

Biological management of root rot disease of sour orange (*Citrus aurantium* L.) caused by *Phytophthora nicotianae*

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Abstract Root rot disease of sour orange (*Citrus aurantium* L.) caused by *Phytophthora nicotianae* was investigated using morphological, molecular, and biological control approaches. Seven *Phytophthora* spp. isolates were obtained from infected citrus roots collected from different locations in Babil Governorate, Iraq. Morphological characterization on PDA medium revealed fast-growing, white to cream-colored, cottony colonies with irregular margins. Pathogenicity tests indicated that *P. nicotianae* isolates Phy1 and Phy5 exhibited the highest disease severity, reaching 74.87% and 71.53%, respectively, after 30 days of inoculation. Molecular identification based on ITS1 and ITS4 regions confirmed the morphological diagnosis. The pathogenic fungus *P. nicotianae* was registered under accession number PV99958.1 and showed 85% sequence similarity with the Moscow isolate (AY773099.1). The biocontrol fungus *Trichoderma harzianum* was registered under accession number MT358869.1 and exhibited 98.44% similarity with the reference isolate (PQ409493.1). In vitro antagonistic assays demonstrated that *Lysinibacillus sphaericus* completely inhibited the growth of *P. nicotianae* (100% inhibition) at a concentration of 160×10^5 CFU ml⁻¹. *Brevibacillus paramycoides* also showed strong antifungal activity, achieving 94.44% inhibition at 8×10^8 CFU ml⁻¹, while *T. harzianum* reduced fungal growth by 91.11%. Greenhouse experiments revealed that biological control agents (*L. sphaericus*, *B. paramycoides*, and *T. harzianum*), in addition to animal organic wastes (cow, sheep, and poultry manure), significantly reduced disease severity and seedling mortality compared with the untreated control. These results indicate that integrated biological control strategies represent a promising and eco-friendly approach for managing root rot disease caused by *P. nicotianae* in sour orange

Keywords: . *P. nicotianae* . *T. harzianum*. *B. paramycoides*. *Citrus*

1. INTRODUCTION

Citrus fruits are among the most important fruit crops, cultivated in over 140 countries across tropical and subtropical regions (Lahlali, 2021). They represent a key horticultural crop with an annual global production of approximately 100 million tons (USDA-FAS). Citrus fruits are highly valued for their nutritional and medicinal properties, as they are rich in vitamins—particularly vitamin C—and serve as a good source of minerals and dietary fiber (Bharate, 2014). Moreover, phytochemicals such as terpenes, limonoids, flavonoids, carotenoids, and hydroxycinnamic acids contribute to the prevention of chronic diseases and cancer (Gyawali, 2014). Citrus production is threatened by bacterial, fungal, and viral pathogens, which can emerge locally and, over time, spread to cause significant economic losses. Chemical pesticides have been widely used to manage these diseases (Fernandes, 2000; Gimenes, 2000; Prabha, 2017); however, their side effects, including pesticide resistance, residue accumulation, environmental pollution, and disruption of ecological balance, are of increasing concern (Pan and Dong, 2018). Plant pathogens remain a major challenge for global crop productivity, highlighting the need for effective and sustainable disease management strategies (Farzand and Ayaz, 2021). Among the pathogens affecting citrus, *Phytophthora* spp. cause severe damage, including root rot and gummosis. Biological control using microbial agents provides an effective and environmentally friendly alternative to chemical pesticides, enhancing soil biological activity and stimulating beneficial organisms that suppress pathogens (Lahlali et al., 2022). Accordingly, this study aimed to isolate and identify the causal agent of root rot in citrus and to evaluate the efficacy of selected biocontrol agents, along with local organic fertilizers (sheep, cow, and poultry manure), in controlling *P. nicotianae*.

2. MATERIALS AND METHODS

2.1. Isolation and diagnosis of fungi associated with the roots of bitter orange trees

Samples were collected from the roots of bitter orange trees (Citrus aurantium) in several areas of Babil Province, including Dabla, Musayib Project, Al-Tahmaziyah, Tufail, Kuthay District, Al-Bakrli, and Abi Gharq District. The sampled trees exhibited symptoms of general weakness, including leaf yellowing, significant leaf drop, and asymmetrical symptom development, often affecting one side of the tree. Some severely infected trees were completely dead. Infected tissues showed gummy exudate and rot, particularly near the soil surface. Additional symptoms included vertical cracks in the bark, forming longitudinal strips saturated with gummy exudate, which accumulated in clumps on the soil surface and tree trunks, and bark detachment due to tissue death. The infected samples were then transported to the Graduate Studies Laboratory at the Technical College of Maysan, Middle Euphrates Technical University, for pathogen isolation. Plant tissue segments were thoroughly washed with tap water to remove dust and debris, cut into 0.5 cm pieces, and surface-sterilized in sodium hypochlorite solution for two minutes. The samples were subsequently rinsed with distilled water and dried using sterile filter paper. Five tissue segments were aseptically transferred to each Petri dish containing prepared potato dextrose agar (PDA) and incubated at 25 ± 2 °C for three days. Fungal colonies were purified by transferring the tips of growing colonies to fresh PDA plates. The isolated fungi were initially identified based on morphological characteristics using the classification keys described by Waterhouse et al.

