# Material and Methods

## CHAPTER III

# MATERIALS AND METHODS

The materials and methods used in the present investigations are described below.

### 3.1: Pathological Investigations

#### 3.1.1: Collection of healthy and diseased earheads of sorghum

The diseased and healthy earheads/grain samples of widely cultivated sorghum varieties *viz*., Phule Yashoda, Phule Mauli, CSH-9 and Maldandi were collected in Kharif season in brown paper bags during grain development stages and after harvest from farmer's fields. From each of the districts *viz*., Ahmednagar, Solapur, Latur, Beed, Satara, Sangli, Kolhapur and Pune infected earhead samples were collected. Then variety wise grains were manually separated carefully from each of the earheads. All the grains separated from earheads, irrespective of location of earhead collection, were thoroughly mixed together taking variety into consideration in order to have a composite grain sample.

#### 3.1.2: Symptoms and signs observed on grains in nature due to infection of various pathogens

The visual and microscopic examination of infected grain samples were carried out to study the abnormal, discolored, infected grains and the fungal bodies present within them.

To study the symptoms and signs present with the different diseased (infected) grains, critical visual observations were carried out followed by separation of grains from healthy and diseased sorghum earheads.The different infected grain lots of similar symptoms and signs were made by means of magnifying hand lens under artificial light as well as under stereoscopic microscope. The symptoms and signs appeared in field condition were recorded. The microscopic examination of such infected grains and endosperm, each was carried out and presence of pathogen was recorded. The hand sections of infected grains were made by cutting the infected grains and endosperm into

small pieces. The slides were prepared to examine them under the microscope to know the presence of pathogen in different parts of a grain.

### 3.1.3: Isolation of pathogens

Isolation of fungi associated with sorghum grains was carried out from randomly collected 400 grains of the composite grain samples by standard blotter method (ISTA, 1976).

#### 3.1.3.1: Isolation of ectophytic mycoflora

The infected sorghum grains having similar symptoms and signs of each variety were separated and different grain lots of similar symptoms and signs were prepared. From these lots repeated isolation were carried out by using twenty-five grains of each symptom lot. In each petriplate twenty-five grains having similar symptoms and signs were kept aseptically by maintaining equidistance from each other on three layers of properly moistened sterilized blotters. The plated petriplates were then incubated at room temperature ($27 \pm 2^0$C). The fungal hyphae developing from the infected grains were sub cultured on Potato Dextrose Agar (PDA) slants. The different isolates obtained from different grain lots were labelled by numbering. They were purified by employing single spore and hyphal tip isolation technique. Various cultures obtained were maintained on PDA slants for further study.

#### 3.1.3.2: Isolation of endophytic mycoflora

The infected sorghum grain lots or their infected tissues of endosperm having similar symptoms and signs were disinfected by dipping in 1: 1000 mercuric chloride ($HgCl_2$) solution for 2 minutes followed by three washings with sterile water to remove the $HgCl_2$ from each grain or their tissues used for isolation. Such twenty-five grains or grain tissues per petriplate were kept aseptically by maintaining equidistance from each other in petriplate containing three layers of properly moistened and sterilized blotters.

The plated petriplates were then incubated at room temperature ($27 \pm 2^0$C). The fungal hyphae developing from the infected grains were sub cultured on PDA slant. The different isolates obtained from different grain lots were labelled by numbering and were

purified by employing single spore and hyphal tip isolation technique. Various cultures obtained were maintained on PDA slants for further study.

### 3.2: Identification of pathogens

The pure cultures of different fungal isolates were grown on PDA medium. They were visually observed to record their cultural characters and under microscope to know their morphological characters (45X and 100X). The fungal characters observed were compared with the characters described in literature to identify the different fungal isolates obtained and were also sent to Agriculture College, Pune, (Mahatma Phule Krishi Vidyapeth) for identification and confirmation of pathogens.

