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# Production of deoxynivalenol by *Fusarium graminearum* Schwabe in culture and its toxicity to wheat germlings in relation to virulence

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**Seven isolates of *Fusarium graminearum* obtained from Seed and Plant Improvement Institute were grown on autoclaved barley grains and toxicity of their cultures to wheat germinating seeds was tested using their semi-purified phytotoxins. The extracts of isolates 167, 169, 173 and 179 did not have considerable inhibitory effects on wheat seed germination, whereas that of 161 (a = 0.05) and two other isolates 131 and 164 displayed a significant inhibitory effect on germination. However, no correlation was found between virulence and toxicity of the isolates examined. Also, the effect of light was studied in relation to wheat germling level of tolerance. Six isolates were investigated for their ability of deoxynivalenol synthesis. Thus, their crude extracts were prepared and partially purified, and subjected to thin layer chromatography with chloroform (acetone and hexane) ethyl acetate solvent systems on fluorescent silica gel plates (60 meshes) using a commercial standard deoxynivalenol (1 mg/ml, Sigma Co., USA) along with the samples. The spots of interest were detected and analyzed under ultraviolet (UV) lights (254 and 365 nm). The isolate 161 produced the most amount of deoxynivalenol compared to others. Also, no correlation was found between virulence and toxigenicity of the isolates.**

**Key words:** Phytotoxicity, fusarium head blight, head scab, virulence, deoxynivalenol, mycotoxin, phytotoxin, *Fusarium graminearum.*

## INTRODUCTION

In many areas of the world, the important cereals (corn and wheat) are very susceptible to infection by *Fusarium* species, and infected seeds may be toxic and poisonous for human or animals consuming such products (Bosch et al., 1989). Four *Fusarium* species, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, and *Fusarium sambucinum*, of section discolor produce mainly diacetoxyscirpenol and deoxynivalenol (Desjardins et al., 1993). *F. graminearum* produces a set of chemicals of different biogenic origins and toxicities, and various strains of the fungus produce various mixtures of metabolites such as; deoxynivalenol, 3/15-acetyldeoxynivalenol, zearalenone, culmorin, culmorone, dihydroxycalonectrin, sambucinol, sambucoin, butenolide, and fusarin C (Farber and Sanders, 1986; Greenhalgh et al., 1984, 1986). Of 40 trichothecenes identified, nivalenol and deoxynivalenol are the most predominant mycotoxins contaminating cereal grains (Ueno, 1983). Based on the production of these metabolites and their derivatives, *F. graminearum* strains are divided into two main chemotype groups. One primarily produces deoxynivalenol or its acetylated forms, and the other produces nivalenol and its derivatives (Miller et al., 1991). Isolates of *F. graminearum* recovered

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**Table 1.** Characteristics or *F. graminearum* isolates studied.

| Isolate No. | Host wheat cultivar/line | Geographic location |
|---|---|---|
| 131 | Falat | Garakheil, Gaemshahr |
| 161 | Falat | Dasht-e-Naz, Sari |
| 164 | Hirmand | Rostamklar , Behshahr |
| 167 | Golestan | Chomadzi, Gorgan road |
| 169 | Falat | Sorkhanklateh, Gorgan |
| 173 | Falat | Mohammad abad, Gorgan |
| 179 | Hirmand | Iraqimaalleh, Gorgan |

from Mazandaran province are placed in the first group (Zamani-Zadeh and Khoursandi, 1990).

However, the occurrence of nivalenol producing isolates of *F. graminearum* has been also recorded in Iran (Etaati, 2002). The severity of *Fusarium* infection and of trichothecene contamination increases with wet weather at harvest and with storage under conditions of high relative humidity (Desjardins et al., 1993). Regardless the kind of metabolites produced, these compounds are associated with some disorders such as livestock emesis, diarrhea, decreased feed consumption, skin irritation, hemorrhage, reproductive problems and hematological changes (Abramson et al., 1993). Therefore, their occurrence in cereals is of importance in agriculture and food industries (Yao et al., 1996), and endangers social safety.