(1963), as well as key features reported by Waterhouse et al. (1983), including spore capsules, non-septate hyphae, and the formation of chlamydospores.

2.2. Pathogenicity Assessment of Selected Fungal Isolates from the Roots of Bitter Orange (*Citrus aurantium*) Seedlings.

This experiment was conducted in greenhouses at the Technical College of Maysan under controlled temperature and humidity conditions. One-year-old bitter orange (*Citrus aurantium*) seedlings were grown in plastic pots (25 cm in diameter) containing 2 kg of sterilized soil. The fungal inoculum was prepared for five *Phytophthora* isolates (Phy1–Phy5) using cucumber fruits as a natural medium, following the method described by Satoru and Butler (1987).

Disease severity was evaluated using the following scale:

0 = Roots healthy.

1 (Mild infection) = Discoloration of secondary roots with slight damage to vegetative mass.

2 (Moderate infection) = Discoloration of secondary roots and part of the main root (26–50%), with slight leaf yellowing.

3 (Severe infection) = Discoloration of secondary and main roots, excluding the crown area (51–75%), with severe leaf yellowing.

4 (Very severe infection) = Discoloration of the main root and crown area (75–100%) and complete seedling death

The percentage of disease severity was calculated using McKinney's formula (1923) as follows

$$\text{Injury severity} = \frac{(4 \times 4 \text{ degree plants number} + \dots + 0 \times 0 \text{ degree plants number})}{\text{Total number of plants examined} \times 4} \times 100\%$$

2.3 Isolation of Beneficial Microbial Elements from Soil

Random soil samples were collected from the rhizosphere of healthy bitter orange (*Citrus aurantium*) trees in Babil Province. The trees were selected based on vigorous and healthy vegetative growth. The soil samples were left to air-dry in the laboratory for 24 hours and then sieved through a 1 mm mesh. A series of tenfold soil dilutions (10^{-1} to 10^{-5}) was prepared. One milliliter of the fifth dilution was transferred to sterile 9 cm Petri dishes containing potato dextrose agar (PDA) medium supplemented with tetracycline at 250 mg/L, with four replicates per treatment. The plates were gently swirled to ensure uniform sample distribution and incubated at 25 ± 1 °C for 4 days. Fungal isolates were purified by transferring portions of colony edges with a sterile inoculation needle to fresh PDA plates and incubating at 25 ± 1 °C for 5 days (Matroud et al., 2021). Bacterial isolation from soil by dilution followed the same procedure used for fungal isolation. One milliliter of the fifth soil

dilution was transferred to sterile 9 cm Petri dishes containing sterilized nutrient agar (NA) medium, with four replicates per treatment. The plates were gently swirled to ensure even distribution of the sample and incubated at 25 ± 1 °C for 48 hours. Bacterial colonies were then purified on NA plates and incubated at 37 °C for 72 hours. Pure bacterial colonies were subsequently transferred to nutrient agar slants in test tubes for long-term storage at 4 °C (Lacy, 1997).

2.4. Molecular Diagnosis of Fungi Using Polymerase Chain Reaction (PCR)

The DNA extraction experiment was conducted at the Wahaj Al-Dana Molecular Research Laboratory. Genomic DNA of the biocontrol fungus *Trichoderma harzianum* and the pathogenic fungus *Phytophthora nicotianae* was isolated for diagnostic purposes after culturing the fungi on 9 cm Petri dishes containing potato dextrose agar (PDA) for 7 days. Bacterial cultures were grown on 9 cm Petri dishes containing nutrient agar for 48 hours. DNA extraction was performed using the ZR Fungal/Yeast/Bacterial DNA MiniPrep™ kit (Zymo Research, USA)

2.5. Preparation of *T. harzianum* Biofungicide for the Shade Experiment

The *T. harzianum* inoculum was prepared on millet seeds according to Dewan (1989). The seeds were soaked for 6 hours, air-dried, and then placed at a rate of 50 g per 250 ml glass flask, which were sealed with cotton plugs. The flasks were sterilized at 121°C for 1 hour and the sterilization was repeated the following day. After cooling, each flask was inoculated with five 1 cm discs of seven-day-old *T. harzianum* mycelium and incubated at 25°C with periodic shaking to ensure uniform fungal distribution and prevent seed clumping

2.6. Preparation of *P. nicotianae* Inoculum for the Shade Experiment

The pathogenic fungus inoculum was prepared according to Satour and Butler (1987) using cucumber fruits as a natural medium. Ripe, soft, and unblemished fruits were thoroughly washed with water and surface-sterilized with alcohol using sterile cotton. A 2–3 cm deep and 6 cm long incision was made in the center of each fruit using a flame-sterilized knife. A portion of a three-day-old P. parasitica colony grown on PDA was inserted into the fruit through the incision using a sterile inoculation needle. The fruits were placed in polyethylene bags and incubated at 30 ± 2 °C for 3–5 days. After abundant fungal growth on the fruit surface, the fruits were cut into equal-sized pieces, and 2–3 pieces were transferred into 250 ml flasks containing 50 ml of sterile water. The flasks were sealed with sterile cotton and incubated at 30 ± 2 °C to allow spore germination, after which the inoculum was ready for use.

