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Some Chemical and Biological Methods to Control Pepper Root Rot Disease in Iraq

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ABSTRACT

Background: Pepper growing fields in the Babil and Wasit governorates in Iraq, are suffering from a decline in production due to the spread of root rot diseases. The use of biological control techniques, such as the use of beneficial bacteria in the rhizosphere and natural plant extracts, along with chemical fungicides, has recently contributed to reducing the damage of plant pathogens globally.

Methods: In this research, the main cause of root rot disease in pepper plants was isolated and diagnosed using polymerase chain reaction technology using (TEF-1α) gene, antagonistic activities of 75 rhizobacterial isolates, natural extracts of garlic and nerium and the fungicides of Promess and Tecto 500 SC were tested *in vitro* against the pathogen, then the superior bacterial isolates were identified. The ability of biological and chemical agents to control the disease was tested under greenhouse conditions according to the completely randomized design (CRD).

Result: The results of isolation showed that the fungus *Fusarium solani* was dominance and the isolate Fsw45 was the most virulent. 3 of 75 rhizobacterial isolates outperformed the pathogen in an antagonistic tests and identified as *Pseudomonas mendocina, P. fluorescens and Staphylococcus kloosii* also the fungicides and botanical extracts showed significant antagonistic activities as well *in vitro*. Under greenhouse conditions, the fungicide treatments excelled in disease control by 100%. As for the 3 bacterial isolates, they achieved a significant reduction in the percentage of disease incidence and disease severity index, their abilities were higher than the nerium and garlic extracts.

Key words: Botanical extract, Fungicide, *Fusarium solani*, Rhizobacteria.

INTRODUCTION

One of the most significant vegetable crops marketed worldwide is pepper (*Capsicum annuum*), a member of the Solanaceae family (Hassan, 2001). Due to its high nutritional content of vitamins, proteins, carbs and salts, pepper production is on the rise globally (Kelley and Boyhan, 2006). The area cultivated with pepper in Iraq reached 988.5 ha in 2017, with a production rate of 13.169 tons/ha (CSO-Central Statistical Organization, 2018). Pepper is exposed to many diseases that result in large economic losses. Root and crown rot disease caused by the fungus *Fusarium solani* is one of the most important diseases that affect the crop cultivation negatively (Bilgili *et al*., 2023). Effective management strategies include crop rotation, sanitation, using disease-resistant cultivars and avoiding overwatering to reduce favorable conditions for its growth. Chemical fungicides may be used as a last resort but are often not a sustainable or environmentally friendly solution (Rosskopf *et al*., 2005). The fungus can affect the host plant at any stage of growth. Infected roots will have reddish-brown lesions along the cortex of the main lateral roots. Vascular discoloration also happens a few inches above and below these lesions. Foliar symptoms include interveinal chlorosis and necrosis, usually on a single branch. As the disease progresses in the roots, the leaves of the entire plant will eventually turn brown and collapse (Coleman, 2016). Environmental stress, such as high temperatures and drought, are among the contributing factors in the emergence of symptoms and signs of disease, especially in the flowering and fruit setting stage (Summerell *et al*., 2003). The framework of environmental protection and usually uses types of botanical extracts which are natural substances derived from plants that have antimicrobial properties and can be used for the control of plant diseases, botanical extracts can act as botanical fungicides, which are chemicals that kill or inhibit the growth of fungal pathogens, or as inducers of systemic resistance, which are substances that stimulate the plant's own defense mechanisms against various pathogens (Sajeena *et al*., 2019), Helpful microorganisms including plant growth-promoting rhizobacteria (PGPR) act as biological control agents through several strategies such as antibiotic production, root colonization, competition for food and space, induction of systemic resistance (ISR), promotion of plant growth or direct parasitism of pathogens (Pandit *et al*., 2022).

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The antibacterial properties of garlic clove extract, in particular Allicin, which is effective against a variety of plant pathogenic bacteria, fungi and oomycetes, are utilized in the management of plant diseases. According to studies by Slusarenko *et al*. (2008), allicin from garlic has been demonstrated to reduce seed-borne Alternaria spp. in carrots, Phytophthora leaf blight in tomatoes and tuber blight in potatoes. An extract from the leaves of the Nerium plant has been shown to prevent plant infections, including Bipolaris oryzae, which causes brown spot disease in rice, from growing mycelially and from germination (Harish *et al*., 2008). Because of their natural origin, efficacy and possible application in organic farming and sustainable agricultural techniques, these extracts are taken into consideration for use in the control of plant diseases. The goal of this study was to identify the agent responsible for the root and crown rot disease of pepper plants in the Iraqi provinces of Babil and Wasit and trying to manage the disease using isolates of helpful rhizobacteria, natural extracts and chemical fungicides.

