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Identification and control the causal agent of root rot disease of cucumber in Iraq

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Abstract: This study aimed to isolate the causal agent of the root rot disease of cucumber and control it biologically. Samples were collected in the cucumber fields in the Diyala and Saladin governorates of Iraq. Isolation test demonstrated associate fungi belong to the twelve geniuses. Fusarium solani exhibited highly percentage of appearance of 82.15% with frequency 54.00%. Seventy-seven isolates identified as F. solani according to their cultural and morphological characteristics while sixty-five isolates of them amplified successfully with specific primer of Fusarium spp using PCR technique. Isolate DF13 was most virulent isolated while exhibited 0% cucumber seed germination in vitro. The bio-agent Bacillus pumilus demonstrated significant inhibition ability against the fungal isolate DF13 in vitro of 100%. Under greenhouse condition B. pumilus decreased the disease incidence and severity to 30.55% and 20.75% respectively compared to the negative control which was 80.50%, 55.00% respectively.

Key words: Fusarium solani, Root rot disease, PCR, Bacillus pumilus.

تشخيص ومكافحة مسبب مرض تعفن جذور محصول الخيار في العراق

صفاء نعمت حسين

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الملخص: تم رصد مرض تعفن الجذور في حقول محصول الخيار في محافظتي ديالى وصلاح الدين في العراق. أظهرت نتائج العزل وجود فطريات تعود الى اثني عشر جنس. اظهر الفطر Fusarium solani اعلى نسبة ظهور بلغت 82.15% وبتكرار 54.00%. سبعة وسبعون عزلة شخصت على اساس صفاتها المزرعية والمظهرية بانها تعود للفطر F. solani، وخمسة وستون عزلة منها تضاعفت ايجابياً مع البوادئ المتخصصة للأنواع التابعة لجنس الفطر Fusarium باتباع تقنية تفاعل البلمرة المتسلسل. العزلة DF13 كانت الاشد ضراوة إذ بلغت نسبة انبات بذور الخيار في معاملاتها 0% مختبرياً. حققت عزلة بكتريا المكافحة الحيوية Bacillus pumilus نسبة تثبيط معنوية ضد العزلة DF13 بلغت 100% مختبرياً. وتحت ظروف البيت الزجاجي حققت العزلة B. pumilus خفض معنوي في نسبة المرض وشدته اذ بلغت 30.55% و 20.75% على التوالي مقارنة مع معاملة السيطرة السالبة التي بلغت 80.50% و 55.00% على التوالي.

الكلمات المفتاحية: Fusarium solani، مرض تعفن الجذور، تفاعل البلمرة المتسلسل، Bacillus pumilus.

Introduction:

Root rot disease of cucumber (Cucumis sativus) is a prevalent disease causes highly economic losses of cucumber in Iraq and worldwide (Vakalounakis,1996). Cucumber is known to contain a large number of important nutrients, such as primarily vitamins A, B and C and various minerals such as manganese and copper (Szalay, 2017). Symptoms of the disease appears depending on the growth stage

of the plant which is interferes with environmental conditions such as high temperature and soil humidity also interferes with farming processes such as plowing and soil rotation before planting, adoption of agricultural cycle, control of regular irrigation during the season, selection of appropriate times for fertilization, planting density (Martyn, 1996). Typical symptoms appear on the shoot system of the plants as a form of wilt and yellowing of the lower leaves and present of brown spots on the crown area which is in contact with the soil surface, and during the fruit holding wilt and yellowing extends towards the top of the plant with drying lower leaves and finally death of the plants, symptoms in root system appear as a rot in the main and subsidiary roots (Ayed et al. 2007). Several fungal species reported to be causal agent of the root rot disease including Fusarim solani, Macrophomina phaseolina, Rhizctonia solani. Toussoun and Snyder (1961) mentioned that all of the cucurbits susceptible to the F. solani. Several spot in the world such as USA reported this disease and indicated that the fungus F. solani was the major causal agent (Snyder and Hansen, 1941). Two specific primers, ITS-Fu-f and ITS-Fu-r, were used by Abd-Elsalam et al. (2003) for identification of Fusarium spp.

