

4. MATERIALS AND METHODS

4.1 COLLECTION OF SEED SAMPLES

One hundred ninety seven seed samples of 7 different crops viz., *Brassica campestris* L., (mustard, 32 samples), *Capsicum annuum* L. (red chillies, 40 samples), *Helianthus annuus* L. (sunflower, 35 samples), *Linum usitatissimum* (linseed, 20 samples), *Pennisetum typhoides* (Burm.) Stapf. & Hubb. (pearlmillet, 20 samples), *Triticum aestivum* L. (wheat, 20 samples) and *Zea mays* L. (corn, 30 samples), were collected from different localities of Punjab and Sindh. These samples were analyzed for seed-borne *Fusarium* species by standard techniques as recommended by International Seed Testing Association (1976).

4.2 ISOLATION OF SEED-BORNE MYCOFLORA

4.2a Standard Blotter Method (Anon., 1976)

Four hundred seeds of each sample were plated on 3 layered moistened blotter discs in 9 cm glass Petriplates @ 25 seeds per plate, except in case of corn and sunflower where 15 seeds were plated. Plates were incubated for 7 days at $22 \pm 1^{\circ}\text{C}$ in Eyela La 1000 low temperature incubator. Incubated seeds were examined under compound light microscope at 4-40X magnifications.

4.2b Deep-freezing Method (Limonard, 1968)

Four hundred seeds of each sample were plated @ 25 seeds per plate and 15 seeds per plate (in case of corn and sunflower) on 3 layers of sterilized moistened blotter

discs in 9 cm glass Petriplates and incubated at $22\pm 1^{\circ}\text{C}$ for 24 hrs. followed by incubation at $-20\pm 1^{\circ}\text{C}$ for 24 hrs. and then at $22\pm 1^{\circ}\text{C}$ for 5 days.

In both methods fungi were isolated and purified on potato dextrose agar (PDA), corn meal agar (CMA) and speziellier nährstoffarmer agar (SNA). The isolated fungi were identified according to Booth (1971), Ellis (1971), Barnett and Hunter (1972), Domsch *et al.* (1980), Nelson *et al.* (1983), Joffe (1986), Pascoe (1990a,b), Nirenberg (1990), Singh *et al.* (1991).

4.3 SEED TRANSMISSION STUDIES

Healthy-looking seedlings of sunflower obtained from samples where occurrence of *Fusarium* spp. was high in standard blotter test were transplanted to 6 in. earthen pots containing sterilized soil to observe seed to seedling transmission of plant pathogenic *Fusarium* spp. that may produce symptoms of disease(s) in later stages of growth. Component plating of different parts of plant was carried out (Baker and Cook, 1974). After observing various symptoms the diseased plants were uprooted, whereas remaining healthy looking plants were uprooted after flowering.

4.4 PATHOGENICITY TEST

Spore suspension from 2 weeks old cultures of test *Fusarium* spp. were adjusted to 50,000 conidia/ml in sterilized dist. water. This conidial suspension was mixed @ 1:20 in sterilized moistened (20%) corn meal-sand medium (Hashmi, 1988) and

incubated at $25 \pm 1^\circ\text{C}$ for 21 days. Freshly prepared corn meal-sand inocula of *Fusarium* spp. were mixed with sterilized soil in different percentages viz., 0.5, 1, 2, 5 and 10% to detect virulence of pathogen at various inoculum levels. All experiments alongwith control were carried out in triplicates.

Different components of healthy and infected sunflower seedlings viz., roots, hypocotyl, stem, leaves and inflorescence were washed in tap water, surface sterilized with 2% NaOCl for 5 min and transferred to PDA containing Penicillin and Streptomycin. Plates were incubated for 5 days at $22 \pm 1^\circ\text{C}$ to confirm infection and colonization by fungi in components of seedlings (Baker and Cook, 1974). Infection and colonization percentages were calculated as follows:

$$\text{Infection \%} = \frac{\text{No. of plants infected by a pathogen}}{\text{Total no. of plants}} \times 100$$

$$\text{Colonization \%} = \frac{\text{Total no. of pieces infected by a pathogen}}{\text{Total no. of pieces}} \times 100$$

4.5 SOIL USED FOR ARTIFICIAL INFESTATION

Soil used for artificial infestation was obtained from the experimental plots of the Department of Botany, University of Karachi. The soil was sandy loam (sand:silt:clay, 70:21:9), pH ranged from 7.1-7.9 with 42% maximum moisture

holding capacity (Keen and Raczkowski, 1921). The soil was screened through 2 mm sieve, moistened and sterilized at 15 psi for 1 hr before use.

