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RESEARCH **Open Access**

Isolation and molecular identification of *Rhizoctonia solani* and *Fusarium solani* isolated from cucumber (*Cucumis sativus* L.) and their control feasibility by *Pseudomonas fluorescens* and *Bacillus subtilis*

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Abstract

This study was conducted to isolate and molecularly identify two pathogenic fungi namely *Rhizoctonia solani* and *Fusarium solani* implicated in root rot and seedling damping-off disease of cucumber, *Cucumis sativus*. Besides, the efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* as bacterial agents in controlling these two pathogens was also evaluated in vitro and in a greenhouse pot experiment.

Results of polymerase chain reaction (PCR) amplification and nucleotide sequence analysis using BLAST demonstrated that *R. solani* isolate was genetically different from the *R. solani* isolates in the National Centre for Biotechnology Information (NCBI). Therefore, it was recorded in GenBank under the accession number MK105921. *P. fluorescens* and *B. subtilis* showed a complete inhibition of the mycelial growths of *R. solani* and *F. solani* in vitro. In the pot experiments, soil treatment with a suspension of *P. fluorescens* and *B. subtilis* before planting significantly reduced the damping off of cucumber seedlings caused by *R. solani* and *F. solani*. This study suggests that these bacterial antagonists could have a good potential as biological control agents to protect cucumber plants from the infection with *R. solani* and *F. solani*.

Keywords: Pathogenicity, *Bacillus subtilis*, *Pseudomonas fluorescens*, Biological control, *Rhizoctonia solani*, *Fusarium solani*, PCR amplification

Background

Cucumber, *Cucumis sativus* L., is one of the most important vegetable crops worldwide but, unfortunately, is affected by many pathogens in the field causing serious yield losses (Mohammed and Hasan 2018). *Rhizoctonia solani* Kühn and *Fusarium solani* (Mart.) Sacc. are devastating pathogens that cause damping-off diseases of cucumber plants either in the greenhouses or in the fields (De Curtis et al. 2010). Both *R. solani* and *F. solani* have a wide host range and are capable to resist the extreme environmental conditions. In addition, they are capable to remain for a long period in soil and plant residues (Šišić et al. 2018).

The accurate diagnosis of plant pathogenic fungi is one of the urgent needs due to its importance in reaching rapid and efficient disease management systems to reduce or prevent damages caused by the fungal infections (Balodi et al. 2017). In previous studies, morphological characteristics are widely used in diagnosing many pathogenic and non-pathogenic fungi (Rezaee et al. 2018). Although identification of fungi based on morphological characteristics may sometimes give accurate results, many researchers do not rely on these characteristics because they require high experience in the field of classification, especially when dealing with closely related fungal groups such as *Fusarium* spp. In addition to its need for time and effort, morphological methods are sometimes inaccurate

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due to some factors such as the nature of growth, light, darkness, humidity, and some other factors that affect the sizes, shapes, and colors of spores and fungal colonies. Therefore, many researchers have shifted to other methods such as polymerase chain reaction (PCR), which is one of the molecular techniques used to target a specific region of the organism's genome that can show the genetic relationships between the fungal isolates to support the morphological identification. This technique has been found to be one of the most important, accurate, and rapid techniques in the detection and identification of many microorganisms including fungi, bacteria, and viruses (Alhussaini et al. 2016).

Different measures for controlling of plant pathogens have been developed such as the use of resistant varieties (Borrelli et al. 2018), plant extracts (Han et al. 2018), chemical control (Karim et al. 2018), and biological control (Yendyo et al. 2017). Overuse of the chemicals can cause environmental problems, negatively affecting human health and increasing the pest resistance to pathogens. Thus, the need for using new biological approaches as alternative safe control methods has been increasing. (Nicolopoulou-Stamati et al. 2016). Many microbial species such as *P. fluorescens* and *B. subtilis* have been shown to effectively control plant pathogens (David et al. 2018).

Bacillus subtilis had a great potential as biocontrol agent against *Rhizoctonia solani* on maize and pepper plants (Madhavi et al. 2018). Zaim et al. (2018) found that *B. subtilis* was effectively used to suppress 93.67% of the disease caused by *F. oxysporum* f. sp. *ciceris* of chickpea and also improved the plant growth leading to increased plant height, root length, and fresh and dry weights of shoot and root. Notz et al. (2002) reported that *P. fluorescens* was effectively reduced root rot disease of bean (*Phaseolus vulgaris*) caused by *Sclerotium rolfsii* and *Fusarium* wilt of tomato caused by *F. oxysporum* f. sp. *lycopersici* and wheat caused by *F. oxysporum*. The objective of the present study was to isolate and molecularly identify *R. solani* and *F. solani*, the causal agents of cucumber seedlings damping off, and to evaluate the efficacy of *P. fluorescens* and *B. subtilis* as biocontrol agents against these fungi.

