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Aggressiveness of Certain *Fusarium graminearum* Isolates on Wheat Seedlings and Relation with their Trichothecene Production

[1]M.R. Asran and [2]M.I. Eraky Amal
[1]Department of Plant Pathology, Faculty of Agriculture, University of Sohag, Sohag, Egypt
[2]Department of Plant Pathology, Faculty of Agriculture, University of Assiut, 71526, Assiut, Egypt

Abstract: *Fusarium* fungi, including *F. graminearum*, cause seedling blight, foot rot and head blight diseases in wheat resulting in yield loss. Trichothecene mycotoxins are a group of toxic fungal secondary metabolites. This group of toxins is found associated with *Fusarium* diseases in wheat in numerous countries worldwide. Five isolates of *F. graminearum* were examined for their degree of disease severity (virulence) and trichothecene production on Sakha 69 wheat cultivar. In greenhouse experiments, following soil infestation all isolates caused pre and post-emergence death of wheat seedlings. Dry weight of infected seedlings was reduced as compared with uninoculated control seedlings. The *F. graminearum* isolates resulted in varying degree of disease severities in seedlings. All tested isolates caused seedling blight symptoms. However, they differed in their degree of pathogenicity. The reaction of wheat cultivars against seedling blight varied. While cv. Sakha 69 was the most susceptible (35.22%), cv. Giza 164 exhibited the highest level of resistance (14.61%) and cv. Giza 168 showed a moderate degree of resistance (23.17%). The *F. graminearum* isolates were examined with regard to possible relation between seedling blight severity and trichothecene production. The trichothecenes contents were detected by using gas chromatography equipped with an Electron Capture Detector (GC-ECD). The amount of trichothecenes produced by the various isolates on autoclaved oat grains ranged from 1393-57081 μg kg^{-1} ground grain. All isolates produced trichothecene *in vitro* but differed significantly in their level of production. The highest amounts of total trichothecene were detected in grains inoculated with isolate Fg 4.3 (57081 μg kg^{-1} ground grain) while grains inoculated with isolate Fg 33 and 18.7 had the lowest amounts of total trichothecenes. There was a close relationship between the degree of disease severity and trichothecene concentration.

Key words: *Fusarium graminearum*, seedling blight, wheat cultivars, trichothecene, mycotoxins, pre and post-emergence, virulence

INTRODUCTION

Wheat is one of the most important feeding crops in Egypt and many other countries in the world. It is primarily grown as a food crop but the straw is also used for industrial products as feed for livestock. Wheat is subjected to relatively large number of diseases during its growing seasons which attack all plant parts causing serious losses in crop productivity (Bakr, 1997).

Fusarium graminearum Schwabe (teleomorph *Gibberella zea* (Schwein.) Petch) has received an overwhelming attention over time. The reason attributed to this attention is that *F. graminearum* has been found to be the most dominant pathogen in many wheat growing areas (McMullen *et al*., 1997; Gilbert and Tekauz, 2000). It survives in soil and is seed-transmitted, it may causes reduction of germination through seed decay and seedling blight.

Variation in virulence among the *F. graminearum* isolates has been reported (Carter *et al*., 2000; Walker *et al*., 2001; Wanyoike, 2002). The physiological factors leading to different virulence levels among isolates are still unclear although synthesis and secretion of cell wall regarding enzymes as well as the production of mycotoxins have been assumed to play an important role (Desjardins and Hohn, 1997).

Trichothecene mycotoxins are a group of toxic fungal secondary metabolites characterized by a sesquiterpenoid structure with a double bound at the C-9/C-10 position and an epoxide ring at the C-12/C-13 position. Trichothecene derivatives, such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15- acetyldeoxynivalenol (15-ADON), nivalenol (NIV), T-2 toxin and HT-2 toxin were found associated with *Fusarium* diseases in wheat in numerous countries worldwide. It is reported that a trichothecene deficient

Corresponding Author: M.R. Asran, Department of Plant Pathology, Faculty of Agriculture, University of Sohag, Sohag, Egypt

F. graminearum isolate generated by gene disruption was pathogenic on wheat and rye but was less pathogenic than the wild-type isolate (Proctor *et al*., 1995; Riungu *et al*., 2008). These findings indicate that DON formation is not required for pathogenicity but may play a role in the virulence of *F. graminearum*. Furthermore, isolates of *F. graminearum* have been isolated which did not form DON although they possessed some degree of virulence (Szecsi and Bartok, 1995; Wanyoike, 2002). The objectives of this study were to investigate the relationship or interactions between virulence and trichothecene production of the fungal isolates.

