

Investigation of the Effectiveness of *Clonostachys rosea* (Sch.) Schroers & Samuels on Root Rot Caused by *Fusarium solani* (March.) Sacc. in Pistachio (*Pistacia vera* L.)*

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Abstract: This study was conducted in the Phytopathology Laboratory and controlled-environment growth chamber of Siirt University during 2022-2023 to evaluate the efficacy of *Clonostachys rosea* against root rot caused by *Fusarium solani* in pistachio (*Pistacia vera* L.). The study material included *F. solani* KY1, previously isolated from pistachio roots and identified as pathogenic, along with *C. rosea* isolates F8, F9, and F10, also obtained from the pistachio root region, and the LO41 isolate obtained from the Phytopathology Laboratory collection. According to the results of the experiment evaluating the antagonistic activity of volatile metabolites produced by *C. rosea* isolates, F10 was the most effective, inhibiting the pathogen by 9.04%. In the dual culture assay, the LO41 isolate exhibited the strongest antagonistic effect against the pathogen, with 17.20% inhibition. However, regarding the antagonists' ability to overgrow and parasitize the hyphae of the pathogen, all isolates, except *C. rosea* F9, exhibited markedly stronger hyperparasitic activity. In the pot experiment conducted to evaluate the efficacy of the antagonists in soil artificially contaminated with *F. solani*, the disease severity in the positive control (*F. solani* KY1) was 72.50%. Following the application of *C. rosea* isolates F10, F8, LO41, and F9, disease severity was reduced to 20.00%, 27.50%, 37.50%, and 52.50%, respectively. The pathogen suppression rates of the antagonists were 72.41%, 62.06%, 48.27%, and 27.58%, respectively, indicating that *C. rosea* F10 was the most effective isolate. Consequently, the capability of *C. rosea* as a biological control agent against *F. solani*, a major soil-borne pathogen in pistachios, was further investigated.

Keywords: Pistachios, root rot, *Clonostachys rosea*, *Fusarium solani*, biological control

1. Introduction

Pistacia vera L., commonly known as pistachio and belonging to the Anacardiaceae family, is a tree species that produces edible seeds enclosed in a hard shell and is cultivated mainly between the 30th and 45th parallels. This species is native to a broad region extending from Asia Minor (Türkiye) through Syria, Iran, and Lebanon to Afghanistan (Salas-Salvadó et al., 2011).

As of 2023, 51.8% of global pistachio production is accounted for by the United States, 23.6% by Iran, and 13% by Türkiye. These three countries contribute approximately 89% of the global production. Other major producing countries include China and Syria, which rank fourth and

fifth, respectively. The United States, the leading producer, recorded a total production of 676.000 tonnes, followed by Iran with 308.000 tonnes, Türkiye with 176.000 tonnes, China with 81.000 tonnes, and Syria with 45.000 tonnes (Anonymous, 2025).

Pistachio production is constrained by numerous factors, including alternate bearing, environmental conditions, improper fertilization and irrigation practices, physiological limitations, cultivar selection, pests, and diseases. The prevalence and impact of diseases vary according to regional and climatic conditions. In particular, pathogens responsible for root rot, crown rot, and wilt diseases are of significant concern. In general, several pathogens are known to cause such diseases

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in pistachios worldwide, including *Eutypa lata* (Pers.) Tul. & C. Tul., *Armillaria mellea* (Vahl) P. Kumm., *Fusarium* spp Link., *Rhizoctonia solani* J.G. Kühn., *Cytospora terebinthi* Bres., *Gibberella zeae* (Schwein.), *Phymatotrichopsis omnivora* (Shear) Hennebert, *Phytophthora cryptogea* Pethybr. & Laff., *Phytophthora citricola* Sawada, and *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.), *Phytophthora capsici* Leonian, *Phytophthora nicotianae* (Breda de Haan), *Phytophthora palmivora* Butler., *Sclerotinia minor* Jagger, *Sclerotinia sclerotiorum* (Lib.) de Bary, and *Verticillium dahliae* Kleb. (Chitzanidis, 1995; Michailides et al., 1995; Eskalen et al., 2001; Türkölmez et al., 2015; Aydın, 2019; Aydın et al., 2023, 2024). These pathogens typically damage the roots and root collars of saplings and mature trees, leading to desiccation and their eventual decline. Several *Fusarium* species, including *F. proliferatum*, *F. equiseti*, and *F. solani*, have been reported to cause diseases in various plants, particularly in pistachio nurseries (Triki et al., 2011; Nouri et al., 2018; Crespo Palomo et al., 2019; Aydın and Ünal, 2021; Aydın et al., 2023, 2024). In disease surveys conducted in pistachio orchards in Siirt Province, Türkiye, *Fusarium solani* was frequently isolated and identified as a major pathogen (Aydın et al., 2023). *Fusarium* species generally cause seed rot, root rot, seedling blight, and wilt in cultivated plants. In the advanced stages, wilting and desiccation occur as a consequence of vascular tissue damage (Arie, 2019).

