How to cite: Aydoğdu, M. & İ. Kurbetli, 2024. *In vitro* and *in vivo* characterization of charcoal rot disease (*Macrophomina phaseolina*) in sesame. Ege Univ. Ziraat Fak. Derg., 61 (2): 151-163, https://doi.org/10.20289/zfdergi.1432773

Research Article
(Araştırma Makalesi)

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Keywords: Field symptoms, fungal disease, fungal traits, *Sesamum indicum*

Anahtar sözcükler: Tarla belirtileri, fungal hastalık, fungal özellikler, *Sesamum indicum*

Ege Üniv. Ziraat Fak. Derg., 2024, 61 (2):151-163
https://doi.org/10.20289/zfdergi.1432773

In vitro and *in vivo* characterization of charcoal rot disease (*Macrophomina phaseolina*) in sesame

Susamda kömür çürüklüğü hastalığı (*Macrophomina phaseolina*)'nın laboratuvar ve tarla şartlarında karakterizasyonu

Received (Alınış): **06.02.2024** **Accepted** (Kabul Tarihi):**23.04.2024**

ABSTRACT

Objective: This study aimed to characterize charcoal rot pathogen (*Macrophomina phaseolina*) of sesame *in vitro* and *in vivo.*

Material and Methods: In 2017, two isolates of *M. phaseolina* were isolated from symptomatic sesame plants in Aksu district of the Western Mediterranean region of Türkiye. Pathogenicity tests were conducted using cv. Muganlı-57 in a greenhouse. Micromorphological characteristics (colony growth, colony color, mycelium, and microsclerotium) and chlorate sensitivity of the isolates were determined *in vitro*. Effects of temperature on colony growth and symptoms of charcoal rot in the field were also examined in detail.

Results: Significant (P<0.01) differences were detected in microsclerotium length, microsclerotium width, and colony growths of the isolates on minimal medium. Both isolates were characterized as chlorate-sensitive. On minimal medium, Aksus1 isolate displayed a feathery growth pattern, while Aksus2 isolate showed a restricted growth pattern. Temperature significantly (P<0.01) affected colony growth. The highest colony growths were detected at 35°C, while the lowest ones were found at 40°C.

Conclusion: The results may provide new insights into the epidemiology of the charcoal rot disease. To the best of our knowledge, this is the first detailed characterization of microscopic traits and charcoal rot symptoms on sesame in the field in the Western Mediterranean region of Türkiye.

ÖZ

Amaç: Bu çalışma susam bitkisinde kömür çürüklüğü patojeninin (*Macrophomina phaseolina*) laboratuvar ve tarla şartlarında karakterize edilmesini amaçlamıştır.

Materyal ve Yöntem: 2017 yılında, *M. phaseolina*'nın iki izolatı Batı Akdeniz Bölgesi'nde Aksu lokasyonunda hastalık belirtisi gösteren bitkilerden izole edilmiştir. Patojenisite testleri serada Muganlı-57 çeşidi kullanılarak yapılmıştır. İzolatların mikromorfolojik özellikleri (koloni gelişmesi, koloni rengi, miselyum ve mikrosklerot) ve klorat hassasiyetleri laboratuvar koşullarında belirlenmiştir. Sıcaklığın koloni gelişmesi üzerine etkisi ve tarlada kömür çürüklüğü hastalığının belirtileri ayrıca detaylı olarak incelenmiştir.

Araştırma Bulguları: Minimal ortamda izolatların mikrosklerot uzunluğu, mikrosklerot genişliği ve koloni gelişmelerinde önemli (%1) farklılıklar tespit edilmiştir. Her iki izolat klorata hassas olarak karakterize edilmiştir. Minimal ortamda Aksus1 izolatı tüylü bir gelişme deseni gösterirken, Aksus2 izolatı kısıtlanmış bir gelişme deseni göstermiştir. Sıcaklık izolatların koloni gelişmelerini önemli (%1) derecede etkilemiştir. En yüksek koloni gelişmeleri 35°C' de tespit edilirken, en düşük gelişmeler 40°C' de bulunmuştur.

Sonuç: Sonuçlar kömür çürüklüğü hastalığının epidemiyolojisinin anlaşılmasına yeni katkılar sunmaktadır. Bildiğimiz kadarıyla, bu Türkiye'nin Batı Akdeniz Bölgesi'nde susam bitkilerinde kömür çürüklüğü hastalığının mikroskobik özelliklerini ve tarlada belirtilerini detaylı olarak karakterize eden ilk çalışmadır.

INTRODUCTION

Sesame (*Sesamum indicum* L.), belongs to *Pedaliaceae* family, is regarded to be the oldest oilseed crop that is native to Africa (Ram et al., 1990). It is mainly grown for its seed and oil in the seed. Sesame seed contains a high amount of oil (60%), protein (25%), and methionine (Nayar & Mehra, 1970). Due to the climatic requirement (110 to 150 frost-free days), it has been cultivated in warm and temperate regions in 69 countries. Sudan, India Tanzania, Myanmar, and Nigeria are top producers with 1119026, 817000, 700000, 641729, and 440000 tonnes of sesame productions, respectively. With 17657 tonnes of sesame production, Türkiye is 34th in the world (FAO, 2023). Breeding studies related to sesame have been conducted in Türkiye (Yılmaz, 2022), however, one of the major biotic stress factors limiting sesame production is charcoal rot disease caused by *Macrophomina phaseolina* (Tassi).