Materials and methods

Sampling and fungal isolation

Root samples were collected from some diseased cucumber plants showing typical symptoms of damping off, growing in some plastic tunnels located in the desert farms in Najaf province, Iraq. The samples were transferred to the laboratory of plant diseases at the Faculty of Agriculture, Kufa University, Iraq, for isolation of fungal pathogens. Collected roots were washed by tap water, cut into small pieces, sterilized with NaOCl (1%) solution for 2 min, and washed with sterile distilled water to remove any residues of NaOCl. Root pieces were then dried using filter papers to remove any excess water and transferred to Petri dishes containing potato dextrose agar (PDA) media, supplemented with chloramphenicol antibiotic at a concentration of 200 mg/L. All

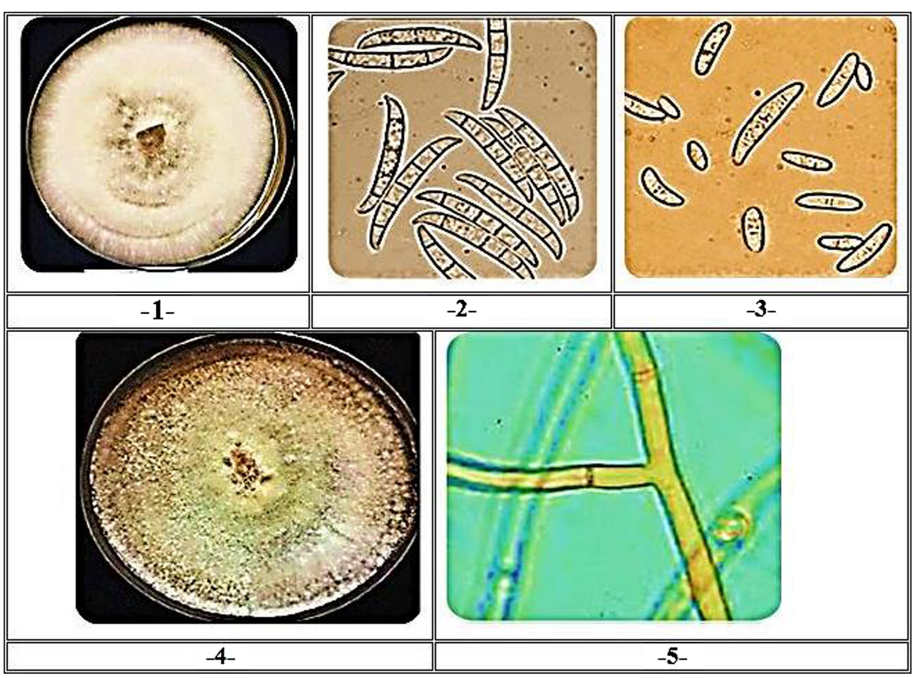

Fig. 1 Morphological characteristics of mycelial growth (**1**), macroconidia (**2**), and microconidia (**3**) of *F. solani* and mycelial growth of *R. solani* (**4** and **5**) on PDA

Petri dishes were incubated at a temperature of 25 ± 2 °C for about 4 days.

Morphological identification

The appeared fungi were purified and maintained on the same medium (PDA) and were used for morphological identification by microscopic examination.

Molecular characterization

The isolated fungi were molecularly identified using PCR technique and determining the nucleotide sequences as follows:

DNA extraction

From each fungal isolate, 50–100 mg of fresh 5-day-old colonies were taken by a sterile scalpel and transferred into an Eppendorf tube for DNA extraction using a specific extraction kit (Zymo Research, Cat. No. D6005), following the manufacturer's instructions. The quality and quantity of DNA extracted from each isolate were measured by a UV spectrophotometer (Thermo Scientific, Germany). DNA was then stored at − 20 until use.

PCR amplification and DNA sequencing of rDNA-ITS region

The internal transcribed spacer (ITS) region of *R. solani* isolates were amplified, using the universal primers ITS1 (TCCGTTGGTGAACCAGCGG) and ITS4 (TCCT CCGC TTATGATATGC) (White et al. 1990) using *Taq* DNA polymerase (Roche, Cat. No. 11146 173 001). The final volume of each PCR reaction mixture (sample) was 20 μl containing; 2 μ1 10 × PCR buffer, 1 μl of each primer (10 pmol), 2 μl dNTPs (2 mM), 3 μl template DNA (30 ng/μ1), 1 unit *Taq* polymerase, then completed to 20 μl by adding nuclease-free sterile distilled water. PCR amplification was performed using the following conditions: initial denaturation at 94 °C for 1 min followed by 35 cycles each consisting of final denaturation at 94 °C for 30 s, annealing temperature at 55 °C for 30 s, initial extension for 1 min, and final extension at 72 °C for 5 min (White et al. 1990. PCR-amplified products were electrophoretically separated on a 1% agarose gel for 140 min at 80 V and 400 mA and visualized with ethidium bromide under UV illumination, and images were captured using Vilber Lourmat, Taiwan, gel documentation system.