4.6 MEDIA USED

4.6a Spezieller Nährstoffarmer Agar (SNA) (Nirenberg, 1976)

One liter of medium in dist. water contained 1g KNO₃, 1g KH₂PO₄, 0.5g MgSO₄·7H₂O, 0.5g KCl, Glucose 0.2g, Sucrose 0.2g and 15g agar. Cultures grown on this low nutrient medium were used for morphological study and for producing conidial suspension of *Fusarium* spp. Sporulating cultures were obtained by placing autoclaved filter paper strips at the periphery of actively growing colonies.

4.6b Malt Yeast Glucose Peptone Agar (YM) (Wickerham, 1951)

This medium was used to obtain cell suspension and for *in vitro* interactions of yeasts with *Fusarium* spp. Broth was made with 3g dried malt extract, 3g dried yeast extract, 5g mycological peptone and 10g D-glucose in 1L double dist. water and adjusted to pH 4.2. Twenty g agar was added for solid medium.

4.6c Yeast Extract Sucrose Agar (YES) (Frisvad and Filtenborg, 1983)

Yeast extract sucrose agar was used for the production of mycotoxins by *Fusarium* spp. The medium contained 150g sucrose, 20g yeast extract and 15g of agar. Final volume of the medium was adjusted to 1 liter.

4.6d Corn Grits Agar (CGA) (Thrane, 1986)

Corn grits agar was another medium extensively used for the production of mycotoxins by *Fusarium* spp. Seventy-five g of corn grits were boiled for about 2 hrs. and filtered. After adding 15g agar the final volume was adjusted to 1L by dist. water and autoclaved. Solution of trace metals containing $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 ppm) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (100 ppm) was added before pouring into the Petriplates.

4.6e Rice Meal Agar (RMA) (Thrane, 1986)

Rice meal agar was used for the production of mycotoxins by *Fusarium equiseti*. Rice meal was prepared by grinding 75 g parboiled rice grains for 3 minutes. This rice meal was boiled for about 2 hrs. and filtered. After adding 15 g agar the final volume was adjusted to 1 L by dist. water and autoclaved. As in corn grits agar, solution of trace metals was added before pouring into the medium.

4.7 IN VITRO INTERACTION BETWEEN KILLER YEASTS AND *FUSAIRUM* SPP.

In vitro interactions of killer strains viz., Y179-*Bensingtonia miscanthi*, Y21-*Bullera pseudoalba*, Y169-*Bullera megalospora*, YF19-*Lipomyces starkeyi*, Y16-*Pichia anomala* and Y20-*Sporidiobolus ruineniae* were carried out with *Fusarium* spp. These yeast species were obtained from Karachi Mycological Herbarium Collection (KMHC), University of Karachi. A loopful of yeast culture was

streaked in triplicate plates on malt-yeast-glucose-peptone agar (YM) and a 5 mm disc of the test *Fusarium* spp. was inoculated 70 mm away from the streak. Three Petriplates of each pathogen were inoculated as control (i.e. without streak of yeast strain).

In another set of experiment, yeast culture was streaked on YM and incubated at $25\pm 1^\circ\text{C}$ and the test fungus was inoculated opposite to the streak after 2,4,6,8 and 10 days. Each interaction was carried out in triplicate and assessed using a key based on four separate modes of interacting colony growth as given below:

- A. Mutually intermingling growth where fungi grew into one another without any macroscopic sign of interaction.
- B. Slight inhibition where the fungi approached each other and a narrow demarcation line, ca. 1-2 mm, between the two colonies was clearly visible.
- C. Mutual inhibition at a distance of $> 2\text{mm}$
- D. Interaction where the killer yeasts killed pathogenic fungus growing over it and subsequently mycelium near the yeast colony was also killed. The killed mycelium of pathogenic fungus became water soaked and occasionally dark brown discoloration appeared in the medium.

Observations were made from 3rd to 9th day of inoculation, growth of fungal colonies was measured and inhibition zone was recorded. Final data were recorded when the fungi had achieved an equilibrium after which there was no further

alteration in the growth pattern. Inhibition of pathogen growth was calculated in percentage as:

$$\text{Inhibition \%} = \frac{r_2 - r_1}{r_2} \times 100$$

Where r_2 denotes radial growth of the pathogenic fungus in control plate and r_1 denotes its growth in plate opposed by the killer yeast (Fokkema, 1973).

