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RESEARCH PAPER

Identification of Races 1, 2, 4 and 8 of *Fusarium oxysporum* f. sp. *dianthi* in Turkey by Using Molecular Markers

İlknur POLAT [1] Aytül YILDIRIM[2] İlker KURBETLİ[3] Ayşe Serpil KAYA[3] Uğur KAHRAMAN[3] Emine GÜMRÜKCÜ[3] Köksal AYDİNŞAKİR[3]

[1] Elmalı Vocational School, Akdeniz University, 07700, Antalya, Turkey
[2] Pendik Veterinary Control Institute, 34890, İstanbul, Turkey
[3] Batı Akdeniz Agricultural Research Instiute, 07100, Antalya, Turkey

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Corresponding Author
E-mail: ilknurpolat@akdeniz.edu.tr

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Abstract

Fusarium oxysporum f.sp. *dianthi* (Prill and Delacr.) (*Fod*) is a causal agent of wilt disease of carnation (*Dianthus caryophyflus* L.) with its different physiological races. Although eleven physiological races of *Fod* have been reported in the world, a polymerase chain reaction (PCR)-based diagnostic tool was developed for identification of *Fod* races 1, 2, 4, and 8, which are the most commonly found in many countries. On the other hand, there is no information about which races are found in Turkey. A total of 91 isolates were collected from plants with Fusarium-like symptoms in the most grown carnation areas in Antalya, İzmir, Isparta and Yalova provinces and all isolates were identified using molecular methods. As a conclusion, the results showed that *Fod* isolates collected from the most carnation grown provinces of Turkey consist mostly of race 1, 2 and 8. This is the first study reporting *Fod* races causing wilt disease on carnation plants in Turkey.

1. Introduction

Carnation (*Dianthus caryophyllus* L.) is one of the highly valued plant species among the cut flowers in Turkey and worldwide. The biggest carnation exporters in the world are Colombia (52.78%), the Netherlands (23.04%), and Turkey (5.75%), respectively (Kazaz et al., 2020). Export values of all three countries between 2015-2019 have increased by years. Colombia alone carries out more than half of the world's carnation exports. According to the data of 2018, carnation is the most produced cut flower in Turkey with an area of 494 ha. According to the data of the same year, 607 070 350 branches of carnations were produced in Turkey. The provinces where the most carnation is produced in Turkey are Antalya (54.11%), İzmir (31.83%), and Isparta (13.07%) (Kazaz et al., 2020).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (Prill and Delacr.) with its different physiological races, is responsible for the most devastating disease on carnation cultivation worldwide (Vergara et al., 2007; Poli et al., 2013; Castano et al., 2014; Deng, 2018). Moreover, it is widely seen and constitutes economic damage in Turkey (Tezcan et al., 2004; Coskuntuna and Yıldız, 2006; Arıcı and Kazaz, 2013; Arıcı et al., 2018; Kazaz et al., 2020; Atakan and Özgönen Özkaya, 2020). Breeders in the world have been developing carnation cultivars resistance to *Fod* because it is the most destructive pathogen on carnation (Scovel et al., 2001). On the other hand, the genetic basis for race resistance and pathogen virulence have been studied (Chiocchetti et al., 1999; Sarrocco et al., 2007; Werner and Irzykowska, 2007; Poli et al., 2013; Castano et al., 2014). Until today, eleven races of *Fod* have been identified in different countries (Chiocchetti et al., 1999). However, race 3 of *Fod* was recently reclassified as *F. redolens* f. sp. *dianthi* race 3 (Baayen et al., 1997; Bogale et al., 2007). Besides, race 2 is commonly found in all

carnation cultivation areas in the world (Baayen et al., 1997; Bogale et al., 2007; Castano et al., 2014). Furthermore, *Fod* races 1 and 8 were found on carnation cultivation areas in Italy, France, and Spain (Garibaldi et al., 1986; Baayen et al., 1997), while race 4 in the United States, Israel, Italy, Colombia and Spain (Ben-Yephet et al., 1992; Baayen et al., 1997; Sarrocco et al., 2007), races 5, 6, and 7 in Great Britain, France, and the Netherlands (Gabrialdi, 1983; Garibaldi et al., 1986), race 9 in Australia (Kalc Wright et al., 1996), race 10 and 11 in the Netherlands (Baayen et al., 1997).

