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Araştırma Makalesi

Identification of Non-Pathogenic Fungal Agents on cotton Based On Morphological and MALDI-TOF Mass Spectrometry Method

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ABSTRACT

In this study, morphological and Matrix-Assisted Laser Desorption/Ionization (MALDI-TOF) MS identification and comparison of non-pathogenic fungal species isolated from diseased root, leaf and boll tissues of cotton plants were carried out. For this purpose, surveys were conducted in Bağlar, Bismil, Çınar, Eğil, Ergani, Kayapınar, Silvan, Sur and Yenişehir districts of Diyarbakır province where cotton production is intensive between June and September 2020 and 2021. 209 samples of plants showing typical fungal disease symptoms were collected from 75 different cotton production areas. A total of 171 fungal isolates were obtained by isolation, culture and purification procedures from diseased plant tissues in the samples. The 20 isolates that were negative in the host pathogenicity test were identified and compared by morphological (traditional) and MALDI-TOF MS methods. According to the results; *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Aspergillus minisclerotigenes*, *Penicillium nalgıovense*, *Chaetomium globosum*, *Dichotomopilus funicola*, *Arthroderma gloria*, *Pseudogmnoacus pannorum*, *Tichophyton interdigitale*, and *Penicillium* sp. were found to be intensively colonized in different parts of cotton such as leaves, bolls and roots. Again, it was determined that the most common species among the total saprophyte isolates was *Aspergillus niger* with a high similarity rate.

Anahtar kelimeler: Cotton, non pathogen, fungal, isolate

Pamukta Patojen Olmayan Fungal Etmen İzolatlarının Morfolojik ve MALDİ-TOF Kütle Spektrometre Yöntemine Bağlı Tanımlanması

ÖZET

Bu çalışmada pamuk bitkisinin hastalıklı kök, yaprak ve koza dokularından izole edilmekle beraber, patojen özellik göstermeyen fungus türlerinin morfolojik ve Matriks-Destekli Lazer Desorpsiyon/İyonizasyonu (MALDI-TOF MS) yöntemine bağlı teşhis ve karşılaştırması yapılmıştır. Bu amaç için 2020 ve 2021 yılları Haziran – Eylül ayları arasında pamuk üretiminin yoğun olarak yapıldığı Diyarbakır ilinin Bağlar, Bismil, Çınar, Eğil, Ergani, Kayapınar, Silvan, Sur ve Yenişehir ilçelerinde sörveyler yapılmıştır. 75 farklı pamuk üretim alanından tipik fungal hastalık belirtileri gösteren bitkilerden 209 adet örnek toplanmıştır. Örneklerdeki hastalıklı bitki dokularından izolasyon, kültür ve saflaştırma işlemleri yapılarak toplam da 171 adet fungal izolat elde edilmiştir. Ana konukçudaki patogenisite testinde negatif çıkan 20 izolat morfolojik (geleneksel) ve MALDI-TOF MS yöntemiyle teşhis edilmiş ve karşılaştırılmıştır. Sonuçlara göre, *Aspergıllus niger*, *Aspergillus flavus*, *Aspergıllus terreus*, *Aspergillus fumigatus*, *Aspergıllus minisclerotigenes*, *Penicillium nalgıovense*, *Chaetomium globosum*,*Dichotomopilus funicola*, *Arthroderma gloria*, *Pseudogmnoacus pannorum*, *Tichophyton interdigitale* türleriyle beraber *Penicillium* sp. cinsine ait fungus türlerinin pamuğun hastalık belirtisi sergileyen yaprak koza ve kök gibi farklı kısımlarında yoğun olarak kolonize olduğu belirlenmiştir. Yine saprofit izolatlar içerisinde en yaygın türün ise Aspergillus niger olduğu yüksek benzerlik oranı ile tespit edilmiştir.

Key words: Pamuk, patojen olmayan, fungal, izolat.