### 3.3: Pathogenicity test

For proving the Koch's postulate, the pathogens isolated from sorghum grains were tested in the laboratory and in polyhouse conditions by adopting standard methods of pathogenicity test (Joi and Ahmed, 1976 and Bora and Gogoi, 1992). The pathogenicity test was carried out for *Fusarium moniliforme*, *Fusarium oxysporum*, *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum graminicola*. For artificial inoculation, the fungi were grown on PDA medium and spore suspensions were prepared from 10 days old culture, using sterile distilled water. After filtering through Watman filter No. 42, the concentration of spores was adjusted to $1 \times 10^5$ spores /ml. This spore suspension was used as inoculum to inoculate the surface sterilized grain. Apparently healthy grains of Phule Yashoda (grain lot without natural infection) were surface sterilized with 0.1 percent mercuric chloride solution for 2 minutes followed by three subsequent washings in sterile water to remove $HgCl_2$ from grains and then soaked in culture filtrate of respective fungus for 24 hours. Grain soaked in sterile distilled water served as a control treatment.

#### 3.3.1: Pathogenicity test in *in vitro*

The high quality transparent plastic petriplates were disinfected using alcohol. Three white sterile blotting papers moistened with sufficient quantity of sterilized water were kept in each petriplate. Ten inoculated seeds were then placed in each petriplates

containing sterilized moist blotting papers for each test pathogen seperately. The petriplates plated with uninoculated seeds served as a control treatment.

These labelled petriplates were incubated at room temperature ($27 \pm 2^0$C) for five days and when the seeds started germinating the lid of petriplates were opened in aseptic condition. The petriplates were watered with sterilized water as and when required.

Observations were recorded on seed germination after 7 days, discoloration of radical and plumule, seedling mortality if any and seedling length after 10 and 15 days of incubation at room temperature. Healthy emergence of the seedlings from seeds was considered as successful germination.

The tissue isolation from non germinated seeds and mortal (died) seedlings were carried out by employing standard isolation method described earlier (3.1.3).

**3.3.2: Pathogenicity test in *in vivo***

To confirm the pathogenic nature of *Fusarium moniliforme*, *Fusarium oxysporum*, *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum graminicola*, pathogenicity tests were carried out in polyhouse. The earthen pots were thoroughly washed with distilled water, disinfected with 4 per cent formaldehyde solution, sun dried for seven days and then were filled with double sterilized soil.

Then the grains inoculated with respective culture were placed in equidistance at 3-5 cm depth in each pot. The grains not inoculated with any isolate served as a control treatment. The pots were watered as and when required in polyhouse, adopting standard techniques of pathogenicity test.

Observations were recorded on seed germination after 7 days, discoloration of radical and plumule, seedling mortality if any and seedling length after 10, 15 and 20 days of sowing. Healthy emergence of the seedlings from the seeds was considered as successful germination.

The reisolation was carried out from non germinated seeds and died seedlings employing standard techniques described earlier for isolation (3.1.3).

### 3.4: Physiological Investigation

### 3.4.1: To find out the superior media for growth and sporulation of various pathogens

To find out the superior medium for better growth and sporulation of pathogens isolated, three synthetic and four semi-synthetic solid and broth medium with four replications for each test pathogen were used. The five major grain molds isolated *viz*., *Fusarium moniliforme*, *Fusarium oxysporum*, *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum graminicola* were studied.

**A] Semi-Synthetic Media**

**(1)** Potato Dextrose Agar (PDA)

| | |
|---|---|
| Peeled potatoes | : 200.0 g |
| Dextrose ($C_6H_{12}O_6$) | : 20.00 g |
| Agar agar | : 20.00 g |
| Distilled water | : 1000ml |

**(2)** Oat meal agar

| | |
|---|---|
| Oat (Flour) | :100.00 g |
| Agar agar | : 20.00 g |
| Distilled water | : 1000 ml |

**(3)** Maize meal agar

| | |
|---|---|
| Maize (Flour) | :100.00 g |
| Agar agar | : 20.00 g |
| Distilled water | : 1000 ml |

**(4)** Sorghum meal agar

| | |
|---|---|
| Sorghum (Flour) | :100.00 g |
| Agar agar | : 20.00 g |
| Distilled water | : 1000 ml |