With plants, some studies carried out on the effects of trichothecenes have been related to general impressions such as wilting, chlorosis, necrosis or inhibited growth of whole plants or plant organs (Brian et al., 1961). Trichothecenes prevent cell division (Linnainmaa et al., 1979), and reduce plant tissue development, increased by growth regulator substance, clearly through indole acetic acid inhibition (Brian et al., 1961; Cole et al., 1981; Freeman, 1955; Stahl et al., 1973). The inhibitory effect of the *Fusarium* toxins on the *in vitro* germination of wheat seed and the growth of the coleoptiles has been indicated before (Miller et al., 1985; Pakdaman et al., 2003, 2006), and therefore, there might be a possible relation between phytotoxicity of *Fusarium* isolates *in vitro*, and their virulence *in planta*. The main purpose of this study was to evaluate the phytotoxicity of semi-purified extracts of different isolates of *F. graminearum* to investigate the possible correlation between toxin production and their virulence.

## MATERIALS AND METHODS

Seven isolates of *F. graminearum* were obtained from Seed and Plant Improvement Institute, Karaj. Information about these isolates is provided in Table 1. Isolates were grown in Petri plates containing Potato Dextrose Agar (PDA) medium, incubated at 26°C under dark conditions for 3 days. To extract toxins, the method of extraction from whole mass of fungus and culture medium was applied.

A medium containing barley seeds saturated (wetted) with 1% sugar solution was prepared and sterilized at 121°C for 15 min, then inoculated by a 5 $mm^2$ disc of actively growing mycelium of the fungus per bottle of 100 g of grains and incubated in dark, at 26°C for 6 weeks. The seed colonized by each fungal isolate were allowed to completely dry in an oven at 60 to 70°C and then ground, and blended in ethanol:water (3:1) for 1 h on a shaker (180 to 200 rpm). Aliquots of 100 ml of the filtrate were concentrated in an oven at 60 to 70°C, and the concentrated filtrates (50 ml) were defatted twice with normal heptan (50 ml) on a shaker (50 rpm) for 30 min. The lower phase was re-extracted with chloroform (100 ml) on a shaker (180 rpm) and then left in a refrigerator overnight. The upper phase was collected, and then kept in a desiccator at 75°C, then dissolved in ethanol:water solution (3:1) and the final volume was adjusted to 3.5 ml.

Collections were individually poured into small vials and dried completely. Then methanol (400 µl) was added to each vial to re-dissolve the preparation. Thin layer chromatography (TLC) was performed using a solvent system containing 20 ml of ethyl acetate:hexane (3:1). Standard deoxynivalenol sample (1 mg/ml) was chromatographed along with the samples. Amounts used were equal to 2 µl per isolate extract and 5 µl for control. TLC plates were of 60 meshes with a fluorescence of yellow-green colour under wave length of 254 nm. A similar analysis was performed with 20 ml of chloroform:acetone (2:3) solvent system and 10 µl samples prepared by adding 2 ml methanol to dried extracts and 5 µl standard deoxynivalenol (1 mg/ml) blotted along with them.

Toxicity test was based on $NGT_{C80}$ method (Buerstmayr et al., 1997) using a base medium made of water and agar (8 g/L) with 30, 35, 40, 45, 50 and 100 ppm of a mixture containing semi-purified phytotoxins. The wheat cultivar used to bioassay was Attila.

After germination of 80% of seeds in controls without phytotoxic extracts, half of Petri dishes were put, exposed to sun light and others in darkness. Temperature was adjusted at 25°C. There were three Petri dishes per treatment each containing 20 seeds. Data related to the virulence of *Fusarium* isolates used (Figure 1) were provided by Cereals Pathology Division of Seed and Plant Production and Improvement Institute, Karaj. These data had been obtained by the determination of the mortality percentage of a *Fusarium* head blight susceptible wheat cultivar grains inoculated with the spore suspensions of the isolates studied.

