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Original article

Determination of *in vitro* Biocontrol Potentials of Antagonist Bacterial Isolates Against Onion Basal and Root Rot Disease Agent *Fusarium proliferatum*

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Abstract

Various Fusarium species cause significant yield and quality losses in onion (*Allium cepa* L.) plants. Onion basal and root rot, caused by *Fusarium proliferatum,* is an emerging postharvest disease that causes severe economic losses. Although the disease has long been recognized as a major constraint to the production of *Allium* spp., there is insufficient information to support disease management. In recent years, a need has arisen for environmentally friendly, innovative alternative methods to avoid the use of chemical pesticides in the control of diseases that are a problem in agriculture. In this study, the biocontrol efficiency of antagonistic bacterial isolates obtained from bulbs, roots and leaves of healthy onion plants was investigated against *F. proliferatum in vitro.* The antagonistic activity of the bacterial isolates in inhibiting the mycelial growth of the fungal agent was determined by the dual culture assay. The bacterial isolates were identified by morphological, biochemical and proteomic (MALDI-TOF MS) methods. A total of 18 putative bacterial isolates were obtained from the bulbs, roots and leaves of healthy onion plants on selective media. As a result of *in vitro* dual culture assays, only six bacterial isolates (*Bacillus cereus* MK2, *Enterobacter xiangfangensis* MK3, *Bacillus thuringiensis* MK8, *Alcaligenes faecalis* MK9, *Pseudomonas putida* MK16 and *Citrobacter freundii* MK17) significantly suppressed mycelial growth of disease agent (43.89-50.56% inhibition). *Bacillus cereus* MK2 was found to be the most effective bacterial isolate with a 50.56% inhibition rate of mycelial growth. Overall, the results suggest that *Bacillus cereus* MK2 could be used as a potential biocontrol agent for a sustainable and environmentally friendly control strategy for onion fields affected by Fusarium basal and root rot disease. It is necessary to conduct further studies on the effects of the effective bacterial isolates against the pathogen *in vivo* and their mechanisms of action.

Keywords: Onion, Antagonist, Bacillus spp., Biological control.

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INTRODUCTION

Fusarium basal and root rot disease cause economic losses in onion production areas throughout the world by reducing the yield and quality of onions both during the growing season and during storage (Cramer, 2000). Some fungal pathogens have been reported to cause yield losses in onion production areas in Turkey (Özer & Ömeroğlu, 1995; Türkkan & Karaca, 2006). Onion production areas are frequently threatened by soil-borne pathogens such as *Fusarium* spp., *Macrophomina phaseolina, Sclerotinia sclerotiorum*, *Rhizoctonia* spp., and *Sclerotium* spp. (Schwartz & Mohan, 1995). *F. oxysporum* f.sp. *cepae, F. solani, F. acuminatum, F. equiseti, F. culmorum, F. proliferatum, F. subglutinans, F. redolens* and *F. tricinctum* are important Fusarium species causing disease that cause major problems in onion growing areas (Abawi & Lorbeer, 1972; Schwartz & Mohan, 1995; Shinmura, 2002; Stankovic et al., 2007). The most common causal agents of Fusarium basal and root rot are *Fusarium oxysporum* f.sp. *cepae* and *Fusarium proliferatum* (Ghanbarzadeh et al., 2014; Haapalainen et al., 2016; Stankovic et al., 2007).

Fusarium proliferatum is an important pathogen that infects many cultivated plants worldwide and causes serious economic losses in agricultural production. The host range of this pathogen, which adapts very quickly to changes in environmental factors, is quite wide. Disease agents include such as corn (Marín et al., 1998), wheat (Desjardins et al., 2007; Amato et al., 2015), barley (Jurado et al., 2010), rice (Park et al., 2005), asparagus (Seefelder et al., 2002), banana (Jimenez et al., 1993), date palm (Abdalla et al., 2000), garlic (Palmero et al., 2012) and onion (Stankovic et al., 2007) host plants. Fusarium species are also known to produce mycotoxins harmful to plant, human and animal health, such as deoxynivalenol, fumonisin-B1, moniliformin and beauvericin, in addition to their direct effects on the yield and quality of products (Palmero et al., 2012). In addition, *F. proliferatum* has been reported to be an important source of mycotoxin contamination in foods (El-Sayed et al., 2022).