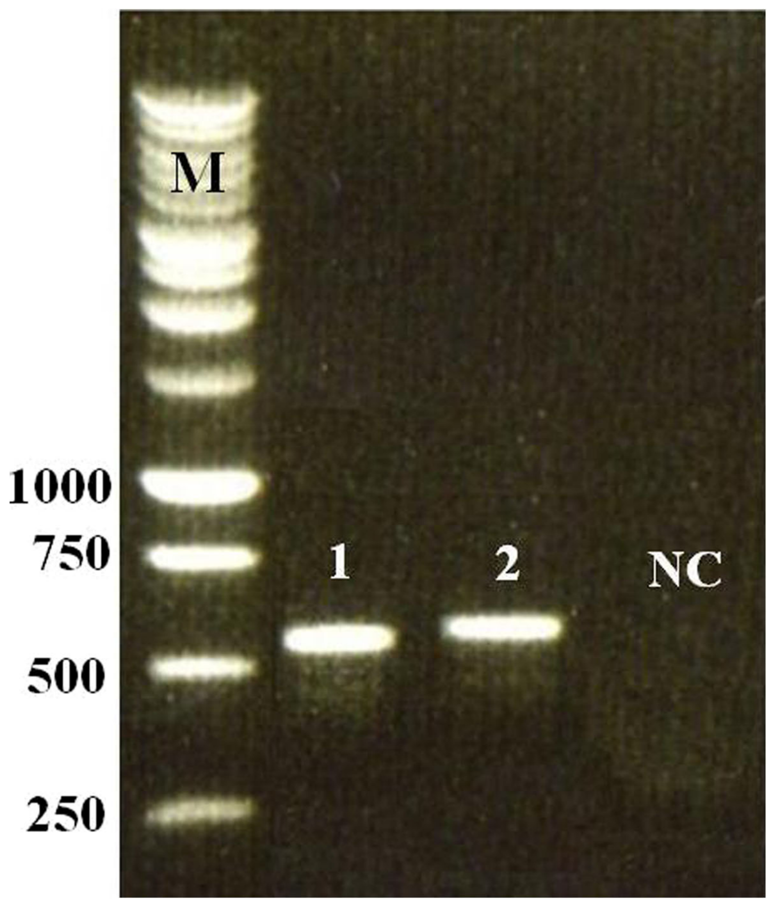

Fig. 2 DNA products amplified by polymerase chain reaction (PCR) from *F. solani* (**1**) and *R. solani* (**2**) isolated from diseased cucumber roots. NC negative control (no template DNA added). M, 1 Kbp DNA ladder marker (Promega, Madison, USA)

For DNA sequencing, the PCR-amplified products were gel-purified using the FavorPrep PCR Purification Kit (Cat. No. FAGCK 001, Favorgen, Taiwan) and sent along with the primer pair (ITS1 and ITS4) to the Macrogen DNA sequencing service in Korea. PCR products were directly sequenced in both directions using the respective forward and reverse primers. The obtained nucleotide sequences were aligned and compared with the sequences belonged to the *R. solani* isolates in the NCBI database using the Basic Local Alignment Search Tool (BLAST) (Zhang et al. 2012). Using the MEGA6 software, multiple alignments of the nucleotide sequences and construction of phylogenetic trees were performed using the neighbor-joining method (Tamura et al. 2013).

Pathogenicity of *F. solani* and *R. solani* to cucumber

Sterilized soil (1 kg/pot) was distributed in 14-cm diameter pots and *F. solani* and *R. solani* isolates separately grown on millet grains were added into the potting soil at 1% (*W*:*W*). The pots were watered and kept for 4 days before sowing. Seeds of cucumber were surface sterilized in 1% sodium hypochlorite solution for 2 min, then were rinsed in sterile distilled water, and sown in the pots (5 seeds/pot). Seeds were also sown in non-infested soil to serve as a control. Four replicates (pots) were established for each treatment, and the pots were randomly distributed in the greenhouse, where they were watered and fertilized as needed. The percentages of pre- and post-emergence damping off were determined after 20 days of sowing. The percentages of seed germination were calculated according to the following formula: seed germination (%) = (number of seeds germinated/total number of seeds) × 100.