MATERIALS AND METHODS

Isolation and Identification the fungi associated with samples

Samples from eight diseased cucumber fields were collected in the Diyala and Saladin governorates in April-May, 2018. Samples were cut into 0.5-1cm segments and washed under running tap water for 30 minute, then surface sterilized in 1% sodium hypochlorite for 2 minute and cultured on the Potato Dextrose Agar (PDA) supplemented with 200 mg\l amoxicillin and incubated at 25±1C° for 7 days, single spore technique was made for each isolate. Isolates were identified to the species level according to their cultural and microscopic morphological characteristics (Booth,1977 , Nirenberg and O'Donnell, 1998, Domsch et al. 2007). The percentage of the appearance and frequency of genera and species were calculated according to the formula below (Marasas et al.,2001):

Appearance (%) = (No. of samples of occurrence/ Total No. of samples) x 100

Frequency (%) = (No. of plant segments of species occurrence/Total No. of segments used) x 100

Pathogenicity test of the F. solani isolates

In vitro experiment was conducted to estimate the pathogenicity ability of the 77 *isolates of F. solani*. sterile cucumber seed (Beta-Alpha cultivar) planted in the petri plate contained PDA (10 seed/plate) and inoculated with 0.5 cm diameter disc of the fungal growth of 5 days age in the center of the plate, each treatment repeated 4 times and incubated at 25 ±1 C ° for 7 days. Percentage of seed germination was calculated according to the formula below (Al-Juboori et al., 2016):

Germination (%) = (No. of seed germinated/Total seed germinated) x 100

Molecular identification of the fungus Fusarium spp by PCR technique

Extract and purification of DNA

Seventy-seven isolates which primarily identified as *F. solani* were grown on the PDA and incubated at 25 ±1 C° for 7 days. Mycelium and spores for each isolate were harvested by using sterile glass rod and transferred into Eppendorf tubes 1.5 ml. DNA of the isolates were extracted according to the protocol described by Hussein and Juber (2014).

Primers preparation

Two specific primers for Fusarium genus, ITS-Fu-f and ITS-Fu-r which designed by Abd-Elsalam et al. (2003), produced by Bioneer company, Coria (Table 1).

Table (1) Primers used to identify Fusarium spp.

Primer	Sequence (5' → 3')	
TS-Fu-f		**5`-CAACTCCCAAACCCCTGTGA-3`**
ITS-Fu-r		**5`-GCGACGATTACCAGTAACGA-3`**

Primers were adjusted to obtain the concentration of 5 pmol/µl, and saved at -20 C° until uses.

Prepare the interactions of the Polymerase Chain Reaction (PCR).

Standard Kits of AccuPower PCR PreMix produced by Bioneer Company, Korea. Each Eppendorf tube consist of the reaction mixture (250 µM dATP,dCTP,DGTP,dTTP, 30 mM KCL, 1.5 mMMgCl2,1U Toq DNA polymeras, 10 Mm Tris-HCL (pH 9.0)). MgCl2 amended to 1.75 mM and 1.5 µl of the primers (TS-Fu-f and ITS-Fu-r) and 3 µl DNA of each sample individually/concentration of 50 ng/µl and 14 µl sterile distilled water, mixed by vortex, and the control prepared by adding 1.5 µl of the primer strands and 17 µl sterile distilled water to the interactions mixture. Eppendorf tubes loaded in to the polymer thermal (ThermoCycler) produced by Biocompare, USA, and program seated according to the method of Hussein (2016) with some modifications, the program included Initial denaturation heating at a temperature of 94 C° for two minutes to separate the double strand of the DNA to single strand, and 30 cycle included; Denaturation at 95 C° for one minute to copy the DNA strand which will be a template for the subsequent reactions, and Annealing at a 58 C° for one minute to linked the primer with DNA template, and Extension cycle at 72 C° for two minutes to elongate the primer which linked with the DNA template, and then final Extension cycle at a 72 C° for 7 minutes.