## RESULTS AND DISCUSSION

With an eye to the importance of trichothecenes, this study was conducted to reveal their (particularly deoxynivalenol) possible role in the virulence of *F. graminearum* on wheat plants. The importance of the identification of virulence factors in a pathosystem and its application in the control of plant diseases has been

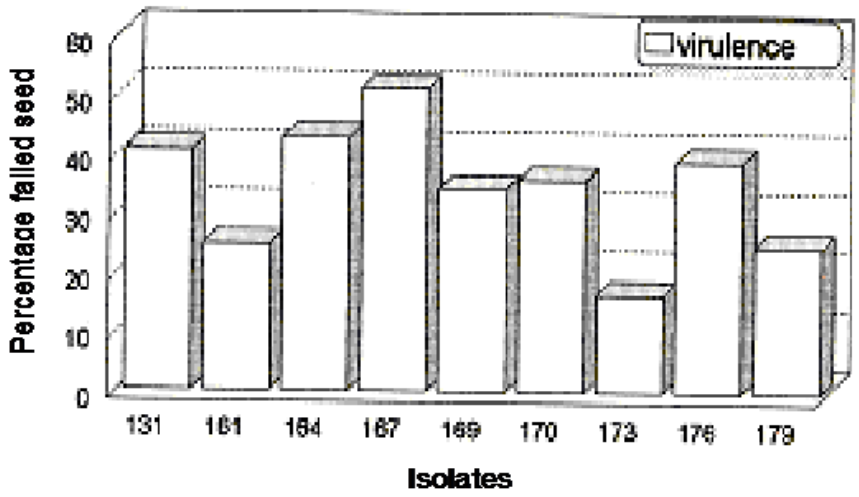


**Figure 1.** The relative virulence of *F. graminearum* isolates based on the percentage of the wheat seed failed to germinate (based on the data from PSSII).

previously discussed (Desjardins et al., 1993).

Based on TLC data, isolate number 161 ($I_{45}$) produced more quantities of deoxynivalenol in barley medium as compared to others. The fluorescent spots related to deoxynivalenol were clearly visible for isolate number 164 ($I_{47}$) and 131 ($I_{42}$). However, other spots related to unknown trichothecenes, were also seen on TLC plates. No attempts were made for their identification.

The fluorescent spots visible under ultraviolet (UV) radiations (254 and 365 nm) were potentially related to deoxynivalenol with $R_f$ quantities approximately equal to 23.26 and 30.08% related to the solvent systems, respectively. Other isolates examined, did not show any spots comparable to deoxynivalenol. Evidently, some isolates produce deoxynivalenol and therefore belong to the Chemotype I.

Also, other spots related to other metabolites were found on the TLC plates, however, because of the lack of the mycotoxin standards, their identification was ignored. It was also notable that the six-month-cultured isolate number 164 produced a compound that was not confronted with other young cultures of this isolate, indicating that with the elapse of time, there are new metabolites that are produced by the same isolate and reach to an extent detectable by TLC. However, the possibility of the release and accumulation of this compound from the plant-based substrate cannot be excluded, although the fungal origin of the spot seems more probable, as a similar spot is found also near to the upper edge of the plate with the isolate number 131 (Figure 2).

Phytotoxicity of various isolates was considerably different (F = 17.09**). Of seven isolates studied, only three namely; 131, 161 and 164 were significantly toxic, and others were not toxic and behaved similar to controls (Figure 2).

Isolates 164 and 131 produced higher amounts of the toxic principal, and their semi-purified toxin extracts considerably inhibited wheat seed germination. Not only inhibitory effects of semi-purified phytotoxins caused the ratio of germinated seeds to the total number of seeds to decrease, but also the growth rates of primitive roots and coleoptile decreased due to the toxicity of fungal extracts (Figure 3). Our results showed that at least some of the isolates originating from Mazandaran province are capable of synthesizing deoxynivalenol which belong to the Chemotype I. This result is in accordance with the results obtained by Zamani-Zadeh and Khoursandi (1995). Occurrence of *F. graminearum* isolates belonging to Chemotype II has been also reported from Iran (Etaati, 2002). The production of trichothecenes such as deoxynivalenol and specially nivalenol by the isolates from Iran is of concern for human and livestock health particularly in Northern provinces, and may cause epidemics when climatic conditions are suitable for rapid growth and development of head scab disease.

