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# CLASS B TRICHOTHECENE CHEMOTYPING IN *Fusarium* SPECIES BY PCR ASSAY

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*Fusarium* isolates are divided into three chemotypes according to produce of class B trichothecenes; 3-acetyldeoxynivalenol (3ADON) 15-acetyldeoxynivalenol (15ADON) and nivalenol (NIV) and 4- acetyldeoxynivalenol (NIV) chemotypes. In this study, chemotyping of seventeen isolates from Turkey and Iran belonging to *F. graminearum*, *F. culmorum*, *F. poae* and *F. pseudograminearum* species were carried out by polymerase chain reaction (PCR) analysis. While all *F. culmorum* and *F. poae* isolates determined as 3ADON, remaining *F. graminearum* and *F. pseudograminearum* isolates were either 3ADON or 15ADON chemotypes. A common band of 583 bp long DNA fragment was amplified in all of *F. culmorum* and *F. poae*, one *F. pseudograminearum* (21F) and four *F. graminearum* (14F, sh14, sh15, sh7) isolates with 3ADON chemotype. However, remaining two *F. pseudograminearum* and four *F. graminearum* isolates with 15ADON chemotype, yielded amplicons that of 863 bp. It was shown that 3ADON was more predominant chemotype from other class B trichothecenes. This is the first report on chemotyping of *F. poae* and *F. pseudograminearum* isolates and also to show presence of 3ADON chemotype in *F. graminearum* isolate from Turkey.

*Key words:* Class B trichothecenes, *Fusarium graminearum*, *F. culmorum*, *F. poae*, *F. pseudograminearum*

## INTRODUCTION

*Fusarium* spp. have pathogenicity on many plant species as well as human and animal. Species infecting cereals cause plant diseases such as head blight of wheat and barley, ear rot of maize, stem rot of carnation, wilt of tobacco, stalk rot, common root rot and foot rot of all cereals

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(BAI and SHANER, 2004). All of the diseases tent to serious agricultural problems including reduction in yield quality and quantity (CHAMPAIL *et al*., 2004; FOROUD and EUDES, 2009). Causal agents of the disease are variable from one agro-ecological region to another. For example, seventeen species have been associated with the head blight. But main causal agent of the disease is *F. graminearum* in many countries (JI *et al*., 2007; HARATIAN *et al*., 2008; MIEDANER *et al*., 2008; SCHERM *et al*., 2013).

*Fusarium* species produce mycotoxins including class B trichothecenes. Three tichothecene metabolite profiles, known as chemotypes, have been detected in *Fusarium* species isolated from diseased crop plants. These profiles are 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON) and nivalenol (NIV) chemotypes (CHANDLER *et al*., 2003; KIMURA *et al*., 2007). Determination of them is carried out by polymerase chain reaction (PCR) based techniques besides conventional approaches. Currently PCR based ones would be preferred to use in chemotyping (JI *et al*., 2007; HARATIAN *et al*., 2008; YÖRÜK and ALBAYRAK, 2012; MERT-TÜRK and GENCER, 2013). Chemotyping via PCR assay is based on the amplification of genes located in *tri5* gene cluster. Eleven genes (*tri1, tri3, tri4, tri7, tri8, tri9, tri11, tri13, tri14, tri16* and *tri101*) are functional in metabolic pathway except *tri5* gene in this cluster. Apart from them, *tri6* and *tri10*, coding transcription factors, and *tri12*, coding a transporter protein, which they were flanking the cluster are also directly related to trichothecene production. Differences in definite genes located in the cluster have made chemotype identification easier. NIV chemotype of *Fusarium* isolates is observed by expression of *tri13* gene. However, three different deletions that of 178, 61 and 37 bp long are resulted in non-functional *tri13* gene. In these circumstances, isolates with non-functional gene are determined as DON chemotype. Insertions with 11 bp repeats found in *tri7* gene were used for differentiation of DON chemotype. Therefore, DON and/or NIV producers were discriminated by amplification of both *tri7* and *tri13* genes. Similarly, *tri3* gene amplification has been used for detection of 3ADON and 15ADON (CHANDLER *et al*., 2003; JENNINGS *et al*., 2004).

