CHAPTER-IV

MATERIAL & METHODS
MATERIAL & METHODS

Rice plant is an important staple food grain, which is widely grown in India. It suffers greatly due to rot disease caused by *S. oryzae* causing considerable loss in yield. At present investigation plant transplanted in the agricultural field of Suriyawan block of Bhadohi district for the studies of different considerable aspect. Different required material and appropriate method related to the all considerable aspect that are taken in present investigation are discussed in sequencing order.

MATERIAL

The Pathogen

Although the disease incitant course in its perfect state *Leptosphaeria salvinii* (catt.) Krause and Webster, Sclerotial state *Sclerotium oryzae* catt. Conidial state *Helminthosporium sigmoideum* (Cav), but in present investigation *S. oryzae* catt. and its propagative and infective unit Sclerotia were used in all the experiments.

The collection of the disease specimens

In order to study the variability and pathogenicity of the stem rot fungus, and detect its virulent strains, and infected plants were collected from different parts of India which are given as follows.
Material and methods

TABLE (4.1) SOURCE OF DIFFERENT ISOLATES OF SCLEROTIUM ORYZAE CATT

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Place of collection</th>
<th>Date of collection</th>
<th>Designation of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IARI (Delhi)</td>
<td>October 1998</td>
<td>CO₁</td>
</tr>
<tr>
<td>2.</td>
<td>Kapurthala (Punjab)</td>
<td>October 1998</td>
<td>CO₂</td>
</tr>
<tr>
<td>3.</td>
<td>Kaul (Hariyana)</td>
<td>November 1998</td>
<td>CO₃</td>
</tr>
<tr>
<td>4.</td>
<td>Raipur</td>
<td>September 1998</td>
<td>CO₄</td>
</tr>
<tr>
<td>5.</td>
<td>Pantnagar (U.P.)</td>
<td>September 1998</td>
<td>CO₅</td>
</tr>
<tr>
<td>7.</td>
<td>Chandoli (U.P.)</td>
<td>November 1999</td>
<td>CO₇</td>
</tr>
<tr>
<td>8.</td>
<td>Sitapur (U.P.)</td>
<td>November 1999</td>
<td>CO₈</td>
</tr>
<tr>
<td>9.</td>
<td>Sonbhadra (U.P.)</td>
<td>November 2000</td>
<td>CO₉</td>
</tr>
<tr>
<td>10.</td>
<td>CRRJ (Orrisa)</td>
<td>October 2000</td>
<td>CO₁₀</td>
</tr>
</tbody>
</table>

Isolation of the pathogen

The infected stem from the above localities harboring the stem rot pathogen *S. oryzae* were washed thoroughly in sterile distilled water and cut in to small piece with the help of sterilized small scissor. These small pieces were then surface sterilized with 0.1% sodium hypochlorite (NaOCl) solution made in sterile distilled water for one minute and washed two time in sterile distilled water. The stem pieces, where the sclerotia were distinctly visible were split open by a sterilized scalped and the sclerotia were surface sterilized as mentioned above. The sclerotia were first separated from stem pieces and then transferred separately by a sterilized inoculating needle inside an aseptic inoculation chamber on sterilized potato dextrose agar.
Material and methods

(PDA) slants in test tubes and incubated at 28±2°C for fifteen days. Care was taken that one PDA slant received only one sclerotium stem piece (2mm) was also kept in PDA slant after surface sterilization.

Maintenance of the pathogen

The mycelial mat and sclerotia of the fungus were subcultured from pure culture tube on fresh PDA slants at 15 days interval and the isolates were maintained separately from sclerotia as well as stem pieces.

The above mentioned isolates were periodically tested on the susceptible rice host cv. Basmati seedlings (30 days old) planted on pots (20cm wide and 30cm depth) under controlled conditions in the net house and re isolated in order to maintain its pathogenic virulence status quo. Each time the culture was compared with its original type under the microscope. Unless otherwise mentioned, the Sonbhadra (CO9) strain was used in all the experiments.

Preparation of inoculums

For the purpose of mass inoculation in the field, the isolates were multiplied on sterilized solidified PDA medium in petridishes (100 mm diameter) under sterile conditions and incubated at 28±2°C for 30 days. For a massive collection of sclerotia, the sterilized cellophane discs were trapped over the PDA surface in the petridishes and the fungus was multiplied on it and incubated as above. When the sclerotia became black in colour, they were scraped out from the cellophane discs, air dried in filter paper and saved in dessicator containing CaC2 for the purpose of inoculation.
Material and methods

**Test rice variety**

Twelve rice variety, were used at the present investigation for study of stem rot resistant under field and pot condition. These were Basmati, Govind, Indrason, IR 36, IR 50, Jaya, Munshuri, Manhar, PantDhan 4, Ratna, Saket and Prasad.

**Surface sterilization of bulk seed**

The seed of all the verities were treated with 0.1 percent sodium hypochloride solution for 1 min and rinsed several times with sterile demineralised water were used, after testing by random sampling from the bulk for the seed born pathogen by agar plate method (Neergard & Saad 1962).

**Scoring System**

Although different scoring system have been suggested by Cralley (1936), Anon (1965, 1966), Chauhan et.al. (1968), Raina et.al. (1980) but in the present investigation the procedures adopted by Krause & Webster (1973) & IARI (1980) were followed.

