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Bioactivity of Bacteria *Bacillus subtilis* and *Pseudomonas fluorescens against Rhizoctonia solani* and *Fusarium solani*, which causes seed rot, seedling on cucumber in greenhouses

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Abstract:

The research contracts with the study of the antagonistic effect of microbe against cucumber disease caused by *Rhizoctonia solani and Fusarium solani*. -- *Bacillus subtilis* and *Pseudomonas fluorescens* were isolated from cucumber field soil. Some of the fungi diagnosing that cause the death of cucumber seeds in the greenhouse in Najaf Governorate. The results showed identification of *R. solani* and *F. solani* was carried out using the PCR technique,with forefronts and back-fronts primers in a range of about 600 pairs to a nitrogen base (Base paris,bp). The analysis showed a new isolation of *Rhizoctonia solani* in the world according to NCBI. The bacteria isolated from the greenhousein some areas of Najaf Governorate.Results showed that *Bacillus subtilis* and *Pseudomonas fluorescens* were used as antifungal agents. Both of them have high resistance against *R. solani* and *F. solani*. Both bacteria *P. fluorescens* and *B. subtilis* have inhibited the growth of pathogenic fungus by 71.41% especially dilution $^{1-}$10..the concentration 20, 25% of bacteria *B. subtilis* showed 100% inhibiting the growth of *R.solani*. As well as the concentration 25% of *P. fluorescens* showed 100% inhibiting the growth of *F. solani*.

Keyword: *Rhizoctonia solani, Fusarium solani,Bacillus subtilis, Pseudomonas fluorescens,* Cucumber

Introduction:

Cucumber has been infected by a large group of pathogens causes of root rot, seed decay and damping, such as *Pythium* spp., *Fusarium solani*, *Phytophthora* spp., *Rhizoctonia solani*, the root rot and seedling disease are the major problems in the field. Many of them have a large family range and most of them have the ability to resist the environmental conditions and can survive in soil and plant part for long time (Agrios, 2007).Classification techniques based on molecular biology have

donated to their accuracy, sensitivity,and ability to detect and study genetic differences and to eliminate the disadvantages of traditional methods in the diagnosis of fungi. Polymerase Chain Reaction (PCR) one of the important techniques and the rapid detection and diagnosis of many living organisms, including microorganisms that include plant fungi (Ramasamy, 2015).The use of biocontrol agents as an alternative to fungicides is growing rapidly in modern agriculture due to the increasing influence of pesticides (Balabaskar and Meera, 2012). The most plentiful bacteria among these organisms and the bacteria that colonize an area around the roots are classified according to their effects in the plant and their interaction with the roots may be pathogenic pathogens or beneficial and stimulating effects of the species and useful species of bacteria such as *Azospirllum, Pseudomonas, Azotobacter, Klebsiella, Enterobacter alcaligenes, Serratia, Bacillus, Burkholderia,* and *Arthrobacter* were stimulating and encouraging plant species. These bacteria were called plant growth stimulating Rhizobacteria (Pieterse *et al*., 2003). Therefore, the study aimed to test the effect of some isolated biocontrol agents from the greenhouses and the roots of the cucumber plant from several areas in Najaf Governorate in case they were added separately or together in the control of rotting seed disease and the death of the cucumber seedlings of *R. solani* and *F. solani*.

Materials and methods:

Fungal pathogenic isolation

Isolation, purification, and diagnosis of fungus causing seed rot disease and the death of cucumber seedlings from the soil of protected crops in some areas of Najaf Governorate.

The fungus that causes seed rot and cucumber seed was isolated from seeds, roots and crown area of cucumber plants by bringing random samples collected from some greenhouses , cucumber cultivars in plant pathology. The infected parts were washed with running water to remove dust and impurities and then sterilized with sodium hypochlorite solution 10% for two minutes, the affected parts were washed with sterile water and then placed on sterile filter paper to remove the free water. After that, the plant parts (roots, seedlings) were cut into small pieces of 0.5-1 cm long and planted in Petri dishes, P.D.A sterile and incubated in the incubator at a temperature of (25 ± 2) ºC and after the growth of fungi was purified and diagnosed, based on the categorical classification (Summerell,2006). fungus's DNA had been individually

extracted and send them to macrogen(south Korean company) for For the purpose of determining the sequence of nitrogenous bases of nuclear acids multiplication of fungal isolates to diagnose isolated mushroom by using the database available at the national center for biotechnology information By a software program named (Basic Local Alignment Search Tool)(Zhwng and eat ,2000) .

