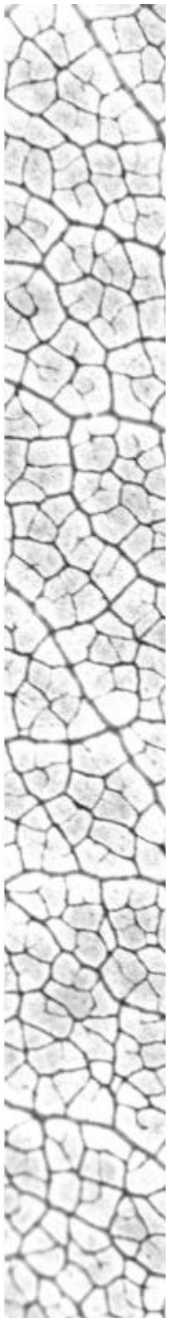

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Isolation and diagnosis of *Fusarium Solani* that causes root rot soybean and evaluating the efficiency of bacteria *Bacillus Subtilus* and *Azotobacter Spp* in controlling the disease

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Abstract
This research was conducted with the aim of studying root rot disease and seedlings death in soybeans that causes by fungus ***Fusarium solani*** in some areas of wasit governorate, isolating the pathogen and obtaining six isolates of *Fusarium solani* isolates from the areas covered by this study and testing its pathogenic ability, evaluating the efficiency of the plant growth promoting Rhizobacteria (PGPR) *Azotobacter spp* and *Bacillus subtilus* isolates, in percentage germination of soybean seeds, reducing percentage of infection and severity of disease and its effect in the length of vegetative and root groups of soy bean plant and comparing the results with beltanol fungicides. The percentage of seed germination treated with *Bacillus subtilis, Azotobacter spp* and beltanol was 95, 95 and 90%, respectively, compared to the comparison treatment 1 (without any addition) that reached 82%. and compared to the comparison treatment 2 (pathogen only) 65%. The treatment of soybean plant with bacteria under study showed a reduction in the infection rate after the trial period, it reached 15 and 10%, respectively, compared to the comparison treatment that included the pathogenic fungus, the infection rate reached 85%.While the severity of the disease incidence in these two treatments was 6.6 and 5%, respectively, compared to the comparison treatment was 42.5%.The biological control and chemical pesticide treatment that used in this study was able to improvement the growth of the plant according to the results of some growth criteria of plant length, vegetative and root groups, and their soft weight.

Keywords: root rot, *Fusarium solani*, PGPR, Beltanol, soybean

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INTRODUCTION

Soybean is the name in Latin (*Glycine max*) this plant belongs to the legume family, it is classified as an oil seed, its seeds contain an oil ratio ranging between17-24% without cholesterol (Sahuki,1991). Soybeans are considered an important food and industrial crops at the global level, as it is included in many strategic industries, such as the pharmaceutical industry, it is distinguished from the other types of pulses because it contains the eight essential amino acids necessary for the human body to make protein, it contains 40% protein and this makes it a source of complete protein, especially for vegetarians. Soybeans are also considered important forage and fertilizer crops, because the foliage and root complex is rich in elements (Hapgood,1987,Friedman and Branood,2001 and Sherif, 2013) ssoybeans are exposed to root rot diseases and seedlings death at the beginning of the planting season, and it suffers from root rot, stems and wilts at all stages of its growth, that causes by the fungus *Fusarium solani* which leads to a decrease in the number of plants in the field, weakening the growth of the remaining plants, and consequently to a decrease in the quantity and quality of crops. *Fusarium solani* is among the most widespread of the plant diseases in the world (Marasas et al,1988 and Burges et al,1981) infected seedling roots will show reddish, brown or dark to light brown discoloration and decayed (Nelson,1997) *Fusarium solani* is one of the major disease of soy bean in Canada and the United states America, these disease cause poor emergence, stunted seedlings (Nelson,1999) in 2002 a survey on the plant

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Table 1. The name, symbol code of the sample, and information about the collection

Serial	Sample location	Sample code	Date of collection	Varaities	Type of Agriculture
1	Al-hai	K1	1/8/2018	Lee74	Open cultivation
2	Al-Muwafaqiya	K2	3/8/2018	Lee74	Open cultivation
3	Al-Dujaili	K3	5/8/2018	Lee74	Open cultivation
4	Al-Numaniya	K4	7/8/2018	Lee74	Open cultivation
5	Husseinieh	K5	10/8/2018	Lee74	Open cultivation
6	Azizia	K6	15/8/2018	Lee74	Open cultivation

profile of soybean root rot in eastern Canada demonstrated that fusarium species represented 68% of fungal disease and yield losses of soybean due to fusarium root rot were estimated to be over 7300 metric tons in Canada and 8600 metric in the united states in1998 (Wrather et al. 2001)The losses increased to 13998 tons in the USA during 2003 to 2005 (Warther and koening, 2006) the wide range of these pathogens and their ability to resist in appropriate environmental conditions and the lack of varieties that are resistant to these diseases made it more difficult to control them (Bolkan and Butler,1974; Backer et al, 2003) therefore, chemical pesticides were relied upon as a means to protect crops from these diseases, but in addition to the negative effects of chemical pesticides on environmental pollution and the cost of control, these diseases was able to develop new strains that are tolerant of these chemicals (Demetrio's et al, 2017).Bacteria is one of the most important components of the biological ecosystem in the soil, as it constitutes the largest proportion of the Hungarian soil regions, its presence and superiority in its number compared to the groups of other neighborhoods of fungi, algae and protozoa. bacterial numbers are affected by many factors, including their type, moisture content, organic matter, temperatures and soil aeration, The agricultural activities carried out by plowing, fertilizing and the amount of sowing (Einallash et al. 2014) bacteria in the soil play an important role in the biochemical transformations that negatively and positively affect the nutrition, health and productivity of the plant, including bacteria that coexist with the roots of leguminous plants, which are among the most important bacterial groups in the soil (James, 2016) The role of these bacteria is not limited to fixing nitrogen only, but extends to some species have opposite activity towards pathogens as well as stimulating them to one or more different criteria for plant growth, these bacteria have been placed in a group known as bio control to resistant many diseases of the plant causes by fungi,the fungus *Fusarium solani* is one of them.