Currently, many biological control agents are commercially registered and extensively used in agriculture. Among these, *Clonostachys rosea* (Sch.) Schroers & Samuels (syn. *Gliocladium roseum*) has gained considerable attention as an effective disease management tool. Initially reported as an aggressive mycoparasite in the late 1950s, *C. rosea* has since been extensively studied for its potential in the biological control of plant diseases (Barnett and Lilly, 1962; Schroers et al., 1999; Aydın and Turhan, 2009; Mouekouba et al., 2014). In brief, *C. rosea* employs several mechanisms to control plant diseases, including the secretion of cell wall-degrading enzymes, synthesis of secondary metabolites such as antibiotics and toxins, and promotion of plant growth (Chatterton and Punja, 2009; Fatema et al., 2018; Wang et al., 2019). *Clonostachys rosea* is widely distributed across diverse habitats worldwide and can be readily isolated (Sutton et al., 1997; Sun et al., 2020). This fungus has been recovered from soil, other fungi, plant debris, and various plant tissues, such as roots, leaves, and flowers (García et al., 2003; Nobre et al., 2005; Jensen et al., 2022; Kulik et al., 2024). Additionally, some isolates have been

obtained from insects and nematodes (Verdejo-Lucas et al., 2002; Haarith et al., 2020). The biocontrol potential of *C. rosea* has been demonstrated against several plant pathogens, such as *Alternaria radicina* A. dauci, *F. graminearum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Phytophthora palmivora*, *Drechslera teres*, *Bipolaris sorokiniana*, *F. verticillioides*, *F. crookwellense*, *Botrytis aclada*, *F. culmorum*, *Moniliophthora roreri*, *Helminthosporium solani*, *Sclerotinia sclerotiorum*, and *Rhynchosporium commune* (Krauss and Soberanis, 2001; Jensen et al., 2004, 2016; Yohalem et al., 2004; Aydın and Turhan, 2009; Kosawang et al., 2014; Schöneberg et al., 2015; Sun et al., 2015; Lysøe et al., 2017; Samsudin et al., 2017).

The management of soil-borne pathogens responsible for decline and dieback in pistachio orchards is challenging, as chemical control measures are generally ineffective or provide limited success. Biological control agents, however, are capable of surviving and colonizing plant tissues for prolonged periods, enabling them to suppress pathogen development and protect plants against disease. Therefore, biological control represents a promising and sustainable approach for the management of soil-borne diseases in pistachio production systems. The study is based on this hypothesis.

In this study, the efficacy of *C. rosea* against root rot caused by *F. solani* in pistachios was evaluated. The aim was to develop an alternative control strategy for this disease.

2. Materials and Methods

This study was conducted in the Phytopathology Laboratory and controlled-environment growth chamber of Siirt University during 2022-2023. In this study, the *F. solani* KY1 isolate, previously obtained from pistachio roots and confirmed as pathogenic (Aydın et al., 2023), the *C. rosea* LO41 isolate maintained in the Plant Pathology Laboratory collection at the Faculty of Agriculture, Siirt University, and *C. rosea* isolates F10, F9, and F8 from pistachio roots were used.

2.1. Evaluation of the antagonistic activity of biological control agents

In vitro experiments were conducted to evaluate the antagonistic activity of the isolates using two methods; assessment of antagonistic activity via volatile metabolites and the dual culture method.

To determine the inhibitory effect of volatile metabolites on *F. solani* KY1, 5 mm diameter agar discs obtained from 7-day-old cultures of *C. rosea*

and *F. solani* grown on potato dextrose agar (PDA) medium were placed at the center of separate Petri dishes containing PDA. The lids were removed, and the dishes were inverted and paired, with *F. solani* positioned on top and *C. rosea* isolates below, and then sealed with parafilm. The Petri dishes were incubated at 24 °C under a 12 h light/12 h dark cycle. In the control treatment, only PDA medium was used instead of the antagonist. Assessment was performed after 5 days by measuring the colony diameter of the test pathogen in both the treatment and control groups, and the inhibition percentage was calculated according to Equation 1 (Dennis and Webster, 1971).

$$\text{Inhibition rate (\%)} = 100 \times (A1 - A2) / A1 \quad (1)$$

Where A1 is the radial growth of the pathogen in the control and A2 is the radial growth in the treatment.

