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Research Article

Occurrence of deoxynivalenol producing isolates of *Fusarium graminearum* species complex associated with head blight of wheat in Moghan area

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Abstract: The severe epidemics of *Fusarium* head blight (FHB) as a devastating disease of cereal crops has occurred on wheat in North and Northwest Iran in recent years. The *in vitro* production of Deoxynivalenol (DON) was qualitatively evaluated in 41 *Fusarium* isolates collected from wheat heads associated with the scab disease, in Moghan area/Northwest Iran. Infected wheat heads were collected during 2004-2007. The isolation of causal agents was carried out using standard methods. According to morphological characteristics and using valid descriptions, all isolates belonged to *Fusarium graminearum* species complex and *F. culmorum* of which the former was dominant. In order to evaluate the potential of DON production in isolates, this mycotoxin was extracted and qualitatively examined by TLC method. The quantification of DON was achieved using HPLC method. TLC results indicated that 54.5% of studied isolates produced DON but there was no significant relationship between this property and cultivars or sub-regions or years. Also based on HPLC analysis, maximum content of DON was detected in *F. graminearum* isolated from cv. Izen green from Moghan Agro-industry company fields in 2004 at the rate of 5827.11 μgkg^{-1}. The results of present study show that DON mycotoxin is produced at various contents by *F. graminearum* isolates on different cultivars and from different origins/ years. Since DON plays a role in pathogenesis and is of paramount importance in contamination of wheat grains, these results give a better insight into the significance of this disease in Northwest Iran.

Keywords: Chemotype, DON, *F. graminearum sensu lato*, Fusarium Head Blight, Northwest Iran

Introduction

Fusarium head blight (FHB, scab), predominantly caused by *Fusarium graminearum* and *F. culmorum*, has become a major limiting factor for sustainable wheat (*Triticum aestivum* L.) production around the world. The incidence of FHB has increased worldwide over the past decades (Goswami and Kistler, 2004). Heavy disease outbreak causes severe losses of yield and reduces grain quality. Plants infected by *F. graminearum* have shriveled grains of significantly lower kernel weight (Bai and Shaner, 2004). Besides causing huge production losses

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and poor baking quality, most of the species causing FHB produce a range of toxic fungal secondary metabolites known as mycotoxins which contaminate the grain (Jennings *et al.*, 2004; Bottalico and Perrone, 2002; Xu *et al.*, 2005; Rodrigues and Naehrer, 2012). Many of these mycotoxins play roles in pathogenesis on wheat (Proctor *et al.*, 2002) and they are harmful to both animals and humans causing a wide range of symptoms of varying severity and are possible immunosuppressant and so these mycotoxins make the infected grain unsuitable for human and livestock consumption (Ehling *et al.,* 1997; Placinta *et al.,* 1999). Mycotoxins produced by *F. graminearum* belong to the zearalenone and trichothecene families. Trichothecenes comprise a class of mycotoxins which include Deoxynivalenol (DON) and its acetylated forms (13ADON, 15ADON), Nivalenol (NIV), T-2 and HT-2 toxins (Bottalico and Perrone, 2002). Trichothecene mycotoxins especially DON are protein synthesis inhibitors for eukaryotic organisms which can subsequently cause delay in plant defence process (Cundilefe and Davis, 1997; Miller *et al*, 1991). Many trichothecenes are associated with FHB, but the predominant one produced by *F. graminearum* is DON which belongs to the type B trichothecenes (Desjardins and Hohn, 1997). Mesterhazy *et al*. (1999) indicated that the total trichothecene toxin-producing capacity of the isolates might be the decisive component of pathogenicity. Many countries have regulations limiting DON content in commodities or foods (FAO, 2004; van Egmond *et al.*, 2007). In 2010, JECFA revised the provisional maximum tolerance daily intake (PMTDI) from 1 $\mu g kg^{-1}$ body weight (bw) for DON only, to a group PMTDI of 1 $\mu g kg^{-1}$ body weight for DON and its acetylated derivatives (JECFA, 2010).

