EFFECT OF DOUBLE-STRANDED RNAS ON VIRULENCE AND DEOXYNIVALENOL PRODUCTION OF *FUSARIUM GRAMINEARUM* ISOLATES

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Abstract: Various viral genomes from plant pathogenic fungi have been reported. The most common of these genomes found are dsRNA which in some fungi are associated with hypovirulence and have been used or proposed as biological control agents. In this study 33 Iranian isolates of *Fusarium graminearum*, were examined for dsRNA infection. In 12 of these isolates, more than three viral fragments measuring 0.9, 1.2, 3, 3.2 and 5 kb were detected. The presence and nature of dsRNA was confirmed by RNaseA and DNaseI treatments. To obtain dsRNA-free isolates, hyphal tip cultures of six isolates were transferred to PDA containing different concentrations of cycloheximide. Three hyphal tip cultures were confirmed to be dsRNA-free. Mycelial growth in dsRNA-containing isolates was less than that of their counterpart cured isolates with no dsRNA. Meanwhile, no significant difference was observed for colony morphology, pigmentation and conidia production of viruliferous and cured isolates. However, the disease severity of the dsRNA-containing isolates was significantly ($p < 0.01$) less than that of the dsRNA-free isolates on susceptible wheat in greenhouse. Deoxynivalenol (DON) production by dsRNA-free and dsRNA-containing isolates was confirmed using HPLC analysis. The range of DON production levels for dsRNA-free and dsRNA-containing isolates varied from 0.07 to 1.6 ppm and 0.06–0.4 ppm, respectively. A very significantly reduced level of DON, up to 50%, was detected in dsRNA-containing derivatives compared to the dsRNA-free isolates.

Key words: *Fusarium graminearum*, mycovirus, dsRNA, deoxynivalenol (DON)

INTRODUCTION

Double-stranded RNA (dsRNA) mycoviruses have been reported in a wide variety of fungi and yeasts (Ghabrial 1998; Varga *et al*. 2003). The sequences currently available in gen banks suggest that the three most common genera of mycoviruses are *Mitovirus*, *Partivirus*, and *Totivirus*. *Mitovirus* species are naked RNA viruses that inhabit mitochondria and have genomes in the range of 2.3 to 3.5 kbp. Members of the family Totiviridae are dsRNA viruses with a linear uncapped genome of 4.6 to 7 kbp and members of the family Partitiviridae contain two dsRNA genomes of 1.4 to 3 kbp (Kwon *et al*. 2007). Mycovirus infections are persistent and generally asymptomatic. In some fungi, however dsRNA mycovirus infections cause distinct morphological and physiological changes. These changes include toxin production, cytological alterations of cellular organelles, virulence associated traits such as growth rate, sporulation, pigmentation, enzymatic activities and hypovirulence (Anagnostakis 1979; Newhouse *et al*. 1983; Boland 1992; Rigling and Alfen 1993; Bottacin *et al*.1994; Varga *et al*. 1994; Magliani *et al*. 1997; Chu *et al*. 2002)

The fungus, *Fusarium graminearum* Schwabe [teleomorph: *Giberella zeae* (Schwein) Petch] (FHB) is an important plant pathogen that causes head and seedling blight of small grains such as wheat and barley. FHB is one of the main diseases of wheat in humid and semi humid areas throughout the world. Sterility of the florets, formation of shriveled seeds, and reduction in grain weight are characteristics of FHB (Goswami and Kistler 2004). In recent years, FHB infections have been associated with direct economic losses due to lower yields and poor grain quality (Windels 2000). Harvested grain is often contaminated with mycotoxins that cause public health problems such as vomiting, skin necrosis, and feed refusal (Chamley *et al*. 1994). Worldwide, deoxynivalenol DON is often detected in high concentrations (Clear and Patrick 2000).

Among *Fusarium* species, dsRNA mycoviruses have been reported to be present in *F. poae* (Fekete *et al*. 1995; Compel *et al*. 1999), *F. oxysporum* (Kilic and Griffin 1998), *F. solani* (Nogawa *et al*. 1996), *F. subglutinans* (Gupta 1991), *and F. proliferatum* (Heaton and Leslie 2004). No morphological alterations or signs of degeneration, however, have been observed to be caused by any of these dsRNA-containing isolates. In the first report of dsRNA infection in *Fusarium graminearum* isolates, results showed that dsRNA causes changes in morphological and pathogenicity phenotypes including reduction in mycelial growth, in-

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creased pigmentation, reduced virulence towards wheat, and decreased production of trichothecene mycotoxin (DON) dsRNA segments of 1.7 to 10 kb (Chu *et al.* 2002). A study that screened Iranian isolates of *F. graminearum* showed that 7% of isolates were viruliferous and contained fragments of 1.5 to 6 kb (Hashemi *et al*. 2004).

