

Integration of *Trichoderma asperellum* the bacteria *Serratia marcescens* and *Sphingobacterium zeae* and eucalyptus extract for the control of okra root rot caused by *Rhizoctonia solani*

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Abstract

*This study aimed to identify the causal agents of okra root rot and evaluate the efficacy of certain biocontrol agents and plant extracts in suppressing the disease caused by *Rhizoctonia solani*. A field survey revealed the disease's presence across all studied fields in Babylon Governorate, with an incidence rate of 17–81% and a severity of 10–44%. Ninety-eight fungal isolates were recovered; *R. solani* was the most frequent 72 isolates, followed by *Fusarium solani* 15 isolates and *Macrophomina phaseolina* 11 isolates, with the *R. solani* isolate Rs6 demonstrating the highest virulence. The bacteria *Serratia marcescens* and *Sphingobacterium zeae* were molecularly identified, showing 100% and 99.7% sequence identity, respectively. Laboratory assays showed 100% inhibition by *Trichoderma asperellum* and *S. marcescens*, and 99.67% by *S. zeae*, while inhibition by eucalyptus extract reached 85.22% for the aqueous extract and 85.78% for the ethanolic extract at a concentration of 4 g/100 ml. In greenhouse trials, biocontrol treatments reduced disease severity and improved plant growth compared to infected plants; the treatment combining aqueous eucalyptus extract with *T. asperellum* resulted in the lowest disease severity 5.24%, whereas the combination of *T. asperellum* and *S. marcescens* yielded the highest values for most growth parameters. These results confirm the potential of integrating biocontrol agents with eucalyptus extract within integrated management programs for okra root rot caused by *R. solani*.*

Keywords: biological control; okra; *Rhizoctonia solani*; *Trichoderma asperellum*; *Serratia marcescens*; *Sphingobacterium zeae*; eucalyptus extract.

1-Introduction

Okra (*Abelmoschus esculentus* (L.) Moench) is an economically and nutritionally important vegetable crop belonging to the family Malvaceae. It is widely grown in tropical, subtropical, and warm temperate regions because of its adaptability and high nutritional value. Its fruits contain proteins and vitamins, particularly vitamins A and C, in addition to fiber, minerals, phenolic compounds, and antioxidants (Islam, 2019; Liu et al., 2021).

Okra is affected by several diseases, particularly those caused by soil-borne pathogens such as *Rhizoctonia solani*, *Pythium spp.*, *Phytophthora spp.*, and *Fusarium oxysporum*. These pathogens cause seed, seedling, and root rots as well as plant wilting, negatively affecting yield and quality (Jukte et al., 2016).

The increasing use of chemical fungicides has been associated with environmental and health problems, including soil and water contamination, accumulation of residues, and the development of fungicide-resistant isolates. This has encouraged the search for safer and more sustainable alternatives. Biological control is among the most important of these alternatives because it relies on beneficial microorganisms or their bioactive products to suppress pathogens and improve plant growth through competition, production of antibiotics and volatile compounds, secretion of lytic enzymes, and induction of plant defenses (Ons et al., 2020; Harman et al., 2021; Zubair et al., 2021).

Trichoderma asperellum is considered a promising biocontrol fungus because of its ability to suppress plant-pathogenic fungi through mycoparasitism and the production of enzymes and antifungal compounds, in addition to inducing plant resistance and improving plant growth (Elshahawy & Marrez, 2024). *Serratia marcescens* and *Sphingobacterium zeae* have also shown promising potential in controlling plant pathogens and promoting plant growth through the production of bioactive compounds, enzymes, volatile substances, and the stimulation of plant defense mechanisms (Akhtar et al., 2025; Yadav et al., 2025).

Despite the large number of studies on biological control agents, studies directly comparing *T. asperellum*, *S. marcescens*, and *S. zeae* for inhibition of *R. solani*, the causal agent of okra root rot, remain limited. Therefore, this study aimed to evaluate the antagonistic efficiency of these agents in inhibiting the radial growth of *R. solani* under laboratory conditions and to identify the most efficient agent for possible inclusion in integrated management programs for okra root rot.

2-Materials and Methods

2-1 Field Survey

A field survey of okra was conducted in Babylon Governorate from 2/9 to 21/9/2025 during the 2025 growing season, and samples were randomly collected from the surveyed fields. Infected plants were placed in polyethylene bags, with the location and date of collection recorded for each sample, and then transported to the Plant Pathology Laboratory, Department of Biological Control Technologies, Al-Mussaib Technical College, Al-Furat Al-Awsat Technical University. Samples were stored at 4°C, and isolation procedures were carried out on the day following collection.

Disease incidence was calculated based on the number of plants showing disease symptoms in each field according to the equation adopted from Al-Juboory et al. (2016):

$$\text{Disease incidence (\%)} = (\text{Number of infected plants} \div \text{Total number of examined plants}) \times 100$$

Root disease severity was calculated using a five-grade disease index as follows:

0 = Healthy plant.

1 = >0–25% of roots affected by rot and showing brown discoloration.

2 = >25–50% of roots affected by rot and showing brown discoloration.

3 = >50–75% of roots affected by rot and showing brown discoloration.

4 = >75–100% of roots affected by rot and showing brown discoloration, or plant death.