MATERIALS AND METHODS

Isolation and identification of the fungi associated with diseased pepper plant

Samples were taken from the roots and crown of pepper plants exhibiting disease signs, samples collected from 26 fields distributed in Babil and Wasit provinces during the period December 2020-May 2021. Fungal growth around the sample segments was purified on PDA and the fungal isolates were tested under a light microscope and identified depending on the morphological characteristics, the percentage of appearance and frequency of fungi isolates were calculated according to the following equations (Hussein and Al Zubidy, 2019).

$$\text{Appearance (\%)} = \frac{\text{No. of samples of occurrence}}{\text{Total no. of samples}} \times 100$$

$$\text{Frequency (\%)} = \frac{\text{No. of plant segments of species occurrence}}{\text{Total no. of segments used}} \times 100$$

Pathogenicity test of the *F. solani* isolates

To determine the pathogenicity potential of the 68 isolates of *F. solani*, an experiment was carried out *in vitro* using pepper seeds cultivar (Anaheim California Chilies) which planted on the PDA (5 seeds/plate) and 0.5 cm of the fungal growth of 5 days old was inoculated in the center of the plate. Each treatment was repeated four times and the plates were then incubated at 25°C for 12 days. The percentage of seed germination calculated according to Bolkan and Butler (1974).

Identification of *F. solani* isolates using PCR assay

DNA of 68 fungal isolates extracted according to the method of (Hussein, 2022). PCR assays were carried out using pair of primers based on transcription elongation factor (TEF-1α) gene of TEF-Fs4f (5′-ATCGGCCACGTCGA CTCT-3′) and TEF-Fs4r (5′-GGCGTCTGTTGA TTGTTA GC-3′) (Arif *et al*., 2012). DNA amplification performed according to the protocol described by Arif *et al*. (2012) with minor modifications which included heating at a temperature. 94°C for 3 minutes and 40 cycles included: Denaturation at 94°C for 1 minutes, annealing at 58°C for 1 minutes, extension at 72°C for 2 minutes, then final extension at 72°C for 7 minutes. PCR product was electrophoresed in a 1.4% agarose gel.

Isolation antagonistic rhizobacteria

Five samples of roots and their rhizosphere soil of intact pepper plants in the fields of Erbil province, northern Iraq were collected, characterized by excellent vegetative and root system. Screening for antagonistic rhizobacteria exhibited by diluting method up to 10^7, 100 µL of the dilution 10^4-10^6 spread on the Nutrient Agar (NA) plates (Produced by HiMedia, India). The plates were incubated at a temperature of 37°C for 48 h. Bacterial growth isolated on the basis of the phenotypic difference of the colonies. Bacterial isolates were purified several times on the same culture medium and stored in the Nutrient broth (Produced by HiMedia, India) mixed with 20% glycerol at -70°C.

Preparing botanical extracts

100 gm of fresh garlic cloves were chopped and steeped in 250 ml of sterile water for 24 hours at room temperature to prepare the natural extraction of the garlic and then the mixture grinded and completed to 1000 ml by adding sterile distilled water and mixed well, To eliminate solid plant debris, the solution was filtered through sterile cheesecloth and placed in a sterile conical flask (Mohana and Raveesha, 2007).

Leaves of Nerium plants (*Nerium oleander*) were collected from local garden in the center of Baghdad province, washed thoroughly and dried completely at 40°C in the oven, dry leaves powder was obtained by using electric grinder and Kept in sterilized containers. For inhibition ability tests, an aqueous extract of Nerium powder were prepared by adding 100 g of the powder to 1000 ml of sterile distilled water in a sterile conical flask with constant shaking at 150 rpm for 72 hours at laboratory temperature, then stored in airtight container for subsequent tests.