In the dual culture method, the antagonists and test pathogens were first cultured on PDA medium for 7 days. Subsequently, 10 mm diameter agar discs were excised and placed opposite each other, 5 mm from the edge of Petri dish. In the control treatment, only the pathogen was placed on one side of the PDA dish and incubated at 24 °C. The experiment was conducted twice, with four replicates for each trial. In the first trial, evaluation was performed after 10 days according to Equation 2 (Vincent, 1947).

$$L = (C - T) / C \times 100 \quad (2)$$

Where, L: Mycelial growth inhibition zone (%), C: Pathogen growth in the control (mm), T: Pathogen growth in the dual culture.

In the second experiment, antibiosis was evaluated based on the time required for the complete inhibition of *F. solani* colony growth. Mycoparasitism was assessed based on the time taken by the antagonist to completely overgrow the *F. solani* colony during the experimental period. Antagonistic activity was further evaluated by measuring the width of the inhibition zone, whereas mycoparasitic activity was rated using the scale proposed by Turhan (1990), considering the rate and density of colonization of the *F. solani* colony by the antagonist.

Very Strong Hyperparasitism (VSH) or Antibiosis (VSA): Inhibition zone wider than 10 mm, or the antagonist has completely overgrown the pathogen colony.

Strong Hyperparasitism (SH) or Antibiosis (SA): Inhibition zone between 7 and 10 mm, or the antagonist showed strong growth over the pathogen colony.

Moderate Hyperparasitism (H) or Antibiosis (A): Inhibition zone between 3-6 mm, or the antagonist has displayed noticeable spread over the pathogen colony.

Weak Hyperparasitism (WH) or Antibiosis (WA): Inhibition zone narrower than 3 mm, or the antagonist has shown very weak growth over the pathogen colony.

Lytic Effect (L): The antagonist causes lysis of the pathogen.

No Effect (O): No visible effect was observed.

2.2. Evaluation of antagonistic efficacy against *F. solani* under pot conditions

2.2.1. Preparation of the potting soil

The potting mix used in this study was prepared by combining garden soil, peat, and sand in equal proportions. The mixture was autoclaved at 121 °C for 15 min in autoclavable bags. The mixture was then aerated, thoroughly mixed, and transferred into pots.

2.2.2. Preparation of the pathogen inoculum

A modified wheat bran culture method was used to prepare the pathogen inoculum (Turhan, 1992). According to this method, 100 g of wheat bran, 40 g of wheat grains, and 10 g of maize flour were mixed with 15 mL of water and placed into suitable glass containers, sterilized in an autoclave at 121 °C for 30 min, and allowed to cool. An actively growing pathogen colony on PDA was aseptically cut into four pieces using an inoculation needle, and each piece was transferred into a separate container containing the bran medium. The inoculated containers were incubated in the dark at 24 °C for 15 days. The resulting inoculum was thoroughly mixed with potting soil at a ratio of 1:20 (w/w).

2.2.3. Preparation of antagonist suspensions

To prepare antagonist suspensions, *C. rosea* isolates were cultured on PDA medium for 10 days. Sterile water was then added, and the cultures were gently scraped with a spatula and passed through a cheesecloth to remove mycelial fragments and obtain a spore suspension. The suspension volume was made up of sterile water, and the spore concentration was adjusted to 1×10^7 spores mL⁻¹ using a hemocytometer. To improve adhesion, 0.05% (w/v) carboxymethyl cellulose (CMC) was added, and Tween 20 was incorporated at three drops per liter to ensure a uniform spore distribution. Finally, the suspensions were agitated on a shaker for 30 min to achieve a homogeneous mixture.

