VIRULENCE OF *Fusarium verticillioides* (SACC.) NIRENBERG (G) ISOLATES ON MAIZE PLANTS ASSOCIATED WITH FUMONISINS PRODUCTION

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**ABSTRACT**

*Fusarium verticillioides* (Sacc.) Nirenberg (G) is a well known phytopathogenic fungus capable to infect a wide range of plants including maize. In addition; its toxic metabolites fumonisin (FB1 and FB2) are associated with Equina Leukoencephalo Malacia and induced hepatotoxic and carcinogenic effects in animals. Twenty two isolates of *F. verticillioides* obtained from corn samples and grown on Barley medium, were added to sterile soil two weeks before sowing maize grains to test their ability to cause corn seedling damping-off. Thirteen isolates caused pre-emergence damping-off, and two of them were significant. In addition one *F. verticillioides* isolate was significantly affecting post emergency and four isolates exhibited the highest disease index. *F. verticillioides* isolates which caused significant pre and post emergence damping-off as well as disease index were found to be fumonisin producers. Fourteen out of twenty two detached and moistened maize leaves showed necrotic lesions when they inoculated by *F. verticillioides* spore suspensions. Further more the crude extracts of the tested *F. verticillioides* isolates showed leaf lesions when they were spotted on the moisted maize leaf cuts, in addition to FB1 standard. Twelve of the pathogenic *F. verticillioides* isolates produced fumonisins (eight; FB1 and four; FB1 & FB2); whereas fumonisins could not been detected in only two of the pathogenic isolates according to TLC. These results suggest the great role of fumonisins that may be played as a virulence factor of *F. verticillioides* on maize.

**INTRODUCTION**

Maize (*Zea mays* L.) is a very important cereal crop grown all over the world due to its high yields per hectare, ease of cultivation and adaptability to different agro-ecological zones, in addition to versatile food uses and storage characteristics (Asiedu, 1989). *Fusarium* is one of the major fungal genera associated with maize in the world as well as in Egypt. *F. verticillioides* is likely to be the most common species isolated worldwide from diseased maize (Munkvold and Desjardins, 1997). Doko *et al.* (1996) reported *F. verticillioides* as the most frequently isolated fungus from maize and maize-based commodities in France, Spain and Italy. Likewise; Orsi *et al.* (2000) found that *F. verticillioides* was the predominant *Fusarium* species on maize in Brazil. In general, very little information is available on *F. verticillioides* occurrence on maize in Egypt. Reports of surveys conducted in some African countries however showed it as the most prevalent fungus on maize (Baba-Moussa, 1998; Kedera *et al.*, 1999). *F. verticillioides* is an endophyte of maize establishing long-term associations with the plant (Baba-Moussa, 1998; Pitt and Hocking, 1999). Symptomless infection can exist throughout the plant in leaves, stems, roots, grains, and the presence of the
fungus is in many cases ignored because it does not cause visible damage to the plant (Munkvold and Desjardins, 1997). This suggests that some strains of *F. verticillioides* produce disease in maize and others do not (Bacon and Williamson, 1992). *F. verticillioides* infects maize at all stages of plant development, either via infected seeds, the silk channel or wounds, causing grain rot during both the pre- and postharvest periods (Munkvold and Desjardins, 1997).

*Fusarium* genus comprises several species including *F. verticillioides* and *F. proliferatum*, which are the most prolific producers of fumonisins (Fandohan et al., 2003).

The fumonisins are a group of economically important mycotoxins and very common contaminants of maize-based foods and feeds throughout the world. Fumonisins are a group of at least 15 chemically related toxic fungal metabolites produced by certain mould species of the genus *Fusarium*, which may colonize cereals, especially maize (Arifio et al., 2009). Fumonisins have emerged as a highly visible animal and human health safety concern since they have been associated with many animal diseases such as leukoencephalomalacia (LEM) in horses (Marasas et al., 1988), pulmonary edema syndrome (PES) in pigs (Harrison et al., 1990; Colvin and Harrison, 1992) and hepatocarcinogenesis in rats (Gelderblom et al., 1988). The objective of this study was to assay the pathogenicity of different *F. verticillioides* isolates and its association with fumonisin productions.