In spite of more than ten *Fusarium* species isolated from diseased cereals, *F. graminearum* was reported as the main causal agent in Turkey (TUNALI *et al*., 2006). Genetic diversity analysis in Turkish *Fusarium* isolates belonging to *F. graminearum* and *F. culmorum* have been studied by different research groups (ARICI and KOC, 2010; GUREL *et al*., 2010; YÖRÜK and ALBAYRAK, 2013). Moreover, chemotyping and chemotype distribution studies have been carried out by using HPLC analysis (TUNALI *et al*., 2006) and PCR assays (YÖRÜK and ALBAYRAK, 2012; MERT-TÜRK and GENCER, 2013). While 3ADON, 15ADON chemotypes and NIV chemotype were reported in *F. culmorum* isolates, 3ADON chemotype was not determined in *F. graminearum* isolates at all. At the same time, *F. pseudograminearum* and *F. poae* species were obtained from diseased crop plants. Since the number of their isolates was very limited, chemotyping of them have not been carried out until now. Similarly, chemotype distribution studies *Fusarium* isolates belonging to only one species, *F. graminearum*, from northern regions of Iran were carried out by researchers (HARATIAN *et al*., 2008; TIZAKI and SABBAGH, 2013). All of three cehemotpes-3ADON, 15ADON and NIV- were reported by them.

Changing environments such as farm practices and crop rotations and climatic conditions can affect spreading of fungal disease, population structure and mycotoxin profiles. Fungal pathogen populations predominating in definite agro-ecological conditions should be screened in terms of mycotoxin production in a certain period of time. Therefore, it was aimed to identify the chemotype profiles of totally seventeen isolates- eight of *F. graminearum*, four of *F.*

*culmorum*, three of *F. pseudograminearum* and two of *F. poae*- newly adding in our culture collection by using PCR assays, in this study.

## MATERIALS AND METHODS

Totally seventeen single spore *Fusarium* isolates including eight of *F. graminearum*, four of *F. culmorum*, three of *F. pseudograminearum* and two of *F. poae* were provided by Dr. Berna Tunali Department of Plant Protection, Agricultural Faculty, Samsun Ondokuz Mayis University and Dr. Bahram Sharifnabi, Department of Plant Protection, College of Agriculture, Isfahan University of Technology. The regions, hosts and codes of *Fusarium* isolates together with chemotypes are given in Table 1.

*Table 1. Fungal isolates used in this study*

| Code | Species | Host | Region | 3ADON/15ADON |
|---|---|---|---|---|
| 14F | F.G.* | Wheat | Sakarya | +/- |
| 15F | F.G. | Wheat | Kastamonu | -/+ |
| sh14 | F.G. | Wheat | Mazandaran | +/- |
| sh15 | F.G. | Wheat | Mazandaran | +/- |
| sh7 | F.G. | Wheat | Mazandaran | +/- |
| sh1 | F.G. | Wheat | Mazandaran | -/+ |
| F49 | F.G. | Wheat | Moghon | -/+ |
| sh13 | F.G. | Wheat | Moghon | -/+ |
| 17F | F.C. | Wheat | Ankara | +/- |
| 18F | F.C. | Wheat | Eskisehir | +/- |
| 19F | F.C. | Wheat | Eskisehir | +/- |
| 20F | F.C.* | Barley | Afyon | +/- |
| 21F | F.PS.* | Wheat | Corum | +/- |
| 22F | F.PS. | Wheat | Samsun | -/+ |
| 23F | F.PS. | Wheat | Cankiri | -/+ |
| 24F | F.P.* | Wheat | Cankiri | +/- |
| 25F | F.P. | Barley | Zonguldak | +/- |

F.G.* means *F. graminearum*, F.C.* means *F. culmorum*, F.PS.* means *F. pseudograminearum* and F.P.* means *F. poae*

Isolates were grown on potato dextrose agar (PDA) plates at 25ºC for six days. Genomic DNA was extracted from six-day-old mycelium by using a genomic DNA isolation kit (Macherey–Nagel, USA). The procedure was based on established CTAB protocol and all steps in nucleic acid extraction were maintained according to the manufacturer's recommendations.