The percentage of disease severity was calculated according to the equation of McKinney (1923) as follows:

$$\% \text{ of severity of infection} = \frac{\frac{\text{Number of plants in Degree (0} \times 0)}{(5 \times 5)} + \frac{\text{Number of plants in Degree (1} \times 1)}{(1 \times 1)} + \frac{\text{Number of plants in degree}}{\text{Total number of examined plants} \times \text{highest degree of infestation}}}{\text{Total number of examined plants} \times \text{highest degree of infestation}} \times 100$$

2-2 Isolation and Identification of Pathogenic Fungi from Okra Roots

Pathogenic fungi were isolated from okra roots showing root-rot symptoms, including wilting, leaf yellowing, poor growth, and brown rotting on the main and lateral roots. Roots were washed under running water for 5 min, cut into 0.5-cm pieces, surface-sterilized with 1% sodium hypochlorite for 2 min, and dried on sterile filter paper. The sterilized pieces were plated on sterile Potato Dextrose Agar (PDA) supplemented with tetracycline at 250 mg/L, using four pieces per plate and four replicates, and incubated at $25 \pm 1^\circ\text{C}$ for 3 days.

The resulting fungal growths were transferred to fresh PDA plates for purification and incubated at $25 \pm 1^\circ\text{C}$ for 7 days. Fungi were identified to the genus level based on cultural and microscopic characteristics, including colony morphology, mycelium, spores, and reproductive structures, according to the taxonomic criteria described by Ellis (1971), Booth (1977), Barnett and Hunter (1998), and Leslie and Summerell (2006).

The frequency of occurrence of each fungus was calculated using the following equation:

$$\text{Frequency of occurrence (\%)} = (\text{Number of isolates of a fungus} \div \text{Total number of fungal isolates}) \times 100$$

2-3 Pathogenicity Test of Fungal Isolates Recovered from Okra Roots

The pathogenicity of seven *R. solani* isolates, five *F. solani* isolates, and three *M. phaseolina* isolates was tested using radish seeds on Water Agar according to Bolkan and Butler (1974). Petri dishes (9 cm diameter) containing the medium supplemented with tetracycline at 250 mg/L were inoculated at the center with 0.5-cm fungal discs taken from the margins of 5-day-old colonies and incubated at $25 \pm 1^\circ\text{C}$ for 3 days. Radish seeds were surface-sterilized with 1% sodium hypochlorite, and 25 seeds were placed in a circular arrangement near the edge of each plate, with three replicates per isolate and a pathogen-free control. Plates were incubated at $25 \pm 1^\circ\text{C}$ until germination was complete in the control treatment, after which germination percentage was calculated as follows:

$$\text{Germination (\%)} = (\text{Number of germinated seeds} \div \text{Total number of seeds}) \times 100$$

2-4 Isolation of Biological Control Agents from Soil

Four rhizosphere soil samples were collected from healthy maize plants with good vegetative growth in the Al-Jilawiya area of Babylon Governorate. Samples were air-dried for 24 h and sieved through a 1-mm mesh. Serial dilutions from 10^{-1} to 10^{-5} were prepared, and 1 mL of the fifth dilution was plated on Nutrient Agar with three replicates per sample. Plates were incubated at 30°C for 48 h.

Bacterial colonies growing on Nutrient Agar were purified and incubated at 30°C for 24–48 h to obtain pure isolates. Isolates were identified based on cultural and microscopic characteristics using the taxonomic keys of Holt et al. (1994) and Brenner et al. (2005). The most efficient isolates were then selected for subsequent biological control assays.

2-5 Molecular Identification of Bacterial Isolates Using PCR

Molecular identification of the two bacterial isolates was carried out at Ghayat Al-Maarifa Molecular Research Laboratory. DNA was extracted from bacterial growth on Nutrient Agar using phenol-chloroform according to Cheng and Jiang (2006), and DNA quality was checked by electrophoresis on 1% agarose gel according to Sambrook and Russell (2001). The DNA was used as a template for PCR with specific primers targeting the 16S rRNA gene according to Mori et al. (2014), in a total reaction volume of 25 μ L. Amplification was performed using an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. Products were stored at 4°C.

PCR products were sequenced in both forward and reverse directions by MacroGen Inc., Seoul, South Korea. Sequences were analyzed using BioEdit v7.1 according to Al-Shuhaib and Hashim (2023) and SnapGene Viewer v4.0.4, and were then compared with NCBI sequences using BLASTn according to Zhang et al. (2000). Isolate S3 was identified as *Sphingobacterium zeae* with 99.7% sequence similarity, whereas isolate S2 was identified as *Serratia marcescens* with 100% similarity. A phylogenetic tree was also constructed using the Neighbor-Joining method in Geneious Prime 2025.2.1.

2-6 Preparation of Inocula of Pathogenic Fungi and the Biocontrol Fungus *T. asperellum*

Fungal inocula were prepared using local millet seeds according to Dewan (1988). The seeds were cleaned, washed, and soaked in water for 6 h. Fifty grams were placed in 250-mL flasks and sterilized by autoclaving. The seeds were then inoculated with 0.5-cm fungal discs of the pathogenic fungi and *T. asperellum* and incubated at 25 $\pm$ 1°C for 14 days with continuous shaking.

Commercial *T. asperellum* Biocontrol® (Biocontrol Technologies, Spain), formulated as a wettable powder (WP) containing 12% (w/w) dry conidia, was used. It was activated in the laboratory by dissolving 1 g of the formulation in 5 mL sterile distilled water, inoculating PDA supplemented with tetracycline (250 mg/L), and incubating at 25 $\pm$ 1°C according to Howell (2003).

