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INTEGRATED MORPHOLOGICAL, MOLECULAR, AND BIOCHEMICAL CHARACTERIZATION OF FUNGAL STALK ROT IN MAIZE

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Abstract

Maize (*Zea mays* L.) is a vital cereal crop in Pakistan, but its production is severely affected by stalk rot disease, a complex condition caused by soil and seed-borne fungi. This investigation was conducted to isolate and characterize the fungal pathogens responsible for maize stalk rot in the Haripur area, and to study their effect on host physiology and metabolism. Fifty symptomatic maize plants were collected from various fields. The results revealed the consistent occurrence of three pathogenic fungi, with *Fusarium moniliforme* and *Macrophomina phaseolina* being the most predominant, and *Fusarium oxysporum* being less frequent. Soil samples from the area also contained *Aspergillus flavus* and *Aspergillus niger*. Sequencing analysis confirmed the morphological identification, showing 100% identity of one isolate with *F. oxysporum* (Accession number PZ049446) and 99.78% identity of another with *Fusarium verticillioides* (syn. *F. moniliforme*) (Accession number PZ039329). Infected plants exhibited significant structural degradation, including a thinner epidermis and vacuolated cells in the leaves. Biochemically, a marked decrease in chlorophyll content was observed in infected plants. Conversely, the activity of defense-related enzymes was significantly increased; peroxidase and catalase activities were substantially higher in infected plants compared to healthy ones, indicating a strong oxidative stress response. Gas Chromatography Mass Spectrometry (GC–MS) metabolite profiling revealed a distinct reprogramming in infected tissues, which accumulated stress-related compounds such as Butanoic acid-3-amino, Cystamine, and 3-nitropropanoic acid etc. In contrast, tissues of controlled plants contained metabolites indicative of normal metabolism, such as Urea, Acetic acid, and Hexanoic acid. Cyclobutanol was found in both control and diseased plants. The integrated approach of morphological, molecular, and biochemical characterization confirmed that *F. moniliforme* and *M. phaseolina* were the primary causal agents of stalk rot in Haripur. This study may be useful for developing the management strategies to control stalk rot disease of maize. Identification of stalk rotting pathogen of maize at molecular level may be useful to diagnose, treat, and control the disease. Farmers could relate the detection of stalk rot disease in maize directly to its management decisions, because early and accurate detection helps reduce yield losses and prevent further spread

Key words: Maize stalk rot; *Fusarium moniliforme*; *Macrophomina phaseolina*; Oxidative stress; Chlorophyll reduction; GC-MS metabolites; Internal Transcribed Spacer region (ITS)

Introduction

Maize is Pakistan's third-largest cereal crop, with production in recent years (2021–2024) fluctuating between 8.2 and over 10 million tons, driven by increased, risky, spring crop cultivation. While production achieved record highs (e.g., 9.5–10.6 million tons) in 2021-2023. The uses of maize are multiplied. It is used as food not only by the rural and urban population, but it is also the principal raw material for animal feeds and poultry rations. Its industrial utilization includes starch, corn oil, corn syrup and maltodextrins, substrates for fermentation products and distilled articles. Maize also contributes the basic raw material for biofuel production, which is the current trend in the whole world towards the use of renewable sources of energy (Bibi *et al*., 2010). However, in spite of its vast usages, maize productivity is always under threat from abiotic stresses i.e., drought and biotic stresses in the form of pathogenic fungi which affect yield and grain quality; overbearingly, in the form of diseases affecting the plant, especially the stalk rot diseases, due to reasons of bio-diversity in their spread, lodging of plants and their direct effect on the grain yield. Throughout the world, maize is subjected to more than seventy diseases which are caused by fungi, bacteria and viruses, with serious pre-and post-harvest losses. In Pakistan, the surveys have revealed that the loss of maize seeds due to fungi may reach up to 66-90% with the mean infection rate of 71.1% in sound seeds and 83.7% in the affected grains (Lal *et al.,* 2025). Stalk rots are often found in previously planted seeds having poor germination, or weak vigor. Also, seeds artificially infected with molds, or with fungi or mycotoxin-producing molds, may be lost in great quantities by spoilage and insect infestation. The effect of improper storage methods further aggravate these conditions, for increased humidity and temperature cause fungi to multiply. The effect on the grain is both quantitative and qualitative. Stalk rot may be regarded as a serious disease of corn, the organisms involved in the causative process being mostly fungi. The degree of disease is most serious when corn is undergoing stress in the grain-filling season. Such stress comes from various causes, most notable of which is lodging of

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diseased stalks, due to weakening and decay, with accompanying small harvestable yield and sacrifice of quality. *Fusarium* stalk rot is possibly the most serious type of stalk rot affecting maize, and is usually caused by *F. moniliforme*, *F. oxysporum*, and *M. phaseolina*. These fungi not only possess the capability to infect maize, but they can also persist in the soil for a long period of time, together with the ability to survive in the crop residues, thus serving as a source of inoculum (Mankvold & Desjardins, 1997). *F. moniliforme,* (teleomorph: *Gibberella fujikuroi*) is very interesting because it serves as a producer of the fumonisins, which are a group of mycotoxins giving rise to cancers in man, and severe disease problems in animals (Nayaka *et al*., 2008). Mucilaginous colonies of *F. moniliforme* are usually white to lavender in color, with septate hyphae and rather simple conidia formation. This fungus is found all over the world and attacks several hosts, such as sorghum, sugar-cane, wheat, banana, cotton, tomatoes and pine-apples (Fandohan *et al*, 2003). In Pakistan, *F. moniliforme* is widely distributed in the maize growing regions of the country (Sitara & Akhtar, 2007; Saleem *et al*, 2012) and causes heavy losses both in the yield of the crop as well as in the quality of the grain. *F. oxysporum* is responsible for the effect which is known as vascular wilt of several crops. Besides producing microconidia and macroconidia, as well as chlamydospores and its various forms, it shows a certain amount of morphological variation depending on various strains. The presence of disease is indicated by the clearing of the veins and diffused epinasty of the leaves, and subsequently causes general wilting and finally death of the plant. Though the *F. oxysporum* is a much less aggressive type than the *F. moniliforme* on maize, it is equally, if not more, responsible for the stalk rot disease (Schisler *et al.*, 2002). In maize, the *M. phaseolina* disturbs the vascular system and interferes with the supply of water and of nutritious food items, and as a result, serious yield losses are caused (Almomani *et al.*, 2013b). In addition to diseases caused by *Fusarium* and *Macrophomina* species, *A. flavus* and *A. niger* are often intimately associated with maize grains during storage (Christensen, 1967; Halloin, 1981). These fungi produce aflatoxins and other mycotoxins destructive to food value. The dual effects of the field fungi (*Fusarium* spp.) and of the storage fungi (*Aspergillus* spp.) highlight the complex etiology of maize diseases and the difficulties of their control (Payne *et al*., 1998). Several workers have attempted to elucidate the pathogenic mechanism and the interrelations of host and parasite involved in stalk rot disease. Ghany (2012) reported that diseased maize plants show an abnormal appearance due to disorganization of epidermal cells, vacuolation and decrease in chlorophyll content, but increased catalase and peroxidase activity. Abe *et al*., (2015) have shown that the *Fusarium* and *Aspergillus* spp. produce extracellular enzymes like cellulases, lipases, proteases, etc., which contribute towards the colonization of the tissue and degradation of the host. Riaz *et al*., (2020) conducted extensive surveys throughout Pakistan and recognized that the most potent and destructive causal agents of potato stalk rot disease are *M. phaseolina*, *F. moniliforme* and *Rhizoctonia solani*. Considering the economic importance of maize and the destructive possibilities of the stalk rot fungi, the present research project was designed to identify fungal pathogens from stalk rot disease affected maize plants of Haripur city of Pakistan. Another objective was to do the biochemical characterization of stalk rot affected plants of maize. The chlorophyll content, activity of catalase, peroxidase enzymes of diseased and control plants were compared. The integrated studies aimed to provide information regarding the pathogens involved in maize stalk rot in Haripur, along with a knowledge of the host biochemical characterization. The work may be useful to develop better strategies for control of stalk rot disease of maize.