The objectives of this study were to determine the effect of dsRNA infections on DON production, growth rate and virulence of *F. graminearum* isolates.

MATERIALS AND METHODS

Fungal isolates

Thirty-three isolates of *F. graminearum* isolated from wheat in the Ardebil, Kerman, Golestan, and Mazandaran provinces of Iran were used in this study (Hashemi *et al*., 2004; Safaie *et al*. 2004). The presence of dsRNA in twenty of these isolates has already been reported (Hashemi *et al*. 2004).

For purification of dsRNAs, fungal mycelium of each isolate was grown in 100 ml of complete medium broth (CMB) at 25°C in an orbital shaker (100 rpm) (Correll *et al.* 1987). After 5 days of incubation, mycelia were collected, frozen and stored at –70°C (Chu *et al*. 2002).

dsRNA extraction

dsRNA was extracted from fungal mycelium using CF-11 cellulose chromatography as described by Zhang *et al*. (1998) with some modifications; mycelial tissue (5–6 g) were frozen in liquid nitrogen then ground to a fine powder in a mortar and pestle and extracted in a mixture of 14 ml 1 x STE, 2 ml 10% sodium dodecyl sulfate (SDS) and 1 ml 2% bentonite. The entire mixture was shaken for 30 min and centrifuged for 15 min at 8 rpm. The supernatant was adjusted to 16.5% ethanol and passed through a CF-11 cellulose (Sigma, Germany) column that bound the dsRNA. After an extensive wash with 1X STE buffer at 16.5% ethanol, the dsRNA was eluted from the column with 1X STE and precipitated with one-tenth volume of 3M sodium acetate pH 5.2 and 2.5 volume of ethanol. The mixture was incubated at –20°C overnight and then centrifuged at 13 000 for 60 min at –10°C. The dsRNA was re-suspended in 30 µl sterile double-deionized water. dsRNA was separated by electrophoresis through 0.8% agarose gels in TBE buffer stained with ethidium bromide and visualized with UV transilluminator.

Enzymatic digestions

To verify the double-stranded nature of the extracted RNA, two digestions were performed separately using RNase A and DNase I as described by Ahn and Lee (2001) and Castro *et al*. (2003). For DNase I digestion, the dsRNA sample was incubated for 15 min at 37°C with enzyme (Fermentas, 1 u/µl DNase 1, RNase-free). For RNase A digestion, the samples were treated with enzyme (Fermentas, 10 mg/ml RNase A, DNase free) in high (0.3 M NaCl) and low (15 mM NaCl) salt concentrations, according to the supplier's instructions.

dsRNA curing

Different *F. graminearum* hyphal tips containing dsRNA were transferred to PDA containing different concentrations (5 ppm to 70 ppm) of cycloheximide. dsRNA infected mycelial plugs of six isolates (F-36, F-38, F-42, F-64, F-68 and F-118) were inoculated on PDA containing 60 ppm of cycloheximide (Sigma, St. Louis, MO, USA) and incubated at 25°C for 10 days. Then twelve hyphal tips of each isolate were transferred to fresh PDA and incubated for 10 days at 25°C. After this period, agar plugs from colony margins were inoculated in 50 ml CMB and incubated at 25°C with shaking at 100 rpm for 5 days. Nucleic acids were purified and separated by electrophoresis as previously described.

Mycelial growth and sporulation

Mycelial growth of dsRNA-containing and dsRNA-free isolates were measured as the diameter of colony on PDA after incubation for 3 days at 25°C (Kousik *et al*. 1994). Conidial were grown in mung bean broth for 9 days at 25°C in a rotary shaker (100 rpm) (Rosewich Gale *et al*. 2002). Sporulation was estimated as described by Ahn and Lee (2001). Fifteen independent experiments were performed to obtain means.

