

6. DISCUSSION

Twelve *Fusarium* spp. were isolated by standard blotter and deep-freezing methods from seeds of seven economically important crops. Seeds of pearl millet highly favored the growth of *Fusarium* spp., where all 12 species were encountered. Fakhrun Nisa and Hashmi (1992) reported same 11 *Fusarium* spp. except *F. anthophilum* on pearl millet. *Fusarium solani* appeared to be the most common species isolated from all crops in both methods, whereas, *F. moniliforme* and *F. pallidoroseum* were common in all crops only in standard blotter method. The isolated *Fusarium* spp. were compared with that reported by Iftikhar *et al.* (1993), Richardson (1979, 1981 and 1983) and Abbas *et al.* (2002) and found that *F. anthophilum* on pearl millet and corn, *F. chlamydosporum* on mustard and capsicum, *F. equiseti* on sunflower and *F. proliferatum* and *F. subglutinans* on mustard and linseed to be newly reported species from Pakistan.

Besides *Fusarium* spp. 61 species belonging to 40 genera of parasitic and saprophytic fungi were also isolated from seed samples of these crops. It may be mentioned that *Absidia corymbifera*, *Aspergillus tamarii*, *Blakeslea* sp., *Cephalophora irregularis*, *Exserohilum holmii*, *Scopulariopsis* sp., *Tritirachium* sp. and *Ulocladium tuberculatum* were newly recorded fungal species from *Capsicum annuum*. Moreover, a *Myrothecium* sp., which is a seed-borne pathogen of sunflower, was isolated from capsicum by both methods. It is possible that in

absence of its own host, it passes a transitory period on capsicum for survival. Hashmi (1988) also isolated *Myrothecium roridum* and *M. verrucaria* from seed samples of capsicum. Similarly *Acremonium* sp., *Arthrobotrys* sp., *Cephalophora tropica*, *Chaetomium crispatum*, *C. spinosum*, *Cladobotryum varium*, *Drechslera bisepata*, *Emericella nidulans*, *Gonatobotrys simplex*, *Humicola* sp., *Mucor* sp., *Myrothecium verrucaria*, *Phialophora* sp. and *Verticillium* sp. were isolated only from sunflower.

Seed transmission of *Fusarium* spp. was observed in healthy-looking seedlings of sunflower obtained from seed samples where occurrence of *Fusarium* spp. was highest. *Fusarium moniliforme*, *F. oxysporum* and *F. solani* were found to be transmitted from seed to plant. *Fusarium chlamydosporum*, *F. equiseti*, *F. pallidoroseum*, *F. proliferatum* and *F. sporotrichioides* were not transmitted in seedlings probably due to low inoculum on the seed. Whereas, *F. anthophilum* and *F. subglutinans*, which were not detected in sunflower seeds were transmitted in sunflower plants. Absence of these *Fusarium* spp. indicates that they were internally seed-borne and directly entered into the system of plants indicating their systemic nature.

On the other hand *Macrophomina phaseolina* (charcoal rot pathogen of sunflower) was isolated from rotted seedlings alongwith saprophytic fungi, whereas, root rot pathogen, *Rhizoctonia solani* was associated with partially wilted seedlings.

Ahmed *et al.* (1994) investigated the transmission and virulence of different pathogens in sunflower and found that *Rhizoctonia solani* and *Macrophomina phaseolina* were lesser pathogenic than *Fusarium oxysporum*.

In pathogenicity experiments, *Fusarium* spp. produced wilting as well as a number of diseases in sunflower plants. It is interesting to note that *F. anthophilum*, *F. chlamydosporum*, *F. equiseti*, *F. longipes*, *F. proliferatum*, *F. scirpi*, *F. sporotrichioides* and *F. subglutinans*, which are not reported as pathogens of sunflower caused significant wilting, seedling-, root-, collar- and stem rots, damping off, stunting and tip burn diseases and reduced the growth of plants. Furthermore, *F. anthophilum*, *F. chlamydosporum*, *F. moniliforme*, *F. oxysporum*, *F. proliferatum*, *F. solani* and *F. subglutinans* were also isolated from healthy-looking plants. Several factors like weather conditions, pH values, soil salinity and virulence of pathogen determine the pathogenicity of *Fusarium* spp.