4.8 *IN VIVO* EFFECT OF KILLER YEASTS ON SUNFLOWER

Effect of killer yeasts viz., Y21-*Bullera pseudoalba*, Y16-*Pichia anomala* and Y20-*Sporidiobolus ruineniae* that showed promising results during *in vitro* interactions were tested on sunflower seedlings by soil amendment method. Killer yeasts were grown in YM broth for 1 week. The cells were harvested by filtration (by haemocytometer) and re-hydrated in double dist. sterilized water at a concentration of 10^7 cells/ml and mixed in sterilized soil at 0.5, 1, 2, 5 and 10% concentrations. Three earthen pots were prepared for each concentration alongwith 3 pots of control. Three surface sterilized seeds of sunflower were sown in each pot and incubated with regular watering. Plants were uprooted after flowering. Root and shoot lengths as well as fresh and dry weights of plants were recorded and component plating was carried out to observe whether yeasts had colonized the plant parts or not.

4.9 BIO-CONTROL EXPERIMENTS

The killer yeasts viz., Y21-*Bullera pseudoalba*, Y16-*Pichia anomala* and Y20-*Sporidiobolus ruineniae* were also used as bio-control agents against *Fusarium*

chlamydosporum, *F. equiseti*, *F. oxysporum*, *F. pallidoroseum*, *F. solani* and *F. sporotrichioides*. These experiments were carried out by soil amendment and seed treatment methods.

4.9a Soil Amendment Method

During soil amendment test 5 different concentrations of killer yeasts viz., 0.5, 1, 2, 5 and 10% were used for each *Fusarium* sp. The best pathogenic level of each *Fusarium* sp. was selected from experiment under section 4.4. Each experiment was prepared in triplicate along with control that received no killer yeasts. Colonization percentages were calculated after taking root, shoot, length and fresh and dry weights of plants.

4.9b Seed Treatment Method

Sunflower seeds were treated with the suspension of killer yeasts, previously prepared in sterilized solution of "Kateera Gum" (a type of gum that instead of swelling, easily dissolves in water), at concentrations of 10^6 , 10^7 and 10^8 cells/ml. Treated seeds were dried and sown in triplicate earthen pots containing sterilized soil amended with 6 different *Fusarium* spp. Component plating of plants was carried out after flowering on PDA and colonization percentages of roots by *Fusarium* spp. were calculated.

4.10 DETECTION OF SECONDARY METABOLITES OF *FUSARIUM* SPP.

4.10a Thin Layer Chromatography (TLC)

Profiles of secondary metabolites of *Fusarium* spp. were detected by thin layer chromatography (TLC) technique. Four mm discs from the center of 12-14 days old culture were removed. A drop of extraction liquid (chloroform: methanol, 2:1 v/v) was placed directly on the disc and the mycelial side of the disc was gently pressed against the application line on TLC plate. The disc was immediately removed from the TLC plate and spots were allowed to dry. Griseofulvin was used as an external standard in all analyses (Frisvad and Filtenborg, 1983).

Chromatographic plates were developed in the following systems:

System I: (Kamimura *et al.*, 1981)

Developing solvent: toluene-acetone-methanol (TAM) (5:3:2, v/v)

Spray 1 20% (w/v) AlCl_3 in 60% ethanol

Spray 2 20% H_2SO_4 in water

Spray 3 0.32% (w/v), 2,4-dinitrophenyl-hydrazine (2-4 DNPH) in 2N HCl

System II: (Filtenborg *et al.*, 1983)

Developing solvent: toluene-ethyl acetate-90% formic acid (TEF) (5:4:1, v/v)

Spray 1 20% (w/v) AlCl_3 in 60% ethanol

Spray 2 20% H_2SO_4 in water (Kamimura *et al.*, 1981)

Spray 3 0.5% (v/v), *p*-anisaldehyde (ANIS) in methanol-acetic acid and conc. H_2SO_4 (17:2:1) (Burmeister *et al.*, 1974)

When front of the system reached a height of 150 mm above the application line on TLC plate, the development was interrupted, plates were dried and examined before and after chemical treatments under visible as well as UV lights at 254nm and 366nm. All retardation factors (Rf) were recorded relative to griseofulvin (relative Rf=1.00).

