MATERIALS AND METHODS

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Four Fusarium spp. viz., Fusarium udum E.Butler (IMI No.334065), Fusarium pallidoroseum (Cooke) Sacc. (IMI No. 334064), Fusarium oxysporum Schlecht (IMI No. 334066) and Fusarium moniliforme J.Sheld. (IMI No.334072), were selected for detailed cultural and pathological studies.

Single spore cultures of the four organisms were prepared by using dummy cutter objective (La Rue Cutter) designed by Key Worth (1959). The cultures were made bacteria free by the method described by Brown (1924). Exenic cultures were made and maintained on potato dextrose agar slants.

Only corning glasswares and B.D.H pure reagents (Analytical grade) were used throughout the investigation. Glassware were cleaned first with "surf" detergent, after that they were rinsed with dilute chromic acid and finally with double distilled water.

To find out the suitable media for growth of the 4 organisms, 25 ml of medium was taken in 150 ml cleaned and oven dried Erlenmayer's conical flasks. They were autoclaved at 15 p.s.i for 20 min. Wherever the medium contained complex substances, which will denature or

decompose at high temperature and pressure were fractional sterilized. For this conical flasks containing medium were subjected to steaming for 30 minutes on three successive days. Inoculation was carried out under asceptic condition in Horizontal Laminar flow (YORCO horizontal laminar flow Type No.YSI-188). Eight to ten days old cultures were used for this purpose. In a few experiments spore suspension containing similar amount of spores was used. Inoculated flasks were incubated at 25 $\pm$ 2°C for 15 days ('CALTON' B.O.D. incubator, Navary Scientific Works Pvt.Ltd.,New Delhi Model NSW 152-12CF). The fungal mat was harvested at the end of incubation period by filtering over previously dried and weighed Whatman's filter paper No.1. Mat was then thoroughly washed with double distilled water, and subsequently dried in an electric oven at 65°C for 50 hours. Dried mats with filter papers were transferred to a desiccator to bring down to room temperature. The filter paper containing fungal mat were rapidly weighed on an electronic single pan analytical balance ("SARTORIUS", Sartoris gmbh gottingen, Germany, Type-1801 accuracy up to 0.0001g). Difference between final and initial weights of filter paper indicated the dry weight of fungal mat alone. Dry weight of mycelial mat and the degree of sporulation were considered as a measure of response of the organisms to different treatments.

There is no noticeable difference among replicates and hence only the average values of three replicates have been recorded and used as quantitative measure for comparing the growth. The change in pH of culture filtrate after incubation period was also recorded. Results were statistically analysed and standard error (S.E.) was calculated by the formula.

$$S.E. = \sqrt{\frac{\text{Mean Square of the error}}{\text{Number of replicates}}}$$

and critical difference (C.D.) was calculated by the formula

$$C.D. = S.E. \times t \times 2$$

where 't' represents probability at 5% level of degree of freedom.

Experiment on the effect of hydrogen ion concentration indicated that pH 5.5 is optimum for both growth and sporulation in all the four Fusarium spp. Similarly the temperature for optimum growth and sporulation was found to be suitable at 25 $\pm$ 2°C. Therefore, initial pH of growth medium in all cultural studies was adjusted to 5.5 by adding 0.1 N HCl or NaOH solution. Similarly temperature was maintained at 25 $\pm$ 2°C

throughout the experiments. pH of the media were determined with the help of Global Digital pH meter.

In order to study the effect of various carbon, nitrogen, and sulphur compounds, the amount of individual substance in the basal medium was calculated and quantity equivalent to that was substituted singly to the basal medium by replacing the original corresponding substance viz. glucose, potassium nitrate, and magnesium sulphate respectively. The amount of poly-saccharide kept similar to that of glucose presented in the basal medium. Medium devoid of glucose, potassium nitrate and magnseium sulphate serve as control for carbon, nitrogen, and sulphur respectively.

