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Screening of Mycotoxin Produced by *Fusarium Verticillioides* and *F. Proliferatum* in Culture Media

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Abstract

This study was conducted to evaluate the production of mycotoxin by *Fusarium verticillioides* and *Fusarium proliferatum* strain in culture media. Results of Fusarium strain speciation by sequencing technique for the αTEF gene regent showed that 16 isolates from 21 belong to *F. verticelloides*, four to *F. proliferatum* and one to *Fusarium solani*. Mycotoxin production test showed that all Fusarium strains obtained produce different levels of T2-toxin, DON, ZEN and fumonisin in culture media with fumonisin toxin having the highest chemotype produced.

Keywords: Chemotypes, Fusarium, PCR

Introduction

According to Braun (2007), maize (*Zea mays* L.) is a staple food for the majority of the world's population as well as wheat and rice. This important food source can be infested by mycotoxin produced by *Fusarium spp. Fusarium verticillioides* (synonym: *F. moniliforme*) belongs to a large genus of filamentous fungi which is prevalent in the midwestern and southeastern United States with some species producing mycotoxins in cereal crops that can affect human and animal. *F. proliferatum*, a closely related fungus also occurs regularly and has been isolated from infected maize (Abbas *et al*., 2002). These species produce high levels of fumonisin (Leslie, 1991, 2004); even in plants that do not show symptoms of infestation.

Plant can become infected in several ways; the most common pathway is via the silk channel. Another manner of spread of *F. verticillioides* is by the European corn borer and other insects such as the corn earworm. The feeding activities of these insects may spread spores to silks or directly to kernels and can create wounds in kernels, which are then colonized by the fungus. *F. verticillioides* and *F. proliferatum*, the most common species associated with maize were found to produce fumonisin and T2-toxin (Nelson *et al*., 1993; Jinanarong, 2000; Matny 2013). According to Ronald *et al*. (1981) *F. monoliforme* grown on cracked corn for 13 days at 28°C was found to produce T-2 toxin at 33 µg/g and vomitoxin at 1.5 µg/g while El-Kady and El-Maraghy (1982) recorded that strains of *F. moniliforme* from Egypt produced ZEA on optimized medium. Matny (2013) also reported that *F. proliferatum* produce ZEN toxin in wheat straw culture.

Materials and method

Source of isolates

Twenty one strain of *Fusarium* spp were isolated from maize (stalk and ear rot) by surface sterilization with 10% sodium hypochlorite for 3 min, and culture in 9 cm Petri dish potato dextrose agar (PDA). The culture was incubated at 25±2^{0}C for 5 days, after which *Fusarium* spp mycelium growth were re-culture by hyphal tip technique in a new PDA Petri dish.

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***Fusarium* spp speciation**

Fusarium isolates were identified by sequencing the translation elongation factor-1 alpha gene (αTEF gene) region while DNA was extracted by CETAP method. PCR was carried out using translation elongation factor-1 alpha gene (αTEF gene) EF1 ^{5}GTGGGGCATTTACCCCGCC^{3} and EF2 ^{5}ACGAACCCTTACCCACCTC^{3}. PCR reaction mixture for TEF gene consisted of 12.8 µl milliQ H_2O, 2.5 µl 10x buffer, 2.5 µl $MgCl_2$, 0.5 µl dNTP mix (10 mM each), 0.8 µl forward primer EF1 (10mM), 0.8 µl reveres primer EF2 (10mM), 0.1 µl Taq (Biotech) and 5 µl DNA sample. All preparation steps were done in ice bath. PCR procedures are as follows: initial denaturation at 95^{o}C for 75 s, denaturation at 95^{o}C for 15s, annealing was at 53^{o}C for 30s, primer extension at 72^{o}C for 30s, (38 cycles), final extension was at 2^{o}C for 30s. The PCR reactions were cleaned up using Ultra Clean TM PCR Clean-Up Kit from MO BIO Laboratories Inc while the product was analyzed using electrophoresis in 1.5% agrarose gel. The reaction mix was prepared by adding 13 µl MilliQ H_2O, 3.5 µl 5X buffer, 0.5 µl primers EF1 or EF2 (prepared separately), 1.0 µl BDT and 2 µl DNA samples. Cycling parameters were 96^{0}C for 2 min 1 cycle, (96^{o}C for 10 s, 50^{o}C for 5 s, 60 ^{o}C for 4 mins) 30 cycles. PCR product was cleaned up using Agencourt Clean SEQ kit, Agencourt Bioscience Corporation. 62 µl of 85% ethanol were added to each PCR product and blend by pipetting seven times the product into a clean PCR plate. The plate was place on SPRI Plate 96R for 3 min to separate beads. The clear phase was transferred into a new clean plate loaded on the detector and send for sequencing in Australian Genome Research Facility Ltd (AGRF) in Brisbane.

