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Determination of Biocontrol Potential of *Bacillus* spp. and *Stenotrophomonas* sp. against *Macrophomina phaseolina* in Sunflower[#]

Özden Salman[1,a,*], Raziye Kocak[2,b], Nuh Boyraz[1,c]

[1]*Department of Plant Protection, Faculty of Agriculture, Selçuk University, 42250 Konya, Turkey*
[2]*Çumra Vocational School, Selçuk University, 42500 Konya, Turkey*
[*]*Corresponding author*

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ABSTRACT

Macrophomina phaseolina is a soil pathogen known as charcoal rot and can cause up to 90% yield loss in sunflower under suitable conditions. The serious damage caused by chemicals used in the control of soil-borne pathogens to the environment and health has become one of the most important concerns in agriculture. Therefore, in our study, it was aimed to determine the *in vitro* antagonistic effects of various bacterial species against *M phaseolina*. A total of 38 bacterial strains were isolated from soil samples in the rhizosphere of *Malva sylvestris* (hibiscus), *Vicia sativa* (vetch), *Cicer arietinum* (chickpea), *Papaver rhoeas* (weasel), *Carlina marianum* (thistle), *Glebionis coronaria* (crown daisy) and *Vicia faba* collected from Urla district of İzmir. All bacterial strains exhibited antibiosis effect under *in vitro* conditions, but it was determined that 5 bacterial isolates among them showed a high inhibition zone and showed an average inhibition potential ranging between 55% and 74%. The most effective bacteria identified at species and genus level by Maldi biotyping (MALDI-TOF MS) were identified as *Bacillus amyloliquefaciens*, *Stenotrophomonas* sp. and *Bacillus cereus* (3 isolates), and these species showed that they can be important biocontrol agents in biological control against *M. phaseolina*.

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Ayçiçeğinde *Macrophomina phaseolina*'ya Karşı *Bacillus* spp. ve *Stenotrophomonas* sp.'nin Biyokontrol Aktivitelerinin Belirlenmesi

MAKALE BİLGİSİ

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Anahtar Kelimeler:
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ÖZ

Macrophomina phaseolina kömür çürüklüğü olarak bilinen bir toprak patojeni olup uygun şartlar altında ayçiçeğinde %90'a yakın verim kaybına neden olabilmektedir. Toprak kaynaklı patojenlerin mücadelesinde kullanılan kimyasalların çevreye ve sağlığa verdiği büyük zararlar tarımda en önemli endişelerden biri haline gelmiştir. Bundan dolayı çalışmamızda, çeşitli bakteri türlerinin *in vitro* da antagonistik etkilerinin *M. phaseolina*'ya karşı belirlenmesi amaçlanmıştır. Toplam 38 bakteri ırkı İzmir'in Urla ilçesinden toplanan *Malva sylvestris* (ebegümeci), *Vicia sativa* (fiğ), *Cicer arietinum* (nohut), *Papaver rhoeas* (gelincik), *Carlina marianum* (devedikeni), *Glebionis coronaria* (taç papatya) ve *Vicia faba* (bakla) bitkilerinin rizosfer kısmındaki toprak örneklerinden izole edilmiştir. *İn vitro* da tüm bakteri ırkları antibiyosis etki sergilemiş fakat bunların arasında 5 bakteri izolatının yüksek inhibiyon zonu oluşturarak ortalama %55-%74 arasında değişen engelleme potansiyeli gösterdikleri belirlenmiştir. Maldi biyotipleme (MALDİ-TOF MS) yoluyla tür ve cins düzeyinde teşhis edilen en etkili bakteriler *Bacillus amyloliquefaciens, Stenotrophomonas sp. ve Bacillus cereus* (3 izolat) olarak tanılanmış ve bu türler *M. phaseolina*'ya karşı biyolojik mücadelede önemli biyokontrol ajanı olabileceklerini göstermişlerdir.