Chromatographic studies were conducted in order to know the utilization pattern of different mono, oligo and poly-saccharides. Solutions containing mono-saccharides were autoclaved at 15 p.s.i for 15 minutes while fractional sterilization was carried out for di- and polysaccharides. These solutions were inoculated with Fusarium at a fixed time for 15 days; so that, 1 to 15 days old cultures could be available on 16th day. Fifteenth day old fungal mats were harvested, dried and

weighed to determine the growth. Filtrate of each day was chromatographically analysed to detect the presence of these sugars or other sugars produced during growth.

The paper chromatograms were run in n-butanol-acetic acid - water (4:1:5 v/v) (Rajan et al;1955), for about 7 hours and they were subsequently dried at room temperature. A mixture of 5 vol of 4% aniline , 5 vol of 4% diphenylamine and 1 vol of orthophosohoric acid (Buchan and Savage, 1952) was used as a spraying reagent. Sprayed chromatograms were again dried and then placed in an electric oven at 110°C for 90 sec., to develop the bands. Rf values of various sugars were recorded and they have been given at appropriate place in the text. Presence of starch and inulin were determined with the help of potassium iodide solution (Ghosh and Tandon 1965).

Rate of utilization of some amino acids by the present organisms were studied by silica gel thin layer chromatographic method. KNO_3 of the basal medium was replaced by different amino acids. They were supplemented singly in a proportion which was equivalent to that of nitrogen present in the basal medium. Rest of the methods were similar to those described for carbohydrates except, that the chromatograms were sprayed with 0.1% ninhydrin in

n-butanol. The sprayed TLC plates were then dried at room temperature and bands were developed by placing in an electric oven at 90°C for 10 minutes.

Seeds of pigeon pea variety DPPA 85-5, LRG 30, and ICP 2376 were procured from pulse directorate, Kanpur and one local variety from market. These varieties were grown in 4 different plots (360 x 120 cm) in our botanical garden. At an interval of 20 days, the plants were harvested for four months period and average length of the roots and shoots as well as their fresh weights were estimated.

The method used for taking out soil samples was similar to that used by Saxena and Mehrotra (1952) and Sarbhoy (1963). Soil samples were collected from all the four plots, where 4 different varieties were growing. Sampling was done separately and randomly in different plots at different depths (15 cm and 30 cm). Samples from all the depths of the same plots were mixed together and they were packed in sterilized polythene bags and brought to the laboratory. Samples were air dried, crushed and passed through 2 mm sieve. Soil dilution plate method, (Parkinson and Thomas, 1965) was employed to isolate various micro- organisms from non rhizosphere soil.

Soil dilution and plate count method (Timonin, 1940) was used for qualitative and quantitative analysis of microflora in the rhizosphere. Roots of five plants with adherent soil were transferred to weighed flask containing 100 ml of sterile distilled water. After thorough shaking, the roots were removed from the flask and suitable dilution of soil suspension were made. One ml of deserved dilution (1:10,000 for fungi and 1:100,000 for bacteria) was plated in sterilized petri dish contained 10 ml of potato dextrose agar medium. Plates were incubated at 25 ± 2°C and were examined after 4 days for fungi and bacteria. The fungal and bacterial colonies were counted taking 5 replicates of three petri dishes. Occurrence of bacteria and actinomycetes and total number of fungi per gram of oven dry rhizosphere soil was recorded.

