

Biological Inhibition of *Thuja* Collar and Root Rot Using Some Antagonistic Bacteria

Shoab Ghadimi¹ and Hadi Rahananadeh^{2*}

¹Master's Student in Plant Pathology, Islamic Azad University, Rasht Branch, Iran

²Department of Agronomy, Rasht Branch, Islamic Azad University, Rasht, Iran, P. O. Box 4415866865, Iran

Received: 06 August 2023

Accepted: 21 February 2024

*Corresponding author's email: rahananadeh20@yahoo.com

Root and collar rot of *Thuja* caused by *Fusarium oxysporum* is one of the important diseases in *Thuja* cultivation. In this research, the effect of eight bacterial strains of *Bacillus licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis*, *B. velezensis*, *Pseudomonas fluorescens*, *P. korensis* and *P. putida* in controlling this disease was investigated in the laboratory. Among them, based on the dual culture test and observation of the inhibition zone, *B. velezensis* and *B. subtilis* had the highest inhibition with 9.33% and 5.6%, respectively. In the study of the effectiveness of non-simultaneous antifungal volatile compounds, *P. fluorescens* had the highest inhibition with 58.33%. The simultaneous volatile compounds of *P. korensis*, *P. fluorescens*, *B. pumilus*, and *B. megaterium* completely controlled the disease agent. In the study of the effect of filtered extracellular liquid metabolites on the growth of the pathogen colony, it was observed that with the increase in the concentration of the metabolites, the inhibition percentage of the growth of the pathogen colony by all bacterial strains increases. The best strains against *F. oxysporum* were *B. velezensis*, *B. subtilis*, and *B. pumilus* strains, which in 25% concentration were 50, 72.27 and 86.67%, respectively, and in 15% concentration *B. pumilus* strain was 66.75% and *B. velezensis* strain with 46.66% inhibition, and at 5% concentration, *B. pumilus* strain with 44% inhibited the growth of the fungal colony. In the protease production test, all isolates were able to produce protease. Only *P. fluorescent* strain was able to produce a siderophore. In the microscopic studies, all the investigated strains caused morphological changes, fusion of different parts of the filaments, and destruction.

Abstract

Keywords: Antagonist, Bacteria, Collar rot, *F. oxysporum*, *Thuja*.

INTRODUCTION

F. oxysporum has a wide host range and causes disease in various crops (Moshayedi and Rahanandeh, 2014). The cause of collar and root rot (*F. oxysporum*) of *Thuja* was first reported in 2022 from the western regions of Gilan province in Iran (Ghadimi and Rahanandeh, 2022). In India in 2007, this fungal agent was reported as the cause of the wilting of the thuja tree (Raghavendra *et al.*, 2007). The use of fungicides leads to environmental pollution, endangering the health of humans and other creatures, and they often have low efficiency in controlling these pathogens. Therefore, researchers have been led to develop and use other environmentally friendly and effective methods for an integrated management of *Fusarium*. The use of biological inhibitors (biological control) is one of the most important methods used in the integrated management of fungal diseases (Rahanandeh *et al.*, 2012; Grosu *et al.*, 2015; Shirmohamadi *et al.*, 2022). The effect of biological control agents can be categorized into two types direct and indirect effects; direct mechanisms include the production of siderophore, the secretion of antimicrobial compounds, or the production of toxic and destructive enzymes (Compant *et al.*, 2005; Rahanandeh *et al.*, 2017). On the other hand, more indirect mechanisms include increasing the resistance of plants, which takes place through two paths acquired resistance (SAR) and induced resistance (ISR) (Doornbos *et al.*, 2012). *Bacillus* species such as *B. amyloliquefaciens* and *B. subtilis* are Gram-positive and spore-forming bacteria and have antagonistic activity against plant pathogens. In addition, *Bacillus* species are suitable for commercialization due to the production of spores that are resistant to adverse environmental conditions such as drought and heat, and they can easily be used in the formulation of sustainable products and as protection against environmental stress (Perez-Garcia *et al.*, 2011). Some strains of *P. fluorescens* have prevented the growth of *F. oxysporum* in the laboratory and the greenhouse, they have caused a decrease in the severity of the disease and an increase in the growth of plants (Ebrahimi Kazemabad *et al.*, 2012). In one of the researches, a direct relationship between the production of antifungal metabolites of bacterial isolates and the reduction of disease has been observed (Ebrahimi Kazemabad *et al.*, 2012). The effect of some isolates of *Pseudomonas* spp., *Bacillus* spp. has been observed in the control of *F. oxysporum* under controlled conditions. The extracellular secretions of *Bacillus* species have inhibitory properties and prevent the germination and mycelial growth of *Fusarium* conidia (Iraqi *et al.*, 2009). It has also been determined that *B. subtilis* strains are very effective in protecting crops against *Fusarium* and *Rhizoctonia* and have greatly increased plant growth (Schisler *et al.*, 2004). According to Zhang *et al.* (2009), *B. subtilis* has fungicidal properties and can be used as a seed coating against root rot caused by pathogens. The production of antibiotics such as mycotoxin, ethiorin A, bacillomycin, basilin, and subsporin by *B. subtilis* species is the main and determining factor in the biological control of plant diseases by this bacteria (Kim *et al.*, 1997). The purpose of this research is to evaluate the identified antagonists against the fungus *F. oxysporum*, which causes collar and root rot of thuja in laboratory conditions.