2.7. Preparation of Bacterial Suspensions of *B. paramycoides* and *Lysinibacillus sphaericus*

The bacterium *B. paramycoides* was obtained from the Pathology Laboratory of the Department of Biological Resistance Techniques, Technical College, Mussib, and had been previously identified, while *L. sphaericus* was previously isolated from soil. Both bacteria were propagated in 500 ml sterile glass flasks containing nutrient broth, sterilized in an

autoclave at 121°C and 1.5 psi for 15 minutes. The medium was then inoculated with the bacteria using a sterile loop from previously prepared nutrient agar cultures aged 48 hours. The flask contents were thoroughly mixed and incubated at 32 ± 1 °C for 48 hours (Al-Karawi, 2021).

2.8. Testing the Antagonistic Activity of *T. harzianum*, *B. paramycoides*, and *Lysinibacillus sphaericus* against *P. nicotianae* on PDA Medium.

The antagonistic activity of the biocontrol fungus *T. harzianum* against the pathogenic fungus *P. nicotianae* was tested using the dual culture technique on 9 cm Petri dishes containing PDA medium, which was sterilized in a steam sterilizer for 15–20 minutes. Each dish was conceptually divided into two equal halves. The center of the first half was inoculated with a 0.5 cm disc of seven-day-old *P. nicotianae*, while the center of the other half was inoculated with a 0.5 cm disc taken from the edge of a seven-day-old colony of *T. harzianum* (Hassan, 2005; Al-Haidari, 2007). All dishes were incubated at 25 ± 2 °C. After the pathogenic fungus in the control treatment reached the edge of the dish, the percentage of inhibition was calculated using the formula described by Montealegre et al. (2003).

$$\text{Fungal growth to inhibit percentage} = \frac{\text{Average diameter of the reference colony} - \text{Average diameter of the treated colony}}{\text{Average diameter of the treated colony}}$$

The antagonistic activity of *B. paramycoides* at a concentration of 6.8×10^7 colony-forming units (CFU)/ml and *L. sphaericus* at a concentration of 16×10^6 CFU/ml against the pathogenic fungal isolate was tested after determining the concentrations that effectively inhibited fungal growth. PDA plates (without antibiotics) were inoculated with 1 ml of each bacterial suspension using a sterile pipette. A 0.5 cm disc from the edge of a five-day-old *P. nicotianae* colony (Phy5) grown on PDA was placed on each plate. Four replicates were prepared for each bacterial dilution. Control plates were left without bacterial inoculation, and 1 ml of sterile distilled water was added to them for comparison.

2.9. Evaluation of the Efficacy of Biocontrol Agents and Organic Fertilizers in Protecting Bitter Orange Seedlings from *P. nicotianae* Infection under Shade Conditions in a Greenhouse

The experiment was conducted in the greenhouse of the Department of Biological Resistance Techniques, College of Technical Sciences, Middle Euphrates Technical University, on 3 June 2025. One-year-old bitter orange (*Citrus aurantium*) seedlings were obtained from the Directorate of Agriculture in Sada al-Hindiya, Karbala, and transplanted into 10 kg plastic pots containing a mixture of soil and animal waste, depending on the treatment, at a ratio of 1:2. The soil was sterilized using commercial formalin (40% concentration) at a rate of 20

ml/liter of water, applying 3 liters of the solution per m³ of soil. The collected soil was spread on nylon, turned over, and covered with transparent nylon under sunlight for 7 days, then left to aerate for three days before being distributed into pots (Al-Karawi, 2021). Local orange seedlings were planted, including a control treatment with soil without animal waste but inoculated with the pathogenic fungus. Appropriate irrigation and fertilization were applied as needed, ensuring that the potted soil was not subjected to drought stress. The experiment was arranged as a completely randomized design (CRD). All pots were placed inside a shade house, and necessary cultural practices such as irrigation and fertilization were carried out as required. The treatments were as follows

1. Plants alone (control).
2. *L. sphaericus* + *P. nicotianae*.
3. *B. paramycoides* + *P. nicotianae*.
4. *T. harzianum* + *P. nicotianae*.
5. Sheep manure + *P. nicotianae*.
6. Cow manure + *P. nicotianae*.
7. Poultry manure + *P. nicotianae*.
8. *P. nicotianae* alone (pathogen control).
9. *L. sphaericus* + sheep manure.
10. *L. sphaericus* + cow manure.
11. *L. sphaericus* + poultry manure.
12. *B. paramycoides* + sheep manure.
13. *B. paramycoides* + cow manure.
14. *B. paramycoides* + poultry manure.
15. *T. harzianum* + sheep manure.
16. *T. harzianum* + cow manure.
17. *T. harzianum* + poultry manure.