MATERIALS AND METHODS

Source of fungal isolates: Five isolates of *F. graminearum* (Table 1) were obtained from the culture collection at the Institute of Phytomedicine, University of Hohenheim, Germany. Three of the isolates previously tested by Asran and Buchenauer (2003) and other two isolates were previously tested by Wanyoike (2002). The isolates had been maintained in soil cultures at 4°C and reactivated by growing them on PDA medium.

Pathogenicity tests under greenhouse conditions: Pathogenicity testes of certain *F. graminearium* isolates were carried out during 2008-2009 wheat growing seasons in greenhouse. For each isolate, three agar discs grown with mycelium were cut using a cork borer and transferred to 50 mL of sterilized liquid potato dextrose medium in 300 mL Erlenmeyer flasks. The inoculated flasks were then incubated for 7 days at 20°C on a rotary shaker at 200 rpm. Four hundred grams of oat grain with 200 mL water were placed in 1L milk glass bottles. The bottles with the grain were autoclaved three times every 24 h for each one h at 121°C and 1.2 bar., Five milliliter of the conidial suspension from the liquid culture containing approximately 2.5×10^5 spore mL^{-1} were added to the grain of each bottle and mixed under sterilized conditions and the bottles were incubated at 20°C for 3 weeks. The bottles were shacked every two days to ensure equal distribution of inoculum.

Inoculum for each isolate was mixed thoroughly with sterilized clay soil at the rate of 3% soil weight then placed in sterilized pots (25 cm in diameter). Sterilization of pots and soil was carried out using 5% formalin solution (30 days before planting date). Non infested soil mixed with 3% sterilized oat grains was used as control. Fifteen surface disinfested wheat grains (cv. Sakha 69) were sown in each pot. Grains were disinfested by dipping in 1% sodium hypochlorite for 2 min. Plants were irrigated as necessary. Four replicates were used for each treatment. During the experimental period until 45 days after sowing, the percentage of germinated seeds, ratio of seedlings that died after germination as well as seedling blight index and dry weight of seedlings of each isolate treatment were determined. The experiment was carried out twice. The different pathogenic effects of fungal isolates on the wheat cv. Sakha 69 were analyzed by ANOVA (LSD range test after analysis) (Gomez and Gomez, 1984).

Table 1: Codes and origin of *F. geraminearum* isolates (Fg) used in the present study

Code	Host plant	Origin
Fg 18.7	*Triticum æstivum*	Sto-Seelfingen*
Fg 4.3	*Triticum æstivum*	Venenberg**
Fg 20.3	*Triticum æstivum*	Biberach*
Fg 33	*Zea mize*	Österreich*
Fg 27.4	*Triticum æstivum*	Vöhringen**

*: Isolates previously tested (Asran and Buchenauer, 2003), **: Isolates previously tested (Wanyoike, 2002)

Calculation of disease index: Disease severity was evaluated using the scale described by Liu *et al*. (1995) ranging from 0 to 5 as follows: 0 = 0%; 1 = 1t o 25 %; 2 = 26 to 50%; 3 = 51 to 75%; 4 = 76 to 100% seedling blight and 5 = whole seedling dead. The data were converted to disease index using the following formula:

$$\text{Disease index (\%)} = \frac{\Sigma(0A + 1B + 2C + 3D + 4E + 5F)}{5T} \times 100$$

where, A, B, C, D, E and F are the No. of plants corresponding to the numerical grade 0, 1, 2, 3, 4 and 5, respectively and 5T is the total No. of plants (T) multiplied by the maximum disease grade 5.