2-7 Evaluation of the Antagonistic Activity of *T. asperellum* against *R. solani* on PDA

The antagonistic activity of *T. asperellum* against the highly virulent *R. solani* isolate Rs-6 was evaluated using the dual-culture technique on PDA. Two 0.5-cm fungal discs, one of *R. solani* and the other of *T. asperellum*, taken from 7-day-old colonies, were placed opposite each other in 9-cm Petri dishes. Four replicates were used, and plates were incubated at 25 $\pm$ 1°C until the control colony reached the edge of the plate. Antagonistic activity was assessed using the five-grade scale of Bell et al. (1982), with treatments considered effective at a rating $\leq$ 2. The percentage inhibition of radial growth of *R. solani* was also calculated according to Montealegre et al. (2003).

Growth inhibition (%) = [(Mean colony diameter of the control – Mean colony diameter of the treatment) $\div$ Mean colony diameter of the control] $\times$ 100

2-8 Evaluation of the Antagonistic Activity of *S. marcescens* and *S. zeae* against *R. solani*

The molecularly identified *S. marcescens* and *S. zeae* isolates were grown in Nutrient Broth at 25 $\pm$ 1°C for 3–4 days, and serial dilutions from 10⁻¹ to 10⁻⁸ were prepared in sterile distilled water. One milliliter of each dilution was inoculated onto PDA, and a 0.5-cm disc of *R. solani* taken from a 7-day-old colony was placed at the center of each plate. Four replicates were used, with sterile water as the control. Plates were incubated at 25 $\pm$ 1°C until the control growth reached the edge of the plate, and growth inhibition was calculated according to Montealegre et al. (2003).

The population density of the two bacterial suspensions was determined using the 10⁻⁶ and 10⁻⁷ dilutions. One milliliter of each was plated on Nutrient Agar in four replicates, and plates

were incubated at 30°C for 24 h. The bacterial count per milliliter was calculated according to Al-Hadithi (1983) using the following equation:

$$\text{Bacterial count/mL} = \text{Number of colonies} \times \text{Reciprocal of dilution}$$

2-9 Plant Extracts

Extracts of five plant species were tested against *Rhizoctonia solani*: colocynth (*Citrullus colocynthis*), cinnamon (*Cinnamomum verum*), Tribulus (*Tribulus terrestris*), eucalyptus (*Eucalyptus globulus*), and olive leaves (*Olea europaea*). Plant samples were collected from Al-Mussaib Technical College, Babylon, during 2025 at the flowering and seed-formation stages, except for cinnamon and colocynth, which were obtained from local markets. Samples were air-dried, ground, sieved, and the resulting powder was stored at 4°C according to Kareem (2000).

The aqueous extract was prepared by mixing 100 g of powder with 200 mL distilled water for 24 h, followed by filtration and centrifugation at 3000 rpm for 10 min, after which it was stored at 4°C according to Gnoatto et al. (2005). The alcoholic extract was prepared by extracting 50 g of powder with 500 mL of 95% ethanol using a Soxhlet apparatus for 6–8 h, followed by filtration, drying at 40–45°C, and storage at 4°C.

The efficacy of the aqueous and alcoholic extracts at concentrations of 1, 2, 3, and 4% against *R. solani* was tested using the poisoned-food technique according to Grover and Moore (1962). The extracts were added to sterile PDA after cooling and poured into 9-cm Petri dishes with four replicates and an extract-free control. Plates were inoculated with a 0.5-cm fungal disc taken from the margin of a 7-day-old colony and incubated at 25±1°C until the control growth reached the edge of the plate. Colony growth was then measured and inhibition percentage was calculated as follows:

$$\text{Growth inhibition (\%)} = [(\text{Control growth} - \text{Treatment growth}) \div \text{Control growth}] \times 100$$

2-10 Evaluation of the Efficiency of *T. asperellum*, *S. marcescens*, *S. zeae*, and Eucalyptus Extract in Reducing *R. solani* Disease Severity under Greenhouse Conditions

The experiment was conducted at Al-Mussaib Technical College on 15/1/2026 using a randomized complete block design (RCBD) and okra cv. Al-Batra. The soil was prepared, pulverized, and leveled, and well-fermented and decomposed animal manure was incorporated. The area was divided into six beds, each 6 m long with 50 cm between beds. Each bed was divided into two rows, with 20 planting holes per row, and three seeds were sown in each hole. Drip irrigation was used, and treatments were applied when plants reached 35 days of age.

Table 1. Experimental treatments

Treatment	Treatment No.
Plant alone	1
Plant + R.s	2
R.s + aqueous eucalyptus extract	3
R.s + alcoholic eucalyptus extract	4
R.s + Beltanol fungicide	5
R.s + T.as	6
R.s + P1	7
R.s + P2	8
Aqueous eucalyptus extract + T.as + R.s	9
Aqueous eucalyptus extract + P1 + R.s	10
Aqueous eucalyptus extract + P2 + R.s	11
Alcoholic eucalyptus extract + T.as + R.s	12

Treatment	Treatment No.
Alcoholic eucalyptus extract + P1 + R.s	13
Alcoholic eucalyptus extract + P2 + R.s	14
T.as + P1 + R.s	15
T.as + P2 + R.s	16
P1 + P2 + R.s	17

*T.as = *T. asperellum*; P1 = *S. marcescens*; P2 = *S. zeae*; R.s = *R. solani*

T. asperellum inoculum carried on local millet seeds was added one week before soil infestation with the pathogen at a rate of 10 g/planting hole according to Al-Samarrai (2002). *S. marcescens* and *S. zeae* were also added to the soil at 25 mL/plant at concentrations of 7.2×10^7 and 6.8×10^7 CFU/mL, respectively, according to Larkin (2004). *R. solani* inoculum carried on millet seeds was applied at 10 g/planting hole in the corresponding treatments.