MATERIALS AND METHODS

Preparation of samples of antagonistic bacteria

To investigate the antagonistic effects, eight bacteria were prepared from Tehran Water and Soil Institute.

Experimental dual-culture

To perform the test, it was done according to Garbeva's method (Garbeva *et al.*, 2008).

After seven days of incubation at 27 °C, the average diameter of the inhibitory halo was measured and the rate of pathogen growth inhibition was calculated according to the method of Huang (Huang *et al.*, 2017).

$$\text{Growth inhibition} = 100 \times \left(\frac{\text{growth diameter of the control plot} - \text{growth diameter of the treatment plot}}{\text{growth diameter of the control plot}} \right)$$

Investigating the effect of non-synchronous antifungal volatile compounds

The amount of 200 µl of the suspension of bacterial isolates was spread on NA and the Petri dishes were kept at 27 °C for 72 h. Then, rings with a diameter of five mm of fungi culture were cultivated in the center of petri dishes containing PDA. Then, the Petri dishes containing the fungus were placed upside down on the Petri dishes containing the antagonist isolates, and the edges of the Petri dishes were placed on top of each other and completely blocked by parafilm tape. Petri dishes were kept at a temperature of 27 °C for one week. In the test of volatile compounds, bacteria and fungi were cultured at the same time, and other test steps were performed as above (Kraus and Loper, 1990; Sedaghatfar *et al.*, 2002). The amount of inhibition of pathogen growth was calculated according to the method of Huang (Huang *et al.*, 2017). This experiment was conducted in the form of a completely randomized design including 8 treatments in 3 repetitions.

Production of extracellular compounds

100 µl of the suspension of bacterial isolates and sterile distilled water were added to the PDA culture medium as a control and spread on the surface of the culture medium with a Pasteur pipette and kept for 72 h at a temperature of 27 °C. Then the isolates were washed from the surface of the culture medium and the Petri dishes were exposed to chloroform vapor for 30 min upside down. Then, a five-mm ring of fungi was planted in the center of each petri dish, observing the sterile conditions. Petri dishes were kept at a temperature of 27 °C and the diameter of mycelium growth was measured after 10 days (Kraus and Loper, 1990). The amount of inhibition of pathogen growth was calculated according to the method of Huang (Huang *et al.*, 2017).

Protease production

Considering the role of protease as one of the biological control mechanisms, the production of this enzyme was evaluated on eight bacterial strains. In this experiment, SMA (Skim milk agar) culture medium containing 5 g of milk powder, 5 g of yeast extract, 4 g of blood agar, and 13.5 g of microbiological agar in one liter. The bacteria were cultured on the surface of the medium in a spot manner and incubated at a temperature of 28 to 30 °C for 48 h. The presence of a colorless halo around the colony is a sign of protease activity. The average radius of this halo in mm was measured in different strains and was used as a criterion to evaluate the ability of protease production in different strains (Maurhofer *et al.*, 1992).