Reaction of some wheat cultivars to *F. graminearum* isolates under greenhouse conditions: Wheat cultivars Sakha 69, Giza 164 and Giza 168 were examined for their resistance to the five *F. graminearum* isolates at wheat growing season of 2008-2009. The wheat cultivars were sown in the pots containing infested soil for each *F. graminearum* isolate as described in the pathogenicity experiments. Seedling blight was determined using a scale rating from 1 to 5 as mentioned above. Experimental design was a split-plot with 4 replicates of each treatment. Cultivars were in the main plots and isolates were in the subplots. The experiment was carried out twice. The different pathogenic effects of fungal isolates on all tested wheat cultivars were analyzed by ANOVA (LSD range test after analysis).

Production of trichothecene mycotoxins by various *F. graminearum* isolates: *In vitro* production of trichothecene was determined by culturing the *F. graminearum* isolates on oat seeds. Oat seeds were

inoculated and incubated as previously described before. Each isolate consisted of three replicates and they were prepared along with two non-inoculated flasks which served as control. The dried infected oat grains were ground, extracted and analyzed for trichothecene content using Gas Chromatography Equipped with an Electron Capture Detector (GC-ECD) as described by Walker and Meier (1998) and Asran and Buchenauer (2003).

Statistical analysis: Data were subjected to statistical analysis using analysis of variance and means were compared using LSD range test as described by Gomez and Gomez (1984).

RESULTS

Pathogenicity of different *F. graminearum* isolates on wheat seedling of cv. Sakha 69 under greenhouse conditions: Five *F. graminearum* isolates were examined for their pathogenicity on wheat seedlings of cv. Sakha 69 under greenhouse conditions. Data in Table 2 shows that all isolates were pathogenic on cv. Sakha 69. In the control treatment, neither emergency of seeds nor disease symptoms in the seedlings were observed which indicated that the sterilization procedure resulted in fungus-free seed and soil. Inoculation of soil with *F. graminearum* isolates reduced emergence of germlings and caused post-emergence seedling death in different extents. While isolate Fg 33 did not significantly reduced emergence of seeds, the other isolates diminished significantly reduced emergence compared with the uninoculated control.

Post-emergence death of seedlings caused by the different *F. graminearum* isolates varied considerably. Isolate Fg 33 caused post-emergence death of seedlings but was not significantly different from uninoculated control while the other isolates caused significant differences in post-emergence seedling death. The highest post-emergence seedling death was exhibited by isolates 4.3, 20.3 and Fg 27 and then isolates Fg 18.7. Compared with the uninoculated control seedlings, inoculation with the different *F. graminearum* isolates resulted in drastically dry weights of the surviving wheat seedlings.

Table 2: Pathogenicity of various *F. graminearum* isolates on wheat seedlings cv. Sakha 69 under greenhouse conditions

Isolates	Emergency (%)	Seedling Killed after germination (%)	Plants dry weight per pot (g)
Fg 18.7	43.00	54.00	0.85
Fg 4.3	12.00	80.00	0.75
Fg 20.3	25.00	80.00	0.87
Fg 33	85.00	10.00	7.25
Fg 27.4	20.00	65.00	1.00
Control	100.00	0.00	15.24
LSD (0.005)	18.20	25.11	3.21

Virulence of *F. graminearum* isolates on wheat using seedling blight rating: Figure 1 show that all tested isolates caused seedling blight disease. The tested *F. graminearum* isolates differed significantly in causing seedling blight symptoms in seedling wheat. The disease index of the inoculated variants ranged from 21 to 88%. The most virulent isolates were Fg 4.3, 27.4 and 20.3 followed by Fg 18.7 while the isolate Fg 33 exhibited the lowest disease index percent.

Reaction of certin wheat cultivars to seedling blight disease under greenhouse conditions: Table 3 reveal that the *F. graminearum* isolates varied in their seedling blight severity. The isolates Fg 4.3 and 27.4 caused the most severe disease index followed by Fg 20.3 and 18.7. The isolate Fg 33 has the lowest virulence.

