CHAPTER - III

MATERIALS AND METHODS

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1. Survey and Collection of disease Samples:

Several plots in villages adjacent to Jaunpur city were regularly surveyed. Plant showing partial area or complete wilting symptoms were carefully callected. While callecting care was taken not to disturb or damage plant root system. The samples were packed in polythene bag and brought back to laboratory for isolations.

2. Isolation of the pathogen and its Koch's Postulates.

A. Isolation of the pathogen:

The plant samples brought to laboratory were thoroughly washed in running tap water to remove adhering soil particles. Subsequently the washed samples were ringed thrice in sterilized water, using sterilized knife/blade, small pieces of plant part showing wilting were cut. The cut pieces were collected in petrideshes for further treatment and isolation. The pieces to acquire disinfestation were washed for one minute in 0.1% $HgCl_2$ solution and then ringed 3-4 times in sterilized water. While cutting the pieces, care was taken to include both healthy and diseased part in each cut pieces sample. The treated pieces were then aseptically transferred in slants employing standard microbiological techniques. Three separate mediawere employed for isolation of the pathogen. They were PDA (a general medium), Czapek's dox agar medium (Reper and Thom, 1949) and Penta chloro Nitro Benzene (PCNB) agar medium (Nash and Snyder, 1962), (both selective media).

The inoculated slants/petri plates were incubated at 28 ± 1^0c

for 4-6 days. As soon as hyphal growth appeared sub culturing was done repeatedly to get pure culture of the organism associated with diseased sample.

B. Microscopic Examination:

Isolated fungi were subjected to microscopic examination and only those showing structural details of *Fusarium spp.* were preserved for further study. The remaining fungi were discarded. In further study, right from colony characters to details of spores were studied. Those similar to characters of *Fusarium udum* were maintained for further study.

C. Multiplication and Preparation of Mass inoculum:

The pathogen was multiplied on sand maize meal medium (Nene *et al.*, 1981). Sand 90%, Maize 10% and water 20% (to moisten the medium) were thoroughly mixed in a tray and placed in a number of 250 ml Erlenmeyer flasks and auto claved twice at 15 Ib pressure for 30 minutes. Each flask was inoculated with three agar blocks cut from the 5 days old culture of the fungus grown on PDA medium in culture plate with the help of sterilized 10 mm cork borer. The flask were inoculated at 25 ± 2^0 C for 15 days and shaken intermittently so as to get a homogeneous growth of the inoculum.

D. Koch's Postulates- (Soil Inoculation Method):

A good amount of field soil was collected, air dried, grind, seived (2 mm porosity) and autoclaved twice at 15 Ib pressure for 30 minutes. Inoculum of the pathogen was thoroughly mixed at the rate of 1% (w/w) with sterilized soil, filled in earthen were pots and watered regularly. After an incubation of one week, at 25^0C for establishment of inoculum, further treatment were done.

Pigeonpea seeds of susceptible variety (Bahar) were

surface sterilized with 0.15% aqueous solution of $HgCl_2$ for one minute followed by subsequent washing with sterilized distilled water. Each pot was seeded with 10 such seeds at equidistance. Suitable check (with out inoculum) was also run simultaneously. The pots were watered daily or as required to maintain soil moisture at field capacity. The plants showing wilting were carefully removed and used for reisolation of the pathogen. Reisolated pathogen purified and further employed for pathogenicity test. Each treatment was replicated five times. The record of percentage of wilted plants was also maintained.

3. Collection of soil samples from rhizoplane, rhizosphere and non-rhizosphere regions:

In order to estimate, enumerate and isolate indiginous soil microflora particularly fungi and actinomycetes, soil samples were collected from root zone and root free soil. The samples were collected from designated plants and plots. After clearing the soil surface from plant free areas, soil samples were collected from 8-10 places depending on the topography of the plot. All the samples collected were mixed thoroughly and finally representative sample was drawn for further study. This was non rhizosphere soil sample.

