2.1 SURVEY

A survey was carried out in certain districts of Central Gujarat and Saurshtra namely 1) Vadodara 2) Bharuch and 3) Jamnagar to find out the different varieties of cotton (Normal / Bt) under cultivation, yield obtained, and associated seed and soil borne diseases.

2.2 ISOLATION OF FUNGI

3 samples from each field were collected from 30 cm depth in clean polythene bags from different locations and at a distance of 50 m and brought to the laboratory for further studies. Both rhizospheric and non rhizospheric soil of the cotton was used for the isolation.

Following method was incorporated for the isolation of different soil fungi:

- **Serial Dilution Technique (Waksman, 1961)**

To study soil mycoflora, serial dilution method is commonly used. The method is based upon the principle that when material containing microorganisms are cultured each viable propagule viz. spore, hyphae, sclerotia etc. will develop into a colony. Hence, the number of colonies appearing on the plates represents the number of living colony forming units (CFU) present in the sample.

10 g of soil sample was dissolved in 100 ml of sterile distilled water. After settling of the soil, 1ml of soil suspension was transferred into tubes containing 9ml of sterile distil water. Similar procedure was repeated 4 times to get the desired dilution.

0.5 ml of soil suspension from each tubes having 10^{-3} and 10^{-4} dilution was spread into the plates containing PDA medium. These plates were incubated at 25±2°C for 7days. Once fungal colonies were formed in PDA plates, each colony was transferred to a new agar slant to obtain a pure culture.

The following culture medium was employed:

- **Potato Dextrose medium: (pH- 6.0-7.0)**

200g of potato were peeled and sliced into small pieces. It was boiled in an autoclave for 40 min in 500 ml of distilled water and then filtered through a cloth. Twenty grams of dextrose was added and total volume was raised to 1000ml.

Borosil glassware and pure reagents supplied by Qualigens or SRL were used throughout the present investigation.

For cultural studies Petri dishes of (100 mm diameter, containing 20 ml agar) were inoculated with a piece of mycelium at the edge kept in diffused daylight at room temperature (20-25^{o}C) and examined at 7 days intervals. Separate slides were prepared and mounted in lactophenol.

LACTOPHENOL – COTTON BLUE STAIN

Phenol : 20 g

Lactic acid : 20 ml

Glycerol : 40 ml

Distilled water: 20 ml

The above mentioned chemicals were mixed and heated at 70 ^{0}C and then 5 ml of 1 % aqueous cotton blue was added.

2.2.1. Identification of fungi based on morphological characters

During field survey the Materials were collected in clean polythene bags from different locations and brought to the laboratory. The identification of fungal cultures was done based on morphological characters of conidia/ spore and final confirmation was done from IARI, New Delhi and Agharkar Research Institute, Pune.

2.3 Isolation of Seed Mycoflora

Before the isolation of the seed mycoflora, cotton seeds were tested for their viability. For seed viability test, four replications of 100 seeds were fully immersed in distilled water for 18 hours to start activity of dehydrogenase enzyme and for the facilitation of the penetration of tetrazolium solution (1% of TTC was used) for three hours at room temperature i.e. 25 ± 2 ºC in dark. After three hours the solution was decanted and excess reagent was removed, the seeds were then rinsed with water and evaluated. Individually the seeds were assessed for their viability. On the basis of staining of embryo, staining of cotyledon assessment on basis of necrosis, assessment on the basis of necrosis, and on the basis of color intensity was done, (Khare and Bhale 2000).

Isolation of seed mycoflora was done using-

(i) Blotter Method and (ii) Agar Plate Method

Blotter Method

In this method the Petri dish was lined with 3 layers of filter paper and then moistened with distilled water, the seeds were then placed and the dishes are kept at 25 ± 2 ^{o}C temperature and the germination of seeds was recorded on 4th and 7th day. Altogether 300 seeds were used (3 replicates of 100 seeds each) for this experiment. However, according to ISTA a sample size of 400 seeds is recommended. If any fungal mycelium was seen associated with seeds it was isolated under sterile conditions in to PDA slants.