The three wheat cultivars reacted differently to the infection by *F. graminearum* isolates. The cv. Sakha 69 was the most susceptible one (35.22%). While cv. Giza 168

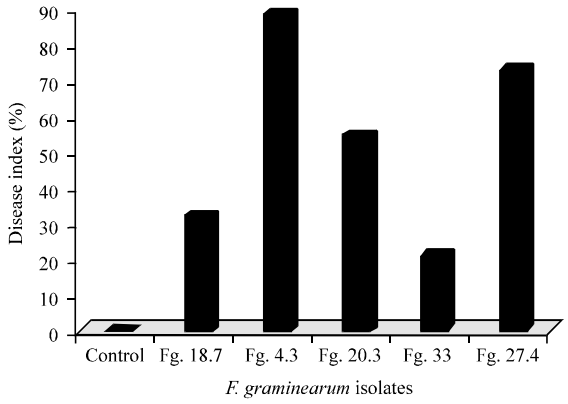

Fig. 1: Seedling blight caused by various *F. graminearum* isolates on the wheat cv. Sakha 69 under greenhouse conditions

Table 3: Disease index of seedling blight in some wheat cultivars after inoculation by several *F. graminearum* isolates

Isolates	Sakha 69	Giza 164	Giza 168	Mean
Fg 18.7	34.52*	11.33	21.65	22.50
Fg 4.3	65.22	26.42	41.62	44.42
Fg 20.3	38.16	19.35	22.98	26.83
Fg 33	21.10	8.22	16.91	15.41
Fg 27.4	52.33	22.33	35.86	36.84
Control	0.00	0.00	0.00	0.00
Mean	35.22	14.61	23.17	

*Disease severity%, LSD (p = 0.05), Isolates (I) = 9.88, Cultivars (C) = 8.66, Interaction (I×C) = 20.11

showed a moderate degree of susceptibility (23.17%), the cultivar Giza 164 exhibited significant lower seedling blight symptoms (14.61 %).

***In vitro* trichothecene contents in inoculated oat grains:** The amount of trichothecenes produced by the various isolates on autoclaved oat grains ranged from 1393-57081 μg kg^{-1} ground grain (Table 4). All isolates produced trichothecenes *in vitro* but differed significantly in their level of production. DON was the principle trichothecene produced by all isolates (range 1200-56338 μg kg^{-1} ground grain). NIV was detected in trace amounts in grains infected by the tested isolates (range 85-102 μg kg^{-1} ground grain). 15-A DON was found in grains infected by all tested isolates except Fg 18.7 isolate. 3-A DON was detected only in grains infected by Fg 4.3 and Fg 27.4. The highest amounts of total trichothecenes were detected in grains inoculated with isolate Fg 4.3 (57081 μg kg^{-1} ground grain) while grains inoculated with isolate Fg 33 and 18.7 had the lowest amounts of total trichothecenes. The other isolates showed intermediate trichothecene production.

Figure 2 also show that grain inoculated by isolate Fg 4.3 the highest disease severity (88 %) and highest concentration of trichothecene (57081 μg kg^{-1} ground grain) were detected. The isolates Fg 18.7 and 33 the lowest disease severity and lowest concentrations of trichothecenes 1717 and 1393 μg kg^{-1} ground grain, respectively) were detected. The results in Fig. 2 suggest relationship between wheat seedling blight disease severity and the trichothecene levels in the inoculated oat grain.

Table 4: Trichothecene contents (μg kg^{-1} ground grain) in oat grain after inoculation with several *F. graminearum* isolates *in vitro*

Isolates	NIV*	DON**	15-A DON	3-A DON	Total
Fg 18.7	92	1522[a]	100	-	1714[a]
Fg 4.3	102	56338[b]	241	400	57081[b]
Fg 20.3	87	3010[a]	-	-	3097[a]
Fg 33	85	1200[a]	108	-	1393[a]
Fg 27.4	96	10113[ab]	250	513	10972[ab]
Control	-	- -	-	-	

*Nivalinol, **Deoxynivalinol-not detectable, Means followed by the same letter in a column are not statistically different by LSD test