For collection of rhizosphere sample, the whole plant root system was dug very carefully. After removing several such plants, the soil adhering to root system was removed by gentle tapping. The soil particles that strictly adhered to root system were carefully removed using gentle tapping and scrapping with knife. The soil sample thus collected served as rhizosphere soil sample and used for further study.

Rhizoplane is the zone where there is very intimate

association between plant roots and the soil. The root systems from which rhizophere soil has already been removed, were used for recovering rhizoplane soil sample. The external surface of the root system was thoroughly scrapped and soil and plant part collected to serve as rhizoplane samples.

4. Enumeration and isolation of Microflora:

In order to estimate, both qualitatively and quantitatively, the status of indiginous microflora (fungi-actinomycetes) in rhizosphere, rhizoplane and non-rhizosphere soils, serial dilution plate technique, was employed. One to ten gram soil sample (depending on quantity available) were mixed in required amount of sterilized water to get 1 : 10 dilution. After thorough shaking, one ml was drawn and mixed in measured amount of sterilized water to get 1 :100 dilution. This way, desired dilution of 10^3 and for actinomycetes a dilution of 10^5 or 10^6 was used. The media used for isolations of these groups of microflora are given below with their ingredients.

Rhizosphere and non-rhizosphere Petri-dishes inoculated with soil suspension were poured with 15 ml sterile, melted but cooled (35^0C) specific agar media of the following composition.

For fungi:- Dextrose 10g, Peptone 5g, KH_2PO_4 1g, $MgSO_4$ 0.5g, Rose Bengal 1 : 15000, Agar-agar 15g, Distilled water 1 litre, P^H 5.5.

For actinomycetes:- Dextrose 2g, Casein 0.2g, K_2HPO_4 0.5g, $MgSO_4$ 0.2g, $FeCl_3$ trace, Agar-agar 15g, Distilled water 1 litre, P^H 6.5-6.8.

For the rhizoplane study the method of Harley and Waid (1955) was employed. Roots were thoroughly washed with sterile distilled water for 10 times. After washing, the adhering water was

removed by sterile blotting paper from the root system and cut off into small pieces of 5 mm by sterile scissors. Five bits were placed into each Petri-dish containing sterilized, cooled, nutrient agar medium. Five replicates were used for zhizoplane.

The plates were incubated at 25^0 C and 30^0 C for fungi, actinomycetes respectively. After an incubation of 6 and 4 days respectively, colonies were counted for calculating the number of each type of organism /g dry soil. Fungi were identified and recorded for calculating their % occurrence.

5. Screening of Biocontrol Agents:

Some dominant rhizosphere fungi like *Aspergillus flavus, A. luchuensis, A. niger, A. terreus, Alternaria alternata, Cladosporium cladosporioides, Penicillium citrinum, Rhizoctonia solani, Trichoderma harzianum* and *T. viride,* which were reported earlier as antagonistic ones against the pathogen (Upadhyay and Rai, 1987) were isolated from the rhizosphere to screen their antagonistic potency. In addition, *Gliocladium virens* and a few bacteria viz. *Bacillus licheniformis* 2042, *B. licheniformis* 2044, *B. thuringiensis* and two unidentified bacteria (obtained from G.B. Pant University of Agr. and Tech. Pantnagar, through the courtesy of Prof. U. S. Singh) were sellected for studying biocontrol potential against the pathogen and suitability in integrated disease management system.

A. Colony interaction:

The colony interaction was studied by inoculating 5 mm agar block of test micro organism with the test pathogen (in triplicate petri dishes) and placing them over solid media, 3 cm apart from each other. The colony interaction between *Fusarium udum*

and test fungi were studied on PDA medium where as the interaction between *F. udum* and bacteria were studied on synthetic medium. The control sets were single and dual inoculated at 25±2^0 C and 32±2^0 C for fungi and bacteria, respectively. In general, the observation was made after 6 days.

