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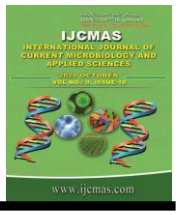

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Cultural, Morphological and Molecular Characterization of *Fusarium verticillioides* causing Maize Ear Rot under Punjab Condition

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A B S T R A C T

Maize ear rot caused by *F. verticillioides* which is prevalent on maize under Punjab conditions. The symptoms appear as white-pink fungal growth and causes "starburst" pattern of white streaks on the kernels. Thirty *Fusarium* isolates were isolated from the infected maize plant from different agro-chemical zones of Punjab. They are characterized on the basis of morphological, cultural characters and growth rate on Potato dextrose agar (PDA). The isolates produced micro-conidial 4.0-33.0 µm x 2.4-3.3 µm on PDA and macrocondia of 37.0-55.0 µm x 4.0-4.2 µm on Carnation leaf agar (CLA). Formation of sporodochia was observed on the Carnation leaf agar (CLA). The production of chalmydospores was not observed in any of the isolates. Isolates were grouped into three categories based on colony color and conidial size. The micro-conidial chains production was also observed on water agar infused with 0.2 perent KCl. Genomic DNA was extracted and was subjected to Polymerase chain reaction (PCR) by using species specific primers (PRO 1 PRO2, VER 1 VER2, VERT 1 VERT 2 and SUB1 SUB2). Out of thirty 27 isolates were confirmed as *F. verticillioides* as they gave single amplicon of 800 bp with primer pair VERT 1 & VERT 2 specific for *F. verticillioides.*

Introduction

Maize (*Zea mays* L). under family Gramineae, is the world's important crop that ranks third after rice and wheat with respect to area and production. It is one of the most versatile emerging crops having wider adaptability under varied agro-climatic conditions. There are various major constraints in the maize production both abiotic and biotic stress which not only affects the yield but also the quality of the produce as well. Among the biotic stress the various diseases caused by fungi, bacteria, viruses, mycoplasma like organisms and nematodes attack maize crop.

Diseases like root rot, stalk rot, and seedling blight causes heavy losses every year by deteriorating the quality and quantity of grains. Maize crop are attacked by several pathogenic species of *Fusarium* belonging to

section *Liseola* including *F. proliferatum, F. graminearum* and *F. anthophilium* (Baired *et al.,* 2008). Fusarium ear rot (FER) is caused by species within the *Gibberalla fujikuroi* species complex (GFSC), especially *F. proliferatum* and Gibberella ear rot (GER) is caused by *Gibberella zeae*, the telomorphic stage of the *F. graminearum* species complex (FGSC) (Bampi *et al.,* 2012).

Among all the *Fusarium* species, *F. verticillioides* is most prevalent on maize worldwide (Leslie and Summerells 2006). About 7-17 percent of reduction in the quantity and quality of the maize was due to the favourable conditions for the diseases (Nagy *et al.,* 2006). *F. verticillioides* is an endophyte of maize which established long-term associations with the plant (Pitt and Hocking 1999). Due to the disease, the ear yields poor grain quality contaminated with mycotoxins, which represent a threat to both humans and animals (Logrieco *et al.,* 2002; Munkvold 2003). The major symptom of the disease is white-pink fungal growth on kernels and causes "starburst" pattern of white streaks on the cap of the kernel or along the base (Wang *et al.,* 1991). Both the morphological and molecular identification is required for the identification of *Fusarium* species.

The morphological identification of the plant pathogenic fungi is the initial step and the most difficult pace for the identification. The morphological identification is not adequate for the *Fusarium* species due to similar characteristic like mycelia pigmentation and shape of the conidia.

For accurate and rapid identification Polymerase chain reaction (PCR) using species specific primer is becoming one of the most effective method (Zheng and Ploetz 2002).