Four different species-specific SCAR (sequence characterized amplified region) primer pairs (UBC85F/R, OPT18F/R, FPG-F/R and FP82F/R), producing monomorphic DNA bands, were used in the species verification of *F. graminearum*, *F. culmorum*, *F. pseudograminearum* and *F. poae* isolates, respectively (SCHILLING *et al*., 1996; NICHOLSON *et al*., 2004; [Table 2]). PCRs were conducted in a total volume of 25 µl containing; 50 ng genomic DNA, 1.5 mM $MgCl_2$, 1X PCR buffer, 10 pmol of each primers, 0.1 mM each of dNTPs and 1 U *Taq* DNA

polymerase (Promega, USA). PCR conditions were performed at 94ºC for 5 min for predenaturation, 35 cycles at 94 ºC for 1 min, 50-61ºC for 1 min, 72ºC for 1 min for amplification and at 72ºC for 5 min for final extention.

Discrimination of DON and NIV chemotypes was carried out by amplification of *tri13* gene by using two different primer sets, Tri13F/Tri13DONR and Tri13NIVF/Tri13R, respectively (Table 2). All PCRs were done in 25µl reaction volumes containing 25 ng of template DNA, 10 pmol of each primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1xPCR buffer and 1U *Taq* DNA polymerase (Promega, USA). PCR programme had 35 cycles of 94ºC for 30 s, 58ºC for 45 s and 72ºC for 45 s. First cycle and final extension steps were as described above. Further chemotype discrimination of *Fusarium* isolates was maintained by amplification of *tri3* gene. Tri303F/Tri303R and Tri315F/Tri315R primer pairs were used for verification of 3ADON and 15ADON chemotypes (Table 2). PCR components and thermal cycle conditions for amplification of *tri3* gene were the same as described for *tri13* gene. All PCR bands were separated by electrophoresis in 1.5% gels and they were visualised under UV light (Avegene, X-lite 200, Thailand). Moreover relative amplicons sizes were evaluated by image station system (Kodak 4000MM, England).

*Table 2. Species- and chemotype- specific primers and their sequences used for PCR analysis in this study*

| Primer set | Gene description | Size in bp | Sequences (5'-3') | Reference |
|---|---|---|---|---|
| UBC85F/R | SCAR85 marker | 332 (FG[b]) | F: GCAGGGTTTGAATCCGAGAC<br>R: AGAATGGAGCTACCAACGGC | Schilling et al., 1996 |
| OPT18F/R | SCAR2-35 marker | 472 (FC[c]) | F: GATGCCAGACCAAGACGAAG<br>R: GATGCCAGACGCACTAAGAT | Schilling et al., 1996 |
| FPG-F/R | SCAR[a] marker | 779 (FPG[d]) | F: GTCGCCGTCACTATC<br>R: CACTTTTATCTCTGGTTGCAG | Nicholson et al., 2004 |
| FP82F/R | SCAR[a] marker | 220 (FP[e]) | F: CAAGCAAACAGGCTCTTCACC<br>R: TGTTCCACCTCAGTGACAGGTT | Nicholson et al., 2004 |
| Tri13f/R | *Tri13* | 282 (DON[f]) | F: CATCATGAGACTTGTKCRAGTTTGGG<br>R: GCTAGATCGATTGTTGCATTGAG | Chandler et al., 2003 |
| Tri303F/R | *Tri3* | 583 (3ADON[g]) | F: GATGGCCGCAAGTGGA<br>R: GCCGGACTGCCCTATTG | Jennings et al., 2004 |
| Tri315F/R | *Tri3* | 863 (15ADON[h]) | F: CTCGCTGAAGTTGGACGTAA<br>R: GTCTATGCTCTCAACGGACAAC | Jennings et al., 2004 |