Materials and Methods

Study area and sample collection: The research was performed in the Haripur district of Khyber-Pakhtunkhwa, Pakistan, during the maize growing season of September 2018. Fifty (50) maize samples were collected from the fields showing the symptoms of stalk rot disease (Table 1). Soil samples were also collected from same regions (Table 1). The symptomatic and asymptomatic maize plants were uprooted carefully so that stalks, roots and leaves could be recovered. The infected plants were detected by the visible symptoms of yellowing of the leaves, early wilting of the plants, lodging of the stem plants and the presence of necrotic lesions. The samples were brought to the Plant Microbiology Laboratory of the Department of Microbiology, University of Haripur, under sterile conditions in autoclavable polyethylene bags. The infected tissues were also collected from the infected plants along with three control maize plants of the same age and same growth phase from the nearby fields as negative controls. All plant samples were processed on arrival in Microbiology lab. The portions of stalks, leaves, grains and rhizospheric soils were separated for isolating fungi and some other tissues and these were preserved for subsequent biochemical assays at 4°C.

Isolation, cultural and morphological characterization of fungal pathogens: The isolation of fungal pathogens was carried out on Potato Dextrose Agar (PDA) and Czapek Dox Agar (CZA). These medias were prepared according to the manufacturer's protocol. Both the media were sterilized by autoclaving at 121°C for 15 minutes.

For the surface sterilization of maize tissues (stems, leaves and seed), the tissues were immersed in 1% sodium hypochlorite solution for 1 min. The tissues were then thoroughly washed three times, with sterile distilled water, to remove any residual disinfectant. Small pieces of tissue (2 to 3 mm.) were then placed aseptically on PDA and CZA plates. Each sample was plated in triplicate to ensure uniformity of the results. The plates were then incubated in the dark at 25 to 28°C. for a period of 5-7 days. The emerging fungal colonies were taken out and sub-cultured on fresh PDA plates for the purpose of securing pure cultures. The cultures were then kept on PDA slants at 4°C. for preservation purposes. The infected rhizosphere soils from the infected plants was also serially diluted and plated on PDA, with Chloramphenicol as antibacterial agent, for the purpose of isolating pathogenic fungi. Colony morphology, pigmentation, aerial mycelium, and colony colors were noted. Microscopic slides were prepared using the lactophenol blue cotton staining technique. Spores and hyphae were observed under compound light microscope at 40X and 100X magnifications.

Table 1. Samples (Plants and Soil) with their number and location.

Sample number	Location	Color of plant	Color of soil
	Haripur Tehsil (Central Region)		
S1	Ali Khan	Yellow	Brown
S2	Shah Muhammad	Yellow Green	Gray
S3	Moonan	Mustard Yellow	Black
S4	Muradabad	Yellow	Brown
S5	Choora Ali Khan	Pale Yellow	Dark Gray
S6	Khairabad	Yellow Green	Black
S7	Pandak	Mustard Yellow	Dark Brown
S8	Talokar	Yellow	Grey
S9	Muslimabad	Yellow Green	Light Brown
S10	Panian	Light Yellow	Black
S11	Hattar	Pale Yellow	Yellow
S12	Bandi Gullu	Mustard Yellow	Brown
S13	Bhoi Ghar	Yellow	Dark Brown
S14	Ahata	Yellow Green	Dark Gray
S15	Jabbri	Pale Yellow	Black
S16	Sirikot	Pale Yellow	Brown
S17	Galli	Yellow Green	Dark Gray
S18	Amazaie	Yellow	Black
S19	Galli	Pale Yellow	Gray
S20	Chountrai	Light Yellow	Dark Brown
S21	Dara Mohat	Mustard Yellow	Black
S22	Goodwalian	Yellow	Gray
S23	Bakka	Yellow	Dark Gray
S24	Pind Hashim Khan	Light Yellow	Dark Brown
S25	Rehana	Pale Yellow	Brown
S26	Sarai Saleh	Yellow	Gray
	Ghazi Tehsil (Eastern Region)		
S27	Ghazi	Yellow Green	Black
S28	Bharwasa	Pale Yellow	Gray
S29	Hassanpur	Mustard Yellow	Dark Gray
S30	Gahra	Yellow	Yellow
S31	Jammun	Light Yellow	Dark Gray
S32	Jabri	Yellow Green	Black
S33	Jallo	Pale Yellow	Black
S34	Tarbela Colony	Yellow	Brown
S35	Bait Gali	Mustard Yellow	Gray
S36	Mohat	Yellow	Dark Brown
S37	Qazipur	Yellow	Dark Gray
S38	Topi Banda	Pale Yellow	Brown
S39	Shaligram	Yellow Green	Brown
	Khanpur Tehsil (Northern Region)		
S40	Khanpur	Mustard Yellow	Black
S41	Mang	Pale Yellow	Dark Gray
S42	Kot Najibullah	Mustard Yellow	Gray
S43	Kundi	Yellow	Yellow
S44	Qazian	Light Yellow	Black
S45	Bajeeda	Yellow Green	Brown
S46	Jattipind	Pale Yellow	Dark Gray
S47	Kag	Yellow	Brown
S48	Darwaza	Mustard Yellow	Yellow
S49	Ladhar Mang	Yellow Green	Gray
S50	Kotehrra	Yellow	Yellow

Molecular characterization of isolated fungi

DNA extraction & PCR: Genomic DNA was extracted from the isolated fungi using the Gene-JET Genomic DNA Purification Kit (Thermo Fischer Scientific). About 100 mg of the fungal mycelium was taken from the PDA plates and placed in sterile 1.5 mL microcentrifuge tubes. The mycelium was homogenized in 1X CTAB buffer and centrifuged at 12,000 × g for 10 seconds for sedimentation of the cells. The sediments were resuspended in 500 µL of lysis buffer and incubated in a water bath at 37°C for 1 hour. After the incubation period, the suspension was centrifuged at 3000 × g for 10 minutes. The supernatant was discarded, and the sediment was treated with 180 µL digestion buffer and 20 µL proteinase K. After vortexing, it was incubated at 56°C for 45 minutes until complete lysis was attained. RNase treatment (20 µL RNase A, 10 minutes at room temperature) was done for the removal of RNA contamination. Purification of the DNA was done by ethanol precipitation and elution in 200 µL elution buffer. The quality and concentration of the DNA were checked by agarose gel electrophoresis (0.8% agarose). PCR was performed by using Internal Transcribed Spacer1 and Internal Transcribed Spacer4 primers. The PCR products were sent to Alpha genomics for purification and sequencing. Sequences of forward and reverse primers are:

The standard universal fungal ITS primers are:
- ITS1 (forward primer): 5′-TCCGTAGGTGAACCTGCGG-3′
- ITS4 (reverse primer): 5′-TCCTCCGCTTATTGATATGC-3′

Polymerase chain reactions were performed on a Galaxy XP Thermal Cycler (BIOER, PRC). Following chemicals were used.