1. Krause and Webster's system (1973) was used for mass scale observation in the field. In this system, healthy and infected tillers were divided in to five categories based on the amount of disease as follows.
   a. Healthy, no symptoms.
   b. Lightly infected with symptoms and sclerotia on the outer leaf sheath only.
Material and methods

c. Mildly infected with discoloration of and sclerotia in the inner leaf sheath culm green and healthy.

d. Moderately infected, slight to mild discoloration of the culm, interior of the culm healthy.

e. Severely infected, culms infected internally, either collapsed or not.

Each such category was weighted and the disease index (DI) was calculated as follows.

\[ DI = \frac{1(H^n) + 2(L^n) + 3(M^n) + 4(M*n^n) + 5(S^n)}{\text{Total number of tillers examined}} \]

Where

- \( H^n \) = Number of healthy tillers
- \( L^n \) = Number of lightly infected tillers
- \( M^n \) = Number of mildly infected tillers
- \( M*n^n \) = Number of moderately infected tillers
- \( S^n \) = Number of severely infected tillers

In this system, which seems to be a proper and reliable valuation of the stem rot disease, DI= represents all healthy tillers and DI=5 represents all severely infected tillers.

Recently, IARI (1980) developed a Standard Evaluation System (SES) on the basis of dark lesions on the stem near water line, small dark sclerotial bodies on or inside the stem and lodging due to stem rotting. In SES, a 0-9 scale was introduced to measure the disease incidence as follows.
Material and methods

O = No incidence
1 = Less than 1%
3 = 1 5%
5 = 6 25%
7 = 26 50%
9 = 51 100%

This system was used for recording the disease incidence on small number of plants either in pots or in the field condition.

The chemicals

The following ‘analar’ grade chemicals were used during the course of investigation.

(A). TABLE(4.2) THE SALTS (FOR NUTRITION OF ELEMENTS).

<table>
<thead>
<tr>
<th>Name</th>
<th>Empirical formula</th>
<th>Available element</th>
<th>Concentration of element in nutrient solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>NH₄NO₃</td>
<td>N</td>
<td>*</td>
</tr>
<tr>
<td>Sodium dihydrogen</td>
<td>NaH₂PO₄. 2H₂O</td>
<td>P</td>
<td>*</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>K</td>
<td>*</td>
</tr>
<tr>
<td>Magnesium Sulphate</td>
<td>MgSO₄. 7H₂O</td>
<td>Mg</td>
<td>*</td>
</tr>
<tr>
<td>Barium sulphate</td>
<td>BaSO₄</td>
<td>S</td>
<td>*</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>CaCl₂</td>
<td>Ca</td>
<td>*</td>
</tr>
<tr>
<td>Manganese Chloride</td>
<td>MnCl₂. 4H₂O</td>
<td>Mn</td>
<td>*</td>
</tr>
<tr>
<td>Sodium tetraborate</td>
<td>Na₂B₄O₇. 10H₂O</td>
<td>B</td>
<td>*</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>(NH₄)₂MoO₄. 7H₂O</td>
<td>Mo</td>
<td>*</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>CuSO₄</td>
<td>Cu</td>
<td>**</td>
</tr>
<tr>
<td>Ferric Chloride</td>
<td>FeCl₃</td>
<td>Fe</td>
<td>**</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>ZnSO₄</td>
<td>Zn</td>
<td>**</td>
</tr>
<tr>
<td>Sodium silicate</td>
<td>Na₂SiO₃. 5H₂O</td>
<td>Si</td>
<td>**</td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td>CoCl₂. 6H₂O</td>
<td>Co</td>
<td>**</td>
</tr>
<tr>
<td>Nickel</td>
<td>NiCl₂. 6H₂O</td>
<td>Ni</td>
<td>**</td>
</tr>
<tr>
<td>Stannous chloride</td>
<td>SnCl₂</td>
<td>Sn</td>
<td>**</td>
</tr>
</tbody>
</table>

Note (*)The compounds containing N, P, K, Mg, & S were used as the rate of 1,2,3,4,5,6,7,8,9, & 10mg/liter
Material and methods

(**) The compounds containing Ca, Mn, B, Mo, Cu, Fe, Zn, Si, Co, Ni & Sn were used at the rate of 1, 2, 3, 4, 5, 6, 7, 8, 9, & 10 ppm/liter.

(B) TABLE (4.3): THE AGROCHEMICALS (FOR CONTROL OF STEM ROT) LIST OF FUNGICIDES (TRADE NAME) THEIR CHEMICAL NAMES AND SOURCES.