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 onto wheat heads. Three replicates were set up for each treatment and arranged in a completely 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 25°C at 150 rpm. Conidia were harvested and spore suspensions were adjusted to $1x10^4$ conidia/ml. 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 the FHB disease severity was estimated. The experiment was replicated twice. Means were separated at $p < 0.01$ using Duncan's multiple range test.

DON analysis

Growth condition for DON production

dsRNA containing and dsRNA free *F. graminearum* isolates (F-38, F-42 and F-118), originally isolated from wheat grains collected in different wheat production areas in Iran, were used to determine the mycotoxin production by *F. graminearum* in the presence of dsRNAs.

Rice substrate was employed for DON production, according to the method described by Lauren and Agnew (1991). The sterile rice culture medium was inoculated

with 2 plugs from the margin of a 4-day-old grown colony of each isolate on PDA (Potato Dextrose Agar) and then incubated for 5 weeks at 25°C.

Extraction of DON

The mycelial mass and substrate were dried at 50°C. Fifteen grams of dried substrate were ground in a blender extracted as described by Lauren and Agnew (1991) and Jenning *et al*. (2004). All cultivation and extraction experiments were repeated three times. Flasks with rice and without fungal inoculation, received the same culture and extraction treatments as samples (the controls) for the exclusion of interfering compounds that might be confused with the mycotoxins under analysis. A spike and recovery experiment was conducted by adding 10 and 20 ppm of DON to rice culture extract obtained from the control.

DON analysis by HPLC

The HPLC system consisted of a Waters™ 600 pump system with a Waters 2 487 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. 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 in methanol/water (5 : 95).

RESULTS

Among 33 Iranian *F. graminearum* isolates analyzed, 12 of them showed bands after electrophoresis in 0.8% agarose. In most of these isolates, more than three fragments of 0.9, 1.2, 3, 3.2 and 5 kb were detected (Table 1). The dsRNA nature of these molecules was confirmed. All these bands were sensitive only to RNase A in low salt concentration and were resistant to digestion with DNaseI and RNaseA in high salt concentration (not shown).

To understand the physiological role of individual dsRNA molecule, we attempted to cure specific dsRNAs from representative isolates harboring dsRNA molecules. After over 100 trials to obtain dsRNA free isolates with cycloheximide, dsRNA free isolates were recovered from each representative isolate: F-38-1 from F-38, F-118-1 from F-118, and F-42-1 from F-42 (Fig. 1).

Mycelial growth and sporulation

The effect of dsRNA on mycelial growth, colony morphology, and pigmentation was measured on PDA after three days. Significant differences in mycelial growth rates were detected ($p < 0.01$) (Table 2). Mycelial growth in dsRNA-containing isolates was less than that of their counterpart cured isolates with no dsRNA, but the differences were not statistically significant (Fig. 2). Meanwhile no significant differences were observed in colony morphology, pigmentation, and conidia production. Both dsRNA-free and dsRNA-containing subcultures produced 132–160 conidia per ml of mung bean broth.

Table 1. The origin of isolates of *F. graminearum* used in the present study

Isolate	Geographic origin	Detected dsRNA [kbp]
Fg-5	Mazandaran, Sari, Dashtnaz	–
Fg-9	Kerman, Jiroft	–
Fg-15	Golestan, Gorgan	–
Fg-16	Kerman, Jiroft	–
Fg-18	Mazandarn, Bandargaz	–
Fg-22	Golestan, Gorgan	–
Fg-26*	Mazandaran, Sari, Dashtnaz	0.9, 1.2, 3, 3.2, 5
Fg-26	Mazandaran, Gharakhil	–
Fg-27	Golestan, Gorgan	–
Fg-33	Northern area	–
Fg-35*	Ardebil, Moghan, Parsabad	3, 3.2, 5
Fg-36*	Ardebil, Moghan, Parsabad	3, 3.2, 5
Fg-37*	Ardebil, Moghan, Parsabad	–
Fg-37	Golestan, Gorgan	–
Fg-38*	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-42*	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-44*	Ardebil , Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-53*	Golestan, Gorgan	3, 3.2, 5
Fg-57	Golestan, Gorgan	–
Fg-58	Northern area	–
Fg-61	Golestan, Gorgan	–
Fg-63*	Mazandarn, Rostamkola	
Fg-64*	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-68*	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-69*	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5
Fg-73	Mazandaran, Sari, Dashtnaz	–
Fg-76	Ardebil, Moghan, Parsabad	–
Fg-78	Ardebil, Moghan, Parsabad	–
Fg-84	Unknown	–
Fg-85	Northern area	–
Fg-90	Farahabad, Mazandaran	–
Fg-118	Golestan, Gorgan	0.9, 1.2, 3, 3.2, 5
Fg-141	Ardebil, Moghan, Parsabad	0.9, 1.2, 3, 3.2, 5