PCR reagents	Stock Conc.	Working Conc.	Vol/Rec
DNA template			1µL
Forward primer	10µM	0.2 µM	0.4 µL
Reverse primer	10 µM	0.2 µM	0.4 µL
dNTPs	10 mM	0.2 mM	0.4 µL
Buffer	10X	1X	2 µL
$MgCl_2$	25 mM	2.5 mM	2 µL
Taq Polymerase	5	1.5U	0.3 µL
Deionizing water			13.5 µL
Final volume			**20 µL**

The PCR optimized conditions are given in Table 2.

Table 2. Optimized PCR conditions.

Steps	Sub-Cycle	Condition	PCR Cycle
Initial denaturation		95ºC,5 min	1
PCR cycle	Denaturation	95ºC,1min	40
	Primer annealing	59ºC,1min	
	Primer extension	72ºC,1min	
Final extension		72ºC.10mins	1

Sequencing and sequence analysis: The sequences obtained were analyzed using the NCBI BLAST (Basic Local Alignment Search Tool), a bioinformatics tool to compare DNA sequences against a large database of known sequences to find similar or matching sequences

Biochemical characterization

Chlorophyll content estimation dose: Chlorophyll content was measured according to Zhang *et al*., (2022). About 250 mg of maize leaf tissues (control and infected) were homogenized in 5 mL phosphate buffer (pH 6.8). The homogenate was mixed with 80% ethanol, boiled for 10 min, cooled and centrifuged at 14,000 x g for 15 min. Supernatants were collected and absorbance was recorded at 665 nm using UV–Vis spectroscopy. Chlorophyll quantity was calculated from the absorbance values.

Enzyme extraction: Leaf samples (200 mg) were homogenized using 5 mL of phosphate buffer (pH 6.8) and centrifuged at 14,000 × g for 15 minutes. The supernatant was taken as a crude enzyme extract for the subsequent bioassays.

Catalase determination: The catalase activity was determined using the method of Hadwan *et al*., (2024) with some modifications. The reaction mixture contained phosphate buffer, H_2O_2 and enzyme extract and the it was incubated at 25°C for 1 min. Reaction was stopped with 2% H_2O_2. Remaining H_2O_2 was titrated with 0.01 N $KMnO_4$. Enzymatic Activity was expressed as changes in absorbance/time.

Peroxidase assay: The enzyme extract was incubated with phosphate buffer, pyrogallol and H_2O_2 for 5 minutes at 25°C to check the peroxidase activity. The reaction was stopped with 2% H_2SO_4 and the absorbance of the reaction mixture was measured at 420 nm (Agunbiade *et al*., 2021).

Gas chromatography–mass spectrometry (GC-MS) analysis: Metabolite profiling work was done using GC-MS analysis. The following methods were adopted for sample preparation (GC-MS analysis). A sample size of 5 g of dried leaves was taken and were ground, then the leaves were extracted in 70% ethanol. The extracts were centrifuged at 6000 × g for 10 min. The supernatants were filtered and were concentrated before being used in analysis. GC-MS analysis was performed using an HP-5MS capillary column (30m x 0.25 mm x 0.25µm). Injection temp. 25°C, the column oven program was (40-300°C) at the rate of 10°C/min. The final hold was for a period of 25 min. Mass range (40-600 m/z), Ionization energy (70 eV), and Ion Source Temperature (180°C) was used. Identification of the compounds was done having peaks with a relative peak area ≥1% and similarly index ≥85% by comparing with the spectra obtained from the National Institute of Standard and Technology library.

Statistical analysis: All biochemical assays were performed in triplicate. All parameters were expressed as mean ± standard deviation (SD). The differences between control and infected plants were statistically computed by means of student's *t*-test with a significance level of $p \leq 0.05$.

Results

Isolation and morphological identification of fungal pathogens: The fungi were initially identified from their colony characters, color, character of the hyphae and color of the spores; *F. moniliforme*, *F. oxysporum* and *M. phaseolina* (Figs. 3, 2 & 1). The ratio of the isolated fungi was *F. moniliforme*: *F. oxysporum*: *M. phaseolina* = 24: 12:14 (Figs. 3, 2 &1) of the total 50 diseased leaf samples. The *Aspergillus flavus* and *Aspergillus niger* were detected from diseased soil samples, indicating that these pathogenic fungi were present

in the rhizosphere of maize plants, and could contribute to post-harvest contamination. From infected grains *F. moniliforme: F. oxysporum: M. phaseolina* were found in the ratio of 18:14:18, showing those pathogens as the causal agent of grain decay. The infected stalks showed *F. moniliforme: F. oxysporum: M. phaseolina* in the ratio of 22:13:15. *M. phaseolina* formed blackish colonies on PDA, produced by round and smooth microsclerotia superficially arranged on the hyphae (Fig. 1a and 1b). The microsclerotia were compact and pigmented, characteristics of *M. phaseolina*, which is known to form persistent survival organs in soil. *F. oxysporum* produced fluffy white colonies on PDA (Fig. 2a) while the reverse of the colonies exhibited purple to pink coloration (Fig. 2b). The colonies of *F. moniliforme* produced white aerial hyphae with a yellowish center in PDA (Fig. 3a and 3b). *A. niger, A. flavus* (Figs. 4 a-e), *M. phaseolina* and *F. oxysporum* in the soil samples (n = 50) were detected as 20: 16: 8: 6, respectively. The recovery of *A. flavus* and *A. niger* in each soil sample (Figs. 4 a-e) suggest that the opportunistic fungi colonizing the rhizosphere would be instrumental in establishing the diseases in the maize stalks. *F. moniliforme* showed maximum isolation frequency percentage from infected maize leaves as compared to the *M. phaseolina* and *F. oxysporum* (Table 3A). In the infected grains the isolation frequency percentage of pathogenic fungi (*F. moniliforme, F. oxysporum* and *M. phaseolina*) was as; 36:28:36 (Table 3A). The isolation frequency (%) of Pathogenic Fungi *A. flavus*, *A. niger*, *M. phaseolina* and *F. oxysporum* from soil was as; 32: 40: 16: 12 (Table 3B). Sequencing and sequence analysis of two pathogenic fungi resulted in the detection of *F. moniliforme* (Accession number PZ039329) and *F. oxysporum* (Accession number PZ049446), while sequencing was failed for *M. phaseolina* but it was already characterized by morphological and microscopical techniques.

