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Fusarium species isolated from wheat samples showing root and crown rot symptoms in Southeast Anatolia

Güneydoğu Anadolu Bölgesi'nde kök ve kök boğazı çürüklüğü belirtileri gösteren buğday örneklerinden izole edilen *Fusarium* türleri

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ABSTRACT

Wheat is one of the most largely grown cereals in Turkey and it is very important for meet calorie requirements of humans and animals. However, due to numerous fungal diseases suffered, the wheat supply is threatened. *Fusarium* spp., known as the big portion of plant parasitic fungi, causes some quality and quantity problems on wheat production. This large genus has over one hundred sub-species, therefore, it is so difficult to diagnose in species level. Using certain molecular and microscopic approaches, this study aimed at determining the *Fusarium* spp., causing root and crown rot on wheat in Southeast Anatolia. Molecularly, using polymerase chain reaction (PCR) experiment with some species-specific primers and sequencing of some part of ribosomal RNA region after amplification by PCR were used to differentiate the species. Additionally, colony and different spore characteristics of cultures were used to diagnose some *Fusarium* species microscopically. At the end of the study, 143 *Fusarium* isolates including 19 species were obtained from wheat producing areas in Diyarbakır, Şanlıurfa, Mardin, and Adıyaman provinces. Although the most frequently isolated species was *F. proliferatum* at the rate of 17.4%, *F. pseudograminearum* and *F. culmorum*, which were the most important crown rot pathogens, were isolated at 13% of the total isolated *Fusarium* isolates.

INTRODUCTION

Wheat is one of the most important foods in diary diet not only in the world but also in Turkey. It is produced on one third of cultivable fields of Turkey and about 19 million tons in 2014 (TUİK 2015). Southeast Anatolia is the first place in which wheat was cultivated (Heun et al. 1997, Özkan et al. 2002). In this area, wheat is produced at 1 M ha and this amount is equivalent to one eighth of Turkey's total wheat production.

Monoculture is very common for some reasons like farmers' behaviors and land characteristics in wheat producing areas of Turkey. This monoculture system, in which the same crops

grow repeatedly, caused an increase in inoculum density and gradual yield losses. The motive behind these yield losses might be the accumulation of both biotic stress factors like fungi and abiotic stress factors like salinity.

Fusarium genus is one of the biggest groups of plant pathogenic fungi and some species with this large genus might cause some plant protection problems at different stages of wheat growth. *F. culmorum* and *F. graminearum* may cause seedling rot at the earlier stage, and root & crown rot and head blight at the following stages of wheat (Mitter et al. 2006). Root and crown rot diseases of wheat may decrease the yield due to the decrease in plant number per unit, growth failure and grain filling abnormalities (Goswami and Kistler 2004, McMullen et al. 1997). *Fusarium* infections at flowering stage produce weak grains and quality problems due to the emerging mycotoxins that are toxic for humans and animals (D'mello et al. 1999, Klein et al. 1991, Peraica et al. 1999).

Fusarium genus has over one hundred species and their microscopic diagnosis is too difficult and needs high level of experience. Morphological discrimination of some species is difficult either; additionally, it was not possible for some close species like *F. graminearum* and *F. pseudograminearum* until molecular techniques brought a solution for this problem (Aoki and O'Donnell 1999). Because molecular techniques are repeatable, accurate, fast, and easy, they have been commonly used for molecular diagnosis in mycology recently.

Southeast Anatolia hosts a huge irrigation project, by which millions of acres' field will be suitable for irrigation in the near future. Therefore, it is expected that monoculture wheat production will be ended and new crops will be introduced to the cropping system. Determination of current *Fusarium* flora in this region will affect the selection of new crops suitable for new rotation system. This study aimed to find out the *Fusarium* species using molecular techniques in wheat producing areas of Southeast Anatolia where wheat was firstly cultivated in the world.

MATERIALS AND METHODS

Sample collection and obtaining single spore isolates of Fusarium spp.

Surveys were conducted in 2009-2010 years at Diyarbakır, Şanlıurfa, Mardin, and Adıyaman provinces, which are the biggest wheat production areas of Southeast Anatolia within Zadoks scale 30-58 (Zadoks et al. 1974). Each field was selected considering having 3-5 km distance from each other. Totally 408 samples were collected during the survey in Southeast Anatolia (Figure 1). Five samples showing root and crown rot symptoms from each field were brought to laboratory within paper bags and kept at +4 °C until the execution of surface sterilization and isolation. For surface sterilization, each sample was chopped into 1 cm-length particles and dropped in 1% sodium hypochlorite dilution for three minutes, then they were rinsed with sterile distilled water for 3 times. Surface sterilized samples were kept between sterile drying papers at -20 °C until isolation. Agar plate technique with PPA (Peptone PCNB Agar) (Nash and Snyder 1962) growth medium was used to obtain *Fusarium* isolates from samples. Fusarium-like colonies were transferred to SNA (synthetic nutrient agar) medium and incubated at 24±1 °C for 1 week under black light 15/9 h day/night regime for sporulation. Using water agar and microscope, single spore isolation was made. After isolation activities, more than 140 Fusarium-like isolates were obtained from the collected samples showing crown and root rot symptoms.