18. *L. sphaericus* + sheep manure + *P. nicotianae*.
19. *L. sphaericus* + cow manure + *P. nicotianae*.
20. *L. sphaericus* + poultry manure + *P. nicotianae*.
21. *B. paramycoides* + sheep manure + *P. nicotianae*.
22. *B. paramycoides* + cow manure + *P. nicotianae*.
23. *B. paramycoides* + poultry manure + *P. nicotianae*.
24. *T. harzianum* + sheep manure + *P. nicotianae*.
25. *T. harzianum* + cow manure + *P. nicotianae*.
26. *T. harzianum* + poultry manure + *P. nicotianae*.
27. *P. nicotianae* + chemical pesticide

The *P. nicotianae* inoculum was prepared using cucumber fruits as a natural medium, as previously described. The fungal inoculum was added to the pots at a rate of 400 ml per pot. One week after the application of the biocontrol agents, the soil was irrigated and maintained at an appropriate moisture level to ensure the viability of the pathogenic fungus (Al-Faraji et al., 1991). The biocontrol fungus *T. harzianum* was added to the prepared local millet seeds at a rate of 2 g/kg for all treatments requiring its application (Al-Samrai, 2002). *Lysinibacillus sphaericus* was applied at a concentration of 16×10^6 CFU/ml after growth in nutrient broth for 48 hours, using a sterile syringe, at a rate of 25 ml per pot. *B. paramycoides* was applied at a concentration of 6.8×10^7 CFU/ml. The evaluation of the treatments was conducted 60 days after the addition of the pathogenic fungus by estimating the severity of infection, as described in the section on pathogenicity testing of fungal isolates from roots. The infection rate was then calculated and the percentage of infection was calculated according to the following equation

$$100 \times (\text{number of infected plants}) / (\text{total number of plants examined}) = \text{percentage infection rate}$$

Based on the length and wet and dry weight of the vegetative and root mass of the orange trees under trial.

2.10 Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS, 2019) to evaluate the effects of the different treatments on the studied traits. Significant differences between means were compared using the Least Significant Difference (LSD) test at a probability level of $P \leq 0.05$

3.RESULTS AND DISCUSSION

3. Isolation and Diagnosis of the Fungus Causing Root Rot and Stem Hardening in Bitter Orange Trees

Isolation and diagnosis of infected citrus roots in Babil Province (Hilla/Dabla, Kouthi District/Maysib Project, Hilla/Tahmazia, Hilla/Tufail, Hilla/Bakrli, and Hilla/Abi Gharq District) revealed seven isolates of *Phytophthora* spp. Growth on PDA medium was characterized by white to cream-colored colonies with a cottony or fluffy appearance, rapid growth, and irregular margins (Figure 1). The fungus was isolated from the rhizosphere of bitter orange trees exhibiting infection symptoms. *Phytophthora* spp. are among the most destructive soil- and water-borne pathogens, posing a serious threat to citrus trees due to their ability to infect all plant parts, from roots to fruits. Infection manifests in a range of symptoms, including fibrous root rot, stem base rot, gummy exudates on the stem, and seedling collapse, collectively causing significant economic losses in citrus production worldwide (Riley et al., 2024). Zhou (1999) reported that *P. nicotianae* is one of the primary and most aggressive causes of root rot in citrus trees in China. The spread of citrus gummosis in these agricultural areas may be attributed to favorable environmental conditions, including high humidity, poorly drained clay soils, and suitable temperatures, which are essential for fungal proliferation. Additionally, the ability of *Phytophthora* spp. to increase and multiply its reproductive structures contributes to its high virulence and rapid spread



Fig.1. Symptoms of infection appearing on orange trees and on the bark of the trunk when infected with the fungus *Phytophthora* spp.

3.2 Testing the Pathogenicity of *Phytophthora* spp. on Local Bitter Orange Seedlings

The results presented in Table 1 indicate that, 30 days after inoculation, plants treated with isolates Phy1 and Phy5 of *P. nicotianae* exhibited the highest infection severity, reaching 74.87% and 71.53%, respectively, whereas isolate Phy4 showed the lowest severity at 30.74%. When the fungus was re-isolated from infected plants, *P. nicotianae* growth was observed on PDA medium, whereas the fungus was not re-isolated from healthy plants in the control treatment. The data also showed that all pathogenic fungal isolates from different locations caused a significant increase in infection severity compared to the control, which remained symptom-free. This variation in pathogenicity among isolates is attributed to the toxins secreted by the fungus, which play a crucial role in infection and damage to plant cells (Dick and Dobbie, 2003)

Table 1. Effect of some isolates of the fungus *P. nicotianae* on bitter orange seedlings

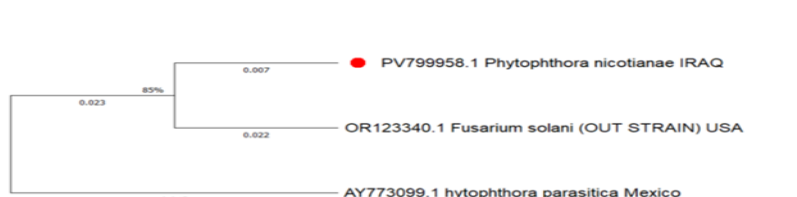
NO	Symbol of isolation	Severity of injury%
1	Phy1	74.87
2	Phy2	45.71
3	Phy3	50.43
4	Phy4	30.74
5	Phy5	71.53
6	Phy6	42.11
7	Phy7	48.52
	L.S.D.	8.739

*(P≤0.05).