The assessment of the interactions were made following the model of Skidmore and Dickinson (1976) for colony interaction. The parameter used for the assessment of colony interaction was percent inhibition of radial colony growth i.e. 100 x (r1 - r2)/ r1 (Fokkema, 1976) : Where r1 denotes diameter of the radial growth of the pathogen towards opposite side and r2 denotes it toward the opponent microorganisms.

B. Effect of culture filtrates of some selected microorganisms on growth of the test pathogen:

The microorganisms as mentioned above were selected and the method described by Upadhyay and Rai (1987) was followed for the study.

Three blocks (5 mm each) of indivisual microorganisms cut from the actively growing margins of 5 days old culture were inoculated separately in 250 ml Erlenmeyer flask containing 100 ml Czapek-Dox medium. After 10 days of incubation at 25±2^0 C the culture filtrates were filtered firsts through whatman filter paper no. 44 and finally through a Seitz filter (45) by vacum pressure to obtain cell free culture filtrates. The metabolites of bacteria were obtained by inoculatting a loopful of bacterial cell in Nutrient broth medium and the self reculture filtrates were obtained following the above method used for fungi. Four ml of culture filtrate was poured in 16 ml auto claved and cooled (40 ^{0} C) Czapek-Dox agar medium for

fungi and in Thornton's medium for bacteria. The petri plate containing medium and culture filtrate was inoculated centrally with a 5 mm agar block of 5 days old culture of the pathogen. The experiment was performed in 3 replicate petri plates for each type of culture filtrate. For control set same quantity of sterilized and cooled distilled water was added in 16 ml of CDA medium. The radial growth of colony was measured after 7 days of incubation.

The percent inhibition was calculated by using the following formula.

$$\text{Per cent growth inhibition} = \frac{C - T}{C} \times 100$$

Where C = Growth in control,

T = Growth in treatment.

6. Screening of Oil-Cake:

4 oil cakes were selected for evaluating their fungitoxicity against the pathogen : neem, coconut, mustard and linseed. The respective oil cakes were collected from oil mills of Jaunpur and Varansi. Firstly the oil cakes were crushed and were ground in electric mixer to obtain their powdered form. The powdered cakes were autoclaved at 15 Ib pressure for 25 minutes. The fungitoxicity of these cakes were tested by poisoned food technique (Flack, 1907). 10, 20 and 30g of each cake was mixed with 90, 80 and 70 ml of sterilized and cooled (40 ^{0} C) CDA medium, respectively to get final concentration of 10, 20 and 30 per cent. Twenty ml amended medium of each concentration was poured in to seperate sterilized petri plate in three replicates and allowed to solidify. Each petri plate was inoculated centrally with a 5 mm agar

block of 5 day old culture of the pathogen. All the plates were incubated at 25±2^0 C. The radial growth of colony was measured after 7 days of incubation and the per cent inhibition was calculated by the formula given earlier.

7. Screening of Essential Oils:

The volatile fungitoxic fractions of most active plants viz., *Eucalyptus citriodora, Eupatorium cannabinum, Callistemon lanciolatus* and *Aegle marmelos* were isolated by hydrodistillation using Clevenger's apparatus (Langenau, 1948). Fresh leaves (500 g) of each plant were washed with water and subjected to hydrodistillation for 4 hr. The isolated essential oils were treated with anhydrous sodium sulphate to remove traces of moisture. Minimum inhibitory concentration at which the oils showed absolute fungitoxicity was determined by the usual poisoned food technique (Flack, 1907). Different concentrations of the oils (100, 200, 500, 1000 and 20000 ppm) were prepared by dissolving the requisite amount in 0.5 ml acetone agar medium separately. The medium of control set contained requisite amount of sterilized water and 0.5 ml acetone in place of the oils. As usual the plates were incubated asceptically with the assay disc (5 mm) of the test pathogen and incubated for seven days at 25±2^0 C. The observation was recorded on the 8th day and per cent mycelial inhibition was calculated by following formula:

$$\frac{C - T}{C} \times 100$$

Where C = Diameter control set,

T = Diameter of treated set.