Figure 1. Research area of the study (four provinces) in Southeast Anatolia

Microscopic and molecular diagnosis of Fusarium spp.

Microscopic diagnosis of some isolates was made using their colony morphology on PDA (potato dextrose agar), macroconidia, microconidia and chlamydospore characteristics (Leslie and Summerell 2006). The rest of unidentified isolates were subjected to molecular diagnosis using species-specific PCR and sequence analysis. To obtain mycelia to be used in DNA isolation, cultures were grown on PDA for 7 days at 24 °C, and mycelia was harvested using sterile scalpel. Mycelia was lyophilized using freeze dryer (Roche) for 12 h within 2 mL sterile tubes and grinded using micro pestle just before starting the DNA isolation. DNA extraction was conducted as described at Motteram et al. (2009), and stock DNA was prepared as 40 ng/µl and kept at -20 °C until PCR amplification. Species-specific PCR experiments were conducted using primer sets (Table 1). PCR reaction mixture was prepared as 25 µl final volume containing 40 ng of fungal genomic DNA, 10 µM each primer,

Table 1. Information about primers used for genus and species-specific diagnosis of *Fusarium* spp. by PCR

Fusarium sp.	Primer Pairs	Primer Sequence	Band Size	References
Fusarium spp.	Fps-F	5- CGCACGTATAGATGGACAAG-3		Jurado et al. (2006)
	Fus-R	5- GGCGAAGGACGGCTTAC- 3		
F. proliferatum	PRO2	5 - TGTCAGTAACTCGACGTTGTTG -3	≈585bp	Jurado et al. (2006)
	PRO1	5 - CTTTCCGCCAAGTTTCTTC -3		
F. verticilloides	VER2	5 - AATTGGCCATTGGTATTATATATCTA - 3	≈578bp	Mulè et al. (2004)
	VER1	5 - CTTCCTGCGATGTTTCTCC - 3		
F. oxysporum	PFO2	5 - CCCAGGGTATTACACGGT -3	≈70bp	Edel et al. (2000)
	PFO3	5 - CGGGGGATAAATGCGG - 3		
F. culmorum	C51 R	5 - CCCTTCTTACGCCAATCTCG - 3	≈570bp	Nicholson et al. (1998)
	C51 F	5 - ATGGTGAACTCGTCGTGGC - 3		
F. equiseti	FE-F	5- CATACCTATACGTTGCCTCG - 3	≈400bp	Mishra et al. (2004)
	FE-R	5- TTACCAGTAACGAGGTGTATG - 3		
F. semitectum	SmibolFM	5 - GCAAAAGCCTCTCGCCAC - 3	≈424bp	Hong et al. (2010)
	Semi1RM	5 - AGGTGTAGAGATATCGCGG - 3		
F. acuminatum	FAC-R	5 - GGGATATCGGCAAGATCG - 3	≈602bp	Williams et al. (2002)
	FAC-F	5 - GGGATATCGGGCCTCA - 3		
ITS	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	≈570bp	White et al. (1990)
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′		

15 mM $MgCl_2$ and 5 mM dNTPs, 0.125 units Taq DNA Polymerase (Thermo, USA). PCR cycling conditions were fixed for the first denaturation to 85 s at 94 °C; denaturation for 35 s at 95 °C; annealing 30 s at 67 °C; extension 60 s at 72 °C for 25 cycle, and final extension for 5 min at 72 °C. PCR products were loaded on 2.5% agarose gels containing ethidium bromide with a concentration of 0.1 µg/ml and electrophoresed in 0.5× Tris-borate-EDTA (TBE) for 1.5 h at 115 V. Gels were photographed under UV light (Quantum ST4, Montreal Biotech). The sizes of the fragments in a gel were compared with GeneRuler 100 bp DNA ladder Plus (MBI).

For undiagnosed isolates with species-specific primer sets, ITS regions were amplified with ITS1 and ITS4 primers and sequenced. Sequencing data obtained from ITS region was analyzed using Geneious genetic analysis tool (Biomatters Ltd, New Zealand). Data was used to make BLAST (Basic Local Alignment Search Tool) analysis and species were determined according to BLAST results.

RESULTS

Fusarium spp. collection obtained from samples showing crown and root rot symptoms were subjected to genus specific PCR experiments and totally 143 isolates were determined as *Fusarium* spp. (Figure 2). Then, all isolates were used to determine their species using some species-specific primer sets (Figure 3). ITS amplification and sequencing studies were made for unidentified isolates. The rest of isolates not yielding any reliable results with molecular techniques were microscopically diagnosed by Prof. Dr. Berna TUNALI.