The aqueous and alcoholic eucalyptus extracts were applied by soil drenching at 40 mL/planting hole, whereas Beltanol fungicide was applied at 1 mL/L of water one day after addition of the pathogen. Crop management and irrigation were carried out as needed. Measurements were taken after 30 days and included root-rot disease severity, fresh and dry weights, and shoot and root lengths.

3-Results and Discussion

3-1Field Survey of Okra Root Rot

The field survey results (Table 2) showed that root rot was present in all surveyed okra fields in Babylon Governorate, with clear variation among locations. Disease incidence ranged from 17 to 81%, whereas disease severity ranged from 10 to 44%. Al-Jilawiya recorded the highest incidence and severity (81 and 44%, respectively), followed by Al-Haidariya (65 and 32%) and Abu Al-Jasim (51 and 26%). Awlad Muslim recorded the lowest values (17 and 10%), while the remaining areas showed intermediate levels.

The high level of disease may be associated with continuous okra cultivation in the same fields, which can increase pathogen inoculum and population density in the absence of an appropriate crop rotation (Pankhurst et al., 2003). Variation among locations may also be attributed to differences in temperature, soil moisture, cultivars, and agricultural practices, as well as injuries caused by hoeing and weeding that increase opportunities for pathogen entry (Agrios, 2005). Poor irrigation management, inadequate drainage, and unbalanced fertilization may further increase plant susceptibility to infection (Abawi & Widmer, 2000). The ability of fungal pathogens to survive in soil through resistant structures also contributes to disease persistence between seasons (Kwak & Weller, 2021). These results agree with Sood et al. (2025) regarding the importance of root-rot pathogens in okra and the association between increased pathogen density and higher disease incidence and severity, with consequent reductions in plant growth and productivity.

Table 2. Field survey of okra root rot in selected areas of Babylon Governorate during the 2025–2026 growing season

Disease severity (%)	Disease incidence (%)	Field area (donum)	Cultivar	Sampling date	Location/Babylon	Sample No.
21	44	2	Sultana	2025/9/2	Al-Mansouri	1
18	34	4	Al-Batra	2025/9/3	Al-Bidah	2
15	27	10	Sultana	2025/9/3	Al-Tahiriyah	3
13	19	4	Al-Batra	2025/9/3	Al-Azzawiyah	4
10	17	4	Lahlouba	2025/9/7	Awlad Muslim	5
44	81	2	Al-Batra	2025/9/7	Al-Jilawiya	6

Disease severity (%)	Disease incidence (%)	Field area (donum)	Cultivar	Sampling date	Location/Babylon	Sample No.
32	65	1.5	Al-Batra	2025/9/9	Al-Haidariya	7
26	51	8	Al-Batra	2025/9/21	Abu Al-Jasim	8

3-2 Isolation and Identification of Fungi Associated with Infected Okra Roots

Isolation and identification results (Table 3) revealed three fungal species associated with okra roots affected by root rot, which was characterized by brown root discoloration and rotting of root parts or the entire root. A total of 98 isolates were obtained. *R. solani* was the most frequent with 72 isolates, followed by *F. solani* with 15 isolates and *M. phaseolina* with 11 isolates. These findings agree with reports by Al-Saadi (1988), Godoy et al. (1990), Al-Haidari (2007), Al-Khafaji (2010), and Emmanuel et al. (2010) concerning the importance of these fungi in okra root rot.

Rhizoctonia solani was characterized by brown colonies and right-angle hyphal branching, with constrictions and septa near the branching points and the formation of brown to dark sclerotia (Parmeter & Whitney, 1970). *Fusarium solani* produced creamy colonies, small single-celled conidia, large falcate multicellular conidia with several septa, and spherical to subspherical rough-walled chlamydospores in terminal or intercalary positions (Booth, 1971). *Macrophomina phaseolina* showed rapid growth, with mycelium changing from white to dark gray or black and forming black sclerotia within the colony (Thirumalachar, 1953).



Figure 1. Pathogenic fungi isolated from okra roots after 7 days of growth on PDA: A = *R. solani*, B = *F. solani*, and C = *M. phaseolina*.

Table 3. Total number and mean frequency of each fungus

<i>R. solani</i>	<i>F. solani</i>	<i>M. phaseolina</i>	Location	No.
10	3	—	Al-Mansouri	1
12	—	4	Al-Bidah	2
—	3	2	Al-Tahiriyah	3
13	1	—	Al-Azzawiyah	4
14	4	—	Awlad Muslim	5
11	—	—	Al-Jilawiya	6
12	—	—	Al-Haidariya	7
10	4	5	Abu Al-Jasim	8
72	15	11	Total frequency for each fungus	
10.29	3.00	3.67	Mean frequency	

3-3 Pathogenicity Test of Fungal Isolates Using Radish Seeds

The pathogenicity test results (Table 4) showed that radish seed germination in fungal-isolate treatments decreased to 0–9%, compared with the control, which recorded 25 germinated seeds and 100% germination. All *R. solani* isolates (Rs1, Rs2, Rs4, Rs5, Rs6, Rs7, and Rs8) completely prevented germination, indicating high pathogenicity, which may be

related to their ability to penetrate tissues and secrete plant cell-wall-degrading enzymes (Bateman & Van Etten, 1969; Xue et al., 2018). For *F. solani*, germination ranged from 5 to 9%, with Fs4 being the most effective and Fs5 the least effective. For *M. phaseolina*, germination ranged from 4 to 8%, with Mp2 being the most effective.