M. *phaseolina,* the causal agent of charcoal rot disease, is a *cosmopolitan fungus* infecting over 500 plant species (Sarkar et al., 2014), but one of economically important hosts of *M*. *phaseolina* is sesame (Amen et al., 2020). Incidence of the disease can reach up to 39% in sesame fields (Balabaskar et al., 2015). The disease causes significant yield losses ranging from 30 to 75% in sesame cultivation (Linhai et al., 2011; Min & Toyota, 2019). *M*. *phaseolina* is a soilborne fungus overwintering in soil or plant debris and penetrates roots and crown. As a result of the infection, blackish microsclerotia occur within vascular bundle and on stem parts of the plant. Microsclerotia can survive in the soil over ten years, making its management difficult (Shekhar et al., 2006; Gupta et al., 2012). For the management of the fungus in sesame cultivation, various studies related to seed treatment (Ahmed et al., 2010), fungicides (Bashir et al., 2017; Karibasappa et al., 2020), soluble silicon (Siddiq et al., 2019), plant extracts (Elaigwu et al., 2017; Savaliya et al., 2015), biofumigation (Amen et al., 2020), intercropping (Ahmed & Ibrahim, 2020), biological control (Radhakrishnan et al., 2017; Abd & AL-Juboory, 2020) and host-resistance (Bedawy & Moharm, 2019; Farooq et al., 2019; Gupta et al., 2020) have been conducted. However, little is known about epidemiology of disease in Türkiye. In this regard, characterization of *Macrophomina phaseolina* isolates may provide new insights into epidemiology and for the management of the charcoal rot in sesame cultivation. Thus, the aims of this study were i) to characterize symptoms of charcoal rot disease in the field (*in vivo*) and ii) to characterize two isolates of *M. phaseolina in vitro*.

MATERIALS and METHODS

Field observations and sampling

In 2017, during surveys, yellowing and wilting symptoms were observed on adult sesame plants in two sesame growing fields in Aksu district of Antalya Province. Four samples from those adult sesame plants showing wilting symptoms were collected and put into paper bags. The samples were brought to the mycology laboratory of Batı Akdeniz Agricultural Research Institute.

Isolation

Stems including crown and roots from wilting plants were initially cleaned. Later, they were chopped to the sizes 1 to 2 cm and soaked in NaOCl 1% for 1.5 minutes and soaked in sterile water. Ensuing drying on filter papers in a laminar flow cabinet, 10 pieces per plate (9 cm) were placed on potato dextrose agar (PDA) and incubated at 25°C for 6 days. Developing fungal colonies on PDA were subcultured.

Identification

Colony growth and morphological features (mycelium and microsclerotium) were examined using

an Olympus BX43 microscope with SC100 digital colour camera. To confirm morphological identification, molecular diagnosis was also done. DNA extractions of two isolates (Aksus1 and Aksus2) were performed using DNA purification kit of Promega. Later, amplifications were done using universal primers ITS-1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS-4 (5' TCC TCC GCT TAT TGA TATGC 3') (White et al., 1990) in a SimpliAmp Thermocycler (Applied Biosystems, USA). Cycling conditions were one cycle at 94°C for three minutes, thirty-five cycles at 94°C for 30 seconds, at 58.5ºC for one minute, at 72°C for one minute and at 72°C for seven minutes. Analysis of sequence was conducted by GENOKS (Ankara, Türkiye). Relatedness of sequences of the two isolates were compared with other sequences displaying a 99 to 100% homology at genbank (NCBI; https://www.ncbi.nlm.nih.gov/nucleotide/). As a result, a phylogenetic tree was constructed using neighbor-joining method in MEGA version 10.0 program.

Pathogenicity test

To fulfill Koch's postulates, soil inoculation method was used in the pathogenicity tests. Initially, sorghum seeds were dipped in water in a 2-L beaker for one night and then the seeds were put in 250-mL Erlenmeyer flasks and autoclaved at 121°C for 30 min in two consecutive days. Later, 6 mycelial plugs (0.5 cm) of 5 day-old- colonies of each isolate were transferred to the seeds in each Erlenmeyer flask and incubated at 25°C for 21 days (Iqbal et al., 2010). A mixture of soil and vermiculite (1:1) was autoclaved at 121°C for 1 hour in two consecutive days. The mixture was put into pots (15 × 20 cm). Inoculum (infected seeds) was homogeneously put in the pots (5%). Afterwards, 5 seeds (cv. Muganlı-57) per pot were sown. For control, seeds autoclaved were put into the pots (Omar et al., 2007).