At the end of study conducted through classical and molecular techniques, 130 of 143 isolates were diagnosed at species level. Results demonstrated that collection included 19 different *Fusarium* species. 11 of these 19 species were identified by molecular techniques. While *F. culmorum*, *F. pseudograminearum*, *F. proliferatum*, *F. oxysporum*, *F. solani*, *F. semitectum*, *F. equiseti*, *F. acuminatum*, *F. verticillioides*, *F. chlamydosporum* and *F. tricinctum* were diagnosed molecularly, *F. crookwellence*, *M. nivale*, *F. subglutinans*, *F. avenaceum*, *F. redolens*, *F. inflexum*, *F. concolor*, *F. heterosporoides*, and *F. dimerum* were diagnosed microscopically (Table 2).

Table 2. Species distribution of *Fusarium* spp. examined in Southeast Anatolia

Provinces	Diyarbakır		Şanlıurfa		Mardin		Adıyaman		Isolate Number	%
Species / Diagnosis Method	Mol.*	Mic.**	Mol.	Mic.	Mol.	Mic.	Mol.	Mic.		
F. culmorum	2		1				2	1	6	4.1
F. pseudograminearum	4		6		2		1		13	9.0
F. crookwellence			1	1					2	1.3
F. tricinctum	4		9		4		4		21	14.6
F. equiseti	1		1						2	1.3
F. semitectum	4		6				4		14	9.7
F. proliferatum	4		10		4		7		25	17.4
M. nivale		4						3	7	4.8
F. acuminatum		3		2		1		2	8	5.5
F. subglutinans		1							1	0.6
F. oxysporum	3		2		3		5		13	9.0
F. verticilloides	1				1				1	0.6
F. chlamydosporum			1						1	0.6
F. avenaceum	2		2				1		5	3.4
F. redolens				2	1			1	3	2.0
F. inflexum				1					1	0.6
F. concolor				1					1	0.6
F. heterosporoides		3							3	2.0
F. dimerum				1		2			3	2.0
Fusarium spp.	3		5		1		4		13	9.0
Total									143	≈100

*Molecularly; **Microscopically

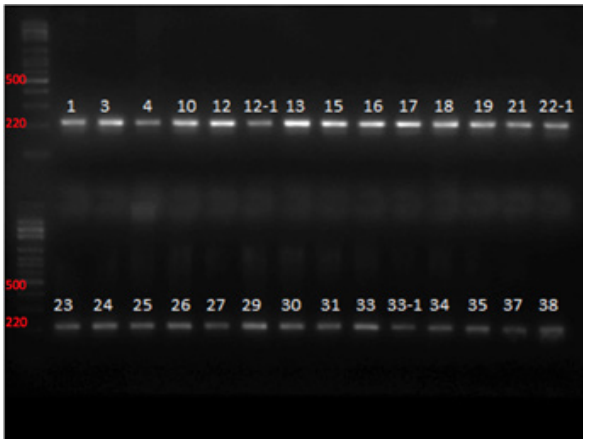

Figure 2. Isolates determined as *Fusarium* spp. after genus specific PCR experiments

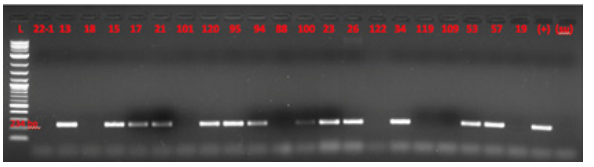

Figure 3. PCR detection of *Fusarium proliferatum*

DISCUSSION

Our results have been consistent with the species found at earlier studies (Aktaş et al. 2000, Arıcı et al. 2013, Aydan et al. 2009, Aydan et al. 2010, Kınacı 1984, Nirenberg 1981, Özer and Soran 1991, Soran and Damgaci 1980, Tunali et al. 2006a, Tunali et al. 2006b, Uçkun and Yıldız 2004, Yılmazdemir 1976) conducted on wheat microflora in Turkey. Within these species, *F. tricinctum* was determined by Uçkun and Yıldız (2004) in wheat growing fields of Aegean Region, but it was determined molecularly at the rate of 13% of total numbers of identified species and firstly reported in this study in Southeast Anatolia. *F. tricinctum* was shown in the weak virulence species of *Fusarium* (Uçkun and Yıldız 2004). This species is known as a part of *F. avenaceum* / *F. acuminatum* / *F. tricinctum* species complex and it is difficult to discriminate. In our study, rate of this complex is about 24%. Although they are not important as root rot pathogens in Europe, they are encountered as serious species causing head blight due to anniatin and moniliformin mycotoxin

produced by these species complex (Kulik 2008). This complex may be important concerning the rising humidity based on increasing irrigable fields with Southeast Anatolian Project.