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Commonly used as</th>
<th>Chemical Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bavistine 50 WP</td>
<td>Fungicide</td>
<td>Methyl 2 benzimidazole carbamate</td>
<td>BASF India Ltd., Bombay</td>
</tr>
<tr>
<td>Blitox 50 WP</td>
<td>Fungicide</td>
<td>Copper oxychloride</td>
<td>Rallis India Ltd., Bombay</td>
</tr>
<tr>
<td>Brassicol 70 WP</td>
<td>Fungicide</td>
<td>Pentachloro nitrobenzene</td>
<td>F.I.D. Parry (India) Ltd., Madras</td>
</tr>
<tr>
<td>Beam 75 WP</td>
<td>Fungicide</td>
<td>5 Methyl 1, 2, 4 triazolo (3,4,b) benzothiazole</td>
<td>Indofil chemicals Ltd., Bombay</td>
</tr>
<tr>
<td>Capton 80 WP</td>
<td>Fungicide</td>
<td>N trichloro methyl thio 4 cyclohexane 1,Z di carboximide</td>
<td>Mysore Agrochemicals Co, Mangalore</td>
</tr>
<tr>
<td>Dithane M 45 80 WP</td>
<td>Fungicide</td>
<td>Zinc ion (2%) and manganese ethylene bisdithio carbamate</td>
<td>Indofil chemicals Ltd., Bombay</td>
</tr>
<tr>
<td>Dithane Z 78 75 WP</td>
<td>Fungicide</td>
<td>Zinc ethylene bis dithio carbamate</td>
<td>Indofil chemicals Ltd., Bombay</td>
</tr>
<tr>
<td>Du Ter 20 WP</td>
<td>Fungicide</td>
<td>Triphenyl tin hydroxide</td>
<td>N.V. Philips duphar amsterdom Holland</td>
</tr>
<tr>
<td>Emisan 6</td>
<td>Fungicide</td>
<td>Methoxyethyl mercury chloride</td>
<td>Excl Industries Ltd., Bombay</td>
</tr>
<tr>
<td>Fongorene 50 WP</td>
<td>Fungicide</td>
<td>1,2,5,6 tetrahydro pyrrol (3,2,1, ij) quinolin 4 one</td>
<td>Hindustan CIBA Geigy Ltd., Bombay</td>
</tr>
</tbody>
</table>
(C) TABLE 4.4: THE COMPOUNDS FOR INHIBITION OF MICROORGANISMS

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical formula</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium hypochlorite</td>
<td>NaOCl</td>
<td>For surface sterilization for isolation</td>
</tr>
<tr>
<td>Torgitol NPX</td>
<td>A nonyl phenyl poly ethylene glycol ether containing 10.5 moles of ethylene oxide</td>
<td>For suppressing the radial growth of rapidly growing fungi and helping the development of slow growing types.</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>(C21H39N7O12)2, (H2SO4)3</td>
<td>For inhibition of bacteria</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>C22H23ClN2O8</td>
<td>For inhibition of bacteria</td>
</tr>
<tr>
<td>Mercuric Chloride</td>
<td>HgCl2</td>
<td>For inhibition of fungal growth</td>
</tr>
</tbody>
</table>

(d) CONSTITUENTS OF DIFFERENT CULTURE MEDIA USED IN VARIOUS EXPERIMENTS

1) Potato dextrose agar
   Potato decoction 200g, dextrose 20.0g, agar 20.0g distilled water to make 1 litter.

2) Oat meal agar medium
   Oat meal 100g, agar 20g distilled water to make 1 litter.

3) Beef extract peptone agar medium.
   Beaf extract 3g, pepton 10g, agar 20g, distilled water 1 litter, pH 7.0.

4) Chitin agar medium
   collidal chitin 2g, agar 20g, distilled water 1 litter, pH 7.0.
Material and methods

(5) Czapek dox+yeast extract medium

agar 15.0g, Na No3 3.0g, K2 HPo4 1.0g, MgSo4 7H2O 0.5g, KCl 0.5g, FeSo4 7H2O 10.0g Sucrose 30.0g, yeast extract 1.0g, Distilled water 1000.0ml, pH 6.8 7.0.

Methods

(1). Physico-chemical analysis of soil:

For the physio-chemical properties of the polluted and unpolluted soil, following appropriate methods were adopted.

Moisture

Soil moisture was estimated at monthly interval and was determined by drying the soil at constant weight in an electric oven at 105°C for 48 hrs.

Mechanical composition

Pipette method was followed to determine the mechanical composition as described by Piper (1944).

Bulk density and percent porosity

Bulk density was determined by dividing the weight of soil by volume of soil and percent porosity by $2.6 \times \frac{D}{2.6}$ where, 2.6 is the absolute specific gravity of soil and D the bulk density.
**Material and methods**

**pH**
Electronic pH meter using a glass electrode at monthly interval measured soil pH.

**Organic carbon:**
The total organic carbon was estimated at monthly interval by rapid titration method as described by Piper (1944).

**Organic matter**
Organic matter was estimated at monthly interval by multiplying the organic carbon with a constant factor of, 7.2.

**Conductivity**
Digital electro conductivity meter with the help of an electrode measured the conductivity of the soil.

**Phosphorus**
Available phosphorus was estimated by chloro stannous reduced molybdo phosphoric blue method (Jackson 1965).

**Potassium and Sodium**
Ten gram of sieved soil was weighted and 50 ml of ammonium acetate was added to it and was shaken will, the solution was left for overnight and then filtered and 50 ml of ammonium acetate was again added to the soil residue. This solution was again shaken and kept for overnight
and then filtered and leachate was collected. The base concentration was read by systronic flame photometer. The concentration of potassium and sodium was calculated on the basis of standard calibration curve.

**Nitrate nitrogen**

Nitrate nitrogen was estimated by phenol-di-sulphonic acid method, Black (1965).