**B] Synthetic Media**

**(1)** Czapek's (Dox) Agar (CzDA)

| | |
|---|---|
| Sucrose ($C_6H_{22}O_{11}$) | : 30.0 g |
| Sodium nitrate ($NaNO_3$) | : 2.00 g |
| Dipotassium hydrogen Orthophosphate ($K_2HPO_4$) | : 1.00 g |

Magnesium sulphate ($MgSO_4.7H_2O$) : 0.50 g

Potassium chloride (KCl) : 0.50 g

Ferrous sulphate ($FeSO_4$-$7H_2O$) : 0.01 g

Agar agar : 20.0 g

Distilled water : 1000.0 ml

**(2**) Richard's Agar (RA)

Potassium nitrate ($KNO_3$) : 10.0 g

Potassium dihydrogen Orthophosphate ($KH_2PO_4$) : 5.00 g

Magnesium sulphate ($MgSO_4$-$7H_2O$) : 2.50 g

Ferric chloride ($FeCl_3$-$6H_2O$) : 0.02 g

Sucrose ($C_6H_{22}O_{11}$) : 50.0 g

Agar agar : 20.0 g

Distilled water : 1000.0 ml

**(3)** Brown's medium (BA)

Dextrose ($C_6H_{12}O_6$) : 2.00 g

Asparagine : 2.00 g

Tribasic potassium phosphate ($K_3PO_4$) : 1.25 g

Magnesium sulphate ($MgSo_4.7H_2O$) : 0.75 g

Agar agar : 20.00 g

Distilled water : 1000.0 ml

The above described solid media were sterilized and 20 ml media was poured into previously sterilized petriplates and allowed them to cool at room temperature ($27 \pm 2^0$C). The culture block (5mm diameter) of test pathogens were cut aseptically with the help of sterilized cork borer (5mm) from 10 days old culture of *F. moniliforme*, *F. oxysporum*, *A. alternata*, *M. phaseolina* and *C. graminicola*. The petriplates were inoculated aseptically by placing culture block in the centre of petriplate separately for each test pathogen and labelled. The petriplates were incubated at room temperature ($27 \pm 2^0$C). There were three replications for recording the radial growth and fourth replication for recording sporulation in each treatment. The radial growth was measured daily up to full growth in any petriplate.

The sporulation count was recorded after 20 days of incubation from the fourth replication. Ten culture blocks of 5 mm diameter were suspended in 10 ml sterile distilled water. It was homogenized, filtered through muslin cloth and a drop from such filtrate was examined under the microscope for spore count. The number of conidia per microscopic field (10X) was recorded from four randomly selected microscopic fields for each test pathogen and sclerotia formation in case of *M. phaseolina*. The data generated for mycelial growth and sporulation were statistically analysed.

All solid media were used as broth media with same ingradients, only omitting solidifying agent agar-agar.

The 50 ml broth medium was poured into each 150ml sterilized conical flasks, plugged with non-absorbent cotton and autoclaved at 1.2 kg $cm^{-2}$ pressure for 20 minutes for sterilization. The flasks were then incubated at room temperature ($27 \pm 2^0C$). After 15 days of incubation mycelial mats were harvested on previously weighted, oven dried whatman's filter paper No. 42. The filter papers with mycelial mats were dried in an oven ($60 \pm 1^0C$) till constant weight was obtained. The dry weight of mycelium was recorded by deducting weight of filter papers. There were three replications for recording the mycelial dry weight and fourth for spore count.

The spore count was recorded from fourth replication after 20 days of incubation of each pathogen. The whole mycelial substrate was homogenized in 50 ml sterile distilled water with the help of homogenizer and was filtered through muslin cloth. A drop of suspension was examined under microscope. The number of conidia per microscopic field was recorded from four microscopic fields randomly selected in each case and sclerotia formation in case of *M. phaseolina*. The data generated for weight of mycelial mat and sporulation were statistically analysed.

**3.4.2: Effect of grain molds on germination of sorghum seed**

Impact of grain infecting fungi on seed germination and seedling vigour was studied by seed inoculation with fungi isolated from naturally infected sorghum grains.