8. Invivo effect of Antagonists, Essential oils (Seed treatment) on per cent Mortality and per cent disease management of wilt of pigeonpea in natural and sterilized soil:

A. Preparation of mass culture of antagonists and the pathogen:

A typical method for making mass culture of antagonists and the pathogen on wheat grains was followed. Clean wheat grains were taken for the purpose. Broken grains were avoided. The grains were prewetted by boiling them in water for 20-30 minutes. This raised the moisture content of the grains to 40-50 percent and at the same time made them soft enough for fungus to grow on it. After boiling excess water was drained of by spreading the grains on wire mesh. Boiled grains were mixed with Gypsum (calcium Sulphate) and chalk powder (Calcium carbonate) at the rate of 2 percent and 0.5 percent respectively on dry weight basis. This would help to check the P^H of the medium and would prevent sticking of grains with one another. The grains were then filled in conical-flask, and mouth of the conical-flask were plugged with non-absorbent cotton. Conical-flaskes with wheat grains were steam sterilized in autoclove at 22 p.s.i. pressure for 1.5-2 hours. This gave uniform temprature of 126.5 ^{0} C which was sufficient to kill bacterial and other contaminations that could have spoiled culture afterwards. Conical-flask with grains were allowed to cool at room temperature and inoculated with pathogen and antagonists by adding 10 discs of 5 mm diameter cut from the margin of the actively growing cultures of antagonists and the pathogen. After inoculation the conical-flask were incubated at 25±2^0 C for 10-15 days for complete growth of the microbes. During the period of incubation the conical-flask were

shaken once or twice to ensure rapid and uniform colonization (Plate3, Fig. 5).

B. Preparation of pots:

Sufficient soil samples from pigeonpea field were collected and brought into laboratory. The soil was air dried at room temperature (30^0 C) for 24 hrs and then ground with the help of pestle and mortar and seived through 2 mm pore size sieve. The soil was well mixed with 1% (w/w) pure inoculum of the pathogen prepared on wheat grains and was taken in plastic pot and kept at room temperature (30^0 C) for one week to allow the pathogen to establish well in the soil. To perform the experiment in sterilized soil, the sample was filled in containers and sterilized in autoclave at 15 Ib pressure for 20 minutes.

The pot containing soil-pathogen inocula mixture was amended with antagonistic microbes, essential oil, oil treated seeds were sown to observe their effects on per cent wilting by the pathogen.

C. Amendment with antagonistic microbes:

Foure antagonists viz., *Aspergillus niger, Penicillium citrinum, Trichoderma harzianum* and *Gliocladium virens* were selected for the present study.

Inocula of these antagonists were prepared on wheat grains (Procedure mentioned in the preceeding pages) and 1%, 2%, and 3% (w/w) pure inocula of each antagonist was mixed in the pot soil (natural and sterilized) infested earlier with the pathogen (1%) for soil infestation.

The pots containg soil-pathogen inocula mixture without antagoinst served as control. Three replicates were maintained for each concentration. Original moisture level was maintained throughout the experiment by adding sterilized distilled water from time to time. Seeds of susceptible variety of pigeonpea (Bahar) were surface sterilized by soaking them in 0.1% aqueous solution of $HgCl_2$ for 1 min. and washed thoroughly several times with sterilized distilled water to remove every trace of $HgCl_2$. The washed seeds were sown in each pot and 10 seedling were allowed to grow per pot. Observation of disease development was made regularly but the final percent wilting of the plants was noted after 45 days of sowing. The per cent seedling mortality and per cent disease control were calculated using the following formulae:

$$\text{Mortality per cent} = \frac{\text{No. of seedling in uninfested pot soil} - \text{No. of seedling in infested pot soil}}{\text{No. of seedling in uninfested pot soil}}$$

$$\text{Per cent disease control} = \frac{\text{Mortality (\%) in Check} - \text{Mortality (\%) in treatment}}{\text{Mortality (\%) in check}} \times 100$$

D. Seed treatment with essential Oils:

Treatment of seeds with the oils was done by the technique of Tripathi and Grover (1977) to find out their potency in control of wilt disease of pigeonpea. Two essential oils of *Aegle marmelos* and *Eucalyptus citriodora*, proved to be strong inhibitor of the pathogen, were used for this study.