Materials and Methods

Fungal Isolation, Purification and Preservation

The ear rot infected samples were collected from different maize growing districts of Punjab (Hoshiarpur, Shaheed Bhagat Singh Nagar, Ropar, Jalandhar, Ludhiana, Gurdaspur and Karputhala). Isolations of the fungi associated with maize ear rot were done from the infected grain samples by using standard procedure. Four surface sterilized grains were placed equidistantly on potato dextrose agar (PDA) in each Petri plate under complete sterile and aseptic conditions. Plates were incubated at 25±2º C in a BOD incubator. The cultures obtained were purified and examined microscopically to identify the fungi associated with ear rot. Different isolates of *Fusarium* spp. were purified by single spore culture method and multiplied on PDA slants for further studies. Finally 30 isolates of *Fusarium* were obtained as F1-F30 and preserved them in slants containing PDA and sterilized soil for further studies.

Cultural and Morphological Characterization

Morphological characteristics

For studying the morphology of micro-conidia, conidiophores and measuring the size, each isolate was grown separately in Petri-dish on water agar amended with KCl. Morphology of macro-conidia and formation of sporodochia were studied using Carnation leaf agar medium (CLA). Slide were prepared and observed under microscope (Leica DM 3000). For studying the macro-conidia and sporodochia, CLA media was prepared as per protocol (Leslie and Summer all 2006). The species were identified morphologically based on "The *Fusarium* Laboratory Manual" (Leslie and Summerall 2006). The size of

conidia was measured for each isolate separately at 40 X by using image analyzer software. For observation of colony color, pigmentation and exudation, 5mm mycelia disc from actively growing culture of each isolate was cut off with the help of cork borer and inoculated on the centre of the petri plate containing PDA medium. After inoculation, petri plates were incubated at the 25± 2° C. Each isolate was replicated thrice. Colony diameter was recorded for 15 days at alternate intervals, thereafter colony color, pigmentation for each isolates and production of exudation was recorded.

Identification of *Fusarium* spp. by PCR using Species Specific Primers

Culture preparation and DNA extraction

Thirty morphologically identified *Fusarium* isolates were grown on 100 ml liquid medium (Potato Dextrose Broth) in 250 ml flasks for 10 days at 25 ± 2°C incubation. After 10 days of incubation the fungal mycelia mat was harvested by filtering with pre-weighed filter paper (Whatman No.1). Genomic DNA was extracted from purified 30 cultures by using mini-prep CTAB method (Murray and Thompson 1980). Quantity and quality of DNA was checked by Thermo Scientific NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Quality of DNA samples was also tested on 0.8 per cent (w/v) agrose gel. Single high molecular weight band represented good quality of DNA.

PCR amplification (Species specific primers)

The genomic DNA of all the 30 isolates were subjected to amplification using the species specific primers namely VERT 1 VERT 2 (Patino *et al.,* 2004), VER1 VER 2, PRO 1 PRO 2 and SUB 1 SUB 2 (Mule *et al.,* 2004). PCR reaction were carried a total volume of 25µl reaction mixture, containing DNA sample, 10X PCR buffer, 2.5 m M $MgCl_2$, 0.25 m MdNTP mix, 20pmol/ml of each forward and reverse primer and 0.40µl (30U/µL) of Taq DNA polymerase was prepared. For PCR, initial denaturation was set at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and primer extension was set 72°C for 1.30 min and final extension at 72°C for 8 min followed by cooling at 4°C. PCR products (10 µl) were loaded at 1.0% Agarose gel containing ethidium bromide along with the standard 1kb bp DNA ladder (Promega) and subjected to gel electrophoresis at 10X TBE buffer at constant voltage of 5Vcm for about 1 hour. After electrophoresis, the gel was visualized under UV transilluminator and photographed using Alpha Innotech Multi Imager gel documentation system software programme from Alpha Innotech, California, USA.