This variation reflects differences in pathogenicity among fungal species and isolates and may be related to genetic and physiological variation, as well as differences in their ability to colonize tissues and produce enzymes, toxins, and secondary metabolites (Leslie & Summerell, 2006; Xu et al., 2015). Overall, *R. solani* isolates were the most effective in inhibiting radish seed germination, confirming the suitability of this assay as a preliminary test for evaluating the virulence of root-disease fungal isolates.

Table 4. Detection of pathogenic fungal isolates using radish seeds

Germination (%)	Mean germination	Location	Isolate code	No.
0.00	0.00	Al-Mansouri	Rs1	1
0.00	0.00	Al-Bidah	Rs2	2
0.00	0.00	Al-Azzawi	Rs4	3
0.00	0.00	Awlad Muslim	Rs5	4
0.00	0.00	Al-Jilawiya	Rs6	5
0.00	0.00	Al-Haidariya	Rs7	6
0.00	0.00	Abu Al-Jasim	Rs8	7
6.00	1.50	Al-Mansouri	Fs1	8
8.00	2.00	Al-Tahiriyah	Fs3	9
5.00	1.25	Al-Azzawi	Fs4	10
9.00	2.25	Awlad Muslim	Fs5	11
6.00	1.50	Abu Al-Jasim	Fs8	12
4.00	1.00	Al-Bidah	Mp2	13
5.00	1.25	Al-Tahiriyah	Mp3	14
8.00	2.00	Al-Haidariya	Mp8	15
100.00	25.00	----	Control	16
3.36	0.84	----	L.S.D 0.05	

*Each value in the table represents the mean of four replicates.

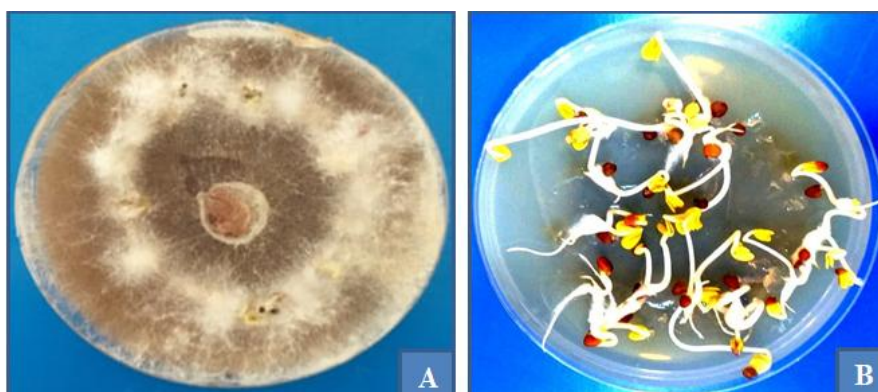


Figure 2. Pathogenicity of the *R. solani* isolate: (A) *R. solani* isolate R6; (B) control treatment, radish seeds alone.

3-4 Results of Isolation of Biological Control Agents

Two bacterial isolates, *S. zeae* and *S. marcescens* strain, were obtained and selected because of their high efficiency in inhibiting growth of the pathogenic fungus *R. solani*. The bacteria occupied the entire culture plate and recorded a first-degree antagonism rating according to the Bell et al. (1982) scale.

3-5 Molecular Identification of *S. marcescens* and *S. zeae* Using PCR

Molecular identification of the two selected bacterial isolates was performed using polymerase chain reaction (PCR) based on amplification of a portion of the 16S rRNA gene, which is widely used for bacterial identification and classification. Electrophoresis of the amplified products showed a single clear DNA band of approximately 440 base pairs (bp), confirming successful amplification and the suitability of the products for nucleotide-sequence analysis.

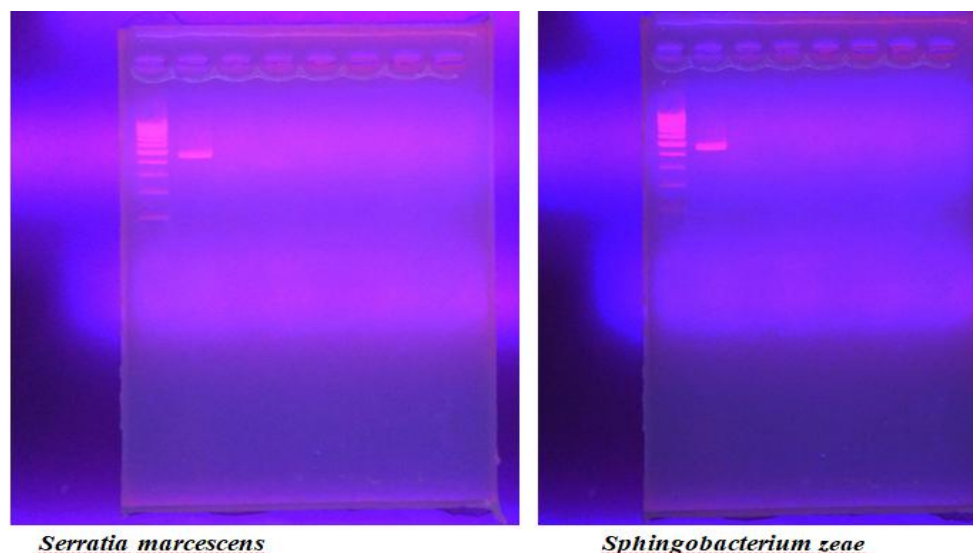


Figure 3. Amplification of a 440-bp DNA fragment of the 16S rRNA gene for species identification using Sanger sequencing. Lane 1: 100-bp DNA ladder; Lane 2: PCR amplification products of the selected bacterial isolates.