Extracellular liquid secretions of antagonist isolates

250 g of potato, 20 g of dextrose were prepared in one liter of distilled water. 50 ml of the above liquid medium were poured into flasks with a capacity of 250 ml and sterilized in autoclave conditions. After cooling the liquid environment, a full loop of 24 h bacterial culture was added to each of the flasks. The flasks containing bacteria were placed on a shaker with 70

revolutions per min at 27 °C for one week, and after passing the liquid medium of the isolates through Whatman No. 1 filter paper and centrifuged at 5000 x g for 20 min, each of the isolates were filtered separately by microbiological 0.22 micron and the extract obtained from each of the mentioned isolates was used in the test of inhibiting the growth of mycelium of fungi. To investigate the effect of the obtained extract, first, the PDA culture medium was poured into tube of 19, 17, and 15 ml, and after sterilization in autoclave conditions, 1 ml (concentration of 5% vol: vol), 3 ml (concentration 15%) and 5 ml (concentration 25%) of the extract of each of the antagonists were added to PDA tubes with a temperature of 45 °C and mixed well and in sterile conditions to sterile Petri dishes. They were transferred. As a control treatment, the liquid culture medium without bacteria that was passed through a 0.22 micron filter was used. After the coagulation of the culture medium, a ring with a diameter of five ml from the fungi culture was planted in the center of each petri dish. The diameter of the filament was measured every day for 10 days after fungi cultivation. The amount of inhibition of pathogen growth was calculated according to the method of Huang (Huang *et al.*, 2017).

Siderophore production

This study was done according to the method of Wheller and Cook (1983). *Pseudomonas fluorescent* isolates were cultured on King B culture medium containing concentrations of 5, 50, and 100 µM of Fe III chloride and were kept for 48 h at a temperature of 25 °C. Then the spore suspension of *Geotrichum candidum* from a 48-h culture on PDA medium was sprayed on the surface of the petri dish containing the antagonist isolate. The absence of mushroom growth around the bacteria indicates the production of siderophore.

Investigating the inhibitory effects of antagonistic strains on a microscopic scale

In addition to measuring the macroscopic effect of antagonistic strains based on their inhibitory properties in Petri dishes, their effects on *Fusarium* fungus were also investigated at the microscopic scale. For this purpose, microscopic samples were prepared from the margin of the colon affected by the metabolites of the antagonistic strains and observed under the microscope with 40x magnification. In this study, the condition of mycelium and mushroom growth were compared to the control treatment.

Statistical analysis of data

The statistical analysis of the data was done using SAS and the categorization of treatments was performed by Duncan Multiple Range Test at 0.1 and 0.5 level of significance.

RESULTS

Characteristics of antagonistic bacteria

The bacteria used in this research were obtained from Tehran Water and Soil Research Institute (Table 1).

Dual-culture test and selection of antagonistic strains

Based on this test, two strains were inhibitory to *F. oxysporum* isolate. The B.ve strain exhibited the highest inhibition at 33.9%, while the B. sub isolate showed 5.6% inhibition, placing it in the next statistical group (Fig. 1).

Table 1. Characteristics of the bacteria used.

Abbreviation of bacteria	Name of bacteria and code
B. li	<i>Bacillus licheniformis</i> (CCSM-B 00587)
B. me	<i>Bacillus megaterium</i> (CCSM-B00702)
B. p	<i>Bacillus pumilus</i> (CCSM-B005220)
B. su	<i>Bacillus subtilis</i> (CCSM-B01449)
B. ve	<i>Bacillus velezensis</i> (CCSM-B484)
P. f	<i>Pseudomonas fluorescens</i> (CCSM-B00102)
P. k	<i>Pseudomonas koreensis</i> (CCSM-B00287)
p. p	<i>Pseudomonas putida</i> (CCSM-B00586)