Chlorate sensitivity

To determine chlorate sensitivity of the isolates, minimal medium with the content of 120 mM potassium chlorate (KClO3) was used. The medium was prepared as described by Pearson et al. (1986). For control, the same medium not containing KClO3 was adjusted. Mycelial plugs (0.5-cm) of the isolates were attached to the centers of both media and kept at 25°C for 6 days. Colony growths of the isolates on the media were daily recorded. The experimental design was established based on completely randomized design with four replicates.

Temperature tests and statistical analysis

To examine the effects of temperature on colony growth of the isolates, mycelial plugs (0.5 cm) of the isolates were put on PDA and kept at 25, 30, 35 and 40°C for 48 hours. Colony growths of the isolates for each temperature tested were recorded. The experiments were carried out based on the completely randomized design with four replications.

Analysis of variance was performed using SAS 9.1 software program. Averages of microsclerotium length, microsclerotium width, and colony growths of the isolates at different temperatures (25, 30, 35, and 40°C) and minimal medium (potassium chlorate containing and not-containing) in the experiments were grouped according to Fisher's least significant difference test ($LSD_{0.01}$).

RESULTS and DISCUSSION

Charcoal rot symptoms in the field

In 2017, two sesame growing fields (30 da) in Aksu district of Antalya Province were examined for presence of charcoal rot disease. During the flowering and pod emergence period, on 24 July, nearly 15 to 20% of adults plants in both fields showed wilting and stunting symptoms (Figure 1).

Figure 1. Symptoms of charcoal rot disease on adult sesame plants in the field (Aksu district, Antalya Province).

Şekil 1. *Tarlada yetişkin susam bitkilerinde kömür çürüklüğü hastalığının belirtileri (Aksu lokasyonu, Antalya İli).*

As the disease progressed, leaf defoliations and then plant deaths with blacking stems were also observed on the symptomatic plants (Figure 2).

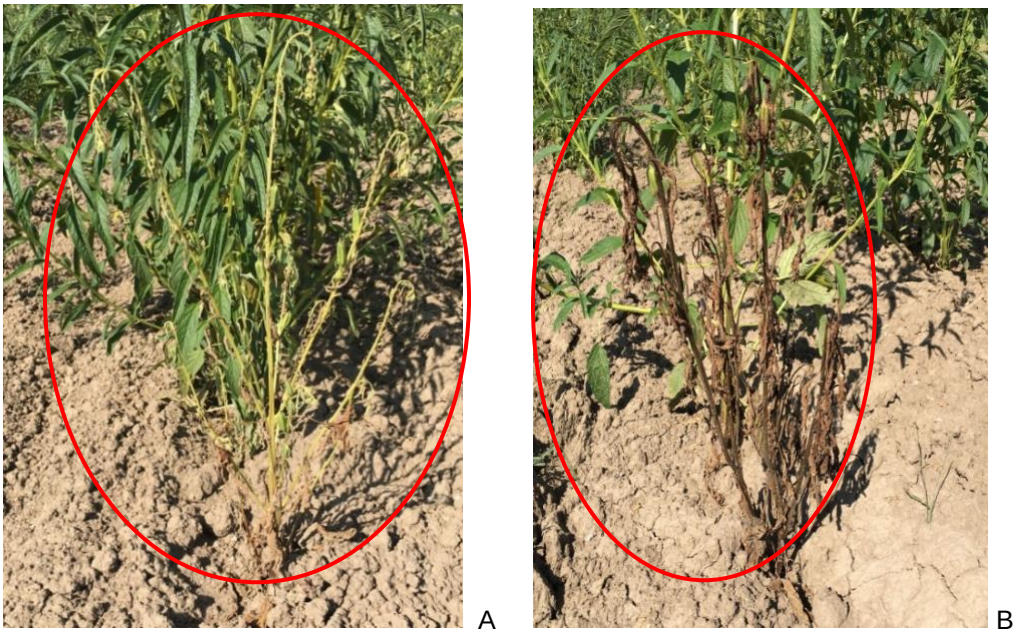

Figure 2. Symptoms of charcoal rot disease on adult sesame plants, (A) Wilting and stunting symptoms of charcoal rot disease on sesame plants, (B) Plant death caused by charcoal rot pathogen (*Macrophomina phaseolina*) in the sesame field.

Şekil 2. *Yetişkin susam bitkilerinde kömür çürüklüğü hastalığının belirtileri, (A) Kömür çürüklüğü hastalığının susam bitkilerinde solma ve cüceleşme belirtileri, (B) Susam tarlasında kömür çürüklüğü patojeni (Macrophomina phaseolina)'nin neden olduğu bitki ölümü.*

In close examination, newly fallen leaves were observed at the beginning of charcoal rot and dry leaves and pods remained on the blacking stems of the adult sesame plants (Figure 3).

Figure 3. Close examination of symptoms of charcoal rot disease on sesame plants in the field, (A) Newly fallen leaves due to charcoal rot disease (with blue arrows) and dry leaves and pods remained on the plant (white arrows), (B) Stem rot caused by *Macrophomina phaseolina* in adult sesame plant.