[a] *ozdensalman@selcuk.edu.tr* *http://orcid.org/0000-0002-7871-4105*
[b] *rkocak@selcuk.edu.tr* *https://orcid.org/0000-0002-8221-0452*
[c] *nboyraz@selcuk.edu.tr* *https://orcid.org/0000-0001-6822-9360*

Introduction

Macrophomina phaseolina (Tassi) Goid, a serious pathogen worldwide, causes significant yield loss in many plants in Turkey (Mengistue et al., 2015; Khan, 2017; Tančić Živanov et al., 2018). These plants include sunflower, soybean, chickpea, sorghum, maize, cotton, beans, beet, cabbage, pepper, melon, strawberry, sesame, peanut and cowpea (Ghosh, 2018). Although it is primarily a soil-borne pathogen, it can be seed-borne in many crops (Mah et al., 2012). This pathogen, known as charcoal-rot in sunflower, also causes seed rot, seedling blight, root and crown rot (Brooker et al., 2007; Bellaloui et al., 2008; Koçak, 2019). In the survey studies conducted in the Aegean Region in 1991, *Macrophomina phaseolina* was reported to be potential pathogens on sunflower (Onan et al., 1992). It was determined by Özer and Soran (1994) that *Macrophomina phaseolina* is among the species that cause wilt in sunflowers. However, charcoal-rot in sunflower was first reported in 2009 in Turkey. In a study conducted in Turkey and Egypt, it was reported that *M. phaseolina* was seen in 70% of oilseed sunflower plants and the disease severity ranged between 10%-50% (Mahmoud and Budak 2011).

M. phaseolina produces sclerotia in host root and stem tissues to survive in adverse environmental conditions. After plant death, sclerotia formation continues until the host tissues dry up, and these structures constitute important sources of inoculum for the reproduction of the pathogen (Baird et al., 2003). Depending on environmental conditions, microsclerotia of the pathogen can survive for 2-15 years on soil and infected plant debris. The increase in plant density and sclerotia population in the soil increases the disease rate. (Kaur et al., 2012; Vasebi et al., 2013).

The severity of infection of charcoal-rot in sunflower has been associated with environmental changes and it has been reported that the losses can reach 60 to 90% under appropriate conditions (Perez-Brandán et al., 2012; Khan, 2017). It has been stated that the disease increases especially in cases of drought and water stress. Under these conditions, the mycelia of the pathogen penetrate deeper into the host plant tissues, causing structural damage and infected plants usually die (Singh et al., 2008; Rayatpanah et al. 2012). Because high temperature and low humidity support disease development (Aegerter et al., 2000). For this reason, one of the most effective ways to reduce losses from the disease is considered to be the use of resistant varieties (Mahtab et al., 2013; Shehbaz et al., 2018). One of the most successful control strategies used for charcoal-rot is soil fumigation. However, this application generates a series of problems such as high cost, environmental pollution, deterioration of the ecological balance of the soil and destruction of the ozone layer (Adhikary et al., 2019).

Biological control as an alternative to control the disease has been shown to be a highly selective method that continues over time (Nico et al., 2005). Numerous studies have focused on the search and selection of antagonist microorganisms on a variety of soil pathogens. Among the most commonly used bioagents are bacterial genera such as *Bacillus*, *Pseudomonas* and *Streptomyces* (Hussain et al., 1990; Adekunle et al., 2001; Singh et al., 2008). These bacteria grow in or on the rhizosphere and on the surface and inside of root nodules. Known as plant growth promoting rhizobacteria (PGPR), these bacteria also function as biocontrol agents against pathogens (Bhattacharyya and Jha 2012; Alijani et al. 2019). They have well-developed mechanisms to inhibit phytopathogens and reduce disease incidence. Antifungal metabolites produced by PGPRs are effective tools for managing plant diseases. In some studies, some endophytic bacteria such as *Pseudomonas* sp. and *Bacillus* sp. were found to have antagonistic effects against *M. phaseolina* (Atef, 2000; Senthilkumar et al., 2009).

It has been reported that *B. cereus* and *B. amyloliquefaciens* species exhibit potent antagonistic activity in the control of *Macrophomina phaseolina*, which infects different agricultural plants (Torres et al., 2016; Sabaté et al., 2017; Caballero et al., 2018; Dave et al., 2021).

B. cereus (E23) was tested against two isolates of M. *phaseolina* (E3, E6) in sunflower and it was observed that it inhibited mycelial growth by 50.83-53.33% (Mahmoud 2010).

In a study in which the antagonistic effects of 37 bacteria isolated from the bean rhizosphere were screened in vitro, it was determined that four isolates (BA97, BN17, BN20 and BR20) identified as *Bacillus* spp inhibited *M. phaseolina* by 62.5-85% (Bojórquez- Armenta et al., 2021).

