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Identification of Seedborne Fungi on Soybean (*Glycine max* L.) Seeds Grown in Mediterranean Region of Turkey

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Abstract: Soybean (*Glycine max* L.) is one of the most valuable oilseed crops in the world. It is not only an oil seed crop and feed for livestock, but also valuable mineral and vitamins sources for the human diet. The soybean yield is affected by various biotic and abiotic stress factors in all growing seasons. Diseases are one of the most significant biotic factors that reduce soybean growth and yield. Fungi are important pathogens affecting yield and quality by attacking plants during the growth period and after harvest. This study was conducted to detect and identify the seed-borne fungi associated with the soybean seed. From this context, 150 soybean seeds were randomly chosen from the experimental fields of Akdeniz University in Antalya province of Turkey. These seeds were sterilized with 70% ethanol for 1 min, followed by 10% sodium hypochlorite for 1 min and then rinsed with sterile water and then placed in Petri plates by using the agar plate method. A total of four seed-borne fungi species namely *Aspergillus* spp., *Penicillium* spp., C*ladosporium* spp. and *Fusarium* spp. were isolated from the soybean seeds. Additionally, Genomic DNAs of these fungal species were extracted and the internal transcribed spacer (ITS) region of ribosomal DNA was amplified with the ITS-1 and ITS-4 primers using a thermal cycler. After sequencing of amplified products, the sequences were aligned. BLASTn analysis of each sequence showed that the sequences of the fungi had the similarity (99%) to the fungal isolates deposited in the GenBank.

Keywords: Soybean, seedborne fungi, seed decay, molecular analysis, ITS region

1. Introduction

Soybean (*Glycine max* L.) is the most commonly produced oilseed crop in the world with more than half of the total oilseed production. The major soybean growers are Brazil (128.5 million tons), the United States (96.7 million tons) and Argentina (48.8 million tons) and they provide more than 80% of 339 million tons total soybean production worldwide (Anonymous, 2021a). Turkey has approximately 35.134 hectare soybean growing area and 155.225 tons of annual soybean production (Anonymous, 2021b). Soybean seeds contain 38% protein, 18% lipids, 30% carbohydrate and 14% other substances (Kim et al., 2021). The seeds are used as oil and protein for human and animal feeding and many chemical products (Ahmad et al., 2014). In addition to this, they are valuable sources of human nutrition with high protein content, essential amino acids, and fatty acids (Maleki et al., 2013; Mutava et al., 2015). The fatty acids are significant in the prevention of diabetes, heart disease and arterial stiffness by decreasing cholesterol levels in the bloods. Soybean diet reported to prevent the risk of various cancers in human due to highly valued phytochemicals (Ahmad et al., 2014).

The soybean yield is influenced by various biotic and abiotic stress factors in all growing seasons. Diseases are one of the most significant biotic factors restricting the growth and yield of soybean. More than 135 microorganisms of 30 species belonging to economically important pathogens groups were associated with soybean diseases (Roy et al., 2001). Among them, fungi are

one of the most important pathogens to attack this crop during the growing period and post-harvest. Many studies have reported the reduction of the quality and yield of soybean seeds by fungi. Fungal pathogens such as, *Aspergillus* spp, *Fusarium* spp, *Alternaria* spp. *Cladosporium* spp. and *Penicillium* spp. and *Phomopsis longicolla* are reported from soybean seed by many researchers and they are frequently found to spread throughout the soybean production areas (Killebrew et al., 1993; Gutleb et al., 2015; Li et al., 2017; Ustun et al., 2018). *Aspergillus* spp. *Penicillium* spp. and *Fusarium* spp. can cause significant quality losses in soybean seeds (Gutleb et al., 2015). Li et al. (2017) reported that the fungus could affect seed germination and seedling growth. Additionally, seed-borne fungi influence negatively seeds in storage conditions. They reduce the germination level and cause the changes in shape, color and biochemical structure of the seeds. Moreover, they produce toxins negatively affecting health in both humans and animals. Seed infected with pathogen is the source of many diseases in the world. Seed-borne pathogens can survive for a long time on them. These seed diseases can be controlled by pathogen-free seed and phytosanitary testing.