Isolation of *P. fluorescence* and *B. subtilis* species associated with cucumber roots and testing their antagonistic ability with each other:

samples were collected from the soil of protected crops surrounding the roots of cucumber plants from different regions of Najaf governorate. 1 g of soil was taken on dry weight basis and each site was isolated and passed through a series of holes in each tube containing 9 ml distilled and sterilized water of the tube after well-ventured to the other tube until the dilution was obtained 10^7, then took 1 ml of this dilution and transferred to a petri dish and then added 20 ml of Nutrient Agar (NA) by 5 replicates. Then stirred the dishes to move the movement of the homogeneity of the suspension with the food medium, and then incubated dishes upside down under the temperature of 28 o for a period of 2-3 days and examined the dishes. The isolates were activated using the activation medium (NB). The bacteria were identified using bio06chemical tests in the Ministry of Science and Technology of Baghdad - Agricultural Research Department - Department of Biotechnologies. The work was on the middle lines winding each in the center of half the dish by taking a swab of the bacterial colony by loop and the work of zigzag line of bacteria in the middle half of the dish and the other half also worked a zigzag line of other bacteria and then incubated at 28 ° C for 24 hours .

Propagation and retention of fungi inoculums isolated from cucumber seeds:

The local millet seeds (*Panicum miliacem* L.) “Proso” Millet were used after being soaked with water and sterilized with autoclave to preparing fungal inoculums. Each flask was individually inoculated by placing 5disc of 0.5 cm diameter for each of the fungi selected for study., flasks were incubated at a temperature of(25 ± 2) ° C for 10 days with shaking every 3 days to ensure ventilation and fungus distribution on all seeds (Dewan, 1989).

Preparation of filtrates form isolated fungi on the P.D.B. medium:

The filtrate of fungi samples used in this study were prepared using Potatoes Dextrose Broth (PDB) . After sterilization of the medium, we inoculated many flasks contains 150 ml of medium with 1 cm diameter of 7-days growth fungi and then incubate for 28 days in the incubator under (25 ± 2) ° C with shaking every 3-4 days, and after 28 days the leachate was collecting and used according to experience. Many flasks was used as a control without inoculation. (Al amari,2011)

Preparation of inoculums and filtrates for *P. fluorescens* and *B.subtilis*:

The bacterial inoculums and filtrates was prepared separately using N.B medium using flasks of 250 ml. Each flask was inoculated with bacterial isolates. The flasks were incubated at (28 ± 2)° C for 48 hours. The filtrates was sterilized using the Millipore filter. The colony formation unit (CFU) was calculated for each bacterial .

Effect of *Rhizoctonia solani* and *Fusarium solani* on seed germination and growth of cucumber seedlings in Petri dishes:

This experiment was carried out by sowing the sterilized cucumber seeds with a sodium hypochlorite solution 10% from stock solution of 6% for 2 minutes then washed with distilled water and planted in petri dishes contain 20 ml of sterile P.D.A, used 10 seeds / dish , the Petridis was inoculated with *R. soalni* and *F. solani* separately at the age of 7 days, with 3 replicates. The seeds were incubated at a temperature of (25 ± 2) ° C. The results was taken after 10 days, by using the following equations:

Percentage of germination= Number of seeds growth/ Total number of seeds* 100

Percentage of rotting seeds= Number of rotting seeds/ Total number of seeds*100

Percentage of dead seedling= Number of dead seedlings/ Total number of seedlings*100

Effect of crud filtrates of *R. solani* and *F. solani* in seed germination and growth of cucumber seedlings in Petri dishes:

Many flask size 250 ml contain 150 ml of sterile (P.D.B.) inoculated with a disc of 0.5 cm of *R. solani* and *F. solani* and leave one as a control, then incubated under(25 ± 2)° C for 28 days with shakings every 3 days. After the incubation period, fungi filtrates were filtered through .

sterile buechner then transferred to the Millipore filtration filter with a 0.20 µm diameter, Under sterile conditions. Ten of sterilized cucumber seeds were planted in each sterilized dish containing a sterilizing filter paper waited with 6 ml of crud filtrate form each isolating fungi and 6 ml of the non-fertilized food medium of each fungi as control. Dishes were incubated under (25 ± 2)° C for 10 days, (Al Ameri, 2011). The percentage of seeds grown, rotting seeds, dead shoots and healthy shoots were calculated according to the equations mentioned previously.

Effective concentration of *P. fluorescens* and *B. subtilis* inoculums which inhibiting the growth of *R.solani* and *F. solani* in petridishes:

Ten test tubes containing 9 ml distilled water were used in this study, 1 ml of bacterial suspension were added to the first tube and then 1 mL from first tube was added to the second tube to obtain a series of dilutions ($^{10-}$ 10------ $^{1-}$10) Many dishes containing PDA free off antibiotic inoculated with 1 ml / dish of each dilution of the bacterial suspension and placed on PDA at the age of five days , three dishes of fungi were left as control using 1 ml of distilled water. The dishes were incubated at (25 ± 2) ° C, results were taken according to the percentage of inhibition using Abbot equation (Shaaban and the sailor, 1993).

% inhibition= The rate of fungal growth in comparison - the rate of fungal growth in treatment / The rate of fungal growth in control *100

Effect of crud bacterial filtrates in inhibiting the growth of pathogenic fungi:

This experiment was carried out by adding to petridishes (5, 10, 15, 20, 25%) of the sterile crud bacterial filtrates using millipore of 0.20 µm diameter under sterile conditions for each type of bacteria, then 20 ml of PDA were added to the dishes . Dishes were inoculated with a disc of *R.solani* and *F. solani* at the age of 7 days, distilled water were adding in the same concentrations above as control, the dishes were incubated at a temperature of (25 ± 2) ° C. The percentage of inhibition was calculated according to previously equation.

