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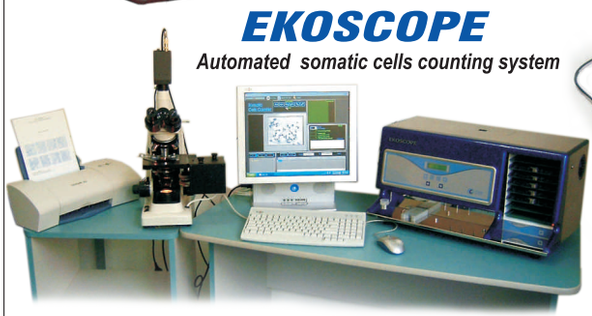

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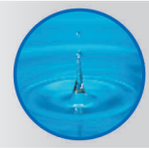

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Agriculture and Environment

Evaluation of deoxynivalenol and virulence in dsRNA containing Fusarium graminearum Isolates

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Abstract. *Double–stranded RNA (dsRNA) viruses in some fungi are associated with hypovirulence and have been used or proposed as biological control agents. To determine the effect of dsRNA on pathogenicity and deoxynivalenol (DON) production by Fusarium graminearum isolates, the causal agent of wheat head blight, three dsRNA–containing and dsRNA–free isolates were screened in this survey. Between the two groups, the disease severity of the dsRNA-containing isolates, however, was significantly ($p<0.01$) less than that of the dsRNA–free isolates on susceptible wheat (cv. Falat) in a greenhouse experiment. DON production by dsRNA–free and dsRNA-containing isolates of F. graminearum was confirmed using HPLC techniques. The range of in vitro DON production levels varied from 0.07 to 1.62 ppm, and 0.06 to 0.4 ppm, respectively. A significantly reduced level of DON up to 50% was detected in dsRNA-containing derivatives than the dsRNA–free isolates. Meanwhile, the range of DON production levels in spikes inoculated with dsRNA–free and dsRNA-containing F. graminearum isolates varied from 0.56 to 0.9 ppm and 0.37 to 0.63 ppm, respectively. These results indicated 27.5% reduction in DON production.*

Keywords: Fusarium graminearum, Deoxynivalenol, dsRNA, hypovirulence

Introduction

Fusarium head blight (FHB) caused by Fusarium graminearum Schwabe (Teleomorph: Gibberella zeae (Schwein) Petch) is a devastating disease responsible for extensive yield and quality losses in wheat in humid and semi–humid regions of the world (McMullen et al., 1997; Champeil et al., 2004). F. graminearum, besides reducing wheat grain quality through FHB, can also produce potent toxins such as the trichothecene deoxynivalenol (DON). The occurrence of DON in wheat grains is of great concern for both human and animal health. Trichothecenes are associated with dermal toxicity, toxic aleukia, food refusal, vomiting, and depressed immune functions (Snidjers, 1990; WHO, 2001). DON is detected the most frequently world–wide and in the highest concentrations (Clear and Patrick, 2000).

Double–stranded RNA (dsRNA) mycoviruses have been described in a wide variety of fungi and yeasts (Ghabrial, 1998; Varga et al., 2003). Mycovirus infections are persistent and generally asymptomatic. In some fungi, however, dsRNA mycovirus infection causes distinct morphological and physiological changes, including toxin production, cytological alterations of cellular organelles, and virulence associated traits such as growth rate, sporulation, pigmentation, and enzymatic activities. In addition, mycoviurses persistently attenuate novel virulence related phenotypes, namely hypovirulence (Magliani et al., 1997; Varga et al., 1994; Newhouse et al., 1983; Boland, 1992; Bottacin et al., 1994; Anagnostakis and Day, 1979; Rigling and Alfen 1993; Chu et al., 2002). dsRNA in some fungi are associated with hypovirulence and have been used or proposed as biological control agents (Chu et al ., 2002).

Results of the first report on dsRNA infection in F. graminearum isolates showed that the dsRNA causes morphological changes, including reduction in mycelial growth, increased pigmentation, reduced virulence towards wheat, and decreased (60 fold) production of trichothecene mycotoxin (DON). The presence or absence of dsRNA was correlated with the changes in pathogenicity and morphology (Chu et al., 2002). A study on screening of Iranian isolates of F. graminearum showed that seven percent of the isolates were viruliferous and contained fragments measuring 1.5 to 6 kb (Hashemi et al., 2004; Aminian et al., unpublished data). Our objectives in this study were to determine the effects of dsRNA infection on DON production of F. graminearum isolates in vitro and greenhouse conditions.