It is considered that *Phomopsis* seed decay of soybean causing the *Phomopsis longicolla* is one of the most economically important disease of soybean. Seedborne diseases caused by many fungal pathogens have increasingly affected soybean production in Turkey. Especially, seed-producing companies and farmers demand to develop efficient control methods against seedborne fungi in soybean production however; there is little knowledge about the fungal species present in soybean seeds in Turkey. For this reason, this study was carried out to identify fungus species in soybean seeds using morphological and molecular methods.

2. Materials and Methods

2.1. Isolation and identification of fungal species

Fungal species were isolated from field-grown soybean seed in the experimental field of Akdeniz University, Antalya, Turkey. Approximately, 150 randomly choosen from seeds that were shown some disease symptoms harvested from the field were sterilized with 70% ethanol for 1 min, followed by 10% sodium hypochlorite for 1 min and rinsed with sterile distilled water, and then placed on Petri plates comprising potato dextrose agar (PDA). Five seeds were placed on each Petri plate and incubated at 24 °C for seven days under 12 hours of light and dark cycles. After the incubation period, the cultures were firstly examined under light microscope and then identified by observing their growth characteristics in the culture and then identified by observing their growth characteristics in the culture (Barnett and Hunter, 1987). The contamination rate (CR) in soybean seeds was calculated according to the Equation 1.

$$CR = NIS/TNS \quad (1)$$

Where NIS is the number of infected seeds, and TNS is the total number of seeds

2.2. Pathogenicity test of the isolates

Pathogenicity testing was conducted using a modified agar slant method in the test tube with PDA amended in vitro conditions (Porter et al., 2015). A piece of mycelium of *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. isolates grown on PDA for 7 days were placed at the bottom of the tubes, and soybean seeds surface-sterilized were placed on the prepared inoculum tubes. After 10-14 days, it was determined that soybean seeds were in decay or not.

2.3. Molecular analyses

Genomic DNA was extracted from fungal single spore cultures by using the modified Cetyltrimethyl Ammonium Bromide (CTAB) method (Catal et al., 2010). The quality and quantity of DNA extracted from fungal isolates were controlled with 1% agarose gel electrophoresis and then they were suspended with sterile distilled water and kept at-20 ºC until use. The rDNA internal transcribed spacer (ITS) regions (ITS-1, 5.8S rDNA subunit, ITS-2) of the fungal isolates were amplified with the ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White et al., 1990). The total volume of the polymerase chain reaction (PCR) reaction mixture was adjusted 20 µl consisting of 11.8 µl water, 2 µl 10X PCR buffer, 1.5 µl $MgCI_2$ (1.5 mM), 1.5 µl of the dNTPs mix (0.25 mM), 0.5 µl each primer (10 pmol), 0.2 µl Taq DNA polymerase (0.5 Units) and 2 µl fungal DNA template. PCR amplifications were conducted by the following: initial denaturation at 94 ºC for 5 min, 30 cycles of 94 ºC for 30 s, 55 ºC for 30 s, 72 ºC for 1 min, and a final extension of 10 min at 72 ºC. The amplification of PCR product was separated on 1.5% agarose gel staining with ethidium bromide by gel electrophoresis and visualized under ultraviolet light. The PCR products were then sent to BMLabosis (Ankara, Turkey) for Sanger sequencing. Sequences results were edited with software Chromas version 2.6.6 with default sequence peak parameters (Technelysium, Australia) and alignment with the BioEdit software

(Hall, 1999) and then these sequences were compared with the retrieved from the nucleotide database in GenBank (Table 1).