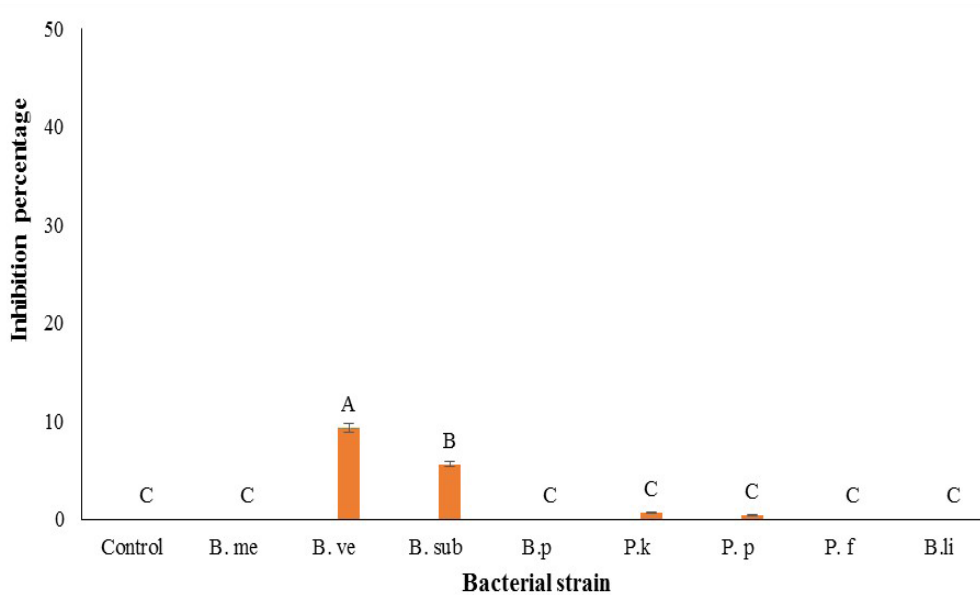


Fig. 1. The effect of antagonistic bacterial strains on the inhibition percentage of the growth of *F. oxysporum*, the causative agent of thuja rot, in the dual-culture test.

The effect of volatile antifungal compounds on bacteria

In the test of the effectiveness of non-synchronous volatile antifungal compounds, strain P.f was the most effective with 58.33 percent inhibition, and other strains were at lower levels based on statistical analysis, and strain B. li was not inhibitory (Fig. 2).

In testing the effect of volatile antifungal metabolites at the same time, B.p., and P.f strains with 50% inhibition of the growth of the disease-causing fungus *F. oxysporum* were the most effective and B. me was the least effective with 8.33% inhibition (Fig. 3).

The effect of the production of diffusible extracellular metabolites of bacterial strains in preventing the mycelium growth of *F. oxysporum*

B. me, B. p, P. f, and P. k strains had a 100 % effect on *F. oxysporum* pathogen compared to the control. B. su isolate with 54.66% inhibition was the least effective isolate in statistical group D (Fig. 4).

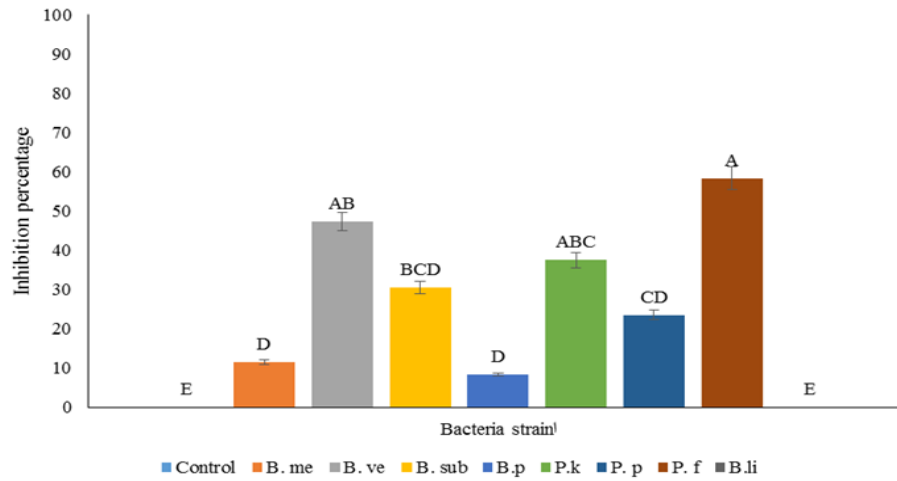


Fig. 2. Inhibition percentage of non-simultaneous volatile compounds of antagonistic bacterial strains.