Fusarium infections have increased in field and storage conditions in the world's important onion growing areas and have been recorded as the most destructive pathogens after harvest. As a result of the isolations, the presence and prevalence rates of *Fusarium oxysporum, F. proliferatum* and *F. redolens*, which are associated with onion basal and root rot disease, were investigated (Haapalainen et al., 2016). There is no effective fungicide against this type of disease agent that causes infection in onions. Beneficial bacteria, which are called biocontrol agents (BCA) in the management of fungal disease agents, have been attracting attention for a long time due to their environmental friendliness, low cost and low dependence on agricultural chemicals (Lin et al., 2013). Certain BCA species provide benefits by protecting the host from infections caused by pathogens by stimulating plant growth and resistance mechanisms (Latz et al., 2018). They do this through several mechanisms such as competition for nutrients and space, production of secondary metabolites, and plant hormone synthesis. Bacteria can use one or more of these metabolites in combination to suppress the pathogen and reduce the development

of resistance (Card et al., 2016). In the literature search, although there are biological control studies on different host plants where the disease agent *F. proliferatum* is a problem, to the best of our knowledge, no biological control study has been found as a basal and root rot agent in onions.

In this study, it was aimed to determine the antagonistic activities of the antagonist bacteria obtained from the healthy bulbs, roots, and leaves of the onion plant against the disease agent *Fusarium proliferatum*, which causes a major problem in onion production areas, *in vitro* conditions.

MATERIALS and METHODS

Isolation of the Fungal Disease Agent *Fusarium proliferatum*

The fungal disease agent *F. proliferatum* was isolated from infected onion bulbs in surveyed onion production areas. Pieces of 5-6 mm in size were cut from the diseased plant tissues with the help of a sterile scalpel. Surface sterilization was performed for 1 minute in 75% ethanol. Disinfected tissue pieces were rinsed with sterile distilled water and dried on sterile blotting papers. Dried pieces were inoculated into PDA medium and incubated at 26°C for 5-7 days. Sections were taken from the growing colonies and purified again on PDA medium (Kara et al., 2023).

Isolation and Selection of Antagonist Bacteria

Bacterial isolates were obtained from surface-disinfected healthy onion bulbs, roots and leaves. The surfaces of healthy plant samples collected from the field were first wiped with 70% ethyl alcohol, then 3% sodium hypochlorite for 5 minutes and rinsed 3 times with sterile water. Surface disinfection completed leaves were crushed in sterile 10 mM $MgCl_2$ solution (pH 7.2) and the resulting suspension was spread onto general [Tryptic Soy Agar (TSA) and Nutrient Agar (NA)] and selective [(King B Agar (KB)] nutrient media (Merck, Germany). In addition, the pieces obtained from onion bulbs were isolated by pressing directly (imprinting method) on the surface of the medium (Aktan & Soylu, 2020; Kara et al., 2020). All petri dishes were incubated at 26°C for 2 days. Purifications were made from bacterial colonies with different morphological appearances that developed on the surface of the media after incubation. Each of the bacterial colonies with different morphological appearances representing the petri dishes was evaluated as a "putative antagonist bacterial isolate".

The obtained putative antagonist bacterial isolates were subjected to a hypersensitivity test (HR) on tobacco plants, soft rot test on potato slices and growth tests at 37°C to determine whether they were plant pathogens. Hypersensitivity (HR) test in tobacco, bacteria that did not cause soft rot in potatoes and did not grow at 37°C were stored at +4°C on petri dishes containing TSA medium until they were identified as "candidate antagonist bacteria" and to be used *in vitro* bioactivity studies

Identification of Bacterial Isolates

Pre-selection of bacteria that did not cause HR in tobacco leaves, soft rot in potatoes and had negative for growth test at 37°C was determined by biochemical tests such as catalase and oxidase activity, gram reaction and fluorescent pigment production in KB medium (Lelliot & Stead, 1987). According to the results of the tests, a preliminary selection of the bacterial isolates was made and the exact species identification was performed by MALDI-TOF MS (Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) (Soylu et al., 2020). Protein isolation was performed from bacterial isolates using the ethanol-formic acid extraction method on pure bacterial colonies taken from pure cultures and grown on media for 24-36 hours, and protein spectra specific to the isolates were obtained with Maldi Biotyper Real-Time Classification (RTC) software (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany). The protein spectra were compared with the spectra of reference bacterial species in the library of the device and identified at the species level with a high-reliability score value (Chalupová et al., 2014).