Physical and chemical managements are not always useful for control of *Fod* due to environmental factors, cost, and limited efficacy. Using resistant cultivars and pathogen-free propagative material offers the most effective approach to Fusarium wilt control (Scovel et al., 2001; Prados-Ligero et al., 2007; Deng, 2018). Race-specific resistance carnation varieties usually show low levels of disease under a range of environmental conditions (Ben-Yephet and Shtienberg, 1997). The determination of races provides relevant information of practical significance (Gabrialdi and Gullino, 1987; Scovel et al., 2001; Prados-Ligero et al., 2007; Baysal et al., 2009; Castano et al., 2014; Polat et al., 2014), which is helpful to breeding programs aimed at selecting resistance cultivars that can reduce the devastating effects of Fusarium wilt (Garibaldi and Gullino, 1987). However, the races of *Fod* are morphologically indistinguishable and pathogenicity tests have been required for race determination (Manulis et al., 1994). Moreover, there are many obstacles such as time-consuming and high dependency on environmental factors etc. (Migheli et al., 1998; Poli et al., 2013). Molecular markers are specific fragments of DNA that can be identified within the whole genome. Nowadays, molecular markers for the determination of *Fod* race 2, race 4, races 1-8 together (Chiochetti et al., 1999), race 1, and race 8 (Migheli et al., 1998) were reported.

The aim of present study was to survey the racial diversity and prevalence of a *Fod* population in the most carnation cultivation provinces in Turkey using specific molecular markers.

2. Material and Methods

2.1. Sampling process

Symptomatic carnation plants showing leaf yellowing and wilting symptoms collected from 91 randomly selected greenhouses in Antalya, İzmir, Isparta and Yalova provinces in Turkey between 2019 and 2020, were sampled (Table 1 and Figure 1). The provinces where the most carnation is produced in Turkey are Antalya (54.11%), İzmir (30.83%), Isparta (13.07%), and Yalova (1.08%) (Kazaz et al., 2020).

2.2. Pathogen isolation

Plants were cut at the stem base and surface sterilization of small pieces of dark discolour vascular tissues were done using 2% (v/v) sodium hypochlorite for 2-3 min. After rinsing two times in sterile distilled water (SDW), tissues were dried on sterile filter paper and placed in Komada's Fusarium semi selective medium containing potato dextrose agar (PDA) and incubated at 25±1°C in 12 h light/dark photoperiod. When hyphae were observed emerging from the tissues, isolates were purified by sub-culturing hyphal tips on fresh PDA. Fungi stored on silica gel were grown on sterile petri dishes on PDA and incubated at 25°C for 7 to 8 days.

2.3. Genomic DNA isolation

Total genomic DNA was extracted from sporangia of *F. dianthi* using a DNA isolation kit (Promega, Wizard Genomic DNA Purification Kit, Madison, US) according to the manufacturer's instructions. Obtained extract was resuspended with DNase-free RNase (Roche Diagnostics, Germany) and checked with a spectrophotometer (Thermo Scientific™ NanoDrop, V, Finland) and quantified in high-resolution agarose gels (1%) using standard lambda DNA for comparison.

2.4. PCR analysis

PCR amplifications specific for *Fod* races 1, 2, 4 and 8 were carried out with the specific primer sets. These primer sets are Ft3 (5′- GGC GAT CTT GAT TGT ATT GTG GTG-3′) / R2.1 (5′-CTT GTT TCT CGA TTT CTG TCT CAC G-3′) to race2, Ft3 / R8.1 (5′-CGA TGA AGT CGG TTT GCG ATT-3′) to jointly race 1 and 8, IMP2 (5′-AAT CCT ATA GAG AAT CTG TGG-3′) / R4.2 (5′-GGT GAT TGG AGG AGG AAT ACC-3′) to race 4 (Chiocchetti et al., 1999) and OPE-08 RAPD (5′-TCA CCA CGG T-3′) to race 1 and 8 (Migheli et al., 1998).