3-5-1 Nucleotide Sequence Analysis and Identification of Bacterial Isolates

After obtaining the nucleotide sequences of the studied isolates, they were analyzed and compared with reference sequences deposited in the NCBI database using BLAST according to Altschul et al. (1990). Isolate S3 was identified as *S. zeae* with 99.7% sequence similarity to a reference isolate from South Korea, whereas isolate S2 was identified as *S. marcescens* with 100% sequence similarity to reference isolates from Japan, the United States, and Germany.

Table 5. Reference nucleotide sequence identification of *S. zeae*

Sample	Identified species	Sequence similarity (%)
1	<i>Sphingobacterium zeae</i>	99.7

Table 6. Reference nucleotide sequence identification of *S. marcescens*

Sample	Identified species	Sequence similarity (%)
2	<i>Serratia marcescens</i> strain	100

3-5-2 Nucleotide Sequence Alignment and Phylogenetic Analysis

Alignment of 16S rRNA gene sequences showed high similarity between the two isolates and the reference sequences, with 99.7% identity for *S. zeae* and 100% for *S. marcescens*, supporting the accuracy of the molecular identification. Phylogenetic analysis also showed that each isolate clustered with reference strains of the same species, confirming their close genetic relationship and the accuracy of their molecular classification. The 16S rRNA gene is an important tool for bacterial identification and classification because it contains conserved and variable regions that facilitate discrimination among species (Janda & Abbott, 2007).



Figure 4 illustrates the phylogenetic trees constructed based on 16S rRNA gene sequences. The numbers shown beside each isolate represent the accession numbers of the reference sequences used in the analysis in the GenBank database. The scale shown below the tree indicates the genetic distance among isolates and reference organisms included in the tree.

The identification results confirmed the presence of *S. zeae* and *S. marcescens* among the bacterial isolates selected from the rhizosphere. Their presence may represent a promising source of biological control agents, particularly because some *Sphingobacterium* species are associated with organic-matter decomposition and nutrient cycling in soil (Kämpfer et al., 2012), whereas *S. marcescens* possesses traits that may contribute to plant growth promotion and pathogen suppression, including the production of siderophores, enzymes, and antifungal substances (Ahemad & Kibret, 2014). These results therefore confirm the successful molecular identification and taxonomic assignment of the two isolates.

3-6Determination of the Population Density of *S. marcescens* and *S. zeae*

The population density of *S. marcescens* and *S. zeae* was 6.8×10^7 and 7.2×10^7 CFU/mL, respectively, which represented sufficient bacterial densities to inhibit the colony of the pathogenic fungus *R. solani* on PDA.

3-7Evaluation of the Efficiency of *T. asperellum*, *S. marcescens*, and *S. zeae* against *R. solani*

The results (Table 7) showed high antagonistic efficiency of all biological control agents in suppressing *R. solani* compared with the control. Growth inhibition reached 100% for both *T. asperellum* and *S. marcescens*. This may be attributed to mycoparasitism, competition, and production of enzymes and antifungal compounds by *Trichoderma* (Abbas et al., 2022; Herrera et al., 2020), and to the production of chitinolytic enzymes and antifungal compounds by *S. marcescens* (Someya et al., 2000). *Sphingobacterium zeae* achieved 99.67% inhibition, which may be related to the production of bioactive compounds and competition for nutrients (Kämpfer et al., 2016). Beltanol fungicide achieved complete inhibition (100%), consistent with Abdulmoohsin et al. (2020).

Table 7. Evaluation of the antagonistic activity of selected biological control agents, *T. asperellum*, *S. marcescens*, and *S. zeae*, against *R. solani* on PDA

Inhibition (%)	Colony diameter (cm)	Treatment	No.
100.00	0.00	<i>T. asperellum</i> + <i>R. solani</i>	1
100.00	0.00	<i>S. marcescens</i> + <i>R. solani</i>	2
99.67	0.03	<i>S. zeae</i> + <i>R. solani</i>	3
100.00	0.00	fungicide Beltanol + <i>R. solani</i>	4
0.00	9.00	Pathogenic fungus alone	5

Inhibition (%)	Colony diameter (cm)	Treatment	No.
0.13	0.012	L.S.D 0.05	

*Each value in the table represents the mean of four replicates.

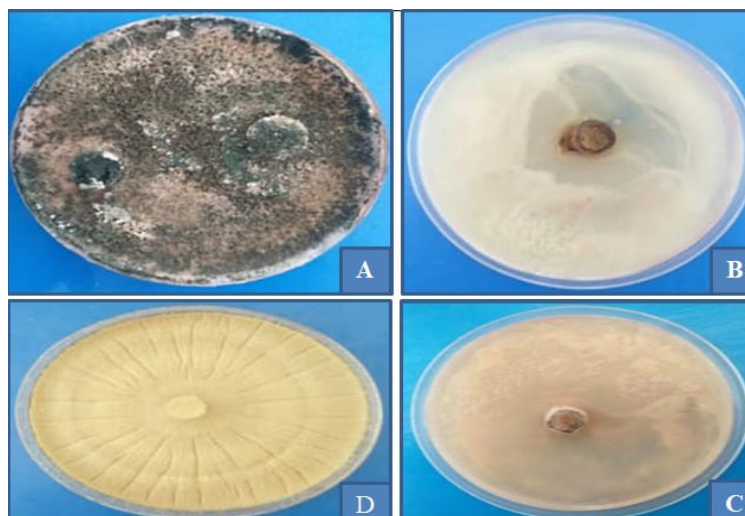


Figure 5. Antagonistic activity of selected biological control agents against *R. solani* on PDA: (A) *T. asperellum*, (B) *S. zeae*, (C) *S. marcescens*, and (D) control treatment, *R. solani* alone.