**(2). Morphological studies of causal organism**

Morphological characters of all ten isolates were compared growing them on PDA medium. Twenty ml. sterilized PDA was poured in each petriplates (9 cm. diameter) and allowed to solidity. Mycelial disc of 5 mm. diameters was cut from actively growing (3 4 days old) culture of the fungus and transferred aseptically in the centre of petriplate and incubated at $28\pm2$°C for 5 days. For each isolates three replications were maintained. Radial growth, colour of the colony, mycelial, sclerotial/conidial character were observed at the end of the incubation period. The size and width of sclerotial and conidia was studied and measured with the help of an ocular micrometer, (Each ocular division measuring 5.6 $\mu$) at 250 $\times$ magnification one hundred slides for each isolates were observed and their average value was presented nearest to the whole number in $m\mu$. 
Material and methods

(3). **PHYSIOLOGICAL STUDIES OF CAUSAL ORGANISM**

(a). **Pathogenicity test**

Twelve rice varieties namely Basmati, Govind, Indrasan, IR 36, IR 50, Jaya, Manshuri, Manhar, Pantdhan 4, Ratna, Saket and Prasad were selected for present study. These variety represented resistant susceptible groups as determined in earlier variety screening test both in pot and in the field under artificial infection with a mixture of ten isolates of *Sclerotium oryzae catt*. (Table ).

**Raising of Plants**

Seed were sown in pots with a diameter of 15 cm. and 25 days seedlings were transplanted in four meter rods rows with spacing of 22×22 cm. and with a basal fertilizers dose of 40 kg. N & 30 kg P₂O₅ per hectare. The same amount of N was again applied in two split doses during the growing season. Two week after flowering the stem were cut at the ground level and leaf sheath were removed fifteen to twenty cm. long piece of stem were cut. So that each stem piece had lower most elongated internode with a node at each end.

**Multiplication of inoculums**

Ten ml of PDA medium sterilized at 7kg/cm² pressure for 20 min was poured into 10 cm petridishes (sterilized at 180 C for 2 hr.) in an aseptic laminar air flow chamber. When cooled single sclerotium from each of the ten isolates (surface disinfested with 0.1% NaOCl for 1 minute) was transferred into separates plates in incubated at 28±2°C for 28 hr. Two mm.
Material and methods

agar block from the leading edge of colonies were scrapped by a flat sterilized needle for stem inoculations. Rice plants of 50 days were incubated by inserting one agar block (2 cm.) with embedded hyphal tip of *S. oryzae* inside the outer leaf sheath 10 cm. above the ground level (on the water line) without any injury to the plant. For each rice entry, ten plants were incubated with each isolated ear marked for separate disease scoring.

**Inoculation**

The cut stem pieces were washed with distilled water and were picked in the middle portion of internode with a bunch of ten inch long alpines. Three-week-old fungus cultured on soil PDA medium was placed on a sterile adsorbent cotton pad moistened with sterile distilled water and their stem pieces of a rice variety were placed over the inoculum in such a way that the wounded internodes came in close contact with the fungus. The wet cotton pad was carefully wrapped around so that the fungus was held in position around the wounded portion of the internode. The inoculated stem pieces were kept in side polythene bag, which were previously sprayed inside with distilled water for maintaining high humidity and incubated separately with each of the ten isolates of *S. oryzae*.

**Scoring infection**

The infection was scored on the extent of formation of sclerotia inside the internode. The length of the internodes showing infection and formation of sclerotia was measured. Evaluation of the degree of resistance or susceptibility and the grading of the variety were done on the basis of the
Material and methods

length of inter node bearing sclerotia. The reactions of the variety to various isolates were classified into five grades according to the scale given in (Table ).

**TABLE (4.5) GRADE OF RESISTANCE/SUSCEPTIBILITY OF RICE VARIETY**

<table>
<thead>
<tr>
<th>Length of stem Bearing Sclerotia (cm)</th>
<th>Grade of resistance/ Susceptibility</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-1.0</td>
<td>Resistance</td>
<td>R</td>
</tr>
<tr>
<td>1.1-3.0</td>
<td>Moderately resistant</td>
<td>MR</td>
</tr>
<tr>
<td>3.1-5.0</td>
<td>Moderately susceptible</td>
<td>MS</td>
</tr>
<tr>
<td>5.1-7.0</td>
<td>Susceptible</td>
<td>S</td>
</tr>
<tr>
<td>7.1 and above</td>
<td>Very Susceptible</td>
<td>VS</td>
</tr>
</tbody>
</table>

(b). Nutritional studies of the pathogen

Various macro elements such as N, P, K, Mg, S and microelements such as Fe, Ca, Zn, Mn, Co, Ni, Sn, Mo, Cu, Si, B were taken in the form of their well known available compounds mentioned previously. The macro elements were used at the rate of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/liter and the microelements were used at the same rates but in ppm/liter. The basal medium consisted of 20 g glucose/liter to which a particular concentration of an element mentioned above was added. Chemicals of analytical grads and Pyrea erlenmeyer flask of 250 ml capacity were used. Hundred ml, of the nutrient medium was taken in each flask was inoculated with single sclerotium of Bhadohi isolates (15 days olds) of the fungus cultured on PDA slants under aseptic condition and incubated for 15 days at 28±2°C under normal day/night cycles. Each treatment was tried in three replications.
Material and methods

After 15 days the mycelial mat was harvested on a pre weighed filter paper and vacuum suction was employed for filtration with the adhering moisture. The filter paper along with fungal mat was dried in hot air oven at 60° for 24 hr and transferred to desiccators for another 24 hrs until a constant weight could be obtained. The dry weight constants for every set in the three flasks was calculated according to the following formula as suggested by Taber and Taber (1970)

\[
M = \frac{3\sqrt{X_1} - 3\sqrt{X_0}}{t_1 - t_0}
\]

Where \( m \) is the dry weight content

\( X_1 \) is the weight at time \( t_1 \).