Determination of Antagonistic Effects of Bacteria *in vitro*

In vitro antagonistic activities of bacteria obtained from healthy bulbs, roots and leaves of onion plants were determined by dual culture tests on a PDA medium (Soylu et al., 2020). In the dual culture tests, the bacterial isolate to be tested was drawn on one end of the media in the petri dish and left to pre-incubate at 26°C for 48 hours. After the bacteria developed, a fresh fungus culture of 5 mm in diameter was placed at a distance of 4 cm from the developing colony and incubated at 26°C. As a control, the fungus was transferred to non-bacterial petri dishes. As a result of the growth of the fungal cultures used as a control to the desired point, fungal mycelial growth (Mu) growing towards the bacteria in all petri dishes containing antagonist bacterial isolates was measured and the % inhibition rates compared to the growth of mycelium (Mk) in the control plates (%Inhibition= ([Mk-Mu] / Mk) *100) was calculated (Soylu et al., 2020).

Statistical Analyzes

All *in vitro* experiments were established according to a randomized plot design, measurements were made for each bacterial isolate in 3 different petri dishes, and the experiment was repeated twice. The data obtained were analyzed by one-way ANOVA using SPSS statistical software (SPSS Statistics 17.0) and the difference between the isolates was determined by the Tukey HSD Test ($P \leq 0.05$).

RESULTS and DISCUSSION

Isolation and Identification of the Fungal Disease Agent

To obtain the fungal culture, isolation was made from onion tubers which were thought to be infected in onion production areas. Sections taken from diseased tissues were planted in petri dishes with PDA medium and incubated at 26°C for 5-7 days. As a result of morphological observations made from the developing colonies, airy micelles of white and violet color were seen on the medium. According to the microscopic examinations; abundant microconidia with thick apical tips, thin, relatively flat and 3-septate macroconidia were observed. Chlamidospores were not observed. As a result of the examinations, the pathogen was identified as *F. proliferatum* (Salvalaggio & Ridao, 2013).

Isolation and Identification of Antagonist Bacteria

A total of 23 candidate antagonist bacteria isolates were obtained from bulbs, roots and leaves of healthy onion plants in onion production areas. To determine whether these isolates are plant pathogens or not, HR in tobacco, soft rot in potatoes and their ability to grow at 37°C were tested. As a result of the tests, out of 23 candidate bacterial isolates, 3 isolates grew at 37°C, 2 isolates caused soft rot in potatoes and these isolates were excluded from the trials due to their potential plant and human pathogenicity. It was decided to carry out dual culture tests with 18 antagonist bacterial isolates that were negative in the HR test on tobacco leaves. As a result of MALDI-TOF MS analyses of 18 bacterial isolates obtained, bacterial isolates have been identified as *Acinetobacter dijkshoorniae Bacillus cereus, Enterobacter xiangfangensis, Raoultella ornithinolytica, Enterobacter cloacae, Klebsiella oxytoca, Bacillus megaterium, Bacillus thuringiensis, Alcaligenes faecalis, Aeromonas veronii, Stenotrophomonas rhizophila, Pseudomonas graminis, Bacillus marisflavi, Lysinibacillus xylanilyticus, Acinetobacter courvalinii, Pseudomonas putida, Citrobacter freundii* and *Pseudomonas flavescens*.

Determination of Antagonistic Effects of Bacteria *in vitro*

The antagonistic activities of 18 antagonist bacterial isolates, which were determined not to be human and plant pathogens as a result of the tests, on the inhibition of mycelial growth of *F. proliferatum* were investigated by *in vitro* dual culture tests (Figure 1). In dual culture tests with antagonist bacteria and fungal pathogen, bacterial isolates inhibited mycelial growth of the fungal pathogen at rates ranging from 1.11-50.56% (Table 1). While five bacterial isolates (MK4, MK10, MK13, MK14 and MK15) tested were ineffective in preventing mycelial growth by falling into the same group as the control group, six antagonist bacterial isolates (MK2, MK3, MK8, MK9, MK16, MK17) showed a high level of antagonistic activity by inhibiting the mycelial growth of the fungal pathogen by 43.89-50.56%. Among the isolates in the same statistical group, the highest antagonistic effect was shown by the bacterial isolate Bacillus cereus (MK2) with 50.56% (Table 1). This isolate was followed by *Citrobacter freundii* MK17, *Alcaligenes faecalis* MK9 and *Bacillus thuringiensis* MK8 with 49.44%, 47.78% and 47.22%,

respectively. *Bacillus marisflavi* MK13, *Aeromonas veronii* MK10, *Raoultella ornithinolytica* MK4, *Lysinibacillus xylanilyticus* MK14, *Acinetobacter courvalinii* MK15, *Acinetobacter dijkshoorniae* MK1, *Pseudomonas flavescens* MK18, *Stenotrophomonas rhizophila* MK11 and *Bacillus megaterium* MK7 were not effective in inhibiting mycelial growth compared to the control (Table 1).