3-8 Efficacy of Aqueous and Alcoholic Plant Extracts in Inhibiting the Growth of *Rhizoctonia solani*

The results presented in Tables (8,9) and 14 showed clear differences in the efficacy of aqueous and alcoholic plant extracts against *R. solani*, with inhibition increasing as the extract concentration increased. Eucalyptus extract was the most effective, showing inhibition rates of 85.22% for the aqueous extract and 85.78% for the alcoholic extract, followed by olive extract with inhibition rates of 69.44% and 79.44%, respectively, and colocynth extract with 66.78% and 63.89%, respectively. In contrast, cinnamon and *Tribulus terrestris* extracts showed no inhibitory effect, as the colony diameter remained 9.00 cm and the inhibition rate was 0%. The superior efficacy of eucalyptus may be attributed to its content of active compounds, including terpenes and 1,8-cineole, whereas the effect of olive extract may be related to its phenolic compounds, particularly oleuropein and hydroxytyrosol. The inhibitory effect of colocynth may be associated with the presence of cucurbitacins (Dawar et al., 2007; Elaissi et al., 2012; Hyldgaard et al., 2012; Liu et al., 2015; Kamaluddin et al., 2022; Al-Obaidi and Al-Khair, 2022). The differences between the two types of extracts may reflect differences in the efficiency of extraction and the concentration of active compounds. The increase in inhibition with increasing extract concentration is consistent with the findings reported by Nazzaro et al. (2013) and Bakkali et al. (2008).

Table 8. Evaluation of the studied aqueous plant extracts for inhibition of *R. solani* growth on PDA

Inhibition (%)	Colony diameter (cm)	Concentration (g/100 mL)	Treatments
44.33	5.01	1	Colocynth
53.00	4.23	2	
58.78	3.71	3	
66.78	2.99	4	
39.67	5.43	1	Olive
47.44	4.73	2	
65.44	3.11	3	
69.44	2.75	4	
0.00	9.00	1	Cinnamon

Inhibition (%)	Colony diameter (cm)	Concentration (g/100 mL)	Treatments
0.00	9.00	2	
0.00	9.00	3	
0.00	9.00	4	
66.78	2.99	1	Eucalyptus
72.00	2.52	2	
79.00	1.89	3	
85.22	1.33	4	
0.00	9.00	1	Tribulus
0.00	9.00	2	
0.00	9.00	3	
0.00	9.00	4	
0.00	9.00	0.0	Control (fungus alone)
0.61	0.059	L.S.D 0.05	

*Each value in the table represents the mean of four replicates.

Table 9. Evaluation of the studied alcoholic plant extracts for inhibition of *R. solani* growth on PDA

Inhibition (%)	Colony diameter (cm)	Concentration (g/100 mL)	Treatments
44.44	5.00	1	Colocynth
50.00	4.50	2	
58.33	3.75	3	
63.89	3.25	4	
48.56	4.63	1	Olive
58.33	3.75	2	
76.33	2.13	3	
79.44	1.85	4	
0.00	9.00	1	Cinnamon
0.00	9.00	2	
0.00	9.00	3	
0.00	9.00	4	
69.44	2.75	1	Eucalyptus
80.56	1.75	2	
81.89	1.63	3	
85.78	1.28	4	
0.00	9.00	1	Tribulus
0.00	9.00	2	
0.00	9.00	3	
0.00	9.00	4	
0.00	9.00	0.0	Control (fungus alone)
0.693	0.0624	L.S.D 0.05	

*Each value in the table represents the mean of four replicates.