Mycotoxins detection

Twenty one fusarium isolates were tested for the production of mycotoxin in wheat seed, corn, wheat straws and millet. They were soaked in water over night. After which water was drain out and materials autoclaved at 121^{o}C, and at a pressure of 1.5 inch/cm^{2} for 30 min. Ten gram of sterile wheat straw in 9 cm dim Petri dish was inoculated with a disc of 1 cm dim of fusarium isolate culture, 7 days old on PDA media. Three replicates of each isolate were incubated at 25 ±2^{o}C for 21 days. The cultures were dried, grounded using coffee grinder and conserved at 4°C. Mycotoxins were extracted using Accelerator Solvent Machine ACE200; 1g of each grinded culture was extracted with 15 ml of Acetonitril: water 85:15. Five hundred µl of each extract was dried under nitrogen flow in 1.5 ml plastic tubes. Fifty five µl of milliQ water were added to each tube and vortex for few seconds each. The toxin in each sample was detected by RIDscreen T-2 Toxin, Zearalenon, Fumonisin RBiopharm AG and Germany and Beacon Deoxynivalenol ELISA kit (Analytical Systems Inc. USA).

Results and discussion

***Fusarium* spp speciation**

Result of DNA sequencing according to GenBank database BLAST searching using individual sequences showed that sixteen isolates belong to *F. verticelloides*, four to *F.proliferatum* and one to *F. solani* (Figure 1).

Mycotoxin production

All Fusarium isolates in this study showed ability to produce mycotoxins in tested culture media. Result of ELISA test showed that DON toxin was produced by all *Fusarium* spp depending on the type of culture and fungal strain. *F. verticelloides* IR93 and IR56 strain produced high concentration of toxin at 2.04 and 2.40 mg/kg on wheat and wheat straw culture respectively, while *F. solani* IR66 produced the highest concentration at 4.45 mg/kg on wheat straw (Figure 1). Aliakbari *et al.* (2011) found that artificial inoculated seed corn with *F. proliferatum* and *F. verticelloides* separately at laboratory conditions produced DON toxin at a mean level of 95. 30 ng/g after 10 days of inoculation.

Both *F. verticelloides* and *F. proliferatum* are known to produce Fumonosin toxin in high concentrations. The results of this study showed that *F. verticelloides* strain was the highest producer of Fumonosin toxin ranging between 5.8-10477.0 mg/kg. The concentration of toxin produced by *F. verticelloides* IR10 and IR46 on millet culture was 10477.0 and 10215.4 mg/kg respectively while *F. verticelloides* IR10 on wheat culture produced 10347.9 mg/kg toxin. One isolate of *F. proliferatum* IR1 produced

high toxin at 9926.6 mg/kg on millet culture (Figure 2). *F. verticillioides* and *F. proliferatum* are by far the most prolific fumonisin producers; they produced the highest amounts of toxins. According to previous research, up to 17900 μg/g of FB1 have been recorded in cultures for *F. verticillioides* and 31000 μg/g FB1 for *F. proliferatum* (Rheeder *et al.*, 2002; Shephard *et al*., 1996).

All Fusarium strains produced T2-toxin on culture media with concentrations ranging between 2.13-20.2 mg/kg. *F. verticelloides* IR93, IR52 and IR10 produced 20.2, 12.1 and 9.34 mg/kg on millet culture respectively while *F. verticelloides* IR57 gave 9.79 mg/kg on corn culture media (Figure 3). A similar research by Varnaitė *et al*. (2006) showed that *F. proliferatum* can produce T2 toxin in artificial culture media.

Zearalenone determination showed that all isolates produced ZEN toxin at concentrations between 0.5-3.4 mg/kg. *F. verticelloides* IR46, IR56 and IR57 produced 3.4, 3.2 and 3.1 mg/kg on wheat grain culture respectively, while IR32 strain on millet culture produced 3.1 mg/kg (Figure 4).

