Research Article

Comparative Evaluation of Different Microbial Biocontrol Agents against *Rhizoctonia solani* Kühn, the Causative Agent of Root Rot of Pepper *Capsicum annuum* L.

Yousra A. Swadi Al-Anzi*, Kadhim Z.K. Al-Karaawi and Iman Jawad Kadhim

Biological Control Technologies Department, Technical College, Al-Mussaib, Al-Furat Al-Awsat Technical University, Babylon, Iraq.

Abstract | Pathogenic fungus *Rhizoctonia solani* Kuhn (Basidiomycota, Cantharellales) is one of the most destructive and economically important ubiquitous soil-borne necrotrophic fungal pest that inflicts injury to a wide range of crop plants. This study isolated and diagnosed some promising fungal and bacterial strains on the basis of ITS1 and ITS4 and 16S rRNA gene sequences, respectively, and assessed the inhibition or antagonistic potential of these microbial biocontrol agents against *R. solani* under laboratory conditions. The pathogenic fungus *R. solani* isolates caused a significant decrease in the germination rate of cucumber seeds on water agar medium as compared to the control treatment. Results of antagonistic bioassays showed a significantly high antagonism between *R. solani* and the biological control agents as compared to the control treatment. The fungal isolate *Trichoderma longibrachiatum* had the highest inhibition (98.0%), followed by the bacterial isolate *Bacillus paramycoides* with an inhibition of 95.33%. Other biocontrol isolates including fungus *Talaromyces oumae-annae*, *Trichoderma harzianum* and bacteria *Bacillus subtilis* exhibited an inhibition from 90 to 93%. It is concluded that all the microbial biocontrol agents tested in this *in-vitro* study, particularly fungal strain *T. longibrachiatum* and bacterial isolate *B. paramycoides*, could be effective and environment-friendly alternates against *R. solani*-induced root rot disease in plants.

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***Correspondence** | Yousra A. Swadi Al-Anzi, Biological Control Technologies Department, Technical College, Al-Mussaib, Al-Furat Al-Awsat Technical University, Babylon, Iraq; **Email:** yousra.ahmed.tcm.33@student.atu.edu.iq
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Introduction

Sweet pepper *Capsicum annuum* L. is one of the most important vegetable crops being cultivated in many countries around the world. Native to Central and South America, its production has been spread to Europe and Asia through Spain and Portugal in the 16th century, about 400 years ago (Chan, 2000; Katz, 2019). Green peppers are widely used as fresh or cooked in many food recipes. Dried peppers are used as spices in cooking and medicine. *C. annuum* fruits exhibit considerable stimulating and carminative

effects which help maintaining health. In addition, it can prevent heart disease and thrombosis and has anti-inflammatory properties (Soare *et al*., 2017).

However, production of *C. annuum* is hampered by a number of factors including different fungal diseases. There are many fungal pathogens which infect vegetables including *C. annuum*. Particularly, soil-borne fungi such as *Rhizoctonia solani* Kühn has been a challenging disease of peppers. It infects the roots, stem base and canopy area of plants hindering the absorption of water, nutrients and salts from the rhizosphere (Mannai *et al*., 2018). Vegetable growers rely predominately on different synthetic fungicides which apart from giving unsatisfactory control manifest various side-effects including human health hazards and environmental contamination (Harris *et al*., 2001).

One of the alternate and environment-friendly solutions to these ecological consequences of synthetic fungicides is the utilization of different microbial biocontrol agents which can suppress the pathogenic organisms in the soil without affecting the beneficial flora of the rhizosphere (Madbouly and Abdelbacki, 2017). These beneficial microbes have a variety of mechanisms for controlling different phytopathogens, including direct parasitism, competition for nutrients, and production of secondary metabolites such as antibiotics and mycotoxins (Kumar and Kaushik, 2012; Aslam *et al*., 2023). One of the important biocontrol fungi is *Trichoderma* which are known for their ability to promote root growth and development, enhance resistance to various environmental conditions, and improve crop productivity and nutrients uptake (Ruangwong *et al*., 2021; Asad, 2022).