Microscopic examination of healthy and infected leaves: Microscopic examination of healthy and diseased maize leaves has shown distinct differences (Fig. 5). The healthy leaves showed increased thickness of epidermal cell layer, dense growth of mesophyll tissue with uniform distribution of chloroplasts and small amount of vacuolation (Fig. 5A). Diseased leaves presented the decreased thickness of epidermis, large ragged vacuoles in epidermal and mesophyll cells, disorganized subsidiary cells, decreased density of chloroplasts (Fig. 5B). These structural changes of the leaves suggested that the influence of the fungus induces a loss of epidermal integrity and hastens the senescence of tissues. This disease was found to be most prevalent during the humid months of June and July, a condition favorable for fungal growth.

Chlorophyll content in control vs. infected plants: Spectrophotometric examination revealed significant decrease in chlorophyll levels in the infected maize plants as compared to control plants (Fig. 6; Supplementary Data, Table 3). The control samples varied for the chlorophyll absorbance in range of 0.733-0.996, whereas the infected plants had absorbance of 0.486-0.650. The mean chlorophyll contents in control leaves was higher as compared to the diseased leaves, where lower values were observed. Decreased chlorophyll levels are due to pathogen-induced depression of photosynthetic activity. Possible reasons are denaturation of chloroplast membranes and inactivation of chlorophyll biosynthetic enzymes (Luhová *et al*., 2006). The decrease in chlorophyll explains the chlorosis observed in infected maize fields, in which the leaves lost their green pigmentation in patches and assumed a pale yellow or necrotic color. An independent sample t-test revealed that the difference of chlorophyll contents between control and diseased leaves was statistically significant ($p \leq 0.05$), indicating that disease significantly reduced chlorophyll content (Fig. 6).

Enzyme activity: peroxidase and catalase: The infected plants showed an increase in the activity of peroxidases (Table 4) and catalase as compared to control plants (Fig. 7 & 8). The control plants showed an average absorbance for catalase in control leaves ranging from 0.225-0.355 and infected plants had considerably higher absorbance 0.405-0.650. The catalase activity in diseased maize leaves was significantly higher than in control plants according to Student's t-test $p \leq 0.05$). Similarly the infected plants produced absorbance values for Peroxidase determination at 420 nm considerably higher than those of control plants, maximum (0.553). The increase in peroxidase and catalase activity of the infected plants suggest that the plants are active in developing their oxidative stress responses. The reaction of fungi on the plants causes the plants to evolve biosynthetic activities to produce reactive oxygen species (ROS), which stimulate the host defense processes. Peroxidases are of great importance in the lignification process and the strengthening of cell walls, while the catalases act as removing agents for the detoxification of hydrogen peroxide to eliminate oxidative damage to the plants (Swarnakumari *et al*., 2011a). Hence the Peroxidase activity in infected maize leaves was significantly higher than that in control plant leaves**,** as determined by Student's t-test $p \leq 0.05$).

Molecular characterization

DNA extraction: Genomic DNA acquired from fungal isolates produced distinct and intact bands upon visualization on agarose gel electrophoresis (Fig. 9). Yields of DNA were adequate for subsequent PCR amplification, with concentrations varying from 50-120 ng/µL.

Table 3A. Isolation frequency of Pathogenic fungi from leaves and grains.

Isolation frequency (%) of pathogenic fungi	*F. moniliforme*	*F. oxysporum*	*M. phaseolina*
From leaves	48	24	28
Grains	36	28	36

Table 3B. Isolation frequency of Pathogenic fungi from soil.

Isolation frequency (%) of pathogenic fungi from soil			
A. niger	*A. flavus*	*M. phaseolina*	*F. oxysporum*
40	32	16	12

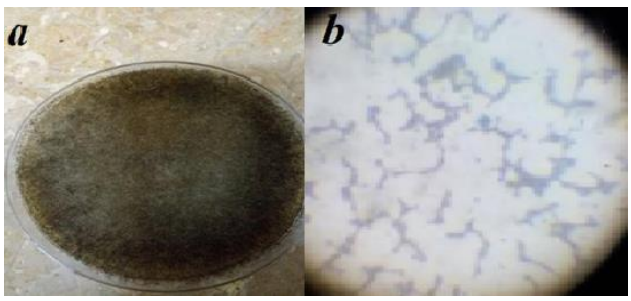

Fig. 1. **a**) Isolation of *M. phaesolina* from infected maize leaves on PDA, **b**) Microscopic view of *M. phaesolina.*

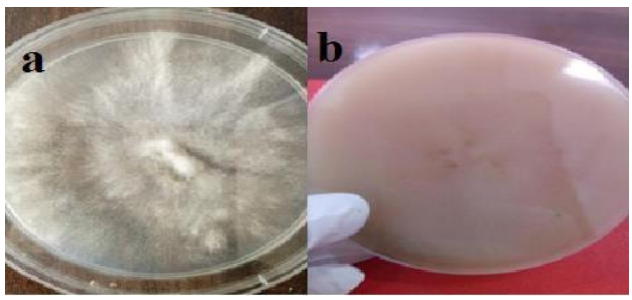

Fig. 2. Detection of *F. oxysporum* **a**) front view, **b**) back view, from infected maize grains on PDA medium.

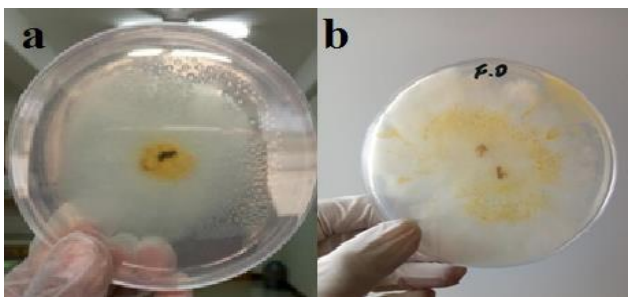

Fig. 3. **a** & **b** *F. moniliforme* detection from infected maize grains on PDA medium.

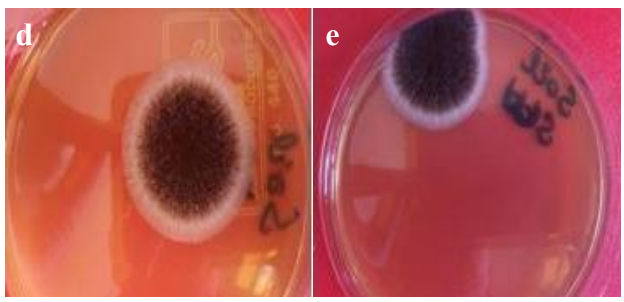

Fig.4. **d, e**; *Aspergillus niger* from rhizospheric soil of diseased maize plants on PDA medium.

Fig. 4. **a**) Isolation of *Aspergillus flavus* from rhizospheric soil of diseased maize plants on PDA medium. **b & c**) *Aspergillus flavus* detection on PDA medium. The antibacterial agent chloramphenicol was added.

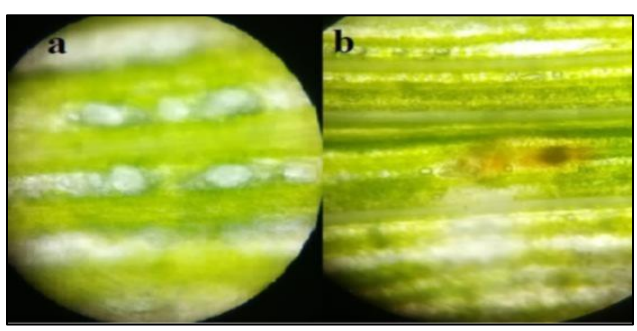

Fig. 5. Light micrographs of leaf of maize. **a**) Healthy leaf with thick epidermis, **b**) Infected leaf with thin epidermis and vacuoles.