SCAR[a] means "Sequence characterized amplified region", FG[b] means *F. graminearum*, FC[c] means *F. culmorum*, FPG[d] means *F. pseudograminearum*, FP[e] means *F. poae*, DON[f] means deoxynivalenol, 3ADON[g] means 3-acetyldeoxynivalenol, 15ADON[h] means 15- acetyldeoxynivalenol

## RESULTS

*Identification of* Fusarium *species*

All isolates belonging to four *Fusarium* species were identified at molecular level on the basis of SCAR amplification. UBC85F/R primer set produced a common DNA band of 332 bp in eight isolates. Consequently, PCR analysis revealed that these eight isolates were belonged to *F. graminearum* species. OPT18F/R primers yielded 472 bp long amplification products in four isolates. As a result, these isolates were verified as *F. culmorum*. Similarly, FPGF/R and FP82F/R primer pairs producing DNA fragments that of 779 bp and 220 bp long provided the confirmation of three isolates belonged to *F. pseudograminearum* and also of two belonged to *F. poae*, respectively. All amplification products are seen in Fig. 1A.

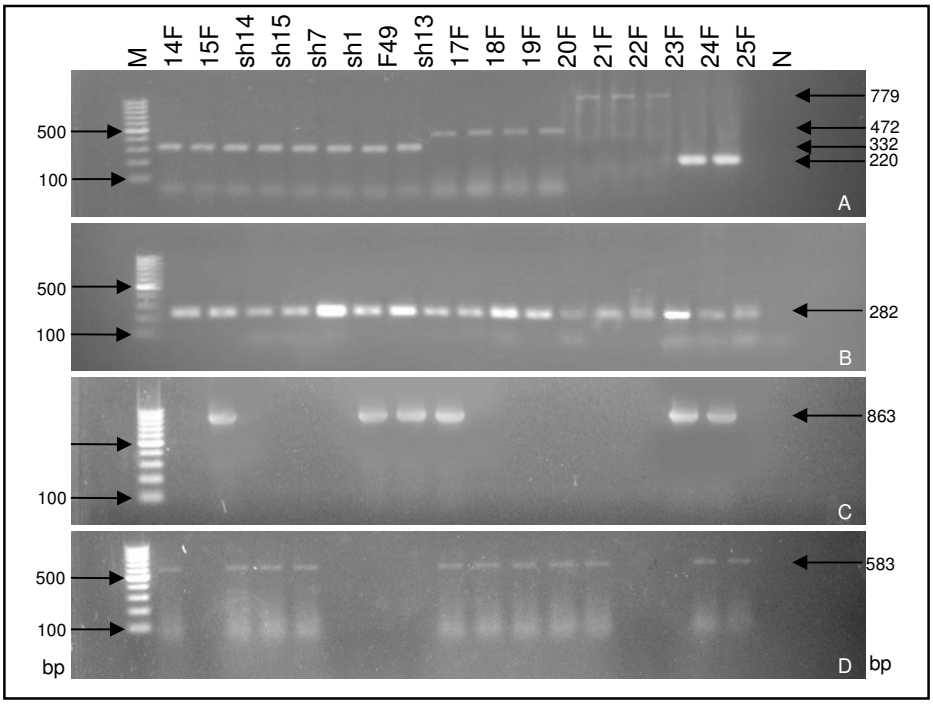


Fig. 1 (A) PCR bands of 779 bp by using primer pair FPG-F/R, 472 bp amplified with OPT18F/R primers, 332 bp by UBC85F/R primer set and 220 bp amplified by using FP82F/R which are specific to genomes of *F. pseudograminearum*, *F. culmorum*, *F. graminearum* and *F. poae*, respectively. **(B)** Common amplicon in size of 282 bp fragment of *tri13* gene amplified from genomes of four species belonging to DON chemotype by Tri13F/Tri13DONR primers. **(C)** 863 bp PCR product obtained with Tri315F/R primers in four *F. graminearum* and 2 *F. pseudograminearum* isolates' genomes. **(D)** 583 bp fragment of *tri3* gene amplified with Tri303F/R primers in genomes of 4 *F. graminearum*, 4 *F. culmorum*, 1 *F. pseudograminearum* and 2 *F. poae* isolates. M: 100 bp DNA marker (Thermo, M0241), N: Negative control, 14F-sh13: *F.graminearum*, 17F-20F: *F. culmorum*, 21F-23F: *F. pseudograminearum* and 24F-25F: *F. poae* isolates.

*Chemotyping of* Fusarium *species*

Generic PCR assay carried out with the Tri13F/Tri13DONR primers revealed 282 bp long DNA band representing DON chemotype in all isolates (Fig. 1B, Table 1). Trichothecene metabolite profiles of *Fusarium* isolates via PCR showed that all isolates produced DON mycotoxin. None of isolates produced any amplification product with Tri13NIVF/Tri13R primer set (data not shown). This finding indicated that these isolates were not of NIV chemotype. Both 3ADON and also 15ADON chemotypes were discriminated according to the *tri3* gene amplification (Table 1). While 11 isolates, produced 583 bp bands, were identified as 3ADON chemotype, remaining isolates, yielded 863 bp amplicon, were belonged to 15ADON chemotype (Fig. 1C and 1D). Hence, *Fusarium* species produced purely DON. Only 3ADON chemotype was determined in *F. culmorum* and *F. poae* isolates, whereas *F. graminearum* and *F. pseudograminearum* isolates showed either 3ADON or 15ADON chemotypes.