PCR amplification to identify race 2, race 1-8 together, and race 4 were conducted as described by Chiocchetti et al. (1999) with some modifications. PCR amplification was carried out in reaction volumes of 25 µl containing 12.5 µl of 2X master mix (AmpMaster Taq master mix), 1 µl 0.5 mM of each primer, 10 µl double distilled water and 3 µl 20 ng of genomic DNA. A DNA Thermal Cycler (Applied Biosystems SimpliAmp Termal Cycler) was used at the cycling parameters included 5 min of denaturing at 94°C, 30 cycles of 3 steps [30 s of denaturing at 94°C, 30 s of annealing at 55°C and 1 min of elongation at 72°C], and 1 cycle of 10 min at 72°C for the extension. On the other hand, PCR amplification to separately identify races 1 and 8 were conducted as described by Migheli et al. (1998) with some modifications. PCR amplification was conducted in 20 µl of reaction volume containing 10 µl of 2X master mix (AmpMaster Taq

Table 1. PCR amplification of genomic DNA performed with race-specific primers: (+) presence of amplicon, (-) absence of amplicon

Province	Isolate code	Ft3-R8.1 (Race 1 and 8)	Ft3-R2.1 (Race 2)	IMP2-R4.2 (Race 4)	OPE-08 (Race 1)	OPE-08 (Race 8)
Antalya	Tm 1-1	-	-	-	-	-
Antalya	Tm 1-2	+	-	-	-	+
Antalya	Tm 2-1	+	-	-	-	+
Antalya	Tm 2-2	+	-	-	-	+
Antalya	AG 1	+	+	-	+	-
Antalya	AG 2-1a	-	-	+	-	-
Antalya	AG 2-1b	+	-	-	-	+
Antalya	S 1-1	-	+	-	-	-
Antalya	S 1-2	-	+	-	-	-
Antalya	S 6-1	-	-	-	-	-
Antalya	S 6-2	+	-	-	+	-
Antalya	S 6-3	-	+	-	-	-
Antalya	S 7-1	+	-	-	+	-
Antalya	S 7-2	-	+	-	-	-
Antalya	S 8-1	-	+	-	-	-
Antalya	S 8-2	-	-	-	-	-
Antalya	Ant	-	-	-	-	-
Antalya	I-4	+	+	-	-	+
Antalya	I-5	+	+	-	+	-
Antalya	I-6	+	+	-	-	+
Antalya	KA 1	+	-	-	+	-
Antalya	KA 2	+	-	-	+	-
Antalya	KA 3	+	-	-	+	-
Antalya	KA 4	+	-	-	+	-
Antalya	AK 1	+	-	-	+	-
Antalya	AK 2	+	-	-	-	+
Antalya	AK 3	+	-	-	+	-
Antalya	AK 4	+	-	-	+	-
Antalya	AK 5	+	-	-	-	+
Antalya	AK 6	+	-	-	+	-
Antalya	HD 1-1	-	-	-	-	-
Antalya	HD 1-2	-	-	-	-	-
Antalya	BT 1-1	+	-	-	-	+
Antalya	BT 2-1	-	-	-	-	-
Antalya	KT 1-1	-	-	-	-	-
Antalya	KT 1-2a	-	-	-	-	-
Antalya	KT 1-2b	-	-	-	-	-
Isparta	YB 1-2	-	-	-	-	-
Isparta	YL 1-2	-	-	-	-	-
Isparta	TT 1	-	-	-	-	-
Isparta	TT 2-1	+	-	-	-	+
Isparta	TT 3-1	+	-	-	-	+
Isparta	TT 3-2	+	-	-	-	+
Isparta	Tm 2-1	-	-	-	-	-
Isparta	Tm 2-2	-	-	-	-	-
Isparta	UT 1-1	-	-	-	-	-
Isparta	UT 1-2	-	-	-	-	-
Isparta	FT 1	-	-	-	-	-
Isparta	FT 3-1	-	-	-	-	-
İzmir	MA 1	+	-	-	+	-
İzmir	MA 2	+	+	-	+	-
İzmir	MS 1	+	+	-	-	+
İzmir	MA 3	+	+	-	-	+
İzmir	MA 4	+	+	-	-	+
İzmir	MA 5	+	-	-	-	+
İzmir	MA 6	+	+	-	-	+
İzmir	MA 7	+	-	-	+	-
İzmir	MA 8	+	+	-	-	+
İzmir	MA 9	+	+	-	-	+
İzmir	MA 10	+	-	-	-	+
İzmir	MA 11	+	+	-	+	-
İzmir	MA 12	+	+	-	+	-
İzmir	MA 13	+	+	-	+	-
İzmir	MA 14	+	+	-	-	+
İzmir	MA 15	+	+	-	-	+

Table 1. PCR amplification of genomic DNA performed with race-specific primers: (+) presence of amplicon, (-) absence of amplicon (continued)