Results and Discussion

Cultural characterization of *F. verticillioides*

On the basis of cultural characters

All the 30 isolates of *F. verticillioides* show cultural variations on the PDA. The color of the colony varied from white to purple and type of mycelium aerial to compact (Plate 1). On PDA medium, isolates formed aerial mycelia and produced violet pigments ranged from a pinkish orange to dark violet (Nithiyaa and Izzati, 2012). Pigmentation in *F. verticillioides* varied from no pigmentation to dark violet or dark margenta on PDA medium (Leslie and Summerell, 2006).

On the basis of colony diameter

The colony diameter was recorded for 15 days at alternate intervals till the fungus fully

covered the Perti dish (Table 2). Most of the isolates (F1, F3, F4-F6, F9-F13, F15-F22 and F24-F3) except four isolates *viz.*, F2, F8, F14 and F23 fully covered the Petri dish after 7 days of inoculation. Potato dextrose agar was used for morphological appearances and colony colorations by Nelson *et al*., 1983. On the basis of colony diameter and average growth rate/day all isolates were categorized into 2 categories (Table 2).

Category I having isolates F3 and F4 with mean colony diameter 60.00-70.00 and average growth rate of 7.50-9.00 mm/day. Category II consisted of isolates F1-F2, F5-F30 with mean colony diameter 70.00-80.00 and average growth rate of 9.10-10.50 mm/day.

Morphological characteristics of *Fusarium* spp.

The average size of microconidia ranged from 4.0-33.0 µm x 2.4-3.3 µm and shape varied from oval to club with flattened base and usually 0-septate. The micro-conidia were abundant in aerial mycelia, formed long chains or false head attached at monophialides branches and cultures did not produce chlamydospores (Li *et al.*, 2006). The average size of macroconidia ranged from 37.0-55.0 µm x 4.0-4.2 µm and the shape were curved, tapered to a point, notched or foot shaped and usually 3 to 5 septate (Plate 2). The fungus produces a copious amount of single-celled micro-conidia and plentiful amount of septate macro-conidia (Li *et al.*, 2006).

The macro-conidia and sporodochia were produced on carnation leaf agar medium by all the isolates. Three types of media i.e., carnation leaf agar (Fisher *et al.*, 1982), potato dextrose agar (Nelson *et al.*, 1983), KCl medium (Fisher *et al.*, 1983) were used for identification of the *Fusarium* species.

The conidial chains having V-shaped pairs appear to give a "rabbit ear" like structure (Leslie and Summerell, 2006). None of the isolate produced chlamydospores in culture. Chains of micro-conidia were present and chalmydospores were absent on the isolates of *Fusarium* (Bashyal *et al.*, 2015).

Table.1 Sequence of Species specific primers

Primer name	Sequence	Product size (bp)	Reference
VERT 1 **VERT 2**	GTCAGAA TCCATGCCAGAACG CACCCGCAGCAAT CCATCAG Specific for *F. verticillioides*	800 bp	Patino *et al* (2004)
VER1 **VER 2**	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA Specific for *F. verticillioides*	578 bp	Mule *et al* (2004)
PRO 1 **PRO 2**	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG Specific for *F. proliferatum*	585 bp	
SUB 1 **SUB 2**	CTGTCGCTAACCTCTTTATCCACAGT ATGGACGTTGGTATTATATCTAA Specific for *F. subglutinas*	631 bp	

Table.2 Categorization of *Fusarium* isolates on the basis of their colony growth on potato dextrose agar medium

Category	Isolates	Colony diameter (mm)	Growth rate/day (mm)
I	F3, F4	60.00-70.00	7.50-9.00
II	F1-F2, F5-F30	70.00-80.00	9.10-10.50

Table.3 Characterization of *F. verticillioides* isolates on the basis of the microconidia, macroconidia and morphological characteristics