* without any dsRNA fragments

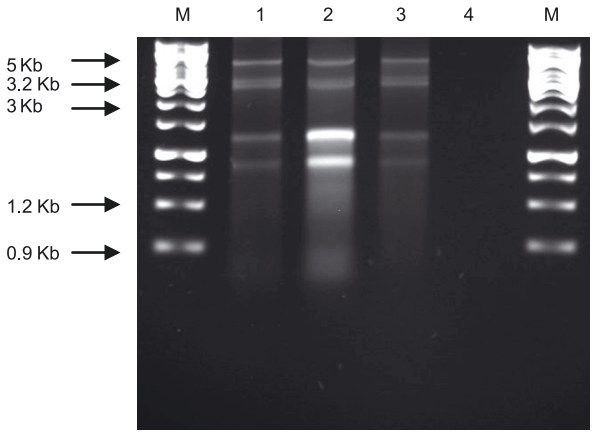


Fig. 1. Double-stranded RNA in *F. graminearum* isolates

M – 1kb DNA ladder; 1 – F-42 isolate; 2 – F-118 isolate; 3 – F38 isolate; 4 – dsRNA-free F-38 isolate

Table 2. Mycelial growth and Pathogenicity of dsRNA-containing and dsRNA-free isolate of *F. graminearum*

Isolate	Colony diameter [cm]	Disease severity
dsRNA-containing F-38	4.9±0.17	43.12±5.96
dsRNA-free F-38	4.9±0.17	69.12±21.69
dsRNA-containing F-42	5.2±0.25	28.94±5.49
dsRNA-free F-42	5.5	81.64±18.34
dsRNA-containing F-118	4.7±0.1	27.17±8.86
dsRNA-free F-118	4.8±0.28	76.75±4.42

