



Effect of bacterial biological factors on reducing incidence and severity of cucumber root rot disease

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This investigation is carried out in the laboratories of the Plant Protection Directorate, Ministry of Agriculture, to evaluate the pathogenicity of eleven fungal isolates obtained from the roots of diseased cucumber plants, and to assess the efficacy of three bacterial strains—*Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus rugosus*—in minimizing the incidence and severity of cucumber root rot induced by *Rhizoctonia solani* under pot conditions. Biologically synthesized nanoparticles, particularly silver nanoparticles (AgNPs), have shown strong antimicrobial activity against a wide range of plant pathogenic fungi. The results showed that all fungal isolates significantly reduced seed germination and increasing intensity of the illness. Isolates of *R. solani* are particularly aggressive, with four isolates completely inhibiting seed germination and causing 100% disease severity. The remaining isolates resulted in germination rates ranging from 30% to 93.3%, and severity levels between 13.3% and 46.7%, compared to the healthy control (100% germination, 0% severity). Nucleotide sequence analysis confirmed the identity of the fungal isolates as *R. solani*, *Fusarium oxysporum*, and *Macrophomina phaseolina*, and the sequences are deposited in GenBank under accession numbers PP892963.1, PQ810062, and PV008703.1, respectively. All three bacterial strains significantly reduced disease incidence and severity, with *B. rugosus* showing the greatest effect, followed by *B. cereus* and *B. thuringiensis*, reducing disease incidence to 6.6%, 26.6%, and 63.3% and severity to 23.3%, 43.3%, and 73.3%, respectively.

Keywords: *R. solani*; Antifungal bacteria; Nanoparticles; Cucumber root rot; *Bacillus rugosus*.

1. INTRODUCTION

Cucumber (*Cucumis sativus*), a member of the Cucurbitaceae family, is one of the most important crops and is grown in multiple regions of the world and of great economic importance. It is cultivated in Iraq during two growing seasons, autumn and spring, under open-field conditions and inside greenhouses. It contains a large percentage of water, up to 96%, carbohydrates, 3% and 1% protein, and some minerals such as iron, calcium, copper, manganese and potassium. It is low in calories and rich in many vitamins [1]. The cultivated area in Iraq is about 2607.4 donum and with a productivity of about 66472 tons [2]. Cucumbers are a very important crop due to the increasing demand for them. Many researches aim to protect the cucumber plant and improve its growth and production by using modern technologies and taking advantage of different sciences [3]. Soil-borne pathogens are representing a major threat to crops, since they develop unnoticed and their symptoms usually appear on the vegetative growth only after the root system has been severely damaged [], which increases their danger. Many of these fungi are capable of infecting multiple plant species, have the ability to resist inadequate environmental conditions, and can stay in the soil and residues of infected plants for a long time [5]. Therefore, cucumber cultivation faces a number of problems, including fungal diseases such as root rot, seed rot, seed death before and after eruption, and wilt diseases caused by a number of mainly pathogenic soil settlements, including *R. solani*, *F. solani*, *F. oxysporum*, *M. phaseolina*, *Sclerotinia sclerotiorum*, *Pythium spp.*, and *Phytophthora spp.* [6-8]. The use of chemical pesticides in the resistance of plant pathogens is the best method, but the exposure of pathogens to electoral pressure as a result of the repeated use of chemical pesticides led to the emergence of resistance in them [9] as well as environmental pollution that occurs due to the frequent use of pesticides and their impact on non-target organisms. The use of chemical control requires labor, equipment and large sums of money [7, 10]. In recent years, nanotechnology has gained considerable attention in plant disease management due to the unique physicochemical properties of nanoparticles [11].

