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Fusarium incarnatum Causing Fusarium Wilt on Protea (*Protea cynaroides* L.) in Turkey

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Abstract: Protea (*Protea cynaroides* L.) is one of the most important ornamental plants commercially cultivated in Turkey and other parts of the world. In May 2017, protea plants heavily showed wilting symptoms were obtained from the protea growing areas at Hatay province of Turkey. Brown vascular tissue in the stems of the infected plants was observed. To determine the causal agent of the disease, necrotic tissue pieces taken from diseased plants were surface-sterilized with 10% sodium hypochlorite, two times rinsed with sterile distilled water, placed on *Petri* dishes containing potato dextrose agar (PDA), and incubated at 24 ºC for seven days. Microconidia were no septate, ovoid, hyaline, single-celled, and 9 to 12 × 1 to 3 µm. Macroconidia were slightly curved, four to six septate, and 27 to 31 × 3 to 5 µm. Based on the morphology and cultural features, the fungus was identified as *F. incarnatum*, which belongs to the *F. incarnatum-equiseti* species complex (FIESC). In addition, a pathogenicity test was made using the apple fruits. The pathogen was re-isolated from inoculated apple fruits and both cultural and morphological characteristics of the pathogen were identical. To confirm the identity of one representative isolate, the internal transcribed spacer (ITS) region including 5.8S rDNA was amplified and sequenced with primers of ITS-1 and ITS-4. Fusarium MLST, Fusarium-ID and GenBank database were used for the identification of sequence. The amplified 465 bp product has 99% nucleotide identity with the sequences of *F. incarnatum-equiseti species complex* of MLST types and deposited in GenBank under accession number (MH005097). According to the literature, this is the first molecular identification of *F. incarnatum* on protea plants in Turkey.

Keywords: *Fusarium incarnatum*, protea, wilting, ITS region, pathogen

1. Introduction

Protea, Leucospermum and Leucadendron, refer to as proteas, are used flower production industry and commonly cultivated taxa in the family. Proteas with the South Africa's national flower is one of the oldest families of flowering plants in the world with over 363 species and also known as sugarbushes (Migliorini et al., 2021). Protea (*Protea cynaroides* L.) as in other plants is attacked by many fungal pathogens, particularly, soil-borne pathogens; *Phytophthora*, *Armillaria* and *Fusarium* have been observed on growing protea plant and its production has been limited economically by these pathogens (Crous et al., 2004). Unfortunately, many diseases have been reported on protea cultivation in the world. Previous studies associated with diseases of cultivated protea, *Colletotrichum gloeosporioides* (Baxter et al., 1983), *Alternaria* spp. and *Batcheloromyces* spp. (Dunne, 2004), *Lophiostoma fuckelii*, *Kabatiella* spp. *Mycospherella* spp., *Phyllachora proteae*, *Septoria grandicipis*, *Teratosphaeria* spp., *Stilbospora proteae*, *Trimmatosotroma elginense*, *Lembosia proteae* and *Verruciporota proteacearum* (Taylor and Crous, 2000), and *Ramularia proteae* (Crous et al., 2000) was found. It was stated that *Phytophthora cinnamomi* causing sudden death in proteas is a soil-borne plant pathogen and is a major disease of cultivated proteas in the world (Dunne, 2004). Other root rot diseases, *Armillaria luteobubalina* (Falk and Parbery, 1995) and *Rosellinia spp* (Forsberg, 1993), have been reported on protea plants worldwide. Stem cankers caused by Fusarium (Swart et al., 1999) and *Botryosphaeria*

species (Crous et al., 2000) were the most frequent pathogen of cultivated protea after *P. cinnamomi*.

Fusarium spp. are filamentous ascomycete fungi (Sordariomycetes: Hypocreales: Nectriaceae) generally causing diseases on many host plants and seriously affecting plant yield in the world (Summerell, 2019). It produce different shapes and sizes of microconidia and macroconidia that are asexual spores. The other spore structure of Fusarium is chlamydospores, which ensure it to survive for many years in extreme conditions (Leslie and Summerell, 2006). Wilt diseases caused by Fusarium species are difficult to control. For this reason, resistance varieties, using the certified seed and healthy seedlings are generally preferred to this disease. The morphological characteristics of Fusarium species have been widely used for identification (Summerell et al., 2003). However, this genus is difficult to identify morphologically. Therefore, the internal transcribed sequence (ITS) of the ribosomal DNA (rDNA) region is widely used for accurate identification and molecular characterization of the Fusarium species by several researchers (Soren et al., 2015; Zarrin et al., 2016), and this is also used in other groups of fungi (Bruns, 2001; Gazis et al., 2011; Du et al., 2012).