Among beneficial soil bacteria genus *Bacillus*, particularly *B. subtilis*, have received special attention, since these have been a crucial part in many biocontrol programs against a wide array of plant pathogens (Dimkić *et al*., 2022; Etesami *et al*., 2023). In addition, some species of fungi such as *Penicillium* and *Talaromyces oumae-annae* are known for their antagonistic activity against many plant pathogens by secreting certain antibiotic compounds and by inducing the systemic resistance in plants by activating several defense signaling pathways (Haque *et al*., 2021; Al-Karawi, 2022; Ren *et al*., 2024).

Keeping in view the ecological consequences of synthetic fungicides and biocontrol potential of above mentioned fungal and bacterial microbes, this study was aimed to compare the antagonistic efficiency of some selected microbial biological control agents against the pathogenic fungi *R. solani*, the causative agent of root rot of pepper *C. annuum* under laboratory conditions.

Materials and Methods

Isolation and pathogenicity test of R. solani

Infected pepper plants with root rot symptoms were randomly collected from different field sites located in Babil Governorate, Iraq. Infected plants were uprooted, placed in sterilized polythene bags and transported to the laboratory for study. The roots were washed with tap water for 3 min to remove attached soil particles. Root segments of approximately 1.0 cm in length were collected and sterilized with a chlorine free 1.0% sodium hypochlorite solution for 2–3 min. Cells are then transferred to sterile distilled water for 2 min, washed thoroughly and placed on sterile filter paper inside a laminar flow hood to remove excess water. Four plant segments were inoculated from Petri plate containing 15–20 ml of potato dextrose agar (PDA) supplemented with tetracycline @ 250 mg/L. The tool was sterilized in an autoclave at 121˚C and 1.5 kg/cm^2 pressure for 15–20 min. The Petri plates were placed in an incubator at 25±1˚C for four days. Fungal growth was examined from infected plant parts, and small portions of each fungal growth were transferred to Petri plates containing PDA and were incubated at 25±2˚C for 7 days. The identification of fungi was carried out based on the morphological characteristics of the fungal colonies, the mycelial nature and using the taxonomic keys of Blazier (2004). The pathogenicity of isolated *R. solani* was tested according to Bolkan and Butler's (1974). Seed germination percentage was calculated using the following equation:

$$Percent\ Germination = \left(\frac{number\ of\ germinated\ seeds}{total\ number\ of\ seeds}\right) \times 100$$

Isolation of biocontrol agents

Soil samples were randomly collected from the rhizospheric profiles of a healthy maize crop located in Babil Governorate, Iraq, and these samples were transferred to the laboratory in sterile polythene bags where these were air dried for 24 h and then were sieved through a 1.0 mm mesh. Using serial dilutions

protocol, soil mixtures were cultured on PDA medium enriched with tetracycline @ 250 mg/L. Petri dishes were gently shaken to ensure uniform sample distribution and then were incubated in a microbiological incubator at 25±1°C for 4 days. Fungal isolates of *Trichoderma* spp. were identified using taxonomic keys of Rifai (1969), while the *Talaromyces oumae-annae* isolate was identified according to the taxonomic key of Samson *et al.* (2001). Bacterial isolates were cultured using nutrients agar and were identified according to the standard taxonomic keys provided by Bergey *et al.* (1984).

Molecular diagnosis of isolated microbes by PCR

These diagnoses were performed at the DNA Genotek Laboratory® for molecular research. DNA of fungi *Trichoderma longibrachiatum*, *T. harzianum*, *Talaromyces oumae-annae* and of bacteria *Bacillus paramycoides* was extracted using ZR Fungal/Yeast/Bacterial DNA MiniPrep™ kit (Zymo Research, USA) following manufacturer's instructions. Primers ITS1 and ITS4 (Martin and Rygiewicz, 2005) and 16S rRNA (Majeed *et al.*, 2015) were used for PCR amplifications for fungal and bacterial detection, respectively. These primers were manufactured by Integrated DNA Technology®, as detailed in Table 1. Each PCR reaction mixture was of 25 µL volume containing Master Mix (iNtRON Biotechnology, Korea), 10 pmole of each primer and template DNA. PCR amplifications were performed in T3000 thermocycler (Biometra, Germany) using thermal protocols as detailed in Table 2. PCR products were visualized on an electrophoresis gel under UV light using gel documentation. In order to analyze the nucleotide sequence of the amplified DNA fragments, PCR amplification products from the isolates were forwarded to Macrogen Co., Ltd., Korea for DNA sequence analysis.