Sequencing and sequence analysis: Isolate S1 showed 100 % identity with *F. oxysporum*, while the isolate S3 was 99.78% identical to *F. verticillioides* (syn. *F. moniliforme*). These results confirmed the morphological identification and confirmed that the *Fusarium* spp. was the most dominant among the stalk rotting pathogens of maize in Haripur (Figs 2 & 3).

GC-MS analysis of biochemical compounds: Differing metabolite profiles between the infected and control maize leaves were evident when the tissues were analyzed by Gas Chromatography-Mass Spectrometry (Figs. 11 and 12, Tables 5 and 6).

Infected leaves: The infected leaves accumulated stress-related metabolites such as 3-nitropropionic acid and Cystamine (Fig. 11). Compounds detected in the infected tissues include: Amino acids and derivatives Alanine, Butanoic acid-3-amino, Cathine, Cystamine. Alcohols and aldehydes: 2-butanol-3-methyl, Heptanal, Dimethylamine. Nitro compounds: 3-nitropropanoic acid. Epoxides: 2,3-epoxybutane. Cyclic alcohols: Cyclobutanol (common to both healthy and infected tissues). These compounds suggest oxidative stress and amino acid catabolism in the infected leaves. 3-nitroproponic acid for example, is a known inhibitor of mitochondrial respiration and could contribute towards necrosis.

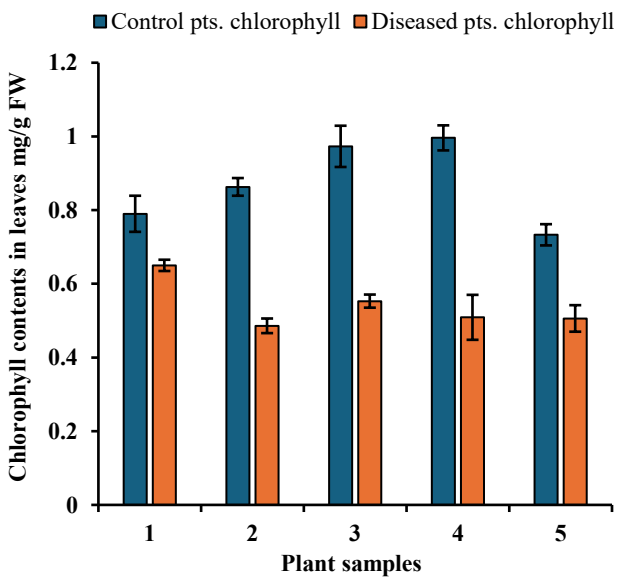

Fig. 6. Chlorophyll content in control and infected maize plants.

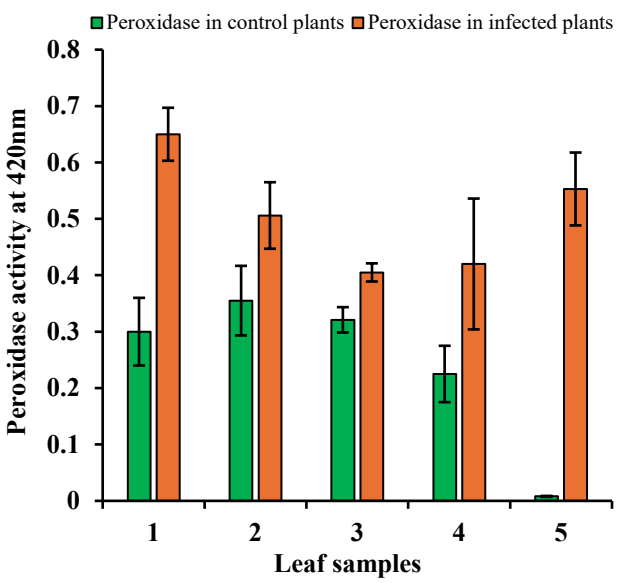

Fig. 7. Peroxidase activity in control and infected maize plants.

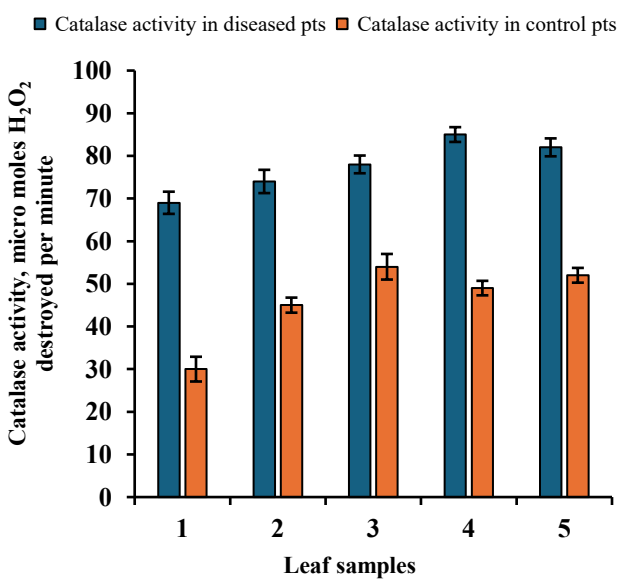

Fig. 8. Catalase activity in control and infected maize plants.

Fig. 9. DNA samples of different stalk-rotting fungal pathogens.

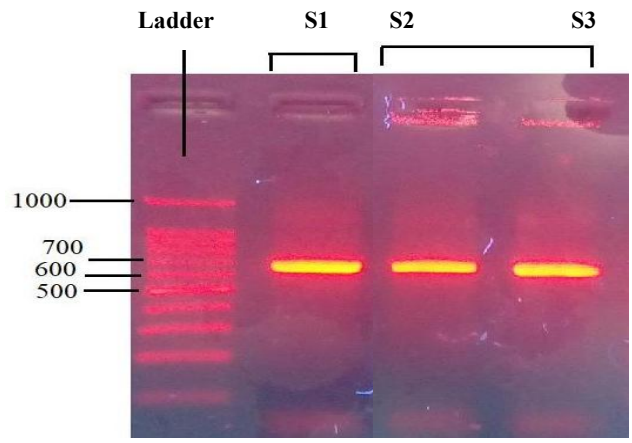

Fig. 10: Amplified products of PCR.

Fig. 13. Typical symptoms on an infected maize plant.

PCR amplification: The PCR amplification of the Internal Transcribed Spacer region generated a discrete band of 650 bp in S1, S2, S3, and S4 isolates (Fig. 10). Identical, intact bands produced by the analysis demonstrated that fungal ribosomal DNA was successfully amplified.