A range of different *Fusarium* species has been associated with the disease but *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum* and *F. avenaceum* appear to predominate depending on climatic conditions (Parry *et al.*, 1995). Nowadays, the most prevalent species world-wide seem to belong to the *F. graminearum sensu lato* also called as *F. graminearum* species complex (FGSC) (Boutigny *et al.*, 2011; O´Donnell *et al.*, 2004; Toth *et al.*, 2005; Starkey *et al.*, 2007). Using morphological characterization, *F. graminearum sensu lato* have been introduced as the dominant FHB species in some parts of Iran such as Northern and North-western provinces of Iran (Golzar *et al.,* 1998; Davari *et al.*, 2006; Zamanizadeh and Khorsandi, 1995; Chehri *et al.,* 2011). On the other hand, Eslahi *et al.,* (2008) and Mousavi-Jorf *et al.,* (2007) have reported other species from FHB-infected wheat heads from Khuzestan province in Southwest Iran. Recently, within the FGSC at least 15 distinct lineages have been recognized based on sequence data, some of which are potentially limited to a certain geographic region or host (Boutigny *et al.*, 2011; O´Donnell *et al.*, 2004; Sarver *et al.*, 2011; Starkey *et al.*, 2007; Davari *et al.,* 2012). In Iran, Davari *et al.* (2013) used the Luminex-Multilocus genotyping (MLGT) assay for separation of FGSC obtained from wheat heads with FHB in North and Northwestern Iran and showed that all FGSC isolates belonged to *F. graminearum s. stricto* (lineage 7).

Moghan, in Northwest of Iran, is one of the major centers of small grains production in Iran and Asia with more than 150,000 ha under wheat cultivation. In recent years, incidence of FHB has increased in Moghan area resulting in huge losses to the grain industry due to reduced yields and mycotoxin production. The aim of present study was to identify causative agents of Fusarium head blight and the examination of DON producing ability of *Fusarium* isolates in different wheat cultivars and regions of this area. Exact knowledge about *Fusarium* species and chemotypes could be useful in the production of resistant varieties and other management strategies in each region.

Materials and Methods

Isolation and identification of fungi: Heads and/or seeds of bread wheat (*Triticum aestivum*) showing symptoms of FHB–brownish discoloration at the base of the floret, a spikelet or even the whole head; occasionally orange sporodochia; white shrivelled or pinkish-red kernels were collected from the fields in seven regions of Moghan (Moghan Agro-Industrial Company/MAC, Old Eslam Abad, New Eslam Abad, Agdam, Dostlukandi, Oltan and Agricultural and Natural Resources Research Centre of Moghan/ARCM) during 2004-2007 (Table 1). Seven cultivars (Atila 4, Atila 50, Tajan, Zagros, Goadloop, Izen green and Gascogen) were grown in those wheat fields. Seeds were surface sterilized using 0.5-1% sodium hypochlorite for 0.5-1 min, rinsed twice with sterile distilled water and finally dried on sterile filter paper in a laminar flow hood (Burgess *et al.,* 1994) and *Fusarium* spp. were isolated from the collected material on Nash & Snyder's medium (Peptone- PCNB-Agar, PPA), as modified by Nelson *et al.* (1983). Single spore cultures were obtained by dilution plating on water agar as described by Leslie and Summerell (2006). For morphological species identification, cultures were incubated on Potato Dextrose Agar (PDA) for colony growth rate and colour assessment and on Synthetic Nutrient-poor Agar (SNA) with sterile filter paper for spore morphology assessment under a light and temperature regime to induce sporulation as previously described (Davari *et al.,* 2006). All species were identified based on descriptions given in Gerlach and Nirenberg (1983), Leslie and Summerell (2006) and Nelson *et al.* (1983).

Extraction of DON, TLC test and HPLC analysis: In order to evaluate the potential of DON production, 33 isolates from different cultivars and regions were selected for TLC test and 28 isolates for HPLC analysis and were assayed according to Jennings *et al.* (2004) and Lauren and Agnew (1991) methods with a little modification by Alizadeh *et al.* (2003).

Extraction of DON: *Fusarium* isolates were grown in rice culture and DON extracted using a method adapted from that of Cooney *et al.* (2001). Rice culture was finely ground and mixed well. A subsample (15 g) from each sample was mixed with acetonitrile/methanol /water (16:1:3; 60 ml) and shaken for 3 h; 2 ml was taken for DON analysis and passed through a clean-up cartridge consisting of a 2 ml syringe packed with a filter-paper disc No.1 Wathman (International Ltd, Maidstone, UK), a 5 ml lump of glass wool and 1 g of alumina/activated carbon (20:1). The sample was allowed to seep by gravity feed through the cartridge and residues in the cartridge were washed out with acetonitrile/ methanol/water (80:5:15; 2 ml). The combined eluate was evaporated (compressed air, 70 ºC) and then resuspended in methanol/water (5:95; 2 ml) and stored at -20 ºC.

