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Original Research

Genetic characterization and virulence of *Fusarium* spp. isolated from chickpea

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Abstract: *Fusarium* wilt causing yield losses in chickpea is one of the most important diseases, and occurs due to some fungi especially *F. oxysporum* and *F. solani.* Molecular studies are important in revealing the genetic characterization and virulence of the pathogen. In this study ten *Fusarium* genotypes isolated from chickpea plants showing symptoms of wilting were used. The ITS region was amplified by using the thermal cycler and genetic similarities and differences among species were revealed by performing sequence analysis of ITS region. Samples except for two of *Fusarium* genotypes; N1, N2, N3, N4, N5, N7, N9 and N10 genotypes showed a close relative to *F. oxysporum*. However, the N8 genotyped was found to be similar to *F. solani*, and interestingly the N6 genotype showed an equal relationship with *F. solani* and *F. oxysporum* so, it could not be fully identified. According to the phylogenetic tree, *F. oxysporum,* N2, N4 and N10 were determined in a separate group and *F. oxysporum* N5, N7 and N9 formed separate another group which showed a close relationship to each other. The severity of disease caused by isolates on ILC-482 chickpea variety varied between 1.25 and 3.50. The close relationship was found between *F. oxysporum,* N2, N4, N5, N7, N9 and N10 isolates was confirmed the result of the pathogenicity test of same isolates. The results revealed that N7 isolate prevented the emergence of plants by causing disease on chickpea seeds and it also indicated the high virulence.

Key words: Chickpea; *Fusarium oxysporum;* Phylogenetic tree; Genetic characterization; Virulence.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the first crop cultivated among edible grain legumes. The origin of chickpea is known as the Eastern Mediterranean area that covers Turkey (1). Chickpea has been grown on 13.200.540 ha area with production 12.200.000 tons throughout the world in 2016. In the same year, Turkey was ranked as the fourth in the world after India, Australia and Pakistan with 395.309 ha planting area and 455.000 tons of production. Fourteen percent of chickpea cultivation areas in Turkey with about 72.800 tons production take place in Diyarbakir, Siirt, Mardin provinces of Southeastern Anatolia region (2).

More than 50 pathogens from different regions of the world have been reported in chickpea plants. Some of these pathogens have been reported causing diseases that lead to significant economic losses in the chickpea production areas (3) such as Ascochyta blight (*Ascochyta rabiei*), *Fusarium* wilt (*F. oxysporum f. sp. ciceris*), black root rot (*F. solani*), collar rot (*Sclerotium rolfsii*), wet root rot (*Rhizoctonia solani*), Phytophthora root rot (*Phytophthora megasperma*), Pythium root and seed rot (*Pythium ultimum*), foot rot (*Operculella padwickii*), and stem rot (*Sclerotinia sclerotiorum*) are important of these diseases, respectively. Most of the other pathogens, except *Ascochyta rabiei*, cause wilt and root rot in chickpea. *F. oxysporum* Schlechtend: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato is the most common pathogens that causes wilt and root rot and it has been reported to cause 10 to 40% economic losses in chickpea production areas (4,5,6).

The pathogen can survive in soil for many years despite the absence of a host plant. Therefore, management is quite difficult (7). A survey carried out in 2001-2002 at 15 different provinces of Turkey revealed that *F. oxysporum, F. solani, F. equiseti, F. semitectum, F. acuminatum, Macrophomina phaseolina* and *Rhizoctonia solani* were the pathogen factors causing wilt and root rot in chickpeas. The most commonly isolated pathogen was *F. oxysporum*, followed by *F. solani* and *M. phaseolina* (8). The wilt caused by *F. oxysporum* is observed in the susceptible chickpea plants 25 days after sowing in the early stage and from sixth and eighth weeks of flowering in the late stage. Petioles, rachis and leaflets drop in the late stage wilted plants, followed by yellowing and necrosis of foliage. Yield losses are higher in the early wilting compared to late wilting. However, the seeds from late-wilted plants are lighter, wrinkled and pale in color than those from healthy plants (9,10,11,12).

The variation in virulence among the isolates of *F. oxysporum f. sp.* led to determination of pathogenic races. Eight different physiological races (0, 1A, 1B/C, 2, 3, 4, 5 and 6) were identified. The races are characterized by their severity on different chickpea varieties of differential sets (13,14,15). The races of number 2 and 3 had been previously reported in Turkey (16).