Stenotrophomonas bacteria mainly consist of clinical strains (Palleroni and Bradbury, 1993), but they can also be obtained from the rhizosphere of various plant species and environmental wastes (Wolf et al., 2002; Di Gregorio et al., 2005; Yang et al., 2006; Heylen et al., 2007; Kim et al., 2009). It has been determined in many studies that *Stenotrophomonas maltophilia* can be used in the biocontrol of plant diseases (Zhang and Yuen, 2000; Zhang et al., 2001; Idris et al., 2007). *S. maltophilia* isolated from the rhizosphere of some plants has been tested against many phytopathogens, including *Macrophomina phaseolina*, and has been shown to be antifungal (Kamil et al., 2007).

It was determined that the *Stenotrophomonas* sp. (AG3) isolate, which is one of the extremophilic bacteria, has a biocontrol effect of 52.2% against *M phaseolina in vitro* (Santos et al., 2021).

Biocontrol agents can be affected by changing climatic conditions, for agricultural sustainability it is necessary to discover new microbial isolates from different habitats that have the potential to adapt to the changing environment. Many medicinal plants, cultivars, and weeds contain a wide variety of microflora, including PGPR, in their rhizosphere. Therefore, the aim of this study is to isolate bacteria from the rhizosphere of different cultivars and to apply their antagonistic effects against *M. phaseolina* in sunflower in vitro and to develop biocontrol strategies.

Materıal and Method

Material

Phytopathogenic Fungus

Macrophomina phaseolina isolate, which was isolated from sunflower and obtained from the culture collection of Selcuk University Mycology Laboratory, was used as pathogen.

Bioagent Bacteria

In the present study, soil samples were taken from the root surface of *Malva sylvestris* (hibiscus), *Vicia sativa* (vetch), *Cicer arietinum* (chickpea), *Papaver rhoeas* (weasel), *Carlina marianum* (thistle), *Glebionis coronaria* (crown daisy) and *Vicia faba* plants in the surveys carried out in Urla District of İzmir in 2021 and stored in bags at 4°C.

Bacillus amyloliquefaciens, *Stenotrophomonas* sp. and *Bacillus cereus* (3 isolate) which isolated from soil samples were used in the study.

Method

Preparation of Phytopathogen Fungus Isolates

Fresh pathogen cultures were grown from slant agar cultures previoulsy stored at 4℃ (Edmunds, 1964; Mihail, 1993). The toothpick was taken from the slant agar and was placed onto the Potatose Dextrose Agar (PDA) meduim treated with antibiotic. The plates were later incubated at 23-25℃ for 7 days.

Isolation and Identification of Bioagent Bacteria Obtained from Plant Rhizosphere

Isolation of beneficial bacterial agents from the soil samples was done according to Saygılı et al. (2006) and the petri dishes were incubated at 27℃ for 24-48 hours. Pure cultures were obtained by streaking the single bacterial colonies with different growth patterns followed by an incubation period as mentioned earlier. The pure cultures were stored in 30% glycerol at -20℃.

After the determination of the antagonistic effect, the bacteria showing 50% or more activity were sent to Hatay Mustafa Kemal University Plant Health Application and Research Center and their diagnosis was made according to MALDI-TOF biotyping.

Evaluation of Antagonistic Effect

The dual culture method was performed to evaluate the antagonistic potential of the bacterial isolates obtained from the soil. 7-days old culture of *Macrophomina phaseolina* and 24-48 hours old cultures of test bacteria were used. The pathogen was placed at the center of the petri dishes containing antibiotic-free PDA, and the test bacteria was streaked in a circle 1.5 cm away from the pathogen followed by an incubation at 25℃ for 7 days. Control petri dishes only contained the pathogen at the center. The mycelial growth of the pathogen was evaluated by measuring the inhibition zone around it, formed by the bacteria. Bacteria that inhibited mycelial growth by 50% or more were recorded. The percent inhibition was calculated as follows;

$$\text{Inhibition (\%)}=\frac{\text{A1-A2}}{\text{A1}}\times 100$$

A1= Mycelial growth (control),

A2= Mycelial growth (treatment) (Tariq et al., 2010).

Results and Discussion

Some medicinally and economically important plants support a large microflora in their rhizosphere, including PGPRs. The amount and activity of microorganisms is a determining factor for the fertility of any soil (Ribeiro, 2011). In our study, a total of 38 bacteria were isolated from hibiscus, vetch, chickpea, poppy, thistle, crown daisy and broad bean plants to investigate the diversity of rhizobacteria and to reveal their activities and tested against *Macrophomina phaseolina* obtained from sunflower to determine their antagonistic effects. 5 bacterial isolates were effective on over 50% of the pathogen and these bacteria were evaluated and characterized according to Maldi biotyping (MALDI-TOF MS), and species were identified as *Bacillus amyloliquefaciens*, *Bacillus cereus* (3 isolates) and *Stenotrophomonas* sp.