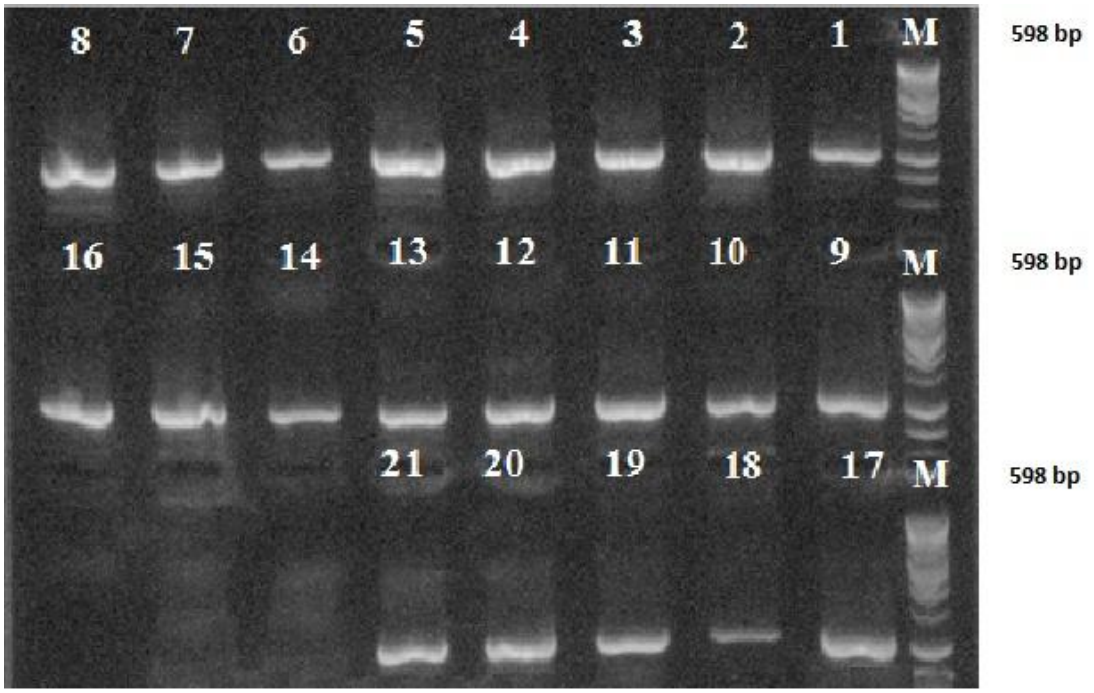

Figure 1: Agarose gel (1.5%) photograph showing PCR amplified products for α-TEF gene for *Fusarium* spp isolates, the product prepared for sequencing test. 1, 2, 3…..21 = the *Fusarium* spp isolates, M= marker