Regarding pathogenic bacteria, a previously identified isolate of *B. subtilis* was obtained from the Pathology Laboratory of the Department of Biotechnology, Technical College, Al-Musayyib, Iraq. *B. paramycoides* on the other hand was isolated from the soil as mentioned above. Both bacteria were mass-cultured in a nutrient broth medium in 500 mL sterile glass bottles in an autoclave at 121°C and a pressure of 1.5 kg/cm^2 for 15 min. The medium was then inoculated with bacteria by taking a sterile sample of bacterial growth on a previously prepared agar medium. The contents of the vials were mixed well and then incubated at 32 ± 3°C for 3–4 days.

Table 1: *DNA sequences of primer pairs used for the detection of ITS gene region of the biocontrol fungi Talaromyces oumae-annae, Trichoderma longibrachiatum and T. harzianum, and for detecting the 16S rRNA gene region in the bacterium Bacillus paramycoides.*

Primer/ Sequence	Tm (°C)	GC (%)	Product size
For fungi			
F 5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50	650 base pair
R 5′-TCCTCCGCTTATTGATATGC-3′	57.8	41	
For bacteria			
F 5'-AGAGTTTGATCCTGGCTCAG-3'	54.3	50.0	1250 base pair
R 5'-GGTTACCTTGTTACGACTT- 3’	49.4	42.1	

Table 2: *Thermal protocol used for the PCR amplification of fungal and bacterial DNA fragments.*

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial denaturation	94°C	3 min.	1 cycle
2	Denaturation	94°C	45sec	35 cycles
3	Annealing	52°C	1 min	
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	7 min.	1 cycle

Evaluation of antagonistic potential of microbial isolates against R. solani

The antagonistic ability of *B. paramycoides* and *B. subtilis* was assessed against the pathogenic fungus *R. solani* on nutrients agar medium using the dilution method (1.0×10^1 - 1.0×10^8 cfu mL^{-1}) post 72 h bacterial inoculations. In brief, 1.0 mL of the bacterial suspension was transferred to 9 cm Petri plate containing nutrient agar medium, with the plate gently shaken to evenly distribute the inoculum (1.0×10^4 - 1.0×10^8 cfu mL^{-1}). Four replicates were maintained for each bacterial dilution. A 7-days old *R. solani* culture disc of 5.0 mm diameter was placed in the center of each plate for each dilution. Four plates were incubated without added bacteria as control. The Petri plates were incubated at 26 ± 1°C for 15 days. The growth rate of pathogenic fungi and the percentage of inhibition were calculated according to the following equation of Montealegre *et al.* (2003).

$$\text{Inhibition of mycelial growth (\%)} = \left(1 - \frac{\text{mycelial growth (diameter) of treatment}}{\text{mycelial growth (diameter) of control}}\right) \times 100$$

The antagonistic ability of the biocontrol fungi *T.*

longibrachiatum, *T. oumae-annae* and *T. harzianum* was determined using double-culture technique (Miftahurrohmat *et al*., 2021) against *R. solani* in 9 cm Petri plates containing PDA medium. The medium was sterilized using an autoclave for 15–20 min and the Petri plate media was divided into two equal halves by drawing a line, with the central portion of one half inoculated with a 7 day-old *R. solani* culture disc, while the other half was inoculated with a fungal disc from the edge of the culture of the biocontrol fungi *T. longibrachiatum*. Same protocol was used for *T. oumae-annae* and *T. harzianum*. All Petri plates were then incubated at a temperature of 25±2 ˚C. Once the growth of the pathogenic fungus reached the edge of the plate in the control treatment, the percentage inhibition was calculated using the above-mentioned equation.