INTRODUCTION

According to archaeological findings, cotton has been cultivated more than five thousand years ago and is the most important fiber plant in the world. This shows that cotton has been the most widely used plant in the field of plant-based textiles since ancient times. Today cotton, which constitutes the raw material of more than 50 industrial branches such as textiles, paper, oil, gunpowder and film, some of which are in the field of nano-technology, is still mostly used in textiles. From this point of view, it is considered to be the world's most important value-added plant that provides jobs and livelihood opportunities for millions of people in the processes from the first planting in the field to harvesting and transformation into finished products in industrial branches (Alamer et al, 2023; Anonymous, 2009; Anonymous, 2017; Anonymous, 2019; Anonymous, 2024a; Anonymous, 2024b)

As a raw material, cotton is used in many industries such as the cotton ginning industry, textile industry, paper industry, oil and feed industry. Considering the continuous increase in the world population, cotton always increases its value as a clothing and foodstuff. On the other hand, cotton is seen as an alternative and pioneering plant for organic production initiated under the leadership of developed countries to compensate for the damages caused by chemical fertilizers and pesticides that have been increasingly used in agricultural production since the 20th century (Harem, 2014; Sharratt and Auvermann, 2014).

Cotton production in Turkey averages around 2,200,000 tons and slightly more than half (55.1%) of the production is obtained in the Southeastern Anatolia region. On the other hand, Turkey ranks sixth in terms of cotton production and fourth in terms of cotton consumption in the world (Kaya, 2020; Shahbandeh, 2019).

Yield losses due to diseases in cotton vary according to the type of agent, region and years. Although the losses caused by general diseases seem to be low, there are disease agents that are specific to some agent types and cause high losses in certain areas. This is important in terms of directly affecting individual producers. According to an evaluation study covering 10 years in the USA, it was determined that cotton suffered an annual average of 3.1% crop loss due to diseases and 27% of these were caused by seedling stage diseases (Devay, 2001).

Accordingly, some fungal agents may be present, both previously unknown and subsequently introduced. On the other hand, cotton may be damaged as a result of the attack or impact of a primary factor, living or non-living, and consequently may be subject to weakness and attack by saprophytic species. There are some saprophytic species that limit the quantity and quality of cotton fiber. Considering cotton production as a whole, saprophytes as well as parasitic species are considered to be important in reducing yield and reducing quality, and it is aimed to determine their species.

About 20 fungal diseases have been identified in cotton, one of the most important cultivated plants for the world and our country. The most important of these are systemic cotton diseases (*Verticillium* and *Fusarium* wilts) and seedling root rot disease agents (*Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., *Thielaviopsis basicola*, *Alternaria* spp., *Aspergillus* spp.), which are known as the most destructive disease in the world, cause significant economic damage in cotton production (Erdoğan, 2009; Yılmaz, 2009).

As a result, as described above, the identification of fungal saprophytic agents isolated from the diseased parts of the roots, root collars, leaves and bolls of cotton is necessary for the continuity of healthy production.

In addition to morphological and pathogenicity testing following cultivation on PDA media, molecular methods based on nucleic acids and spectral methods based on proteins have been used in recent years for the identification of fungi. One of these is the MALDI-TOF mass spectrometry (MS) method. The method, which is derived from the abbreviation of 'Matrix-assisted laser desorption ionization time of flight mass spectrometry' in English, is based on the comparison of protein volumes of living organisms and is performed with MS device. After being subjected to MS, the molecules (proteins, peptides, sugars, polymers, dendrimers, macromolecules) of microorganisms are ionized, and after being passed through a magnetic field, the volumetric sequences (profiles) of the proteins are revealed. The results are compared with reference organisms stored in the database of the device, and depending on the agreement rate, they can be identified at the genus and species level. Recently, MALDI-TOF MS has been used to identify fungi, bacteria, mycobacteria and viruses (Anonymous, 2009; Anonymous, 2019; Vidal et al, 2018; Yılmaz et al, 2014).

It has been reported that the rate of correct identification of fungi on species basis by MALDI-TOF MS method is higher especially in the genus Candida, while it is partially lower in the genera *Fusarium*, *Penicillium* and *Aspergillus* due to the lack of reference spectrum. However, in many recent studies, it is seen that many *Aspergillus* species have been identified using MALDI-TOF MS method and the reference spectrum has been tried to be expanded (Atalay et al, 2016; Li et al, 2016; Li et al, 2017; Park et al, 2017; Vidal et al, 2018).

