Chapter III

Materials and Methods

STUDY AREA :-

SATARA is a district place, located at the foot of Sahyadri ranges toward wastern side. It has provided a very good site for botanical studies because of its peculiar topography and climate. Satara is situated at $16^0.50'$ to $18^0.10'$ N latitude and $73^0.45'$ to $75^0.0'$ E longitude at 696 meters area above mean sea level. Satara district covers 10,0962 sq. km. (Gazzatter of Satara district, 1991). The main hill features in the Satara tract are Ajinkyatara or Satara fort, Yewteshwer and Pateshwer. Satara district is placed at the cushions of the Sahyadri ranges along with *viz.* Mahabaleshwar, Kas and Koynanagar which are rich in vegetation. It provides the warm and moist conditions. These favourable climatic conditions and host availability is responsible for the growth of various types of saprophytic and parasitic fungi.

Rainfall:- In Satara during 2006 average annual rainfall was 1571.1 mm whereas in 2007 it was 1274.7 mm.

Temperature:- Maximum temperature recorded in 2006 was 37.9^0C at the Satara and in 2007 was 38.2^0C, whereas minimum temperature recorded in January 2006 was 12.5^0C and in January 2007 was 13.6^0C. The cold weather is during middle of November to end of February. January is the coldest month. (Meteorological Department of India, Satara.)

Humidity:- Maximum and Minimum humidity values recorded in the year 2006 were 98% and 42%, while in the year 2007 there were 98% and 22% respectively. (Meteorological Department of India, Satara.)

Soil: The soil varies from tract to tract. Mostly it covers the basaltic to laterite which gives rise to red loamy soil, black cotton and clayey soil rich in humus.

METHODOLOGY:-

For the present study 14 different types of crop seeds were used to examine mycoflora associated with them. Seeds always harbour diverse groups of microorganisms. The evaluation of seed microorganisms, seed mycoflora in particular becomes very important for the purpose of evaluation of its planting value (viability), seed treatment, plant quarantine, seed certification etc. Several methods are available for the detection of microorganisms associated with seeds. The selection of methods used for the detection of seed-borne pathogens depends upon purpose of the test. Incubation methods, Blotter Method, Agar Plate Method and Seed Suspension Method have been recommended by the ISTA (1966) for routine examination of crop seeds for fungal infection.

To evaluate seeds for associated mycoflora, seed samples of different crops were collected from farmers, crop fields, local markets in and around Satara. Seeds of cereals, pulses, oil seeds and vegetable seeds were collected for present study.

Plant Materials:-

For the present study 14 types of crop seeds were considered to study evaluate seed mycoflora. Following is the list of plants the seeds of which were considered for evaluation of seed mycoflora.

- **CEREALS**
  - *Sorghum bicolor* L. Moench. (Sorghum)
  - *Triticum aestivum* L. (Wheat)
  - *Zea mays* Linn. (Maize)
- **PULSES**
  - *Cajanus cajan* (L.) Millsp. (Pigeon pea)
  - *Cicer arietinum* L. (Gram)
  - *Pisum sativum* L. (Pea)
  - *Vigna mungo* (L.) Hepper.(Urdbean)
- **OIL SEEDS**
  - *Arachis hypogea* L. (Groundnut)
  - *Glycin max* L. Merril. (Soybean)
  - *Helianthus annus* L. (Sunflower)
- **VEGETABLE SEEDS**
  - *Abelmoschus esculentus* L. (Okra)
  - *Capsicum annum* L. (Chilli)

- *Cyamopsis tetragonoloba* Taub. (Cluster bean)
- *Phaseolus vulgaris* L. (French bean)

Several samples of these seeds were collected and representative seed samples obtained were stored in clean plastic containers. These seeds were used for the study of mycoflora.

Seeds possess mycoflora inside the seed and on the surface of the seed. Mycoflora inside the seed is called endophytic seed mycoflora while mycoflora on the surface and seed is called ectophytic mycoflora.

Methods of Evaluation of seed mycoflora:-

1. Visual Evaluation of mycoflora:-

- Examination of dry seeds without incubation ;
- Examination under a binocular microscope

2. Examination after incubation:-

- Examination after incubation ;
  - I. Blotter Method
  - II. Agar Plate Method
    - Dry Seed Inoculation Method.
    - Seed Suspension Test.