Efficacy of *P. fluorescens* and *B. subtilis* as bio-control agents against *R. solani* and *F. solani* on cucumber

Preparation of fungal inoculums

Clean 250-ml flasks were filled with millet grains and autoclaved at 121 °C for 1 h for two successive days. Five-millimeter diameter discs from the margins of the fungal colonies (*R. solani* or *F. solani*) were added to the flasks. Flasks were incubated at 25 ± 2 °C for 2 weeks and

Table 1 Comparison of the generated sequence of the Iraqi *F. solani*, isolated from diseased cucumber roots, with those of *F. solani* isolates available in GenBank

Fungus	Isolate or strain name	Origin	The most similar sequences in Gen Bank database	
			GenBank accession number	Sequence similarity (%)
F. solani	–	Iraq	–	100
	ITS-5_ITS1	Iraq	KY662484.1	100
	ITS-1_ITS1	Iraq	KY662480.1	100
	FJCE	Mexico	KY013237.1	100
	Fs9 18S	India	KC156601.1	100
	FS8 18S	India	HQ265426.1	100
	FS1 18S	Ireland	HQ265419.1	100
	RFR1-4	China	KY432816.1	99
	G6	China	MF800959.1	99
	VGFS16-3	Canada	MF663682.1	99
	Cc_163	India	KM017142.1	99
	Fs2 18S	India	KC156594.1	99
	Fs2 18S	India	KC156594.1	99
	FS2 18S	Ireland	HQ265420.1	99
	FUS ITS 11 18S	India	HQ384397.1	99
	Y6	China	MH383181.1	99
	Y4	China	MH383179.1	99
	Y1	China	MH383176.1	99
	IGFRIWE9	India	MF171064.1	99
	Ifu05	India	MH015225.1	99
	GG2F6	India	KY419545.1	99
	XJL22	China	KY283800.1	99

F. solani isolated and identified in this study were shaken every 2 days to ensure uniform colonization of the fungus.

Bacterial isolation

Soil samples were collected from the rhizosphere of cucumber non-symptomized plants growing adjacent to the plants that are showing damping-off or wilt symptoms. One gram of each collected sample was suspended in 9 ml of sterile distilled water and serially diluted until getting the dilution of 10^{-7}. One milliliter of each sample was spread on a Petri dish plate containing nutrient agar medium, and all plates were incubated at 37 °C for 24 h. Individual bacterial colonies were picked up using a sterilized loop, transferred to nutrient agar plates, and incubated at 37 °C for 24 h. The developed single colonies were transferred to nutrient agar medium slants and pure cultures were stored in a refrigerator at 4 °C.

The isolated bacterial isolates were identified, using morphological (staining and motility), cultural (Nutrient agar, Cetrimide agar), and biochemical tests (IMIC test, triple sugar iron test, nitrate reduction test, catalase test, casein hydrolysis, oxidase test, starch hydrolysis, lipid hydrolysis, gelatin liquefaction, and carbohydrate tests).

In vitro evaluation of antifungal activity of the isolated bacterial isolates

The antagonistic effects of *P. fluorescens* and *B. subtilis* against *R. solani* and *F. solani* were evaluated in vitro. A streak of either *P. fluorescens* or *B. subtilis* was placed on PDA plates at 28 °C for 1 day; then a mycelial disc (0.5 cm) of either *R. solani* or *F. solani* was placed onto the center of each PDA plate. All plates were incubated at 28 °C until the fungal growth of the control plates reached the edge of the plate. The reduction of the fungal mycelial growths was calculated according to Fokemma (1973). Radial growth of *R. solani* and *F. solani* was recorded and inhibition percent of the growth was calculated according to the following formula:

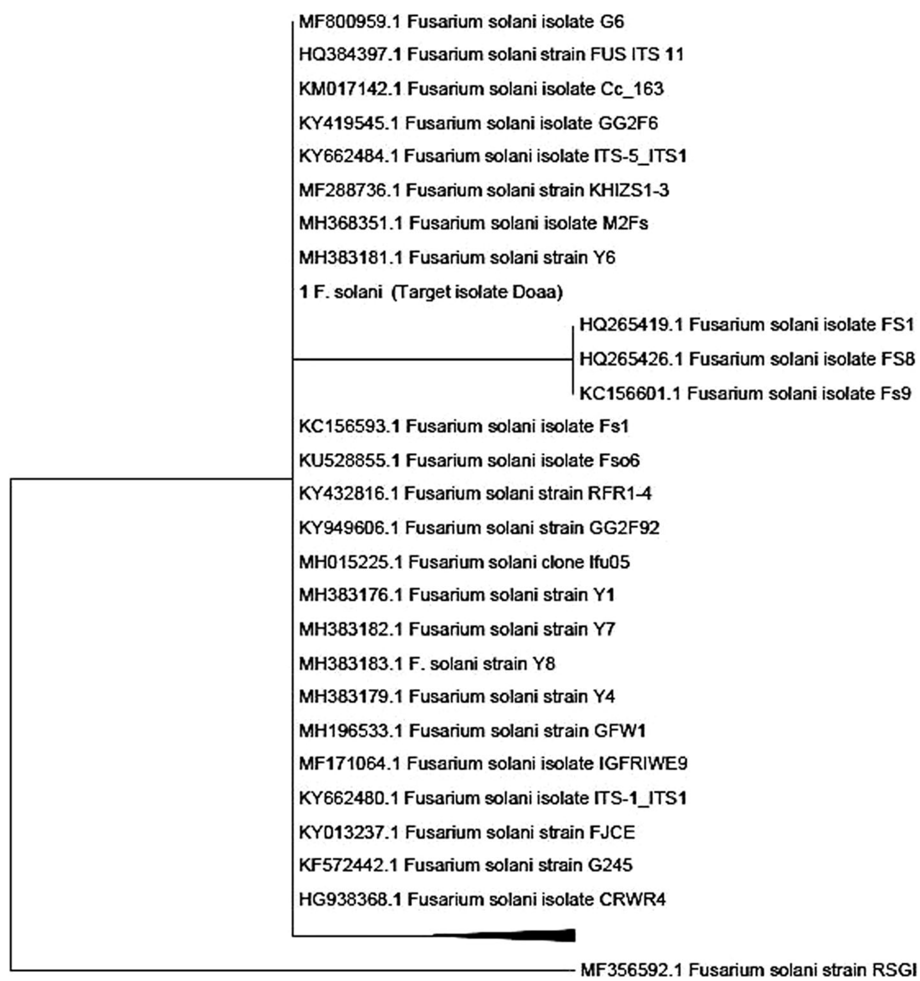

Fig. 3 A phylogenetic tree, generated using the neighbor-joining method showing the genetic relationship between the Iraqi *F. solani* isolate (indicated as Doaa) and other *F. solani* isolates available in GenBank (NCBI)

Table 2 Comparison of the similarity percentages of *R. solani* isolated from diseased cucumber plants, with the other isolates of the same fungus previously registered in GenBank

Fungus	Isolate or strain name	Origin	The most similar sequences in Gen Bank database	
			GenBank accession number	Sequence similarity (%)
R. solani	Doaa*	Iraq	MK105921	100
	Muntadher	Iraq	KX828173.1	97
	IQ34	Iraq	KF372660.1	96
	FJR7	Pakistan	MF716663.1	93
	IQ40	Iraq	KF372662.1	93
	IQ49	Iraq	KF372653.1	93
	IQ35	Iraq	KF372646.1	93
	IQ23	Iraq	KF372645.1	93
	MML4001	India	JX535004.1	93
	RsolTeaIN1	India	KJ466117.1	93
	IQ30	Iraq	KF372657.1	93
	Babylon	Iraq	KY283953.1	91
	Amer	Iraq	MF497741.1	91
	RUPP93 18S	India	JF701784.1	90
	RKNM3 18S	India	KC997793.1	89
	RDLM6	India	JF701717.1	88
	RKNG9	India	JF701745.1	88
	3629	Costa Rica	JX294325.1	88
	R6	Brazil	KY810804.1	88
	R5	Brazil	KY810803.1	88

**R. solani* isolated in this study and registered in GenBank

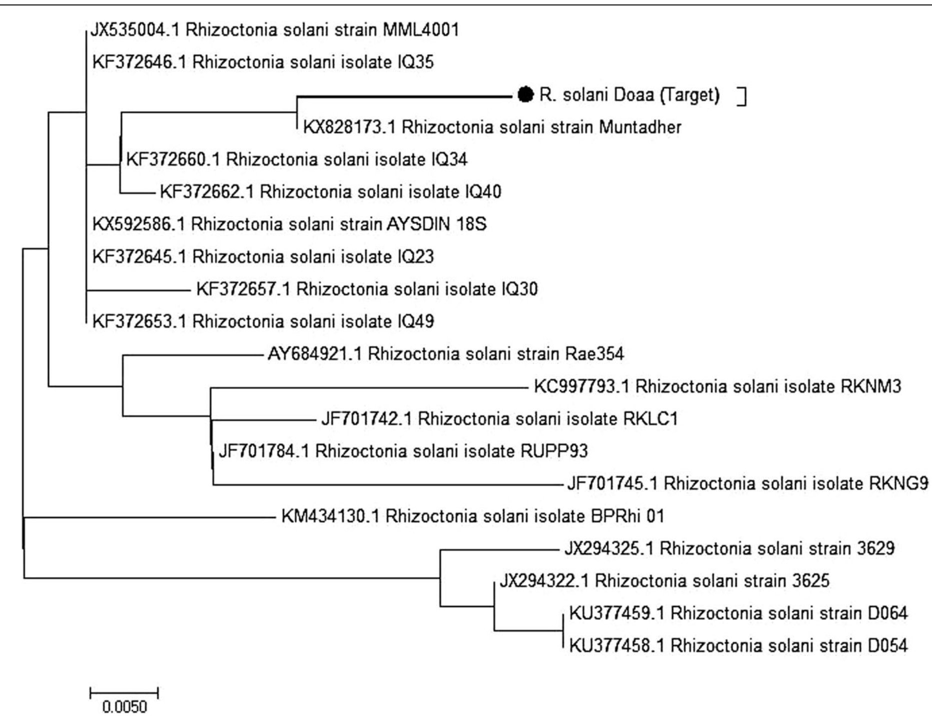

Fig. 4 A phylogenetic tree, generated using the neighbor-joining method showing the genetic relationship among the Iraqi *F. solani* isolate (indicated by black dot ●), with those of other *R. solani* isolates available in GenBank (NCBI)