**MATERIALS AND METHODS**

**Pathogen and inoculum preparation**

Twenty-two *Fusarium verticillioides* isolates were obtained from the stock cultures of Fungal Physiology lab., Dept. of Botany, Fac. of Science, Sohg, Egypt. For each isolate, three agar discs grown with mycelium were cut using a sterile 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 barely grains with 200 ml water were placed in 1L milk glass bottles. The bottles with the grains were autoclaved three times for successive days. Five ml of the conidial suspension of each *F. verticillioides* isolate liquid culture, $1 \times 10^7$ spore ml$^{-1}$, were added to each bottle and mixed under aseptic conditions. The bottles were incubated at 20 °C for 3 weeks and shaken every two days to ensure equal distribution of inoculum.

Inoculum of 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). Pots and soil were sterilized using 5% formalin solution (30 days before planting date). Non infested soil mixed with 3% surface sterilized barely grains was used as control. Five surface disinfested maize grains (cv. T.H. 310) were sown in each pot. Grains disinfectations were done using 1% sodium hypochlorite for 2 minutes. Plants were irrigated when 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 of each
isolate treatment were determined. The experiment was carried out twice. The different pathogenic effects of fungal isolates on the maize cv. T. H. 310 were analyzed by ANOVA (L.S.D. range test after analysis) (Gomez and Gomez, 1984).

Calculations 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 death.

Disease index % = \( \frac{\sum (0A +1B + 2C + 3D + 4E + 5F)}{5T \times 100} \) where, A, B, C, D, E and F are the number of plants corresponding to the numerical grade 0, 1, 2, 3, 4 and 5 respectively and 5T is the total number of plants (T) multiplied by the maximum disease grade 5.

Corn medium:
Twenty two Fusarium verticilliodes isolates were inoculated on polished corn medium in 250 ml Erlenmeyer flasks and incubated at 25 °C for two weeks. The flasks were shaken every other day to insure homogenous distribution of Fusarium mycelia and then incubated for other 5 weeks under 5 °C in darkness to enhance toxin production (Fadl Allah, 1998). Three replicates of five grams of each Fusarium corn culture were weight and left on atmospheric air to be dried. The dried corn cultures were ground and soaked in 50 ml 96% methanol over night. The methanol extracts were decanted and the corn culture was re-extracted twice using 25 ml 96 % methanol each time. The extracts filters were joined and filtered through (Whatman No. 1) filter papers. The crude extract dissolved in the minimal amount of methanol and transferred into cleaned dram vials dried under a stream of nitrogen and stored to be tested for their virulence activity and chemical analyzed.

Corn leaf cuts virulence assay:

1 – Crude extract assay:
Ten µl of dissolved crude extracts of 22 F. verticilliodes isolates, in addition to 10 µl of 96 % methanol (control) were dropped on moistened corn leaf cuts and incubated for 72 hrs at 25 °C and examined for their ability to cause necrotic leaf lesions.

2 – Fusarium verticilliodes spore suspension assay:
All of the collected Fusarium verticilliodes isolates inoculated into PDA slants and incubated at 25 °C for five days. Ten ml sterile distilled water were add to each slant culture and easily agitated using sterile culture needle and vigorously shook to insure homogenous , 1 X 10^-7 spore suspension ml^-1. One ml of each of the spore suspensions were dropped on moistened corn leaf cuts and incubated for 72 hrs at 25 °C in dark to test their pathogenic ability.

Thin layer chromatography (TLC):
Thin-layer chromatographic technique adopted by El- Kady and Moubasher (1982) was employed. The crude extracts (10 µl) were spotted on TLC plate (Aluminium sheet, silica gel, Merck) along with 5 µg each of FB1 and FB2 standards (purchased from SIGMA-ALDRICH). The spots were dried during application with a flow of cold air. and developed using 96% methanol : water (80 : 20, v/v). The developed plates were viewed after spraying with
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50% concentrated sulphuric acid in methanol (Mubatanhema et al., 1999) under short wave (254 nm) and long wave (356 nm) ultra-violet irradiation. Fumonisin B₁ and B₂ appears as slight bluish spots under both near and far UV (Mubatanhema et al., 1999) at Rf 0.7 and Rf 0.61, respectively.