Test for antagonism activity

On the PDA medium, the fungicide, botanical extracts and the poisoned food approach were used to measure the inhibition activity. According to the manufacturer's recommendations, the fungicides Promess (Pr), whose active ingredient is propamocarb hydrochloride (produced by Arysta LifeScience, Belgium) and Tecto 500 SC (Te), whose active ingredient is thiabendazole (produced by Syngenta, Switzerland), at the concentration of 2 ml/l were used. One milliliter of each fungicide (Pr and Te) and 1 ml of each botanical extract of garlic (Ga) and nerium (Ne) were added to the petri plate separately before the plates

were filled with 20 ml of autoclaved PDA and each one had a 50 mm disc of PDA with the fungal isolate (Fsw45) in the middle. All of the plates were incubated at 25°C for 5 days until the control's fungus reached the border of the plates. Inhibition activities of unknown 75 rhizobacterial isolates were screened using dual culture technique, by placing a loop full of each isolates at the side of petri plate and on the opposite side at the similar distance 50 mm disc of fungal isolate placed carefully, plates incubated at 25°C until the fungal growth of the control treatment (Fungus alone) reached the edge of the plates.

Four replicates were amended for each treatment and the entire experiment was repeated twice. Inhibition zone were calculated using the formula described by Mohana and Raveesha (2007).

Identification antagonistic bacteria

Three bacterial isolates which exhibited an inhibition rate higher than 80% against the pathogenic fungus *F. solani in vitro*. Its physiological and microscopic characteristics were studied and then identified using an automated microbial identification system of Vitek2 Compact System technique (produced by bioMerieux's, France).

Greenhouse assay

Inoculum of the fungal pathogen Fsw45 cultivated by growing it on millet seeds (*Pennisetum glaucum*) added at a ratio of 1% (w/w) to autoclaved mixture of soil and peat moss (2/1 v/v) in the 1 kg plastic pots. After 5 days. In each pot, 10 pepper seeds cultivar of (Anaheim California Chilies) were surface sterilized with 1% sodium hypochlorite solution. Inoculum of the bacterial isolates of *P. fluorescens*, *P. mendocina* and *S. kloosii* were prepared on nutrient broth at 28°C with continuous shaking for 48 h, bacterial cells harvested by centrifugation at 10.000 rpm for 10 min and washed them twice with sterile distilled water to remove residual medium components, then cells resuspend in sterile distilled water and cell density adjusted to 10^9 CFU/ml by hemocytometer.

The fungicides Promess (Pr), Tecto (Te) and the botanical extracts garlic (Gr), Nerium (Ne) and the bacterial inoculum of *P. fluorescens* (Pf), *P. mendocina* (Pm), *S. kloosii* (Sk) at the same time as the seeds were planted and at the same dose for the *in vitro* experiment, were placed into the soil as 10 ml/pot., then each 15 days each application repeated again at the same concentration, Percentage of the seed germination was estimated after 15 days of seeds planting. After 70 days of seed transplanting, the percentages of disease incidence (DI) calculated according to Masood *et al.* (2010) and disease severity index (DSI) according to Grau and Radke (1982) and using the scale rating described by Nagao *et al.* (1994).

The dry weight of the plants was measured after 70 days after seed sowing in order to assess the impact of the biological and chemical agents utilized on the plant growth parameter. The greenhouse's pots were dispersed and frequently watered in accordance with a completely randomized design (CRD) with four replications and the entire experiment was repeated twice.

RESULTS AND DISCUSSION

Isolation and identification of the fungi associated with diseased pepper plants

The results of isolation and identification of the fungi associated with diseased pepper samples showed that 15 species of fungi belonging to 7 genera presented (Table 1) and the fungus *F. solani* was predominant, as its appearance rate reached 74.77% with a frequency rate of 57.24% (Fig 1) which representing sixty-eight isolates.

Pathogenicity test of *F. solani* isolates

The results of pathogenicity test of 68 isolates of *F. solani in vitro* showed variation in their pathogenicity on the pepper seed, the seed germination rate ranged 5-95%. Isolated Fsw45 was superior in his pathogenicity ability which exhibited 5% seed germination compared to the control which was 100%.

Molecular identification of *F. solani* isolates

The results of DNA amplification of 68 isolates of *F. solani* based on the transcription elongation factor (TEF-1α) gene showed that 38 isolates positively amplified with the specific primers of *F. solani* and showed clear band at the size of 658 bp, while 30 isolates did not show clear bands on the Agarose gel. Perhaps this is due to the existence of genetic variation between the isolates of the *F. solani*, or these isolates may belong to another species of Fusarium genus.

Isolation antagonistic rhizobacteria

The findings demonstrated that 75 distinct bacterial isolates were obtained from the rhizosphere of samples of healthy pepper plants. The isolates underwent purification and were stored for use in further studies.

Table 1: Fungal isolates associated with the roots and crown of diseased pepper plants.