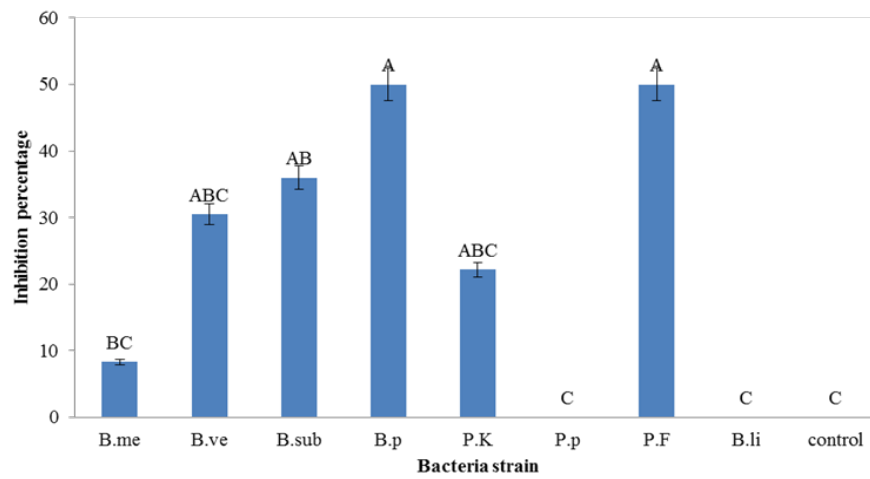


Fig. 3. Inhibition percentage of simultaneous volatile compounds of antagonistic bacterial strains.

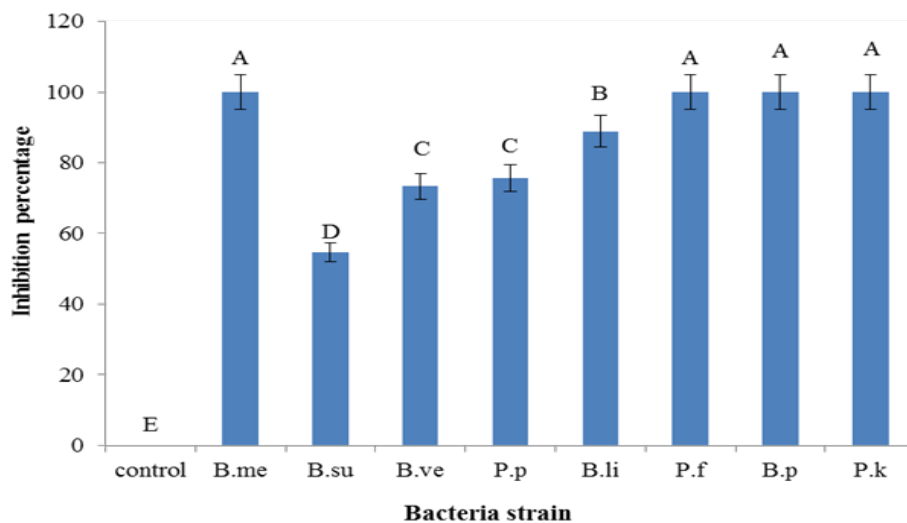


Fig. 4. Inhibition percentage of diffusible extracellular compounds of antagonistic bacterial strains.

Effect of filtered extracellular liquid metabolites of antagonistic bacteria

Increasing the concentration of metabolites, resulted in a higher percentage of inhibition of pathogen growth by all bacterial strains. At a concentration of 25%, the *B. ve*, *B. su*, and *B. p* strains exhibited inhibitions of 50%, 72.27%, and 86.67%, respectively. At a concentration of 15%, the *B. p* strain showed 75.66% inhibition, while the *B. ve* strain showed 46% inhibition. Finally, at a concentration of 5%, the *B. p* strain demonstrated the highest inhibition of fungal growth at 44% (Fig. 5).