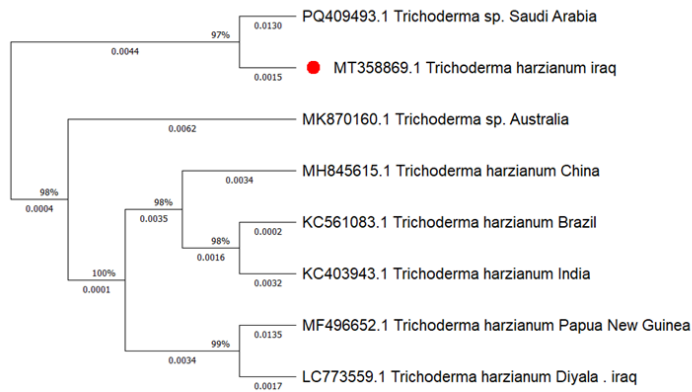
*Each number in the table represents an average of three replicates

3.3. Molecular Diagnosis of Fungi and Bacteria Using Polymerase Chain Reaction (PCR)

The molecular diagnosis of the studied fungi confirmed that the isolates identified morphologically, according to the adopted classification criteria, were consistent with the results obtained from molecular analysis using the ITS1 and ITS4 regions. The pathogenic fungus *P. nicotianae* was registered under accession number PV99958.1 and showed 85% similarity to the Moscow isolate registered under accession number AY773099.1. The biocontrol fungus *T. harzianum* was registered under accession number MT358869.1 and exhibited 98.44% similarity with the reference isolate PQ409493.1



The Neighbor-Joining tree shows the phylogenetic relationship of the fungal isolate *P. nicotianae* isolated in this study from global isolates.



The Neighbor-Joining tree shows the phylogenetic relationship of the *T. harzianum* fungal isolate isolated in this study from global isolates.

3.4. Testing the antagonistic ability of the fungus *T. harzianum* and the bacteria *L. sphaericus* and *B. paramycoides* in inhibiting the growth of the fungus *P. nicotianae* in PDA culture medium.

The results presented in Table 2 indicate the strong antagonistic activity of *Lysinibacillus sphaericus* against the pathogenic fungus *P. nicotianae* at a concentration of 1.6×10^7 colony-forming units (CFU)/ml on PDA medium. The radial growth of the pathogenic fungus was completely inhibited (0.00 cm), corresponding to 100% inhibition, compared to the control, where the radial growth reached 9.00 cm with 0% inhibition. This inhibitory effect is attributed to the rapid growth and spread of the bacteria on the PDA medium, as well as their ability to produce antibiotics and hydrolytic enzymes such as chitinase, which degrades fungal cell wall chitin, and protease, which breaks down fungal cell components. These findings are consistent with Karthick and Mohanraju (2020), who reported that *L. sphaericus* produces various bioactive compounds, including organic acids, antibiotics, and enzymes such as protease, chitinase, and glucanase, which inhibit pathogenic fungi and contribute to plant disease resistance. The isolation of *B. paramycoides* revealed a strong ability to inhibit the growth of the pathogenic fungus *P. nicotianae* on PDA medium, with the inhibition rate

varying according to bacterial concentration. The fifth dilution (8×10^8 CFU/ml) exhibited a high inhibition rate of 94.44%, with a radial growth of 0.5 cm, compared to the control, where fungal growth reached 9.0 cm with 0% inhibition. This inhibitory effect is attributed to multiple mechanisms, including the production of antifungal compounds such as fatty peptides, enzymes that degrade fungal cell walls, and antibiotics that directly kill the pathogen (Al-Anzi, 2024). Similarly, *T. harzianum* significantly reduced the growth of *P. nicotianae*, achieving 91.11% inhibition with a radial growth of 0.8 cm, compared to the control with 9.0 cm growth and 0% inhibition. This effect is attributed to the fungus's multiple well-known mechanisms of pathogen control, including competition for nutrients and space, and the production of biocins and hydrolytic enzymes such as protease, β -1,3-glucanase, and chitinase (Hadar et al., 1978). These findings are consistent with previous studies demonstrating the biocontrol potential of *T. harzianum* against pathogenic fungi (Jaber et al., 2002; Hassoun, 2005; Matloub, 2007) (Figure 2)



Figure 2. Antagonistic activity of the biotic fungus *T. harzianum* against the pathogenic fungus *P. nicotianae*.

Table 2. Testing the antagonistic ability of the fungus *T. harzianum* and the bacteria *L. sphaericus* and *B. paramycoides* in inhibiting the growth of the fungus *P. nicotianae* in PDA culture medium.