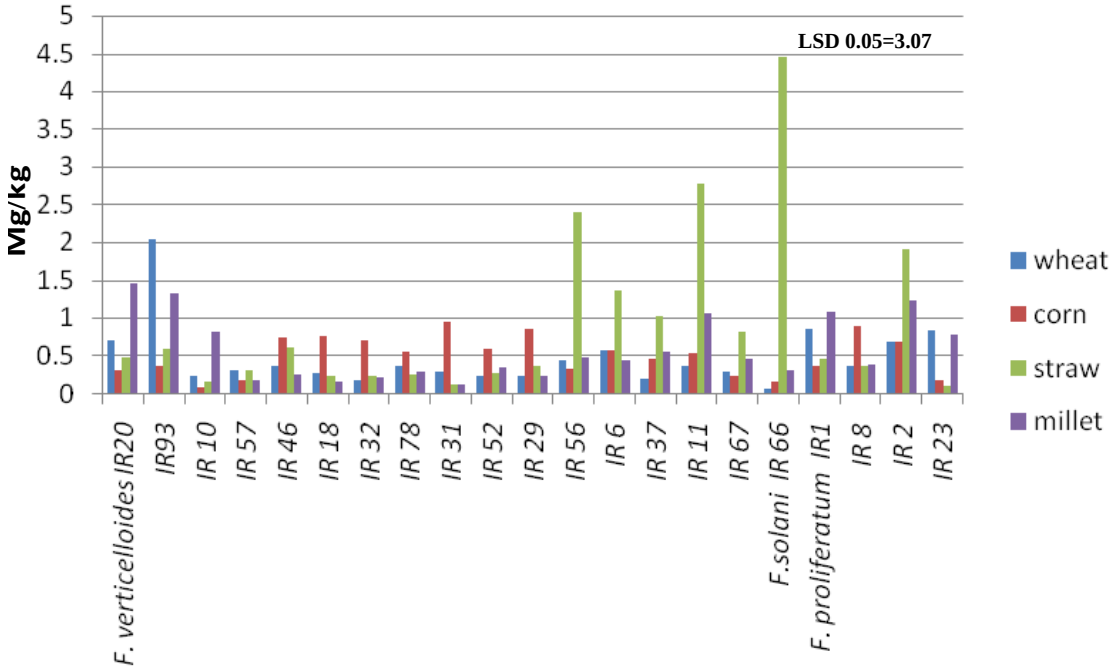

Figure 1: Deoxynivalenol produced by different *Fusarium strain* in wheat, maize, wheat straw and millet culture

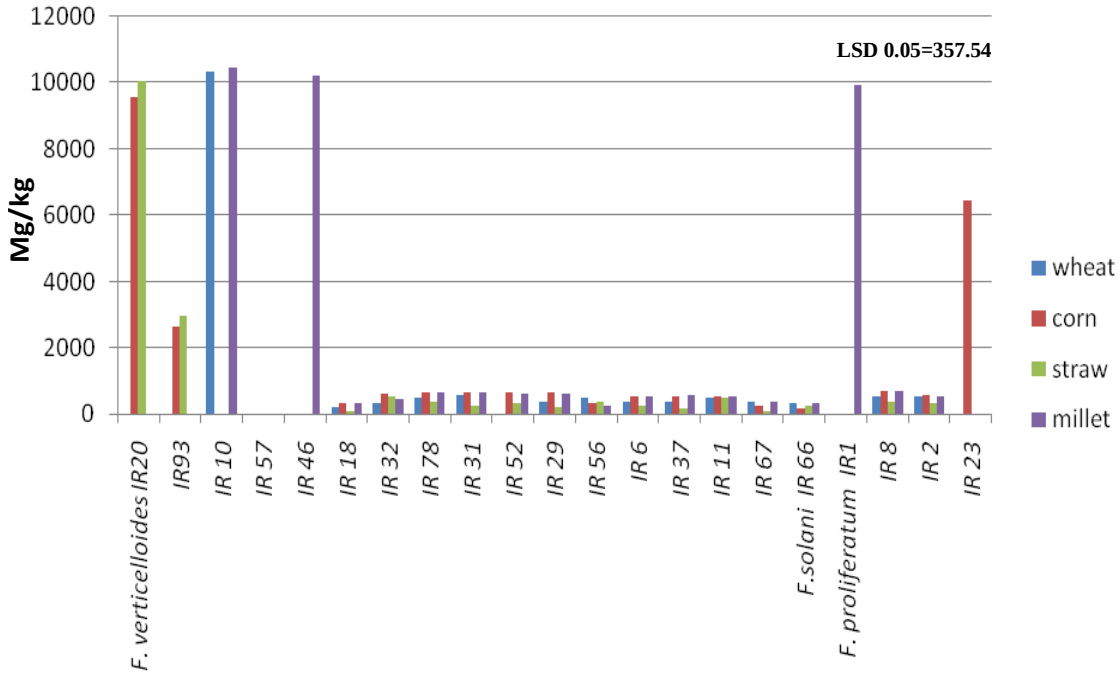

Figure 2: Fumonisin produced by different *Fusarium strain* in wheat, maize, wheat straw and millet culture