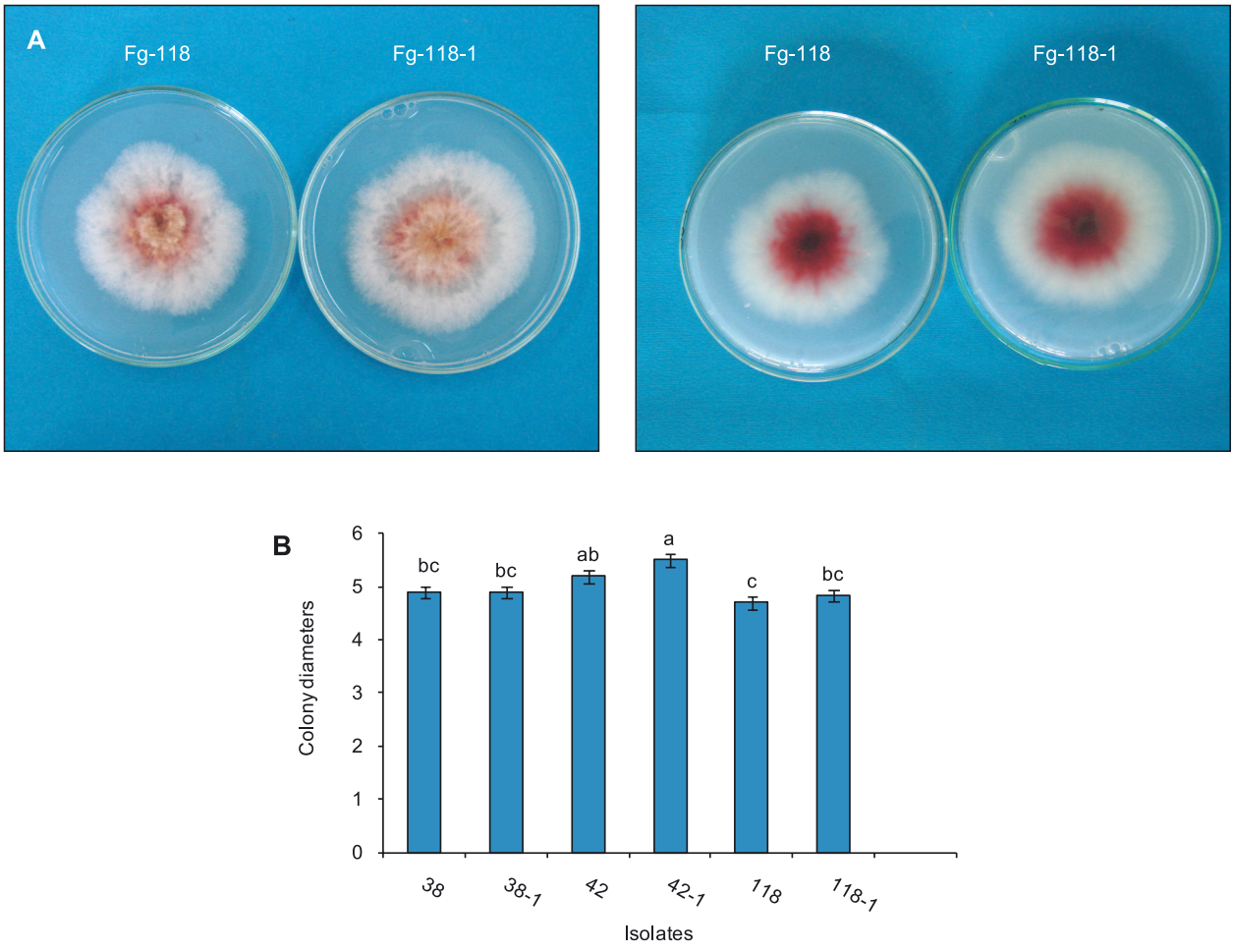


Fig. 2. Cultural morphology and comparison of colony diameter in dsRNA-free and dsRNA-containing *F. graminearum* isolates.

A – Mycelial growth in dsRNA-containing isolates was less than that of their counterpart isolates with no dsRNA, but the differences were not statistically significant

B – Comparison of colony diameter (cm) and standard deviation in *F. graminearum* isogenic isolates grown for 3 days on PDA. Different letters imply statistical differences among isolates at the 1% level

Pathogenicity test

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 was significantly less than that of the dsRNA-free isolates. Disease severity of dsRNA-free isolates on inoculated wheat heads were 69–81%, whereas those of dsRNA-containing isolates were 27–43% (Table 2, Fig. 3).

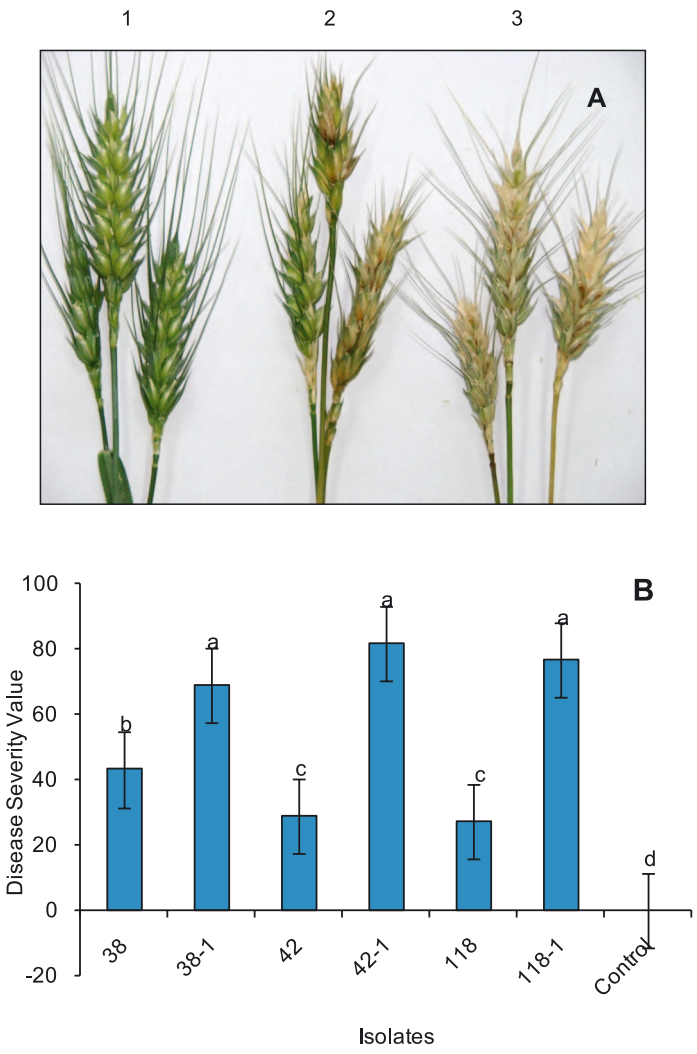


Fig. 3. Pathogenicity test and Disease severity

A – For pathogenicity test conidial suspensions were inoculated onto 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