For treatment, 10g seeds of susceptible variety of pigeonpea (Bahar) were soaked separately for 12 hrs in 0.1 and 0.3% of the *A. marmelos* and *E. citriodora* oils which were prepared in

sterilized water as usual. Ten seeds per pot were sown equidistantly in 36 pots. These pots were filled with natural and sterilized soil infested earlier with *F. udum* (1% w/w). The treatment consisted of 18 pots for each oil, 9 each for natural soil and sterilized soil. Seeds soaked in 0.1, 0.2 and 0.3% of oil were sown in pots. For each concentration 3 replicates were used both for natural and sterilized soil. On the other hand in control, seed soaked in sterilized water only for 12 hrs were similarly sown in 12 pots. The moisture level was maintained throughout the experiment by adding sterilized water from time to time. Further procedures of experiments to record per cent mortality and per cent disease control were the same as described above.

9. Isolation of Rhizosphere Mycoflora of Sterilized and Natural Soil Amended with Potent Antagonists and Effective substances:

The soil samples from root region of plants, grown in natural and sterilized soil infested with the pathogen (1% w/w) and amended with the antagonists and effective substances separately in pots, were collected for initial (when plants were in seedling stage) and for final samplings (when plants were 45 or more than 45 days old showing complete symptoms of wilting).

Rhizosphere soil samples were collected for each type of amendments and their concentration separately in triplicate in sterilized polythene bags. One g soil from each pot was taken separately into 250 ml conical flask containing 100 ml sterilized distilled water. The flask were shaken on electrical shaker (120 throws min^{-1} and 1.5 cm displacement per throw) to get a homogeneouse

suspension and there after 1 : 1000 (for fungi) and 1 : 100000 (for bacteria) dilutions were prepared in sterilized distilled water. Three replicates for each sample and dilution were inoculated with 1 ml aliquot of soil suspension separately for fungi and bacteria. Seperate sterilized pipettes were used for each dilution. The inoculated petri-dishes were poured separately with melted and cooled 25 ml martin's and Thornton's media for fungi and bacteria respectively. The plates were incubated separately at 25 ± 2^0 C and 37 ± 1^0 C for isolation of fungi and bacteria respectively and the colonies were recorded after seven days of inoculation.

10. In vitro studies on Integrated Effects of the Antagonists and some Effective Substances on Colony Growth of *F. udum*

A. Selection and preparation of the antagonists and test substances:

Four antagonists viz., *Aspergillus niger*, *Gliocladium virens*, *Penicillium citrinum* and *Trichoderma harzianum*, essential oil of *Aegle marmelos*, and the fungicides MeMc and the Bavistin, which were found to be most potent against *F. udum* on the basis of in vitro and vivo screening, were selected for the above study.

B. In vitro test:

LD_{50} concentration of essential oil, and fungicides was calculated by plotting inhibition or survival (percentage) curve against the concentrations of each substances on a semi log graph paper. The survival curve was drawn with the help of replica (french curve). The LD_{50} concentration of essential oil (200 ppm) and of fungicides MeMc (0.75 ppm) and Bavistin (0.70ppm) was selected for the above test.

The LD_{50} concentration of essential oil, and fungicides were integrated with 20% metabolites of different antagonists in different combinations and the additive were mixed into molten and cooled PDA medium and the following combination were prepared:

1. Antagonist + essential oil.
2. Antagonist + fungicides.

The additives with PDA were poured in petri-plates and each petri-plate was inoculated centrally with 5 mm block of actively growing colony of *Fusarium udum*. Three replicates for each combination were taken. The control petri plate was set using only nutrient medium without any additive. The radial growth of *F. udum* was measured in treated and control petri plates after seven days in incubation. The per cent growth inhibition was calculated with the following formula:

$$\text{Per cent inhibition} = \frac{dc - dt}{dc} \times 100$$

Where dc = diameter of control set,

dt = diameter of treated set.