Statistical analysis

This research was carried out in a completely randomized design (CRD) and the statistical program GenStat (Payne, 2009) was used to analyze the data to study the effect of different treatments on the studied traits. Significant differences between means were compared using the least significant difference (LSD) test.

Results and Discussion

Isolation and identification of R. solani

Field samples collected from the diseased parts of pepper plants (*i.e.* from roots and areas adjacent to the trunk) revealed the presence of four *R. solani* fungal isolates. Microscopic examination showed a clear difference in their growth rate, sclerotia formation and fungal mycelial density, in addition to differences in the colony color ranging from brown to light brown with short and numerous cells. The fungal mycelium exhibited numerous ramifications at perpendicular and intersecting angles with the original fungal mycelium, as well as cellular constriction in the branching area and the formation of depressed barriers on the branches near the point of origin. It was observed that neither spores nor asexual conidia were formed as described by some previous studies (Stalpers and Andersen, 1996; Ajayi- Oyetunde and Bradley, 2018).

Pathogenicity test with isolated R. solani on lettuce seedlings

According to pathogenicity test, *R. solani isolate* exhibited a differential effect on lettuce seed germination as shown in Figure 1. This antagonistic potential of *R. solani* corroborates the findings of previous studies (Adesina *et al*., 2009). Inhibition of lettuce seed germination would be due to the fact that fungus *R. solani* produces an array of enzymes that aid in the breakdown of host cell walls, such as pectinase, pectin methylesterase, cellulase and phosphatase (Dillard, 1987; Verwaaijen *et al*., 2017; Laevens *et al*., 2024).

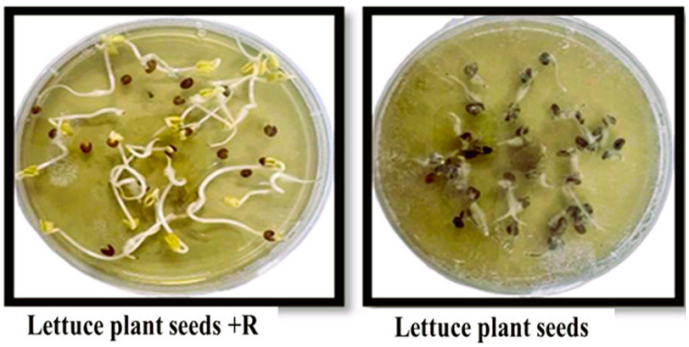

Figure 1: *Pathogenic or antagonistic ability of the fungus R. solani isolate on the seeds of lettuce plant.*

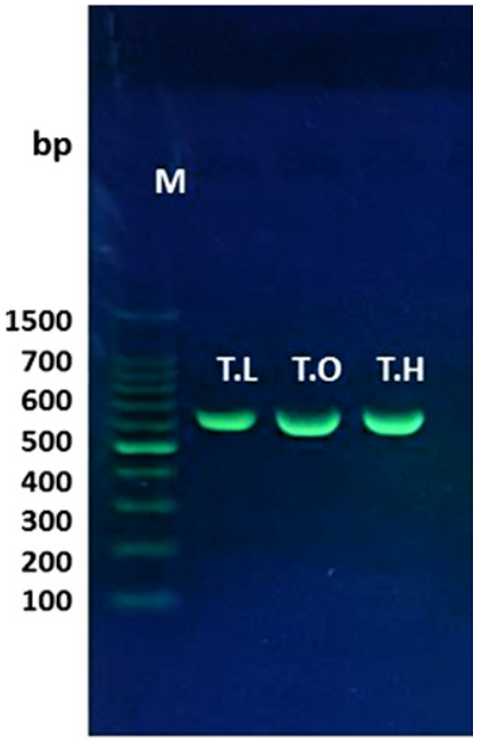

Figure 2: *Inhibition of pathogenic fungus R. solani by some biocontrol agents (Bacillus paramycoides and B. subtilis, Talaromyces oumae-annae, Trichoderma longibrachiatum and T. harzianum) and synthetic fungicide Beltanol (hydroxyquinoline sulphate) on PDA agar medium under laboratory conditions.*