2.4. Phylogenetic analysis

Nucleotide sequences of the Internal transcribed spacer (ITS) gene region of the fungi showing homology by the BLAST search program were designated as reference sequences that were obtained from the DNA database of GenBank in National Center for Biotechnology Information (NCBI). The determined nucleotide sequences of the isolates in this study and reference sequences were subjected to multiple nucleotide sequence alignment using the MEGA 7 software (Kumar et al., 2016). According to the aligned sequences, a phylogenetic tree was formed using this program to analyze the relationship between the isolates and reference isolates. Phylogenetic tree construction was performed using the neighbor-joining method (Saitou and Nei, 1987) based on the nucleotide sequence of the ITS gene region. Codon positions contained were $1^{st}+2^{nd}+3^{rd}$ noncoding. All positions involving gaps and missing data were ignored. Bootstrap values were inferred from 1000 replicates for clade reliability of the phylogenetic tree.

Table 1. Reference sequences obtained from the DNA database of GenBank in NCBI

Species	Isolate/Strain	GenBank accession no
Cladosporium cladosporioides	AC1*	MH125284
Cladosporium cladosporioides	DETSC1B	KT877405
Cladosporium cladosporioides	CBS112388	HM148003
Cladosporium cladosporioides	CBS113738	HM148004
Cladosporium cladosporioides	CBS143.35	HM148011
Cladosporium cladosporioides	CBS11398	HM148024
Cladosporium cladosporioides	CBS117134	HM148156
Cladosporium cladosporioides	CBS117153	HM148157
Cladosporium cladosporioides	CBS674.82	HM148014
Phomopsis vaccinia	CBS160.32	AF317578
Fusarium proliferatum	RU1**	MH013301
Fusarium proliferatum	CBS 246.61	MH869604
Fusarium proliferatum	CBS 240.64	MH870056
Fusarium proliferatum	CBS 186.56	MH869117
Fusarium proliferatum	CBS 266.54	MH868863
Fusarium proliferatum	CBS 265.54	MH868862
Fusarium proliferatum	CBS 189.38	MH867442
Fusarium proliferatum	CBS 264.54	MH868861
Fusarium proliferatum	CBS 181.35	MH867142
Fusarium proliferatum	CBS 181.30	MH866554
Fusarium proliferatum	CBS 181.31	MH866624
Fusarium solani	MRC 2565	MH582420

*: *Cladosporium cladosporioides* AC1 isolate, **: *Fusarium proliferatum* RU1 isolate are sequenced in this study

3. Results and Discussion

In this study, some disease symptoms were observed on soybean pods in the experimental field. The soybean pods and seeds with the disease were collected to detect (Figure 1). The results demonstrated that a total of four seed-borne fungal species were found in 150 seeds attained from different randomly picked soybean varieties in the experimental area at Akdeniz University. Based on the morphologic features, these fungi species *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. were identified on soybean seeds (Figure 2). Among the 150 soybean seeds tested, 18 seeds (12%) were confirmed to be not contaminated while 132 seeds (88%) were contaminated with fungi. A total of 33 (25%) out of the 132 soybean seeds were infected with multiple fungi. In this study, 11 seeds were contaminated by both *Aspergillus* spp. and *Cladosporium* spp., and 10 seeds were contaminated with *Fusarium* spp. and *Cladosporium* spp., and seven seeds were contaminated with *Aspergillus* spp. and *Penicillium* spp., and five seeds were contaminated with *Fusarium* spp. and *Penicillium* spp. The tested soybean seeds were found to be infected with every four fungal colonies at different rates. The infection rate of *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. were 42%, 38%, 10%, and 0.9% in soybean seed respectively. Many studies have been carried out to investigate fungal pathogens in soybean seeds and the result of these studies, *Fusarium* was found as the most frequency species in infected seeds (Pedrozo et al., 2015; Chiotta et al., 2016; Pedrozo and Little, 2017). In conjunction with this, we found that *Fusarium* spp. was detected to have a high frequency in soybean seeds. Similiarly, the current

Figure 1. Symptoms of soybean pods (a) and soybean seeds (b)