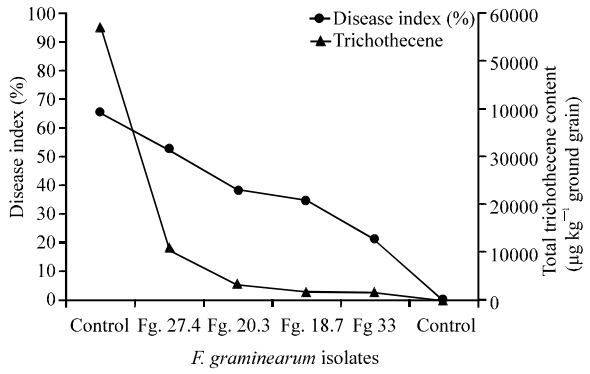

Fig. 2: Relationship between wheat seedling blight disease index in greenhouse and the trichothecene levels in the inoculated oat grain *in vitro*

DISCUSSION

For better understanding of pathogenicity and virulence mechanisms of *F. graminearum* involved in seedling blight disease of wheat pre and post-emergence as well as the severity of wheat seedling blight caused by the different isolates and the capability of the isolates to produce trichothecene mycotoxins were investigated. The isolates of *F. graminearum* used in this study were found to be highly variable in their pathogenicity towards wheat. The results of this investigation revealed that all isolates caused pre-and post-emergence death of wheat seedlings. The isolates however, varied considerably in seedling blight disease severity. Dry weight of infected seedlings was markedly reduced compared with uninoculated control seedlings. It has been known over a long time that the virulence of *F. graminearum* isolates is genetically variable (Mesterhazy, 1978; Miedaner and Schilling, 1996; Carter *et al*., 2000; Walker *et al*., 2001; Wanyoike, 2002). The alternating lifecycle of *F. graminearum* with a parasitic and saprophytic phase in crop rotations with a high percentage of cereals a long with a large genetic diversity have been linked to this large variation in virulence (Miedaner *et al*., 2000). Variation in virulence of *F. graminearum* isolates has also been reported on maize (Carter *et al*., 2000; Asran and Buchenauer, 2003) and on wheat and rice (Carter *et al*., 2000). The wheat cultivars responded differently to the *F. graminearum* isolates. The cultivar Sakha 69 was the most susceptible one. While cv. Giza 168 showed a moderate of susceptibility, the cv. Giza 164 exhibited significant lower seedling symptoms. These results are partially in agreement with those reported by Eraky (1993) and Allam (1994).

F. graminearum isolates can produce trichothecenes such as NIV, DON, 15-A DON and 3-A DON (Wanyoike, 2002; Asran and Buchenauer, 2003). Results from the analysis of the trichothecenes *in vitro* studies show that *F. graminearum* isolates produced trichothecenes at varying amounts. The principal trichothecene produced by all isolates was DON. Isolates were clearly different on the basis of their ability to produce trichothecenes. Two isolates (Fg 4.3 and 27.4) consistently produced high trichothecene levels while others produced low levels. These results confirm previous findings by other authors that the abilities of *F. graminearum* isolates to produce

trichothecenes varied considerably (Mesterhazy *et al*., 1999; Miedaner *et al*., 2000; Wanyoike, 2002; Asran and Buchenauer, 2003). On the other hand, some authors suggested that DON formation is not required for pathogenicity; it may be due to gene structure (Cumagun *et al*., 2007). The results of this investigation indicate that there is a relation between disease severity caused by the *F. graminearum* isolates and the trichothecenes concentrations (Fig. 2). Trichothecenes production therefore, may be regarded as an essential factor of virulence for spreading of *F. graminearum* in wheat seedlings. Similar results have been reported by Wanyoike (2002) testing trichothecene- producing and non- producing isolates of *F. graminearum* for their ability to cause head blight in wheat. Less virulent isolates produced low amounts of trichothecenes. These results showed that these mycotoxins play a vital role in the virulence mechanism of *F. graminearum*. Significant positive relationships have also been demonstrated between head blight severity and trichothecenes content of wheat caused by *F. = graminearum* (Desjardins *et al*., 1996; Wanyoike, 2002) and also demonstrated between seedling blight of corn (Asran and Buchenauer, 2003).

CONCLUSION

It could be concluded that trichothecene contents, especially DON derivatives are playing the most important role in pathogenesis of *F. graminearume*.

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