Seed germination was carried out by Paper towel method (Sahu and Agarwal, 2003). Seedling Vigour Index (SVI) was determined on the basis of seed germination as

well as shoot and root length of seedlings. Vigour index was calculated by using following formula (Thippeswamy and Lokesh, 1997).

Seedling Vigour Index (SVI) = (mean root length + mean shoot length) × % of seed germination.

Healthy grains of Phule Yashoda variety were artificially inoculated with each of nine fungal species separately and mixture of nine fungi together. For artificial inoculation, healthy grains of Phule Yashoda were treated by soaking them for 24 hr into culture filtrate of respective fungus obtained from the 15 days old fungal culture grown on PDA medium at 27 ± $2^0$C. Then, these inoculated grains were used for seed germination and SVI study. Uninoculated grains served as control treatment for comparison.

**Fungal species used for artificial inoculation of grains**

i. *Fusarium moniliforme*
*ii.* *Fusarium oxysporum*
iii. *Colletotrichum graminicola*
*iv.* *Aspergillus niger*
*v.* *Aspergillus flavus*
*vi.* *Alternaria alternata*
*vii.* *Macrophomina phaseolina*
*viii.* *Curvularia lunata*
*ix.* *Phoma sorghina*
x. Mixture of mycoflora
xi. Untreated control

Fifty grains of respective treatment were placed on germination paper moistened with distilled water. Second sheet of germination paper was placed on first sheet followed by moistening it carefully. Both sheets were rolled along with wax coated paper and were incubated in seed germinator (27 ± $2^0$C) for seven days. At the end of incubation, rolled towel papers were carefully opened and germinated and not germinated seeds were counted from each of the treatments. Healthy emergence of the seedlings from the seeds

was considered as successful germination. Four replications each of 100 grains were maintained for each of the treatment.

### 3.5: Management of Sorghum Grain Molds

#### 3.5.1: *In vitro* screening of plant extracts against pathogenic fungi

The effect of phytoextracts of different plant species (Table-3.1) were tested *in vitro* by Poisoned Food Technique (PFT) to know their inhibitory effect on the growth of *Fusarium moniliforme*, *Fusarium oxysporum*, *Alternaria alternata*, *Macrophomina phaseolina* and *Colletotrichum graminicola*.

A healthy fresh plant part i.e., leaves, seed, bulbs and rhizomes were taken, washed thoroughly with fresh water and finally rinsed with sterilized distilled water. Fifty gram of plant parts were cut into small pieces and minced with the help of a grinder by adding 50 ml sterilized distilled water. The phytoextracts were filtered through double-layer muslin cloth in 150 ml conical flasks and plugged with non-absorbent cotton. These filtered extracts were autoclaved at 1.2 kg $cm^{-2}$ pressure for 20 minutes. Autoclaved extracts were individually added into previously sterilized PDA at 10 per cent (i.e. 2 ml extract + 18 ml PDA $plate^{-1}$) and mixed thoroughly at the time of pouring in the previously sterilized petriplates. The petriplates were inoculated aseptically after solidification by placing 5 mm diameter mycelial disc at the centre cut aseptically with cork borer from 15 days old pure culture of *F. moniliforme, F. oxysporum, C. graminicola, M. phaseolina* and *A. alternata*. Three repetitions of each treatment were maintained. The plate without phytoextract served as control. The petriplates were incubated at 27 $\pm$ $2^0$C temperature up to full mycelial growth in any treatment. The observations on radial mycelial growth were recorded by averaging two diameters of colony at right angles to one another and subtracting 5 mm of the mycelial discs. The Percent Growth Inhibition (PGI) was calculated by using the formula given by Vincent (1947).

$$PGI = \frac{(C-T)}{C} \times 100$$

Where,

PGI = Percent Growth Inhibition

C = Average diameter of mycelial colony of control (cm)

T = Average diameter of mycelial colony of treatement (cm)

Data obtained on percent growth inhibition were subjected to statistical analysis.