TLC test: Following extraction and clean up, the extracts were qualitatively examined by Thin Layer Chromatography method for DON. For this purpose, 15 µl of extracts were spotted in TLC plates (TLC, Merck, TLC Aluminium sheets, 20 × 20, Silica gel 60F254), together with specific standards (Sigma, USA) separately. Plates were placed in a solution containing toluene/acetone (7: 12). After drying, each plate was analysed under UV light at 254 nm. DON appears as bluish fluorescent spots (Fig. 1).

HPLC analysis: Quantification of DON was by high performance liquid chromatography. HPLC separation was performed using a luna C18-column (150 mm × 3.9 µm) (phenomenex, USA at a flow rate of 1 ml/min. A 40 µl amount of sample was injected. The mobile phase was methanol/water (5:95). The effluent was monitored at 220 nm. DON contents of the extracts were determined by comparison with external standards (Figs. 2, 3).

Statistical analysis: Kruskal-Wallis test was performed to compare the ability and amount of DON production by sub-regions or wheat cultivar or years in SAS program (SAS Institute, 1999).

Results

Identification of fungi: Based on morphological characteristics of isolates obtained from infected heads, 62 isolates belonged to *F. graminearum* species complex and one isolate to *F. culmorum.*

TLC Test: TLC results indicated that 54.54% of studied isolates (18 of 33) produced DON. DON producing and non-producing isolates are shown in Table 1.

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Table 1 DON production of Fusarium isolates examined from Moghan area by TLC test (++: high content, +: presence and -: absence of DON) and HPLC analysis.

Isolate no.	Origin of samples	Wheat cultivar	Year	Species	TLC test	Extracted DON contamination by HPLC (ppb)
F1	MAC	Gascogene	2004	*F. graminearum*	-	0.00
F3	MAC	Gascogene	2004	*F. graminearum*	+	105.19
F4	MAC	Izen green	2004	*F. graminearum*		5827.19
F5	MAC	Atila 50	2004	*F. graminearum*	-	
F8	AD	-	2005	*F. graminearum*	-	
F9	DLK	Tajan	2006	*F. graminearum*		115.30
F10	MAC	Gascogene	2004	*F. graminearum*	-	
F11	MAC	Gascogene	2004	*F. graminearum*	+	215.23
F12	MAC	Zagros	2004	*F. graminearum*	+	223.15
F15	MAC	Goadloop	2004	*F. graminearum*	+	182.96
F16	DLK	Tajan	2006	*F. graminearum*	+	704.48
F17	MAC	Atila 4	2004	*F. graminearum*	-	
F20	MAC	Tajan	2004	*F. graminearum*		0.00
F21	MAC	-	2004	*F. graminearum*	+	313.83
F22	MAC	Zagros	2004	*F. graminearum*	-	
F23	MAC	Izen green	2004	*F. graminearum*	+	173.45
F24	OL	Atila 4	2006	*F. graminearum*	+	
F25	MAC	Goadloop	2004	*F. graminearum*		1081.78
F26	MAC	Gascogene	2004	*F. graminearum*	-	
F27	MAC	Gascogene	2005	*F. graminearum*	-	0.00
F29	MAC	Zagros	2004	*F. graminearum*	+	250.42
F34	ARCM	Atila4	2007	*F. graminearum*	+	115.14
F35	ARCM	Atila4	2007	*F. graminearum*	+	697.72
F36	MAC	-	2004	*F. graminearum*	+	759.91
F37	MAC	-	2004	*F. graminearum*		231.00
F41	OEA	Atila 4	2004	*F. graminearum*	+	278.01
F43	ARCM	Atila 4	2007	*F. graminearum*	-	0.00
F45	NEA	Atila 4	2005	*F. graminearum*	++	
F46	NEA	Atila 4	2005	*F. graminearum*	-	
F47	NEA	Atila 4	2005	*F. culmorum*	-	0.00
F48	NEA	Atila 4	2005	*F. graminearum*		178.88
F49	NEA	Atila 4	2005	*F. graminearum*	-	
F50	ARCM	Atila4	2007	*F. graminearum*	+	209.94
F51	ARCM	Atila4	2007	*F. graminearum*	+	148.36
F52	NEA	Atila 4	2004	*F. graminearum*		817.73
F53	NEA	Atila 4	2004	*F. graminearum*	-	
F55	NEA	-	2005	*F. graminearum*		0.00
F57	DLK	Tajan	2006	*F. graminearum*	-	
F75	ARCM	Atila 4	2007	*F. graminearum*	+	101.10
F76	ARCM	Atila 4	2007	*F. graminearum*	++	
F77	ARCM	Atila 4	2007	*F. graminearum*	-	0.00