Province	Isolate code	Ft3-R8.1 (Race 1 and 8)	Ft3-R2.1 (Race 2)	IMP2-R4.2 (Race 4)	OPE-08 (Race 1)	OPE-08 (Race 8)
İzmir	MA 16	+	-	-	-	+
İzmir	İ-1	+	+	-	-	+
İzmir	İ-2	+	+	-	+	-
İzmir	İ-3	+	+	-	+	-
İzmir	İ-4	+	+	-	-	+
İzmir	İ-5	+	-	-	-	+
İzmir	İ-6	+	-	-	+	-
İzmir	U-1	+	-	-	-	+
İzmir	U-2	+	-	-	-	+
İzmir	U-3	+	-	-	+	-
İzmir	U-4	+	-	-	+	-
İzmir	U-5	+	+	-	+	-
İzmir	U-6	+	+	-	+	-
İzmir	U-7	+	+	-	-	+
İzmir	U-8	+	-	-	-	+
İzmir	U-9	+	+	-	-	+
Yalova	K-1	+	-	-	+	-
Yalova	K-2	+	+	-	-	+
Yalova	M-1	+	+	-	-	+
Yalova	M-2	+	+	-	+	-
Yalova	YK 1-2	-	-	-	-	-
Yalova	YK 2-1	+	-	-	+	-
Yalova	YK 2-2	+	-	-	+	-
Yalova	YK 3-1	+	-	-	+	-
Yalova	YK 5-1	+	-	-	+	-
Yalova	YK 5-2	+	-	-	+	-

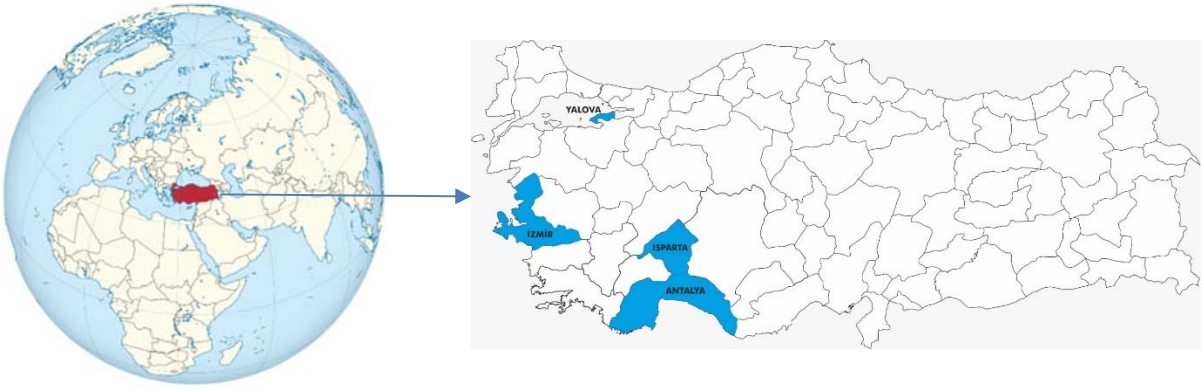

Figure 1. *Fusarium oxysporum* f.sp. *dianthi* sampled in the carnation cultivation provinces of Turkey.
(Antalya: 262.5 ha production area, 11.7 ha sampled area, and 37 isolate; İzmir: 159.6 ha production area, 9.6 ha sampled area, and 32 isolate; Isparta: 51.4 ha production area, 3.5 ha sampled area, and 12 isolate; and Yalove: 3.5 ha production area, 1.0 ha sampled area, and 10 isolate).

master mix), 1 µl 0.5 mM primer, 5 µl double distilled water and 4 µl 20 ng of genomic DNA. PCR conditions were conducted at 94°C for 2.5 min followed by 45 cycles of 94°C for 30 s, 1 min at 36°C, 72°C for 2 min, and followed by final extension of 5 min at 72°C. PCR products were separated on 2.5% high-resolution agarose gel in 1X TAE buffer at 100 V for 3.0 h. A 100 bp DNA ladder (Vivantis) was used as molecular standard. The fragment patterns were photographed under UV light (ENDURO GDS Gel Documentation System) in dye (EZ-ONE N472-KIT, Ambresco) for further analysis. All PCR experiments were repeated three times to confirm the reproducibility of the banding patterns.