RESULTS AND DISCUSSION

1- Test of Pathogenic ability

The soil infestation with the twenty two different *F. verticillioides* isolates showed that; thirteen isolates were pathogenic on corn cv. "T.C. 310", (Table 1). Emergence of seeds was diminished between 80 to 96.67 %. While 20 isolates did not significantly reduced emergence of seeds; only two isolates (4 and 19) diminished significantly seed emergence compared two the un-inoculated control.

Post-emergence death of seedlings caused by the different *F. verticillioides* isolates was varied. While, *F. verticillioides* isolate (19) caused significant post-emergence death of seedlings, isolates (2, 3, 4, 5, 6, 7, 9, 10, 13, 14, 17 and 21) caused post-emergence death of seedlings but not significantly different from the un-inoculated control.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Emergence (%)</th>
<th>Seedling killed after germination (%)</th>
<th>Disease index (%)</th>
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<tr>
<td>1</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>86.67</td>
<td>12.00</td>
<td>16.67</td>
</tr>
<tr>
<td>3</td>
<td>90.00</td>
<td>20.00</td>
<td>19.20</td>
</tr>
<tr>
<td>4</td>
<td>80.00</td>
<td>22.22</td>
<td>21.30</td>
</tr>
<tr>
<td>5</td>
<td>86.67</td>
<td>13.33</td>
<td>18.36</td>
</tr>
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<td>6</td>
<td>96.67</td>
<td>18.12</td>
<td>12.23</td>
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<tr>
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<td>93.33</td>
<td>21.16</td>
<td>15.36</td>
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<td>8</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
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<td>93.33</td>
<td>18.12</td>
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<td>96.67</td>
<td>15.66</td>
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<td>11</td>
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<td>21</td>
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<tr>
<td>22</td>
<td>100.00</td>
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<td>Control</td>
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</tr>
<tr>
<td>L.S.D. 0.05</td>
<td>18.09</td>
<td>25.07</td>
<td>19.02</td>
</tr>
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</table>
Four *F. verticillioides* isolates (4, 19, 14 and 3) exhibited the highest disease index ranging from 19.20 to 21.30%. Disease index incited by isolates (17, 10, 21, 6, 9, 13, 7, 2, 5 and 3) exhibited the lowest disease index ranging from 10.36 to 19.20%, the values were not significantly different (at p < 0.05) compared to the un-inoculated control. Results obtained by Bacon et al., 1994 agreed our results. Asran and Buchenauer, 2002 also reported considerable variability in pathogenicity of *Fusarium moniliforme* isolates on corn seedlings. Marases et al., (1984) and Nelson et al., (1992) found that *F. verticillioides* is the major pathogen of maize world wide causing seedling, stalk and ear rots.

2- Pathogenic effect of *F. verticillioides* isolates tested on corn leaf cuts

Data in Table 2 indicated that fourteen *F. verticillioides* isolates were pathogenic on corn leaves causing leaf lesions. While 2 isolates (11 and 20) caused low pathogenic ability, of which 5 *F. verticillioides* isolates (2, 3, 9, 17 and 21) were moderately pathogenic and 7 isolates (4, 5, 7 10, 13, 14 and 19) were highly pathogenic.

Table 2: Effect of spore suspension and crude extracts of *F. verticillioides* dropped on moistened corn leaf cuts incubated for 72 hrs under ambient temperature.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Spore suspension</th>
<th>Crude extracts</th>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
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</tr>
<tr>
<td>2</td>
<td>++</td>
<td>++</td>
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<tr>
<td>3</td>
<td>++</td>
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<td>+++</td>
<td>+++</td>
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<td>8</td>
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<td>9</td>
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<td>10</td>
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<td>14</td>
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<tr>
<td>Control</td>
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</table>

+++ = Highly pathogenic
++  = Moderately pathogenic
+   = Low pathogenic
-   = Non pathogenic
The rest of the isolates were non-pathogenic. Also, the crude extracts of 14 *F. verticillioides* showed corn leaf lesions ranging between low pathogenic (11 and 20) to moderately pathogenic (2, 3, 4, 5, 9, 10, 13, 14, 17, 19 and 21) in addition to one isolate (7) showed high pathogenic ability.

