2.</td>
<td>Indofil Z-78</td>
<td>Zinc ethylenebisdithiocarbamate</td>
</tr>
<tr>
<td>3.</td>
<td>Fytolon</td>
<td>Copper oxychloride</td>
</tr>
<tr>
<td>4.</td>
<td>Difolatan</td>
<td>N-1,1,2,2 Tetrachloro ethyltho-cis -4-cyclohexane -1, 2-dicorboximide</td>
</tr>
<tr>
<td>5.</td>
<td>Triforin</td>
<td>N,N-bis (1-formamido-2,2,2-trichloro ethyl) – piperazine</td>
</tr>
<tr>
<td>6.</td>
<td>Captan</td>
<td>N-Trichloromethylthio -4-cyclohexane 1, 2dicarboximide</td>
</tr>
<tr>
<td>7.</td>
<td>Thiram</td>
<td>Tetramethyl –thiram disulphide</td>
</tr>
<tr>
<td>8.</td>
<td>Derosal</td>
<td>Methyl-2-Benzimidazole Carbae (Carbendazim)</td>
</tr>
</tbody>
</table>
17.2.1 *In vitro* evaluation

Poisoned food technique was used for the evaluation of eight commonly used fungicides. Fungicides were incorporated in PDA medium after sterilization and poured into presterilised petridishes. After solidification, 5mm disc of pure culture of *A. alternata* was placed in the centre of the poisoned medium. Petridishes were incubated at 25 ± 1°C. Observation were recorded on the radial growth and colony diameter after 5 and 8 day of incubation. In control, no fungicide was mixed with PDA. The discs were placed inverted to make contact of fungus with the medium.

17.2.2 Seed Treatment

Management of chilli disease incited by *Alternaria alternata* was tried by seed dressing fungicides. Seed Sample No. 27 obtained from *Pandurna* was used. The sample had maximum natural infection (31.0%) of the fungus as detected by Standard Blotter Method. Observations were recorded on the associated *A. alternata* after 8 days of incubation.

17.2.3 Fungicidal spray Trial

IGFRI, Jhansi form Five fungicides, Indofil M-45 (0.25%), Indofil Z-78 (0.25%), Fytelon (0.25%), Difolaton (0.25%) and Triforin (0.15%) were used as spray. Observations were recorded after three sprays starting from flowering to ripening and incidence of the fruit rot was recorded.