Agar Plate Method

In this method, hundred seeds in four replictes were placed on potato dextrose agar. Before placing in Petri plate, seeds were surface sterilized with 0.1% mercuric chloride solution for one or two minutes and washed thrice with sterile distilled water to avoid surface contaminants and for the isolation of internal mycoflora, which were placed at equidistance in

Petri plates. These plates were then incubated for seven days. Different fungal colonies were examined under microscope for further identification.

2.4 Isolation of Arbuscular Mycorrhizal Fungi

Soil samples were collected from different locations of cotton growing area (Plate I) to study the natural distribution of AM Fungi. Soil was dug out with a trowel to a depth of 30 cm. Three representative samples were taken in clean polythene bags, labelled and stored until they were processed further. The number of AM spores found in rhizospheric and non rhizospheric soil are depicted in Tables 6-9. Isolation of AM Fungal spores from the rhizospheric and non rhizospheric soil of cotton was done.

- **Wet Sieving and Decanting Method (Gerdemann and Nicolson, 1963)**

The method includes mixing of 100g soil with 1litre of tap water and then decanting it in a stack of sieves. In the present case 5 sieves ranging from 63 to 500 µm were used. The AM spores were separated on different sieves, based on their size. They were then picked up by hypodermic syringe using a stereo microscope. Permanent slides were prepared in polyvinyl alcohol glycerol. Similar types of 5 spores were placed in a vial containing 0.05% streptomycin sulphate for further use.

- **Mounting of AM spores**

AM spores were mounted with polyvinyl-lacto-glycerol (PVLG) and were examined for their various characteristics and fungi were identified using the standard keys (Schenck and Perez, 1990; Mehrotra and Baijal, 1994)

Ingredients	Quantity
Lactic acid	100 ml
Glycerol	10 ml
Polyvinly alcohol (PVA)	16.6 g
Distilled Water	100 ml

PVLG is used to mount the spores semi -permanently on glass slides. For the best results, mounted specimens should not be studied for 2-3 days after they were mounted to give time for spore contents to clear. Whole spores will change color, generally darkening to varying degrees, and shrink or collapse with plasmolysis of spore contents. Broken spores are also needed to mounted as discrete layers of the spore wall or flexible inner walls of broken spores will swell to varying degrees and appear fused after long storage in some instances.

It is most important to mix all ingredients in a dark bottle before adding the polyvinyl alcohol. The PVA should have the following properties: 99-100% hydrolyzed, and a viscosity of 24 - 32 centipoise in a 4% aqueous solution at 20^{o}C. The PVA is added as a powder to the other liquid ingredients. The PVA dissolves slowly, and then only when placed in a hot water bath (70 - 80^{o}C). The solution will be clear in 4-6 hours. The solution was prepared by the mixture in the evening and letting it incubated in the water bath overnight. PVLG stores well in dark bottles for approximately one year. According to Koske in 2013 (http://invam.wvu.edu/methods/recipes), PVA powder can be added to the water, followed by autoclaving for 15 minutes. The lactic acid and glycerin are added, and the solution then is stored at room temperature for at least 24 h before using.

- **Storage of AM spores**

Isolated spores were collected in 0.1% Streptomycin sulphate solution and were preserved in small injection vials at low temperature, in a refrigerator (10-15^{0}C).

2.4.1. Identification of AM spores

Spores are the main structures used for the Identification of AM fungi. AM fungi were identified by using following morphological structures i.e. color, size, shape, wall structure, presence or absence of hyphae, surface ornamentations, nature and size of subtending hyphae

and bulbous suspensor attachment. For identification Manual of Scheneck and Perez (1990) and Mehrotra and Baijal (1994) and identification key of http://invam.caf.wvu.edu was used. Spores were photographed using Leica DME Research Microscope. The details are presented in Table 10.