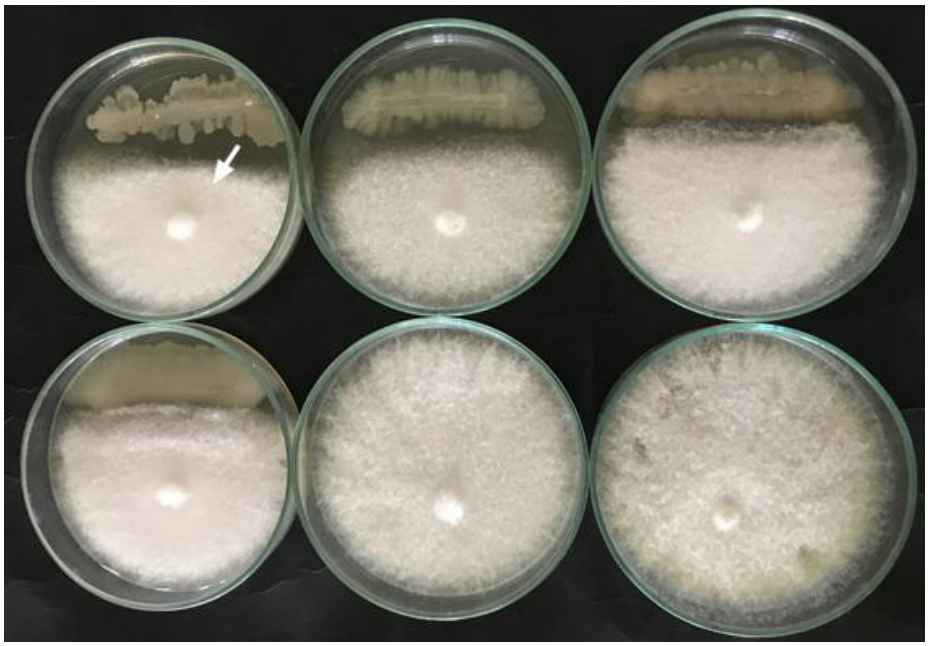

Figure 1. Antifungal activities of different antagonist bacterial isolates on the inhibition of mycelial growth (arrow) of the onion basal and root rot disease agent *Fusarium proliferatum.*

Table 1. Antifungal activities of different antagonist bacterial isolates on the inhibition of mycelial growth of the onion basal and root rot disease agent *Fusarium proliferatum.*

İsolates	**Species**	**MG (mm)[a]**	**IMG (%)**
MK1	*Acinetobacter dijkshoorniae*	55.33d-f	7.78
MK2	*Bacillus cereus*	29.67a	50.56
MK3	*Enterobacter xiangfangensis*	33.67a	43.89
MK4	*Raoultella ornithinolytica*	59.33fg	1.11
MK5	*Enterobacter cloacae*	52.33b-d	12.78
MK6	*Klebsiella oxytoca*	50.67bc	15.56
MK7	*Bacillus megaterium*	54.33b-e	9.44
MK8	*Bacillus thuringiensis*	31.67a	47.22
MK9	*Alcaligenes faecalis*	31.33a	47.78
MK10	*Aeromonas veronii*	60.00g	0.00
MK11	*Stenotrophomonas rhizophila*	54.67c-e	8.89
MK12	*Pseudomonas graminis*	50.33b	16.11
MK13	*Bacillus marisflavi*	60.00g	0.00
MK14	*Lysinibacillus xylanilyticus*	57.00e-g	5.00
MK15	*Acinetobacter courvalinii*	57.00e-g	5.00
MK16	*Pseudomonas putida*	32.67a	45.56
MK17	*Citrobacter freundii*	30.33a	49.44
MK18	*Pseudomonas flavescens*	55.33d-f	7.78
	Control	60.00g	

MG: Mycelial Growth; MGI: Mycelial Growth Inhibition

[a] The mean mycelial growth was based on the measurements of 3 replicate plates, recorded at 5 days after inoculation. Mean values followed by different small letters within the column are significantly different according to the Tukey Test ($P<0.05$).

Although antagonistic activities of abiocontrol agent bacteria against *Fusarium proliferatum* were investigated in different host plants (Bjelic et al., 2018, De la Lastra et al., 2021, Duan et al., 2021, Duan et al., 2022, Baard et al., 2023), no study was found on onion.