All isolates of *Fusarium* spp. were tested for toxins and secondary metabolites and compared with their standards (Hashmi, 1988). Toxins and secondary metabolites used as standard were bostrycoidin, *cis*-dihydrofusarubin, vomitoxin (deoxynivalenol), diacetoxyscirpinol, griseofulvin, isomarticin, marticin, moniliformin, nectriafurone, zearalenone and zearalenol (Table 4.1, Fig. 4.1-2).

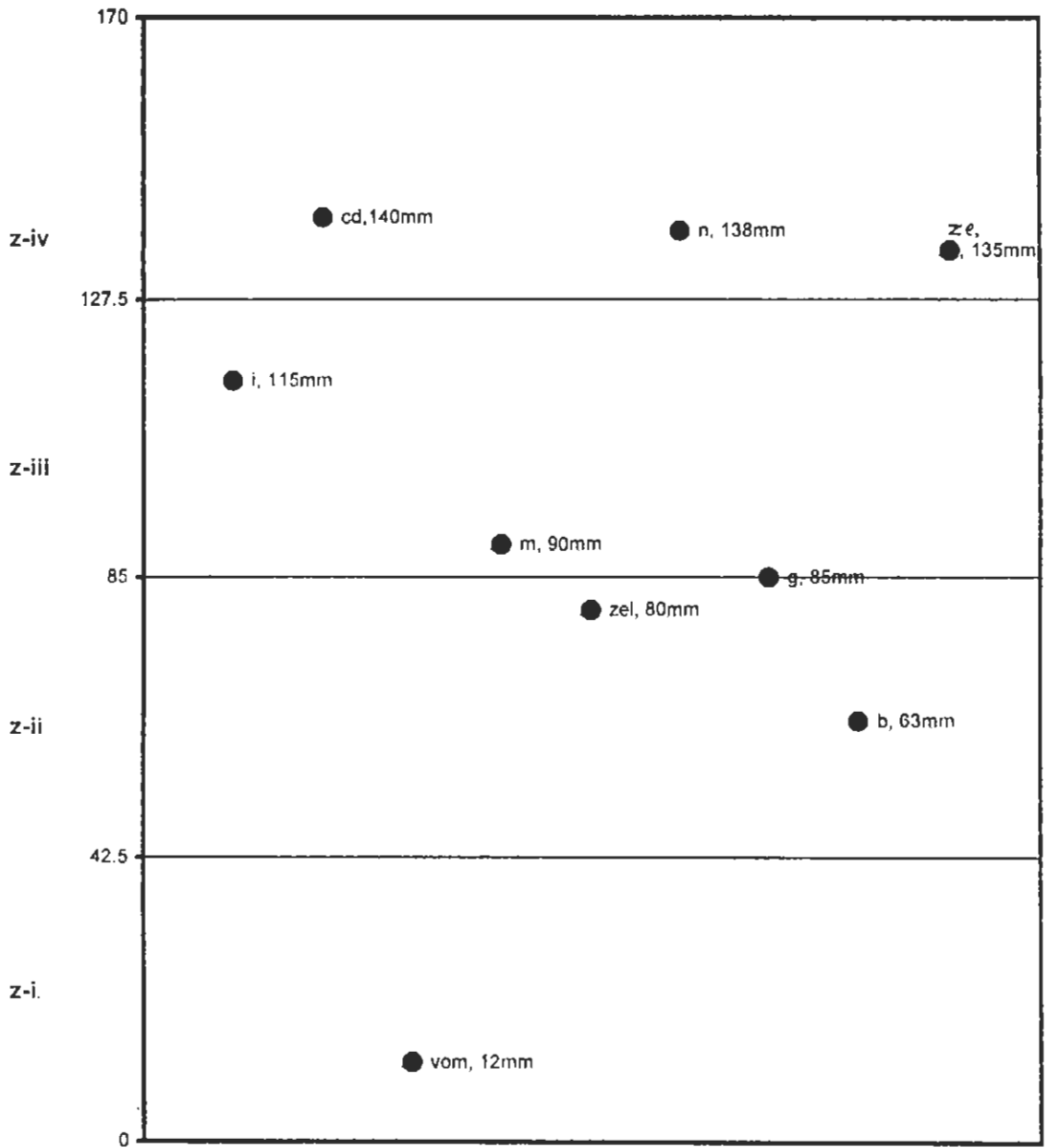
4.10b Column Chromatography

For column chromatography, mass cultivation of *Fusarium* spp. viz., *F. chlamydosporum*, *F. equiseti* and *F. pallidoroseum* was carried out in corn grits and rice grits broth media. Thirty L of broth medium were inoculated by a *Fusarium* sp. and incubated for 30 days with continuous shaking. The cultures were then filtered by 4G sintered glass funnel fitted with 1L flask. Secondary metabolites were extracted from culture filtrates of *Fusarium* spp. by twice partitioning with absolute chloroform in one-liter glass separating funnel. Using rotary evaporator solvent was evaporated, the mixture of secondary metabolites was collected in a separate vial and weighed. This mixture was dissolved in

Table 4.1: Characteristics of standard secondary metabolites

Secondary Metabolites	Relative Rf values in		Treatments	Fluorescing color
	TAM	TEF		
<u>Mycotoxins</u>				
Vomitoxin (deoxynivalenol)	0.88	0.14	20%AlCl ₃	Light blue
Griseofulvin	1.00	1.00	366nm UV	Bright blue
Moniliformin	0.27	ND	2,4-DNPH	Brown
Nectriafurone	ND	1.62	20%AlCl ₃	Green
Zearalenone	1.66	1.58	254nm UV	Blue green
Zearalenol	1.22	0.94	254nm UV	Blue green
<u>Pigments</u>				
Bostrycoidin	ND	0.74	20% AlCl ₃	Orange
<i>cis</i> -Dihydrofusarubin	1.77	1.64	366nm UV	Bright green
Isomarticin	0.12	1.35	254nmUV	Yellow orange
Marticin	0.13	1.05	366nmUV	Pink