2.5. Percentage Root Colonization by Root Clearing and Staining Technique (Phillips and Hayman, 1970)

The roots were washed and rinse in several changes of tap water. Then add 10% KOH and autoclave at 120^{o} C for 15 min. Decant KOH and rinse with water to remove KOH. Acidify roots by adding 1% HCl for 5 min. Decant HCl, do not rinse with water because the specimens must be acidified for proper staining. Add 0.05% trypan blue in lacto glycerol and simmer for 10 min. Decant stain and add lacto glycerol. Examine under microscope for mycorrhizal colonization.

2.6 Mass Multipication of AM fungi

50: 50 sand + soil mixture was sterilized for 1 hr. After cooling down fill the pots (30 cm dia) upto $3/4^{th}$ with this mixture. AM spores were sterilized by keeping in 200 ppm Streptomycin sulphate solution for 5 min. After disinfection, the spores were thoroughly washed in sterilized distilled water and were used as inoculant for initiating mycorrhization in maize plant. Add AM spores (20 spores in each pot) in center and spread one layer of soil (3cm) over to it. Sow the five maize seeds and again spread one layer of soil (2cm) to it and add water to it. The pots were kept in green house under constant observation. After 90 days, soil containing AM spores was used to inoculate nursery seedlings. Further, after 90 days, the maize roots were cut into pieces of 1cm and were mixed with the soil for preparing the AM inoculum. This incoulum was also used in plots containing garden soil without any fertilizer for mass multiplication of AM spores.

2.7 Mass culture of Fungi

For mass culture, the fungi were inoculated in to maize meal sand medium (Singh 1977). In 250ml conical flask, 150 g of sand and 4.5 g of crushed maize grains. 20ml of distilled water was added. The 150 g sand maize meal mixture gives 65% saturation. Then the flasks were autoclaved for 30 min at 15 *p.s.i* and were then inoculated with agar inoculum discs (Garrett, 1936) with 10 day old cultures flasks were incubated for 21 days at room temperature and in between they were shaken so that the fungus gets distributed equally inside the flask. When the fungal mycelium was well grown then it was ready to be incorporated in the soil. 5 g of upper 10cm inoculum was used per pot and mixed with the pot soil. Then seeds of Cotton varieties were sown. The pots were then watered once in a day to maintain appropriate moisture and then the effect of fungus on plant biomass was observed.

2.8.Biomass Study:

Plant Analysis:

Morphological and Biochemical Analysis of the Plants.

The plants were harvested on 15^{th}, 30^{th}, 45^{th}, 60^{th}, 75^{th} and 90^{th} day and morphological and biochemical analyses of *G. herbaceum* L. were considered in three replicates. The parameters that were taken into considerations during the plant analysis were as follows:

(A) Morphological Parameters:

I. **Shoot Length (cm):** At every harvest shoot length of all three varieties of G. *herbaceum* were noted.

II. **Root Length (cm):** At every harvest the root length of the plants was noted.

III. **Number of leaves per plant**: At every harvest number of leaves per plant was counted.

(B) Biochemical Parameters:

I.Fresh weight /plant (g):

The whole plant after harvesting was washed with water properly and air dried till the water evaporates from the plant surface. This plant was then cut to separate root and shoot and was weighed on the accurate digital balance. This procedure was repeated with 3 plants and the average of all the plants was calculated.

II.Dry weight /plant (g):

After noting the fresh weight of these plants , they were kept in oven for drying at 60^0 C for 48 hr separately and then the plant was weighed again and the average dry weight was calculated.

III. Estimation of Total Chlorophylls (Sadasivam and Manickam, 1996)

Weigh 1g of fresh leaf tissue and grind in the mortar. Add a pinch of $CaCO_3$ and the tissue was grinded to make fine pulp with the addition of 20 ml of 80% Acetone. The acetone extract was centrifuged at (5000 rpm for 5 min) and transfer the supernatant to a 100ml volumetric flask. The same procedure was repeated till the residue became colourless. The volume of volumetric flask was make up to 100ml with 80% acetone. The absorbance of the solution was read at 645 and 663 nm against the solvent (80% acetone) as blank in the spectrophotometer.