**Table 3.1: Evaluation of various botanicals against sorghum grain molds**

| Sr. No. | Common name | Botanical name | Plant part used | Conc. (%) |
|---|---|---|---|---|
| 1. | Tulsi | *Ocimum sanctum* | Leaves | 10 |
| 2. | Neem | *Azadirachta indica* | Seeds | 10 |
| 3. | Karanj | *Pongamia pinnata* | Leaves | 10 |
| 4. | Giripushpa | *Glyricidia maculate* | Leaves | 10 |
| 5. | Bougainvillea | *Bougainvillea spectabilis* | Leaves | 10 |
| 6. | Garlic | *Allium sativum* | Bulb | 10 |
| 7. | Ginger | *Zingiber officinale* | Rhizomes | 10 |

### 3.5.2: *In vitro* screening of antagonists against pathogenic fungi

Eight known antagonists viz., Trichoderma viride, Trichoderma harzianum, Trichoderma longibrachyatum, Aspergillus niger, Aspergillus flavus, Chaetomium globosum, Gliocladium virens and Bacillus subtilis (Table 3.2) were tested in in vitro to know their antagonistic effect against Fusarium moniliforme, Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina and Colletotrichum graminicola.

Three different methods which were employed in the study are as under:

**[A] Dual culture method (Dennis and Webster, 1971)**

The petriplates containing 20 ml PDA medium were inoculated aseptically with each pathogen seperately along with the test organism (antagonist) by placing 5 mm diameter culture blocks at 70 mm apart from each other. Three repetitions of each treatment were kept and the petriplates with only pathogen at centre served as control. All the plates were incubated at 27 $\pm$ $2^0$C temperature. Observations on colony diameter were recorded up to full growth of pathogen or test organism in any treatment. Percent Growth Inhibition (PGI) was worked out by using the formula given by Vincent (1947).

$$PGI = \frac{(C-T)}{C} \times 100$$

Where,

PGI = Percent Growth Inhibition
C = Average diameter of mycelial colony of control (mm)
T = Average diameter of mycelial colony of treatement (mm)

**[B] Pathogen at periphery (Asalmol *et al*., 1990)**

Mycelial discs of 5 mm diameter of the pathogens and the test organism (antagonist) were cut uniformly with the help of a cork borer and were inoculated aseptically by placing these culture blocks in petriplates containing 20 ml PDA. Four mycelial discs were kept radially 35 mm away from the centre of the petriplates for each test pathogen separately and one culture disc of the test organism (antagonist) was inoculated in the centre of the same petriplates simultaneously. Three repetitions of each treatment were kept and the petriplates with only pathogen at centre served as a control. Inoculated plates were incubated at 27 $\pm$ $2^0$C temperature and the radial growth were recorded after the complete coverage of plates with full growth of pathogen or test organism in any treatment. The Percent Growth Inhibition (PGI) was worked out by using the formula given by Vincent (1947) as mentioned in the section 3.5.2. (A) of this chapter.

**[C] Pathogen at centre (Asalmol *et al*., 1990)**

Each petriplate containing 20 ml PDA medium were inoculated aseptically by transferring a 5 mm diameter mycelial disc of each pathogen separately in the centre and four discs of the test organism (antagonist) were placed 35 mm away radially from the centre in the same petriplate simultaneously. Each treatment was repeated three times.

The plates inoculated in the centre with only pathogen served as control. The plates were incubated at 27 $\pm$ $2^0$C temperature. Observations on radial growth of the pathogen and the test organism were recorded after the complete coverage of plates with full growth of pathogen or test organism in any treatment. The Percent Growth Inhibition (PGI) was worked out by using the formula given by Vincent (1947) as mentioned in the section 3.5.2 (A) of this chapter.

**Table 3.2 Evaluation of various antagonists against sorghum grain molds**

| Sr. No. | Antagonist |
|---|---|
| 1. | *Trichoderma viride* |
| 2. | *Trichoderma harzianum* |
| 3. | *Trichoderma longibrachyatum* |
| 4. | *Aspergillus niger* |
| 5. | *Aspergillus flavus* |
| 6. | *Chaetomium globosum* |
| 7. | *Gliocladium virens* |
| 8. | *Bacillus subtilis* |

### 3.5.3: *In vitro* screening of fungicides by Poisoned Food Technique (PFT) against pathogenic fungi

Poison Food Technique (PFT) as per Nene and Thapliyal (1993) were employed to study the efficacy of various fungicides. The principle involved in PFT is to poison the nutrient medium with a fungicide toxicant and allow the test fungus to grow on it, finally record the extent of growth. Screening of promising fungicides against *Fusarium moniliforme, Fusarium oxysporum, Alternaria alternata, Macrophomina phaseolina* and *Colletotrichum graminicola* was done by PFT technique in *in vitro* (Table- 3.3).