In summary, protea is a valuable ornamental plant mainly grown as cut flower and used for home gardens and landscaping throughout the world. The wilting symptom has been recently observed on protea plants in Turkey. Therefore, the current study was conducted to determine the pathogen causing wilting symptom observed on protea cultivation.

2. Materials and Methods

2.1. Isolation and morphological identification

In May 2017, diseased protea plants showing wilting symptoms were obtained from the grown protea plants at Hatay province of Turkey. These infected protea plants were firstly observed brown vascular tissue in the stems and blackening formation of the stem and root crown. To determine the causal agent of the disease, infected plant tissue were firstly cut sterile scalpel and then surface-sterilized with 10% sodium hypochlorite for 1 min, two times rinsed with sterile distilled water, placed on *Petri* dishes containing potato dextrose agar (PDA) and incubated at 24 ºC for seven days under 16/8 hours light/dark conditions. Single spore was obtained by serial dilution method and it was grown on PDA (Leslie and Summerell, 2006). Additionally, colony and conidia characteristics of the isolate were recorded.

2.2. Pathogenicity tests

Pathogenicity tests were conducted on apple fruits using the mycelial plug technique. In detail, five healthy apple fruits were firstly sterilized with 96% ethanol and then a piece of the peel using sterile bisturi was removed. Lastly, it was applied on the wound 10 mm diameter mycelial plugs are taken from *Fusarium* isolate obtained from seven-day old culture grown on PDA and subsequently covered with parafilm, and incubated at 24 ºC. As a control, only sterile PDA plugs were applied on apple fruits as described above. Samples from the inoculated apple fruits were taken and the pathogen was re-isolated.

2.3. DNA extraction and amplification

Total genomic DNA obtained from a single spore isolate of a representative fungal isolate was extracted by using modified Cetyltrimethyl Ammonium Bromide (CTAB) protocol (Catal et al., 2010). The extracted DNA was checked on agarose gel staining with ethidium bromide to control for the quality and concentration and then they were kept in sterile distilled water and stored at -20 ºC until use. The ITS region including 5.8S rDNA was used to identify the pathogen and this region was amplified with ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA TATGC-3') primers (White et al., 1990). Total 25 µl reaction mixture containing; 2.5 µl 10 x PCR buffer, 2 µl $MgCI_2$, 2.5 µl of the dNTPs mix, 0.1 µl Taq DNA polymerase (Sigma Aldrich, Missouri, USA), 1.25 µl of ITS1 primer, 1.25 µl of ITS4 primer, 1 µl DNA template, 14.4 µl distilled water was prepared. PCR amplification of the ITS region was performed by Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA). PCR was carried out as follows; initial denaturation at 94 ºC for 5 min, 30 cycles of 94 ºC for 30 s, annealing temperature 55 ºC for 30 s, 72 ºC for 1 min, and a final extension of 10 min at 72 ºC. The PCR products were separated in 2% agarose gel staining with ethidium bromide and visualized through UVP UVsolo touch gel imaging system under UV light. Also, amplicon sizes were determined against a 50 bp DNA ladder. Sequence analysis of the PCR products was done by the BM laboratory system (Ankara, Turkey).

2.4. Molecular identification and phylogenetic analysis

Phylogenetic analysis of the isolates was conducted by the MEGA7 software (Kumar et al., 2016) based on the ITS sequence data. BLAST analysis of the sequence data was made in both

National Center for Biotechnology Information (NCBI) GenBank, Fusarium-ID and Fusarium multi locus sequence typing (MLST) database to compare with the Fusarium isolates retrieved from the database. Later the sequence data with other Fusarium sequences retrieved from the GenBank database were aligned by using the MEGA7 software. The phylogenetic tree of the isolates was constructed by using the Neighbor-Joining method (Saitou and Nei, 1987) and pairwise distances calculated using Tamura-Nei Model. All gaps were eliminated and bootstrap value was obtained from 1000 replications. Reference isolates used in this study are given in Table 1, and the sequence of *Cladobotryum mycophilum* GenBank (accession number KP267826) was included as an out-group for the phylogenetic analysis.

Table 1. Sequences of the reference isolates obtained from the DNA database of GenBank in NCBI

Species	Isolate/Strain	GenBank accession number
FIESC MLST type 15-a	NRRL 43619	GQ505748
FIESC MLST type 23-b	NRRL 13379	GQ505680
FIESC MLST type 25-a	NRRL 22244	GQ505685
FIESC MLST type 26-a	NRRL 26417	GQ505687
FIESC MLST type 18-a	NRRL 31167	GQ505697
FIESC MLST type 15-a	NRRL 32175	GQ505698
FIESC MLST type 21-b	NRRL 32865	GQ505703
FIESC MLST type 25-c	NRRL 32868	GQ505706
FIESC MLST type 26-b	NRRL 28714	GQ505693
FIESC MLST type 25-b	NRRL 32993	GQ505709
FIESC MLST type 15-a	NRRL 43619	GQ505748
Fusarium incarnatum	TR1*	MH005097
Cladobotryum mycophilum	PE70CD	KP267826