1. Visual Evaluation of mycoflora:-

Visual evaluations were carried out by following methods:-

- **Examination of seeds without incubation: -**

Examination of dry seeds is a quick method for detection of seed–borne pathogens. The seeds from representative samples were observed by unaided eye to find out the difference in size or colour and accordingly the data were recorded.

- **Examination under a binocular:-**

The untreated seeds were examined under a stereobinocular bright-field microscope to make out external symptoms and contaminants present on the surface of seeds.

2. Examination after incubation :-

Examination after incubation were carried out by following methods.

❖ **Examination after incubation:-**

Two methods, Blotter method and Agar Plate method are recommended by ISTA for routine examination of crop seeds for fungal infection. For each experiment five to six seeds were placed in petriplates. Each type of crop seeds were inoculated in triplicates and incubated. And average of three such observations was taken. Identification is based on fungal morphology developed during incubation on the seed surface, on Blotters or on colony characteris on an agar medium.

I. Blotter Method:-

Doyer (1938) was first who used filter paper for identification of seed borne pathogens. The Blotter Method is a simple method used for detecting the mycoflora associated with seeds. It is used for detecting those fungi which are able to produce mycelial growth and fruiting structures during incubation.

Blotters were sterilized and soaked in sterile distilled water. They blotters were placed in sterilized petriplates. Excess water stimulates bacterial growth. Four to six seeds were placed equidistant from one another. After placement of seeds, culture plates were incubated at room temperature (28^0C - 32^0C) for five to ten days. Slides were prepared for necessary observations.

II. Agar Plate Method:-

Muskett and Melone (1941) first used this method. Agar plate method can be used for the detection of the seed–borne fungal pathogens. Fungi which are not easily detectable in Blotter Method can be detected by this method. This method is expansive but less time consuming than blotter method and is of two types.

- **Dry Seed Inoculation method:-**

Dry seeds were picked up from the representative samples with sterile forceps, kept over the surface of agar and slightly pressed into the medium. Five to six such seeds were inoculated equidistantly on each plate and incubated under room temperature (28^0C) for five to ten days. Slides were prepared for observation of fungi.

- **Seed Suspension Test:**

The washing test is a qualitative test. This test is used only for detection of that type of pathogen which adheres to the seed surface in the form of identifiable spores.

For this method five to six seeds were put in one ml. of sterile distilled water, and shook well in order to bring the spores and fragments of hyphae into suspension. The suspension along with the seeds was poured on each agar plate. The suspension was spread with spreader. These plates were incubated at room temperature for 5 to 10 days till the growth of fungi. Slides were made wherever necessary.

Dry seeds as well as seed suspension were placed on CZ medium and these plates were incubated at room temperature. Three such replicates were prepared and each experiment was repeated three times and the fungi that developed were identified. This study was divided into two groups:

- Ectophytic or externally seed borne fungi.
- Endophytic or internally seed borne fungi.

- **ECTOPHYTIC MYCOFLORA: -**

Five to six seeds of each sample were placed on Czapek's (Dox) agar medium. To avoid bacterial growth streptomycin sulphate was added in the medium. For the study of ectophytic mycoflora fungal medium used for growth of all fungi were:

Czapek's (Dox) Agar :

Sodium nitrate	----------	3.0 gm.
Potassium dihydrogen phosphate	----------	1.0 gm.
Magnesium sulphate	----------	0.5 gm.
Potassium chloride	----------	0.5 gm.
Ferrous sulphate	----------	0.01 gm.
Sucrose	----------	30.0 gm.
Agar	----------	20.0 gm.
Distilled water	----------	1000 ml.
pH	----------	6.5

- **ENDOPHYTIC MYCOFLORA :**

The seeds were surface sterilized in mercuric chloride (1:1000) for five minutes. The seeds were then washed 4 -6 times with sterile distilled water to remove the traces of mercuric chloride. Five to six sterilized seeds of each sample split open using sterile forceps were placed on Czapek's (Dox) agar. To avoid bacterial growth streptopenicillin added in the medium. All the plates were inoculated in triplicate and the experiment was repeated thrice. It took longer time for endophytic fungi to appear on the medium.

3. Identification of seed mycoflora:-

Fungi on seeds appeared more readily. Slides were prepared for observation. The fungi were identified up to generic level using the "Illustrated Genera of Fungi Imperfecti" by Barnett (1973) and identification up to species level was done with the aid of Ellis (1976), Subramanian (1961), Tandon (1968), and Kamat (1959) Thom and Raper key for *Aspergillus* were used from Subramanian (1961) etc. Photomicrographs of the culture and slides were taken.