Material and methods

1. Fungal isolates

Three dsRNA containing and three dsRNA free *F. graminearum* isolates (F38, F42, and F118) originally isolated from wheat in Ardebil and Golestan provinces, Iran were used to study the presence or absence of the dsRNAs affected mycotoxin production by *F. graminearum* (Hashemi et al., 2004; Aminian et al., unpublished data).

2. In vitro DON analysis

2.1. Growth condition for DON production

Rice substrate was employed for DON production, according to

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method described by Lauren and Agnew (1991). The rice sterile culture medium was inoculated with two plugs from the margin of 4-day-old growing colony of each isolate on PDA (potato dextrose agar) and then incubated for 5 weeks at 25 ºC.

2.2 Extraction and clean up

The mycelial mass and substrate were dried at 50 ºC. Fifteen grams of dried substrate was ground in a blender extractor as described by Lauren and Agnew (1991) and Jening et al. (2004). Briefly, each ground sample (15g) was extracted with 60 ml acetonitril : methanol : water (16: 1: 3) in a 250- ml Erlenmeyer flask for 3h on a rotary shaker (170 rpm), and the extract was filtered through Whatman (Maidston, Kent, England) no. 1 filter paper. A glass tube (30 cm+1 cm id) was plugged with glass wool and dry–packed with alumina/carbon (20: 1 ; 1g) and then 2g prewashed cation exchange resin added to form a mini-clean up column. The filtrate was applied to this column and allowed to drain under gravity and the eluant collected. For further clean up, 10 ml of first column elute was passed through mini column made by packing glass tube (30 cm + 1cm id) with small glass wool plug and alumina/carbon (1: 1 ; 1g). The elute was then allowed to drain by gravity. The second column elute was evaporated at 70 ºC till dried. Residues were dissolved in methanol/water (10: 90; 2ml) and maintained at rest for 1 h at room temperature. Then, the residues were dissolved in methanol/water (5: 95; 2 ml) and acetonitril–methanol (3: 1; 2 ml) and evaporated at 70 ºC. Final residues were dissolved in methanol/water (5: 95; 2 ml) and kept in the refrigerator at 4 ºC until analysis. All cultivation and extraction experiments were repeated three times. Flasks with rice, albeit without fungus inoculum, received same culture and extraction treatments as samples (controls) for the exclusion of interfering compounds that might be confused with the mycotoxins under analysis.

3. DON analysis by HPLC

The HPLC system consisted of a Waters™ 600 pump system with a Waters 2487 uv detector. Chromatographic separation was performed on a Nova-park C_{18} column (15 cm, 3.9 µm particle size). The mobile phase consisted of methanol / water (5: 95, v/v) at a flow rate of 1 ml min^{-1}. The detector was set at 220 nm with an attention of 2 AUFS. Injection volume was 50 µl. Quantification was relative to external standards of 1-4 µg ml^{-1} in methanol/ water (5:95).

4. Pathogenicity

Five seedlings of wheat (Cv Falat) were grown in the greenhouse for 12 weeks. To determine the effects of dsRNAs on host fungus virulence, conidial suspensions of dsRNA–containing and dsRNA-free isolates (F-38, F-42 and F-118) were inoculated on to wheat heads. Three replicates were set up for each treatment and arranged in a complete randomized design.

Conidial inoculum was produced in mung bean broth (Rosewich Gale *et al*., 2002). Nine days before head inoculation, a plug of *F. graminearum* of each isolate was used to inoculate a flask containing 50 ml of mung bean broth. Flasks were incubated for seven days at 25ºC at 150 rpm. Conidia were harvested and spore suspensions were adjusted to 1×10^4 conidia ml^{-1}. Ten microliters of this suspension were used to inoculate a single, centrally located floret on five wheat heads per treatment at anthesis. Heads inoculated with sterile distilled water served as controls. Then, the plants were placed in a plastic bag for 3 days to maintain high relative humidity. Inoculated wheat heads were evaluated after 10 days and FHB severity disease was estimated. The experiment was replicated twice. Means were separated at $p<0.01$ using Duncan's multiple range test.