Replication assay

PCR amplifications products were size fractionated on the 1.5% agarose gel using 100 pb DNA ladder (Azura Genomics Inc.,USA). Replication results were conducted by exposing the agarose gel to the UV light at the wavelength 320 nm in the UV Transilluminator machine produced by Biocompare, USA.

Inhibition Test in vitro

Inhibition ability of the biological control agent of *B. pumilus* (Bp) (Obtained from the University of Al Mustansiriyah, Environmental Engineering department, Iraq), was tested against the *F. solani* isolate DF13 using plate poisoning technique on the PDA media according to the method described by Hussein (2018) with four replicates. Series of the bacterial inoculum concentration of 10^4,10^5,10^6,10^7,10^8 CFU/ml were tested. Inhibition percentage calculated according to the formula below:

Inhibition (%) = (Fungal growth diameter in control– fungal growth diameter in treatment) /Fungal growth diameter in control) ×100

Biological control of the root rot disease of cucumber

Under greenhouse conditions, plastic pots 15 cm diameter filled with 1 Kg sterile mixture of soil and compost, inoculum of the *F. solani* (DF13 isolate) prepared by adding disc (0.5 cm diameter) of the mycelial growth on the PDA age 5 days to the 1 Kg of disinfected Sorghum seed *(Sorghum bicolor*) and incubated for 14 days at 25 ±1 C°. 10 mg of the fungal inoculum added to the pots and irrigated with distilled sterile water, after three days disinfected cucumber seed (Beta-Alpha cultivar) planted as 5 seed/pot, and irrigated with distilled sterile water, 10 ml of the bioagent inoculum *B. pumilus* (Bp) grown on the nutrient broth with concentration of 10^7 CFU/ml added in the same time of the seed planting, each treatment repeated 4 times and control treatments irrigated with distilled sterile water. All the pots arranged in the greenhouse according to the completely randomized design. After 45 days disease incidence were calculated according to the formula below (Masood et al. 2010):

Disease Incidence (%) = (No. of infected plants/Total No. of plants assessed) x 100

And disease severity measured based on the disease rating scale described by (Hussein, 2014) and calculated according to the formulas below (McKinney, 1923):

Disease Severity (%) = ((No. of infected plants x their infected degree)/ (Total tested plants x higher infected degree)) x 100

Data were analyzed by estimated analysis of variance ANOVA using GenStat Discovery edition3 software.

Results

Isolation and identification of the pathogen

The results of the isolation showed that 15 species of the fungi were associated with the samples (Table 2). *The most* predominant fungus was *F. solani* which exhibited percentage of appearance and frequency of 82.15% and 54.00% respectively. *F. solani* showing white mycelium growth on the PDA, with brown pigment in the verse side of the plate (Fig. 1). Three types of the spores shown under compound microscope investigation were spindle to cylindrical shape of macroconidia divided to 4-7 cells by crosswise barriers and microconidia were oval, renal and spindle shape, some divided by crosswise barrier to two equal cells, produced from long monophilides growth laterally on the aerial mycelium and chlamydospore which produced as a single cell or pairs on the lateral small branch or formed in the middle of the hypha. These criteria agreed with what described by Booth (1977).

Table (2) Fungi associated with the cucumber plant samples

Fungus name	Appearance (%)	Frequency (%)
Alternaria alternata	19.11	**9.54**
Aspergillus flavus	17.00	**13.33**
A. niger	18.63	**12.66**
A. oryzae	8.66	**1.16**
Cladosporium cladosporioides	11.22	**1.33**
Curvularia lunata	15.25	**4.64**
Drechslera hawaiiensis	11.83	**6.00**
Eurotium berbanbrum	6.42	**1.00**
Fusarium oxysporum.	16.64	**9.66**
Fusarium solani.	82.15	**54.00**
Macrophomina phaseolina	10.12	**7.20**
Pencillium commune	16.33	**5.33**
Phoma pinodella	7.55	**1.66**
Tichoderma harzianum	7.55	**1.36**
Ulocladium atrum	9.71	**3.75**