Fungus	Appearance (%)	Frequency (%)
Alternaria alternata (Fres.) Keissler	12.15	7.71
A. brassicicola	6.54	2.34
A. raphanin	4.67	1.75
Alternaria sp.	8.41	2.45
Aspergillus niger Van Tieghem	16.82	8.18
Aspergillus sp.	14.95	7.24
Fusarium oxysporum Schlesht	13.08	7.94
F. solani (Mart.) Sacc.	74.77	57.24
F. sporotrichioides	4.67	1.05
F. redolens	2.80	0.58
Fusarium sp.	10.28	6.31
Pencillium sp.	12.15	5.14
Rhizctonia solani (Kuhn)	9.35	5.37
Rhizopus sp.	11.21	9.23
Pythium sp.	8.41	5.61

Antagonism assay

The radial growth of the fungus *F. solani* on the PDA was significantly inhibited by the botanical extracts of nerium and garlic in the poisoning culture media test, with inhibition percentages of 80.56% and 77.78%, respectively, compared to the control, which filled the plates after 7 days of incubation, both the fungicides Promess and Tecto showed 100% inhibition (Fig 2).

Dual culture test of 75 bacterial isolates against the fungal isolate Fsw45 showed variation in their inhibition abilities, three bacterial isolates of B15, B38, B71 were superior which exhibited 87.46%, 84.29%, 83.81% inhibition percentage respectively without significant differences between them.

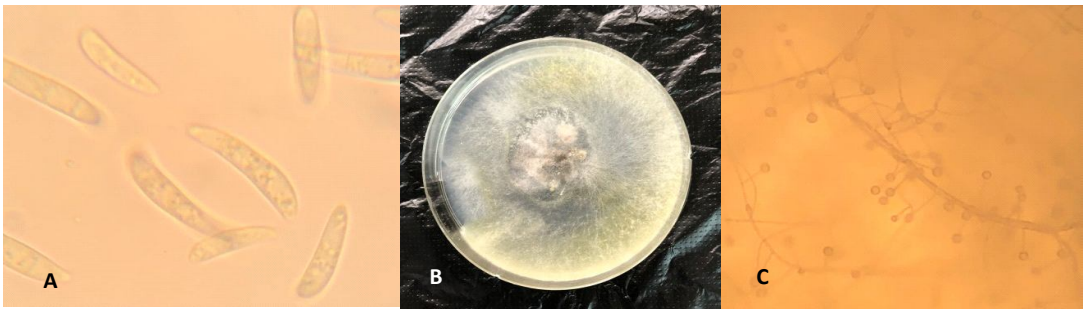
Identification antagonistic bacteria

Identification results of 3 rhizobacterial isolates of B15, B38 and B71 which exhibited higher antagonistic activities against the pathogen using Vitek2 compact system technique, showed that they belong to the bacterial species of *Pseudomonas mendocina, P. fluorescens* and *Staphylococcus kloosii* respectively. Morphological screening of the bacterial isolates showed that both the isolates of *P. mendocina* and *P. fluorescens* were Gram-negative, motile, rod-shaped, aerobic bacterium, with cell diameter of 1.5-2.5 µm in length and 0.5-1.0 µm in width, arranged in single, pairs and short chains, colonies shape were circular, smooth, convex and translucent with a diameter of 2-4 mm after 48 hours of incubation at 37°C on nutrient agar, colony color of *P. mendocina* was yellowish white while *P. fluorescens was* creamy white.

S. kloosii was Gram-positive, non-motile, cocci-shaped, aerobic bacterium, with cell diameter of 0.5-1.0 µm, arranged in single, pairs and clusters, colonies shape were S-type, smooth, convex and shiny surface ranged with diameter of 3-6 mm after incubation for 48 h at 37°C on nutrient agar with yellowish color.