Şekil 3. *Kömür çürüklüğü hastalığının tarlada susam bitkilerindeki belirtilerinin yakın incelemesi, (A) Kömür çürüklüğü hastalığı nedeniyle yeni düşen yapraklar (mavi oklar) ve bitkide kalan kuruyan yapraklar ve kapsüller (beyaz oklar) (B) Yetişkin susam bitkilerinde Macrophomina phaseolina 'nın neden olduğu sap çürümesi.*

Isolation and identification

As a result of the isolation procedure, two isolates of *Macrophomina phaseolina* were obtained from those symptomatic plants in the field observations. Initially, colonies of both isolates were light in color and then became black. However, colony color of AkSus1 isolate was darker than AkSus2 isolate but aerial mycelium (over 2 mm) of AkSus2 isolate was higher than AkSus1 isolate (Figure 4).

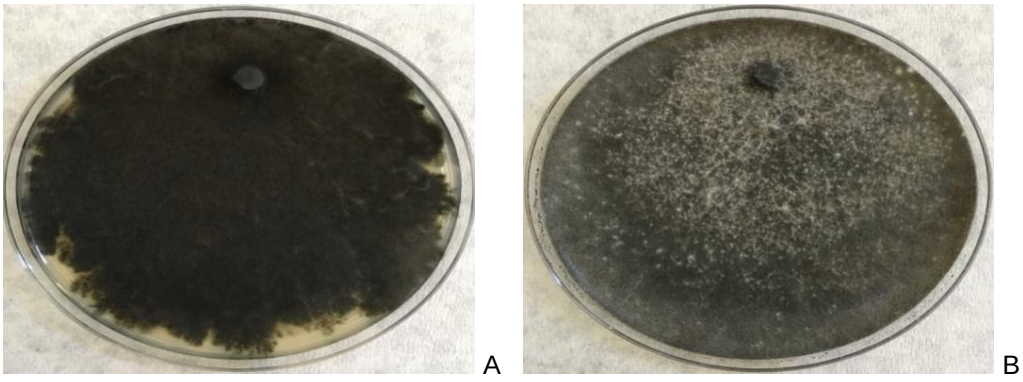

Figure 4. Colony growths (5-day-old) of *Macrophomina phaseolina* isolates on PDA, (A) *M. phaseolina* isolate Aksus1, (B) *M. phaseolina* isolate AkSus2.

Şekil 4. *PDA üzerinde Macrophomina phaseolina izolatlarının beş günlük koloni gelişmeleri, (A) M. phaseolina'nın Aksus1 izolatı, (B) M. phaseolina'nın Aksus2 izolatı.*

Hyphae of both isolates were brown to black, septated and branched. Microsclerotia were brown and then became brown to black. The microsclerotia of both isolates were irregular, however, they were generally spheroid and oblong. Microsclerotia of AkSus1 and AkSus2 isolates were 34.58 × 210.04 µm (average: 98.61 × 135.58 µm) and 37.62 × 233.68 µm (average: 115.45 × 152.36 µm) in diameter, respectively (Figure 5). These micromorphological features were similar to those of *Macrophomina phaseolina* (Dhingra & Sinclair, 1978).

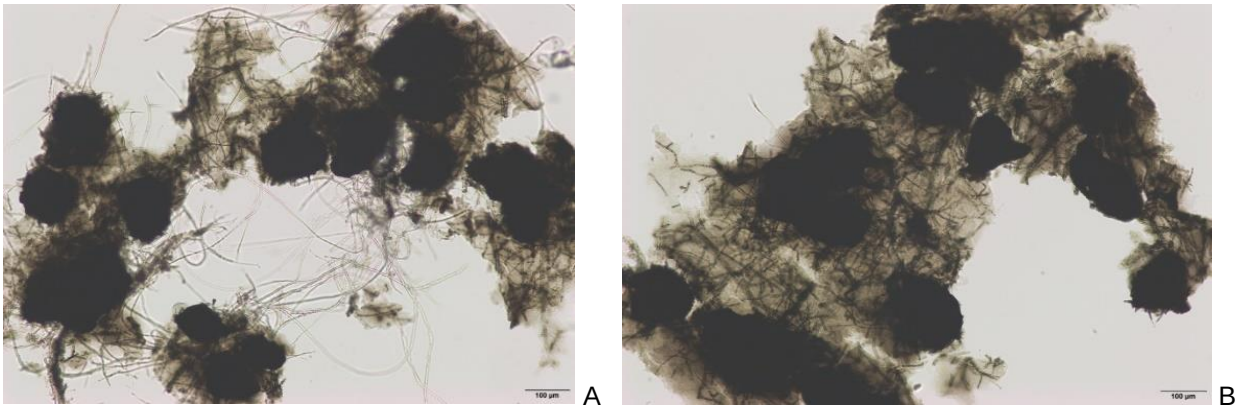

Figure 5. Micromorphological features of both isolates of *Macrophomina phaseolina,* (A) Microsclerotium and mycelium of AkSus1 isolate, (B) Microsclerotium and mycelium of AkSus2 isolate (10×).