CATEGORY	Colony Color*	Isolates on PDA	Types of mycelium	Micro-conidia size range (µm)**	Macro-conidia size range (µm)**	No. of Septa	Sporodochia***	Chains****
GRP I	Milk white, White, Cotton candy, Pearl	F(1,2,9,15,19,29) F(14,20,30)F(5,23), F(3,21,25)	Compact to Aerial Compact Aerial Aerial to Fluffy	(4.00-34.60) x (2.103.30) µm	(37.10-48.60) x (4.004.10)µm	3-5	+	+
GRPII	Tyrian purple, Viola purple, Plum purple, Dull purple, Blue Lotus, Lipstick purple	F(8,17) F(10,26,27) F(12,16), F(22,28), F(6,18,24), F(7)	Compact, Compact, Aerial, Aerial, Aerial, Compact	(4.10-27.40) x (2.40-2.50)µm	(37.40-42.90) x (4.00-4.10)µm	4-5	+	+
GRPIII	Maroon, Egg plant	F(4,11,13)	Aerial	(8.40-33.00) x (2.50-2.80)µm	(37.50-39.70) x (4.00-4.10)µm	4-5	+	+

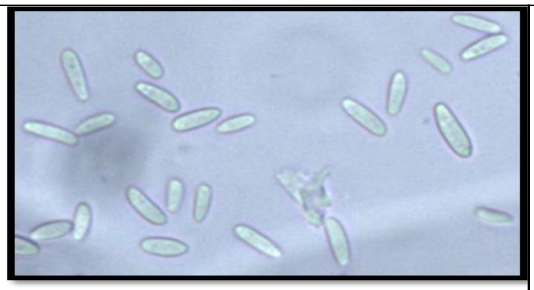
*As per colour chart from website www.W3schools.com (-Absent, +Present)
**On PDA medium
*** Carnation leaf agar (CLA) medium
****On water agar with 2 % KCl (-Absent, +Present)

Plate.1 Colony color of different isolates of *F. verticillioides*

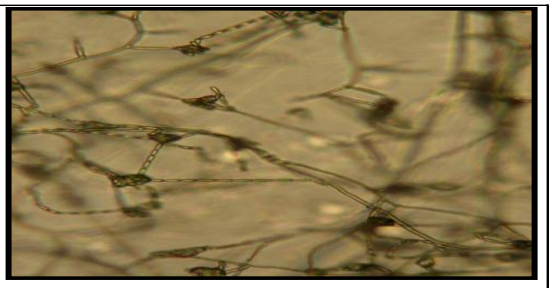
GRP I (White) **GRP II (Purple)** **GRP III (Maroon)**

Reverse on PDA

Plate.2 Morphological features observed under the microscope

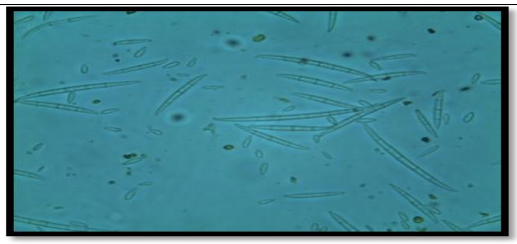


(A) Micro-conidia



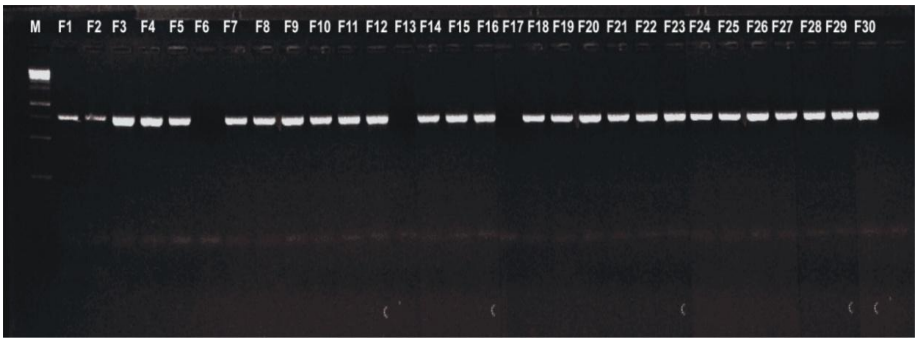
(B) Micro-conidial chain

(C) Sporodochia



(D) Macro-conidia

Plate3 DNA amplification of *F. verticillioides* obtained by species specific primer VERT1 and VERT2