Table 3 Effects of *R. solani* and *F. solani* on seed rot, and seedlings damping-off disease of cucumber on PDA

Fungal isolate	Seed rot (%)	Seedling damping off (%)
R. solani	100	0.0
F. solani	20.0	100
Control	0.0	0.0
$L.S.D_{0.05}$	16.31	23.07

growth reduction (%) = [(growth in control – growth in treatment)/growth in control] × 100

Effect of *B. subtilis* and *P. fluorescens* on cucumber seed germination and damping-off disease in pots

A pot experiment was designed using small pots containing reasonable weight (300 g.) of sterilized soil (sandy loam). *R. solani* and *F. solani* grown on millet grains were mixed with the potting soil at 1% *W*:*W*. After 3 days, bacterial suspensions containing 1×10^9 CFU/ml of the tested bacterial isolates were also added, and the infested pots were irrigated for 5 days before sowing. Ten cucumber seeds were sown in each pot with 3 replicates (pots) for each treatment in completely randomized design (CRD). The experiment included 10 treatments namely non-infested soil (control), soil treated with *R. solani* only, soil treated with *F. solani* only, soil treated with *B. subtilis* only, soil treated with *P. fluorescens* only, soil treated with *R. solani* + *B. subtilis*, soil treated with *R. solani* + *P. fluorescens*, soil treated with *F. solani* + *B. subtilis*, and soil treated with *F. solani* + *P. fluorescens*. Pots were kept under greenhouse conditions till the end of the experiment (3 weeks of sowing). The percentage of seed germination and damping off of cucumber seedlings were determined at the end of the experiment.

Results and discussion

Isolation and identification of *F. solani* and *R. solani*

Results of the cultural and morphological characteristics showed that *R. solani*, isolated from diseased cucumber plants, showed slightly melanized hyphae and irregularly shaped and brownish sclerotia. Moreover, microscopic observation showed that the hyphae branch at a 90° angle and constriction of hyphae and formation of septa at a short distance from the point of the hyphal branches' origins and absence of clamp connection, conidia, and rhizomorphs (Fig. 1). Similar results concerning morphological characters of *R. solani* were reported by several researchers (Moni et al., 2016; Desvani et al., 2018).

F. solani isolated from the infected cucumber roots produced sparse to abundant, white creamy mycelium (Fig. 1). Macroconidia are sickle-shaped with a slightly blunted apical end blunt and have 3 to 4 septa on average. Microconidia are abundant, oval to kidney-shaped, and formed in false heads on very long monophialides (Ke et al. 2016).

For confirmation of the morphological identification of *F. solani* and *R. solani*, PCR amplification of DNAs extracted from these isolates showed the possibility of amplifying PCR products with sizes ranging between 600 and 650 bp using the ITS1–ITS4 primers (Fig. 2).

The PCR-amplified ITS region (ITS1, 5.8S rDNA, and ITS4) of each *F. solani* and *R. solani* isolates were sequenced and the generated nucleotide sequences were subjected to a BLAST search. Molecular identification results confirmed the morphological identification of the tested isolates. The comparison of the whole ITS region (ITS1, 5.8S rDNA, and ITS4) of the *F. solani* isolate with those previously deposited in the GenBank revealed that the nearest genetic similarity (100%) of the generated ITS sequence was with *F. solani* from Iraq (KY662484.1 and KY662480.1) (Table 1). As well, close phylogenetic relationships also appeared with some *F. solani* isolates from India (MF800959, HQ384397.1, KM017142, and KY419545.1) (Fig. 3).

Comparison of the sequence obtained from *R. solani* with the other *R. solani* isolates deposited in GenBank showed that the highest genetic similarity was 97 and

Fig. 5 Effect of the Iraqi *R. solani* and *F. solani* isolates on seed germination and seedling damping-off disease in cucumber