Effect of *R. solani* and *F. solani* on seed germination and growth of cucumber seedlings in plastic pots:

The millet seeds (*Panicum millaceum* L.) was used for growth of *R. solani* and *F. solani*.

Soil was autoclaved for one hour, then left for 24 hours and then re-sterilized in the same manner (Dewan *et al*., 1994). One gram from millet seeds of each fungi

were added to 200 g of sterile soil and placed in 9 x 12 cm plastic pots. Control treatment used without adding fungi inoculums, all treatments sowing with 10 sterilized seeds per pot , the pots irrigated as soon as needing (Azerjawi, 2011). Percentage of germination of seeds and dead plants was calculated 5after 10 days of planting according to the equations mentioned previously.

Results and discussion:

Isolation and diagnosis of fungi causing seeds rot disease and the death of cucumber seedlings from the soil of protected crops in some areas of Najaf province

Results of this study from dead seedlings showed isolation of two pathogenic fungi, *F. solani* and *R. solani* isolated from seedlings that have been infected in the field. The isolation and diagnosis fungi that causes seeds rot disease and death of seedling plants were diagnosed in the laboratories of the plant protection Department, Faculty of Agriculture-Kufa University., and Karbala university. Polymerase Chain Reaction technique (PCR) was used to confirm diagnosis Figure (1).

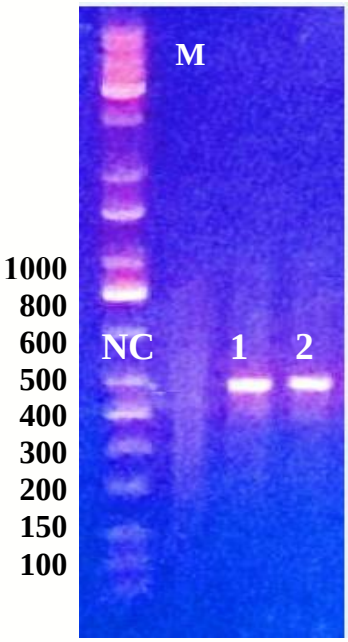

Figure (1): DNA multiplied by polymerase chain reaction (PCR) from *F. solani* (1) *R. solani* (2) and, M = DNA ladder marker. NC: Comparative treatment (without adding DNA to other PCR components).

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 R. solani*

Effect of fungi that causes *R.solani* and *F. solani* on seed germination and growth of cucumber seedlings in petri dishes:

The results of Table (1) showed that the treatment of *R. solani* led to 100% rotten seeds of cucumber plant compared to 20% of *F. solani* in 5 days and then increased to 100% after 10 days, compared to control of 100% germination which significantly different with other treatments. These results were justified by (Emmanual *et al*., 2010). The variability of the ratios between the complete rot of *R. solani and F. solani* fungi were due to the environmental conditions or the virulence of the isolation used in this study. *R. solani* was produce a number of enzymes such as Cutinase, Cellulose, and Protease, which have a significant effect on embryo, rotting and preventing seeds from germination (Alwan and Faris, 2010) . The high percentage of dead plants after seed rot of *F. solani* was due to the ability of this fungus to produce Metabolic substances such as toxins and enzymes (Kilot *et al*., 2009). The isolates were differ in their virulence ability and parasitism , since the highly pathogenic isolates could be covered seeds with their mycelium and did not allow them to germinate (Aboud *et al*., 2001).

Table (1) Effect of *R. solani* and *F. solani* on the germination and seeds rotten in Petri dishes.

Treatments	Germination	% Rotten seeds	Dead Seedling%
Control	**100**	**0.0**	**0.0**
Rhizoctonia solani	**0.0**	**100**	**0.0**
Fusarium solani	**80.0**	**20.0**	**100**
L.S.D=0.05	**16.31**	**16.31**	**23.07**