In this study, *F. acuminatum* isolates were able to be diagnosed through PCR, while *F. avenaceum* and *F. tricinctum* samples were distinguished by sequence analysis only. Similarly, Harrow et al. (2010), could not diagnose these species using PCR based techniques targeting mitochondrial SSU region; but using β-tubulin (βTUB) and translation elongation factor 1α (EF1α) regions could distinguish these three species. Also, Kulik (2008), found out that it was not possible to diagnose using IGS region of rDNA of *F. acuminatum*, *F. nuragi* and *F. tricinctum*.

It was seen that 13% of species determined in this study were *F. pseudograminearum* or *F. culmorum* although in a previous study conducted by Tunali et al. (2008) in Southeast Anatolia demonstrated that this rate reached up to 23%. It is thought that this rational decrease might arise from sample size differences between two studies. However, the number of samples evaluated in this study was higher than that of the above-mentioned study. While the most important species was *F. culmorum* in Tunalı's study, *F. pseudograminearum* was the most important one in current study. This species difference between two studies might be arising from the differences in temperature and humidity due to the increasing irrigable fields in the last two decades in Southeast Anatolia. Likewise, some studies demonstrated different climatic requirements for *F. culmorum* and *F. pseudograminearum* (Poole et al. 2013). According to this study conducted in the USA, *F. pseudograminearum* was observed at low altitudes, under low moisture levels, and warmer conditions, while *F. culmorum* was seen in places with moderate and high moisture, high altitude and under colder conditions. In the same study, it was mentioned that dispersion of species might vary according to years, and incidence of these species could vary across the years as a response to short term climatic changes.

One of the important species determined in Diyarbakır and Adıyaman was *Microdochium nivale* known as "snow mold" and observed in cold seasons on the winter wheat (Cockerell 1997, Parry et al. 1995, Smiley and Patterson 1996). Finding *M. nivale* in these provinces coincided with the known climatic requirements of these species. Although it has been known that this species was sometimes problematic and was firstly recorded in East Anatolia in Turkey (Demirci and Dane 2003), and isolation rate could reach up to 15%; in current study, it was isolated firstly in Southeast Anatolia and its isolation rate was found as 5%.

F. proliferatum, *F. acuminatum*, *F. verticillioides*, *F. oxysporum*, and *F. equiseti* isolated during the survey have been known as natural members of wheat microflora and they show weak pathogenicity on wheat (Aktas et al. 1996, Aktas et al. 1997, Bentley et al. 2006, Demirci and Dane 2003, Tunali et al. 2006a). It has been thought that the other *Fusarium* species isolated from Southeast Anatolia are natural members of soil microflora. Increasing amounts of irrigable fields through the Southeast Anatolian Project may affect the *Fusarium* flora in the wheat production areas and may change the crop pattern grown in the region. Introducing new crops together with irrigation may raise the new soil pathogens particular to these new crops and cause a decrease in the importance of wheat *Fusarium* microflora causing problems on the wheat production in Southeast Anatolia.

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ÖZET

Türkiye'de insan ve hayvan beslenmesinde kalori ihtiyacının çok önemli bir bölümünü karşılayan buğday ülkemizde en geniş alanda yetiştirilen tahıldır. Buğday arzını tehdit eden birçok fungal hastalık olmasına rağmen, *Fusarium* türleri buğdayda kalite ve kantite problemlerine neden olan önemli bir patojenik gruptur. *Fusarium* cinsine ait 100'den fazla tür vardır ve bu yüzden tür düzeyinde ayrımları kolay değildir. Bu çalışma Güneydoğu Anadolu Bölgesi'nde buğdayda kök ve kök boğazı çürüklüğüne neden olan *Fusarium* türlerinin belirlenmesini amaçlamıştır. Tür düzeyinde moleküler teşhisler için bazı tür-spesifik primerler ve bazı ribozomal RNA bölgelerinin dizilenmesi ile gerçekleştirilmiştir. Bunlara ek olarak bazı türlerin teşhisleri, koloni ve spor karakterleri kullanılarak, mikroskobik olarak yapılmıştır. Çalışma sonunda Diyarbakır, Şanlıurfa, Mardin ve Adıyaman illeri buğday üretim alanlarında 19 farklı türe ait 143 *Fusarium* türü belirlenmiştir. En yaygın tür %17.4 ile *F. proliferatum* olmasına rağmen, en önemli buğday kök boğazı çürüklüğü patojenlerinden olan *F. pseudograminearum* ve *F. culmorum* izole edilen *Fusarium* türlerinin %13'ünü oluşturmuştur.

Anahtar kelimeler: buğday, kök ve kök boğazı çürüklüğü, *Fusarium*, PCR

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