\( X_0 \) is the weight at time \( t_0 \).

The growth rate of fungus from the day of incubation up to the day of harvest for a particular concentration of the element was represented in growth units by growth rate unit curve.

(c). STUDY OF PARASITE RELATIONSHIP IN VARYING REGION OF ENVIRONMENT CONDITION:

(i). Temperature

Response of the 10 isolates of the pathogen to different temperature, were studied on PDA in three replicates in relation to growth. Mycelia dise (5 mm. diameter) of each isolates was aseptically placed in the centre of the petriplate having 20 ml, solidified medium. The petriplates were incubated at 15°, 20°, 25°, 30°, 35°, and 40°C. Radial growth of the colony was measured after 5 days and data were analyzed statistically (Panse and Sukhutme 1967).
Material and methods

(ii) Effect of PH

PDA was prepared and different pH namely 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were adjusted with the help of 0.1 N HCl and 0.1 N NaOH using BDH universal strip (Range 4.0 11.0). Twenty ml. sterilized PDA was poured in sterilized petriplates.* Mycelial dise (5 mm. diameter) of each isolates was aseptically placed in the center of the petriplates. Radial growth of colony was measured after 5 days and statistically analyzed.

(d). Mode of infection and disease development

To study the developmental stages of infection process steam of the healthy, highly susceptible cultivar Bashmati variety were used. The stems were surface sterilized with 0.1% mercuric chloride, washed with sterile distilled water thrice and were inoculated with single sclerotium at the middle of inter node. The inoculated stem pieces were placed in humid chamber and incubated at 28±2°C. Samples were taken at an intervals of 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64, 68 and 72 hrs killed and fixed in FAA solution (formaldehyde 37.40%) 10ml, ethyl alcohol (95%) 50 ml, glacial acetic acid 5 ml and distilled water 35 ml. Whole mount method was employed to study the specific stage (S) of the infection. The peelings were taken from the inoculated portion collected at different intervals and were passed through 50%, 30% alcohol followed by the distilled water, stained in 0.1% aqueous aniline blue for 2 minutes, dehydrated through 30%, 50%, 70%, 90% and absolute alcohol by keeping them for 2 minutes in each grade. The peelings were cleared in xylene alcohol series i.e. 1:3, 1:1, 3:1 pure xylene and finally mounted in Canada balsam. Microphotographs of different developmental stage were taken.
Material and methods

(e). Survival of the pathogen in adverse condition of temperature and depth

Survivals of the fungus were studied under laboratory and field condition by exposing the infected stubbles at different temperature and depth in the soil.

(i). Survival of the fungus at different temperature

Infected rice stubbles were collected at the end of season and cut into pieces of approximately 2 cm. The pieces were kept at 10°C, 15°C, 20°C, 25°C, 30°C and 35°C at room temperature. The percent germination of sclerotia was recorded immediately after collection and determined subsequently by isolation of the fungus from the samples kept at each temperature at monthly interval till positive result were obtained. Sclerotia were drawn from each treatments surface sterilized with 0.1% mercuric chloride solution for 30-45 second, washed 2-3 times in sterile water and transferred aseptically to PDA plates containing 25 ppm, Diethen M 45+ Rose Bengal. Ten sclerotia were inoculated in each petriplate and incubated at 28±2°C for 48 hours. The germinated sclerotia were observed and counted under microscope. The percent germination was calculated for each treatment.

(ii). Survival of the fungus at different depth in soil

The survival of the fungus under field condition was studied keeping stubbles in nylon fine net and burying them in soil at the depth of 5 cm, 10 cm, 15 cm and at the soil surface. The percent germination of sclerotia was observed in each treatment by the appropriate method at monthly interval and compared with the initial percent germination.
(4). **SOIL AMENDMENTS IN RELATION TO THE DISEASE**

(a). **Application of chemical fertilizers N.P. and K**

Highly susceptible variety Basmati, were sown in raised nursery beds and 30 days old seedling were transplanted in pots containing 10 kg of field soil at the rate of three seedling per pots sixty four treatment combination of N.P. and K (0.80, 160 and 240 ppm of NX 0.60, 120 and 180 ppm of PX 0.60, 120 and 180 ppm of K) were included in the experiments. Each treatment was replicates three times. The doses of P and K was applied at the time of transplanting, whereas N in the form of ammonium sulphate was applied in two common doses 75% at transplanting and 25% after five weeks. The plants were inoculated with the stem rots fungus based on the appropriate method. Three weeks old stem rot fungus cultured on soil, oats meal medium was used for inoculation. The plants were inoculated by placing the inoculums at the base of the plant and in between the tiller, which were pricked with a bunch of ten one inch long alpine. The inoculation, were carried out twice during the season i.e. at the tillering and stem elongation stages observation on the incidence of stem rot were recorded at the time of harvest on maturity by examining all the tiller after splitting them open.