Biologically synthesized nanoparticles, particularly silver nanoparticles (AgNPs), have shown strong antimicrobial activity against extensive range of plant pathogenic fungi [12]. Several studies have reported that nanoparticles can enhance the efficiency of biological control agents and inhibit fungal growth through different mechanisms such as disruption of cell membranes and induction of oxidative stress. Therefore, integrating nanotechnology with biological control strategies may provide an effective and environmentally friendly approach for managing soil-borne diseases such as cucumber root rot caused by *Rhizoctonia solani* [13]. In order to achieve the goal that everyone seeks to reach, which is to reduce the incidence of pathogens and find new means and methods in combating plant pathogens because chemical pesticides are expensive and not environmentally friendly, the importance of biological control to combat plant pathogens has emerged as an easy way that does not cause environmental imbalance [14], as well as it is based on the joint action of different resistance methods to reduce the damage of these pathogens. Therefore, biological control has gained wide acceptance around the world and enjoys continuous encouragement and wide and advanced application in the field. It works to reduce the risk of crop damage caused by pathogens [8]. It has become one of the most important pillars in resisting these pathogens, including the use of anti-pathogenic biota and leading to reduce the density and activity of pathogenic communities. It has used a number of bacteria species that encourage Plant Growth Promoting *Rhizobacteria* (PGPR) in several mechanisms and has been found to accompany the roots of different plants in the Rhizosphere region, such as *Bacillus spp.*, *Antinomies sp.* *Pseudomonas spp.* and *Azotobacter sp.* These biological agents are highly antagonistic to pathogens and are catalysts for plant growth; beneficial bacteria live free in the soil[15, 16, 17,18, 19]. Based on this concept, this study aimed to diagnose Some fungal species associated with the roots

of cucumber plants and obtain environmentally friendly biological control agents bearing the specifications of biocides against cucumber root rot disease.

2. EXPERIMENTAL

2.1. Isolating and identification some of fungi that cause cucumber root rot

The fungi are obtained from the roots of infected cucumber plants, which Indicated the symptoms of the disease from the deterioration and yellowing of the vegetative part, the wilt and death of plants, the discoloration of the roots with a dark brown color, and the presence of ulcers and rotting roots, which are collected from several fields of cucumber plants from Baghdad Governorate, Salah Al-Din Governorate, Basra Governorate, Sulaymaniyah Governorate, and the necessary information is recorded on them (the name of the governorate and the region /date of collection). The samples are preserved in polyethylene bags and transferred to the laboratories of the Ministry of Agriculture /Plant Protection Department. The next day, the fungi associated with the root system are isolated. The roots are washed under running water (tap water) for 15 minutes to remove all plankton and impurities on the surface of the roots. The roots are cut into small pieces of length (0.5-1.0) cm, sterilized superficially with a sodium hypochlorite solution at a concentration of 1%. After that, the roots are washed with sterile water for 2 minutes, dried on Whatman No.1 sterile filter paper for the purpose of removing excess water. The pieces are planted by means of sterile forceps of four pieces per petri dish with a diameter of 9 cm containing 15-20. cm-3 of Potato Dextrose Agar (PDA) culture medium (200g potatoes, 20g dextrose, 15 g agar, 1-liter distilled water) sterilized with buffers under 121 °C and a pressure of 1.5 kg cm-2 for 20 minutes with added of the antibiotic buffer device Ceftriaxone 200 mg L-1 to reduce the growth of bacteria.

The dishes are incubated in the incubator at a temperature of $25 \pm 2^{\circ}\text{C}$. After four days, the fungi are purified in a single spore method for *Fusarium spp.* The rest of the fungi are purified by removing a part of the edge of the fungal growth and replanted in other petri dishes. The dishes are incubated at a temperature of $25 \pm 2^{\circ}\text{C}$ for 5 days. Fungal identification is carried out phenotypically according to colony features and the nature of the mycelium, spores, and structures that they form based on the approved taxonomic keys [20].