A significant difference was observed among various concentrations effects of toxin (F = 14.33**) on coleoptile growth. Means comparative analysis by Duncan test ($\alpha$ = 0.05) demonstrated that the concentrations more or less than 50 ppm not only had not caused the growth to decrease, but also they had stimulated the growth as an apparently positive factor, an effect that has been observed before (Alizadeh and Zhange, 1998) personal communication. Semi-purified toxic extract (50 ppm) from the culture of the isolate number 164 reduced the growth of wheat coleoptile in accordance with the results obtained by Brian et al. (1961) and Wakulinski (1989).

Interactions between light and concentrations, variety and concentrations and among all the factors were highly significant ($\alpha$ = 0.01). Our results are in agreement with the studies of Buerstmayr et al. (1997) and Packa and

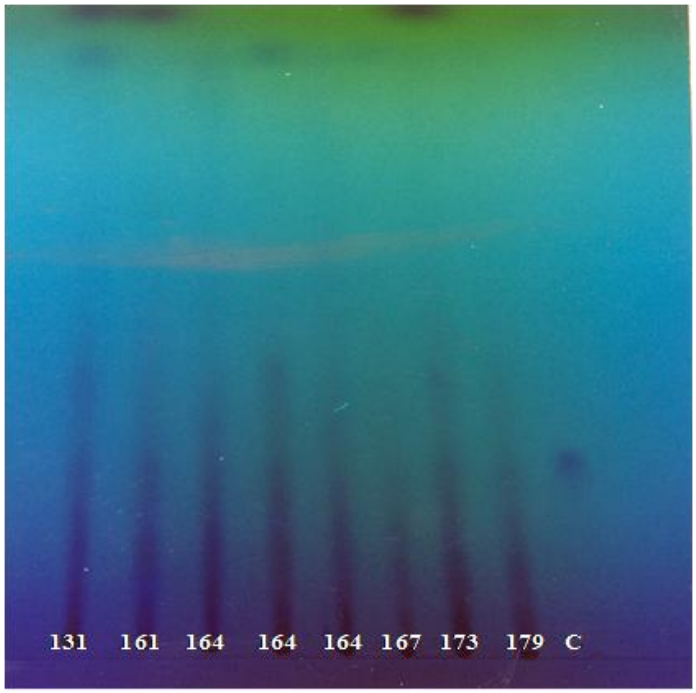


**Figure 2.** Thin layer cheomatography with chloroform-aceton; the isolate number 164, from left to right: synchronously cultivated with other isolates, 6 months old, and freshly cultured.

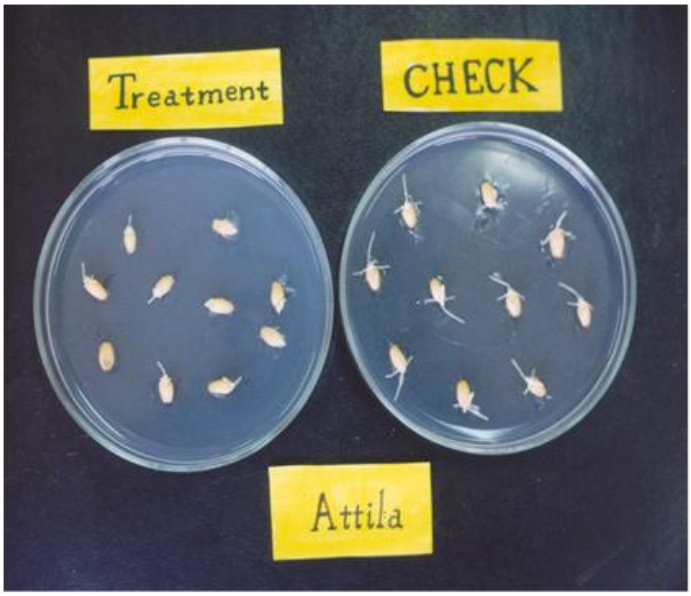


**Figure 3.** Effect of *F. graminareaum* phytotoxic extract (50 ppm) on seed germination in wheat susceptible cultivar Attila.