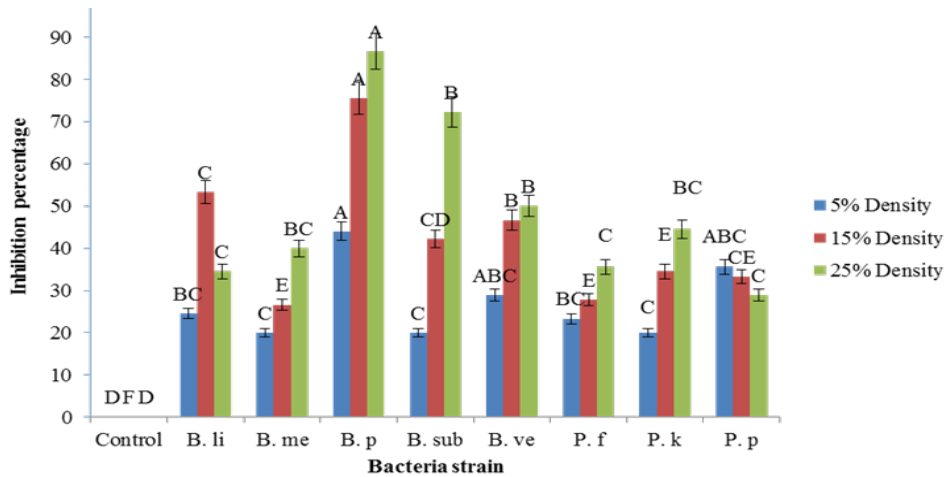


Fig. 5. The effect of filtered extracellular fluid metabolites of bacterial strains on the growth inhibition rate of *F. oxysporum* fungi.

Protease production test

All isolates were able to produce protease. In this test, *B. ve* bacteria had the largest halo diameter of 8.167 mm and *B. su* had the smallest halo diameter of 1.667 mm (Fig. 6).

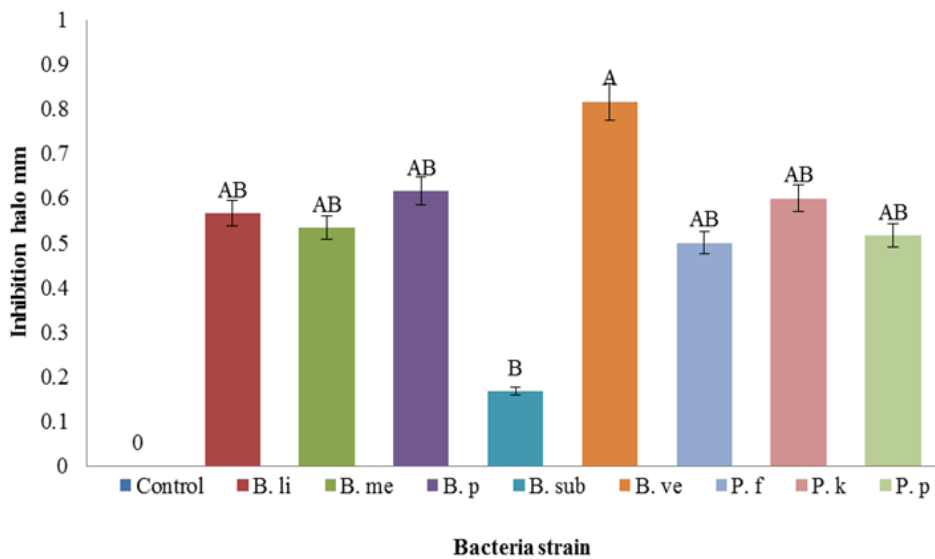


Fig. 6. The amount of halo formed by bacterial isolates in the protease test.

Siderophore production

P. f strain was able to produce siderophore at concentrations of zero, 25, 50, and 100 micromol of iron III chloride on King-B culture medium.

The effect of antagonistic bacterial strains on the microscopic characteristics of pathogenic fungi

In the microscopic examination of all the investigated strains, morphological changes such as the formation of a series of vesicles at the end of the filaments and near the end of the filaments and also along the length of the filaments, lightening of the tip of the hyphae and changes in the amount of pigment production as well as complexity and to co-irradiation of different parts of the root and destruction of the root became the disease (Fig. 7).



Fig. 7. Deformation and vesiculation of fungal hyphae due to antagonistic bacteria.