Figure 1: Detached corn leaf inoculated with *F. verticillioides* spore suspension\(^1\) and *F. verticillioides* crude extracts \(^2\) incubated for 72 hrs under room temperature (in the right). Leaf cut in the left was untreated control.

3- Detection of fumonisin B1 and B2 produced by tested *F. verticillioides* isolates using TLC.

Data of this study are presented in Table (3) and indicated that fumonisin B1 was produced by 12 isolates of *F. verticillioides* (2, 3, 4, 5, 7, 9, 10, 12, 13, 17, 19 and 21). However, fumonisin B1 and B2 were obtained only by 4 isolates (3, 4, 13 and 19), while production of either Fuminisin B1 or fumisin B2 was not detected for the other tested *F. verticillioides* isolates. These results suggest the great role of fumonisins that been played in virulence of *F. verticillioides* on maize. Fumonisins are the most important group of mycotoxins synthesize and secreted by *F. moniliforme* (syn. *F. verticillioides*) assumed to be involved in pathogenesis of *F. verticillioides* and causing seedling blight in maize (Desjardins *et al.*, 1995). They also supported the hypothesis that fumonisins may play a role in virulence of *F. verticillioides* but is not necessary sufficient on maize seedling. Also, Asran and Buchenauer (2002) suggested that fumonisins may act as a virulence factor to spread of *F. moniliforme* on maize. Fumonisins can cause necrosis in maize seedlings (Lamprecht *et al.*, 1994) and maize callus (van Asch *et al.*, 1994).
1992) as well as apoptosis in tomato protoplasts (Wang et al. 1996). On the other hand, Abbas and Boyette (1992) could not detect any toxicity in maize seedlings after treatment with fumonisins up to 1000 µg/ml.

**Table (3):** Thin layer chromatographic analysis (TLC) of the crude extract produced by the tested *F. verticillioides* isolates.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Fumonisin B1</th>
<th>Fumonisin B2</th>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2</td>
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<td>3</td>
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<td>4</td>
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**REFERENCES**


القدرة المرضية لبعض عزلات الفطر

نباتات الدرجة الشامية واعلاقة ذلك بإنتاج الفيروسين

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يعتبر الفطر Fusarium verticilliodes مردلاً ببعض الفيروسات ذات التأثير الضار على العديد من الحيوانات. وقد أجري هذا البحث بهدف تقييم القدرة المرضية لبعض عزلات الفطر واعلاقة إنتاج الفيروسين، فتم الحصول على 22 عزلة من عزلات الفطر المزعولة من الدرجة الشامية وتم تبنيها على بيئة الشعير لإختبار قدرتها المرضية على نباتات الدرجة الشامية.

وقد ظهرت النتائج كما يلي:

* أظهرت 14 عزلة قدرتها على إحداث إصابة لنباتات الدرجة الشامية منها 13 عزلة قادرة على إحداث وفاة للنباتات قبل ظهورها فوق سطح التربة. وعزلة واحدة لها تأثير معنوي في موت الورود بعد ظهورها فوق سطح التربة.
* أظهرت 4 عزلات من هذه العزلات المرضية قدرتها على إحداث لقاح الورود في درجة مماثلة
* وذلك لأن النباتات التي أتت بعد ظهورها فوق سطح التربة.
* كما أظهرت النتائج ظهور الفيروس في نباتات عسر الصعاب عند تغذيتها سواء بملعقة الجراثيم أو مخلع الجراثيم من هذه العزلات التي حالت الإصابة.

وقد أظهرت النتائج أيضاً أن من هذه 14 عزلة المرضية للنباتات المدينة 12 عزلة تنتج الفيروسين (8 عزلات تنتج FB1 و4 عزلات تنتج كلا من FB1 & FB2) أثناء نمو الفطر في عزلتين فقط من العزلات المرضية لنباتات الدرجة الشامية مما يعني أن هناك F. verticilliodes فرعًا كبيراً للفيروسين كعامل هام من عوامل القدرة المرضية للفطر على نباتات الدرجة الشامية.

قام بتحكيم البحث:

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