CHAPTER THREE

MATERIALS AND METHODS

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The fruit-vegetables tomato and brinjal constitute the two favourable cash crops of the farmers in the opulent irrigated area of Kolhapur District since they fetch quick and lucrative returns vis-a-vis sugarcane, the ubiquitous and long standing crop of Krishna-Panchaganga basins in the district.

Mainly five popular and high yielding varieties of tomato : *Lycopersicum esculentum* Mill. Viz., 'Vaishali', 'Rupali', 'Rashmi', 'Dhanshri' and 'Rajashri' are in vogue. The former three are Indo-American hybrid varieties while the latter two are the cultivars released by Mahatma Phule Krishi Vidyapeeth, Rahuri (M.S.).

In case of brinjal : *Solanum melongena* L. too, five important varieties viz., 'Manjari gota', 'Krishna', 'Vaishali', 'Mahyco-56' and 'Krishnakath' are commonly cultivated.

Selection of Varieties :

For the investigation of rhizosphere fungi the extensively cultivated 'Rupali' cultivar of tomato and 'Mahyco-56' hybrid cultivar of brinjal were selected after a detailed survey of cropping practices in this district.

Area of Cultivation and Schedule of Cultivation :

The seeds of both the varieties were procured from the

market. These varieties were grown as winter crops and for this purpose the seeds were sown on 29/10/1994 in specially preapred beds in Botanical Garden, Shivaji University Campus, Kolhapur, to raise the saplings. In order to avail two different types of soil, cultural and environmental conditions the saplings were then transplanted at the two following localities :

1. Botanical Garden, Shivaji University Campus, Kolhapur .
2. A private farm at Chinchwad (Tal. - Shirol, Dist : Kolhapur).

The detailed records of watering schedule, application of fertilizers, weeding and plant protection schedule at both these localities for both these varieties were meticulously maintained and followed.

Collection of Rhizosphere Soil Samples :

During the growing season, the plants at different stages of growth (30, 60, 90, 120 and 150 days old plants) were dug out, at 3 to 4 places in respective fields, alongwith a block of soil intact around the root system, by means of a sterile spatula. The roots were slowly shaken to remove the soil adhering to them. The samples were taken in polythene bags and brought to laboratory within 12 hours. They were air dried and sieved through 2 mm mesh sieve. Five grams of soil was used for experimental work and the remaining parts of soil samples were preserved. The soil samples were properly

labelled. The pH of the soil samples was determined by Elico pH meter model LI - 10 T.

Collection of Non-rhizosphere Soil Samples :

Simultaneously, alongwith rhizosphere soil samples, non-rhizosphere soil samples were collected from the area left between the plants. The samples were air dried, sieved and kept in polythene bags. They were properly labelled.

Isolation of Fungi :

The occurrence of fungi in rhizosphere and non-rhizosphere soil samples was studied by using soil dilution plate technique.

The sieved soil sample was thoroughly mixed and from it one gram soil was weighed quickly. This was then suspended in 250 ml conical flask containing 100 ml sterile distilled water so as to get the stock solution. The flask was vigorously agitated on a shaker for about 15 minutes and then allowed to stand till the soil settled.

From this stock solution one ml of soil suspension was transferred by means of sterilized 1 ml pipette to test-tube containing 9 ml of sterile distilled water which gave a dilution of 1:10. By repeating the process, further serial dilutions of 1:100, 1:1000, 1:10,000, 1:1,00,000 were obtained. All the dilutions were thoroughly shaken again and then 1 ml of each of these dilutions was poured on plates under aseptic conditions. Each plate was gently swirled to ensure uniform distribution of suspension.

Selection of Medium :

It is difficult to select a single suitable medium for isolation of the soil fungi because of their diverse requirements for growth in culture hence different media were tested for obtaining maximum results. Dextrose-Peptone Agar (D.P.A.) and Czapek's Dextrose Agar (C.D.A.) were found to be most suitable for fungal count. Among these two, Dextrose-Peptone Agar was found to be ideal for fungal isolations and their counts. The composition of Dextrose-Peptone Agar (Waksman's soil special medium) is given below :

Composition of Dextrose-Peptone Agar :

1.	Dextrose	10.0 g
2.	Peptone	05.0 g
3.	Potassium phosphate (Monarch)	01.0 g
4.	Magnesium sulphate	00.5 g
5.	Agar	25.0 g
6.	Distilled water	1000.0 ml

The pH of medium was adjusted between 4.0 and 4.5 by means of 1N H_2SO_4 or 1N H_3PO_3 or by 2N NaOH. The medium was sterilized at 15 lbs pressure for 25 minutes and before pouring the medium in the petriplates 80 mg streptomycin sulphate per litre of medium was added in the medium as an antibacterial agent.

Counting of Fungal Colonies :

After the gentle swirling, the inoculated plates were incubated at room temperature. The numbers of fungal colonies appeared in petriplate on the fifth day of incubation were counted. Most of the colonies appeared on the third and fourth day and very few appeared on fifth day. Colonies appearing after five days were considered secondary and hence were not included in the total count. Some of the slow growing fungi might have been excluded by this method. However, the appearance of secondary colonies derived from original sporulating colonies made the count after five days unreliable. Using Practica-MTL-50 camera coloured photographs of culture plates were taken on fifth day of incubation.