N0	Treatment	Percentage growth rate of pathogenic fungi	Percentage of inhibition
1	<i>L.sphaericus</i> + <i>P.nicotianae</i>	0.00	100%
2	<i>B.paramycoides</i> + <i>P.nicotianae</i>	0.5	94.44%
3	<i>P.nicotianae</i> + <i>T.harzianum</i>	0.8	91.11
4	<i>P. nicotianae</i> alone (comparison)	9.00	00.00
	<i>L.S.D.</i>	0.722	8.637

*Each number in the table represents an average of three repetitions.

3.5. Evaluation of the Efficacy of Biological Control Agents and Organic Fertilizers on Infection Severity by P. nicotianae and Growth Parameters of Bitter Orange Seedlings under Greenhouse Shade Conditions in Babil Province.

The results presented in Table 3 indicate that the biological control agents, including the bacteria *L. sphaericus* and *B. paramycoides*, the fungus *T. harzianum*, and organic fertilizers (cow, sheep, and poultry manure), effectively reduced the severity of seedling blight and stem rot caused by *P. nicotianae* compared to the control treatment (plants alone). The most effective treatment was the combination of *L. sphaericus* and sheep manure in the presence of the pathogenic fungus, which reduced infection severity to 4.23%. This was followed by the combination of the biocontrol fungus *T. harzianum* and sheep manure with *P. nicotianae*, achieving a significant reduction in infection severity to 5.75%, compared to 75.33% in seedlings inoculated with the pathogenic fungus alone.

The superiority of synergistic interactions in reducing disease severity can be attributed to the cooperative effects between the biocontrol agents used in this experiment, as they work together to stimulate the plant's systemic resistance against pathogens (Latha et al., 2009). Synergy between biocontrol agents is also considered a strategic approach for managing plant diseases (Saravanakumar, 2007). This effect is primarily due to the ability of *L. sphaericus* to secrete enzymes that degrade fungal cell walls, such as chitinase and β -1,3-glucanase, in addition to producing antimicrobial compounds that inhibit pathogen growth (Zhou et al., 2022; Hassan et al., 2023). When *T. harzianum* interacts with organic residues, it demonstrates comparable effectiveness in reducing infection severity. This confirms its ability to compete with the pathogen in the rhizosphere, in addition to secreting hydrolytic enzymes such as chitinase and protease that degrade fungal cell walls. Moreover, *T. harzianum* can secrete auxins and citrates, which enhance root growth (Abd El-Rahman et al., 2021; Harman et al., 2021). These results are consistent with several studies showing that synergistic interactions between biocontrol agents are more effective in reducing the severity of plant pathogens than the use of a single biocontrol agent (Latha et al., 2009). Treatments with *L. sphaericus*, *T. harzianum*, and *B. paramycoides* individually against the pathogenic fungus *P. nicotianae* resulted in significant reductions in infection severity, reaching 10.63%, 13.36%, and 15.28%, respectively, compared to 75.33% in seedlings treated with the pathogenic fungus alone. Species of the genus *Trichoderma* play a vital role in biological control, which has made them the focus of numerous studies in this field (Pandya and Saraf, 2010). These fungi are highly adaptable to environmental conditions and generally grow faster than many plant pathogens. They can effectively compete with pathogens for limited nutrients, thereby inhibiting their growth. This mechanism is crucial for controlling soil-borne and foliar diseases (Howell, 2007). The effectiveness of these mechanisms may be enhanced through synergistic interactions, and the success of biological control depends on factors such as the fungal strain, the crop species, and environmental conditions, including nutrient availability, temperature, and iron concentration (Harman et al., 2004). Among microorganisms,

Trichoderma spp. are considered highly beneficial as they serve as biocontrol agents worldwide, managing soil-borne diseases, promoting plant growth, and stimulating systemic resistance in plants. Plant resistance is attributed to competition with soil-borne pathogenic fungi for nutrients, direct antagonism, antibiotic production, and the induction of systemic resistance, as well as the stimulation of plant growth through the production of growth-promoting molecules (Maurya et al., 2008).

The biocontrol bacterium *B. paramycoides* is known to suppress plant pathogens through the production of enzymes and secondary metabolites, in addition to stimulating systemic resistance in plants (Sahoo et al., 2021; Kim et al., 2024). Members of the genus *Bacillus* are effective agents for biological control against a wide range of plant pathogens, including fungi that are highly sensitive to their antimicrobial compounds. These bacteria inhibit the metabolic activities of competing soil microorganisms, stimulate plant growth, enhance productivity, and improve overall plant quality (Ghazadeh et al., 2008; Kinsella et al., 2009). Furthermore, treatments combining sheep and cow manure with the pathogenic fungus *P. nicotianae* resulted in infection rates of 17.54% and 20.34%, respectively, compared to the control treatment inoculated with the pathogen alone. The addition of animal waste to soil is an effective strategy to reduce fungal disease severity and improve plant growth, as these amendments enhance the soil's physical and chemical properties, increase organic matter and nutrient content, and activate beneficial microorganisms that contribute to pathogen suppression (Zheng et al., 2021)