Calculations:

The amount of chlorophyll present in the extract mg chlorophyll per gram tissue was calculated using the following equations:-

Chlorophyll-a (mg/g tissue) : $[\ 12.7(A_{663}) - 2.69(A_{645})] \times \frac{V}{1000 \times W}$

Chlorophyll-b (mg/g tissue) : $[\ 22.9(A_{645}) - 4.68(A_{663})] \times \frac{V}{1000 \times W}$

Total Chlorophyll (mg/g tissue) : $[\ 20.2(A_{645}) + 8.02\ (A_{663})] \times \frac{V}{1000 \times W}$

Where,

A= Absorbance at specific wavelength

V= Final volume of chlorophyll extract in acetone

W= Fresh weight of tissue extracted

2.9 Effect of Fungal Cultural Filtrates on cotton seed germination

Agar blocks of equal size (5 mm) was cut from the actively growing margin of the individual species of soil fungi namely *Aspergillus niger, Fusarium oxysporum, Gliocladium virens, T. viride* and *T. harzianum*, and inoculated separately into the 250 ml conical flasks containing 100 ml sterile potato dextrose broth. The flasks were incubated at 25 ± 2^0C for 15 days. After incubation, the fungal cultures were filtered through Whatman filter paper No.1. The filtrates were transferred to conical flasks and stored at 4°C for further use.

2.10 Antagonistic Effect

To study the antagonistic effect, an experiment was laid out in petriplates poured with sterilized PDA. Solidified medium in the petri plates was inoculated by placing the discs (5 mm diameter) of bioagents culture. It was exactly opposite to this disc of test fungus (7 days old culture) were placed in such a manner that both organisms would get equal opportunity for their growth. The experiment was conducted with four replications and one control plate containing only test fungus. The growth rate of both test fungus and antagonistic fungi were recorded at 24 h intervals. Assessment was made when the fungi had achieved an equilibrium after which there was no further alternation in the growth. These plates were then incubated at 25 ± 2^0C. Observations were recorded after seven days of inoculation on area covered by the antagonist fungi and pathogen and percent growth inhibition was calculated as per formula (Skidmore and Dickinson,1976).

The percentage inhibition of growth was calculated as follows.

$$\text{Percent inhibition} = \frac{r - r_1}{r} \times 100$$

where, r = colony diameter of pathogenic fungi in control set

r_1 = colony diameter of pathogenic fungi in treated set

2.11. Bio – control of Pathogenic fungi (Poisoned Food Technique)

Effect of aqueous leaf and Methanolic extracts were obtained by Soxhlet Extraction method of 7 plants was observed on test organisms. It was tested on 3 different pathogenic fungi. The healthy leaves of *Annona reticulata, Balanites roxburghii, Cochlospermum religiosum, Gliricidia sepium, Ferronia limonia, Sapindus emarginatus* and *Tephrosia jamagerensis* were collected and washed well and dried in oven at 60 ^{0}C for 48h. The dried leaves were powdered and stored in plastic bags. Twenty grams of leaf powder was extracted in a Soxhlet extractor with 200 ml methanol for 8 hours. The extract was concentrated then the residue was treated with 20 % of methanol. It was added to dry residue and water soluble compounds were filtered out. The leaf extracts were mixed with appropriate volume of medium (PDA) to obtain concentrations ranging from 5 to 25 % in the final volume of 100 ml of medium. This 100 ml medium was dispensed into 100 mm Petri plates with triplicates **(**Nene and Thapliyal, 1979).

Fungal isolates of was placed in the centre of each plate. Control sets were also prepared without plant extract. The plates were incubated at 25 °±2°C and growth of colony was measured after 7 days of inoculation. The radial growth of mycelium was measured at two points along the diameter of the plate and the mean of these two readings was taken as the diameter of the colony. The growth of the colony in control sets was compared with that of various treatments and the difference was converted into percent inhibition by following formula

$$\text{Percent inhibition} = \frac{\text{Diameter of control set - diameter of treated set}}{\text{Diameter of control set}} \times 100$$