2.2. Effect of some pathogenic fungi on germination of cucumber seeds and plants their plants in pots

The pathogenicity of 11 obtained of fungi, seven isolates of *Rhizctonia solani*, three obtained of *Fusarium spp.*, and one obtained of *Macrophomina phaseolina* is tested, which proved its high pathogenicity on the seeds of cucumber plants in the laboratory[6].Pathogenic isolates are grown on local millet seeds *Panicum miliaceum* in flasks, placed in each flask with a capacity of 500 cm³ 100 g of seeds and added to it 300 cm³ of water, soaked for 6 hr. , then poured the excess water, Sterilized with autoclave under 121 °C and a pressure of 1.5 kg / cm² for 15 minutes, each flask is pollinized with three tablets with a diameter of 5 mm taken from near the edges of isolate farms grown on the PDA medium, The flasks are maintained at $25 \pm 2^{\circ}\text{C}$ for 14 days and shaken at intervals of 2–3 days to ensure ventilation and distribution of the mushroom pollen to the seeds. The experiment is carried out according to Completely Randomized Design (CRD) with three replicates for each pathogenic mushroom using plastic pots with a diameter of 20 cm and a height of 15 cm and a capacity of 2.5kg of mixed soil, placed in each pot 2 kg of soil and planted with seeds of cucumber plants, a glorious variety of five seeds per pot with a comparison treatment without pathogenic fungi. The pathogenic fungus is added to the soil at a rate of 1% w / w and then the pot is watered and followed up. The

percentage of germination is calculated 10 days after planting the seeds and severity of infection is evaluated 30 days after planting using a five-level disease scale:

0 = Healthy plant, 1 = Secondary root rot, 2= Secondary root rot + Main root rot + Beginning stem base rot, 3= Secondary root rot + Main root rot + Entire stem base rot, 4= Entire plant death.

The severity of the disease (%) is calculated following the McKinney equation [21].

$$\text{Injury Severity Percentage} = \frac{[\text{Number of plants in grade 0} \times 0] + \dots + [\text{Number of plants in grade 4} \times 4]}{\text{Total number of plants inspected} \times 4} \times 100$$

2.3. Identification of some fungal isolates associated with cucumber roots using PCR technology

Three isolates of fungi are Identification, R5, F2 and M, which proved highly pathogenic. The fungi are Identification to the level of the species according to the molecular method nucleotide sequencing analysis of the gene 5.8s rRNA. A swab is used from the newly developed pure colony for each fungal isolation genomic DNA is extracted using the ready-made kit to extract DNA. The ITS region of the 5.8 SrRNA gene is replicated using the initial PCR [20]. With the use of the primers, ITS1-ITS4 Universal Primer, the sequences of which are shown in Table (1) to determine the elected isolates for the ITS region and equipped by (Integrated DNA Technologies company, Canada) [22].

Table 1 Nucleotide sequences of innate prefixes.

Primer	Sequence	Product size
Forward ITS1	5'- TCCGTAGGTGAACCTGCGG -3'	650 base pair
Reverse ITS4	5' TCCTCCGCTTATTGATATGC-3'	

After being stained with the dye (Intron Korea red stain), the PCR result is separated using electrophoresis on agarose gel (1.5%) and the DNA genomic packages are displayed using ultraviolet UV light with a wavelength of 302 nm. After acquiring the 8S rRNA, the nitrogenous bases of the amplified gene are identified by directly interacting with the PCR product and the 10 µl concentration. Five genes are duplicated by sending a 25 µl PCR product and a 10 µl 10-bit concentration to the National Biotechnology Laboratory (NCBI) via the Basic AI Localization Search Tool (BLTAS).

2.4. Effect of bacterial biocontrol agents in reducing the percentage and severity of infection with *R. solani* causing cucumber root rot disease in the pot.

Three isolates of bacteria that have been shown to have a high ability to inhibit fungal growth in vitro are selected: *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus rugosus*, which are confirmed not to be pathogenic to humans in the Central Public Health Laboratory and are molecularly Identification and deposited in the gene bank. The sequence of nitrogenous bases is deposited in the Gene Bank under accession numbers PP935479.1, PP935461.1 and PP934669.1, respectively.