Metabolites detected in the control leaves: Control leaves contained metabolites indicative of normal nitrogen assimilation, like Urea and Hexanoic acid. Well-nourished leaves of maize were found to contain compounds such as: Diglycoamine, Urea, Acetic acid, 1-pentanol-4-amino, Ethane diamide, Hexanoic acid, Pentanal, 2-hexanol, and Cyclobutanol (Fig. 12, Table 7). Here, the metabolites are indicators of unharmed metabolism and the pathways of

assimilation of nitrogen. The fact that cyclobutanol is present as an indicator in healthy tissues and is also to be found in tissues affected by the action of the fungus, indicates that it has the right of precedence in the list of the metabolites indicating unharmed metabolism found present in maize, nevertheless the presence of a greater number of those un-normal products which indicate an attacked state of vitality in the diseased tissues. The presence of compounds associated with metabolic stress, such as Cystamine and 3-nitropropionic acid in the diseased tissues, indicates that the metabolism in these parts has been further re-metabolized.

Present study on maize and pathogen interactions showed that infection changed the plant's metabolism. Infected maize plants had different levels of amino acids, organic acids, and volatile compounds. This research is in accordance with Sun *et al*., (2022), where maize infected with *Fusarium* species showed increased activity in amino acid and organic acid pathways. This suggests that the plant is adjusting its metabolism to defend itself. In cases of stalk rot caused by *Fusarium*, there are changes in amino acids and other secondary metabolites that are linked to stress responses. Our study also found organic acids and lipid-related volatile compounds such as acetic acid, hexanoic acid, pentanal, and heptanal. These compounds may be associated with oxidative stress and damage to cell membranes in infected maize tissues. In addition, amines (such as dimethylamine) and small alcohols. Although these are not reported as often, they may indicate changes in nitrogen metabolism and volatile signaling, which are important parts of the plant's defense system (Stirling *et al*., 2024).

Field observations and symptoms of stalk rot: In field examinations of stalk rot, it was established that stalk rot was prevalent in fields where little or no irrigation water was provided. The infected plants lodged before maturity, and were likely to be poorly produced, the ears being small and the grain poorly filled. Visual symptoms in the field included yellowing (chlorosis), wilting, and necrotic lesions on leaves and stalks, which progressively worsened with disease development until lodging occurred (Fig. 13). The symptoms of stalk rot were prominent during the months of June and July, which corresponded with periods of high humidity. It can be seen that stalk rot is an important economic factor in the Haripur valley.

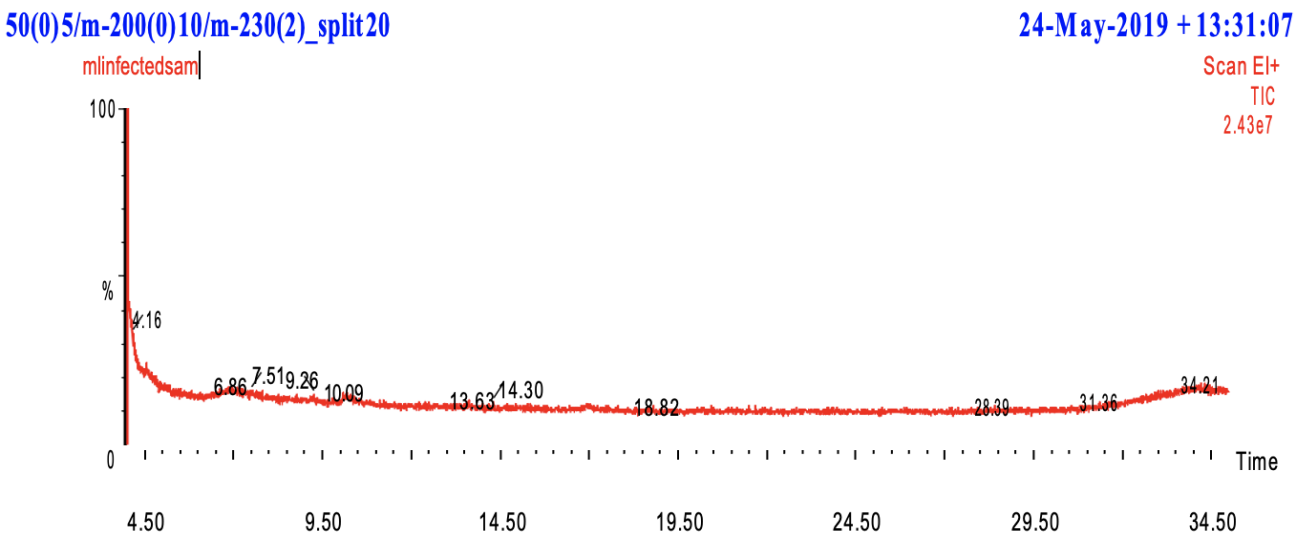

Fig. 11. GC-MS spectrum for ethanolic extracts of infected maize leaves.

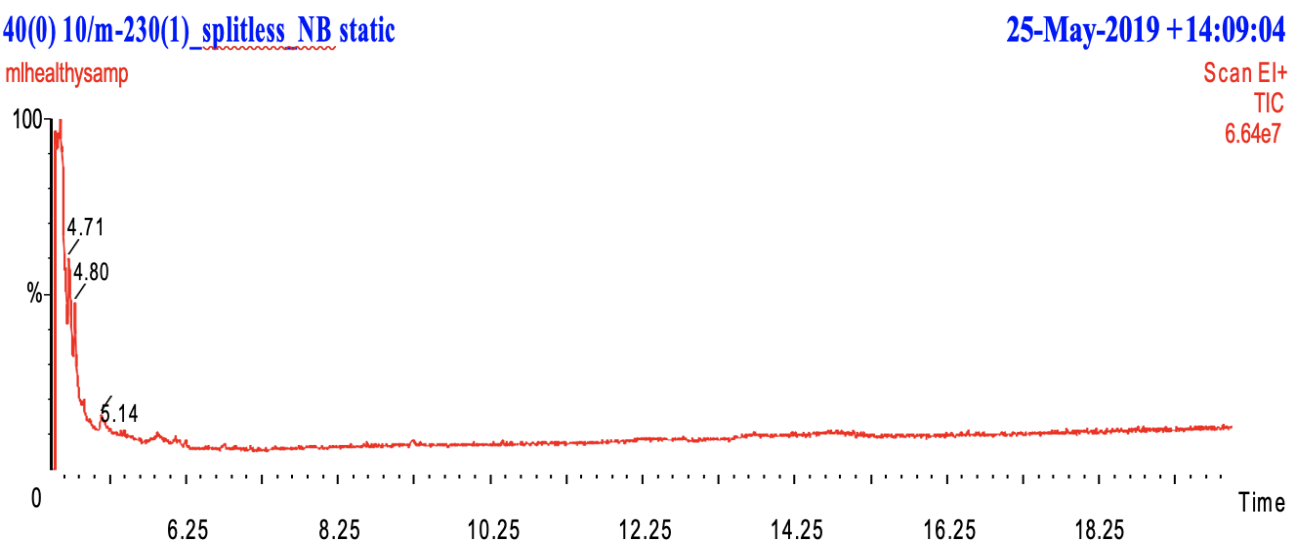

Fig. 12. GC-MS spectrum for ethanolic extracts of control maize leaves.

Table 4. Chlorophyll contents in control and infected maize plants.

S. No.	Control plant	Infected plant
1.	0.790nm	0.650nm
2.	0.863nm	0.486nm
3.	0.973nm	0.553nm
4.	0.996nm	0.509nm
5.	0.733nm	0.506nm

Table 5. Peroxidase activity in control and infected maize plants.