The results presented in Table 3 indicate that the biological control agents, including the fungus *T. harzianum*, the bacteria *B. paramycoides* and *L. sphaericus*, as well as organic fertilizers (sheep, cow, and poultry manure), significantly improved the growth parameters of bitter orange seedlings compared to the control treatment (pathogen alone). The most effective treatment was the combination of *L. sphaericus* with sheep manure in the presence of *P. nicotianae*, resulting in the highest growth values: shoot length 73.22 cm, shoot fresh weight 62.34 g, shoot dry weight 21.23 g, root length 59.65 cm, root fresh weight 42.53 g, and root dry weight 17.16 g. This was followed by the combination of *L. sphaericus* with cow manure in the presence of the pathogen, which also significantly increased growth parameters, reaching shoot length 73.11 cm, shoot fresh weight 61.89 g, shoot dry weight 18.34 g, root length 57.34 cm, root fresh weight 41.99 g, and root dry weight 16.97 g, compared to the control treatment values of shoot length 65.53 cm, shoot fresh weight 35.51 g, shoot dry weight 19.31 g, root length 25.62 cm, root fresh weight 31.56 g, and root dry weight 18.98 g. Many studies have demonstrated that synergistic interactions between biocontrol agents lead to higher yields compared to the use of a single agent. The observed enhancement in growth parameters of bitter orange seedlings under synergistic treatments is attributed to the cooperative effects of the biological resistance mechanisms provided by the fungi and bacteria used in the experiment.

Regarding treatments with individual biocontrol agents, the combination of *L. sphaericus* with the pathogenic fungus *P. nicotianae* produced the highest growth parameters for bitter orange seedlings compared to the control. In this treatment, shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight were 57.96 cm, 52.11 g, 20.96 g, 36.95 cm, 26.47 g, and 9.56 g, respectively. This effect is attributed to the ability of this bioactive bacterium to promote plant growth, enhance vegetative and root development, and increase both the quantity and quality of production. *L. sphaericus* colonizes the rhizosphere in a non-parasitic manner, creating a healthy microbial environment around the roots that facilitates better plant growth. Treatment with *T. harzianum* in the presence of the pathogenic fungus *P. nicotianae* resulted in a significant increase in growth parameters, including shoot length, root length, and shoot and root fresh and dry weights, which were 55.86 cm, 48.95 g, 18.97 g, 36.85 cm, 25.76 g, and 15.84 g, respectively, compared to the treatment with the pathogen alone. Biocontrol agents are highly effective in protecting plants from pathogenic fungi due to the production of numerous bioactive compounds, including antibiotics (Harman, 2000). The biocontrol fungus *T. harzianum* acts as a competitor to fungal pathogens, particularly under nutrient-limited conditions (Mansoori et al., 2013). It has a strong ability to stimulate systemic resistance in plants, enhancing their defense against a wide range of pathogens, including fungi, bacteria, and viruses, by activating hormonal signaling pathways such as salicylic acid, jasmonic acid, and ethylene (Martínez-Medina et al., 2013). Additionally, *T. harzianum* produces enzymes that degrade the cell walls of pathogenic fungi, including proteases, and efficiently utilizes soil nutrients to colonize the rhizosphere (Tziros et al., 2007). Treatment with *B. paramycoides* in the presence of the pathogenic fungus *P. nicotianae* showed high efficiency in improving the growth parameters of bitter orange seedlings compared to the control treatment. Shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight reached 53.95 cm, 49.62 g, 18.12 g, 32.74 cm, 22.32 g, and 11.31 g, respectively. This improvement is attributed to the ability of *B. paramycoides* strains to compete with fungal pathogens for nutrients, thereby limiting their growth and colonization in the rhizosphere. In addition, these bacteria stimulate systemic resistance in plants, enhancing their intrinsic defense mechanisms against fungal pathogens and improving their tolerance to infection (Chakraborty et al., 2022). Similarly, the treatment combining *L. sphaericus* with sheep manure resulted in a marked enhancement of seedling growth compared to the control treatment. The recorded values for shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight were 75.21 cm, 65.53 g, 23.63 g, 62.57 cm, 45.73 g, and 19.23 g, respectively. The addition of animal waste in combination with beneficial bacteria contributed to enhancing plant systemic resistance by increasing the activity of defense-related enzymes such as peroxidase and cathepsin. Khalifa et al. (2023) reported that the integration of cow or sheep manure with *Trichoderma* and *Bacillus* biocontrol agents significantly reduced the severity of wilt and root rot diseases and markedly increased the length and biomass of vegetative and root systems. Furthermore, El-Sobky et al. (2024) demonstrated that organic fertilizers not only serve as a source of nutrients but also create a favorable environment for the proliferation of beneficial microorganisms that

secrete antifungal compounds. Therefore, the combined application of organic fertilizers and biological control agents represents a sustainable and effective strategy for managing soil-borne diseases.