Isolation and molecular identification of microbial biocontrol agents

PCR product of fungal DNA amplified utilizing ITS1 and ITS4 primers was electrophorized on sucrose gel and it exhibited a distinct band of molecular weight of

650 bp (Figure 2). This detection validates the efficacy of these primers in amplifying rDNA nucleic acid for fungal species as evidenced by literature (Korabečná *et al*., 2003). Similarly, for the bacterial strain examined, the electrophoresis on sucrose gel displayed a band of molecular weight of 1250 bp (Figure 3) when employing 16*S* rRNA cloning primers. This finding also affirms the capability of these primers to amplify rDNA nucleic acid of bacteria as demonstrated in various studies (Cardinale *et al*., 2004; Mirtalebi *et al*., 2019).

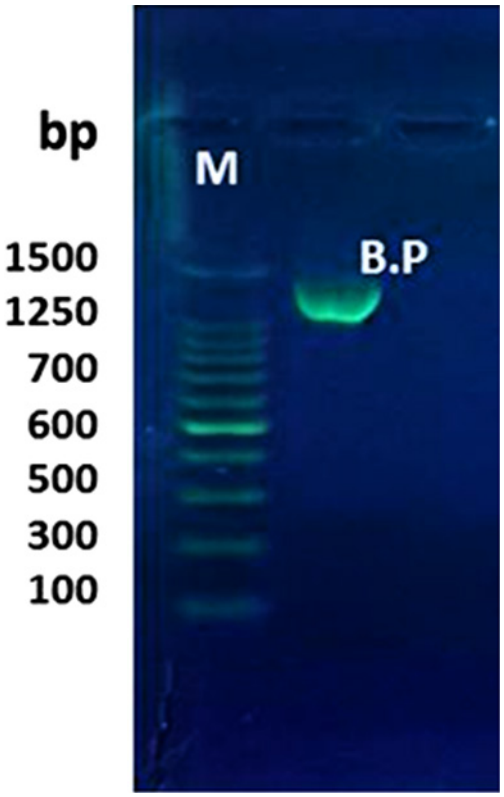

Figure 3: *Electrophoresis gel image of the PCR amplified DNA product of biocontrol fungi Talaromyces oumae-annae (T.o), Trichoderma longibrachiatum (T. l) and T. harzianum (T.h.).*

Nucleotide sequencing study

Nucleotide sequence analysis of the fungus *T. longibrachiatum* showed 98% similarity with the global isolates available in GenBank NCBI. In addition, the phylogenetic tree of the fungus *T. longibrachiatum* (Figure 4) revealed that the Iraq isolate corresponded 98% with the isolates from Moldova and 99% with the isolates from China, Egypt, Mexico and Belgium. The genomic DNA sequences of fungal isolates have been deposited in the global repository GenBank (NCBI) with accession number PP564406 assigned, serving as a point of reference for Iraq, the Middle East, and worldwide (Figure 4). Nucleotide sequence results for the fungus *T. oumae-annae* showed that this isolate had a 100% similarity with the worldwide isolates available in GenBank database (NCBI). In addition, the phylogenetic tree of *T. oumae-annae* fungi (Figure 4) showed that the Iraq isolate corresponded 100% with the isolates from Iran, Ethiopia and Europe and 94% with isolates from China and Malaysia. These fungal nucleotide sequences are deposited in the GenBank database with the accession number PP576344, establishing itself as a key reference for Iraq, the Middle East, and globally (Figure 4). The results of the nucleotide sequences for the fungus *Trichoderma harzianum* revealed a 98% similarity with the diverse isolates available in GenBank NCBI.