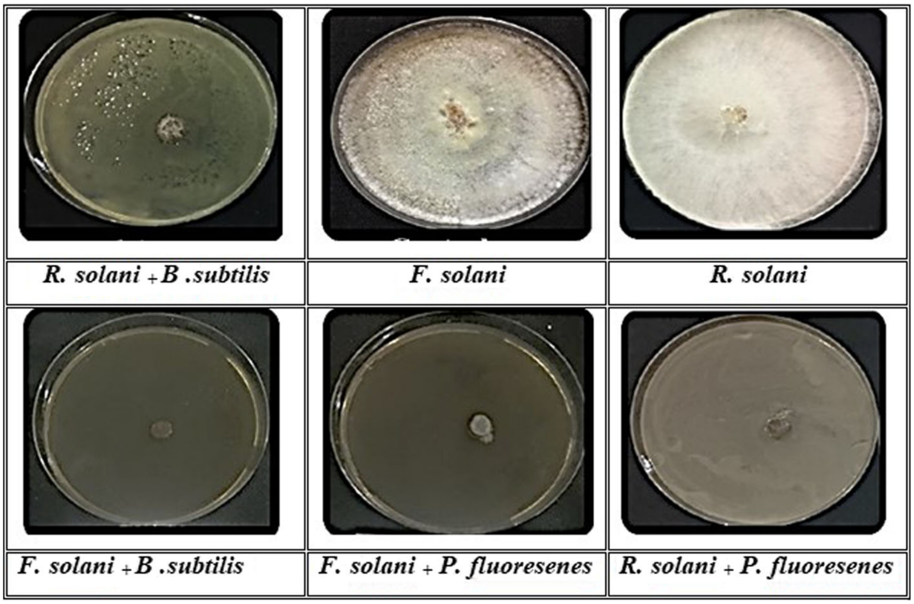

Fig. 6 Effect of *P. fluorescens* and *B. subtilis* on the inhibition of the radial growth of *R. solani* and *F. solani*

96% with the *R. solani* isolates previously identified in Iraq (KX828173.1 and KF372660.1, respectively). Minimum nucleotide sequence similarity (88%) for this *R. solani* isolate was observed with *R. solani* isolates identified in India (JF701717.1 and JF701745.1), Costa Rica (JX294325.1), and Brazil (KY810804.1 and KY810803.1). The *R. solani* isolate identified in this study also showed genetic differences ranged 89–93% with the other *R. solani* isolates formerly identified and deposited in NCBI (Table 2 and Fig. 4).

Results of the nucleotide sequence analysis using BLAST demonstrated that *R. solani* were genetically different from the other isolates and not previously registered in GenBank; therefore, it was recorded in GenBank under the accession number MK105921. This newly identified *R. solani* isolate may be more dangerous and devastating for economic crops. Polymerase chain reaction (PCR) technology was used in this study to diagnose the isolates of *F. solani* and *R. solani* due to its high accuracy in the diagnosis of many organisms, including pathogenic and non-pathogenic fungi such as *F. solani*, *R. solani*, *Alternaria alternata*, and *Aspergillus* spp. (AL-Abedy et al. 2018; Khan et al. 2018).

Pathogenicity of *R. solani* and *F. solani*

R. solani and *F. solani* isolated in this study from infected cucumber roots were found to be pathogenic and had the ability to infect cucumber seedlings 10 days after inoculation (Table 3 and Fig. 5). The tested *F. solani* and *R. solani* isolates caused 20 and 100% seed rot and 100.0 and 0.0% seedling damping off, respectively, compared to the control treatments. These results are in agreement with previous ones reported that *R. solani* and *F. solani* are highly pathogenic fungi causing a significant reduction in seed germination of many vegetable crops including cucumber (Al-Fadhal et al. 2018). Variability in seed rot percentages caused by *R. solani* and *F. solani* may be due to the differences in their virulence, the speed of growth, the nature of parasitism, and the sensitivity of the plant species for these pathogenic fungi (Desvani et al. 2018). *R. solani* produces a number of enzymes such as cutinase, cellulose, and protease, which have a significant effect on seed germination (Karima and Nadia 2012).

Antagonistic activities of *P. fluorescens* or *B. subtillis* against *R. solani* and *F. solani*

In vitro investigations showed that *B. subtilis* and *P. fluorescens* showed a high reduction of the radial

Table 4 Effect of *B. subtilis* and *P. fluorescens* on damping-off disease caused by *F. solani* and *R. solani* in pots

Treatment	% seed germination	% seedling damping off
F. solani	96.60	93.30
R. solani	96.60	68.50
P. fluorescens + F. solani	84.50	20.42
P. fluorescens + R. solani	80.55	19.44
B. subtilis + F. solani	100	0.00
B. subtilis + R. solani	100	0.00
B. subtilis	100	0.00
P. fluorescens	98.00	0.00
Control	100.0	0.00
$L.S.D_{0.05}$	7.806	8.401