2.2.4. Establishment of the pot experiment

Pot experiments were conducted using the Siirt pistachio cultivar to assess the efficacy of the antagonists against *F. solani* KY1. Seeds harvested in the same year were stored in the dark at 4 °C for 4-5 months. Before sowing, the seeds were soaked in water for 12 h and then dried on absorbent paper to enhance germination. For surface disinfection, the seeds were treated with 2% NaOCl for 5 min and rinsed twice with sterile water. After soaking in the prepared spore suspensions for approximately 60 min, three seeds per pot were sown into 5 L pots containing either contaminated or sterile soil, which had been prepared one day prior. The experiment included a positive control (contaminated soil + untreated seeds), a negative control (sterile soil + untreated seeds), and four antagonist treatments with contaminated soil, arranged in a randomized block design with a total of six treatments and four replicates per treatment. The trial was conducted in the phytopathology laboratory under a controlled-environment chamber at 25-30 °C, with irrigation and routine maintenance performed periodically throughout the experiment.

2.2.5. Evaluation of pot experiments

Throughout the pot experiment, the shoots were regularly monitored, and any plants that dried out were recorded. At the end of the experiment, on July 25, 2023, before even completing one year, all plants were removed and evaluated using the following 0-5 scale.

(0) No lesions: plants healthy, (1) Damage to ≤20% of the roots or slight wilting of the upper part of the plant, (2) Damage to 21-40% of the roots, loss of some lateral roots, or wilting of one-third of the upper parts, (3) Damage to 41-60% of the roots, (4) Damage to 61-80% of the roots, loss of most lateral roots, or wilting of half of the upper parts, (5) Damage to 81-100% of the roots, loss of all lateral roots, or complete wilting of the upper parts.

Disease severity for each assessment was calculated as a percentage based on the scale values using the Townsend-Heuberger formula (Equation 3), and the mean values were subsequently determined.

$$\text{Severity of disease} = \frac{\text{Total } (n \times V)}{ZN} \times 100 \quad (3)$$

According to Equation 3, n represents the number of plants in each damage category, V denotes the damage severity divided into groups, N is the total number of plants evaluated, and Z corresponds to the highest scale value.

The efficacy of the treatments was evaluated by comparing these values with those of the positive control using the Abbott formula (Equation 4).

$$\text{Percentage effect (\%)} = \frac{(X-Y)}{X} \times 100 \quad (4)$$

Where, X mean disease severity in positive control plots (%), Y mean disease severity in treated plots (%).

2.3. Statistical evaluations

The collected data were analyzed by ANOVA (analysis of variance), and differences among means were compared using the Least Significant Difference (LSD) test. All statistical analyses were conducted using the JMP 13 software.

3. Results

3.1. The antagonistic activity of biological control agents

In this study, the effects of *C. rosea* isolates as biological control agents against the soil-borne pathogen *F. solani*, the causal agent of root rot in pistachios, were evaluated. The results of the assessment of the antagonistic activity via volatile metabolites are presented in Table 1. As shown in Table 1, although all treatments were assigned to the same statistical group, no significant differences in efficacy were observed among them. Among the isolates, *C. rosea* F10 showed the highest inhibition (9.04%), followed by *C. rosea* LO41 (7.68%) and *C. rosea* F9 (6.77%). In contrast, *C. rosea* F8 exhibited the lowest antagonistic activity (4.86%) (Table 1).

Table 1. Inhibitory effects of volatile metabolites produced by *C. rosea* isolates on *F. solani*

Treatments	Mean colony diameter (mm)*	Inhibition (%)
<i>C. rosea</i> F10	70.05 a	9.04
<i>C. rosea</i> F8	73.27 a	4.86
<i>C. rosea</i> LO41	71.10 a	7.68
<i>C. rosea</i> F9	71.80 a	6.77
<i>F. solani</i> KY1	77.02 ab	
Standard error	1.21	
P-value	<0.001	

*: Values followed by the same letter in column do not differ significantly at $p < 0.01$

The initial results of the evaluation of the antagonistic activity using the dual culture method are presented in Table 2. The examination of Table 2 indicates the formation of two statistically distinct groups. The highest antagonistic activity against the pathogen was observed in *C. rosea* LO41 (17.20%), followed by *C. rosea* F8 (15.28%) and *C. rosea* FG9 (14.44%). These three isolates exhibited similar effects and were thus grouped together. In contrast, *C. rosea* F10 exhibited the lowest antagonistic activity, with an inhibition rate of 6.01% (Table 2).

The results of the second experiment, which evaluated the antagonistic activity using the dual

culture method, are presented in Table 3. As shown in Table 3, *C. rosea* isolates generally exhibit hyperparasitic behavior. Among them, *C. rosea* F8 and F10 demonstrated notably stronger hyperparasitic activity (VSH) than the other isolates (Table 3).