Fusarium chlamydosporum, *F. pallidoroseum*, *F. solani* and *F. sporotrichioides* caused tip-burn, collar-, seedling- and stem rots in 15-day old sunflower seedlings. Pizzinatto and Menten (1991) reported the same pathogenic nature of these *Fusarium* spp. in cotton seedlings. Wilting and root rot symptoms were observed after 30 to 45 days in sunflower plants by *F. chlamydosporum*, *F. equiseti*, *F. pallidoroseum* and *F. solani*. Kapoor and Kar (1988) reported wilting in tomato by same *Fusarium* spp. *Fusarium equiseti* and *F. longipes* reduced growth and

produced stunting in sunflower seedlings. Similar results were observed by Soleimany *et al.* (1993) on cotton seedlings due to pathogenic activities of *F. pallidoroseum* and *F. equiseti*.

Apart from pathogenicity, experiments were also carried out for the biological control of *Fusarium* spp. In the present study *in vitro* antagonistic activity of 6 killer yeasts was detected against 12 seed-borne pathogenic *Fusarium* spp. Y21-*Bullera pseudoalba*, Y16-*Pichia anomala* and Y20-*Sporidiobolus ruineniae* showed significant antagonistic activity. Most pronounced activity was exhibited by Y21-*Bullera pseudoalba* against *Fusarium anthophilum* and *F. solani* (D type interaction) where the killer yeast not only antagonized but also killed the mycelium of the pathogen. Y20-*Sporidiobolus ruineniae* also inhibited *F. solani* significantly (D type interaction). It may be mentioned here that biological control activities of species of *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula*, *Saccharomyces*, and *Saccharomyces* are known against several plant pathogens (Suzzi *et al.*, 1995; Schisler *et al.*, 1995; Wilson *et al.*, 1993) but information about these killer yeast species is rather scanty. However, recently Mushtaq (2002) used these killer yeasts as antagonistic agents against plant pathogenic sclerotial fungi such as *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*.

Another significant point is that when these killer yeasts were applied in soil they increased growth and vigor of sunflower plants. Mushtaq (2002) also found

similar results of these killer yeasts on corn. Ehteshamul-Haq and Ghaffar (1992) reported plant growth promoting rhizobacteria (*Bradyrhizobium japonicum*) to enhance plant growth and vigor of sunflower as well as bio-control activity against soil borne pathogens. When these killer yeasts were mixed with soil infested with *Fusarium* spp. they significantly reduced infection and colonization percentages. Killer yeasts could be used in various fermentation processes since they can eliminate sensitive spoilage yeasts species and produce dominant population at the end of the process and the biomass of yeast, which is a byproduct could be used for the control of plant diseases.

In the present study the TLC profiles of secondary metabolites of 12 *Fusarium* species have indicated their definite parameters in day light as well as under UV light (254-366 nm). These metabolites showed consistent profiles in a particular *Fusarium* sp. when agar plug method (Filtenborg and Frisvad, 1980) was used alongwith the developing system TEF (Filtenborg *et al.*, 1983) before and after chemical treatments. The sensitivity of the method seems to be adequate for the differentiation of isolates of 12 *Fusarium* spp. tested since it proved useful for the screening of extracellular pigments and mycotoxins.

For identification of species of *Fusarium* use of macro- and micromorphological criteria as well as various secondary metabolites and colors produced in the thallus or secreted into the medium have been

emphasized (Filtenbrg *et al.*, 1983; Thrane, 1986). Most of the secondary metabolites are produced consistently by the isolates of a given species, irrespective of geographic and substrate source. Like morphology, the production of secondary metabolites are a part of the differentiation process in fungi since particular metabolites may be produced by diverse groups of fungi or even higher plants (Samuels, 1984; Moss, 1984). Production of the same mycotoxins may not necessarily indicate a close relationship between different fungi (Frisvad and Filtenborg, 1983), however, a complete profile of secondary metabolites seems to be species specific. The chloroform:methanol (2:1) system appeared to be sufficient for optimum extraction of toxins and other secondary metabolites. It may be mentioned that this solvent has been used for lipid extraction (Hanahan, 1960). Release of the toxins from the mycelium may be due to the polar solvent breaking the protein-lipid bonds in membranes by denaturing the proteins, with the less polar solvent helping to dissolve the lipids (Hanahan, 1960).