DISCUSSION

The fungus *F. oxysporum*, which cause of thuja collar and root rot, is one of the important factors that reduce thuja plant production and cultivation per unit area in Iran, since the pathogen is soil-borne and the ineffectiveness of chemical poisons in controlling soil-borne pathogens, there is a need to use methods replacement is required (Ghadimi and Rahanandeh, 2022). In many researches, the high antagonistic effect of *Bacillus* bacteria in controlling pathogenic fungi has been proven. *Bacillus* BAS23 isolate controlled more than 12% of rice pathogenic fungi and caused a decrease of more than 12% in the dry weight of the mycelium of pathogenic fungi (Saechow *et al.*, 2018). In this research, *B. ve* and *B. su* isolates showed the greatest inhibition of the growth of pathogenic fungi in the dual-culture method. In the test related to the effect of volatile metabolites, most of the isolates were effective. Isolate *P. f* with 58.33% showed the greatest inhibitory effect on the mycelium growth of the pathogenic fungus. Also, in this study, after opening the Petri lid and placing the fungus at room temperature, the pathogenic fungus was able to start its growth again, and after 9 days, it filled the Petri dish, which indicates the effect of the static fungus on the fugitive metabolites produced by *Pseudomonas* bacteria, and also indicates that the presence and survival of the bacteria is necessary to continue controlling the pathogenic fungus. One of the biocontrol factors in the antagonistic bacteria *Pseudomonas*

and *Bacillus* has been the production of volatile compounds in various research (Elshahat *et al.*, 2016). Volatile compounds produced by *B. subtilis* caused vacuolization and swelling of *Rhizoctonia solani* mycelium (Fiddaman and Rossall, 1993). Extracellular compounds produced by the *Pseudomonas* strain can cause deformation and destruction of the mycelium of fungi (Gupta and Verhoeven, 2001). In *B. subtilis* bacteria, extracellular compounds are considered to be a more effective mechanism than volatile substances in preventing the activity of pathogenic agents. The production of these compounds in the cultivation environment depends on the presence of some substances. By adding D-glucose or a combination of carbohydrates along with peptone, the production of these substances in the environment increased (Fiddaman and Rossall, 1993). In the current research, it was also shown that all antagonistic bacterial strains can produce extracellular compounds. In studies related to siderophore production, *P. f* isolates were able to produce siderophore. Siderophore production can both act as a growth stimulant and control the disease through the competition mechanism (Ahmadzadeh and Sharifi Tehrani, 2021). The biocontrol ability of *Pseudomonas* is related to the production of siderophore (Loper and Schroth, 1986), volatile compounds, and antibiotics (Elshahat *et al.*, 2016; Rahanandeh *et al.*, 2017). In the test related to the production of protease enzyme, all isolates were able to produce this enzyme, one of the important mechanisms in the biological control of pathogenic fungi is the production of extracellular enzymes, which mainly aim to destroy the cell wall and plasma membrane of the pathogen. They become chitinase enzyme produced by *Bacillus* bacteria is one of the most important chitin degrading enzymes. Therefore, by producing this enzyme, *Bacillus* bacteria destroy the cell wall of the pathogenic fungus and prevent its growth (Shoda, 2000; Rahanandeh *et al.*, 2012).

CONCLUSION

Considering the importance of thuja collar and root rot disease in the country and the lack of a suitable management method to control this disease, the present study was conducted to evaluate the effect of *Bacillus* and *Pseudomonas* isolates on the disease-causing fungus. The bacterial isolates used in this research could inhibit *F. oxysporum* in laboratory tests. Therefore, based on comprehensive investigations and considering prevalence of *Fusarium* disease common in the region, the use of these bacteria can be used as a soil treatment, as recommended an efficient method in sustainable agriculture to manage *Fusarium* diseases and improve thuja growth. However, it is necessary to evaluate the positive and negative characteristics of these bacterial strains as well as their effect on non-target organisms before making recommendations. Additionally, since each of these isolates employs a specific mechanism to control the pathogenic agent, it is advisable to use a combination of them for controlling this fungus. Furthermore, it is important to assess the effect of the bacterial combination on each other and the host plant.

ACKNOWLEDGMENT

The authors would like to thank Engineer Mahsa Moshayedi, Technical Officer of plant pathology Clinic, Islamic Azad University, Rasht Branch.

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How to cite this article:

Ghadimi,Sh. & Rahananadeh, H. (2024). Biological Inhibition of *Thuja* Collar and Root Rot Using Some Antagonistic Bacteria. *Journal of Ornamental Plants*, 14(2), 105-115.

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