This experiment is carried out in the Department of Plant Protection/ Ministry of Agriculture according to the completely random design (CRD with three iterations for each transaction using plastic pots with a diameter of 20 cm and a height of 15 cm and a capacity of 2.5kg mixed soil, placed in each pot 2 kg soil and planted with seeds of cucumber plants, 10 seeds of the plant *Cucumis sativus* is planted for each pot and the experiment contained the following treatments:

- 1- *R.solani*
- 2- *Bacillus cereus*
- 3- *Bacillus cereus*+ *R.solani*
4. *Bacillus thuringiensis*
5. *Bacillus thuringiensis*+ *R. solani*
6. *Bacillus rugosus*
7. *Bacillus rugosus*+ *R.solani*
- 8- Healthy cucumber plants

The percentage of infection for each treatment is calculated according to the equation

$$\text{Percentage of Injury} = \frac{\text{number of dead plants}}{\text{total number of plants}} \times 100$$

After 30 days following transplantation, the percentage of the injury's severity is determined using the five-degree pathological index. As in the preceding paragraph, the McKinney equation is used to determine the proportion of the injury's severity.

Silver nanoparticles (AgNPs) are synthesized using bacterial culture filtrates as reducing and stabilizing agents. Briefly, bacterial cultures are grown in nutrient broth and incubated at 28 °C for 48 h. The cultures are centrifuged and the supernatant is mixed with silver nitrate (AgNO₃) solution (1 mM). The reaction mixture is incubated in the dark until a color change from pale yellow to brown is observed, indicating the formation of AgNPs. The synthesized nanoparticles are preliminarily confirmed using UV–Visible spectroscopy. The antifungal activity of the synthesized nanoparticles is then evaluated against *Rhizoctonia solani* under laboratory and pot conditions.

3. RESULTS AND DISCUSSION

3.1. Isolating and Identification some of fungi causing cucumber root rot

The results of isolation and Identification from the roots of infected cucumber plants that showed symptoms of yellowing of leaves, ulcers, withering of the plant and rotting of the roots, The results showed that the most abundant fungus is the fungus *R. solani*, as it appeared in most of the samples from which it is isolated, followed by the fungus isolates *Fusarium* spp. and one isolate of *M. phaseolina* fungi. The pathogenicity of 11 isolates of fungi is tested, seven isolates of fungi *R. solani*, three isolates of fungi *Fusarium* spp, and one isolate of fungi *M. phaseolina*, which proved its high pathogenicity on the seeds of cucumber plants in the laboratory [6]

3.2. Effect of some pathogenic fungi on germination of cucumber seeds and plants their plants in pots

The results of the pathogenicity test for 11 isolates of fungi, seven isolates of the fungus *R. solani*, three isolates of the fungus *Fusarium* spp, and one isolate of the fungus *M. phaseolina*, showed that all isolates of fungi have caused a significant reduction in the rate of seed germination and infection of cucumber plants compared to the comparison treatment in which the germination rate reached 100% and the severity of infection 0%. *R.solani* mushroom isolates have significantly excelled in reducing the percentage of germination, as isolates R1, R3, R4 and 5R prevented the germination of seeds in full and the severity of infection is 100% compared to other fungal isolates treatments, as the percentage of germination of *R. solani* mushroom isolates reached 63.3%, 30 and 40% and the severity of infection is 46.7, 36.7 and 16.7% for R2, R6 and R7 isolates respectively, while the germination ratios of *Fusarium* spp (F1-F2-F3) mushroom isolates reached 73.3%, 30% and 93.3%, and the severity of

infection is 20 , 40 and 10% respectively , As for the isolation of the fungus *M. phaseolina*, the germination rate is 86.6% and the severity of the infection is 13.3% .These results are consistent with what a number of researchers have found that the fungus that causes root rot has a negative impact on the germination rate. The infection rate ranges from minor to severe, which leads to the complete destruction of the crop and causes up to 100% in severely infected fields when the conditions are appropriate [23]. The difference in pathological ability between the tested isolates may be due to genetic and environmental differences, or the difference may be due to the ability of fungi to secrete toxins and seed-dissolving enzymes, and the failure of germination and seed rot may be due to the ability of these fungi to secrete enzymes, toxins and toxic metabolites that cause these symptoms [24, 25]. Many studies have shown the susceptibility of fungi *R. solani*, *F.spp* and *M. phaseolina* to the production of toxins and hydrolyzed enzymes that have the ability to analyze the components of the plant cell wall and thus contribute to the penetration of the pathogenic fungus and its arrival in the cytoplasm of the target cell. These enzymes are Cellulase, pectinase, Protase, amylase and others [26] , and this fungus has the ability to produce several types of plant toxins such as fungal toxins *R.solani*, Phenyl acetic acid, P or M-hydroxyl derivatives, *phaseolina* mushroom toxins, such as Paseolinone, Botryodiplin, Phomenone, Gantigone and *F.spp* toxins such as DON, Zeralin (ZEN), Fusaric acid, Javanici, Phedpeptinoxin [24 , 26, 27]. These results are consistent with several studies that have indicated the ability of these fungi to reduce the germination rate of a large number of plant families compared to the comparison treatment [23, 24,28].These results also agreed with what is recorded by [23] as the fungus *F.solani* and *R.solani* are isolated and Identification from the roots of infected cucumber plants in the province and had a high pathological capacity as it caused a significant reduction in the germination rate of cucumber seeds.