*: *Fusarium incarnatum* TR1 isolate is sequenced in this study

3. Results and Discussion

Fusarium species are important plant pathogens in many plants all over the world (Summerell et al., 2010; Srivastava et al., 2018; Shan et al., 2019). *Fusarium* spp. was reported as a significant pathogen of the protea plants worldwide (Swart et al., 1999). In this study, symptoms of the cortex tissue of protea were observed (Figure 1a) and the identification of five Fusarium isolates was isolated from the showing of the brown vascular tissue on protea plants (Figure 1b). The isolates obtained from the single spores were morphologically characterized (Figure 1c). Morphologic features of the isolates such as mycelium, microconidia and macroconidia are shown in Figure 1d. Microconidia were no septate, ovoid, hyaline, single-celled, and 9 to 12 × 1 to 3 µm (Figure 1e). Macroconidia were slightly curved, four to six septate, and 27 to 31 × 3 to 5 µm (Figure 1d). According to the cultural developments and morphologic features of isolates, five Fusarium isolates were identified as *F. proliferatium* belonging to the *F. incarnatum-equiseti* species complex (FIESC) (Leslie and Summerell, 2006).

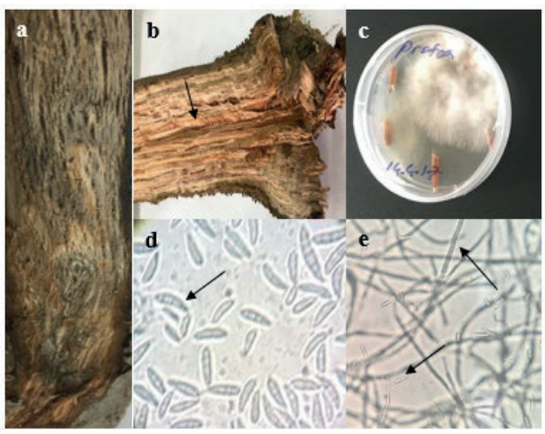

Figure 1. Symptoms and morphological features of *Fusarium incarnatum* isolate TR1. Symptoms of the cortex tissue of protea (a), brown symptoms observed on vascular tissue (b), development of the colony on potato dextrose agar (PDA) (c), macroconidia (d), microconidia and mycelium (e)

The isolate from protea plants showing wilting symptoms investigated pathogenesis against apple fruit in the laboratory conditions. According to the pathogenicity test results, fruit rot was observed on apple fruits inoculated with mycelium plugs of Fusarium, on the other hand no symptoms were observed on negative control (Figure 2). The fungus was re-isolated and its morphologic characteristics in accordance with the same culture of *F. incarnatum* were isolated from this study.

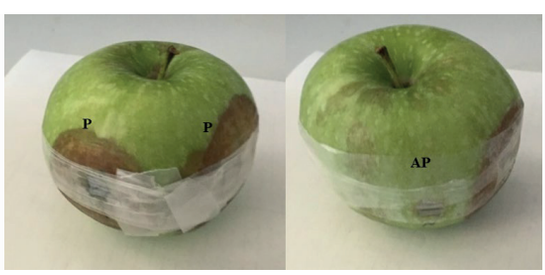

Figure 2. Apple fruits inoculated with P; *Fusarium proliferatum* and AP; agar plug

Previous studies suggested that molecular analysis is generally preferred for characterization and identification of Fusarium species, and provides easy detection of the differences in this species (Nayyar et al., 2018; Guan et al., 2020). Combinations of both morphological observations and molecular characters are necessary to accurately identify Fusarium species (Zhu et al., 2014; Chang et al., 2018; Guan et al., 2020; Kintega et al., 2020). Many researchers (O'Donnell, 2000;

Geiser et al., 2004; Geiser et al., 2005; O'Donnell et al., 2009, 2010) conducted molecular studies relating to the phylogenetic relationship of Fusarium species. Likewise, it was stated that an ITS of the rDNA gene has been widely used for the identification and phylogenetic analysis of Fusarium species by many researchers (Nilsson et al., 2010; Schoch et al., 2012; Jedidi et al., 2018; Minati and Mohammed-Ameen, 2019; Moummi et al., 2020). In line with the above studies, the ITS region was used to identify Fusarium species causing the wilting symptom in this study. Molecular findings from this study showed that PCR product of approximately 465 bp was obtained from the amplification of DNA of the isolate with ITS-1 and ITS-4 primers. The amplified fragment was sequenced and later it was deposited in GenBank under accession number MH005097. Sequence data set was generated from 12 isolates including 10 species FIESC retrieved from the GenBank, one species *F. incarnatum* isolate TR1 obtained from this study, and out-group.