3. Results and Discussion

In this study, 82 greenhouses in 4 provinces (Table 1) were sampled. A total of 91 isolates displaying Fusarium morphology were isolated (Figure 1). All collected isolates did not differ in terms of their macroscopic and microscopic characteristics. Amplification with the primer pair Ft3/R2.1, Ft3/R8.1, IMP2/R4.2 and primer OPE-08 RAPD resulted in amplicons of about 564, 295, 1315 and 2000 bp, corresponding to race 2, jointly race 1 and 8, race 4, and separately race 1 and 8, respectively. During the research process, the following *Fod* fungal disease races were analyzed: 1 isolate race 4, 32 isolates race 2, 65 isolates race

1 and 8 (32 isolates of them race 1 and 33 isolates of them race 8) (Table 1). Analysis with specific primers showed a prevalence of 71.43%, 1.09%, 35.16%, 35.16% and 36.26% for jointly race 1 or race 8, race 4, race 2, race 1 and race 8, respectively. Moreover, 1 isolate presence race 4, 5 isolates displayed only Fod race 2, 16 isolate race 8, 22 isolates had only race 1, while *Fod* race 2 and race 1 or race 8 were located together in 27 isolates. However, nineteen isolates were not determined by molecular markers.

Fusarium wilt (*Fod*) is responsible for the most serious and severe disease affecting carnation and economically important species complex within the genus Fusarium in the world (Vergara et al., 2007; Werner and Irzykowska, 2007; Poli et al., 2013; Castano et al., 2014). Similarly, *F. dianthi* was determined as the Fusarium species affecting carnation in Turkey (Tezcan et al., 2004; Arıcı et al., 2018; Arıcı and Kazaz, 2013; Atakan and Özgönen Özkaya, 2020). This study has made an important contribution to the determine of the *Fod* races except that it is first report of detection of *F. dianthi* races on carnation in Turkey.

Determination of the races provides relevant information of practical significance for developing resistance breeding programs and management for disease control (Gabrialdi and Gullino, 1987; Scovel et al., 2001; Prados-Ligero et al., 2007). However, the races of *F. dianthi* has morphologically indistinguishable and race determination requires pathogenicity tests (Manulis et al., 1994). Moreover, it has many obstacles such as time consuming and affected by high dependency on environmental factors etc. (Migheli et al., 1998; Poli et al., 2013). Molecular markers are specific fragments of DNA that can be identified within the whole genome. Nowadays, molecular markers for the determination of *Fod* races 2, 4, and, jointly, 1 and 8 (Chiocchetti et al., 1999), 1 and 8 (Migheli et al., 1998) were reported.

Of ninety one isolates, 32 were identified as belonging to race 1, 32 were identified as belonging to race 2, 33 isolates were identified as race 8 and one isolates were identified as race 4 indicating that *Fod* race 1, 2 and 8 was spatially more widespread than *Fod* race 4 in Turkey. However, race 2 is the most widespread in all areas of carnation cultivation in the world (Ben-Yephet et al., 1992; Chiocchetti et al., 1999; Prados-Ligero et al., 2007; Zahiri et al., 2013; Castano et al., 2014, Poli et al., 2014; Deng, 2018). Race 4 is found in carnation cultivars in the United States, Colombia, Spain, Italy, and Israel (Chiocchetti et al., 1999; Bogale et al., 2007). On the other hand, races 1 and 8 are reported in France, Italy, Iran, Colombia and Spain (Katan et al., 1989; Manulis et al., 1993; Baayen et al., 1997; Bogale et al., 2007; Zahiri et al., 2013; Poli et al., 2013). However, survey studies carried out in greenhouse cut flower carnation areas in Izmir province of Turkey, it was determined that 67 of 84 *Fod* isolates were *Fod* race 2 and 16 were *Fod* race 1/8 (Cer, 2021). The biggest reason for the races to be seen in many countries is migration.

4. Conclusion

In this paper, we show that specific molecular markers can efficiently be used for determination of the race 1, 2, 4 and 8 between and among isolates of *Fod*. Overall, the results showed that *Fod* isolates in the most carnation cultivation province of Turkey consist mostly of race 1, 2 and 8. However, some isolates did not generate any amplification by using molecular markers the reason is that we could not determine molecular as may belong to other races. Yet, highly specific molecular markers are required to better identification among other races isolates of *F. dianthi.* Therefore, molecular markers for identifying these isolates could be developed in future studies.

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