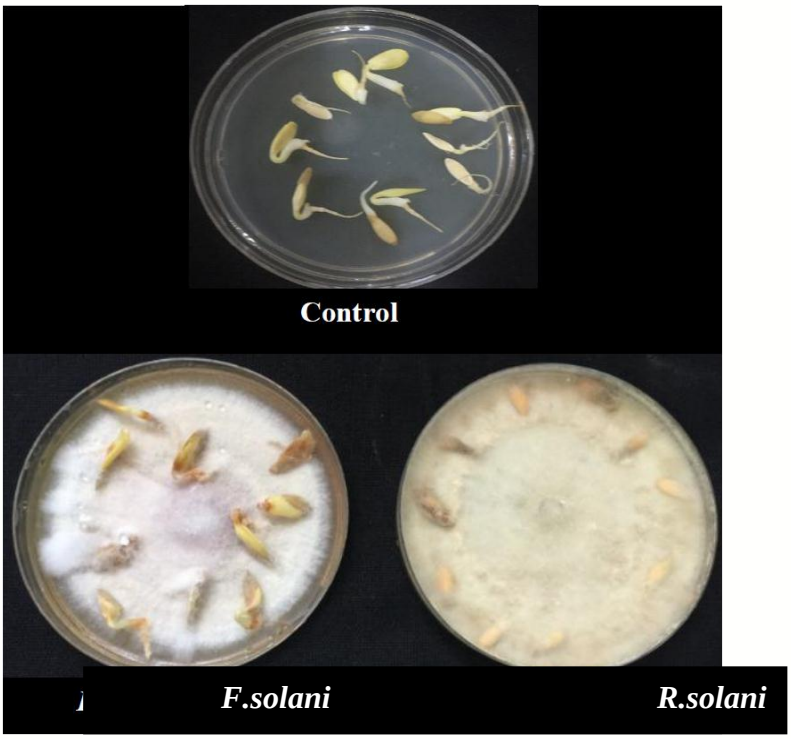

Image (1) shows the germination of cucumber seeds on the PDA medium in the treatments of control and *R. solani* and *F. solani*.

Crud filtrates effect of *R. solani* and *F. solani* on germination of cucumber seeds in Petri dishes:

As shown in (Table 2) that the crud filtrates of *R. solani* and *F. solani* decreased germination of cucumber seeds germination on the filter paper in Petri dishes, where the germination rate was 0.00 and 10%, respectively, compared to 96.66% of control . The percentage of rotting seeds was 100 and 90%, respectively, with highly significant differences to control of 3.33%, Figure (2). This was since the fungi filtrates have many toxins, in addition to many enzymes, like proximides and some fatty and amino acids, including salicylic acid, which caused the seeds rot and damage. The filtrates was not boiled before treated the seeds and this confirm the effect of enzymes as well as the results of many researchers, including (Dewan *et al*, 2007).

Table (2) Effect of crud filtrates of *R. solani* and *F. solani* on germination of cucumber seeds within 10 days *in vitro*.

Treatments	%	
	Germination	Rot seeds
Control	96.66	*3.33
R. solani	0.00	100
F. solani	10	90
L.S.D 0.05	2.106	21.06

* Each number in the table represents the rate of three replicates.

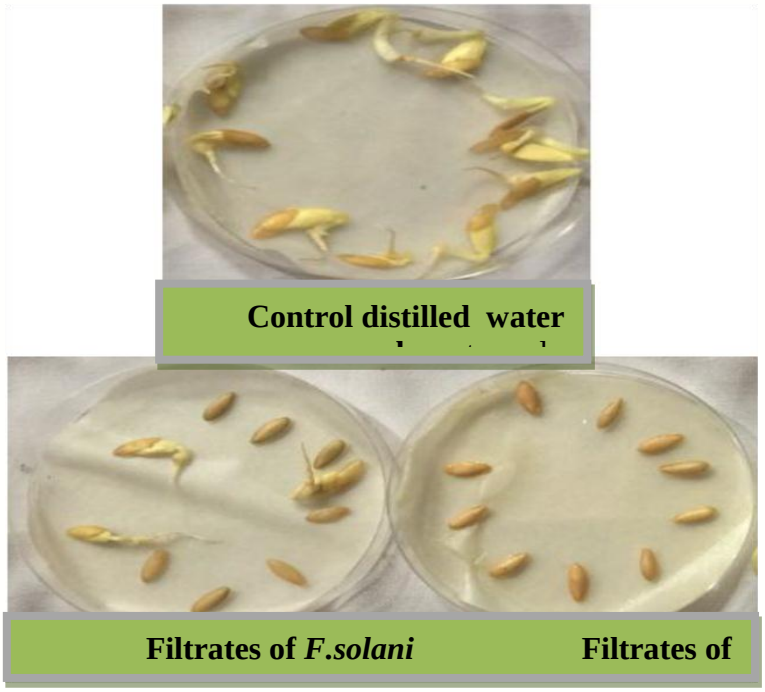

Image (2) Effect of crud filtrates of *R. solani* and *F. solani* on germination of cucumber seeds in Petri dishes and on PDA within 10 days.

Isolation, Diagnosis and Antagonisms of *B. subtilis* and *P. fluorescens* :

The antagonisms results of *B.subtilis* and *P. fluorescens* showed no antagonism between them. The growth of the above bacteria did not affect in the same dish. These results were encourage us for selection these bacteria in the biological control study of the fungi *R. solani* and *F. solani* used in the study (Al Janabi, 2017).

The active concentration of *P. fluorescens* and *B. subtilis* which inhibits the growth of *R. solani* and *F. solani* isolated from cucumber seedling :

The results showed in table (3) that the $^{1-}$10 x 1 suspension dilution of *B. subtilis* and *P. fluorecsens* inhibiting 100% the growth of above fungi . This dilution was differ significantly with all others except ($^{2-}$10 x1 dilution) of *F. solani*. The table

also showed that *R. solani* was not inhibited by the two type of the bacteria above with the (10^{-10} x 1) dilution .