S. No.	Control plants	Infected plants
1	0.300nm	0.650nm
2	0.355nm	0.506nm
3	0.321nm	0.405nm
4	0.225nm	0.420nm
5	0.008nm	0.553nm

Table 6. GC-MS analysis of infected maize leaves.

S. No.	Compound name	Retention time	Molecular weight (g/mol)	Formula	CAS	Match score
1.	Alanine	4.16	89	C3H7O2N	56-41-7	980 (Excellent)
2.	Butanoic acid , 3amino	4.16	103	C4H9O2N	541-48-0	920 (Excellent)
3.	Cathine	4.98	151	C9H13ON	492-39-7	935 (Excellent)
4.	Cystamine	4.98	152	C4H12N2S2	51-85-4	900 (Excellent)
5.	2-Butanol, 3Methyl	10.09	88	C5H12O	598-75-4	965 (Excellent)
6.	3-Nitroproponaic acid	10.09	119	C3H5NO4	504-88-1	915 (Excellent)
7.	Heptanal	28.39	114	C7H14ON	111-71-7	940 (Excellent)
8.	2,3Epoxybutane	28.39	72	C4H8O	3266-23-7	910 (Excellent)
9.	Cyclobutanol	28.39	72	C4H8O	2919-23-5	870(Good)
10.	Dimethylamine	4.16	45	C2H7N	124-40-3	890(Good)

Table 7. GC-MS analysis of control maize leaves.

S. No.	Compound name	Retention time	Molecular weight (g/mol)	Formula	CAS	Match score
1.	Diglycoamine	4.71	105	C4H11NO2	929-06-6	985(Excellent)
2.	Urea	4.71	60	CH4O2N2	57-13-6	940(Excellent)
3.	Acetic acid	4.71	132	CH34O4N2	585-05-7	915(Excellent)
4.	Cyclobutanol	4.80	72	C4H8O		945(Excellent)
5.	1pentanol, 4amino	4.80	103	C5H13ON	927-55-9	970(Excellent)
6.	Ethane diamide	4.80	88	C2H402N2	471-46-5	955(Excellent)
7.	Hexanoic acid	5.14	116	C6H12O2	142-62-1	925(Excellent)
8.	Cycloserine	5.14	102	C3H6O2N2	68-41-7	915(Excellent)
9.	Pentanal	4.80	86	C5H10O	110-62-3	930(Excellent)
10.	2-Hexanol	5.14	102	C6H14O	626-93-7	890(Good)

Discussion

The present investigation on stalk rot disease of maize (*Zea mays* L.) in Haripur, Pakistan, was carried out with the preliminary objectives of obtaining detailed morphological, molecular and biochemical studies of the causal organisms and reactions of host plants. The results showed that *F. moniliforme*, *M. phaseolina* and *F. oxysporum* were the predominant pathogens associated with diseased plants of maize, but *Aspergillus* species were also isolated from soil. These results are in accordance with the reports of Riaz *et al.,* (2020) and Singh *et al*., (2012), wherein *Fusarium* and *Macrophomina* species have been recognized as the most destructive causes of stalk rot in maize in Pakistan in the maize-growing areas. It is also significant that *A. flavus* and *A. niger* are present since these fungi tend to increase the losses in maize due to the toxic metabolites produced by these fungi as is particularly noted under storage conditions when high humidity and inadequate storage conditions result in the contamination of the grain with different toxins (Halloin, 1981; Payne *et al*., 1998). The importance of *F. moniliforme* in maize samples of Haripur is of considerable significance since this pathogen produces fumonisins, which are known to be carcinogenic mycotoxins and have been associated with diseases in lower animals and also effect human health (Nayaka *et al*., 2008).The endophytic character of this fungus permits it to grow in the tissues of maize asymptomatically and later develop into stalk and ear rot phases, respectively, which renders detection and control increasingly difficult (Munkvold & Desjardins, 1997). The *M. phaseolina* fungus is known to produce microsclerotia which results in the long-time survival through the winter of the pathogen in the soil and ensures recurrence of the disease year after year in such crop rotations (Almomani *et al*., 2013). The wide host range of *M. phaseolina* limits crop rotation as a management option, since it can persist upon a considerable number of other hosts.

The detection of *Fusarium oxysporum* in maize samples of Haripur show that soil of selected field may be contaminated with this pathogen, since it is a soil-borne fungus. It may be the reason for wilting of selected Haripur plants. Its detection confirms that the stalk rot disease symptoms observed in the maize plants are likely due to this pathogen alongwith *M. phaseolina* and *F. moniliformae*. The anatomical differences that were found in infected maize leaves showed further the harmful influence of these pathogens. The control maize