Table 2. Antagonistic activity of *C. rosea* isolates against *F. solani* as determined by the dual culture method

Treatments	Mean colony diameter (mm)*	Inhibition (%)
<i>C. rosea</i> F10	39.05 ab	6.01
<i>C. rosea</i> F8	35.20 a	15.28
<i>C. rosea</i> LO41	34.40 a	17.20
<i>C. rosea</i> F9	35.55 a	14.44
<i>F. solani</i> KY1	41.55 ab	
Standard error	0.87	
P-value	<.001	

*: Values followed by the same letter in column do not differ significantly at $p < 0.01$

3.2. Antagonistic efficacy against *F. solani* in pot experiments

In trials conducted with pistachio seeds treated with antagonists in artificially infested soil, the mean disease severity across replicates, resulting statistical groups, and efficacy of the treatments are presented in Table 4. As shown in Table 4, the differences in mean disease severity among the treatments were statistically significant. The disease severity in the positive control (*F. solani* KY1) was 72.50%. The corresponding disease severities for the antagonist treatments were 20.00% for *C. rosea* F10, 27.50% for F8, 37.50% for LO41, and 52.50% for FG9, respectively. Compared with the positive control, F10 was the most effective antagonist, achieving 72.41% efficacy, whereas F9 exhibited the lowest efficacy (27.58%) (Table 4). Figure 1 shows pistachio seedlings treated with *C. rosea* F10 and F8, along with positive control plants, after their removal from the pots.

Table 3. In vitro antagonistic effects of *C. rosea* isolates against *F. solani*

Antagonists	1. Repetition	2. Repetition	3. Repetition	4. Repetition
<i>C. rosea</i> F10	VSH	SH	VSH	SA
<i>C. rosea</i> F8	VSH	VSH	VSH	SA
<i>C. rosea</i> LO41	SA	VSH	SA	SA
<i>C. rosea</i> F9	SA	H	SA	H

SA: Strong antibiosis, H: Moderate hyperparasitism, SH: Strong hyperparasitism, VSH: Very strong hyperparasitism

Table 4. Evaluation of disease severity, grouping, and efficacy of treatments in pistachio seed trials under artificially infested soil conditions

Treatments	Mean disease severity (%)*	Treatment efficacy (%)
<i>C. rosea</i> F10	20.00 d	72.41
<i>C. rosea</i> F8	27.50 cd	62.06
<i>C. rosea</i> LO41	37.50 c	48.27
<i>C. rosea</i> F9	52.50 b	27.58
<i>F. solani</i> KY1 (control)	72.50 a	
Standard error	4.28	
P-value	<.0001	

*: Values followed by the same letter in column do not differ significantly at $p < 0.01$

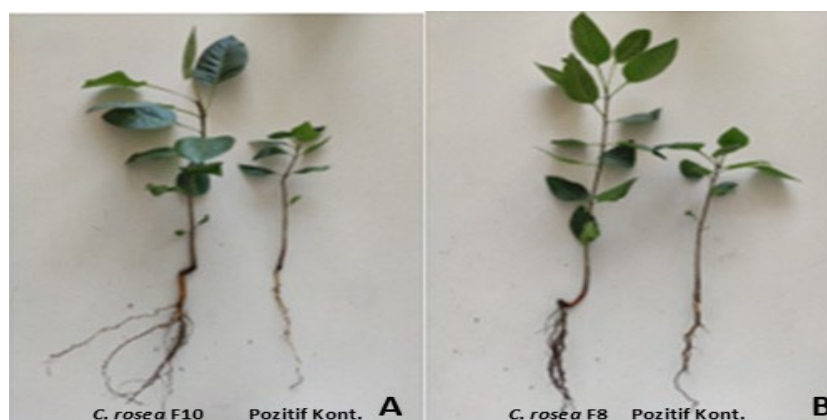


Figure 1. Pistachio seedlings treated with *C. rosea* F10 and positive control plants (A), and seedlings treated with *C. rosea* F8 and positive control plants (B)

4. Discussion and Conclusion

Pistachio production is an important source of income for both the Turkish economy and agricultural sector. However, various constraints during the production process, including unfavorable environmental conditions and pest and disease pressures, limit productivity. The use of non-certified, potentially infected seeds and saplings, along with the establishment of orchards in soils infested with soil-borne pathogens, further exacerbates disease-related issues.