The study of dilution on the percentage of inhibiting *R.solani* and *F. solani* by *B. subtilis* and *P. fluorecsens*, showed that the dilution 10^{-1} inhibited the growth of the above fungi 100% and significantly differ than other treatments .

The results showed also that *F. solani* was significantly inhibited more than *R. solani* according to percentage of inhibition 82.92% and 57.86% respectively at probability level of 0.05.

P. fluorecsens was significantly better than *B. subtilis* in inhibiting the growth of fungi above and the rate of percentage inhibition was 71.41% and 69.38% , respectively at probability level of 0.05. Pictures (3) and (4), and this may be return to the production of some antibiotics such as lipopeptide and amphisin and the production of some enzymes surrounding the walls of fungal cells such as endochitinase enzyme by *P. fluorecsens* (Waeli, 2004).

This result was consistent with many studies that demonstrated the efficacy of *P. fluorescens* isolates in the inhibition of *R. solani* on medium (Kazempour, 2004).

B. subtilis also has ability of producing metabolic and organic compounds , indol acetic acid , hydrogen cyanide and antibiotic (Hillel, 2005 ; Jafar , 2011) .

Table (3) Effect of different dilutions of *P.fluoresenes* and *B. subtilis* on the inhibition of *R. solani* and *F. solani* in the laboratory on the PDA medium.

Types of Bacteria	Treatments	% inhibition		% Dilution effect	% bacterial effect
		R. solani	*F. solani*		
Bacillus	1-1	100	100	100.00	69.38
	2-2	92	99.17		
	3-3	90.83	96.52	96.72	
	4-4	88.21	93.98		
	5-5	86.84	89.76	94.93	
	6-6	73.89	82.36		
	7-7	26.26	72.96	91.10	
	8-8	22.5	63.98		
	9-9	7.64	52.98	85.89	
	10-10	0	47.58		
Pseudomonas	1-1	100	100	73.02	71.41
	2-2	97	98.72		
	3-3	95.03	97.32	53.82	
	4-4	87.80	94.38		
	5-5	75.91	91.05	44.53	
	6-6	53.76	82.05		
	7-7	38.15	77.9	34.33	
	8-8	16.87	74.75		
	9-9	4.48	72.20	29.58	
	10-10	0	70.74		
Effect of fungi		57.86	82.92		
L.S.D=0.05	Bacteria=0.754 dilution = 1. 85 interaction 3.372 fungi = 0.754				