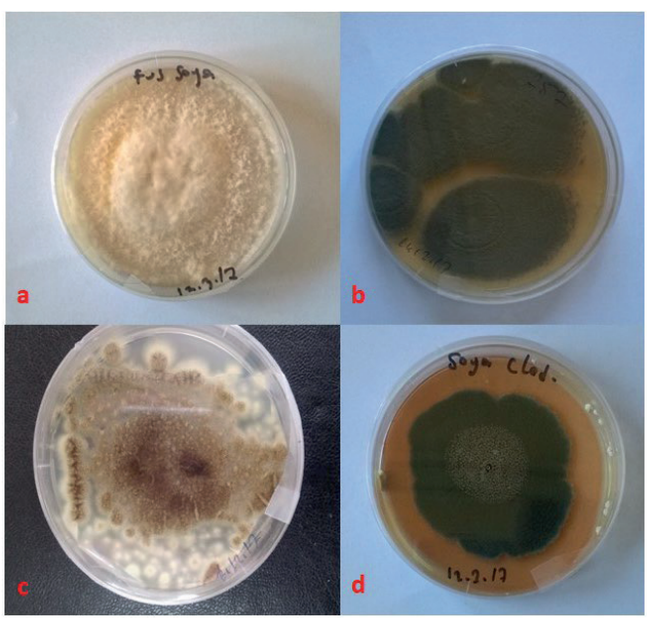

Figure 2. Fungi isolated from soybean seeds: The culture images of *Fusarium proliferatum* (a), *Penicillium* spp. (b), *Aspergillus* spp. (c), and *Cladosporium cladosporiodes* (d)

study showed that *Cladosporium* spp. was the more dominant species in seeds. Molecular studies were done by PCR. According to the pathogenicity test results, fungal mycelia of *F. proliferatum* (RU1) and *C. clodosporioides* (AC1) isolates caused the seed rot. The other isolates, *Aspergillus* spp. and *Penicillium* spp, not cause seed rot. PCR amplification of the ITS region with the ITS-1 and ITS-4 primers were amplified about 500-550-bp fragment (Figure 3). Amplified PCR products belonging to *Cladosporium* spp. and *Fusarium* spp. were sequenced. The sequences for the *C. cladosporiodes* AC1 and *F. proliferatum* RU1 isolates were recorded in the NCBI database with accession numbers MH125284 and MH013301, respectively. Two sequences were subjected to a BLAST search in the GenBank database (NCBI) and multiple alignments were performed using the BioEdit software (Hall, 1999). Blast analysis confirmed that AC1 isolate shows 99% similarity to the sequences of the *C. cladosporiodes* isolates (Figure 3) in GenBank (GenBank accession no KT877405 – HM148015). Similarly, the RU1 isolate shows 99% identity to the sequences of *F. proliferatum* isolates (Figure 4) in GenBank (GenBank accession no MH869604-MH866624). The phylogenetic tree was constructed by using isolates retrieved from the Genbank (Table 1).

Soybean diseases caused by the seedborne fungi both decrease seed quality and provide the primary inoculum for seedborne pathogens (Liu et al., 2016). Related to this, the movement of the seedborne fungal pathogens in soybean that is spread out through the infected seed across the world brings economic losses in soybean production (Munkvold, 2009). The identification of seedborne fungal disease associated with soybeans is important for the management and the