Koczowska (1987), which have demonstrated the production of toxin(s) by *Fusarium* in culture resulting in reduced germination and primary root and stem (coleoptile) growth (Figure 4).

Desjardins et al. (1996), Proctor et al. (1995), Eudes et al. (2001) and Dyer et al. (2005) have shown a correlation between phytotoxic trichothecenes and virulence. With our experiments, no correlation was generally found ($R = 0.51^{ns}$)

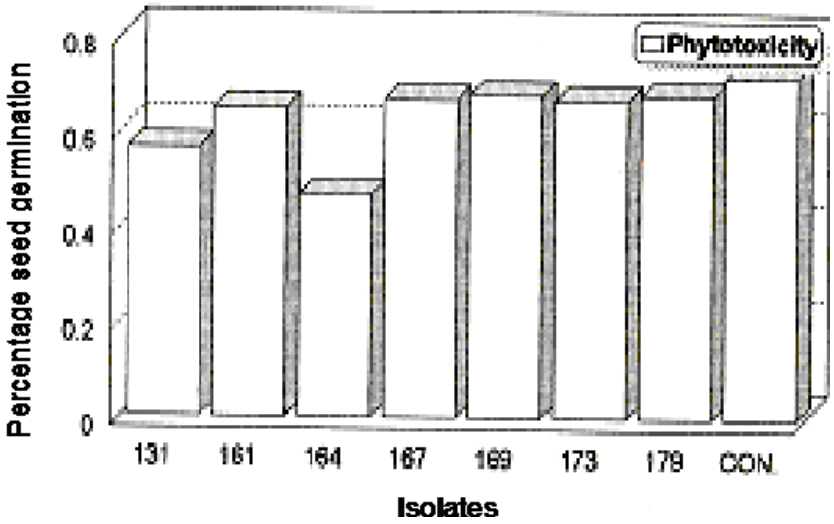


**Figure 4.** The relative phytotoxicity of *F. graminearum* isolates based on their semi-purified extract potentials for wheat seed germination inhibition.

between isolate phytotoxicity and virulence, so that the isolate number 161 produced the most amount of dissolved organic nitrogen (DON), but it was not so toxic overall. In contrast, the isolate number 164 with lower DON production potential caused the most phytotoxicity. It seems that the reason of this controversy is the effect of the DON concentration on its toxicity, and also the different genetic bases of different isolates. The influence of mycotoxin chemotype in determination of pathogenicity of *F. graminearum* strains on a particular host plant has been previously reported (Carter et al., 2002). Such an effect of *F. graminearum* genetic diversity of trichothecene production on the pathogen virulence has been documented with strains from Nepal (Desjardins et al., 2004). However, some other factors may involve in virulence. For example, one of the factors concerned to this result may be that any cultivar has its own potentials for tolerance of toxins; therefore, it is necessary to test both the characteristics with each given cultivar. It is also known that different isolates of *F. graminearum* have various potentials for production of mycotoxins *in vitro* (Miller et al., 1983; Neish and Cohen, 1981) and in the field (Thiel et al., 1982). Our results also are in accordance with that of Gang et al. (1988), and this interpretation is fortified by Miedaner et al. (1997).

Regardless of concentrations, germlings had more growth when exposed to the sun light, and this is in accordance with the results obtained by Yao et al. (1996) that the head scab resistant cultivars were capable of fungal toxin conversion to a Compound X through its biodegradation, a compound which was no longer effective on wheat tissues, but able to prevent *Gibberella zeae* spore germination.

**Abbreviations: TLC,** Thin layer chromatography; **DON,** dissolved organic nitrogen.

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