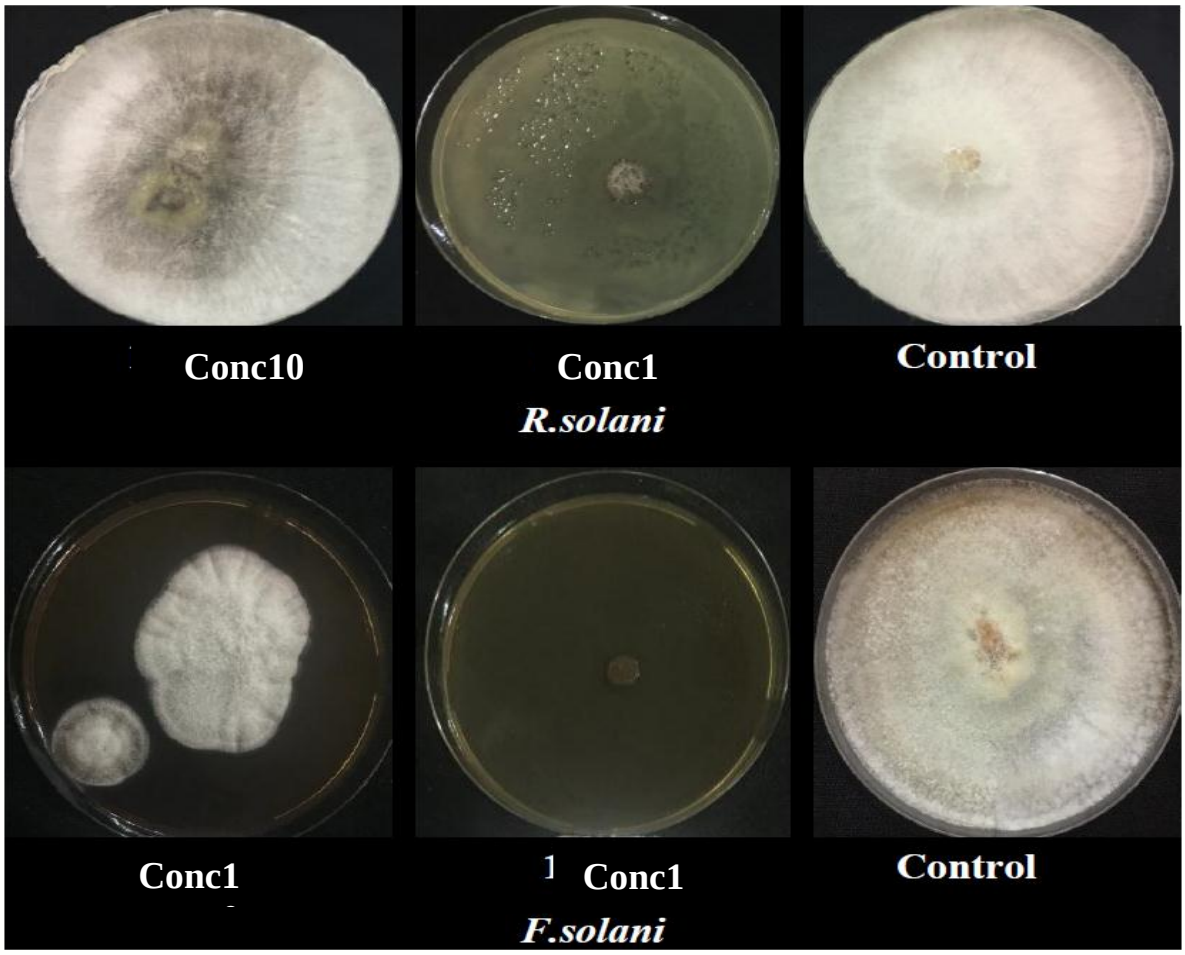

Image (3) Effects of different dilutions *B.subtilis* in inhibition of *R. solani* and *F. solani* in the laboratory on the PDA medium.

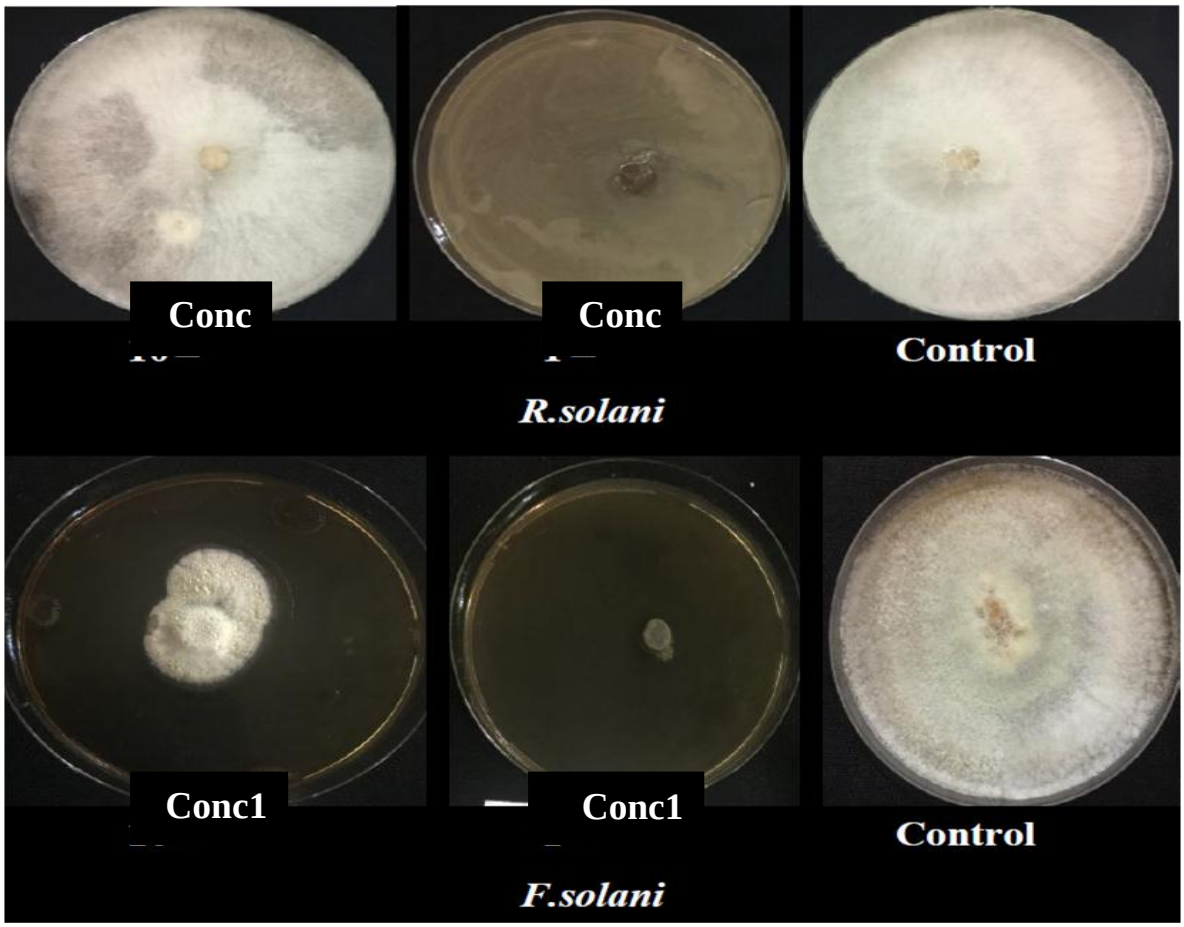

Image (4) Effects of different dilutions of *P. fluoresenes* in the inhibition of *R. solani and F. solani* in the laboratory on the PDA.