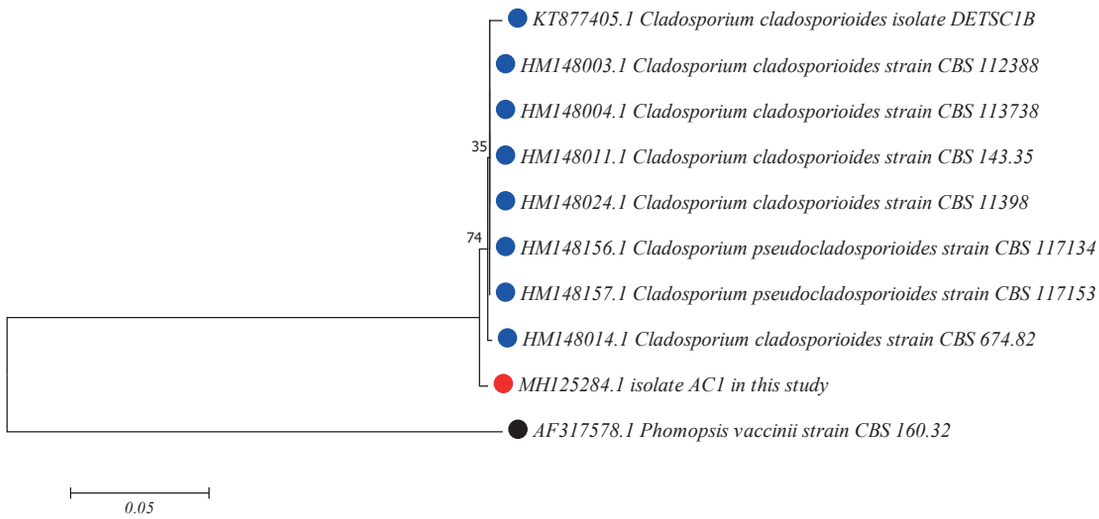

Figure 3. Phylogenetic tree for the *Cladosporium cladosporiodes* isolate AC1, and related *Cladosporium cladosporiodes* isolates based on the neighbor-joining analysis of ITS region sequence using MEGA 7 program*

*: The numbers at the nodes state the bootstrap value for 1.000 repetitions. The 0.05 scale bar shows substitutions per nucleotide position, the outgroup is *Phomopsis vaccinia*

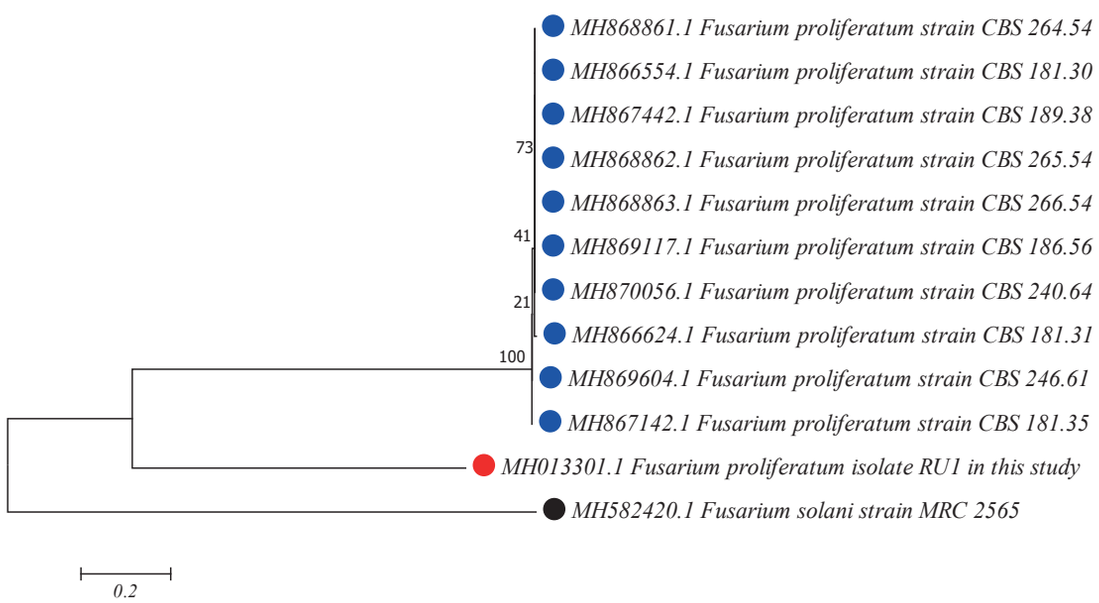

Figure 4. Phylogenetic tree for the *Fusarium proliferatum* isolate RU1, and related *Cladosporium cladosporiodes* isolates based on the neighbor-joining analysis of ITS region sequence using MEGA 7 program*