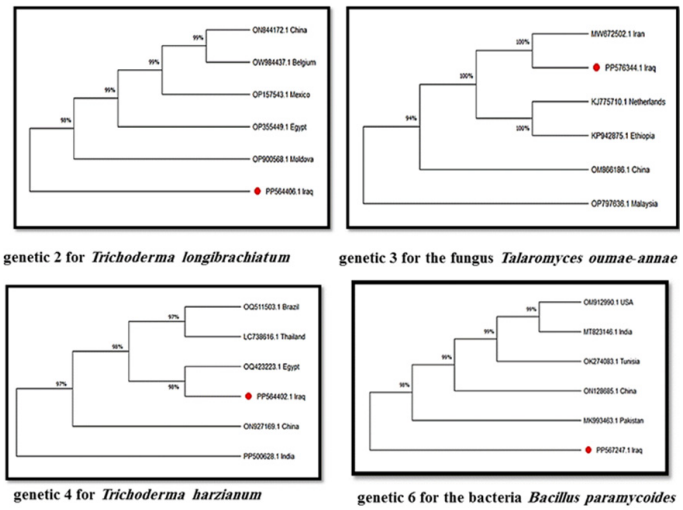

Figure 4: *Electrophoresis gel image of the PCR amplified DNA product of biocontrol bacterium Bacillus paramycoides (B. p.).*

Moreover, the phylogenetic tree analysis of *T. harzianum* (Figure 4) indicated that the Iraqi specimen displayed a 98% match with isolates from Egypt and a 97% resemblance with isolates from China, Brazil, India, and Thailand. Another set of fungal nucleotide sequences were deposited in GenBank under accession number PP564402, further solidifying it as a significant reference for Iraq, the Middle East and worldwide research (Figure 4). Regarding the nucleotide sequencing based phylogenetic analysis of bacterial isolates, it was found that bacterial isolate *B. paramycoides* exhibited a 98% match with the global bacterial isolates available in the NCBI GenBank. Additionally, the phylogenetic tree of the *B. paramycoides* bacteria (Figure 4) demonstrated a 98% alignment with the bacterial isolates from Pakistan and a 99% alignment with the isolates from USA, India, Tunisia and China. The nucleotide sequences of the bacteria were deposited in the global GenBank organization with the accession number PP567247, establishing it as a reference for Iraq and the Middle East (Figure 4).

Table 3: *Inhibition of pathogenic fungus Rhizoctonia solani by some biocontrol agents (Bacillus paramycoides and B. subtilis, Talaromyces oumae-annae, Trichoderma longibrachiatum and T. harzianum) and synthetic fungicide Beltanol (hydroxyquinoline sulphate) under laboratory conditions.*

S. No.	Treatments	Colony diameter (cm)	Scaling ratio (%)
1	*Bacillus paramycoides* + *Rhizoctonia solani*	0.3	95.3
2	*Trichoderma longibrachiatum* + *Rhizoctonia solani*	0.1	98.0
3	*Rhizoctonia solani* + *Talaromyces oumae-annae*	0.5	93.3
4	Pathogenic fungi alone	9.0	0.0
5	*Trichoderma harzianum* + *Rhizoctonia solani*	0.8	92.0
6	*Bacillus subtilis* + *Rhizoctonia solani*	0.6	91.0
7	*Rhizoctonia solani* +Beltanol	0.0	100
LSD (P ≤ 0.05) Colony diameter = 0.151		Scaling ratio = 1.779	

In-vitro antagonistic potential of microbial biocontrol agents against R. solani

Laboratory bioassays carried out to assess the inhibition potential of the isolated fungal and bacterial strains against phytopathogenic fungus *R. solani* revealed a high antagonistic capacity of all these the biocontrol microbes. All these fungal and bacterial isolates exhibited a significant inhibition of the growth and proliferation of *R. solani* as compared to the control treatment (Table 3 and Figure 5). All tested fungal strains of *Trichoderma* and *Talaromyces* genera showed a high antagonistic potential and inhibition effect reached the edge of the plaque, after four days of the inoculation of pathogenic fungi (*R. solani*). The treatment with *T. longibrachiatum* showed the highest inhibition *i.e.* 98.0%. These results corroborate the fact that fungi such as *Trichoderma* and *Talaromyces* are important biological control agents against phytopathogens (Kakvan *et al.*, 2013; Abbas *et al.*, 2025). These fungi have various antagonistic properties against plant pathogens and are crucial for plant growth and production, in addition to their ability to stimulate plant resistance to different diseases and other pests (Harman and Uphoff, 2019; Thambugala *et al.*, 2020; Xie *et al.*, 2021; Abbas *et al.*, 2025).