Current molecular techniques are used for easier and more reliable diagnosis of pathogen fungi. The (Internal Transcribed Spacer) ITS gene region is widely

used for fungal diagnosis. The primers used in the reproduction of the ITS1 gene region are specific for fungi. These primers are nested in three edges of the SSU (Small subunit) gene of the rDNA. ITS4-ITS5 primer pairs were identified as the universal primers and complement to the 5 ends of the LSU (Large subunit) gene (17,18). 18S rDNA region relatively slowly evolves and used to compare distant related organisms. Non-coding regions (ITS and IGS) evolve more rapidly and it is used for comparing species of a single genus or strains of a species. Some regions of 28S rDNA differ between species (19). DNA sequences such as ITS are suitable for the diagnosis of a single species among all other species (18). Since sequences are in the form of repeating units and tend to be variable among species, and similar within the species, many investigators select sequences from the ITS region to develop species-specific probes (17,20,21).

In this study, the genetic proximity/similarity relationships of pathogens and DNA sequence-based isolates were determined by macroscopic, microscopic diagnosis and genetic characterizations of *Fusarium* isolates isolated from plants showing signs of wilt in some chickpea fields located in Siirt, Diyarbakır and Mardin provinces.

Materials and Methods

Fusarium isolates

Ten *Fusarium* genotypes used in this study were isolated from diseased chickpea plants with symptoms of wilt incidence in April and May from Siirt, Mardin and Diyarbakır provinces of Turkey. The isolates were maintained on potato dextrose agar (PDA, 38 g and sterile water, made up to 1 litre). Single spore isolations of the cultures were made on potato dextrose agar (Merck, Germany) and stored at 4 °C for further use. Genus based identification of isolates were performed both macroscopically and microscopically considering the identification key of Booth and Anonymous (22,23). Species based identification was made according to DNA sequence based on molecular methods. The areas where the chickpea plants were collected and the identification of the isolates are presented in Table 1.

Genetic Characterization of *F. oxysporum*

Genomic DNA isolation from Fungi

In this study ten isolates of *F. oxysporum* were grown in Potato Dextrose Agar (PDA, Merck) medium have been incubated for one week, and then purified using single spore technique. DNA isolation for each isolate was then performed using the CTAB (cetyl trimethyl ammonium bromide) method (24). DNA samples were run on a 1% agarose gel to measure the qualitative quality of isolated genomic DNA. In addition, the quantitative quality of DNA samples was measured using a Nanodrop (Thermo) device. The DNAs were kept at -80 °C until the be used for PCR amplification.

Table 1. *Fusarium* isolates used in the study, and regions of isolation.

Isolate No	Area of Isolation
Fusarium N1	Central Village/Diyarbakır
Fusarium N2	Central Village/Diyarbakır
Fusarium N3	Central Village/Diyarbakır
Fusarium N4	Kezer/Siirt
Fusarium N5	Kezer/Siirt
Fusarium N6	Kızıltepe/Mardin
Fusarium N7	Kızıltepe/Mardin
Fusarium N8	Kezer/Siirt
Fusarium N9	Central Village/Diyarbakır
Fusarium N10	Central Village/Diyarbakır

Implementation of Polymerase Chain Reaction (PCR)

PCR was achieved with total reaction volume of 50 µL. For this purpose, total volume was set at 50 µL by adding 6 µL of 25 mM $MgCI_2$, 1 µL of 10 mM dNTP, 2 µL of 10 pmol each reverse and forward primers, 0.7 µL of Taq DNA Polymerase, 5 µL of 10X Taq Buffer, 2 µL of BSA, 4 µL of template DNA and 29.3 µL from dH_2O. The PCR components were kept on ice throughout the study. In addition, negative controls were used to check for contamination during PCR studies. Primers sequences used in the study were: ITS4 TCC TCC GCT TAT TGA TAT GC and ITS5 GGA AGT AAA AGT CGT AAC AAG G. PCR products were analyzed in agarose gel of 1.5% concentration. PCR product (10 µL) was mixed with 3 µL of loading dye and loaded into gel wells using a micropipette. 100 bp DNA leader was used to determine the length of PCR product (Figure 1). The agarose gel was run for 30 min with an electrical voltage of 90 volts. The samples were then transferred to gel imaging device and analyzed under UV light. The PCR products were sent to IONTEK company to obtain DNA sequences of the reproduced regions at the desired length (500-600 bp).

Bioinformatic analysis for phylogenetic tree

All sequences obtained were BLASTed in NCBI to verify results belongs to our isolated. Then BioEdit software was used to process the sequences of resulting ITS gene region. DNA sequences were processed in BioEdit software and consensus sequences were generated. In order to make sequences ready for alignment, the sequences were converted to fasta format. Then the sequences were aligned using Clustal W software. To construct the phylogenetic tree, MEGA 7.0 software was used. Distance-based Neighbor Joining (NJ) method showed the relations of the isolates with each other.