## DISCUSSION

Incidence of fungal disease depends on not only species of fungi but also toxigenic effects of mycotoxins produced by pathogen. Identification of *Fusarium* isolates at species level is very important in diagnosis. Conventional techniques used in species-specific identification could lead to false positive results. CARTER *et al*. (2000) excluded two isolates from their studies because of this reason. They showed that two isolates initially identified as *F. graminearum* by conventional approaches belonged to *F. napiforme* and *F. fujikuroi* with rDNA analysis. MARASAS *et al*. (1985) also reported that approximately one half of toxigenic *Fusarium* strains were incorrectly named in the literature. Therefore, reliable and accurate diagnosis of isolates has a big importance in terms of reduction false positive results in genetic characterization studies. Nowadays, SCAR markers are effectively used in species-specific identification of isolates which were already identified according to morphological characteristics. In this study, four different SCAR markers (UBC85, SCAR 2-35 and two different SCAR[a]) were amplified by PCR technology for species-specific diagnosis. After species identification of all isolates, chemotyping of them was carried out also by PCR based methods.

Chemotyping is an approach which is used for determination of mycotoxins. It is crucial in agriculture since mycotoxins produced by causal agents of head blight display destructive effects on cereals. These toxic molecules are also harmful on both humans and animals, because they inhibit protein synthesis (BAI and SHANER, 2004; FOROUD and EUDES, 2009). In addition, they cause serious disease and they have lethal effects (BAI and SHANER, 2004). Therefore, chemotyping of fungal isolates is very important strategy in terms of protection of health. PCR based methods are effectively used in chemotyping as well as traditional ones. But, traditional techniques are time consuming and inconclusive. Also, they need a large amount of endotoxin content (TUNALI *et al*., 2006; HARATIAN *et al*., 2008). As a result, molecular techniques and usage concomitantly of traditional and molecular techniques provide acquisition of accurate and reliable results (CHANDLER *et al*., 2003; JENNINGS *et al*., 2004). Most of PCR based strategies for differentiation of the mycotoxins produced by different *Fusarium* species were based upon amplification of certain genes located in *tri5* gene cluster. The *tri5* gene cluster consists of twelve genes, as it is well known. Among them, since *tri7*, *tri3* and *tri13* genes had clear differences in sequence data, they were directly targeted for amplification by PCR assay in differentiation of chemotypes produced by species and isolates. While NIV producers possess the functional copy of the *tri7* gene encoding 3-acetyltrichothecene 4-O-acetyltransferase, DON