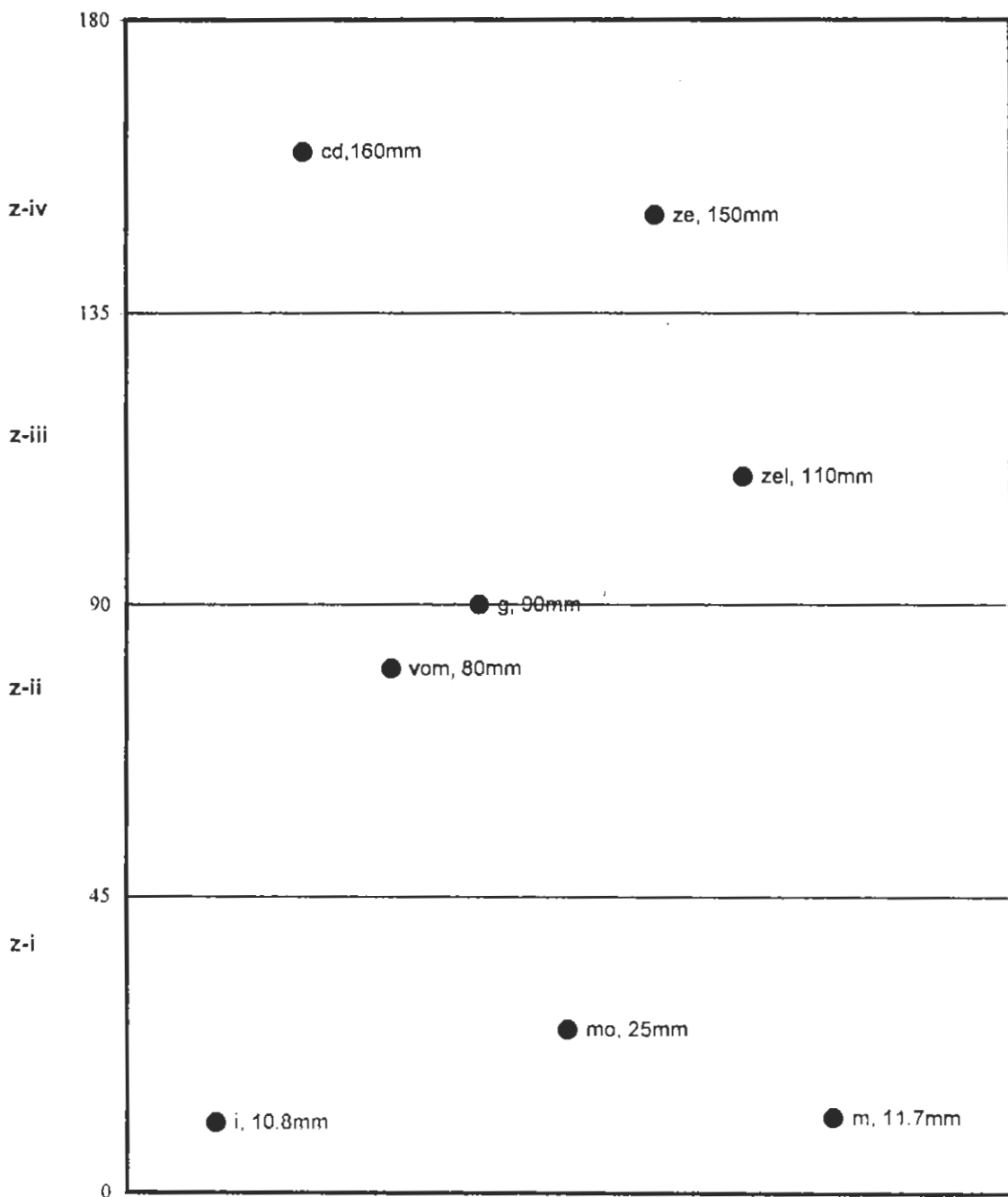
ND= Not detected



b : bostrycoidin cd : *cis*-dihydrofusarubin
 g : griseofulvin i. : isomarticin
 m : marticin n : nectriafurone
 vom : vomitoxin ze : zearalenone
 zel : zearalenol

z-i to z-iv : zones of TLC each at 42.5 mm

Fig. 4.1: Replica of TLC of standard secondary metabolites in TEF.



cd : *cis*-dihydrofusarubin g : griseofulvin
 i : isomarticin m : marticin
 mo : moniliformin vom : vomitoxin
 ze : zearalenone zel : zearalenol

z-i to z-iv : zones of TLC each at 45 mm

Fig. 4.2: Replica of TLC of standard secondary metabolites in TAM.

chloroform/methanol (50:50) and taken out in a 100 ml glass plate. A sufficient amount (about five-times than organic compound) of column silica gel 60G was added and allowed to dry before loading into the column. Column running was started with absolute hexane and collected in 25 ml glass vials. At least 500 ml of pure hexane was run. Solution in the vials were concentrated by rotary evaporator and checked by TLC.

If there was no evidence of any compound on TLC, the polarity of column system was increased by gradual addition of chloroform in hexane from 3 to 100%. After 100% chloroform, absolute methanol from 3 to 20% was gradually added to increase polarity of the column system and finally the column was washed with 100% absolute methanol. Different fractions obtained in the vials were checked and the similar fractions were mixed on the basis of their TLC pattern. Major fractions thus obtained were labeled, weighed and stored at low temperature to avoid desiccation.