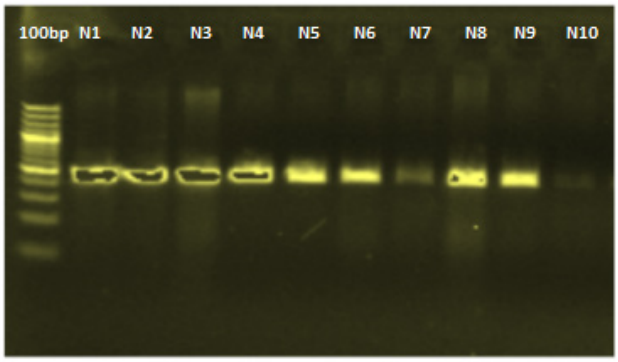

Figure 1. Agarose gel image of PCR-amplified ITS gene region of *Fusarium* isolates from chickpea.

Assessment of virulence of *F. oxysporum* isolates

Sensitive chickpea variety of ILC-482 was used in the virulence study. The seeds were sterilized with 1% NaOHCl for 1.5 min then allowed to dry. The method developed was used in inoculum preparation and potting studies (25). For inoculum preparation, chickpea flour (5 g) and fine sand (45 g) were mixed and placed into 250 ml glass bottles. Discs from 7-10 day old *Fusarium* cultures grown on Potato Dextrose Agar (PDA) at 22±24 °C were transferred to these bottles and incubated for two weeks. After the incubation, 60-70 g inoculum was mixed with 2 kg of sterilized soil in 25 cm pots. Four days later, 10 chickpea seeds were planted in each pot and allowed to grow at room temperature with 30-50% humidity. The experiment was established with three replications for each isolate. Sterilized soil without any chickpea seeds and pathogens was used in control pots.

Disease assessment was performed six weeks after inoculation and 0-4 scale (0 = 0%; 1 = 1 - 33%; 2 = 34 - 66%; 3 = 67 - 100%; 4 = Dead Plant) based on acropetally yellowing and necrosis was used. In the sensitive chickpea cultivars, the isolates having the value of 3.1-4.0 are considered as highly virulent (HV), the isolates with 1.1-3.0 scale values are moderately virulent (MV) and the isolates with 0.0-1.0 value are low virulent (LV) or non-pathogen (26).

Results

Genetic characterization of *Fusarium* isolates

The ITS primer pairs designed and were used for molecular identification of fungus varieties isolated from chickpea plants and for amplification of ITS region on ribosomal DNA (17). Genomic DNA of different *Fusarium* species was amplified into a region of about 500-600 bp length using specific primers (Figure 1). The bands were very clean and a reaction without band called flour-specific was obtained The band of tenth sample obtained was slightly weak. This sample, however, was also sufficient to evaluate after sequencing process.

Originality or similarity index and dendrogram data obtained by genetic characterization studies of *F. oxysporum* revealed the genomic similarities and differences of studied chickpea *Fusarium* isolates. The sequence information of ITS region provided an identification for relationships of close/distant taxa relatives. Thus, extensive information has been obtained on genetic relatives of the studied isolates. In this study, genetic similarities and differences among ten genotypes of *Fusarium* isolated from chickpea. ITS sequence of Four different *Fusarium* species (*F. sambucinum, F. oxysporum, F. solani* and *F. equiseti*) obtained from NCBI (National Center for Biotechnology Information) and strains belonged to these species were used to perform a reliable molecular diagnosis of the isolates (Figure 2). The *Fusarium* isolates; N1, N2, N3, N4, N5, N7, N9, and N10 used in this study have close relation with *F. oxysporum*, and N8 isolate was more similar to *F. solani*. However, N6 isolate showed an equal relation with *F. solani* and *F. oxysporum*, here of a complete molecular diagnosis of N6 isolate has not been performed. According to the phylogenetic tree, a distant relation was observed between the isolates studied and

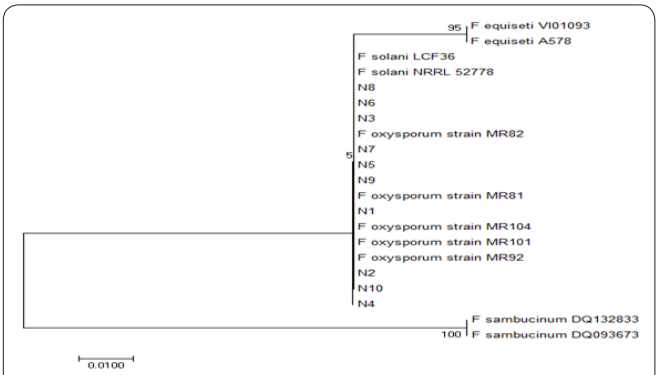

Figure 2. Phylogenetic tree based on neighbor-joining analysis using the ITS nuclear DNA region.

the species of *F. sambucinum* and *F. equiseti* (Figure 2). Comparison of isolates with each other showed that N2, N4 and N10 isolates have closer relationship with each other, while N5, N7 and N9 isolates had a closer relationship. Similarly, N3, N6 and N8 isolates had closer relations with each other (Figure 2).