Field experiments were conducted at botanical garden. Soil was ploughed to a depth of 25 to 30 cm, levelled to prepare a seed bed, and irrigated by flooding. The plots were covered (solarized) for 45 days (April 15th to June 30th 1989) continuously with ultraviolet-stabilized 0.03 mm, 0.06mm and 0.135 mm thick transparent polyethylene sheets (Katan et al.,1983). The experimental design was a randamized complete block with treatments (solarized - irrigated, non-solarized-irrigated) replicated five times

concurrently. Each replicated plots had a dimension of 3.65 x 1.22 M. To avoid contamination among treatment a 30 cm border was left between plots. Solarized and non-solarized plots were irrigated ones in a week time until the plastic sheets were removed. Soil moisture content was measured with the help of soil moisture meter (Type DN-33, Eleco Private Limited, Hyderabad). Soil temperature (°C) were measured in three replication,in plots using immersion type thermometer (accuracy $\pm$ 0.5°C) at depths of 6 and 12 cms. Coloured polyethylene sheets viz. Red, Black, Blue and Green were procured from local market and solarization was conducted for 45 days. Irrigation, replication, mode of temperature and moisture recording were same as mentioned above. Pre-solarization sampling for microflora study was done 12 hours after irrigation and post-solarization sampling was soon after removing the polyethylene mulching.

Green leaves of 'Neem' (Azadirachta indica A.Juess. 'Aak' (Calotropis procera R.Br.) and 'Nilgiri' (Eucalyptus globulis Labill) were collected and chopped into small pieces. 5.0 Kg. leaves per plot were incorporated and polyethyelene sheets of 0.06 mm thickness were covered for 45 days. Presence and absence of micro-organisms were recorded.

In thermal-death point study, capillary tubes of about 5 cm length were filled with spore suspension in sterilized distilled water and fuse sealed at both ends. These capillary tubes were then fastened to the lower end of a thermometer which was then dipped in water bath and maintained at a particular temperature for 10 min. Capillary tubes were then crushed with the help of sterilized forceps in petridishes containing solidified sterilized P.D.A. medium. Absence of the growth indicated that, spores died at that particular temperature.

For pathogenecity tests, studies were conducted in pots. 22.5 cm diameter earthen pots were used for this purpose. In order to incorporate fungus to the pot soil, cultures of Fusarium species were made in 3% maize meal sand medium (Singh 1977). This was prepared in 250 ml. conical flasks. Each flask was first filled up by 150 g.of sand and maize meal mixture, (150 g of dry clean sand + 4.5 g of crushed maize grains). 20 ml of distilled water was carefully added (100 g dry sand holds 20 ml water at saturation). So, 20 ml for 150 g sand maize meal mixture gives about 65% saturation). Such flasks were then autoclaved for 30 min. at 20 p.s.i and were later inoculated with agar inoculum discs (Garrett, 1936) from a colony margin of 10 days old cultures of Fusarium on

P.D.A. Flasks were incubated for 30 days at room temperature and in between shaken after 15 days to distribute the fungus inside the flask. When the above flasks containing 3% maize meal culture of Fusarium species were well grown, they were then ready for incorporation to soil. 5 gms of the inoculum were blended per pot containing garden soil of pH 6.5. Moisture content of the soil were maintained throughout the experiment. After 48 h of inoculation of fungus to soil, seeds of pigeon pea varieties were sown in pots, in such a way that each pot contains 5 seeds. These pots were placed under direct sun light. Degree of wilting was recorded up to a period of 2 months. Small parts of wilted plants were collected and brought to laboratory. These parts were first washed in water and then dipped in 0.5% mercuric chloride solution for 30 sec. They were washed in sterilized double distilled water. These surface sterilized root, stem and color regions were split into 4 pieces with the help of a sterile scalpel under asceptic condition. The splited pieces were placed on sterilized P.D.A. medium contained in petri dishes. Petri dishes were then incubated for 10 days at 25 $\pm$ 2°C. After 5 days, isolation and sub-culturing were done from advancing mycelial margin. These sub-cultures were made pure and identified with the help of morphological characters.