Ajilogba et al. (2013) investigated the antagonistic activity of bacterial isolates belonging to B. *amyloliquefaciens, B. cereus, B. pumilus and B. subtilis* species against *Fusarium solani* causing Fusarium wilt in tomatoes under *in vivo* and *in vitro* conditions. As a result of *in vitro* analysis, *B. amyloliquefaciens* inhibited the development of the disease agent with a high rate of 95.2%. This isolate was followed by *B. pumilus, B. subtilis* and *B. cereus* isolates with 70.46%, 82.1% and 55.7% inhibition rates, respectively. *In vivo* analysis showed that *B. cereus* was the isolate that inhibited the disease agent at the highest level (81.2%). Baard et al. (2023) investigated the biocontrol potential of plant bacterial isolates against *Fusarium oxysporum, Fusarium proliferatum, Fusarium culmorum* and *Fusarium*

verticillioides. Bacterial endophytes were isolated from *Glycine max* L. leaves (B1), *Brassica napus* (B2), *Vigna unguiculata* (B3) and *Glycine max* seeds (B4) and identified using 16S rRNA PCR sequencing. As a result of phylogenetic analysis, bacterial isolates were identified as *Bacillus subtilis* (B1) and *Bacillus tequilensis* (B2-B4). All bacterial isolates produced substantially indole acetic acid (IAA), siderophore and protease activity. *In vitro* dual culture tests of these isolates significantly inhibited ($p < 0.05$) mycelium of *F. proliferatum* > *F. culmorum* > *F. verticillioides* > *F. oxysporum*. The results showed that these bacterial isolates are good biocontrol candidates against selected Fusarium species. Duan et al. (2022) investigated the antagonistic activity of *Bacillus vallismortis* HSB-2 isolate against *Fusarium oxysporum, Fusarium moniliforme, Fusarium proliferatum,* and *Fusarium solani*, which are the pathogens of apple trees, *in vitro* conditions. In dual culture tests, they determined that the bacterial cells and fermented cell suspension cultures showed significant antagonistic activity on the inhibition of mycelial growth and germination of spores of fungal agents. In conclusion, HSB-2 has the potential to protect apple plants from the four *Fusarium* species, improve soil microbial community, increase soil enzyme activity and promote plant growth. As a result of the study, it was suggested that *B. vallismortis* HSB-2 could be used as a biological control agent in the control of disease agents. Duan et al. (2021) investigated the antagonistic activity of the *Bacillus amyloliquefaciens* QSB-6 isolate against *Fusarium proliferatum, Fusarium solani, Fusarium verticillioides, Fusarium oxysporum, Alternaria alternata, Aspergillus flavus, Phoma* sp., *Valsa mali, Rhizoctonia solani, Penicillium brasilianum* and *Albifimbria verrucaria, in vitro* conditions. In dual culture tests, they determined that the bacterial and fermented suspension showed significant antagonistic activity on the inhibition of mycelial growth and germination of spores of fungal agents. As a result, it has been suggested that *B. amyloliquefaciens* QSB-6 can be used as a biological control agent in promoting plant root growth and in the control of disease agents seen in apple plants. De la Lastra et al. (2021) used *Streptomyces fradiae* Hvs6, *Bacillus paralicheniformis* Hvs2 and *Bacillus velezensis* FC37 as putative BCA isolates against *F. proliferatum, F. oxysporum f. sp. asparagi* and *F. redolens* associated with asparagus dieback death syndrome disease. While FC37 and Hvs2 isolates were the most effective isolates for the control of pathogenic *F. proliferatum* and *F. oxysporum* f. sp. *asparagi*, none of them showed antagonistic activity against *F. redolens* isolate. Bjelic et al. (2018) identified *Fusarium tricinctum, F. oxysporum* f. sp. *cepae, F. proliferatum, F. acuminatum* and *F. verticillioides* as causal agents of root and stem rot in garlic plants. The antifungal activity of four bacterial isolates identified as *Bacillus subtilis* against fungal agents was investigated under *in vitro* conditions. These isolates inhibited mycelial growth up to 71% under *in vitro* conditions and caused a 58% reduction in rot symptoms of garlic bulbs under in vivo conditions.

Conclusion

As a result, antagonistic bacteria were isolated from healthy onion tubers, roots and leaves taken from onion production areas and their antagonistic activity against *Fusarium proliferatum*, the causal agent of Fusarium bottom rot disease, was investigated. When the results were evaluated, *Bacillus cereus* was found to have the highest antagonistic activity against the fungal pathogen. By using beneficial BCA, it is aimed to reduce the use of chemical substances, which are used very much today and which adversely affect environmental factors and human health.

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