Şekil 5. *Her iki Macrophomina phaseolina izolatlarının mikromorfolojik özellikleri, (A) AkSus1 izolatının mikrosklerot ve miselyumu, (B) AkSus2 izolatının mikrosklerot ve miselyumu (10×).*

Comparing size of both isolates, there were significant (P<0.01) differences in both microsclerotium length and width of the isolates. Microsclerotium size of Aksus2 isolate was significantly bigger than Aksus1 (Figure 6).

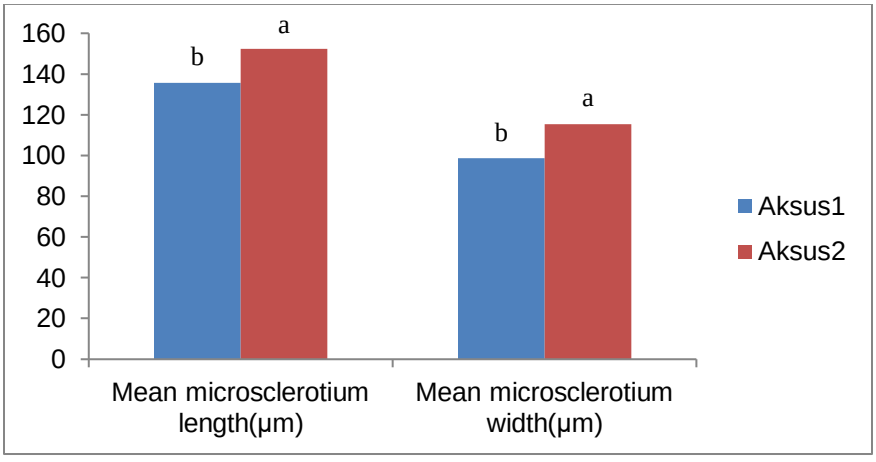

Figure 6. Comparison of microsclerotium sizes of the *M. phaseolina* isolates.

Şekil 6. *M. phaseolina izolatlarının mikrosklerot büyüklüklerinin kıyaslanması.*

Sequence sizes of AkSus1 (acc. no MH593869) and AkSus2 (acc. no MH593870) isolates were 500 and 510 bp respectively and they were registered in the GenBank (http://www.ncbi.nlm.nih.gov). Based on the morphological and molecular data, the isolates were identified as *Macrophomina phaseolina* (Tassi) Goid. In addition, relatedness of the isolates was compared with other *Macrophomina phaseolina* isolates in the Genbank (Figure 7).

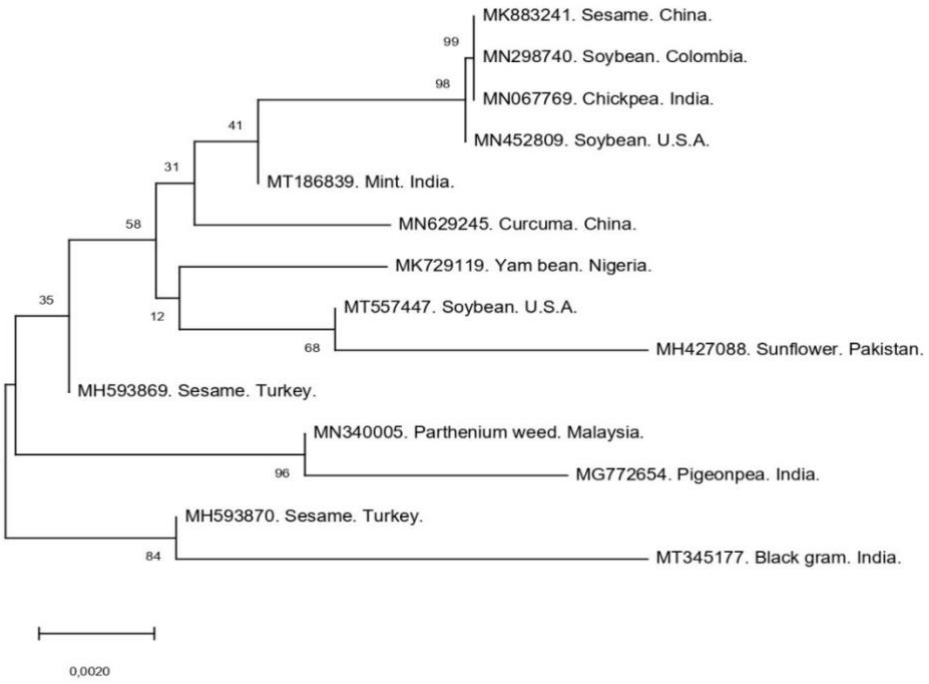

Figure 7. Phylogenetic tree showing relatedness of our *M. phaseolina* isolates (MH593869 and MH593870) with other *Macrophomina phaseolina* isolates in different hosts in the Genbank (https://www.ncbi.nlm.nih.gov).