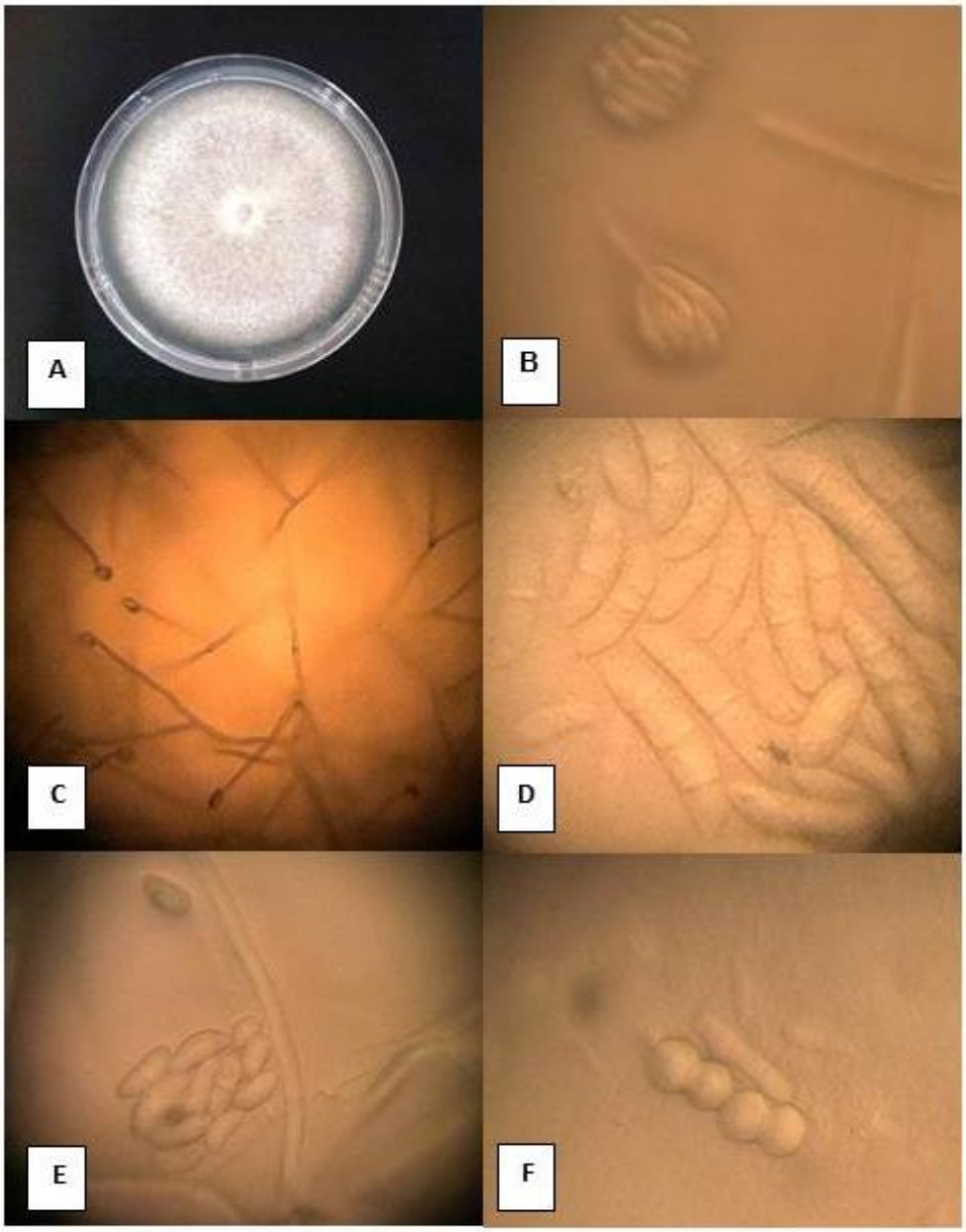

Fig (1) Cultural and microscopic characteristics of the fungus *F. solani*

(A) Growth of the fungal colony on the PDA (B) Long Monophialides conidiospher X40 (C) Long Monophialides conidiospher X10 (D) Macroconidia X40 (E) Microconidia X40 (F) Chlamydospore X40