M. phaseolina is a destructive pathogen that hosts more than 500 plants worldwide, especially in agriculture, and causes root rot. Therefore, antagonistic microorganisms such as bacteria and fungi are an alternative source to control these pathogens (Kim et al., 2003). It was determined that 13% of the bacteria we used in our experiment were effective against *M. phaseolina.* The efficacy of bacteria against the pathogen ranged from 55-74% (Table 1). In a similar study, 19.6% of 290 bacteria isolated from the rhizosphere of different plants were found to have antagonistic effects against the pathogen (Malleswari, 2014).

The antagonistic potential of the isolates assessed by the dual culture test by restricting pathogen growth and creating a zone of inhibition against the antagonist (Figure 1). *Bacillus amyloliquefaciens* (IEB1) and *Stenotrophomonas* sp. (IGL1) formed an inhibition zone in the petri dish and remained within the bacterial circle striked on the petri dish. Other bacterial isolates have reached the bacterial limit or slightly exceeded this limit as mycelium.

Bacillus bacteria species are accepted as safe biological agents and they have been effective in different antagonistic studies (Mallesh et al., 2009). These bacteria show an excellent effect against plant pathogens as they contain antimicrobial cyclic lipopeptides (iturins, fengicins and surfactins), enzymes (chitinase and ß-1,3-glucanases) and antifungal volatile organic compounds (VOCs) (Bakhshi et al., 2018).

It was determined that *Bacillus amyloliquefaciens* IEB1 isolated from hibiscus showed the highest antagonistic activity under *in vitro* conditions among the bacteria used in our study. Ji et al. (2013) tested *Bacillus amyloliquefaciens* CNU114001 against 12 fungal pathogens. In the dual culture experiment against *Colletotrichum orbiculare, Fusarium oxysporum, Penicillium digitatum* and *Pyricularia grisea*, it was determined that mycelial growth of pathogens inhibited more than 70% as compared to the control petri dishes.

Table 1. Bioagents with antagonistic action against *Macrophomina phaseolina*

Antagonistic Bacteria	Isolate Code	Plant Species from which the bacteria are isolated	% Effect Against Pathogen
Bacillus amyloliquefaciens	IEB1	Hibiscus (Malva sylvestris)	74
	IFG3	Vetch (Vicia sativa)	55
Bacillus cereus	IDV2	Thistle (Carlina marianum)	62
	INH	Chickpea (Cicer arietinum)	66
Stenotrophomonas sp	IGL1	Common poppy (Papaver rhoeas)	70