MATERIALS AND METHODS

The fungal isolates constituting the material of the study were obtained from the production areas where fungal disease symptoms were observed on roots, leaves and bolls in Diyarbakır center and districts where cotton production is intensive (Bağlar, Sur, Çınar, Bismil, Yenişehir, Ergani, Eğil, Kayapınar, Silvan). Samples were taken from different parts of the cotton plant such as roots, leaves and bolls where typical symptoms of fungal agents such as browning, wilting and decay were observed and all kinds of culture media,tools,equipment and diagnosis of isolated formed the material of the study. The surveys were carried out in the cotton production areas of the province between 2020-2021 in the period between the post-flowering and boll setting periods of the plants.

Based on the prominent symptons in the samples brought to the laboratory after being preserved with ice molds in the cooling unit, isolation studies were carried out as soon as possible for the samples that were thought to be diseased. In the isolation process of the samples showing obvious signs of disease, these parts of the plants showing signs of disease were first exposed to tap water for 30-45 seconds (s), The tissues were cleaned from coarse dirt and then cut into 3-5 millimeter (mm) lengths to include diseased and viable tissue, immersed in 2% sodium hypochlorite (NaOCl) solution for 2 minutes (min), rinsed twice in sterile distilled water and then dried with sterile blotting paper and transferred to the media. Potato Dextrose Agar (PDA) was used as general medium for isolations. Potato Dextrose Agar (PDA) media were prepared according to the manufacturer's recommendation and after sterilization in autoclave at 121 ˚C for 15 min, the samples were transferred to sterile Petri dishes in sterile environment and the mouths of the Petri dishes were covered with parafilm.

In order to determine whether the purified fungal isolates were plant pathogens or not, they were brought to the climate chamber of Dicle University Faculty of Agriculture for pathogenicity test. The soil used for the pathogenicity test carried out within the scope of the pot experiment was treated with 36% formaldehyde for sterilization and kept in sealed polyethylene bags for 48 hours and then the soil was aerated for 48 hours to prevent residue in the soil. 1 liter plastic pots were used in the study. For the sterilization of the pots, the pots were kept in tubs filled with water containing 1% NaClO for 1 day, rinsed and dried. The sterilized pots were filled with sterile soil. Then, the roots of the cotton plant, which were previously planted and germinated in the vials, were slightly shaved and transferred to the pots after 45-60 s in the containers containing the isolates transferred to 10 milliliter (ml) of pure water at a certain rate.

Stonvil 468 seed variety, which is a susceptible variety and widely used in the region, was used and the development of the diseases was followed. For the sterilization of the cotton seeds used in the experiment, the seeds were kept in 1% NaClO solution for 3 minutes and then passed through sterile pure water 6 times and dried on blotting paper.

After the seeds germinated in viols became seedlings, they were transferred to sterile pots and incubated in climate chamber conditions at 25-28 ˚C and 60-80% relative humidity. In cotton plants, browning of the inoculated roots started to be observed within 4-5 weeks. Depending on the decay in the roots, yellowing of the leaves, growth retardation, yellowing, shedding and finally drying of the plants were observed and these were considered as positive reactions.

Figure 1. Images of Saprophytic Fungi in PDA Medium

At the end of the 2-7 day incubation period, fungal growth was checked and recorded. The samples with negative pathogenicity tests were identified by conventional methods and purified for MALDI-TOF analysis for molecular identification and identified in the Department of Plant Health of Hatay Mustafa Kemal University.

RESULTS AND DISCUSSION

In the study, a total of 209 plant samples were collected from different plant tissues from samples with typical fungal disease symptoms on roots, leaves and bolls in Diyarbakır center and districts where cotton production is intense (Bağlar, Sur, Çınar, Bismil, Yenişehir, Ergani, Eğil, Kayapınar, Silvan). The fungal isolates obtained from the collected samples as a result of isolation and purification studies were subjected to pathogenicity tests on plants grown in the climate chamber and ın such a way as to represent the regıon where ıt ıs obtaıned ın the plant 20 samples were selected from the isolates with negative results. The highest number of fungal isolates were obtained from leaf (8), followed by root (6) and boll (6).