*: The numbers at the nodes state the bootstrap value for 1.000 repetitions. The 0.2 scale bar shows substitutions per nucleotide position, the outgroup is *Fusarium solani*

development of the resistant varieties. Chang et al. (2020) found that the genus of the *Fusarium* was the dominant seedborne fungi (55%) in seeds of 12 soybean cultivars collected from the three growing regions of China, which were followed by *Colletotrichum*, *Alternaria*, *Phomopsis* and the other genus. Furthermore; different *Fusarium* species, *F. fujikuroi*, *F. incarnatum*, *F. proliferatum*, *F. verticillioides*, and *F. asiaticum* were molecularly identified and *F. fujikuroi* (51.2%) was found more prevalent in soybean varieties growing in China. In a similar study conducted by Escamilla et al. (2019), seedborne pathogens *F. equiseti*, *F. chlamydosporum*, and *F. proliferatum* were isolated from the on sprout soybean seeds. It was reported that *F. proliferatum*

producing many mycotoxins were highly toxic for grains (Wicklow et al., 1987). It was stated that *P. citrinum*, *C. cladosporioides*, and *Phoma* spp. are significant storage fungus of soybean seeds (Pitt et al., 1994; Kim et al., 2013) however, *Phoma* spp. is ubiquitous species and rarely pathogenic and generally observed on diseased and dead plant materials (Kövics et al., 2014). In our study, *Cladosporium cladosporiodes* and *Fusarium proliferatum* were identified from the soybean seeds and the frequency of them had more dominant than *Aspergillus* spp. and *Penicillium* spp. The frequency of seedborne pathogens in seed samples generally varies depending on the environmental conditions, host genotype, and agricultural practices. Levic et al. (2012) reported that a total of 41 species of fungi were isolated from barley, corn, soybean, and sunflower seeds collected in different locations of Serbia. Species belonging to genera *Alternaria*, *Chaetomium*, *Epicoccum*, *Fusarium*, *Penicillium*, and *Rhizopus* are isolated from these seeds and consequently, *Alternaria* species, *F. oxysporum*, *F. semitectum*, and *F. sporotrichioides* were prevalent on soybean seeds. In addition to this, a total of five fungi species mainly *Aspergillus flavus*, *Aspergillus niger*, *Fusarium* spp., *Penicillium* spp., and *Rhizopus* spp. were detected and among which *A. flavus* and *A. niger* were highly observed on soybean seeds (Alemu, 2014). According to Ahammed et al. (2006), germination of soybean seed was significantly reduced by *Fusarium* spp. *Rhizoctonia* spp. and *Alternaria* spp. Moreover, they reported that *Alternaria* spp., *Aspergillus flavus*, *Aspergillus niger*, *Curvularia lunata*, *Fusarium* spp., *Rhizoctonia* spp., *Rhizopus stolonifer*, *Penicillium* spp. were found to be associated with the seeds of the soybean. Ahmed et al. (2016) conducted a similar work to identify fungal species on soybean cultivars and they reported that *Aspergillus* spp., *Curvularia* spp., *Fusarium* spp., *Penicillium* spp., and *Phomopsis* spp. isolated from the fifteen soybean cultivars. Results of this study were similar to the previously reported studies that were mentioned above (Ahammed et al., 2006; Levic et al., 2012; Alemu, 2014). There is little knowledge about the fungal species related to soybean seeds in Turkey. For this reason, this study is planned to detect seedborne fungi on seeds. In the present study, seedborne fungi species on soybean seeds were successfully identified on both morphological and molecular levels.

4. Conclusions

Major yield-limiting factors for soybean production are seedborne diseases that are extensively observed in different regions of the world. The current study demonstrated that fungi species *Aspergillus* spp., *Penicillium* spp., *Cladosporium* spp., and *Fusarium* spp. were determined in soybean seeds. Based on the sequences analysis of the ITS region, *Cladosporium cladosporiodes* and *Fusarium proliferatum* that were pathogenic species on soybean seeds were identified from naturally infected soybean seeds in Antalya province western region of Turkey. This study is one of the few studies to determine the seedborne fungi in soybean seeds in Turkey. According to high prevalence of seed-borne diseases on the samples we examined, there is a need for routine seed testing to control the disease spread. These findings can contribute to the next research conducted with the soybean seed disease management and resistance breeding studies.

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