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 2). 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% (Fig. 4, Table 3).

Table 3. Deoxynivalenol DON production by dsRNA–free and dsRNA-containing *F. graminearum* isolates on rice substrate

Isolate	DON production [ppm][1]	DON reduction [%][2]
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	

[1] values and standard deviation; [2] mean recovery was 85%

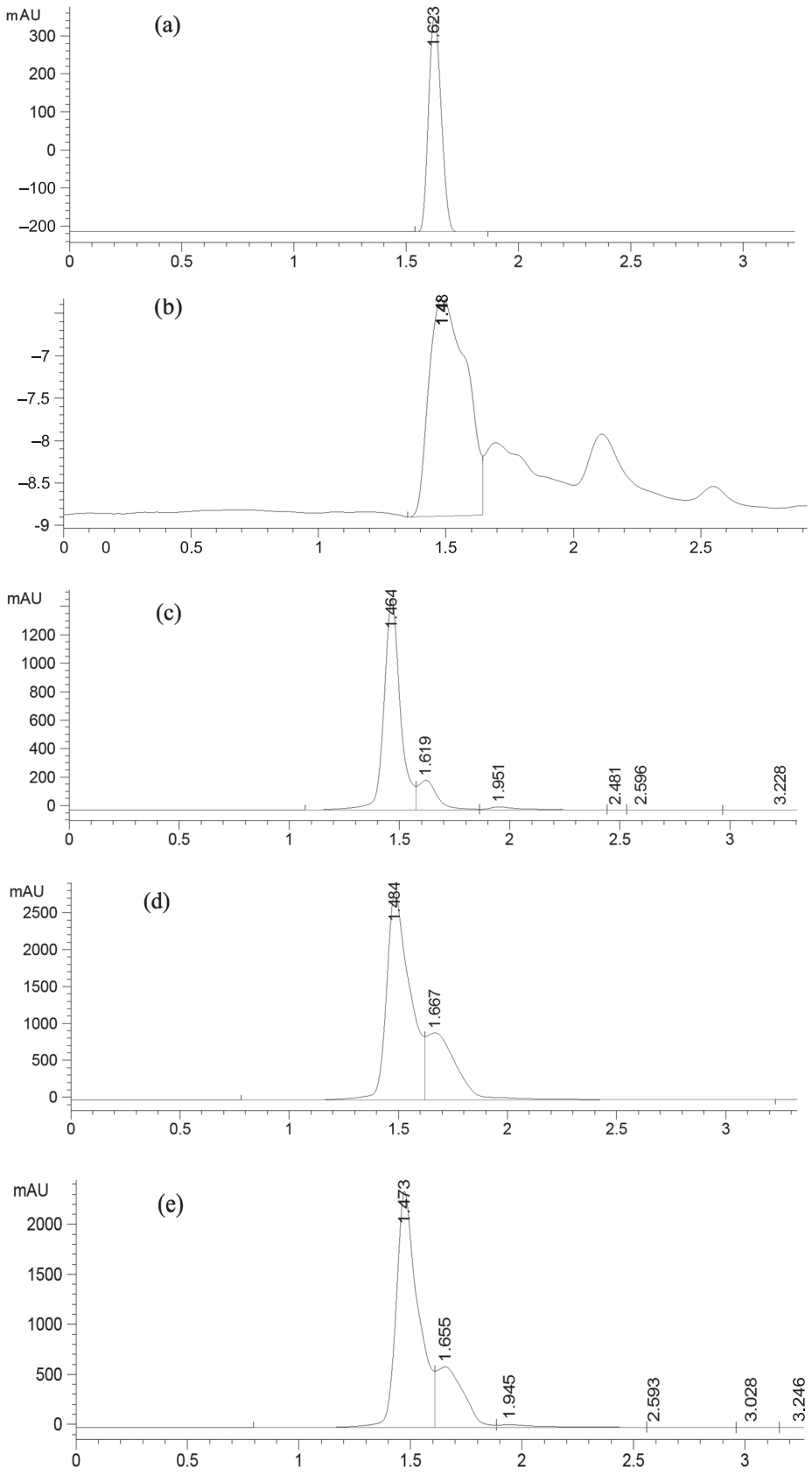
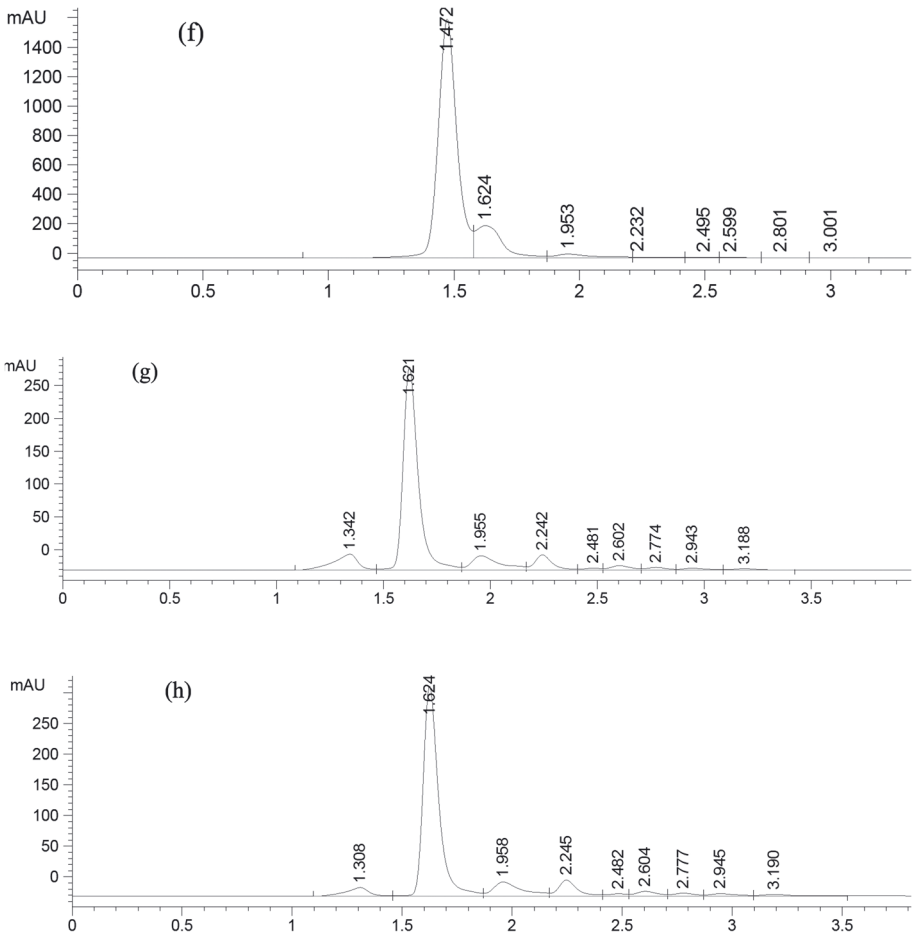