Table 2 The effect of some of pathogenic fungi on the germination of cucumber seeds and their plants in pots.

Isolation	%germination	severity of infection %
R1)(<i>R. solani</i>	0.0	100.0
<i>R. solani</i> (R2)	63.3	46.7
<i>R. solani</i> (R3)	0.0	100.0
<i>R. solani</i> (R4)	0.0	100.0
<i>R. solani</i> (R5)	0.0	100.0
<i>R. solani</i> (R6)	30.0	36.7
<i>R. solani</i> (R7)	40.0	16.7
<i>Fusarium spp</i> (F1)	73.3	20.0
<i>Fusarium spp</i> (F2)	30.0	40.0
<i>Fusarium spp</i> (F3)	93.3	10.0
M1)(<i>M. phaseolina</i>	86.6	13.3
Control	100.0	0.0
L.S.D (0.05)	16.12	11.76

3.3. Identification of some fungal isolates associated with cucumber roots using PCR technology

The results of the polymerase chain reaction (PCR) showed that the DNA chain is amplified from three isolates of the most pathogenic fungi (R5, F2 and M1) using the primers ITS1 and ITS4, which are purified by electrophoresis for the results of the PCR using agarose gel by the appearance of DNA pieces with a molecular weight between 500 – 750 base pairs (bp) (Figure 1). The DNA sequences that

are duplicated for the its region of the three fungal isolates are identified as belonging to the fungus (R5 *R. solani*, (F2) *F. oxysporum*, (M1) *M. phaseolina*. The sequence of nitrogenous bases for the three isolates is deposited in the National Center for Biotechnology Information (NCBI) under the accession numbers PP892963.1, PQ810062 and PV008703.1.

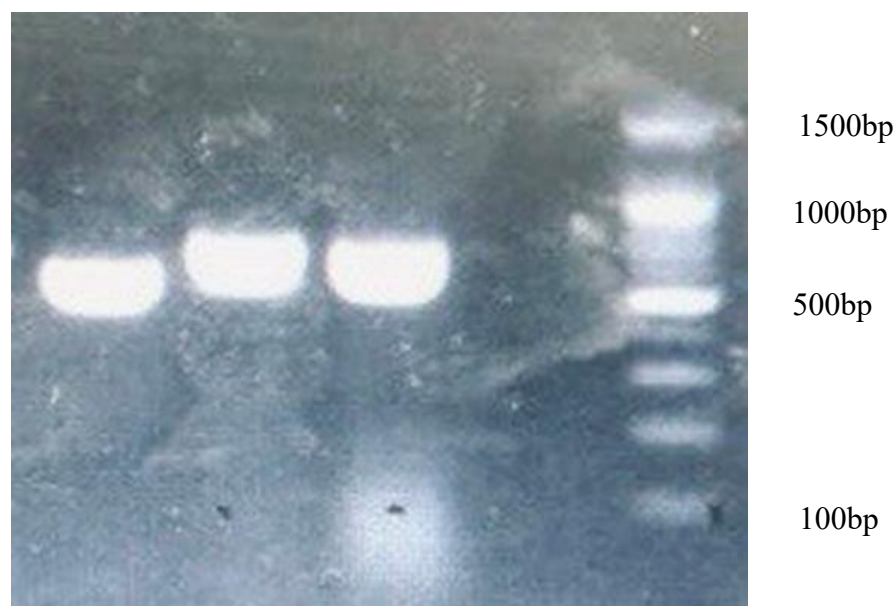


Figure 1 Bands resulting from DNA electrophoresis.