11. Integrated Management of wilt Disease in Field:

To conduct the integrated management of wilt disease in the field, same formulations were used as invivo integrated management of wilt disease in glass house.

To perform the experiment, a field of 20m X 20m sq was selected in the Agricultural Research farm of T.D.College, Jaunpur, where pigeonpea crop was regularly grown showing symptoms of wilting. Fifteen plots of 1 X 1 sq meter was made for each combination.

Each plot was made sick with the pathogen by amending

150g of mass inoculum of the pathogen on wheat grains/m^2 and plots were left for 20 days and light watering was done to establish the pathogen in the sick plots. Hundred fifty gram mass inoculum of the antagonist and oil-cakes of *Azadirachata india* were amended into the plots and left for 10 days.

To amend the fungicide in the plots, seeds of susceptible variety (Bahar) treated with Bavistin (1%) were sown in each plot at the rate of 20 seeds per plots (10 seeds per row). The plots were arranged in randomized complete block design with three replication per treatment. The sick plots without antagonist, oil-cakes and Bavistin treated seeds served as control.

The percent mortality and percent disease management were calculated by the formula mentioned earlier after 60 days of sowing.

Constituents of Different Culture Media in Various Experiments:

Different media and their constituents are given below which have been used for different experiments during the study.

Czapek-Dox Agar Medium (Raper and Thom, 1949)

$NaNO_3$	2g
KH_2PO_4	1g
$MgSO_4$	0.5g
KCI	1g
$FeSO_4.7H_2O$	0.066g
Sucrose	30g
Yeast extract	0.5g
Distilled water	1000ml
P^H	5.6

Martin's Agar Medium (Martin, 1950)

Dextrose	10g
Peptone	5g
KH_2PO_4	1g
$MgSO_4.7H_2O$	0.5g
Rose Bengal	0.066g
Agar-agar	18g
Distilled water	1000ml
pH	5

Nutrient Broth Medium

Beef extract	3g
Peptone	5g
Distilled water	1000ml
pH	7.3

Potato Dextrose Agar Medium

Potato	200g
Dextrose	20g
Agar-agar	18g
Distilled water	1000ml
Streptomycin	30 µg/ml
(added after autoclaving)	

Penta Chloro Nitro Benzene (PCNB) Agar Medium (Nash and Snyder, 1962)

$MgSO_4.7H_2O$	0.5g
KCI	1g
$NANO_3$	2g
NH_2PO_4	1g
$FeSO_4$	0.066g
Yeast extract	0.5g
Sucrose	30g
Agar-agar	18g
Distilled water	1000ml
P^H	5.6
PCNB 75% WP	1g
Malachite green	Trace

Synthetic Medium for the Growth of Bacteria and Fungi both

K_2HPO_4	1g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	0.1g
NaCl	0.1g
$FeCl_3$	0.002g
KNO_3	0.5g
Asperagine	0.5g
Mannital	2g
Sucrose	20g
Agar-agar (Bacto difco)	12g
Distilled water	1000ml
P^H	7

Thronton's Medium (Thronton, 1922)

K_2HPO_4	1g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	0.1g
NaCl	0.1g
$FeCl_3$	0.002g
KNO_3	0.5g
Asperagine	0.5g
Mannital	1g
Agar-agar	15g
Distilled water	1000ml
P^H	7.4
Mycostatin (added after autoclaving)	25 µg/ml

Statistical Analysis:

The data recorded were subjected to statistical analysis wherever required by applying analysis of Variance (ANOVA) and Critical Difference (CD) and were calculated by standard methods (Goon *et al.*, 1986).