Effect of crud filtrates of *P. fluorescens* and *B. subtilis* in inhibiting the growth of *R. solani* after 48 hours of growth in Petri dishes:

The results in table (4) indicated that the concentration 20% and 25% of *P. fluorescens* and *B. subtilis* was significantly higher than other concentrations in inhibition of *R. solani* after 48 hours which reached to 100% . The same result of 100% inhibition got it in mean concentration .

P. fluorescents has exceeded significantly on *B. subtilis* in inhibition of *R. solani* by 90% compared with 77.91% , image (3) and (4) , and this was belong to the bacterial crud filtrates that contains an enzymes, toxins or phenolic compounds or alkaloids which effect on fungi growth (Akhtar *et al*., 2010). The mechanism of inhibition of *P. flourescens* is due to its production of inhibitory enzymes for many fungi such as Chitinase, B-1,4glucanase , protase, and this lead to antagonism action against *R. solani*. These results were agree with many studies that have demonstrated the efficiency of *P.flourescens* in inhibition of pathogenic *R. solani* (Jassem, 2007).

Table (4) Effect of crud filtrates of *P. fluorescens* and *B. subtilis* in inhibitiog of *R. solani* after 48 hours in Petri

%Concentration (Filtrate / medium)	% *R. solani* inhibition		% Mean concentration
	Bacillus	***Pseudomonas***	
5	**50.33**	**75.00**	**62.66**
10	**66.26**	**85.00**	**75.63**
15	**73.00**	**90.00**	**81.50**
20	**100.00**	**100.00**	**100.00**
25	**100.00**	**100.00**	**100.00**
% Bacteria effect	**77.91**	**90.00**	
L.S.D=0.05	**Concentration = 4.593, Bacteria = 2.905, Interaction = 6.495**		

Effect of *P. fluorescens* and *B. subtilis* in inhibiting the growth of *F. solani* after 6 days in Petri dishes:

The results of table (5) showed that the highest concentration 25% of *P. fluorescens* and *B. subtilis* was significantly higher than other concentrations in inhibition of *F. solani* which reached 100% and 96.30% respectively , while the effect of mean concentration of 25 % was the higher than other treatments.

P. fluorescens was also significantly superior to the *B. subtilis*, with 86.07% and 69.87% Respectively, image (5) and (6). This result came in line with many studies that demonstrated the efficacy of *P. fluorescens* in inhibition of pathogenic fungi *F. solani*, which caused the seedling diseases (Waeli, 2004).

Table (5) Effect of *P. fluorescens* and *B. subtilis* in inhibition of *F. solani* after 6 days in Petri dishes.

% Concentration (filtrate / medium)	**%** of ***F. solani*** Inhibition		**%** Mean concentration
	Bacillus	***Pseudomonas***	
5	**38.33**	**63.52**	**50.92**
10	**38.36**	**87.50**	**62.93**
15	**87.20**	**88.62**	**87.91**
20	**89.20**	**90.73**	**89.96**
25	**96.30**	**100.00**	**98.15**
%Effect of bacteria	**69.87**	**86.07**	
L.S.D=0.05	**Concentration = 1.811, Bacteria= 1.14, Interaction= 2.561**		