Treatment with *L. sphaericus* in combination with cow manure significantly enhanced the growth parameters of bitter orange seedlings compared to the control treatment. Shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight reached 75.50 cm, 63.34 g, 22.54 g, 61.52 cm, 44.23 g, and 18.31 g, respectively. Similarly, treatment with *B. paramycoides* resulted in high growth performance of orange seedlings compared to the control, with shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, and root dry weight of 70.25 cm, 60.96 g, 18.65 g, 56.34 cm, 39.88 g, and 15.32 g, respectively. Treatment with the chemical fungicide Kriptanol also reduced the severity of infection caused by the pathogenic fungus *P. nicotianae* in treated orange seedlings compared to the pathogen-only treatment, which positively reflected on plant growth parameters. These results are consistent with previous studies demonstrating the high inhibitory efficacy of Kriptanol against pathogenic fungi, where its application significantly reduced disease severity and consequently enhanced vegetative growth. This effect is attributed to the nature of Kriptanol as a broad-spectrum, systemic, and preventive fungicide with a strong ability to disrupt pathogen cell walls and inhibit spore germination. Gershon et al. (2006) reported that the active ingredient of this fungicide operates through a chelation mechanism by binding to essential mineral ions required for fungal enzymatic activity, thereby disrupting vital metabolic processes and preventing fungal reproduction and spread within host tissues.

Table (3) shows the evaluation of the efficacy of biological control agents represented by the fungus *T. harzinum*, the bacterium *B. paramycoides*, and the bacterium *sphaericus*. L and animal waste (poultry, sheep, cows) on the severity of infection with the pathogenic fungus *P. nicotianae* and some growth parameters of orange seedlings in a greenhouse under shade conditions in the province of Babel.

Root sum weight (gm)		Total root length (cm)	Total vegetable weight (gm)		Total vegetative length (cm)	Severity of injury %	Treatment *	no
dry	Soft		dry	Soft				
15.32	26.47	36.95	20.96	52.11	57.96	10.63	<i>Phy.n +L.s</i>	1
19.23	45.73	62.57	23.63	65.53	75.21	0.00	<i>A + L.s</i>	2
18.31	44.23	61.52	22.54	63.34	75.5	0.00	<i>B + L.s</i>	3
17.55	42.73	60.53	21.74	62.12	75.1	3.33	<i>C + L.s</i>	4
15.84	25.76	36.85	18.97	48.95	55.86	13.86	<i>Phy.n + T.h</i>	5
16.87	42.22	59.45	21.23	62.5	73.12	0.00	<i>A + T.h</i>	6

16.23	41.98	58.99	19.95	61.89	72.88	0.00	<i>B + T.h</i>	7
15.79	41.32	57.34	19.23	61.22	71.45	4.21	<i>C + T.h</i>	8
11.31	22.32	32.74	18.12	49.62	53.95	15.28	<i>Phy.n + B.p</i>	9
15.32	39.88	56.34	18.65	60.96	70.25	0.00	<i>A + B.p</i>	10
15.21	38.98	55.12	17.33	59.67	69.99	0.00	<i>B + B.p</i>	11
15.11	37.76	54.99	17.11	59.22	68.56	0.00	<i>C + B.p</i>	12
17.16	42.53	59.65	21.23	62.34	73.22	4.23	<i>+ A + L.s</i> <i>Phy.n</i>	13
16.97	41.99	57.34	18.34	61.89	73.11	6.33	<i>+ B + L.s</i> <i>Phy.n</i>	14
15.28	39.87	56.48	17.36	59.97	71.87	8.52	<i>+ C + L.s</i> <i>Phy.n</i>	15
14.22	37.33	55.34	19.97	59.36	69.98	5.75	<i>+ A + T.h</i> <i>Phy.n</i>	16
12.41	35.21	53.73	18.76	58.64	68.56	7.32	<i>+ B + T.h</i> <i>Phy.n</i>	17
11.97	34.87	51.44	17.88	57.99	67.55	9.63	<i>+ C + T.h</i> <i>Phy.n</i>	18
14.77	35.87	52.77	15.87	59.78	68.55	6.97	<i>+ A + B.p</i> <i>Phy.n</i>	19
13.86	34.75	51.63	14.55	57.43	66.69	6.32	<i>+ B + B.p</i> <i>Phy.n</i>	20
12.97	32.89	49.78	12.79	55.64	63.68	10.43	<i>+ C + B.p</i> <i>Phy.n</i>	21
9.21	26.34	34.39	11.93	49.56	52.99	17.54	<i>A + Phy.n</i>	22
6.43	25.49	31.84	9.77	44.23	49.64	20.34	<i>B + Phy.n</i>	23
6.23	23.54	29.37	7.44	39.34	35.94	21.87	<i>C + Phy.n</i>	24
18.98	31.56	25.62	19.31	35.51	65.53	0.00	The plant alone	25
5.66	17.34	21.53	9.77	22.43	26.46	75.33	<i>Phy.n alone</i>	26
19.49	42.95	59.45	27.59	50.21	81.53	11.34	<i>+ Phy.n</i> <i>Pesticide</i>	27
3.85	6.91	8.37	4.93	8.77	9.25	5.48	<i>L.S.D.</i>	

.($P \leq 0.05$) *

T.h * = *T. harzinum* * *Phy.n* = *P. nicotianae*

* *A* = sheep manure, *B* = cow manure, *C* = poultry manure

**L.s.* = *sphaericus. L* * *B.p* = *B. paramycoides*

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