MAC: Moghan Agro-industry Company; OEA: Old Eslam Abad; NEA: New Eslam Abad; AD: Agdam; DLK: Dostlukandi; OL: Oltan; ARCM: Agricultural and Natural Resources Research Centre of Moghan.

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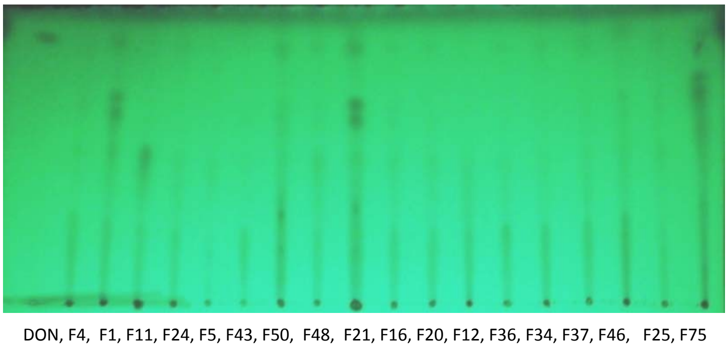

Figure 1 Mycotoxin assay by TLC. Lane 1: Standard samples of DON, Lanes 2-18: Toxin extraction from different isolates of *F. graminearum* species complex.

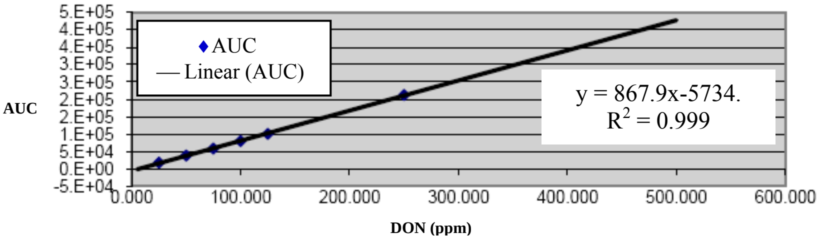

Figure 2 Calibration curve of different concentrations of Deoxynivalenol.

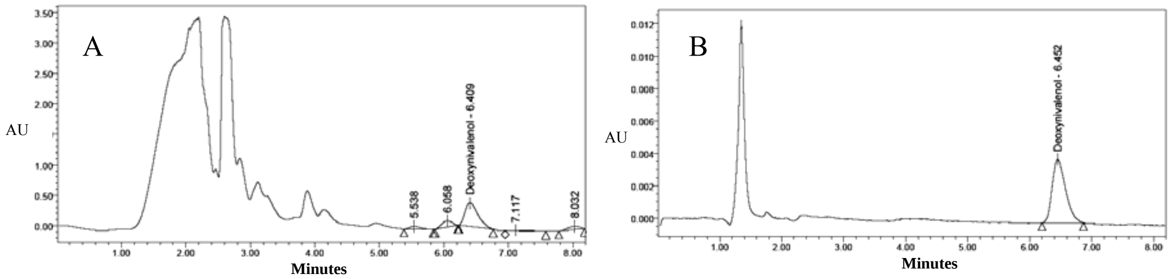

Figure 3 HPLC chromatogram of **A**: isolate F4 of *F. graminearum* and **B**: Standard DON (1500 ngml^{-1}).

HPLC Evaluation: Based on HPLC analysis, maximum content of DON belonged to *F.graminearum* isolated from cv. Izen green in Moghan Agro-industry Co. fields in 2004 (F4) with 5827.11 µgkg^{-1} and minimum amount (101.11 µgkg^{-1}) belonged to an isolate of this species recovered from cv. Atila 4 of this region in 2007 (F75). Overall, much difference was observed between DON producing ability of studied isolates. Also some isolates did not produce this trichothecene (Table 1).