producers have non-functional copy of *tri7* with a repeated insertion of 11 bp in intron regions of the gene. Also, *tri13* gene encoding P450 monooxygenase is necessary for NIV production. Similarly, DON-producing isolates carry non-functional *tri13* copies with three deletions. The *tri3* gene is responsible for coding C-15 acetylase. Expression of *tri3* is carried out in all chemotypes; 3ADON, 15ADON and NIV (KIMURA *et al*., 2007; ALEXANDER *et al*., 2011). Whereas *tri3* is possessed full-length in 15ADON and NIV producers, 3ADON-producing isolates include *tri3* with a deletion. Therefore, we used *tri3* and *tri13* gene sequence information in order to classify of *Fusarium* species into NIV, 3ADON and 15ADON chemotypes.

In this study, according to amplification of *tri13* gene, we obtained that *Fusarium* isolates originated from Turkey and Iran were only DON chemotype. In addition, we showed that isolates in DON chemotype were either 3ADON or 15ADON producers. DON was predominant chemotype in previously published similar studies (CHANDLER *et al*., 2003; JENNINGS *et al*., 2004; JI *et al*., 2007; YÖRÜK and ALBAYRAK, 2012; SCHERM *et al*., 2013). All of *F. culmorum* isolates were of 3ADON chemotype. As our knowledge this is the first report showing 3ADON chemotype of *F. graminearum* isolates from Turkey. Moreover, there was no report in literature about class B trichothecenes production in *F. poae* and *F. pseudograminearum* isolates from Turkey. Therefore, this is the also first report about chemotyping of both of two species. These findings provide updated information about distributions of class B trichothecenes. Similar studies including especially low number of fungal isolates could be informative about chemotype distribution in both local and global areas. Therefore, chemotyping of newly sampling isolates is very important. Results obtained from this study could be indirectly useful in pathogen characterization, controlling disease development and mycotoxin contamination.

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# HEMOTIPING KLASE B TRIHOTECENE U VRSTAMA FUZARIUMA PRIMENOM PCR METODA

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Izvod

Izolati *Fusarium-a* su podeljeni u tri hemotipa prema proizvodnji klase B trichotecene. U ovim istraživanjima vršen je hemotiping sedamnaest isolata iz Turske i Irana, koji pripadaju *F. graminearum*, *F. culmorum*, *F. poae* and *F. pseudograminearum* vrstama primenom metode lančane reakcije polimeraze ( PCR). Dok su svi isolati *F. culmorum F. poae* determinisani kao 3ADON, preostali *F. graminearum* and *F. pseudograminearum* izolati su bili ili 3ADON ili 15ADON hemotipovi. Zajednički fragment DNK dužine 583 bp je umnožen u svim *F. culmorum* i *F. poae*, jedan *F. pseudograminearum* (21F) i 4 *F. graminearum* (14F, sh14, sh15, sh7) izolata sa 3ADON hemotipa. Međutim, preostala dva *F. pseudograminearum* i četiri *F. graminearum* izolata sa 15ADON hemotipom, dali su amplicone dužine 863 bp. Pokazano je da je 3ADON bio više predominantan hemotip u odnosu na druge klase B trichothecenes. Ovo su prvi rezultati i saopštenje o hemotipingu *F. poae i F. pseudograminearum* izolata kao i rezultati koji pokazuju prisustvo 3ADON hemotipa u *F. graminearum* isolatima iz Turske.

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