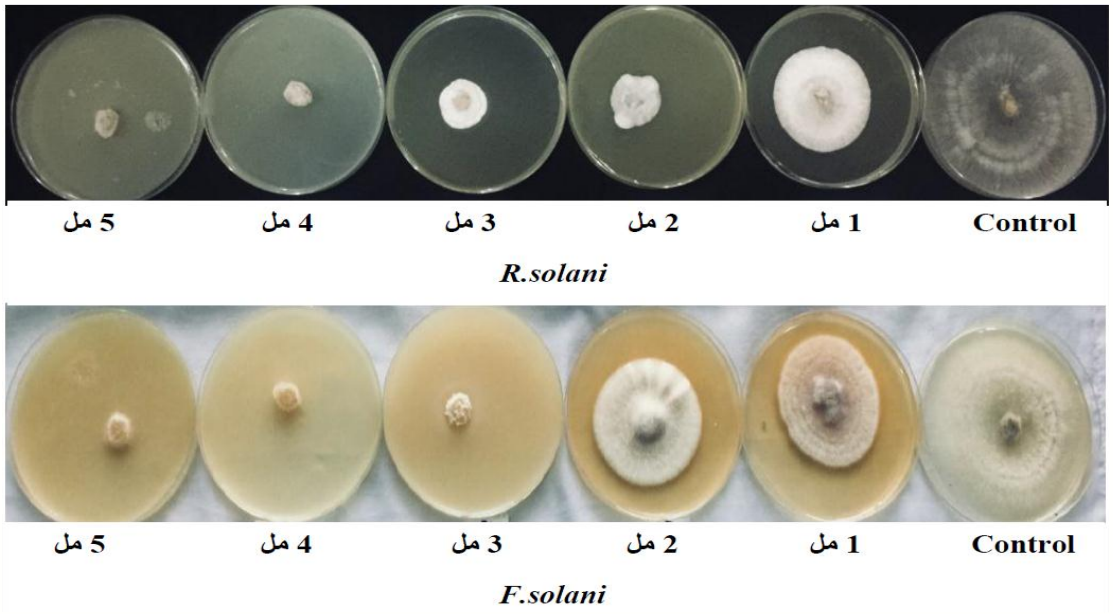

Image (5) Effect of crud filtrates of *B. subtilis* in inhibiting the growth of *R. solani* and *F. solani* in Petri dishes.

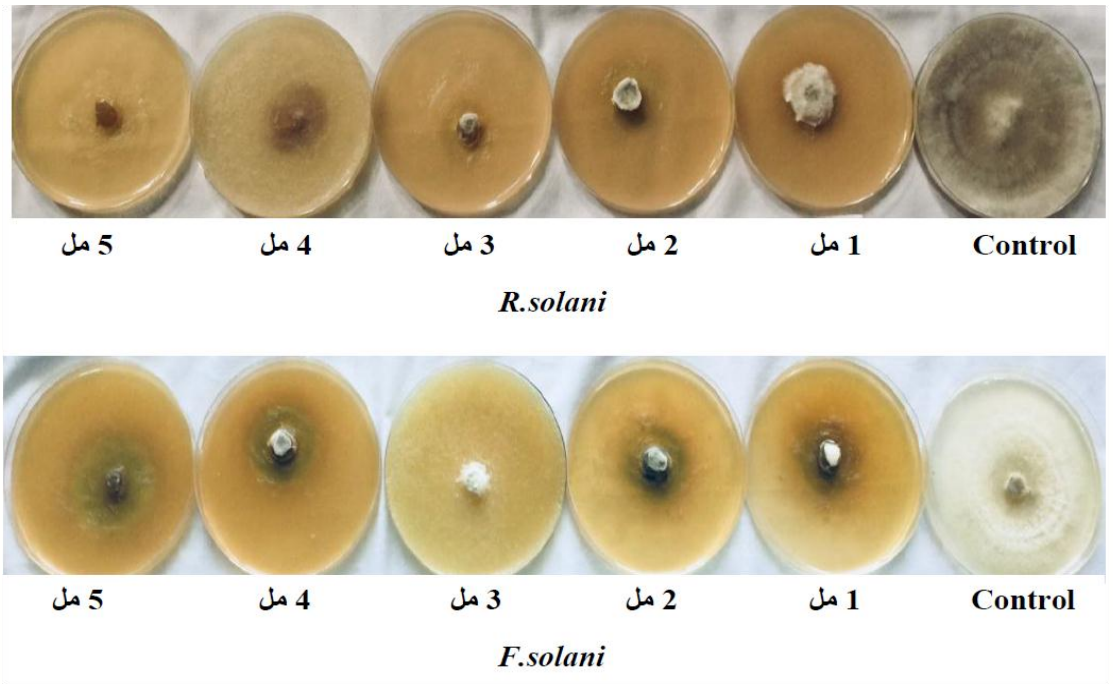

Image (6) Effect of crude filtrates of *P. fluorescens* in inhibiting *R. solani* and *F. solani* in Petri dishes.

Effect of *R. solani* and *F. solani* on seed germination and growth of cucumber seedlings in plastic pots at laboratory:

The results shown in table (6) that the percentage of seed germination after 5 days was 96.6 % and the fungi *R. solani* and *F. solani* does not have sever effect on germination .

After 10 days these two fungi had resulted in the death of cucumber seedlings, we found that *R. solani* was more effective than *F. solani* in the percentage of dead seedlings were 93.3% and 68.5% respectively compared to control of 0% dead seedling, image (7) , and this belong to the ability of *R. solani* to secrete the enzymes and toxic materials that lead to embryo death and germination failure according to (Weinhold *et al*., 1996).

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Table (6) Effect of *R. solani* and *F. solani* on germination and death of cucumber seedlings in pots at laboratory.

Treatments	%	
	Germination	dead seedlings
Control	100.0	0.0
Rhizoctonia solani	96.6	93.3
Fusarium solani	96.6	68.5
L.S.D=0.05	**9.42**	**34.30**

Image (7) Effect of *F. solani* and *R. solani* on germination and death of cucumber seedlings in the pots after 10 days.

In conclusion, studies were showed that *R solani* and *F.solani* isolated from seeds and roots of cucumber seeds were responsible for seed rot disease and seedling death of cucumber plant. The results indicated that a new strain of *R. solani* was registered at the National Center for Biotechnology Information (NCBI) at first time and was shown to have high pathogenicity for cucumber plant, 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. Further studies should be carried out to identify and separate the active substances in the metabolic products of the bacterial species used in this study .

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