Table 1.Comparison of morphological identificatıon results and MALDI-TOF MASS spectometer identificatıon results of the ısolates

No	Plant Isolated from	Diagnostic Results of the Isolates based on MALDI-TOF Mass Spectrometry Method (Species, Genus names)	MALDI-TOF Similarity Index	Diagnostic Results of Isolates based on Traditional (Morphological) Method (Species, Genus names)	Plant Tissue Isolated from
1	Cotton	Aspergillus flavus	1,228	Aspergillius sp.	Root
2	Cotton	Chaetomium globosum	1,170	Unidentified	Root
3	Cotton	Aspergillus niger	2,061	Aspergillus niger	Leaf
4	Cotton	Aspergillus terreus	1,061	Aspergillius sp.	Cocoon
5	Cotton	Aspergillus niger	2,317	Aspergillus niger	Leaf
6	Cotton	Penicillium nalgiovense	0,977	Penicillium sp.	Cocoon
7	Cotton	Dichotomopilus funicola	1,086	Unidentified	Cocoon
8	Cotton	Arthroderma gloria	1,061	Unidentified	Leaf
9	Cotton	Pseudogmnoacus pannorum	0,937	Unidentified	Leaf
10	Cotton	Aspergillus niger	1,56	Aspergillus niger	Cocoon
11	Cotton	Aspergillus niger	1,285	Aspergillus niger	Leaf
12	Cotton	Aspergillus fumigatus	1,10	Aspergillus sp.	Leaf
13	Cotton	Aspergillus niger	1,036	Aspergillus niger	Cocoon
14	Cotton	Aspergillus fumigatus	1,087	Aspergillus sp.	Root
15	Cotton	Aspergillus fumigatus	1,097	Aspergillus sp.	Root
16	Cotton	Penicillium sp.	1,056	Penicillium sp.	Leaf
17	Cotton	Tichophyton interdigitale	1,119	Unidentified	Leaf
18	Cotton	Aspergillus sp.	0,993	Aspergillus sp.	Cocoon
19	Cotton	Aspergillus fumigatus	1,057	Aspergillus sp.	Root
20	Cotton	Aspergillus minisclerotigenes	0,927	Unidentified	Root

Fungal isolates, which were determined to be non-pathogenic by the host pathogenicity tests, were tried to be identified by MALDI-TOF analysis method.

However, as is well known, the MALDI-TOF method involves the ionization of proteins of microorganisms by laser pulses that are passed through electromagnetic tubes. As the ions, which gain speed in proportion to their mass, hit the detector at different times, signals are generated and these signals generate the mass spectra of the proteins. These spectra are compared with pre-existing spectra in the database to identify the genus and species (Pelit et al, 2017; Vidal et al., 2018; Yılmaz et al., 2014).

In their study, Kim et al. (2014) confirmed 26 Candida spp. isolates identified as C. famata with VITEK-2 by using the MALDI-TOF MS method and confirmed them with gene sequence analysis. In the study, it was stated that the strains identified by VITEK-2 as C. famata showed 100% homology with C. guilliermondii in gene sequence analysis, and MALDI-TOF MS correctly identified 21 of the strains, but could not identify 4 isolates at an 'acceptable level'.

Atalay et al. conducted a similar study in 2016 and as a result of this study, it was stated that it was evident that MALDI-TOF and rep-PCR methods showed a positive agreement with the traditional method in identifying Aspergillus species (Atalay et al., 2016).

It is reported that the rate of correct identification of fungal agents on a species basis is generally higher in candida genus fungi in MALDI-TOF molecular analysis method, while dermatophytes, Aspergillus, Penicillium and Fusarium genus fungi are identified at a lower rate due to the lack of reference spectrum (Özcan et al., 2016).

CONCLUSION

As a result of the study conducted with non-pathogenic fungi isolated from different cotton production areas and different plant tissues, especially Aspergillus genus fungi came to the fore.

According to the MALDI-TOF identification results obtained in this study, the majority of the fungal isolates were Aspergillus (65% of all samples) followed by Penicillium (10% of all samples). On the other hand, the results of the Traditional Method (identification based on macroscopic and microscopic based morphological characteristics) were highly similar to MALDI-TOF.

The conventional approach for identifying fungi was found to be effective, particularly at the species level, when contrasted with MALDI-TOF, a molecular identification technique. Nevertheless, when considering time, the expedited outcomes of MALDI-TOF analysis are advantageous in hastening scientific inquiries. However, the limited database for diagnosing fungal agents and the substantial expense of molecular diagnostics are drawbacks.

Conflict of Interest: The authors of the article declare that they have no conflict of interest.

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