Fig. 4. HPLC chromatograms of rice inoculated with *F. graminearum* isolates

(a) standard DON (100 ppm), (b) control rice substrate extract, (c) Rice extract inoculated with dsRNA-containing F-118 isolate (diluted to 1/25), (d) dsRNA-free F-118 (diluted to 1/5), (e) dsRNA-containing F-42, (f) dsRNA-free F-42 (diluted to 1/25), (g) dsRNA-containing F-38, (h) dsRNA-free F-38



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DISCUSSION

Approximately 36.3% of *F. graminearum* isolates surveyed (12 out of 33) were infected by dsRNAs. In most isolates, more than three fragments of 0.9, 1.2, 3, 3.2 and 5 kb were detected. The number and size of dsRNAs in fungi are very diverse and highly variable, depending upon the fungal species (Rogers *et al.* 1986; Varga *et al*. 1994; Elias and Cotty 1996). In different phytopathogenic fungi, dsRNAs with sizes ranging between 800 bp to 15 kb were found (Howitt *et al*. 1995; Castro *et al*. 1999; Preisig *et al*. 2000; Papp *et al*. 2001; Robinson and Deacon 2002). The presence of multispecies of dsRNA in a single isolate has probably caused multiple infection by more than one type of virus or the frequent generation of deletion products from a large dsRNA molecule or infection by mycoviruses with segmented genome (Buck 1986; Ahn and Lee 2000).