There were not significant correlations between the DON-producing ability and wheat cultivar (G = 6.4474, P = 0.3750) or sub-region (G = 6.4474, P = 0.3750) or year (G = 3.9274, P = 0.2694) at $P < 0.05$. Also, no significant correlation was observed between the amount of DON produced by isolates and sub-region (G = 3.0853, P = 0.5437), cultivar (G = 10.6342, P = 0.1004) and year (G = 7.9506, P = 0.064).

Discussion

The great majority of the FHB causing *Fusarium* species in Iran proved to be *F. graminearum s. lato,* which also is the most prevalent FHB-agents elsewhere in the world (Boutigny *et al.*, 2011; O´Donnell *et al.*, 2004;

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Starkey *et al.*, 2007). Between 15 known lineages, *F. graminearum sensu stricto* (lineage 7) was dominant species in whole of wheat cultivars and throughout Moghan area (Davari *et al.,* 2013) and it is dominant in other regions too (O'Donnell *et al*., 2004; Ramirez *et al*., 2007; Reynoso *et al.,* 2011; Zhang *et al.,* 2012).

Trichothecenes are structurally diverse family of mycotoxins that induce mycotoxicoses in humans and animals and enhance the virulence of some *Fusarium* species on some plant hosts. DON or vomitoxin is the most prevalent trichothecene in *Fusarium* species that has a significant role in pathogenesis of fungus and it is a serious health threat (Cumagun and Miedaner, 2004; Leslie and Summerell, 2006). Based on the results, around half of the examined isolates were able to produce DON, so belonged to chemotype I (Miller *et al.*, 1991), this diversity in DON production is similar to Gale *et al.* (2003) results as only some isolates of *F. graminearum s. lato* in North America had such a capacity. Also, Geraldo *et al.,* (2006) revealed that 33% of *F. graminearum s. lato* isolates have DON producing ability in southern Brazil. Zamanizadeh and Khorsandi (1995) evaluated 19 isolates of *F. graminearum s. lato* and *F. culmorum* obtained from infected heads of wheat in Mazandaran province using HPLC and revealed that all *F. graminearum s. lato* isolates have DON producing ability as well as ZON. In Golestan, assessment of natural zearalenone contamination in wheat samples using HPLC revealed that the levels were below the advisory levels for zearalenone in wheat (Karami-Osboo and Mirabolfathi, 2008). Also biomarker studies in the Golestan area indicated that both fumonisins and DON levels are relatively low or not detectable in local women (Turner *et al*., 2012). The chemotypes appear to differ in geographical distribution, with both DON and NIV chemotypes reported in several countries of Africa, Asia, and Europe (Miller *et al.* 1991; Jennings *et al.,* 2004) but only the DON chemotype was reported in North America (Mirocha *et al.* 1989). DON is more prevalent than NIV in some countries such as Italy and South Africa (Prodi *et al.,* 2009; Boutigny *et al.,* 2011). This clear difference was observed between *F. graminearum* populations in Iran, too. For example, Davari *et al.* (2013) evaluated the chemotypes of *F. graminearum s. str.* obtained from Ardabil (Northwest of Iran) and Golestan (North of Iran) provinces with a Luminex-Multilocus genotyping (MLGT) assay and showed that isolates differ significantly in their toxins as NIV was the prevalent toxin in Golestan province while DON in Ardabil province. Haratian *et al.* (2008) tested six isolates from Moghan for length polymorphisms of the *Tri13* gene by PCR indicating three DON and three NIV producers, while five isolates from Golestan were only of the NIV-type. The reason for the differences in the distribution of the two chemotypes is unknown and it is possible that differences in the distribution of alternative hosts, soil type, cultivar, cropping practice or temperature may all play a part (Jennings *et al.,* 2004; Toth *et al.,* 2005).

Also, the results of present study show that DON mycotoxin is produced in various contents by different isolates of *Fusarium* on various cultivars and in different regions and it can play a role in pathogenesis and contaminates wheat grains during FHB epidemic years. The mycotoxin producing ability in isolates showed a wide range (101.1-5828 μgkg^{-1}). No significant relation observed between toxigenic potential of *Fusarium* isolates and wheat cultivar or origin or year of isolation of fungi and it is similar to Alvarez *et al*. (2009) results. They did not observe significant relation between toxigenic profile of *F. graminearum s. str.* isolates and sub-regions from wheat in Argentina.