The synthesized silver nanoparticles (AgNPs) showed noticeable antifungal activity against *Rhizoctonia solani*. The formation of nanoparticles is indicated by the color change of the reaction mixture and confirmed by UV–V is spectroscopy. Application of the synthesized nanoparticles significantly reduced fungal growth and disease development compared with the untreated control. The combined use of bacterial strains and nanoparticles enhanced the suppression of cucumber root rot, suggesting that nanotechnology can improve the effectiveness of biological control agents in managing soil-borne plant diseases [29].

3.4. Effect of bacterial biocontrol agents in reducing percentage and severity of infection with *R. solani* causing cucumber root rot disease in the pot.

The results showed that all the bacterial biological control agents used in this experiment had a significant impact on reducing the percentage and severity of on cucumber root rot disease compared to the comparison treatment contaminated with the pathogenic fungus alone Table (3). The infection rate ranged from 23.3 to 73.3% and the severity of infection ranged from 6.7 to 63.3% compared to the control treatment in which the infection rate that reached. 96.7% and the severity of the injury is 93.3% compared to the control treatment, in which the severity of the injury is 93.3% and the incidence rate is 96.7%. The treatment of the pathogenic fungus *R.solani* with the bacterium *Bacillus rugosus* achieved high efficiency in reducing the incidence of infection and severity of the disease, followed by the treatment of the pathogenic fungus *R.solani* with the bacterium *Bacillus cereus*, and then the treatment

of the pathogenic fungus *R.solani* with the bacterium *Bacillus thuringiensis*, as the severity of infection reached 6.7, 26.7 and 63.3, and the incidence rate is 23.3, 43.3, 73.3, respectively. Several studies have indicated that root bacteria have high antagonistic efficiency with many pathogens and increase the susceptibility of the soil to suppress pathogens, especially those causing root rot [23]. Their effect may be due to the high susceptibility of these bacteria to competition for food and place, especially in the area surrounding the roots, where the appropriate environment for the presence of nutrients secreted from the roots [3, 8]. The effectiveness of bacteria in resisting the pathogen *R.solani* may also be due to the activity of these bacteria in enhancing the construction of the walls of the host cells and making the plant tissue more cohesive, which weakens the pathogen's susceptibility to the invasion of plant tissue [30-32]. It also has the mechanism of inducing systemic resistance in plants with an active role in resisting pathogens, as it strongly provokes defense reactions within the plant or increases the activity of enzymes or phytoalexins such as Peroxidase, Chitinase, glucanase and Polyphenol oxidase [33-36].

Table 2 The effect of bacterial biological agents in reducing the percentage and severity of infection with the *R. solani* in the pot.

Treatment	Percentage of Infection	Severity of Infection%
<i>R .solani</i>	96.7	93.3
<i>Bacillus cereus</i>	0.0	0.0
<i>Bacillus cereus</i> + <i>R. solani</i>	43.3	26.7
<i>Bacillus thuringiensis</i>	0.0	0.0
<i>Bacillus thuringiensis</i> + <i>R. solani</i>	73.3	63.3
<i>Bacillus rugosus</i>	0.0	0.0
<i>Bacillus rugosus</i> + <i>R. solani</i>	23.3	6.7
control	0.0	0.0
L.S.D	7.52	12.31

4. CONCLUSIONS

This study confirmed that fungal isolates associated with cucumber root rot significantly reduced seed germination and increased disease severity, with *Rhizoctonia solani* being the most aggressive pathogen. Molecular analysis verified the identity of the main pathogens involved. The tested *Bacillus* strains effectively reduced disease incidence and severity under pot conditions. Among them, *Bacillus rugosus* showed the highest biocontrol efficiency, followed by *B. cereus* and *B. thuringiensis*. These findings highlight the potential of *Bacillus* spp. as effective and eco-friendly alternatives for managing cucumber root rot.

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