5. Analysis of DON production in spike

The DON analysis was done using the methodology described in the previous section except that spikes of each treatment were mixed and finally ground with liquid nitrogen. A 3 g sub-sample was extracted by mixing with acetonitril: methanol: water (16: 1: 3) in a 250- ml Erlenmeyer flask for 5 h on a rotary shaker (170 rpm) and then filtered through filter paper Whatman No.1. A glass tube (30 cm + 1 cm id) was plugged with glass wool and dry–packed with alumina/carbon (20: 1 ; 1g) and then 2g prewashed cation exchange resin added to form a mini-clean up column.

The full extract was applied to the column and allowed to drain under gravity and the eluent collected. For further clean up, all of the first column eluent was passed through mini column made by packing glass tube (30 cm + 1cm id) with small glass wool plug and alumina / carbon (1:1;1g) and allowed to drain by gravity. The second column eluent was evaporated at 70 °C till dried. Residues were dissolved in methanol / water (10: 90) and maintained at rest for 1 h at room temperature. Then, residues were dissolved in methanol / water (5: 95; 2 ml) and acetonitril–methanol (3: 1) and evaporated at 70 °C. Final residues were dissolved in methanol/water (5: 95) and kept in the refrigerator at 4 °C until analysis. Three replicates were set up for each treatment and all incubation and extraction experiments were repeated twice.

Results

In vitro DON analysis

The relative levels of DON production by dsRNA–free and dsRNA-containing isolates varied between 0.07 to 1.62 ppm, and 0.06 to 0.4 ppm, respectively (Table 1). DON was detected at significantly lower levels in dsRNA-containing derivatives than the dsRNA–free counterpart isolates. The reduction in DON production ranged from 5.4%-77.1%.

Pathogenicity

Head blight symptoms were observed on all wheat plants inoculated with the dsRNA–free and dsRNA-containing F. graminearum isolates. The disease severity of the dsRNA-containing isolates, however, was significantly less than that of the dsRNA–free isolates. Disease severities of dsRNA–free isolates on inoculated wheat heads were 69% - 81%, whereas those of dsRNA-containing isolates were 27%-43%, when inoculated with ten microliter suspensions of 10^4 conidia per ml, respectively. No symptoms were observed in the control inoculation (Figure 1).

DON production in spike

The amount of DON produced in the spikes inoculated with dsRNA–free and dsRNA-containing F. graminearum isolates are shown in Table 2. The relative levels of DON production by dsRNA–free and dsRNA-containing isolates varied between 0.56 to 0.9 ppm, and 0.37to 0.63 ppm, respectively. DON was detected at significantly lower levels in dsRNA-containing derivatives than the dsRNA–free counterpart isolates. The reduction in DON production ranged from 21.7 to 33.9% (Table 2).

Discussion

Studies on the role of virus infection on toxin production by certain fungal species have produced a variable pattern. However, a

Table 1. DON production by dsRNA–free and dsRNA-containing Fusarium graminearum isolates on rice substrate

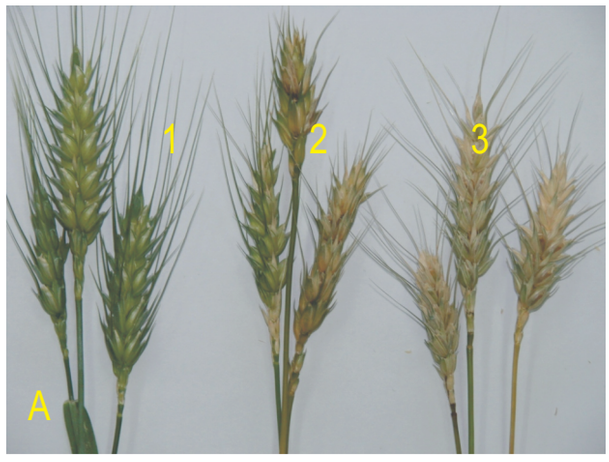
Isolate	DON production [ppm][a]	DON reduction [%][b]
dsRNA-containing F-38	0.069 ± 0.015	5.4
dsRNA-free F-38	0.073 ± 0.018	
dsRNA-containing F-42	0.37 ± 0.049	77.1
dsRNA-free F-42	1.62 ± 0.059	
dsRNA-containing F-118	0.41 ± 0.26	74.6
dsRNA-free F-118	1.62 ± 0.11	

a- values and standard deviation, b- mean recovery was 85%

Table 2. DON production by *F. graminearum* isolates on wheat spikes during greenhouse assay