The curing mechanism is still unknown, but it has been recorded that cycloheximide inhibits RNA synthesis (Bottacin *et al*. 1994; Elias and Cotty 1996). In fungi that have multiple dsRNA segments, the cure is frequently partial and the smaller fragments are lost (Robinson and Deacon 2002; Ikeda *et al*. 2004). In this study, all of dsRNA fragments with different sizes were cured.

Our results with *in vitro* rice culture showed that isolates carrying the dsRNA produced much less mycotoxin (DON) than the dsRNA free isolates. Also the disease severity of the dsRNA-containing isolates was significantly less than that of the dsRNA–free isolates. The results of Chu *et al*. (2002), 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). Our results showed that only growth rates were affected by dsRNAs.

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 is a complicated process, so additional studies are required to confirm these results.

Head blight reduces grain yield. Harvested grain is often contaminated with mycotoxins 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 for feed (Chamley *et al*. 1994; Windels 2000).

There is also concern about the public health implications of exposure to Fusarium mycotoxins, such as feed refusal, vomiting and skin necrosis (Foster *et a*l. 1986; Chamley *et al*. 1994). If the dsRNA fragments in F-38, F-42 and F-118 are transferable to dsRNA-free isolates through hyphal fusion with a high incidence. 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* can achieved.

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POLISH SUMMARY

WPŁYW DWUNICIENIOWYCH RNA NA WIRULENCJĘ I WYTWARZANIE DEOKSYNIWALENOLU PRZEZ IZOLATY GRZYBA *FUSARIUM GRAMINEARUM*

Wirusowe genomy występujące u grzybów patogenicznych dla roślin były przedmiotem dotychczasowych doniesień w literaturze. Najczęściej wykrywano genomy dsRNA, wykazujące hipowirulencję u niektórych grzybów, stąd też proponowano ich wykorzystanie jako czynników biologicznego zwalczania. W prezentowanej pracy, badaniom poddano 33 irańskie izolaty grzyba *Fusarium graminearum* na infekcję dsRNA. W przypadku 12 izolatów patogena wykryto ponad 3 fragmenty wirusowe dsRNA o długości 0,9; 1,2; 3,0; 3,2 oraz 5 kb. Zarówno obecność, jak i charakter dsRNA zostały potwierdzone działaniem RNazy A i DNazy I. W celu uzyskania izolatów grzyba wolnych od dsRNA, końcówki strzępek grzybni przeszczepiono na pożywkę PDA z dodatkiem cykloheksamidu w różnych stężeniach. W efekcie wykryto 3 kultury grzyba wolne od dsRNA. Obserwowano również słabszy wzrost grzybni zawierających dsRNA w porównaniu do izolatów pozbawionych fragmentów wirusa. Biorąc pod uwagę zarówno morfologię kolonii patogena, pigmentację oraz wytwarzanie konidiów, nie stwierdzono istotnych różnic pomiędzy izolatami wirusonośnymi, a izolatami wolnymi od wirusa. Jednak w warunkach szklarniowych doświadczeń zaobserwowano istotnie słabsze porażenie ($p < 0,01$) siewek podatnej odmiany, w przypadku infekcji wirusonośnymi izolatami grzyba pszenicy, w porównaniu do porażenia siewek tej samej odmiany, izolatami grzyba wolnymi od wirusa. Wytwarzanie deoksyniwalenolu (DON) zarówno przez wirusonośne jak i wolne od wirusą dsRNA izolaty *F. graminearum*, potwierdzono przy pomocy analizy HPLC. Koncentracje wytwarzanego DON przez izolaty wolne od dsRNA oraz zawierające dsRNA były zróżnicowane i wynosiły odpowiednio 0,07–1,6 ppm oraz 0,06–0,4 ppm. Wykryto istotnie obniżony poziom wytwarzanego DON do 50% u izolatów grzyba zawierających pochodne dsRNA, w porównaniu do izolatów wolnych od dsRNA.