Zearalenone, zearalenol and its derivatives were detected from *F. chlamydosporum*, *F. equiseti*, *F. pallidoroseum*, *F. proliferatum*, *F. solani*, *F. sporotrichioides* and *F. subglutinans*. Zearalenone is an anabolic and uterotropic metabolite produced by various species of *Fusarium* and has been observed as a natural contaminant of crop seeds (Siame and Lovelace, 1994; Sydenham *et al.*, 1990). Zearalenol is a derivative of zearalenone, presumably more actively estrogenic than the parent zearalenone

(Ueno and Tashiro, 1981). Zearalenone and its derivatives were previously reported from *F. equiseti*, *F. moniliforme* and *F. pallidoroseum* isolated from spices (Hashmi and Thrane, 1990) and from *F. proliferatum*, *F. solani* and *F. subglutinans* from cereals (Fakhrun Nisa, 1998) Among trichothecens, vomitoxin was detected from isolates of *F. moniliforme* and *F. subglutinans*. Fakhrun Nisa (1998) also detected vomitoxin from these *Fusarium* spp., whereas, Dalcerro *et al.* (1997) reported it from 80% wheat samples contaminated by *F. graminearum*.

More than 30 trichothecenes derivatives have been isolated from fungal cultures, but so far only 4 of them *viz.*, diactoxyscirpenol, T-2 toxin, vomitoxin and nivalenol have been identified as natural contaminants (Morooka *et al.*, 1972; Mirocha *et al.*, 1976). Vomitoxin is sometimes found concomitantly with zearalenone (Pathre and Mirocha, 1978; Vesonder *et al.*, 1981). It produces vomiting and feed refusal in swine (Vesonder *et al.*, 1973; Yoshizawa and Morooka, 1973) and has been detected in wheat breakfast cereals, wheat flour, bran, cookies, crackers and baby cereals (Hart and Braselton, 1983; Scott, 1983). In the present study also vomitoxin has been detected with zearalenone and appears as a potential contaminant of capsicum, corn and wheat. Trichothecenes are thought to be somehow involved in diseases in man e.g., red-mold disease in Japan and alimentary toxic aleukia in Russia (Pathre and Mirocha, 1979; Ueno, 1980).

Among naphthoquinone pigments, nectriafurone and *cis*-dihydrofusarubin were detected from *F. oxysporum*, *F. moniliforme*, *F. proliferatum*, *F. solani* and *F. subglutinans*. Both of these pigments were detected in isolates of *F. subglutinans* (Fakhrun Nisa, 1998). Isomarticin was detected in isolates of *Fusarium pallidoroseum*, marticin in *F. proliferatum* and bostrycoidin from several isolates of *F. moniliforme* and *F. solani*. A large number of isolates of *Fusarium* spp. produced secondary metabolites that could not be identified by comparing with several known toxins and pigments. The characteristic colors and retardation factors (Rf) of such unknown metabolites greatly enhanced their diagnostic value. Isolates of *F. chlamydosporum* consistently produced characteristic reddish brown and brown pigments before and after the spray of $AlCl_3$ in visible light that may be used as a taxonomic character in its identification.