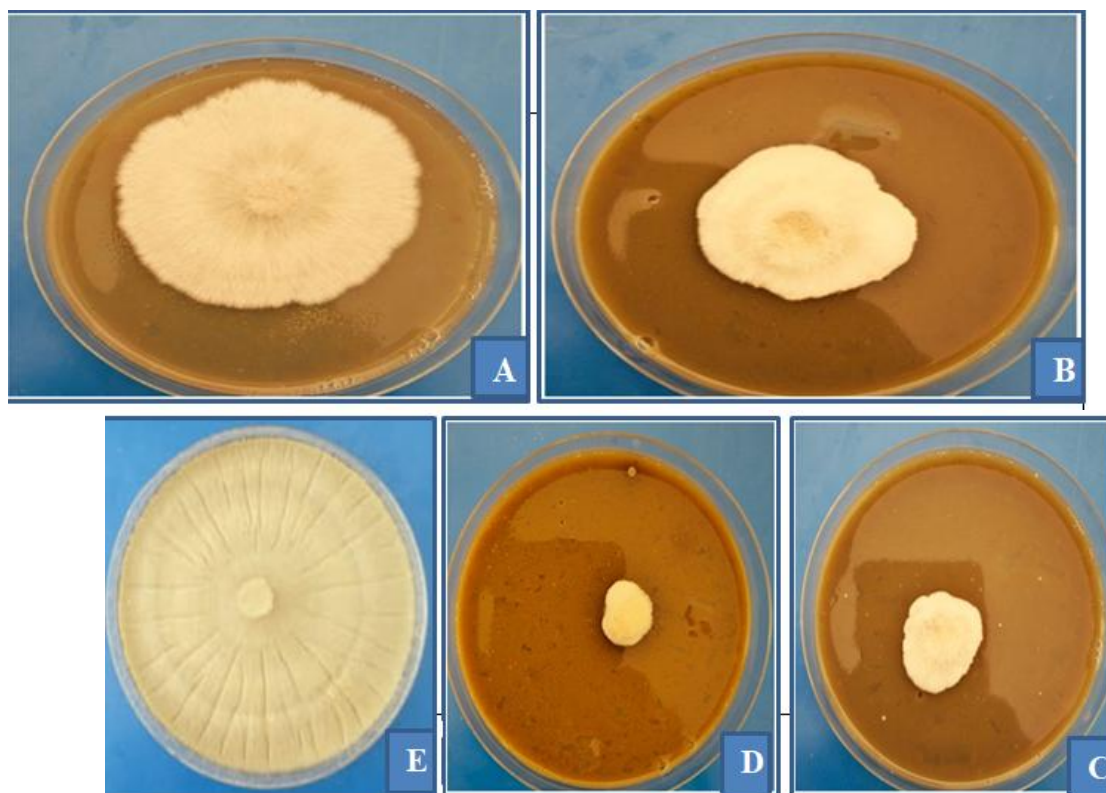


Figure 6. Effect of different concentrations of alcoholic eucalyptus extract on inhibition of *R. solani* growth: (A) 1 g/100 mL, (B) 2 g/100 mL, (C) 3 g/100 mL, (D) 4 g/100 mL, and (E) control treatment (*R. solani* alone).

3-9Evaluation of *T. asperellum*, *S. marcescens*, *S. zeae*, and Aqueous and Alcoholic Eucalyptus Extracts for Reducing *R. solani* Disease Severity and Improving Okra Growth under Greenhouse Conditions

The results in Table (10) showed clear differences among treatments in disease severity and growth characteristics. Inoculation with *R. solani* increased disease severity to 74.44% and markedly reduced growth compared with healthy plants as a result of the pathogen's effects on roots and on water and nutrient uptake (Jones & Dangl, 2006; Taiz et al., 2015).

Biological control treatments and eucalyptus extracts reduced disease severity and improved growth. The treatment aqueous eucalyptus extract + *T. asperellum* + *R. solani* recorded the lowest disease severity (5.24%), followed by *T. asperellum* + *S. marcescens* + *R. solani* and *T. asperellum* + *S. zeae* + *R. solani* (6.22%). For growth traits, *T. asperellum* + *S. marcescens* + *R. solani* was superior, recording the highest values for shoot and root length and fresh and dry weights: 160.04 cm, 221.50 and 99.04 g, 24.45 cm, and 24.38 and 13.22 g, respectively. The treatment alcoholic eucalyptus extract + *T. asperellum* + *R. solani* also showed high efficiency, with disease severity of 6.55%.

Individual treatments also improved plant performance compared with infected plants. Disease severity was 8.25% with *T. asperellum* and 12.25% with both *S. marcescens* and the fungicide. The effects of biological control agents may be related to competition, production of pathogen-inhibiting compounds and enzymes, and induction of plant defenses (Lugtenberg & Kamilova, 2009; Vacheron et al., 2013). The efficacy of eucalyptus may be attributed to its active phytochemicals, antimicrobial effects, and induction of systemic resistance (Cowan, 1999; Cowan, 2009; Walters et al., 2013). These findings agree with Al-Malki (2014) regarding the effectiveness of integrating biological control agents and plant extracts to reduce the impact of *R. solani* and improve okra growth.

Table 10. Effects of *T. asperellum*, *S. marcescens*, *S. zeae*, and aqueous and alcoholic eucalyptus extracts on *R. solani* disease severity and selected shoot and root growth traits of okra under greenhouse conditions

Root weight (g)		Root length (cm)	Shoot weight (g)		Shoot length (cm)	Disease severity (%)	Treatment*	No.
Dry	Fresh		Dry	Fresh				
2.23	10.78	8.17	15.48	126.56	105.73	0.00	Plant alone	1
0.89	3.37	2.53	10.57	63.67	57.23	74.44	Plant + R.s	2
1.66	7.67	4.03	11.32	119.23	111.37	16.99	Aqueous eucalyptus extract + R.s	3
1.78	9.72	6.17	13.22	120.63	107.47	15.34	Alcoholic eucalyptus extract + R.s	4
1.33	6.86	4.23	13.52	115.47	113.03	12.25	R + fungicide	5
4.44	13.44	12.45	31.91	140.30	127.65	8.25	R.s + T.as	6
3.00	12.33	11.34	23.79	139.47	125.27	12.25	R.s + P1	7
2.00	10.18	8.33	16.58	130.63	123.20	10.25	R.s + P2	8
4.98	13.72	14.45	37.64	144.03	133.25	5.24	Aqueous eucalyptus extract + T + R.s	9
3.29	12.63	12.23	25.51	135.23	121.20	10.33	Aqueous eucalyptus extract + P1 + R.s	10
2.95	11.52	9.87	18.48	133.77	118.60	16.5	Aqueous eucalyptus extract + P2 + R.s	11
5.50	14.92	15.04	51.86	161.40	155.38	6.55	Alcoholic eucalyptus extract + T + R.s	12
2.50	11.31	11.37	22.98	138.63	120.37	16.33	Alcoholic eucalyptus extract + P1 + R.s	13
1.99	9.97	10.98	19.46	127.13	119.23	15.23	Alcoholic eucalyptus extract + P2 + R.s	14
13.22	24.38	24.45	99.04	221.50	160.04	6.22	T + P1 + R.s	15
7.45	16.99	17.23	54.15	163.23	157.50	6.22	T + P2 + R.s	16
11.05	22.17	21.78	68.21	173.33	140.77	8.66	P1 + P2 + R.s	17
0.238	0.464	0.403	1.488	2.016	1.815	0.735	L.S.D 0.05	

*T = *T. asperellum*; P1 = *S. marcescens*; P2 = *S. zeae*; R = *R. solani*.

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