Pure Cultures of Fungal Isolates :

It was observed that fungal isolates exhibit a keen competition for development in the initial medium and colonies appearing on this medium generally do not grow and develop to their full extent and hence M_2 Agar was used. The composition of M_2 Agar is as follows :

Composition of M_2 Agar Medium :

1.	Glucose	10.00 g
2.	Yeast extract	05.00 g
3.	Glycerol	10.00 g
4.	Potassium dihydrogen phosphate	00.10 g
5.	Magnesium Sulphate	00.05 g

6.	Agar	25.00 g
7.	Distilled water	1000.00 ml

Single colonies of different fungi were picked up from D.P.A. petriplate cultures and transferred aseptically on M_2 agar slants. They were numbered properly for further studies. For transfer of fungi from colonies in the plates to M_2 agar slants, flamed sterilized chrome wire or chrome loop was used. At the time of transfer, the test-tube containing the slant or organisms was held near the flame. This helps in avoiding the contamination during transfer. It is essential to note that, to obtain pure cultures, the transfer of colonies must be done strictly under aseptic conditions and to achieve this, the process of transferring the colonies was done swiftly and quickly to minimize the contamination.

After sufficient growth of isolates in the slants the macroscopic observations of colonies such as external features, texture, colony colour, colour on reverse, growth characteristics, zonations and variations were recorded. Using Practica-MTL-50 Camera coloured photographs of slants were also taken.

Preservation and Maintenance of Cultures :

Various methods of preservation and maintenance of cultures have been recommended in different standard works. The pure cultures of different fungi isolated from soil and rhizosphere were maintained by repeated transfers under aseptic conditions on M_2 agar slants. These slants were preserved in

mineral oil and the cotton plugs were wrapped by aluminium foil.

Identification of Isolates :

The micropreparations (slides) of each cultures have been made by direct mounts from pure cultures using cotton blue as stain and Poly Vinyl Alcohol (P.V.A.) as mounting medium. The composition of polyvinyl alcohol mounting medium used is as follows :

Composition of mounting medium :

I.	Polyvinyl Alcohol	11.0 g
2.	Glycerol	10.0 ml
3.	Phenol (distilled)	25 drops
4.	Lactic acid	25 drops
5.	Distilled water	100.0 ml

The photomicrographs of slides were taken using Leitz 307-107.015 and Jenavel photomicrography units.

The fungal organisms were identified using uptodate and standard references such as Gilman (1957), Barnett and Hunter (1960), Barron (1968), Subramanian (1971), Ellis, M.B. (1971-1976), Kendrick (1971) etc.

The Perishable plant products such as fruits and vegetables are severely affected by diseases in field caused by various pathogens. Moreover, these products during their transit and storage are attacked more severely by several types

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of fungal pathogens causing rots, blemishes and other diseases. This poses a grave concern for the growers as well as the vendors. An attempt has been made in this work to study some of the fungal diseases of fruits and vegetables received in the markets.

For this purpose a preliminary survey of fruits and vegetables in various markets was done. The diseased materials were collected from some major important markets at Kolhapur, Jaysingpur and Ichalkaranji. The diseased samples were collected sufficiently in seperate polythene bags and brought to the laboratory. The symptoms were recorded and photographs were taken immediately. The samples were then incubated for a few days.

The fungal pathogens that developed were isolated by using Potato Dextrose Agar (P.D.A.) culture medium. The composition of Potato Dextrose Agar medium used was as follows :

Composition of Potato Dextrose Agar :

1.	Potato	200.00 g
2.	Dextrose	20.00 g
3.	Agar	15.00 g
4.	Distilled water	1000.00 ml

Potatoes were peeled off and shreded into small pieces. These pieces were boiled in 500 ml water for about an hour on a hot plate. It was filtered through two layered muslin cloth.

In another 1000 ml conical flask agar was melted in 500 ml water to which the dextrose was added. The filtered potato fluid was added into the molten agar and the volume was adjusted to 1000 ml with water. The flask containing medium was plugged with non-absorbent cotton wrapped in cheese cloth and autoclaved at 15 lbs (121°C) pressure for half an hour. 30 mg streptomycin sulphate was added to the cooled liquid medium before pouring it in petridishes, whenever desired.

Inoculation of Diseased Samples :

The diseased part of the sample, particularly the one near the advancing margin of the lesion, was cut into small pieces aseptically. These pieces were surface sterilized by dipping in 0.1% $HgCl_2$ solution for about 1 minute. Then the pieces were repeatedly washed in sterile distilled water, blotted with sterile blotting paper and then inoculated on the plates containing the potato-dextrose agar nutrient medium aseptically. These plates were incubated at room temperature, the growth of the pathogen was monitored and after sufficient growth the diagnostic features of the culture were noted. The photographs of culture plates have also been taken.

Identification of Fungal Pathogens :

Morphological characters of various fungal pathogens were studied in detail from micropreparations (slides) made directly from the colonies on the infected fruits and vegetables as well

as their cultures. Cotton blue was used as stain while Polyvinyl Alcohol (P.V.A.) was used as mountant. The identification of fungal organisms was done by referring various monographs, research papers and other literature such as Barnett and Hunter (1960), Subramanian (1971), Ellis, M.B. (1971-1976), Kendrick (1971), Rao, V.G. (1968-1980) etc. All the cultures, semipermanent slides, materials were properly labelled, numbered and deposited in mycological Herberium, Dept. of Botany, Shivaji University, Kolhapur (M.S.) under Nos. WIF (FUNGI OF WESTERN INDIA).