Şekil 7. *M. phaseolina izolatlarımızın (MH593869 and MH593870) Gen bankasındaki (https://www.ncbi.nlm.nih.gov) farklı konukçulardaki diğer M. phaseolina izolatları ile ilişkisini gösteren filogenetik ağaç.*

Pathogenicity tests

As a result of the soil inoculation method, charcoal rot symptoms occurred on seedlings of cv. Muganlı-57 in inoculated pots. Both isolates were re-isolated from stems of the sesame plants in the inoculated pots, fulfilling Koch's postulates (Figure 8).

Figure 8. Pathogenicity test of *Macrophomina phaseolina* in sesame (cv. Muganlı-57), (A) Wilting plants inoculated with *Macrophomina phaseolina*, (B) Stem rot caused by *Macrophomina phaseolina* in inoculated pot.

Şekil 8. *Macrophomina phaseolina 'nın susamdaki (Muganlı-57 çeşidi) patojenisite testi, (A) Macrophomina phaseolina ile inokule edilen bitkilerde solgunluk, (B) İnokuleli saksılarda Macrophomina phaseolina nedeni ile oluşan sap çürüklüğü.*

Effects of temperature on the isolates of *Macrophomina phaseolina*

In the temperature tests, temperature significantly (P<0.01) affected colonial development of both isolates (Table 1).

Table 1. Analysis of variance for effects of temperature on the isolates of *Macrophomina phaseolina in vitro*.

Çizelge 1. *Macrophomina phaseolina izolatlarına sıcaklık etkisi için varyans analiz tablosu.*

Source	D.F.	Mean of squares	F value	P>F
Isolate	1	38.06**	153.60	P<0.01
Temperature	3	637.56**	2572.75	P<0.01
Isolate × Temperature	3	0.64	2.59	0.0760
Error	24	0.247		
Total	31			

CV(%):1.18 D.F. : Degree of freedom

Colony growths of Aksus1 at 25, 30, 35 and 40°C were 37.27, 48.22, 52.65 and 34.10 mm, respectively, while they were 35.72, 46.22, 50.8 and 31.07 mm in the Aksus2 isolate, respectively. Comparing both isolates, colony growths of Aksus1 isolate were significantly (P<0.01) faster than Aksus2 isolate at each temperature tested (Figure 9).

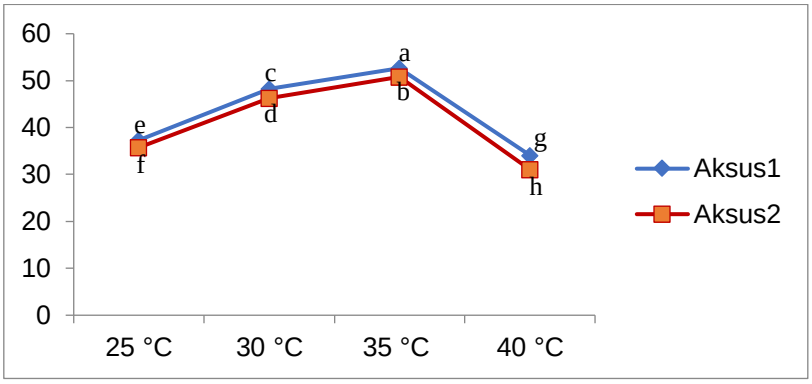

Figure 9. Colony growth (mm) of the isolates of *Macrophomina phaseolina* at 25, 30, 35 and 40°C on PDA for 48 h ($LSD_{0.01}$: 0.69).

Şekil 9. *Macrophomina phaseolina izolatlarının PDA'da 25, 30, 35 and 40°C'lerdeki 48 saatlik koloni gelişmesi (mm).*

Chlorate sensitivity of the isolates of *Macrophomina phaseolina*

That minimal medium with potassium chlorate significantly (P<0.01) affected colonial developments of both isolates. Additionally, isolate, medium and isolate × medium interactions were significant (P<0.01) statistically (Table 2).

Table 2. Analysis of variance for chlorate resistance of *Macrophomina phaseolina* isolates.

Çizelge 2. *Macrophomina phaseolina izolatlarının klorat dayanıklılıkları için varyans analiz tablosu.*

Source	D.F.	Mean of squares	F value	P>F
Isolate	1	75.69**	931.57	P<0.01
Medium	1	3214.89**	39567.9	P<0.01
Isolate × Medium	1	28.62**	352.28	P<0.01
Error	12	0.081		
Total	15			

CV (%) : 1.26 D.F. : Degree of freedom.

Colony growths of both isolates on minimal medium were given in figures 10 and 11.