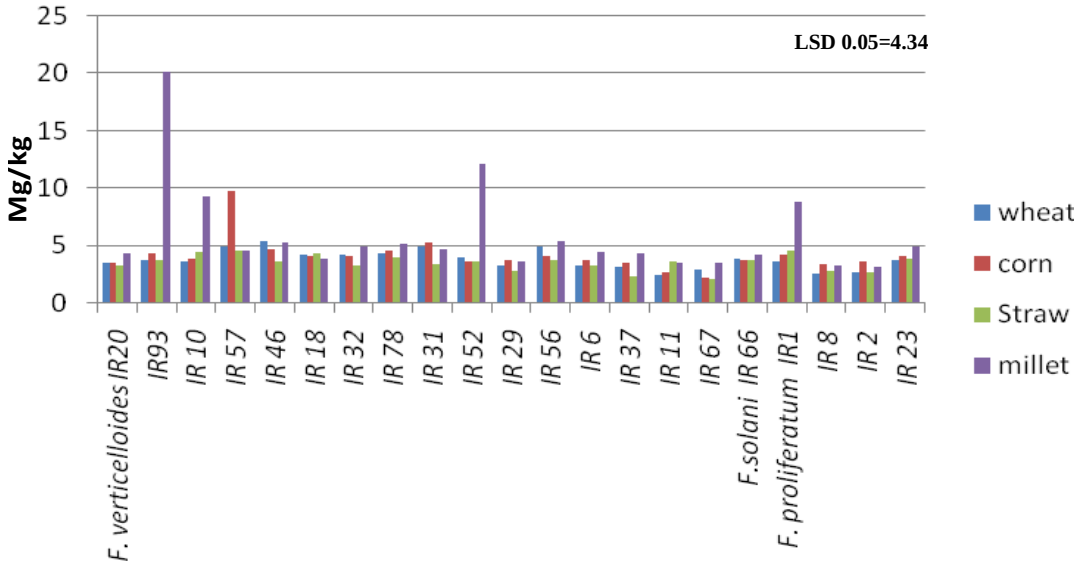

Figure 3: T2-toxin produced by different *Fusarium strain* in wheat, maize, wheat straw and millet culture

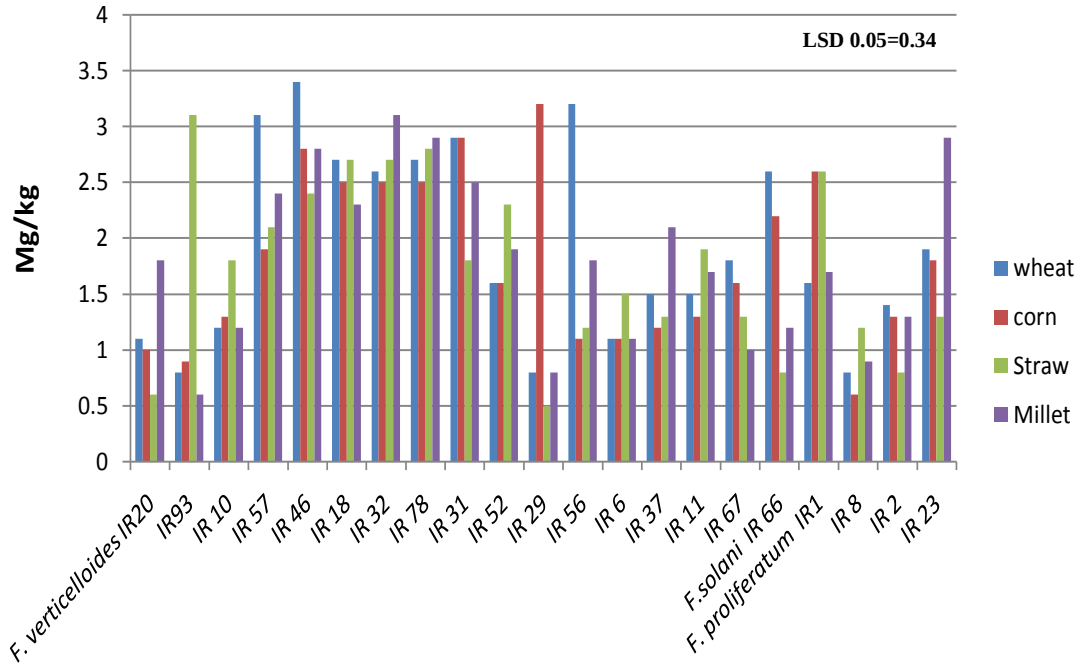

Figure 4: Zearalenone produced by different *Fusarium strain* in wheat, maize, wheat straw and millet culture

Conclusions

Fusarium spp have being known to produce mycotoxin which infest many cereals crop such as maize and wheat especially under bad storage conditions. The results of this study showed that *F. verticelloides* and *F. proliferatum* have the ability to produce high concentration of Fumonisin in growing cultures, amounting to more than 10000 mg/kg as well as in producing other kinds of mycotoxin such as Zearalenone, T2-toxin and Deoxynivalenol in different concentrations depending on the culture media and the fungal strain. This poses a serious

problem to human and animal diet as this infected grain can be blended with clean grain for food/feed production.

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