Assessing the virulence of *F. oxsporum* isolates

The pathogenicity test was established as a pot study using sensitive ILC-482 chickpea variety. The results obtained in this study were given in Table 2.

All the isolates of *F. oxysporum* caused disease in susceptible ILC-482 chickpea variety (Table 2). The isolates of N1, N5, N6 and N7 with various virulence degree collected from Diyarbakır-Merkez, Siirt-Kezer and Mardin-Kızıltepe regions had high degree of virulence and other isolates moderate degree of virulence. Pieces of roots and root crown of the plants showing symptoms of disease were collected, surface sterilized with sodium hypochlorite (NaOCI) and placed into PDA medium. The colonies grown were examined under a microscope and consequently, all isolates were retrieved.

Soil inoculation method was used in this study. In this method, the inoculum grown in a mixture of sand/chickpea flour was mixed to the sterile soil in a specific ratio and then the seeds were planted in this soil. Due to the inoculum surrounding the soil, seeds cannot germinate at all or germinate but cannot emerge.

Table 2. Disease severity and virulence degree of *Fusarium* isolates used in the study.

Isolates	Severity of Disease	Degree of Virulence
Fusarium oxysporum -N1	3,50	HV*
Fusarium oxysporum- N2	1,25	MV
Fusarium oxysporum -N3	1,50	MV
Fusarium oxysporum -N4	1,25	MV
Fusarium oxysporum -N5	3,75	HV
Fusarium -N6	3,00	MV
Fusarium oxysporum -N7	3,50	HV
Fusarium solani -N8	1,50	MV
Fusarium oxysporum -N9	2,50	MV
Fusarium oxysporum -N10	1,50	MV
Control	0.00	-

*Highly virulence (HV), Moderately virulence (MV) and Low virulence (LV).

If seedlings emerge they died soon after due to root decay or plants exerted typical wilt. The pots that were inoculated with N7 isolate, usually had seeding that did not emerge or the plants died due to the root rot. Infected seedlings in the pots where other isolates were inoculated had lighter in color compared to the healthy seedlings and some of the leaves had fallen. Some of infected seedlings had laid over soil surface and when the infected seedlings were removed from the soil, their body was cut off.

Discussion

Given the characteristics of ideal site to be selected for PCR amplification (27), the genes where ribosomal RNAs are coded (rRNA) contain most of these criteria and can be analyzed extensively. Designing the primers for the reproduction of rDNA sites has considerably facilitated the taxonomic studies of fungi (17).

Dissimilarity index and dendrogram data obtained in the genetic characterization study of *Fusarium* spp. revealed the genomic similarities and differences of chickpea fungi isolates. The sequence of ITS gene region in the *Fusarium* genus is highly variable (28). Reproduction of ITS regions ensured the definition of relationships between relatives of distant taxa. Thus, detailed information on genetic characteristics of the isolates studied has been obtained. The information obtained in such studies is crucial in solving the taxonomic problems of the genera that will be studied based on molecular phylogenetic and revealing the position at molecular level in fungi systematic. Previous studies on defining many complex species have reported that the ITS region has a rather informative or low nucleotide sequence variation (29,30). Reliable, accurate and rapid diagnosis can be obtained by using the correct primer pairs and thermal cycler amplification of the ITS region of the rDNA (17).

The phylogenetic tree obtained revealed that *F. oxysporum* and N2, N4 and N10 isolates appear to have a closer relationship with each other. The virulence of these isolates was between weak and moderate virulence (OV) (1.25-1.50). The N5, N7 and N9 isolates also showed a closer relationship between each other. High virulence of these isolates has been indicated in the phylogenetic tree. Therefore, the results concluded that disease-causing impacts of *F. oxysporum* isolates, which have close affinity, on chickpea plants are similar. Different pathogen species of *F. oxysporum* in chickpea have been reported (15).

The number and nature of races may change over time. The isolates investigated in this study as close relatives are presumably in the same or close races. The ITS gene region has been proven as a useful gene site for identifying the current *Fusarium* species (31,28). They pointed out the high variability of *F. sambucinum* species using ITS gene region (31). The wilt symptoms caused by *F. oxysporum* in chickpea plants are usually seen 3-4 weeks after sowing (11,12). However, some of the seeds in pots where *F. oxysporum* N7 isolate applied have infected prior to emerging. Emerging of seedlings in other pots was in the normal course. This case indicated the differences in virulence and symptom pattern of N7 isolate relative to other isolates investigated. Further studies are also needed for the genetic characterization of N7 isolate, and virulence effect and severity in chickpea plant.

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Interest conflict
The authors declare that they have no conflict of interest.

Author's contribution
We gratefully declare that all authors participated in the study design, data collection, data analysis, manuscript preparation and revision and agree to be accountable for all aspects of the work.

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