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

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ABSTRACT

Fusarium verticilliodes (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. verticilliodes* 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. verticilliodes* isolate was significantly affecting post emergency and four isolates exhibited the highest disease index. *F. verticilliodes* 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. verticilliodes* spore suspensions. Further more the crude extracts of the tested *F. verticilliodes* isolates showed leaf lesions when they were spotted on the moisted maize leaf cuts, in addition to FB1 standard. Twelve of the pathogenic *F. verticilliodes* 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. verticilliodes* 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 verticilliodes* 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. verticilliodes* isolate liquid culture, $1X10^7$ spore ml^{-1}, were added to each bottle and mixed under aseptic conditions. The bottles were incubated at 20 °C for 3 weeks and shacked 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 disinfestations 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 % = $\sum$(0A +1B + 2C + 3D + 4E + 5F) / 5T X 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 verticillioide*s 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 shacked to insure homogenous , 1 X 10^{-7} spore suspension ml^{-1}. One ml of each of the spore suspensions were dropped on moisted 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 FB_1 and FB_2 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 viwed after spraying with

50% concentrated sulphuric acid in methanol (Mubatanhema *et al.*, 1999) under short wave (254 nm) and long wave (356 nm) ultra-violet irradiation. Fumonisin B_1 and B_2 appears as slight bluish spots under both near and far UV (Mubatanhema *et al.*, 1999) at R_f 0.7 and R_f 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 (1): **Corn seedlings damping-off caused by *Fusarium verticillioides* tested under greenhouse conditions**

Isolate No.	Emergence (%)	Seedling killed after germination (%)	Disease index (%)
1	100.00	0.00	0.00
2	86.67	12.00	16.67
3	90.00	20.00	19.20
4	80.00	22.22	21.30
5	86.67	13.33	18.36
6	96.67	18.12	12.23
7	93.33	21.16	15.36
8	100.00	0.00	0.00
9	93.33	18.12	12.50
10	96.67	15.66	10.32
11	100.00	0.00	0.00
12	100.00	0.00	0.00
13	86.67	12.00	14.36
14	83.33	22.22	19.52
15	100.00	0.00	0.00
16	100.00	0.00	0.00
17	96.67	15.19	10.36
18	100.00	0.00	0.00
19	80.00	32.04	21.30
20	100.00	0.00	0.00
21	96.67	11.11	12.22
22	100.00	0.00	0.00
Control	100.00	0.00	0.00
L.S.D. 0.05	**18.09**	**25.07**	**19.02**

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.

Isolate No.	Spore suspension	Crude extracts
1	-	-
2	++	++
3	++	++
4	+++	++
5	+++	++
6	-	-
7	+++	+++
8	-	-
9	++	++
10	+++	++
11	+	+
12	-	-
13	+++	++
14	+++	++
15	-	-
16	-	-
17	++	++
18	-	-
19	+++	++
20	+	+
21	++	++
22	-	-
Control	-	-

+++ = 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.

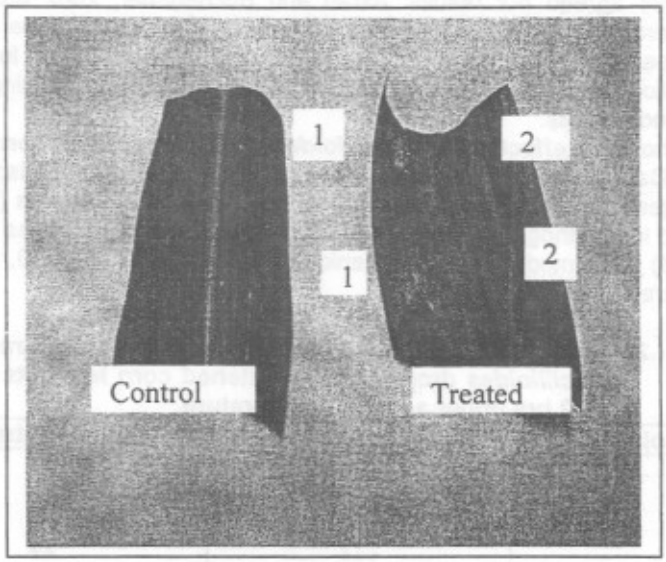

Figure 1: Detached corn leaf inoculated with *F. verticillioides* spore suspension[1] and *F. verticillioides* crude extracts [2] incubated for 72 hrs under room temperature (in the right). Leaf cut in the left was untreated control.

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, fumonisinB1 and B2 were obtained only by 4 isolates (3, 4, 13 and 19), while production of either Fuminisin B1 or fuminsin 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. verticilliodes* 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.*,

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.

Isolate No.	Fumonisin B1	Fumonisin B2
1	-	-
2	+	-
3	+	+
4	+	+
5	+	-
6	-	-
7	+	-
8	-	-
9	+	-
10	+	-
11	-	-
12	-	-
13	+	+
14	+	-
15	-	-
16	-	-
17	+	-
18	-	-
19	+	+
20	-	-
21	+	-
22	-	-

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القدرة المرضية لبعض عزلات الفطر *Fusarium verticillioddes* على نباتات الذرة الشامية وعلاقة ذلك بإنتاجها للفيمونسين

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يعتبر الفطر *F. verticillioddes* من الفطريات التى تصيب العديد من المحاصيل الزراعية ومنها محصول الذرة الشامية. وهذا الفطر من الفطريات المنتجة للسموم مثل الفيمونسين FB1والفيمونسين FB2 ذات التأثير الضار على العديد من الحيوانات. وقد أجرى هذا البحث بهدف تقدير القدرة المرضية لبعض عزلات الفطر وعلاقتها بإنتاج الفيمونسين ، فتم الحصول على ٢٢ عزلة من عزلات الفطر المعزولة من الذرة الشامية وتم تنميتها على بيئة الشعير لإختبار قدرتها المرضية على نباتات الذرة الشامية

وقد ظهرت النتائج كما يلى :

* أظهرت ١٤ عزلة قدرتها على إحداث إصابة لبادرات الذرة الشامية منها ١٣ عزلة قادرة على إحداث موت للبادرات قبل ظهورها فوق سطح التربة وعزلة واحدة لها تأثر معنوى فى موت البادرات بعد ظهورها فوق سطح التربة.
* أظهرت ٤ عزلات من هذه العزلات الممرضة قدرتها على إحداث لفحة البادرات بدرجة معنوية وذلك للبادرات التى تبقت بعد ظهورها فوق سطح التربة .
* كما أظهرت النتائج ظهور البقع الموضعية على أوراق النباتات عند تلقيحها سواء بمعلق الجراثيم أو بمستخلصات الفطر من هذه العزلات التى أحدثت الإصابة.
* وقد أظهرت النتائج أيضاً أن من هذه الـ ١٤ عزلة الممرضة للنبات وجد عدد ١٢ عزلة تنتج الفيمونسين(٨ عزلات تنتج FB1 وعدد ٤ عزلات تنتج كلاً من (FB2 & FB1 بينما لم يظهر إنتاج الفيمونسين فى عزلتين فقط من العزلات الممرضة لنباتات الذرة الشامية مما يعنى أن هناك دوراً كبيراً يلعبه الفيمونسين كعامل هام من عوامل القدرة المرضية للفطر *F. verticillioddes*على نباتات الذرة الشامية .

قام بتحكيم البحث

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