Significant spots of compounds were purified either by TLC or through pencil column. Bands of compounds were scratched and dissolved in appropriate solvent, like chloroform or methanol. High Performance Thin Layer Chromatography (HPTLC) was used to confirm purity of the compounds.

4.11 SPECTRAL ANALYSES OF SECONDARY METABOLITES

The low resolution EIMS (Electron Ionization Mass Spectroscopy) was recorded on a Varian Mat 312 double focussing mass spectrometer connected to DEC PDP 11/34 computer system. High Resolution Electron Ionization Mass Spectroscopy (HREIMS) was recorded on JEOL, the MS Route JMS 600H Japan and JMS Hx110 JEOL, Japan GC-HP5890. The FAB+ve ion was also recorded on JEOL. The MS Route JMS 600H Japan. The UV spectra (Ultra Violet spectroscopy) were recorded on a Shimadzu UV 240 instrument. The IR spectra (Infra Red Spectroscopy) were recorded on a Jasco-1 IR spectrophotometer. The ¹H NMR spectra (Proton Nuclear Magnetic Resonance) were recorded in CDCl₃ or in CD₃OD at 300 and 400 MHz on Bruker AM-400 and AMX-300 NMR spectrometers with TMS as internal standard.

4.12 EXPERIMENTAL DESIGN AND ANALYSES OF DATA

A computer package SPSS for Windows, version 10 was used for statistical analysis. Using this program standard error was calculated for the data of seed-borne *Fusarium* spp. isolated from different crops. During experiments on pathogenicity, transmission and biocontrol of *Fusarium* spp., a randomized complete block design with 3 replicates of each treatment was used and data were analyzed by analysis of variance (ANOVA).