The total number of healthy and infected tiller in each plant were recorded and the percentage of infection was calculated. Finally the mean percentage of infection in each treatment was worked out. The percentage of infection was transformed into angle \( \sin^{-1} \sqrt{\% \text{ infection}} \).
Material and methods

(b). **Application of green and organic manuring:**

A pot experiments examined the effect of green and organic manuring for the study of percentage of infection of the test fungus *S. oryzae*. The experiments were done in pots with 3.0 kg soil in each pot. Soil artificially infected with sclerotia of *S. oryzae* at 5 sclerotia/g of soil. For the study of green manuring fresh *Azolla bipinata* was used at control, 0.1%, 0.5% and 1.0% wt/wt in soil. For the study of organic manuring we take neem cake at a different concentration such as control, 0.1%, 0.5% and 1.0% wt/wt in soil. Uniform amount of water (100ml) was added every alternate day and left for decomposition for 15 days. 30 days old for seedlings of rice variety were transplanted in each pot result were noted in percentage of infection. Reading, were recorded every 10 days up to 80 days respectively.

(c). **Effect of soil moisture at various level**

For the study of soil moisture on percentage infection of disease development, different levels of moisture were maintained on oven dry basis of soil. All the moisture levels were kept constant by weighing each pot every day and adding the required quantity of water to maintained the original level of moisture. Like the other experiments observation were recorded at various moisture % level, 15, 20, 25, 30 and 40%. The percentage of infection, were calculated by appropriate method, which are described above. The sclerotia was thoroughly mixed with the soil in each of the pot before the incorporation and maintaination of the moisture in pots.
Material and methods

(d) Application of soil pH.

In this experiment the effect of soil pH on the percentage infection of disease was studied. Experiments were carried out in pots. Thoroughly washed pure quartz sand of 0.5 1.0 mm particles were used. The pots were filled with 1kg of sterilized quartz sand in each. The standard nutrient solution suggested by Hewitt (1952) was used and the pH of the solution was adjusted to desired level. 30 days old seedling of rice were transplanted in each pot. Results were noted in percentage of infection. Reading, were recorded two and half month and one week interval at pH 6.5 at 8, weak.

(5). QUANTITATIVE ANALYSIS OF OTHER SOIL MICROORGANISM AND THERE EFFECTS ON SCLEROTIAL MORTALITY.

The low land of rice field affected by stem rot was chosen to conduct the experiment. The stem rot sick plot was (10×10m), twenty five days old healthy seedling of Bashmati raised in the nursery were transplanted in the field with a spacing of 10×15 cm in the first week of August during Kharif from 1998 to 1999. During the end of season the moist soil samples were collected from this field as par the method previously describe first week of November every year for quantitative analysis of soil fungi, bacteria, actinomycetes and sclerotial population of *S.oryzae* by dilution plate technique method.

**Soil dilution technique**

This technique was modified from that of Maciejowski (1962). The waring blender along with lid was cleared thoroughly by treatment with boiling water. Three hundred ml of strile 0.1% water agar and 30.0g of soil samples collected from field were mixed, stirred thoroughly and passed
Material and methods

through 0.5 mesh, sieve to remove the debris and blended for one minutes. One ml of this soil suspension (1:10) was added to 99 ml of 0.1% sterile water agar and sterilized 125ml screw capped prescription bottles giving dilution of 1:10,000 ($10^4$) and 1:1,00,000 ($10^5$) respectively. On ml of dilution ($10^5$) was added to 9ml of 0.1% sterile water agar giving a dilution of 1:10,00,000 ($10^6$).

As suggested, by Tuite (1969) soil dilution of 1:10,000 ($10^4$). 1:1,00,000 ($10^5$) and 1:10,00,000 ($10^6$) were taken in respective suitable media for satisfactory identification of soil fungi, actinomycetes and bacteria respectively. The pH of media was adjusted to 7.0 in every cause.

**Estimation of other soil fungi**

For the estimation of other soil fungi the PDA medium with pH 7.0 was prepared as before and sterilized at 7kg/cm² pressure for 20 min. After autoclaving 20ml of the above selected dilution ($10^4$), 1,000 ppm. of nonionic surfactant torgitol NPX, 35ppm of both streptomycin sulfate and aureomycin were added (after Glassman 1948; Hornby and Ultrup 1967; Stien and Watson 1965 a, b) to 250ml PDA medium at 45°C thoroughly stirred manually and equally divided and spread over 10 sterilized petridishes (100m diameter).

The petridishes were incubated at 28±°C for 120 hr. The different fungal colonies/plate were counted and identified under the microscope. The number of fungal colonies that spread were averaged and multiplied by the dilution factor to give the number of colonies/g soil.
Material and methods

The percentage of each fungal colony was determined as follows

\[
\text{% of colonization} = \frac{\text{No of colonies of particular fungus species}}{\text{Total No of all fungal colonies}} \times 100
\]

Estimation of bacteria

The phenomenon for estimation of bacteria was the same as that of fungi, but instead of PDA (incorporated with bacterial and fungal suppressants), only the beef extract peptone agar medium (nutrient agar) with the following composition and selective soil dilution of 1:10,00,000 (10^6) were used (Tuite, 1969).