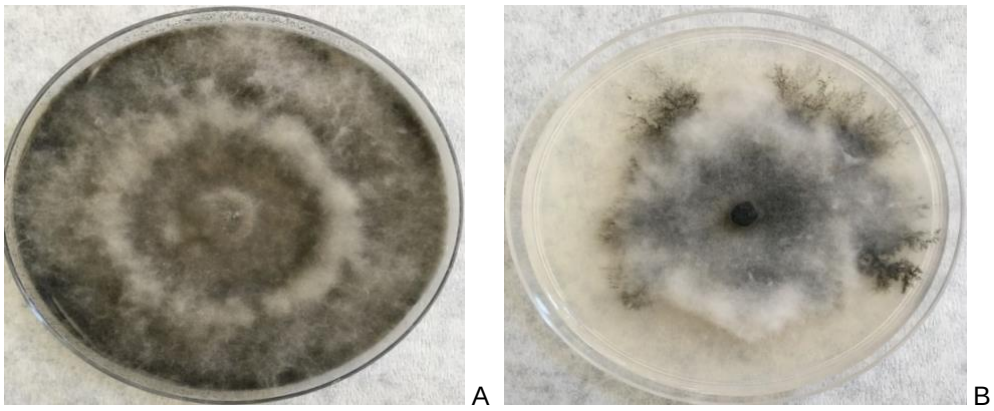

Figure 10. Chlorate sensitivity of *Macrophomina phaseolina* Aksus1 isolate, (A) Colony growth of Aksus1 isolate on minimal medium without potassium chlorate (KClO3), (B) Feathery growth pattern of Aksus1 isolate on minimal medium with KClO3.

Şekil 10. *Macrophomina phaseolina Aksus1 izolatının klorata hassasiyeti A) Aksus1 izolatının potasyum klorat içermeyen minimal ortamda koloni gelişmesi, (B) Aksus1 izolatının potasyum klorat içeren minimal ortamda tüylü gelişme deseni.*

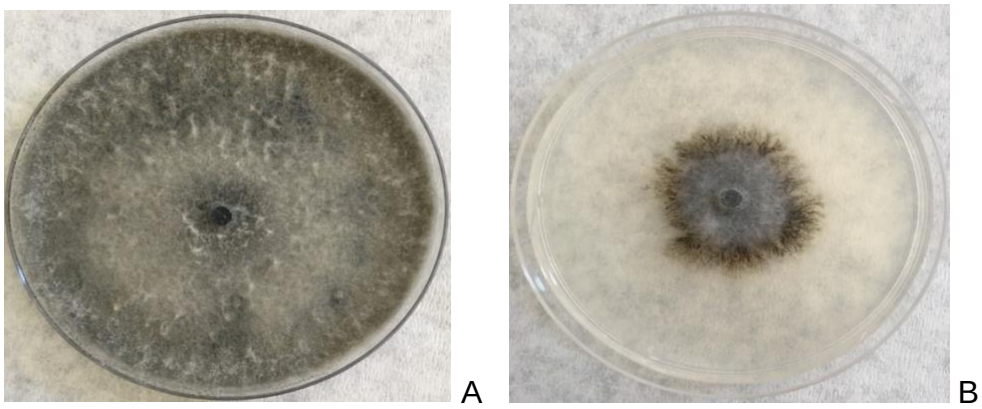

Figure 11. Chlorate sensitivity of *Macrophomina phaseolina* Aksus2 isolate, (A) Colony growth of Aksus2 isolate on minimal medium without KClO3, (B) Restricted growth pattern of Aksus2 isolate on minimal medium with KClO3.

Şekil 11. *Macrophomina phaseolina Aksus2 izolatının klorata hassasiyeti A) Aksus2 izolatının potasyum klorat içermeyen minimal ortamda koloni gelişmesi, (B) Aksus2 izolatının potasyum klorat içeren minimal ortamda kısıtlı gelişme deseni.*

Both isolates were characterized as chlorate-sensitive. Aksus1 isolate displayed a feathery growth pattern on minimal medium containing potassium chlorate, while Aksus2 isolate showed a restricted growth pattern on minimal medium containing potassium chlorate. The differences in their colony growths were statistically significant ($P<0.01$). However, the colonial development of Aksus1 isolate on the minimal medium without KClO3 was 37.60 mm, whereas it was 35.92 mm in Aksus2 isolate (Figure 12).

Karibasappa et al. (2020) reported that after flowering period sudden wilting was the most common symptoms of charcoal rot of sesame and stems became black in severe infections caused by *M. phaseolina*. Likewise, in the present study, during flowering and pod emergence period, on 24 July, wilting and stunting symptoms were observed. As the disease progressed stem discolorations on adult plants bearing most of the leaves and pods were also observed. In addition, leaf defoliations were detected. In fact, these symptoms expressed by the sesame plants might be related to the disease severity of the charcoal rot. Apart from this, environmental factors such as temperature and relative humidity may play an important role in the disease severity of charcoal rot of sesame. However, there is limited information available about them. In this regard, Deepthi et al. (2014) stated that increasing

temperature and decreasing relative humidity were closely associated with lesion sizes caused by *M. phaseoliona* on sesame. Considering environmental conditions prevailing at growing season of sesame, the Mediterranean climate (mean maximum temperature ranging from 21.4 to 34.1°C) prevailing in Antalya province may serve as an ideal condition for charcoal rot disease (Anonymous, 2023a, b), implying that the disease may cause serious economic damages to sesame production in the region. Likewise, Karibasappa et al. (2018) underscored that charcoal rot of sesame decreases yield a greater extent in high-temperature areas. Moreover, growing conditions like irrigation may play a role in the disease development. In this context, it was reported that sesame cultivated under rainfed conditions displayed more charcoal rot incidence than that of under irrigated conditions (Balabaskar et al., 2015).