In the Southeastern Anatolia Region, the expansion of pistachio production areas and the increasing incidence of soil-borne diseases have been associated with the establishment of orchards in areas previously used for polyculture farming. Several soil-borne pathogens, such as *Rhizoctonia solani*, *Fusarium* spp., *F. solani*, and *Macrophomina phaseolina*, are known to cause economically important diseases in pistachio trees (Aydın and Ünal, 2021; Aydın et al., 2023, 2024). *Fusarium* species can infect a diverse range of crops, including wheat, maize, strawberry, barley, beans, cucurbits, and anise (Nganje et al., 2004; Leslie and Summerell, 2013; Beccari et al., 2022). In particular, *F. solani* is known to damage numerous forest species and a wide range of fruit trees, including pistachios (Arie, 2019).

In agriculture, chemical control is often considered one of the most practical and rapid techniques for controlling plant diseases and pests. However, soil-borne pathogens such as *F. solani* are typically polyphagous and possess a wide host range, making chemical control difficult or economically unfeasible. Consequently, biological control has emerged as an important alternative. Numerous antagonistic microorganisms are used in biological control, among which *C. rosea* has received considerable attention. This fungus, isolated from various plant tissues, has been reported to be an effective biocontrol agent (Barnett and Lilly, 1962; Shravan et al., 2025).

In this study, three isolates of the antagonist *C. rosea* obtained from pistachio roots, along with the LO41 isolate derived from soil, were evaluated against *F. solani*, the causal agent of root rot in pistachios. These results indicate that these antagonists were not effective in inhibiting *F. solani* through volatile metabolites. Since volatile metabolites produced by antagonists are associated with antibiotic activity, this suggests that their production is limited. Similarly, in the dual culture assay, the formation of inhibition zones resulting from the interaction between the antagonist and pathogen was limited. Observations and evaluations based on the scale proposed by Turhan (1990),

considering the rate and density of antagonist colonization of the pathogen colony, indicated that antagonist growth was initially slow during the first week but subsequently increased, leading to complete overgrowth of the pathogen. *Clonostachys rosea* F8 initially formed a slight inhibition zone and later spread over the pathogen. Similarly, *C. rosea* LO41 first produced an inhibition zone and subsequently colonized the pathogen, effectively suppressing its growth. An antagonist developed against *F. solani* on PDA medium is considered a hyperparasite if it continues to advance, overgrows the pathogen colony, and, upon microscopic examination, is observed to envelop and penetrate the hyphae of the pathogen. Microscopic observations in the present study confirmed that the antagonists exhibited distinct parasitic characteristics. Similarly, Aydın and Turhan (2009) reported that although *Gliocladium roseum* (syn. *Clonostachys rosea*) isolates appeared to grow slowly and weakly on *Rhizoctonia solani* colonies, and microscopic examinations revealed strong and aggressive hyphal interactions, including envelopment and penetration of the pathogen. In addition, *C. rosea* has been reported to possess a high mycoparasitic capacity and is an effective biological control agent against numerous plant pathogenic fungi (Roberti et al., 2008; Nygren et al., 2018).

In the pot experiment evaluating the efficacy of the antagonists against *F. solani* KY1, the antagonist treatments were found to be effective at varying levels. Compared to the positive control, *C. rosea* F10 was the most effective isolate, achieving an efficacy of 72.41%. As this is the first study to evaluate these antagonists in pistachios, this level of efficacy is considered promising for the management of this pathogen. Previous studies have also demonstrated the effectiveness of these antagonists against diseases in other crops as well. For example, Çevik et al. (2022) reported that *C. rosea* exhibited high suppression of pathogens causing Verticillium wilt and early blight in tomatoes. Furthermore, *C. rosea* possesses endophytic capabilities, rapidly colonizing plant tissues and providing effective control against a wide range of fungal plant pathogens (Jensen et al., 2004; Sun et al., 2020).

In conclusion, in vitro studies demonstrated that *C. rosea* exhibits a certain level of antagonistic activity against *F. solani*, primarily through mycoparasitism of the pathogen's mycelia. Pot experiments further revealed that disease severity in plants infected with *F. solani* was higher in the untreated control than in those treated with *C. rosea*, indicating that the antagonist effectively suppressed the pathogen.

Ethical Statement

The authors declare that ethical approval is not required for this research.

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Author Contributions Statement

Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing-Original Draft, Writing-Review & Editing, *M. YILDIRIM*; Conceptualization, Project Administration, Funding Acquisition, Methodology, Supervision, Writing-Review & Editing, *M.H. AYDIN*. All authors have seen, read and agreed to the published version of the manuscript.

Statement of Conflicts of Interest

The authors declare no conflicts of interest.

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