Pathogenicity test of F. solani isolates

Results of the pathogenicity test showed variation in the pathogenicity ability between the isolates. The seed germination percentage ranged between 0.0% to 97.5% (Table 3), and the most virulent isolate was DF13 which exhibited 0.0% seed germination.

Table (3) Pathogenicity ability of the *F. solani* isolates on the cucumber seed germination

Isolate	Seed germination (%)	Isolate	Seed germination (%)	Isolate	Seed germination (%)
DF01	82.0	DF27	60.0	SF02	47.5
DF02	77.5	DF28	35.0	SF03	45.0
DF03	47.5	DF29	37.5	SF04	60.0
DF04	75.0	DF30	57.5	SF05	82.0
DF05	57.5	DF31	60.0	SF06	50.0
DF06	50.0	DF32	45.0	SF07	60.0
DF07	77.5	DF33	40.0	SF08	75.0
DF08	62.5	DF34	97.5	SF09	75.0
DF09	57.5	DF35	97.5	SF10	75.0
DF10	55.0	DF36	60.0	SF11	75.0
DF11	45.0	DF37	77.5	SF12	37.5
DF12	60.0	DF38	75.0	SF13	57.5
DF13	0.0	DF39	65.0	SF14	75.0
DF14	60.0	DF40	45.0	SF15	50.0
DF15	47.5	DF41	82.0	SF16	57.5
DF16	87.5	DF42	57.5	SF17	67.5
DF17	60.0	DF43	35.0	SF18	52.5
DF18	82.0	DF44	50.0	SF19	52.5
DF19	50.0	DF45	52.5	SF20	45.0
DF20	57.5	DF46	75.0	SF21	57.5
DF21	67.5	DF47	62.5	SF22	60.0
DF22	45.0	DF48	82.0	SF23	82.0
DF23	77.5	DF49	70.0	SF24	37.5
DF24	55.0	DF50	67.5	SF25	47.5
DF25	62.5	DF51	77.5	SF26	37.5
DF26	47.5	SF01	75.0		
L.S.D. (0.05) = 9.8					

Molecular identification of Fusarium spp by PCR technique.

The results showed that 65 isolates of the 77 *Fusarium solani* isolates were amplified positively with the specific primers of the Fusarium spp (Table 4). Bands showed up at the 398-400 bp (Figure 2), and these results agreed with Hussein (2019).

Table (4) Molecular identification of the Fusarium spp isolates

Isolate	Result of Amplification	Isolate	Result of Amplification	Isolate	Result of Amplification
DF01	+	DF27	-	SF02	+
DF02	+	DF28	-	SF03	+
DF03	-	DF29	+	SF04	+
DF04	+	DF30	-	SF05	+
DF05	+	DF31	+	SF06	+
DF06	+	DF32	-	SF07	+
DF07	+	DF33	+	SF08	+
DF08	+	DF34	+	SF09	+
DF09	+	DF35	-	SF10	+
DF10	+	DF36	+	SF11	+
DF11	-	DF37	+	SF12	+
DF12	+	DF38	+	SF13	+
DF13	+	DF39	+	SF14	+
DF14	+	DF40	+	SF15	+
DF15	-	DF41	-	SF16	+
DF16	+	DF42	+	SF17	+
DF17	+	DF43	-	SF18	+
DF18	+	DF44	+	SF19	+
DF19	+	DF45	+	SF20	+
DF20	+	DF46	+	SF21	-
DF21	+	DF47	+	SF22	+
DF22	+	DF48	+	SF23	+
DF23	+	DF49	+	SF24	+
DF24	+	DF50	+	SF25	+
DF25	-	DF51	+	SF26	+
DF26	+	SF01	+		