Many studies show that the Fusarium MLST (O'Donnell et al., 2010, 2012) and Fusarium-ID (Geiser et al., 2004; Park et al., 2010; O'Donnell et al., 2012) databases have been used to compare Fusarium isolates phylogenetically. In agreement with the previous studies, *F. incarnatum* obtained from the protea was searched in Fusarium MLST and Fusarium-ID database for phylogenetic studies. According to the result of the phylogenetic analysis, the ITS sequence of *Fusarium incarnatum* isolate TR1 had 99% similarity to the sequences of isolates of FIESC in GenBank including Fusarium MLST type 15-a, 18-a, 21-b, 23-b, 25-a, and 26-b (Figure 3).

According to the previous studies conducted to relate with the protea plants, many pathogens were reported on the cultivated protea in different regions of the world (Knox-Davies, 1981; Knox-Davies et al., 1987; Swart et al., 1999; Crous et al., 2000; Denman et al., 2003; Dunne, 2004; Lubbe et al., 2004; Crous et al., 2013; Burgess-Tan et al., 2019). A study conducted by Crous et al. (2011) stated that many of the fungi associated with diseases of protea identified morphologically and molecularly again. Similarly, the findings of this study reveal that *F. incarnatum* is firstly identified in Turkey and has negative effects on the development of the protea plants.

In summary, taking the results obtained from the current study into consideration, identification of the *F. incarnatum* and its pathogenicity on protea helped to elucidate the causal agent associated with wilting symptoms. In particular, the first identification of *F. incarnatum* in Turkey is important to reduce the risk of transmission of the pathogen between other regions. Moreover, this will provide us with management strategies linked with the pathogen, and to apply phytosanitary methods to impede the spreading of this disease.

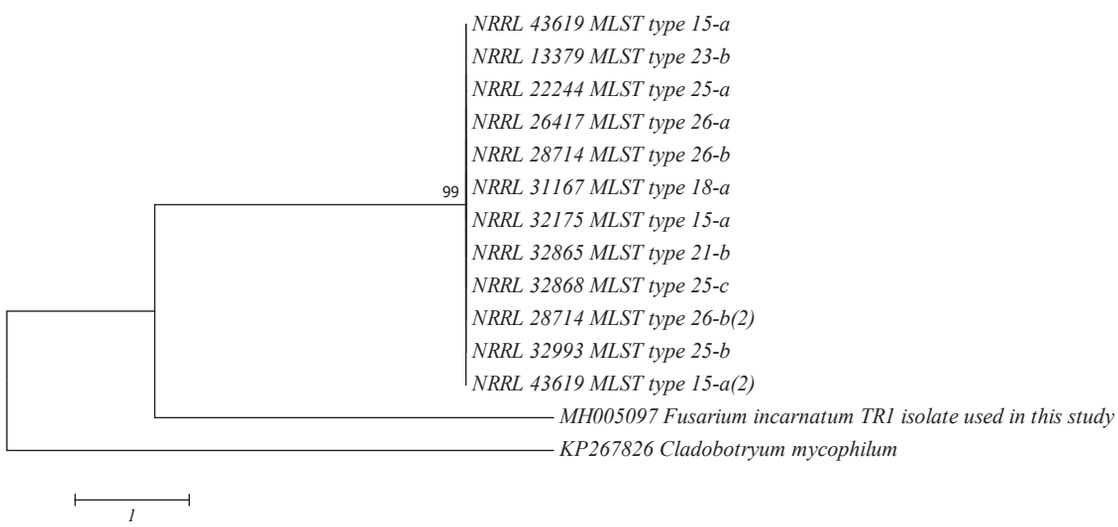

Figure 3. Phylogenetic tree constructed based on ITS gene region. 12 reference sequences from members of the *Fusarium incarnatum*-equiseti complex (FIESC) isolates were used for the phylogenetic tree and *Cladobotryum mycophilum* was used as the out-group. The reference sequences were obtained from the *Fusarium* MLST database and GenBank.

4. Conclusions

Fusarium species are among the most important pathogen in the world. Many Fusarium species are well defined, however, there are many species that are pathogenic on plants, which are still not identified or characterized. A combination of morphological and molecular methods is used to identify Fusarium species accurately. Based on morphological observation and ITS sequence data, the causal agent of the wilting disease of protea, was identified as *F. incarnatum*. The findings of the current study is revealed the importance of the pathogen and its effective disease management. According to the literature, this is the first identification of *F. incarnatum* causing wilting disease of protea in Turkey. More studies should be conducted by using the more gene regions to study the identification of Fusarium isolates at the species complex and quarantine measures must be taken to prevent the spread of the causal agent.

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Declaration of Conflicts of Interest

No conflict of interest has been declared by the author.

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