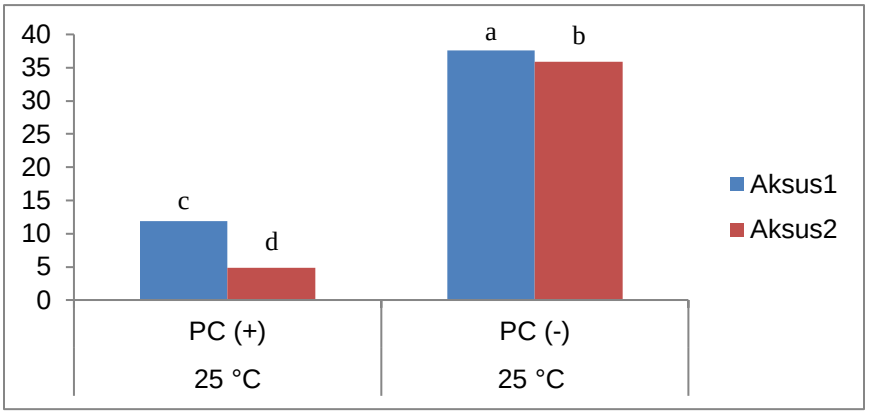

Figure 12. Colony growths of the isolates of *Macrophomina phaseolina* on minimal medium containing potassium chlorate (PC+) and without potassium chlorate (PC-) at 25°C for 48 h ($LSD_{0.01}$:0.43).

Şekil 12. *Macrophomina phaseolina izolatlarının potasyum klorat içeren (PC+) ve potasyum klorat içeren içermeyen (PC-) minimal ortamında 25°C'de 48 saat sonra koloni gelişmeleri.*

Comparing the isolates, the highest colony growths were detected at 35°C, whereas the lowest ones were determined at 40°C in the present study. With regard to colony growth of *M. phaseolina*, in a study, *M. phaseolina* isolates from sunflower in the mideast and southern part of Italy grew better at temperatures ranging from 30 to 35° but their growth were the weakest at 15 and 40°C (Manici et al., 1995). In this regard, Akhtar et al. (2011) reported that temperatures varying from 30 to 35°C was optimum for mycelial growth and microsclerotium production of *M. phaseolina* isolates of sesame in Pakistan.

With regard to genetic relatedness, both isolates were on different clades in the phylogenetic tree in the present study, indicating genetic diversity of *M. phaseolina* isolates even if taken in the same location. In this regard, Salahlou et al. (2016) reported that there was a high genetic diversity among *M. phaseolina* isolates of sesame. In fact, genetic diversity may not be related to the origin of isolates or hosts. Likewise, Sarr et al. (2014) found no correlation among genotype, host and geographic location of 189 *M. phaseolina* isolates from different hosts and countries. As a result of the genetic diversity, micromorphological features such as microsclerotium size, colony growth and chlorate sensitivity might be variable among *M. phaseolina* isolates. In the present study, we found significant differences in microsclerotium sizes of the isolates. Similarly, differences in colony growths of the isolates were also significant, implying virulence differences as well. In this regard, Vinothini et al. (2020) found that virulence of *M. phaseolina* isolates was positively correlated with their growth rate. In the present study, both isolates of *M. phaseolina* exhibited a chlorate sensitive phenotype and there was no any pycnidia on the plant tissues showing the disease symptoms. Likewise, Mihail & Taylor (1995) reported that *M. phaseolina* isolates displaying chlorate sensitive phenotype did not form or rarely formed pycnidia on the host tissues, corroborating our field observations.

CONCLUSION

To the best of our knowledge, this is the first detailed characterization of charcoal rot symptoms on sesame in the field in the Western Mediterranean region of Türkiye. In addition, the present study provided new insights into epidemiology of the charcoal rot disease. However, further studies regarding control of the disease are needed to mitigate the damage caused by charcoal rot pathogen in sesame cultivation areas.

Data Availability

Data will be made available upon reasonable request.

Author Contributions

Conception and design of the study: MA; sample collection: MA, İK; analysis and interpretation of data: MA; statistical analysis: MA; visualization: MA, İK; writing manuscript: MA.

Conflict of Interest

There is no conflict of interest between the authors in this study.

Ethical Statement

We declare that there is no need for an ethics committee for this research.

Financial Support

This study did not receive any specific funding.

Article Description

This article was edited by Editor Prof. Dr. Galip KAŞKAVALCI.

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