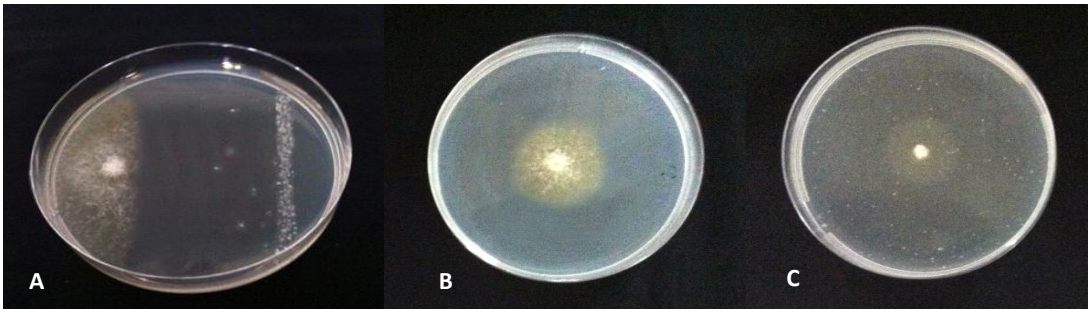
Greenhouse assay

Greenhouse experiments indicated that the both fungicide of Promess and Tecto were superior in controlling root and crow rot disease of pepper significantly, while they conducted 100% seed germination in the presence of the pathogen compared with the positive control (Rsw45 alone) which exhibited 65% seed germination (Table 2) and in comparison to the positive control, which showed disease incidence and severity indexes of 95% and 73% respectively, both fungicides showed 0% disease incidence. Thiabendazole which is the active ingredient of Tecto 500 SC is a systemic fungicide and Propamocarb hydrochloride which is active ingredient of Promess is semi-systemic fungicide, systemic fungicides are chemicals that can be absorbed by plants and protect them from fungal pathogens, they can act on different aspects of fungal metabolism, such as cell wall synthesis, sterol



Macroconidia × 100 (a) Fugal colony on the PDA medium (b) Long conidiophore × 10 (c).

Fig 1: Morphological characteristics of *F. solani.*



Fungal isolate Fsw45 × Bacterial isolate B38 (A), Fsw45 × Garlic extract (B), Fsw45 × Fungicide Promess (C).

Fig 2: Antagonism activities of chemical and biological agents against *F. solani.*

biosynthesis, respiration, or DNA replication, both of the fungicides are acropetal which move upward in the plant through the xylem (Ayesha *et al*., 2021). Thiabendazole works by inhibiting the enzyme fumarate reductase, which is essential for fungal metabolism (Gilman *et al*., 1990), it can control diseases caused by fungi, such as blue mold, green mold and stem end rot in citrus fruits (Upadhyay *et al*., 1980). Propamocarb hydrochloride has low toxicity and low risk of resistance development (Propamocarb Hydrochloride, 2004).

The botanical extract of nerium showed biocontrol activities higher than garlic extract, while it exhibited 90%, 55%, 45% percentage of seed germination, disease incidence and disease severity index respectively compared to the garlic extract which exhibited 87.5%, 65%, 50.5% respectively. Botanical extracts can act as botanical fungicides, which are chemicals that kill or inhibit the growth of fungal pathogens, or as inducers of systemic resistance, which are substances that stimulate the plant's own defense mechanisms against various pathogens (Sajeena *et al*., 2019). The botanical extract of Nerium, also known as oleander, is a plant that has been used for the management of plant diseases caused by fungal pathogens, Harish *et al*. (2008) indicated that Nerium exerts can achieve high inhibition percentage to the mycelial growth and spore germination of *Bipolaris oryzae*, the causal agent of brown spot disease in rice and It can also reduce the incidence of brown spot disease in rice by 53% and increase the yield by 18% when sprayed twice on rice plants in field conditions. It is also effective against other fungal pathogens, such as *F. oxysporum*, *R. solani*, *Sclerotium rolfsii* and *P. aphanidermatum* (Sajeena *et al*., 2019). Slusarenko *et al*. (2008) mentioned that garlic extract contains Allicin, a volatile antimicrobial substance that is produced when garlic tissues are damaged and the substrate alliin mixes with the enzyme alliin-lyase. Allicin can penetrate the fungal cell membrane and interfere with the protein function by reacting with the free thiol groups and he mentioned that using garlic extract before or after inoculation decreased the severity of leaf blight in tomatoes by 50% to 60% and prevented *P. infestans* from growing *in vitro*.