The different bacterial colony character were observed and identified in each petridish along with the very few fungi and actinomycetes. The average of all kind of colonies was determined and was multiplied by the dilution factor to give their number/g soil. The percentage of each bacterial colonies was determined by using the following formula.

\[
\text{% of colonization} = \frac{\text{No. of colonies of a particular bacterium (species)}}{\text{Total No. of all colonies}} \times 100
\]

Estimation of Actinomycetes

The procedure for estimations of actinomycetes was same as that of fungi, but their the selected soil dilution of 1:1,00,000 (10^5) was added to the only Chitin agar medium having the following composition (Lingappa and Lockwood, 1960, 1962).
Material and methods

**Preparation of colloidal Chitin**

For the preparation of colloidal chitin, it was washed five times alternately for 24hr. at a time with 1N NaOH and 1N HCl solution and then four time with 95% ethanol. This process removed nearly 40% of the original material and a white product was obtained. Then 15g of cleaned chitin was moistened with acetone and dissolved in 100ml of cold conc HCl stirring for 20 min in an ice broth. The thick syrupy solution thus obtained was filtered into 2 liters of stirred, cold distilled water by passing through thin glass wool pad in a Buckner funnel. The colloidal suspension thus obtained was sediment by allowing it to sand and yielded colloidal chitin, which was washed thoroughly with distilled water several time and stored in a refrigerator for use.

After incubation as before the actinomycetes colonies in each petridish were identified and counted along with very few fungi and bacteria. Its average numbers were calculated per gram of soil and the colonization percentage of actinomycetes was determined as follow.

\[
\text{\% of colonization of actinomycetes} = \frac{\text{No. of colonies of actinomycetes}}{\text{Total No. of all colonies}} \times 100
\]

**Isolation of sclerotia of *S. oryzae* from soil and its motality**

The sclerotia were isolated from soil by wet sieving method as mentioned previously, its number was determined per gram of soil and subject to study of germination. The mortality population/g soil was find out on the basis of non germinated sclerotia as follow

\[
\text{Mortality \% of sclerotia} = \frac{\text{Non germinated sclerotia}}{\text{Total sclerotia tested}} \times 100
\]
Material and methods

(6). EFFECT OF SOIL POLLUTANTS ON TEST PATHOGEN

(i). Effect of pollutants on colony growth of *Sclerotium oryzae* and some dominant rhizosphere fungi.

(a). Raw sludge treatment

Raw sludge was mixed in Czapek dox+ yeast extract medium at the concentration of 1000 μg/ml, 1500 μg/ml, 2000 μg/ml, and 2500 μg/ml separately and then the medium was autoclaved, medium without raw sludge serves as a control.

(b). Ammonia gas treatment

The test pathogen of the present investigation was fumigated with a mixture of air and ammonia gas in the exposure chamber. The dominant rhizosphere fungi and test pathogen were exposed to a known concentration of pollutant in this chamber. Continuous circulation of air gas mixture keeps in the internal environment of the chamber equal to the ambient level. The gas produced in this chamber was 5.0 μg/cm.

5 mm discs of the test fungi were centrally placed in petriplates containing czapak dox + yeast extract medium. The lid of the plates was removed and inoculated bottom plates were exposed to the gas in chamber for 10, 20 and 30 minute.

(c). Cement dust treatment

Cement dust was mixed in czaek dox + yeast extract medium at the concentration of 500 μg/ml, 1000 μg/ml, 1500 μg/ml, 2000 μg/ml, and 2500 μg/ml separately and then the medium was autoclaved. Medium without cement dust serve as control.
Material and methods

5mm disks cut from actively growing margins of the respective fungi were inoculated in petriplates containing sterilized cooled czapek dox+ cement dust and only with czapek dox + yeast extract medium the plate were then incubated at 25±2°C to determine the growth of the test fungi. The radial growth of the test fungi were measured after 24 hrs of incubation for 3 days. The radial growth was recorded by the method describe above.

(II) Effect of pollutant on germination on sclerotia of the test pathogen and some dominant rhizosphere fungi

(a). Raw sludge treatment

Raw sludge was mixed in liquid czapek dox medium in different proportion as described under the experiment 1. The sclerotial germination was recorded by following formula.

\[
\text{% inhibition of sclerotial germination} = \frac{\text{Number of sclerotia germinated in control} - \text{Number of sclerotia germinated in treatment}}{\text{Number of sclerotia germinated in control}} \times 100
\]

(b). Ammonia gas treatment

Czapek dox liquid medium were exposed in the gas chamber as mentioned under experiment 1. The treated liquid medium was distributed in cavity slide placed in sterilized petriplates. 0.1 ml of the sclerotial suspension of the test fungi and the test pathogen was added to each cavity slide and incubated at 25±2°C. After 24 hrs the sclerotia with treated
Material and methods

Medium were mounted on clean slide and present inhibition of sclerotial germination as mentioned above.

(c). Cement dust treatment

Cement dust was mixed with liquid czepek dox medium in different proportion as mentioned under experiment 1. The treated liquid medium was distributed in cavity slides placed, in sterilized petriplates 0.1 ml of the conidial suspension of the test fungi and test pathogen was added to each cavity slides and incubated at 25±2°C. After 24 hrs, the sclerotia with sclerotia treated medium were mounted and percent inhibition was calculated as described above.

Localities for pollutant soil samples

The soil collected from the localities and the physico-chemical properties mentioned in the beginning of the thesis in chapter material and method. For specific pollutant soil samples are polluted due to application of the under mentioned substances in agricultural field of the following respective localities.

Phoolpur

There is a factory namely Phoolpur fertilizers Ltd. which discharge effluent which contain chiefly ammonium hydroxide (90 105 mg/l) ammonium chloride (fixed) (35 45 mg/l), sodium chloride (450 mg/l) total dissolved solid (more than 1000 1200 mg/l) total suspended solid (about 20 mg/l) and oil and grease (2 3 mg/l) (personal communication).