For fusaric acid detection, Fusarium species were grown for 20 days in 100 ml of Asthana and Hawker's liquid medium in 500 ml Erlenmeyer flasks. The culture filterates were collected and centrifuged at 2000 rpm for 20 min. The clear supernatant was taken and the pH was adjusted to 4.0 by adding 2 N HCl. Hundred milliliter of the filterate was mixed with equal volumes of ethylacetate atleast for 4 times in a separating funnel allowing 15 min for each extraction. All ethyle acetate extracted were mixed and evaporated to dryness. One to two milliliter of ethanol was added to dissolve the residue. Drops of known volume (0.005 ml) of filterates as well as an index solution of fusaric acid were kept on Whatman's filter paper No.1. Spotted chromatogram were run ascendingly for 10 to 12 hrs, in butanol, formic acid and water (75:15:10). Chromatograms were dried and sprayed by bromophenol blue (0.04% in 90% ethylalcohol). Fusaric acid gives a yellow colour.

Eight to ten days old Fusarium cultures on PDA were used for morphological studies with the help of scanning electron microscope. A small portion of the culture along with the medium was taken for specimen preparation. The primary fixation was carried out with 3% glutaraldehyde Glutaraldehyde fixative is prepared in 0.2 M cacodylate buffer. (Sodium cacodylate $(Na(CH_3)_2.3H_2o)$ 42.8 gm in 6.9

ml N HCl and made up to 1000 ml with double distilled water). To prepare fixative, 60 ml of 25% glutaraldehyde solution was made up to 250 ml with double distilled water and 250 ml of 0.2M cacodylate buffer added to it. To this solution add 12.5 mg of anhydrous $CaCl_2$. The specimens were very delicate and soft, so secondary fixation was omitted. After 4 hrs. of primary fixation, the specimens were washed in 0.1M cacodylate buffer. Then they were trimmed to the proper size and stored in buffer.

Dehydration was carried out in acetone series of 30, 50, 70, 80, 90 and 95% solutions. Series were prepared from filtered dry acetone. Specimens were kept in each grade for 15 min. Two change of 15 min each were given in 100% dry acetone. The whole process was done at 4°C. Critical point drying was performed on polaron E3000. The procedure for it is given below:

- circulated cold water in the apparatus to bring the chamber to 20°C,
- fill the specimen loading basket with dry acetone.
- load the specimen in chamber and close the apparatus tightly,
- allow the liquid CO_2 into the chamber by opening the inlet valve connected to CO_2 cylinder. Open the vent valve to avoid back pressure,
- with the vent valve slightly opened to maintain the

level of the liquid, open the drain valve to remove acetone. This process of 'flushing' has to be carried out two-three times for 3-5 mins.each in order to drain off all the acetone from the specimen chamber,

- after flushing is completed fill the chamber with liquid CO_2 and leave for one hour to allow impergnation,
- drain off the whole liquid CO_2 and refill the chamber with liquid CO_2,
- close the inlet valve and allow the liquid CO_2 to fall to the level of the specimen boat,
- warm up the chamber by running warm water (36-38°C) and when the temperature rises to 32.5°C the liquid CO_2 would have evaporated and drying completed. (critical point of CO_2= 31.5°C at 1100 p.s.i),
- carefully and gradually vent the CO_2 to avoid condensation,
- remove the specimen from the chamber.

Oried specimens were then mounted on aluminum stub (specimen holder) with conducting paint and stored in a desiccator. The stub along with specimens were sputter cotted using gold as target. Balzer SCD020 sputter device was used for this. The entire process was carried out under vacuum in an inert atmosphere using argon gas.

Current - 21.5 m.A

Pressure - 0.05 mbar

Gas - argon

Distance between cathode and specimen - 30 mm

Time 1 min.

The specimens were observed under Philips SEM-501B scanning electron microscope having a capacity to magnify X80,000 and resolving power of 5 nm. Photographs were taken using polaroid camera with 125 mm fast film (400 ASA). Exposed films were developed in D-76 fine grain developer at 18°C for 9 min, washed twice in tap water and fixed in Agfa 301 fixer (20°-24°C) for 30 mins. Again washed in running water (20°-24°C), rinsed in the wetting agent (Agfa A913) and dried. Prints were taken on Sterling normal glossy photopaper.