The biocontrol activities of the 3 rhizobacterial isolates of *P. mendocina, P. fluorescens* and *S. kloosii* showed significant differences in reducing the disease, while they exhibited 95%, 92.5%, 100% percentage of seed germination respectively and they conducted 40%, 35%, 55% disease incidence respectively and they exhibited 30.3%, 28.3%, 41.8% disease severity index respectively. *P. mendocina* and *P. fluorescens* are a bacteria that can act as a biological control agents of plant diseases caused by fungal pathogens, since they can produce several antimicrobial compounds, such as phenazine-1-carboxylic acid, pyrrolnitrin and 2,4-diacetylphloroglucinol, that can inhibit the growth and development of fungi, they can also induce systemic resistance in plants, which is a mechanism that enhances the plant's own defense responses against various pathogens (Someya *et al*., 2013). According to Bonaterra *et al*. (2022), *P. mendocina* produces phenazine-1-carboxylic acid and pyrrolnitrin, which have antifungal and nematicidal activities, these compounds also induce systemic resistance in tomato plants by activating the salicylic acid and jasmonic acid signaling pathways. *P. fluorescens* shown a considerable ability to treat *P. ultimum* caused carrot root rot disease, as well as *Verticillium dahlia*, the disease-causing agent for eggplant wilt (Panpatte *et al*., 2016). *S. kloosii* recorded by Sevim *et al*. (2015) as biocontrol agent against stored product pests.

Table 2: In a greenhouse, control the pathogen that causes pepper root and crown rot.

Treatment	Seed germination (%)	Disease incidence (%)	Disease severity index (%)	Dry weight g/plant
Negative control (Plants alone)	100.00	0.00	0.00	20.55
Positive control (Rsw45 alone)	65.00	95.00	73.00	5.26
Pr alone	100.00	0.00	0.00	19.90
Te alone	100.00	0.00	0.00	20.85
Ne alone	100.00	0.00	0.00	21.36
Ga alone	100.00	0.00	0.00	20.95
Pf alone	100.00	0.00	0.00	23.15
Pm alone	100.00	0.00	0.00	22.75
Sk alone	100.00	0.00	0.00	22.80
Pr + Rsw45	100.00	0.00	0.00	19.00
Te + Rsw45	100.00	0.00	0.00	19.60
Ne + Rsw45	90.00	55.00	45.50	13.77
Ga + Rsw45	87.50	65.00	50.50	10.55
Pf + Rsw45	95.00	40.00	30.30	18.00
Pm + Rsw45	92.50	35.00	28.30	17.25
Sk + Rsw45	100.00	55.00	41.80	17.12
LSD (0.05)	3.50	4.30	19.25	1.45

There is no clear evidence that this bacterium has been previously used as a biological agent against plant pathogens and it may use for the first time in this study.

All of the biological and chemical agents used in this experiment showed significant effects in suppressing root and crown rot disease of pepper, but the chemical pesticides Promess and Tecto achieved high effectiveness compared to the rest of the treatments.

The results showed also significant increase in the dry weight of pepper plants when bacterial isolates were added to the plants in the absence of the pathogen, but with the addition of the pathogen. There was no significant increase in the plants' dry weight across all treatments. On the contrary, all the treatments which consist of the pathogen, the average of plant's dry weight decreased significantly, which indicates the negative impact of the pathogen on the vital functions of plants. The role that PGPR increasing plant growth parameters such as dry weight of plant is to provide a natural and sustainable way of enhancing the nutrient uptake, water availability and stress tolerance of plants. PGPR can also produce various phytohormones, such as auxins, cytokinins, gibberellins and ethylene, that can regulate the plant growth and development (Bhattacharyya and Jha, 2012). *P. fluorescens* recorded as siderophore-producing bacterium, has ability to increase the dry weight of rice by 15%, cucumber by 20% and carrot by 10% (Riaz *et al*., 2021) and *Bacillus subtilis* recorded as phosphate-solubilizing bacterium, has ability to increase the dry weight of tomato by 40%, potato by 30% and sunflower by 25% (Bhattacharyya and Jha, 2012).

CONCLUSION

Despite the presence of some soil-borne pathogenic fungi in the soil of pepper fields in the governorates of Babil and Wasit, the dominance was of the pathogenic fungus *F. solani*. Both of the fungicides (Promess and Tecto 500 SC) showed efficiency in inhibition the pathogenic fungi isolate Fsw45 *in vitro* alongside with 3 rhizobacterial isolates of *P. mendocina*, *P. fluorescens*, *S. kloosii* and plant extracts of garlic and nerium. Under greenhouse conditions, both chemical fungicides were able to control the disease by 100%, as for bacterial treatments and plant extracts, although they were able to reduce the percentage of disease incidence and severity significantly but they did not reach the rate achieved by both fungicides. The 3 bacterial isolates were able to enhance plant growth by increasing the dry weight of plants in the absence of the pathogen. However, in the presence of the pathogen, all chemical and biological factors did not achieve an increase in plant growth indicators.

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Conflict of interest

The author declares that they have no conflicts of interest.

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