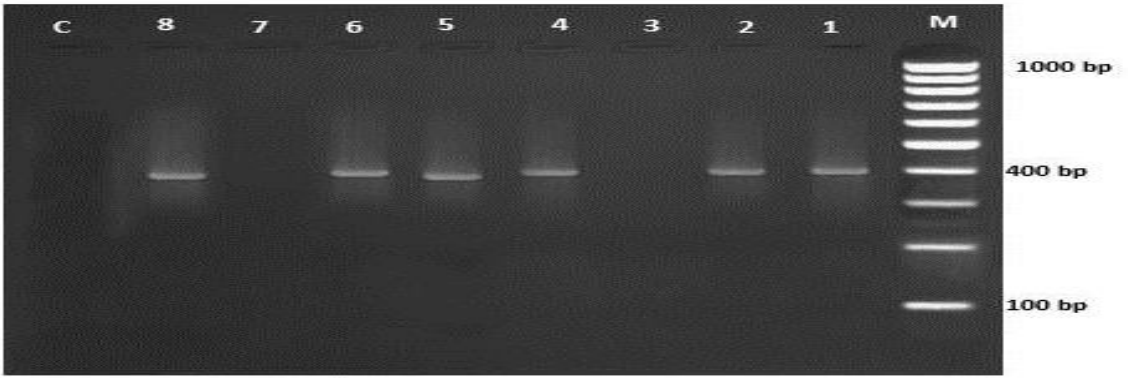

Figure (2) PCR products amplification with specific primer of Fusarium genus on the 1.5% agarose gel

M= (100 bp NDA ladder), (1-8) DNA of the Fungal isolates 1= DF9, 2= DF10,3= DF11, 4=DF12,5=DF13,6=DF14,DF=15,DF=16, C= Control

Inhibition Test in vitro

Results of the inhibition ability of the bioagent bacteria *B. pumilus* (Bp) against the fungal isolate DF13 *in vitro* indicated that the inoculum concentration of 10^7 and 10^8 exhibited 100% inhibition percentage (Table 5) compared with the control (Fungus alone) which filled the plate after 7 days of incubation (Figure 3). While the inoculum concentration of 10^4,10^5 and 10^6 ranged between 65.9% to 72.2%.

Table (5) Effect of the bioagent *B. pumilus* on the root rot disease of the cucumber under greenhouse conditions.

No.	Treatment	Inhibition (%)
1	Control (DF13 Alone)	**0.0**
2	DF13 x Bp 10^4	**56.9**
3	DF13 x Bp 10^5	**63.8**
4	DF13 x Bp 10^6	**72.2**
5	DF13 x Bp 10^7	**100.0**
6	DF13 x Bp 10^8	**100.0**
	L.S.D. (0.05)	**4.0**