leaves contained an unbroken epidermis and compact intercellular material, whereas infected tissue displayed a thin epidermis, enlarged vacuoles and an abnormal arrangement of layers of cells (Fig. 5). Such structural damage may be related to the action of the hydrolytic enzymes concerned in their action, produced by the fungi, positively, endo-glucanases, pectinases, and proteases, which break down the host's tissues and facilitate colonization of the pathogens (Abe *et al.*, 2015). Similar changes have been observed by Ghany (2012) who stated that there is disorganization of the epidermis, and an increase in vacuolation in diseased maize leaves. A distinct decline in chlorophyll was noticed in the infected plants, as compared to the controls (Fig. 6), indicating that in the infected plant and saprophytic action may have prevented the photosynthetic function. The cause of the chlorosis due to the action of the certain pathogens is probably due to thinned chloroplast membranes due to action and blocking the action of the chlorophyllous biosynthetic enzymes (Luhova *et al*., 2006). The increased peroxidase and catalase activities in diseased maize plants as compared to controls (Figs. 7 & 8), suggested that maize plants induced oxidative stress protective mechanisms upon infection. Fungal invasion is known to produce active oxygen metabolites during the interaction of the host and pathogen. Catalases detoxify hydrogen peroxide, diminishing the oxidative damage, while peroxidases take part in the synthesis of lignin and strengthen the plant cell walls, which impede the spread of pathogens (Swarnakumari *et al*., 2011b). The increase in the activity of these enzymes in diseased plants indicated that the host attempted to resist the establishment of the pathogen. This observation is in accordance with previous work, where it was found that activities of peroxidase and catalase extensively increased in plants attacked by pathogenic fungi. This could be indicative of their activity as biochemical indices of stress conditions exerted by pathogens (Akhtar *et al*., 2016). The molecular identification yielded confirmation of the morphological data, thus diminishing the uncertainties involved with variations in culture conditions on the morphology of the colonies. Sequencing of the Internal Transcribed Spacer region has shown 100% identity to *F. oxysporum* and close similarity to *F. verticillioides*. Molecular confirmation is most necessary, since different *Fusarium sp.* possess considerable morphological plasticity leading to unreliability of the use of morphology for their correct identification (Abd-Elsalam *et al*., 2003; Singha *et al*., 2016). Furthermore, phylogenetic analysis placed the isolates within established *Fusarium* lineages (Figs. 14 and 15), indicating that the pathogens isolated from Haripur are genetically related to globally distributed *Fusarium* populations. The metabolomic evaluations performed by GC-MS strategies demonstrated new insights into the metabolic remodeling of maize following pathogen infection. In contrast to healthy tissues, diseased tissues accumulated several stress-related metabolites such as Butanoic acid-3-amino, Cystamine, and 3-nitropropanoic acid etc. These metabolites may communicate the more profound alterations in amino acid metabolism, and secondary metabolite formation occurring during disease. The 3-nitroproponic acid is a mitochondrial toxin, may also invade necrotic symptoms. This compound may have disrupted energy metabolism. The accumulation of Cystamine indicated the involvement of sulfur-related metabolic pathways, may be involved in the detoxification of reactive oxygen species. The presence of Alanine indicated alterations in nitrogen metabolism which are often coincident with stress responses in plants. Control plants, on the other hand, had metabolites present in the plant tissue metabolites which show normal nitrogen assimilation and lipid metabolism such as Urea, Acetic acid, Hexanoic acid, and 2-Hexanol. Cyclobutanol was present readily in both healthy and diseased tissues. The findings of these analyses are consistent with earlier reports that the infective processes of pathogens result in the alteration of the metabolite pool of the host organism. The dominance of *F. moniliforme* and *M. phaseolina* would suggest that resistance to these two pathogens should be emphasized in the breeding programs. The index of detection of stalk rot disease must be improved since plant density and water stress could have increased disease incidence in Haripur. Balanced fertilization is also of utmost importance since excess nitrogen fertilization has been associated with an increased susceptibility to *Fusarium,* while sufficient potassium increases resistance due to its effect in solidifying plant cell walls (Bibi *et al*., 2010). Biological control also appears to be promising. Antagonistic fungi such as *Trichoderma* spp. and beneficial bacteria like *Bacillus subtilis* have been reported to restrain *Fusarium* and *Macrophomina* by competition, antibiosis and the production of systemic acquired resistance (Shafique *et al*., 2016). Further, post-harvest measures are important since *Aspergillus* spp. may take part in storage contamination. The drying of maize to safe moisture contents, an increase in aeration of storage bins, and regular checks for mycotoxins are measures that could lessen losses and safeguard food safety (Saleem *et al*., 2012). The novelty of this study lies in the multidisciplinary approach taken. Using this integrated investigation the causes of stalk rot infection were not only confirmed, but information on the biochemical markers of the organism was obtained. The study has also indicated the regional trends of maize stalk rot in Haripur, where maize production is so important from the point of view of the living of so many people, which has otherwise been little known about so far from the point of view of research into plant pathological problems. The study is not without limitations. The investigation is from only one cropping season and seasonal variation might affect both the incidence of the pathogens and the severity of the disease symptoms. By the duration of the sampling over more than one year, a true appraisal of the epidemiology of the disease in the locality would be obtained. The molecular identification of the fungi in this case has been entirely the result of Internal Transcribed Spacer sequence analysis, but would be even more secure in this respect if identification was carried out based on a multi locus sequence analysis. Also, while GC-MS has been useful in defining the leaf metabolite changes, it would be preferable in the future that the biochemical sampling should be extended to flora

and grain, where a more representative picture of the incidences of disease and the storage of mycotoxins might be gained. The stalk rot in maize produced in Haripur may be due to various complex environmental conditions also besides the soil borne and opportunistic fungi. The *F. moniliforme* and *M. phaseolina* being the principal organisms involved, which have caused great structural disturbance of the tissues involved, with a marked decrease in the chlorophyll content. From a control point of view we need to determine the best means of growing resistant strains of maize, giving the crops better agronomic support, the use, where possible, of biological warfare systems which are known, and use the environment friendly methods of storage, of the crops produced, are vital steps necessary in obtaining a better state of control in the areas affected with stalk rot. This work has been able to indicate a line of approach in the studies both from the point of view of the diversity of the pathogens met with in the investigation and the various aspects of the biochemical multidimensional activity and responses of the host systems, while at the same time giving us foundation for further research into the overall study of the sustainable means of control for this disease in maize grown in the other places of world. This study focusses on the limited pathogen diversity as multiple genera or species could contribute to stalk rot disease in different regions. Samples were collected from only Haripur region and at specific climatic conditions while the results might not represent stalk rot disease dynamics at different climatic conditions.

Fig. 14. Phylogenetic tree generated using nucleotide sequence information of the Internal Transcribed Spacer region of the conserved ribosomal DNA of Fusarium isolates.

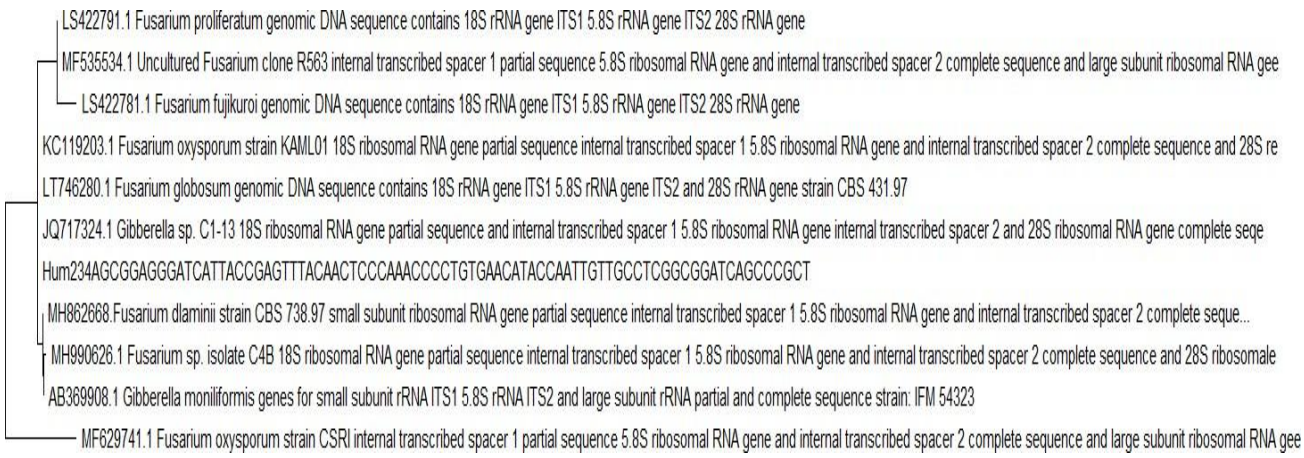

Fig. 15. Phylogenetic tree generated using nucleotide sequence information of the Internal Transcribed Spacer region of the conserved ribosomal DNA of Fusarium isolates.

Conclusion

F. moniliforme (Accession number PZ039329), *M. phaseolina* and *F. oxysporum* (Accession number PZ049446) were detected as the primary causal agents for stalk rot of maize in Haripur in summer 2018. *Aspergillus niger* and *Aspergillus flavus* was also identified in the infected soil samples. Infection was associated with certain characteristic anatomical damage and reduction of chlorophyll and enhanced activities of Catalase and Peroxidase. Some stress-related compounds, were also found in diseased samples after GC-MS analysis. The integration of morphological, genetic and biochemical approaches provided pathogen identification, but also indications of host-pathogen interactions, thus providing a basis for finding strategies in the future for minimizing maize stalk rot.

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