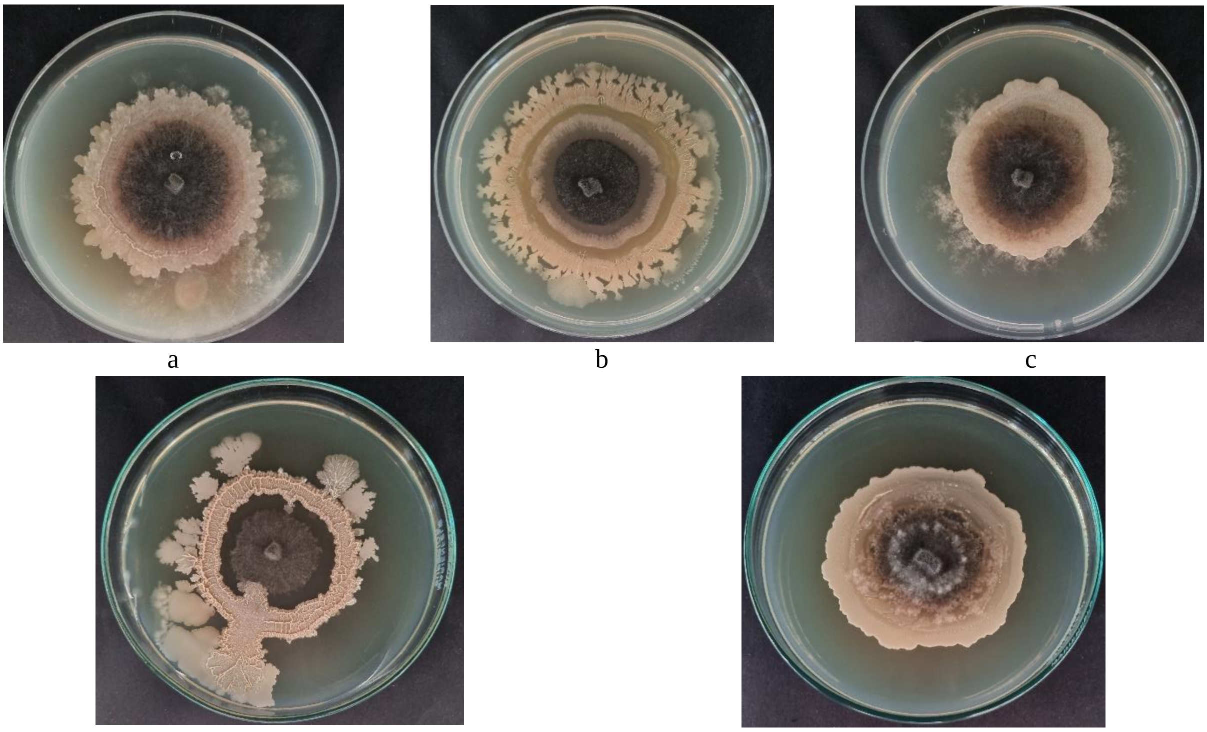

Figure 1. Petri view of effective bacteria against *Macrophomina phaseolina* in sunflower
a-IDV2, b-IGL1, c-IFG3, d-E1, e- INH

In our experiment, 3 isolates of *Bacillus cereus* were used as IFG3 isolate obtained from vetch, IDV2 isolate from thistle and INH isolate from chickpea. The isolate with the highest antagonistic activity against the pathogen was INH with 66% efficacy, followed by IDV2 (62%) and IFG3 (55%). The chitinase activity of *Bacillus cereus* YQ308 has inhibited the growth of *F.oxysporum, F.solani and Pythium ultimum* (Chang et al., 2003). *Bacillus cereus* UW85 reduced the seedling mortality rate of alfalfa plants, caused by *P. megasperma* f.sp medicaginis, to 0% (Handelsman et al., 1990).

The number of biocontrol agents in agriculture is growing and new strains are being discovered. Barely discovered genera such as *Stenotrophomonas* sp. from extremophilic bacteria appear to be effective in the biological control of phytopathogens. Their effects on the pathogen are thought to be related to their ability to secrete polyamines, chitin and lytic enzymes (Santos et al., 2021). *Stenotrophomonas* sp. IGL1 isolated from common poppy showed 70% efficacy against the pathogen. It has been reported that disease severity is reduced in germinated soybean seeds infected with *M. phaseolina* treated with *Stenotrophomonas sp* (Santos et al., 2021). *Stenotrophomonas maltophilia* C3 strain used as a biocontrol agent against plant diseases due to its chitinase activity and this bacterial strain inhibited the conidy germination of *Bipolaris sorokiniana* and similarly suppressed the disease in field conditions (Zhang et al., 2000). In various studies, data have been obtained that *Stenotrophomonas maltophilia* will be used in biological control against fungal diseases such as *Rhizoctonia solani*, *Verticillium dahliae*, *Magnaporthe poae, Pythium ultimum*, *Fusarium graminearum* (Hayward et al., 2009).

It appears that *Bacillus* species and extremophilic bacteria can potentially be used in the biocontrol of *M. phaseolina* as well as in the biological control of various plant pathogens. In our experiment, *Bacillus amyloliquefaciens* was effective against the pathogen by 74%, followed by *Stenotrophomonas* sp., which was isolated from common poppy with 70%, which is known to be effective especially against drought stress. It was determined that 3 isolates of *Bacillus cereus* also showed significant effects. Since some species of *Stenotrophomonas* are human pathogens, it is important to determine that which species of this genus belongs to future studies.

Trichoderma, Aspergillus, Penicillium, *Bacillus* and *Pseudomonas* are promising species in the control of *M. phaseolina* (Lodha and Mawar, 2020). This study shows that species containing many antifungal components also have an inhibitory effect against soil-borne diseases. Excellent alternative bioagents can be used which can limit the widespread use of chemical pesticides in the control of plant diseases. The results also show that the use of antimicrobial species with various applications in agriculture will increase in the future and the portfolio of biological agents will expand and new strains can be used.

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