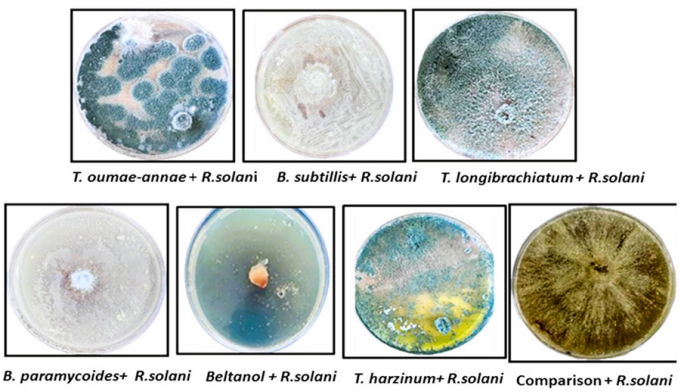

Figure 5: *The phylogenetic tree analysis of Trichoderma harzianum and Bacillus paramycoides.*

Most fungi including *Trichoderma* spp. show a high effectiveness against plant pathogens and have differential mechanisms of competition for space, nutrients and parasitism (Adnan *et al.*, 2019). The antagonistic organisms directly remove nutrients from the pathogens, and the resistance mechanism involves the production of metabolic substances that prevent the pathogens from invading plant tissues (Baker and Paulitz, 1996; Stracquadanio *et al.*, 2020; Adeleke *et al.*, 2022). Apart from the above-mentioned fungal isolates, the bacteria *B. paramycoides* as well showed a high inhibition of 95.33%, followed by the treatment with the mold *T. oumae-annae*, which showed an inhibition of 93.33%. In addition, the treatment with the fungus *T. harzianum* showed a considerable inhibition (92.0%) of *R. solani*, and the treatment with the bacteria *B. subtilis* showed an inhibition of 91.0%. These results are in line with the findings of El-Sersawy *et al.* (2021) demonstrated the effectiveness of *Bacillus* isolates against *R. solani* and against other fungal diseases such as against *Fusarium oxysporum*-induced and *R. solani*-induced wilt in soybean plants (Jain *et al.*, 2017; El-Sersawy *et al.*, 2021; Maral-Gül and Eltem, 2024). Similarly, our results validate the results of Singh *et al.* (2021) and Lotfalinezhad *et al.* (2024) reporting the biocontrol efficiency of *T. oumae-annae* and *T. harzianum* against *R. solani*.

Conclusions and Recommendations

On the basis of overall study results, it is concluded that the microbial biocontrol agents tested in this *in-vitro* study could be effective and environment-friendly alternates for combating against the incidence of *R. solani* on plants. Particularly, fungal strain *T. longibrachiatum* and bacterial isolate *B. paramycoides* effectively reduced the severity of pathogenic fungus *R. solani*-induced root rot disease in plants. However,

field evaluation of these microbial biocontrol agents and their interaction with other beneficial non-target fauna and flora constitute the future perspectives of this study.

Acknowledgements

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Novelty Statement

All the microbial biocontrol agents we tested in this in-vitro study, particularly fungal strain *T. longibrachiatum* and bacterial isolate *B. paramycoides*, could be used as effective and environment-friendly alternates against *R. solani*-induced root rot disease in plants.

Author's Contribution

Yousra A. Swadi Al-Anzi , Kadhim Z.K. Al-Karaawi and Iman Jawad Kadhim: Conceived of the original idea, developed the theoretical and performed the statistical analysis for experimental data and discussed the results and contributed to manuscript writeup.
Yousra A. Swadi Al-Anzi and Kadhim Z.K. Al-Karaawi: Verified the analytical methods.
Kadhim Z.K. Al-Karaawi and Iman Jawad Kadhim: Worked for lab analysis and supervised the project.

Conflict of interest
The authors have declared no conflict of interest regarding the publication of this research work.

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