The amount of DON did not show significant differences by sub-regions or cultivars or years. Maximum content of DON belonged to *F. graminearum* isolated from cv. Izen green in Moghan Agro-industry company fields in 2004 with 5827.11 μgkg-1 and minimum amount (101.11 μgkg-1) was relevant to an isolate of this species recovered from cv. Atila 4 of this region in 2007 (Table 1).

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These assays, along with those developed elsewhere, are useful tools with which to increase our understanding of the factors that influence FHB and, ultimately, our ability to control this disease and eliminate the risk of mycotoxin contamination of grain and foodstuffs. The assays presented in this study provide information about the capacity to produce DON in different cultivars and regions that can assist epidemiological studies of toxin producing *Fusarium* isolates and are useful for defining contamination of wheat grains in the field. Also, the Ardabil and Golestan provinces in the Northwest and North of Iran have the highest rates of oesophageal cancer in Iran (Islami *et al.,* 2009; Sadjadi *et al.,* 2003) and already since long ago a link has been established between this type of cancer and mycotoxin consumption (Marasas *et al.,* 1979; Kamangar *et al.,* 2009). Therefore, Agricultural crop screening and mycotoxin producing ability assessments would be useful for health in these regions.

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تولید زهرابه قارچی داکسی‌نیوالنول توسط جدایه‌های مختلف کمپلکس گونه‌ای *Fusarium graminearum* sensu lato عامل سوختگی سنبله گندم در منطقه مغان

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چکیده: بیماری بلایت فوزاریومی سنبله (FHB) گندم از بیماری‌های بسیار مهم و مخرب غلات دانه‌ریز به‌شمار می‌رود و طی سال‌های اخیر در استان‌های شمالی و شمال‌غرب کشور روی محصول گندم به سطح اپیدمی رسیده است. به‌منظور بررسی تولید زهرابه قارچی داکسی‌نیوالنول (DON) در ۴۱ جدایه فوزاریوم که طی سال‌های ۱۳۸۳ تا ۱۳۸۶ از سنبله‌های آلوده گندم از نواحی مختلف منطقه مغان جداسازی شده بود، این پژوهش انجام گرفت. مطابق ویژگی‌های ریخت‌شناختی و با استفاده از کلیدهای معتبر، همه جدایه‌ها متعلق به کمپلکس گونه‌ای *F. graminearum* و *F. culmorum* تشخیص داده شد که گونه اول به‌عنوان گونه غالب بود. به‌منظور ارزیابی تولید زهرابه DON در جدایه‌های منتخب، این زهرابه با استفاده از روش‌های استاندارد استخراج شد. برای تعیین کیفیت DON استخراج شده از روش کروماتوگرافی لایه نازک (TLC) و برای تعیین مقدار آن در عصاره از کروماتوگرافی فاز مایع با کارآیی بالا (HPLC) استفاده گردید. نتایج آزمون TLC نشان داد که ۵۴/۵ درصد جدایه‌ها قادر به تولید DON هستند، اما رابطه معنی‌داری بین قدرت تولید زهرابه قارچی و رقم گندم یا سال جداسازی یا ناحیه پراکنش جدایه‌ها دیده نشد. نتایج حاصل از تجزیه HPLC نیز نشان داد که بیشترین مقدار DON مربوط به جدایه *F. graminearum* جداسازی شده از رقم ایزن‌گرین مزارع کشت و صنعت مغان در سال ۱۳۸۳ با مقدار ۵۸۲۷/۱۱ میکروگرم بر کیلوگرم می‌باشد. با توجه به نتایج این تحقیق می‌توان گفت که زهرابه قارچی DON در مقادیر مختلف توسط جدایه‌های *Fusarium* روی انواع ارقام گندم و در نواحی و سال‌های مختلف در منطقه مغان تولید می‌شود. با توجه به نقش این زهرابه در بیماریزایی و خطرات بهداشتی آلودگی بذور گندم به این زهرابه، نتایج حاضر لزوم توجه بیشتر به این بیماری در شمال‌غرب ایران را نشان می‌دهد.

واژگان کلیدی: تیپ شیمیایی، DON، *F. graminearum sensu lato*، سوختگی فوزاریومی سنبله، شمال غرب ایران

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