Based on the morphological characteristics isolates were grouped into 3 categories (Table 3). GRP I contain Milk white, White, Cotton candy and Pearl colored colony producing isolates with size of microconidia ranging from (4.00-34.60) x (2.10-3.30) µm with no septa and macroconidia ranging from (37.10-48.60) x (4.00-4.10)µm with 3-5 septa. GRP II comprised of Tyrian purple, Viola purple, Plum purple, Dull purple and Blue Lotus, Lipstick purple colored colony with size of microconidia ranging from (4.10-27.40) x (2.40-2.50)µm with no septa and macroconidia ranging from (37.40-42.90) x (4.00-4.10)µm with 4-5 septa. GRPIII consisted of Maroon and Egg plant producing isolates with size of microconidia ranging from (8.40-33.00) x (2.50-2.80) µm with no septa and macroconidia ranging from (37.50-39.70) x (4.00-4.10) µm with 4-5 septa (Plate 1). On the basis of the cultural and morphological data, it was confirmed from "The *Fusarium* Laboratory Manual" that the all isolates are *F. verticillioides.*

Molecular characterization of *F. verticillioides* by species specific primers

For further confirmation, all the 30 isolates of *F. verticillioides* were subjected to PCR by using species specific primers VERT1 VERT2, VER1 VER2, PRO1 PRO2 and SUB1 SUB2 (Table 1). Out of the 30 isolates of DNA, got amplified with set of primers VERT1 VERT2 and gave a single amplicon of 800bp (Plate 3). None of the isolate got amplified with the primers specific for *F. proliferatum* and *F. subglutinas.* So, confirmation was made from the morphologically as well as the molecular characterization that the species isolated from the sample is *F. verticillioides*

The pathogen was isolated from the infected samples of maize ear rot collected from different districts of Punjab. All the thirty isolates obtained are characterized on the basis of cultural, morphological and molecular methods. The studies revealed that maize ear rot is caused by *F. verticillioides* in Punjab and no other *Fusarium* spp were detected. However, the maize ears are also infested with the other species of *Gibberella fujikuroi* complex, including *F. proliferatum* and *F. subgultinans* (Augin *et al.*, 2013). With reference to "The *Fusarium* Laboratory Manual," the cultural characteristics (colony color, colony diameter, pigmentation, exudation, type of mycelium) and morphological characteristics (shape and size of micro and macro conidia, presence of micro-conidia in chain and presence or absence of chalmydospores or sporodochia) revealed that maize ear rot is caused by *F. verticillioides* (Leslie and Summerell 2006). From the four different types of species

specific primers VERT1/ VERT2, VER1 /VER2, PRO1/ PRO2 and SUB1 /SUB2, only VERT1 /VERT2 gave the positive results. VERT1 and VERT2 primers are specific for *F. verticillioides* based on Intergeneric spacer region of Ribosomal DNA (Mirete *et al.*, 2004; Patino et al 2004; Kaur *et al.*, 2014) and have efficiency to discriminate among various other *Fusarium* spp. Out of 30 isolates, 27 isolates gave the 800 single amplicon with the VERT1/VERT2 species specific primers. Sreenivasa *et al.*, (2008) and Dissanayake *et al.*, (2009) also identified *F. verticillioides* from Indian maize kernels by using VERT1 and VERT2 primer pair. Conclusively, it can be determined that *F. verticilloides* is a predominate *Fusarium* spp. associated with the disease in Punjab.

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