Three secondary metabolites from *F. chlamydosporum*, 1 from *F. equiseti* and 2 from *F. pallidoroseum* were isolated by column chromatography and identified by their physical and spectral characteristics. All of these metabolites were found newly isolated from these *Fusarium* spp. The metabolite I was isolated from *Fusarium chlamydosporum* and identified as Bis(2-methylpropyl)ester or (Diisobutyl phthalate). This metabolite is a plasticizer and commercially produced by Aldrich (15264-1) and Fluka (80130) Chemical Companies. It is a derivative of phthalic acid that is industrially produced by the oxidation of 1,2-Dimethylbenzene BLQ00-J and

Naphthalen BNK69-U found in *Gibberella fujikuroi* [*Fusarium moniliforme*] (Matsuda and Kikkawa, 1957). Dibutyl ester (dibutyl phthalate), which is an isomer of this metabolite is known to be produced by *Penicillium bilaii* (Savard, *et al.*, 1994).

Another metabolite was isolated from *F. chlamydosporum* and identified as cyanidin 3-(xylosylarabinoside). This metabolite is an anthocyanin and cyanidin derivative glycoside that has been previously isolated from fruits of *Cyathodes dealbata* (family Epacridaceae) (Jarman and Crowden, 1973). Glycosides are well known antibiotics, which are produced by fungi (Coval, *et al.*, 1995; Schneider, *et al.*, 1995) and bacteria (Hanada, *et al.*, 1980; Argoudelis, *et al.*, 1987). Okada *et al.* (1993) isolated antibiotic BE 29602 related to this class of compounds from *Fusarium* spp. that showed strong antifungal and cytotoxic activities.

Lotusine E was the third metabolite isolated from *F. chlamydosporum*. It was a cyclopeptide alkaloid that has been previously isolated from *Zizyphus lotus* (family Rhamnaceae) (Ghedira, *et al.*, 1995). It is noteworthy that members of family Rhamnaceae produce cyclopeptic alkaloids such as pubescine A (Tschesche, *et al.*, 1980), Sativanine D and F (Shah, *et al.*, 1985) and dacehuine S5 (Han, *et al.*, 1987). However, it seems to be newly reported from *Fusarium chlamydosporum*.

Metabolite IV was isolated from *F. equiseti* and identified as melitric acid B. The metabolite is related to rosmarinic acid derivatives that are widely distributed in plants of family Labiatae. Agata *et al.* (1993) isolated melitric acid B from above ground parts of *Melissa officinales*. This plant is used as folk medicine under the name of balm and lemon balm for the treatment of chronic bronchial catarrh, feverish cold headaches and tension.

Metabolite V was isolated from *Fusarium pallidorozeum* and identified as musacine K. It is a known antibiotic of *Streptomyces griseoviridis* DSM-7429 that shows significant antibacterial activity (Susanne *et al.*, 1994). It may be mentioned here that some other musacine antibiotics such as musacine A (2,3-Dihydroxypropyl ester), musacine B2 (stereoisomer of musacine B1), musacine C, D, E and F are also known to be produced by *Streptomyces griseoviridis* (Schneider *et al.*, 1996).

Metabolite VI, radulanin A was a bibenzyl compound, which was isolated from *Fusarium pallidorozeum*. Asakawa *et al.* (1978) isolated this and some other related compounds for the first time from *Radula variabilis* (family Radulaceae of Liverworts) and identified them with the characteristic 7 carbon heterocyclic ring structure. In further studies on *Radula javanica* he isolated other compounds, radulanin J and L (Asakawa, *et al.*, 1991). *Radula* species possess allergenic properties (Le Coulant and Lopes, 1956), however

activity of radulanin compounds has not been hitherto proved in allergic contact dermatitis.

Seed-borne pathogens cause different types of damages, which are not always recognized by users, such as seed death, seedling and plant abnormalities or decreased seed vigor. Once harmful fungi, pathogenic as well as toxigenic, have been listed, it is important to define for each of them the methods to be used for their detection and identification (Neergaard, 1979). When basic knowledge of the fungus and mycotoxin(s) is obtained progress in the prevention and control could be rapid. There is undoubtedly worldwide contamination of the seed with a variety of mycotoxin-producing fungi and there is little doubt that mycotoxins are a probable source of naturally occurring carcinogens in humans (Diener *et al.*, 1981). Concerted effort could be made to avoid such contaminants using seed health technology.