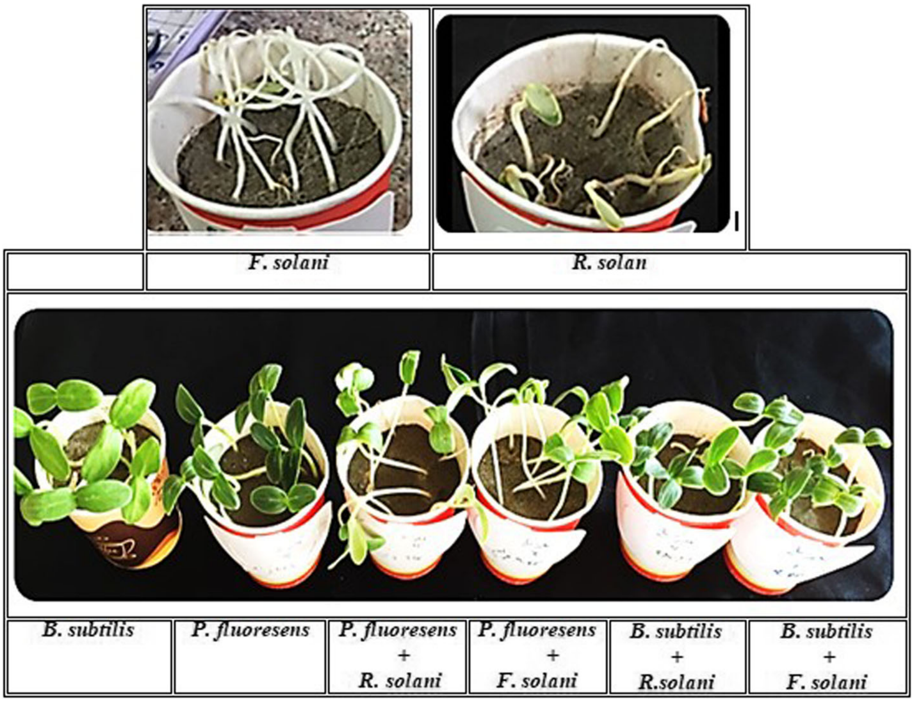

Fig. 7 Effect of *B. subtilis* and *P. fluorescens* on damping-off disease caused by *F. solani* and *R. solani* in the pots

growth of both *R. solani* and *F. solani* (100% growth inhibition) (Fig. 6). The potentialities of the bacterial species used in this study could be attributed to their ability to secrete hydrolytic enzymes or antifungal metabolites. As reported by Montealegre et al. (2003), *B. subtilis* can secrete several antifungal metabolites such as bacitracin, subtilin, bacillin, and bacillomycin which have an inhibitory effect on many fungal pathogens. Sarhan et al. (2001) also indicated that *B. subtilis* inhibited the mycelial growth of *F. solani*. It was also found that *P. fluorescens* is able to secrete antifungal metabolites, e.g., lipopeptide cyclic as well as several hydrolytic enzymes such as chitinase, endochitinase, β-1, 4 glucanase, β-1,3 glucanase, lipase, and protease which have an inhibitory effect on fungal pathogens (Saad 2006).

In the pot experiments, it was found that *B. subtilis* and *P. fluorescence* were very effective in reducing the severity of damping-off disease of cucumber seedlings caused by *F. solani* or *R. solani* (Table 4 and Fig. 7). The production of antifungal metabolites is considered as the main mechanism of antifungal activity of *P. fluorescens* and *B. subtilis* against *F. solani* and *R. solani* (Karkachi et al. 2010). Manikandan et al. (2010) reported that *P. fluorescence* is an effective biocontrol agent in controlling several plant pathogens such as *R. solani*, *F. solani*, and *P. fluorescens*. It also has the ability to suppress fungal root diseases through different mechanisms including production of antibiotics, bio-surfactants, toxins or lytic enzymes, induction of systemic resistance, and competition for colonization sites, minerals, and nutrients (Erdogan and Benlioglu 2010). Besides, it also possesses some of the other mechanisms such as antibiotic production and spore formation and the production of siderophore (Nielsen et al., 1998). The inhibitory effect of *P. fluorescens* and *B. subtilis* against the phytopathogenic fungi might be due to the production of hydrolytic enzymes that can degrade cell walls, several cyclic lipodepsipeptides, and iron-chelating siderophores (Kim et al. 2008). Mansoori et al. (2013) reported that *P. fluorescens* isolates can superimposed the *Bacillus* isolates in reducing wilt disease of cotton. This could be due to the various antagonistic mechanisms of *P. fluorescens* such as antibiosis, siderophore production, hormone production, and inducing systemic resistance in host plants.

Conclusion

B. subtilis and *P. fluorescens* isolated in this study shown their potentiality in reduction of the damping off of cucumber seedlings. In addition, future studies may be required to determine the antagonistic activity of *B. subtilis* and *P. fluorescens* under greenhouse and field conditions as well as the antagonistic effects on other diseases of economic importance in Iraq.

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Authors' contributions
All authors were equally contributed to this work by designing, conducting, and analyzing all data reported in this manuscript. The final manuscript was written, read, and approved by all the authors.

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Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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