All the glasswares used in the study were sterilized at $160^0$C for one hour in hot air oven. PDA medium 100 ml was distributed in 250 ml capacity Erlenmeyer flasks and sterilized at 15 psi for 15 minutes in an autoclave. To each flask, the weighed quantity of

test fungicide as per the desired concentration was mixed under aseptic conditions of Laminar Flow Bench and the mixture was homogenized thoroughly by gentle shaking and was poured in previously sterilized petriplate. Each fungicide was replicated thrice. The cultures of test fungi *viz.*, *A. alternata*, *F. monoliforme*, *C. graminicola, M. phaseolina* and *F. oxysporum* were grown on PDA for 10 days and the fungal discs of 5 mm diameter were cut with the help of sterilized cork borer and transferred aseptically in the center of each petriplate containing poisoned PDA medium. The medium without fungicide mixture and inoculated with fungal disc served as a control. The plates were incubated at 27 ± $2^0$C in incubator and observations for colony diameter were recorded daily at 24 hours interval up to full growth of the pathogen in any treatment.

Percent inhibition of growth was calculated by following formula of Vincent (1947).

$$PGI = \frac{(C-T)}{C} \times 100$$

Where,

PGI = Percent Growth Inhibition
C = Average diameter of mycelial colony of control (mm)
T = Average diameter of mycelial colony of treatement (mm)

Data obtained on percent growth inhibition were subjected to statistical analysis.

**Table-3.3: Systemic and non-systemic fungicides tested for their bio- efficacy against sorghum grain molds**

| Sr. No. | Technical name | Trade name | Conc | Chemical name | Manufacturers |
|---|---|---|---|---|---|
| 1. | Triadimefon | Bayleton 25 % WP | 0.1 | (1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1-2-triazole-yl)buton –2-one | Bayer Cropscience India Ltd, Mumbai |
| 2. | Difenconazole | Score 25 % EC | 0.1 | 1-((2-(2-chloro-4-(4-chlorophenoxy)phenyl)-4-methyl-1, 3-dioxolan-2-yl)methyl)-1H-1, 2, 4-triazole | Syngenta India Ltd., Mumbai |
| 3. | Chlorothalonil | Ishaan 75% WP | 0.1 | 2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile | Rallis India Ltd., Rallis House, Mumbai |
| 4. | Metalaxyl 8% + Mancozeb 64% | Master 72 % WP | 0.1 | N-(2-6-Dimethyl-Phenyl) N-( Methoxy-acetyl)-alanine methyl ester + Zinc ion and manganese ethylene bisdithiocarbamate | Rallis India Ltd., Rallis House, Mumbai |
| 5. | Carbendazim | Bavistin 50 % WP | 0.1 | Methyl-1-H-benzimidazole carbamate-2-yl carbomate | BASF India Ltd., Mumbai |
| 6. | Hexaconazole | Contaf plus 5 % SC | 0.1 | (RS)-2-$C_2$, 4 -dichlorophemyl-1- (14 -1, 2, 4 -triazole-1-41) hexan-2-01 | Rallis India Ltd., Rallis House, Mumbai |
| 7. | Propiconazole | Tilt 25 % EC | 0.1 | 1-[2-(2,4-dichlorophenyl) -4-propyl-1,3 dioxolan-2yl-methyl-1]-1H1,2,4 triazole | Syngenta India Ltd., Mumbai |
| 8. | Copper oxychloride | Blitox 50 % WP | 0.15 | 3Cu(OH)2CuCl2 | Rallis India Ltd., Rallis House, Mumbai |
| 9. | Control | - | | | |