The agricultural field of this localities are irrigated with this effluents, thus ammonium hydro oxide polluted soil mentioned any where in the thesis does not implies the ammonia hydro oxide only also other content mentioned above.
Material and methods

Bhadohi

Raw sewage from Bhadohi city is discharged in agricultural field of this, localities at irregular interval and used in agricultural field.

Dala Cement Factory

Cement dust emitted continuously in the atmosphere from Dala cement factory and deposited over soil surface in the vicinity of this factory.

Statistical analysis of data

The data collected during present investigation was subjected to F test and analysis of variance and mentioned separately the chapter statistical analysis.

(7) Germination of sclerotia in the leaf exudates of twelve rice varieties

Pathogenicity test of twelve rice varieties Basmati, Govind, Indrason, IR 36, Ir 50 Jaya, Manshuri, Manhar, Pantdhan 4, Ratna, Saket, and Prasad was conducted previously and varieties were identified resistant, moderately resistant, moderately susceptible, susceptible and very susceptible according to the disease response to different isolates. The result and condition of different isolates against the disease response are cited in chapter “Physiological study of causal organism” under the heading pathogenicity test of causal organism.

For collection of leaf exudates the seedlings of each variety were developed in separate earthen pots (10 kg capacity) filling with soil from the field. Pots having two weeks old seedlings were brought to glass chamber, flooded water and kept over night. Leaf exudates were collected from individual seedlings leaf tip early in the morning (6.00 a.m.) by means of dropper in small tubes (5 ml). The tube with cotton plugs, were kept in the
Material and methods

refrigerator immediately for different analysis. Three replications were used for collecting exudates from each variety. The total sugar different exudate was determined by using anthrone as the analytical reagent and taking reading in spectrophotometer at 660 nm (Beroad 1975). The detection of amino acid was done by descending paper chromatogram method using whatmann no 1 filter paper and comparing the Rf values with standard amino acid set (Block et al 1958).

The sclerotia of different isolates were germinated in exudates and slides were prepared with distilled sterile water (control) in separates groove slides. These slides were kept for four hour in moist chamber made of petridishes with moist blotting paper lining the inner sides of either plate at room temperature (25°C) during daytime for germination of spores.

(8). APPLICATION OF FUNGICIDES

(a). In Vitro studies

Twelve fungicides namely Bavistin, Beam, Blitox 50, Brassical, Capton, Dethan M 45, Diethan Z.78, Due Ter, Emisan, Fongorene, Thiram and Topsin m, were tested against the fungus by poisoned food technique (None & Thapliyal 1979). Desired concentration of 25, 50 and 100 PPM were screened against the fungus. The common and chemical names and sources of availability of these fungicides have been given in materials and method chapter table( ). The required amount of each fungicides was throughly mixed in melted, cooled medium and 20 ml of medium was
Material and methods

poured in each petriplate. Medium without chemical served as control. Mycelial disc of 5 mm diameter from actively growing culture was transformed at the, centre of each petriplate aseptically. The petriplates were incubated at 28±2°C for 5 days. The radial growth was recorded in each treatment and data were analyzed statistically. Table ( )

Effect of combination of fungicides on growth of the fungus

Two fungicides, Dithane M 45 and Dithane Z 78 were not inhibitory to the fungus at 25 and 50 ppm. Therefore these were selected for further listing against the fungus by poisoned food technique in the following combination.

1. Dithane M 45 25 and 50 ppm.
2. Dithane Z 78 25 and 50 ppm.
4. Control Basal Medium

After incorporating chemicals in these combinations in the medium a mycelial disc of 5 mm was inoculated in petriplate and incubated for 5 days at 28±2°C. The radial growth was measured. The petriplates were exposed in the air for an hour so that common contamination may have access and grow. These were recorded.

The same combinations of fungicides were further tested to observe the recovery of the sclerotia after mixing them in sterilized and un sterilized soils. The soil was air dried powered and sieved though the 50 mesh sieve. It was sterilized at 105 kg/em² for 45 minutes on two consecutive days.
Material and methods

Five mg sclerotia were mixed in one hundred mg sterilized and unsterilized soils separately. Twenty mg soil mixed with sclerotia was spread on solidified medium incorporated with chemicals. A control was maintained for comparison. A set of three petriplates was maintained for each treatment. Petriplates were incubated at 28±20 for 48 hours. The terminated sclerotia contaminants in were rewarded and data analyzed statistically in Table ( ).

(b). In Vivo Studies

The field experiment for fungicides control of stem rot disease was conducted, with highly rot susceptible cultivar Bashmati for two consecutive kharif season (1999 2000) by using five fungicides found most effective in vitro condition namely Bavistin, Topsin M, Diethan Z–78, Brassicol and Imison. The field was laid out in a randomised block design with three replication each with a plots of 5×5n. and 6 treatments including control (fig ).

The seed of Bashmati varieties were shown in the nursery after surface sterilization and twenty five days old healthy seedling were transplanted plots uniformly for all the treatments with a spacing of 10×15 cm. Total amount of 150 kg N, 80 kg P2O5 and 60 kg K2O was used during growing season in each year. Since the disease severity under natural condition was not adequate, attempts were taken to inoculate the plant artificially. The sclerotia multiplied in the laboratory were just heated on the surface of water in each plots at the rate of 5g/plot and helped to increase the inoculation potential and disease syndrome. The solutions (0.2%) of the fungicides were sprayed at the rate of 1000 liter of spray fluid/ha \(^1\) in three