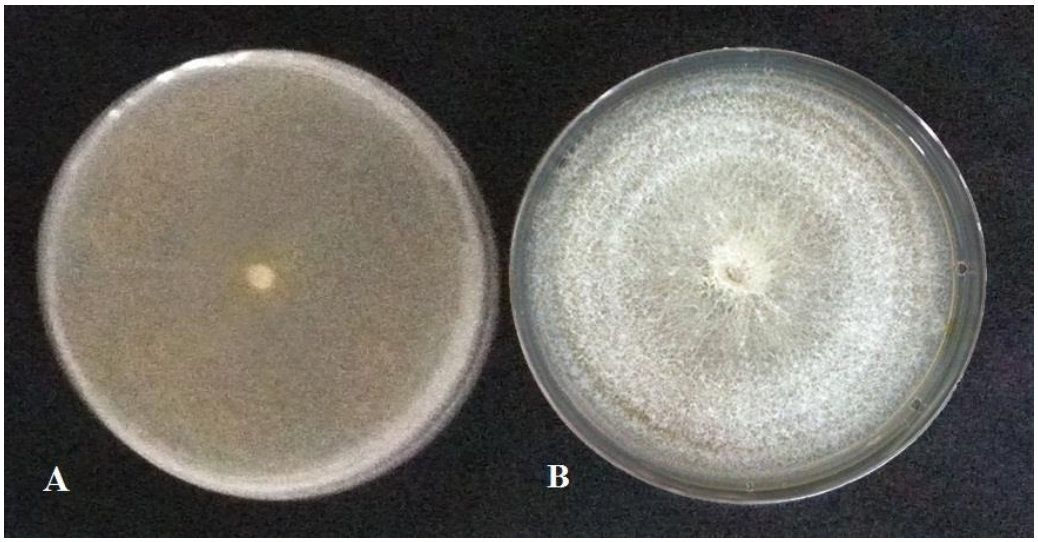

Figure (3) Inhibition ability of the bioagent *B. pumilus* against phytopathogen *F. solani*

A. F. solani (DF13) x B. Pumilus (Bp) B. Control (DF13)

Biological control of the root rot disease of cucumber

Results showed that the disease incidence and severity reduced to 30.55% and 20.75% respectively in the bioagent *B. pumilus* treatment (Table 6) compared to the negative control which were 80.50% and 55.00% respectively, this study agreed with the study of Heidarzadeh and Baghee-Ravari (2015) whom indicated that *B. pumilus* reduced 73.00% of the Fusarium wilt disease of tomato and increased the root and shoot system. Presence of *B. pumilus* in the rhizosphere area of plant prevent Fusarium spp and Rhizoctonia spp from spore germination (USEPA, 2012). Beneficial bacteria to the plants are referred usually to plant growth promoting rhizobacteria (PGPR), it can be known as beneficial species that live in the rhizosphere and colonize plants and promote plant growth by inhibiting the effectiveness of microorganisms harmful and producing growth regulators (Bhattacharyya and Jha, 2012). The functions of bacteria can be summarized to three points: synthesis of special compounds for plant growth, increased nutrient uptake of soil, and reduction or prevention of pathogens (Hayat et al., 2010). PGPR works to promote plant growth in two mechanisms, directly and indirectly, for the direct mechanism, which is the ability to produce enzymes such as 1-Aminocyclopropane-1-carboxylate deminase (ACC-deminase), which in turn reduces the level of ethylene in the roots which is reflected positively on root growth parameters, the ability to produce plant hormones such as auxins, cytokinins and gibberellins, fixing atmospheric nitrogen, processing and dissolving essential nutrients such as phosphorus and iron, enhancing plant susceptibility to dry environmental conditions, provide the plants with vitamin B, pantothenic acid, thiamine, riboflavine and biotin, also production of VOCs, the induction of systemic resistance in the plant, and the promotion of symbiotic relationships between plants and other useful microorganisms such as mycorhize. The indirect mechanism depends mainly on their susceptibility and biological control of plant pathogen microorganisms in the rhizosphere such as bacteria and fungi through the production of antibiotics, siderophores and enzymes such as B-1,3-glucanase, chitinases and cyanide (Hayat et al., 2010, Bhattacharyya and Jha, 2012).

Table (6) Effect of the bioagent *B. pumilus* on the root rot disease of the cucumber under greenhouse conditions.

No.	Treatment	Incidence (%)	Severity (%)
1	Control	0.00	**0.00**
2	DF13 Alone (Negative control)	80.50	**55.00**
3	*B. pumilus* (Bp) Alone	0.00	**0.00**
4	DF13 + Bp	30.55	**20.75**
	L.S.D. (0.05)	12.7	**7.09**

Conclusions

The main causal agent of the root rot disease of cucumber plants in the Diyala and Saladin governorates of Iraq is the fungus *F. solani*. The bio-agent *B. pumilus* showed significant biological control ability against this pathogen and could use it as an ecofriendly method to control plant disease.

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