Isolate	DON production [ppm][a]	DON reduction [%][b]
dsRNA-containing F-38	0.63 ± 0.11	27
dsRNA-free F-38	0.9 ± 0.25	
dsRNA-containing F-42	0.54 ± 0.037	21.7
dsRNA-free F-42	0.69 ± 0.014	
dsRNA-containing F-118	0.37 ± 0.05	33.9
dsRNA-free F-118	0.56 ± 0.034	

a- values and standard deviation, b- mean recovery was 85%



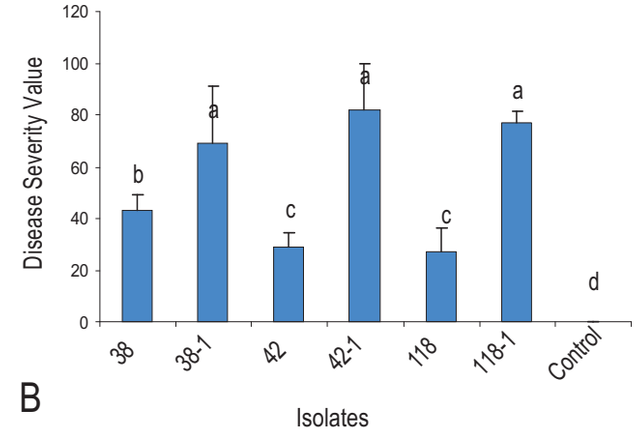


Figure 1. Pathogenicity test and Disease severity
(A) for pathogenicity test conidial suspensions were inoculated on to wheat plants. Plants infected with dsRNA–free spores of F-118 (3) showed more severe symptoms than those infected with dsRNA–containing spores of F-118 (2). Water injected in control plants (1)
(B) comparison of disease severity means of dsRNA–free and dsRNA-containing *F. graminearum* isolates. Different letters imply statistical differences among isolates at the 1% level.

correlation between toxin production (pathogenicity) and the presence of a virus particle has been reported in *Helminthosporium maydis*, the causal agent of southern corn blight (Bozarth et al., 1972). Studies with Periconia circinata, the causal agent of root and corn rot of sorghum, have failed to detect any association between toxin production and the presence or absence of virus particles (Dunkle, 1974).

In our study rice substrate was employed for *in vitro* DON research. Mycotoxins of the genus *Fusarium* are generally produced when cereals, usually rice and maize, are used as a solid substrate for growth. Such cultures have to pass through extraction and purification process prior to identification by thin layer chromatography, gas liquid chromatography or high performance liquid chromatography (Geraldo et al., 2006).

Our results showed that isolates carrying the dsRNA containing *F. graminearum* isolates produce much less mycotoxin (DON) than do the dsRNA free isolates in both rice culture in vitro condition and wheat spike in greenhouse. Also, the disease severity of the dsRNA-containing isolates was significantly less than that of the dsRNA–free isolates. These results are similar to those reported by Chu et al. (2002). They showed that the dsRNA causes reduced virulence towards wheat and decreased (60-fold) production of trichothecene mycotoxin (DON). The presence or absence of dsRNA was correlated with the changes in pathogenicity and morphology (Chu et al ., 2002).

Trichothecene production has a role in virulence; therefore dsRNA containing F. graminearum isolates caused much slower disease development on infected wheat plants. Development of disease, however, is a complicated phenomenon, so additional studies are required to confirm these results.

Head blight reduces grain yield, and harvested grain is often contaminated with mycotoxins that have a trichothecene structure including DON, nivalenol and zearalenone (Marasas et al., 1984). Direct economic losses that can result from the pathogen, including

lower crop yields and poor grain quality, have become common (Windels, 2000) for feed (Chamley et al., 1994). There is also concern about the public health implications of exposure to Fusarium mycotoxins, such as feed refusal, vomiting and skin necrosis (Chamley et al., 1994; Foster et al., 1986). If the dsRNA fragment in F-38, F-42 and F-118 are transferable to dsRNA–free isolates through hyphal fusion with a high incident, and if the virulence level and the mycotoxin production are reduced due to the dsRNA as described above, biological control of diseases caused by *F. graminearum* could be achieved.

Acknowledgments

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