MATERIALS AND METHODS

Collecting of samples

Samples were collected from soybean plants infected with root rot disease from different places from the fields planted with soybean crop in some areas of wasit governorate for the period from 1/8/2018 to 15/8/2018 the samples was placed in polyethylene sac for the purpose of preservation, and we are recording the location and date of collection of the sample, cultivated variety and type of cultivation (**Table 1**) six different isolates were obtained for the fungus that we are researches about it, representing the areas that were collected including samples, the plants were considered infected with root rot disease if apparent symptoms are wilting of plants and their yellowing with brown coloration of the roots and rot of all part of the root or the entire root. The samples was putted in plastic sacs and preserved with a temperature ($4c^0$) in refrigerator in the laboratory for the purpose of conducting subsequent operations on them.

Isolation and diagnosis of fungus *Fusarium solani*

The fungus was isolated from the roots of soybean plants that showed symptoms and signs of disease on the day following the collection process for each of the areas covered by the study, we took small pieces of plant roots length(0.5-1 cm)and sterilized surface with sodium hypochlorite solution for two minutes, then the pieces were washed with sterile distilled water for 2 minutes to get rid of the disinfectant residue and dried with paper sterilized blotting in a steam sterilizer, we plant 5 pieces in each petri dish, 9 cm in diameter containing the agar (PSA) (200 g potatoes, 10 g sugar, 1 g agar in 1 liter of water) and incubate the dishes at a temperature of 25 ± 2 c ^{0} and after 3-5 days the fungi isolates were purified by planting them on the PSA medium by way of cultivating the tip of the fungal thread hyphal and incubated at a temperature of 25 c ^{0}±2 for five days. Taxonomic diagnosis of the fungus under study by upon on (Berge's et al,1981).

The pathogenicity test for *Fusarium solani* isolates

The pathogenic ability of *F. solani* fungi isolates obtained through isolation process was examined, according to the method (Dewan,1989) modified by using the seeds of cress instead of "grace seeds", as the cultivated medium consisting of agar and sterile water (20 g agar and 1 liter of distilled water) and added to it the antibiotic tetracycline, the dishes were inoculated in its center with a diameter disk (0.5 cm) from the fungus grown on the cultivated medium PSA at the age of 7 days, the dishes were incubated at a temperature of 25 c^0±2 for three days,cress seeds were planted locally after sterilized "with a solution of sodium hypochlorite and planted in a circular motion near the edge of the dish at a rate of 15 seeds. The treatments were repeated 5 replicates in addition to the control treatment (without

Table 2. Treatments used in testing the effect of using biological factors and the chemical pesticide used in the study on the percentage of soybean seed germination and the infection percentage and severity of infection of soybean by the fungus under study

Serial	Treatment
1	comparison 1 without any addition
2	Add the pathogen with *Bacillus subtilis*
3	Add the pathogen with Azotobacter bacteria
4	Add only Beltanol
5	Add the pathogen with Beltanol
6	comparison 2 only add the pathogen

fungus) and incubated at a temperature of 25 c ^{0}±2, after seven days the percentage of germination was calculated according to the formula next:

$$Germination\ percentage = \frac{number\ of\ germination\ seed}{total\ count\ of\ planted\ seeds} \times 100$$

And we selected the fungal isolation that showed high pathogenicity and its symbol is Fs4 which means *Fusarium solani* 4.

Effect of bacterial isolates on growth of isolate Fs4 fungi in laboratory on PSA culture medium

The antagonistic susceptibility of the *Bacillus subtilis* and *Azotobacter* isolates obtained from the Plant Protection Department of the Collage of Agriculture - Karbala University, was tested against the isolation of Fs4 on the PSA culture medium, as the method included, Adding 2 ml solution of each bacterial isolates grown on the liquid nutrient broth medium old 2-3 days (sixth dilution) to a petri dish containing the PSA culture medium and moving the dish a molar movement to spread the bacterial trap, then put a 0.5 cm diameter disk from the pathogenic fungus 7 days old at the center of each dish, we used 4 plates for each treatment and left 4 dishes without adding bacteria as a comparison treatment, incubating the dishes at a temperature of 25 c^0±2 after we calculate the growth rate of pathogenic fungi and the percentage of inhibition according to the following formula:

$$\%\ of\ inhibition = \frac{the\ growth\ rate\ of\ fungus\ in\ the\ control\ treatment - the\ growth\ rate\ of\ fungus\ in\ the\ treatment}{the\ growth\ rate\ of\ fungus\ in\ the\ control\ treatment} \times 100$$

Preparing the fungal vaccine for fungal isolation Fs4

Dewan, (1989) method was used to prepare the fungal vaccine using local millet seeds by placing 100 g of those seeds in 500ml glass jugs after cleaning it from the impurities, washing, and steeping them for 6 hours, and sterilizing it by using the autoclave system at a temperature of 121 c ^{0} and pressing 1.5 kg / cm ^{2} for 20 minutes and twice in succession during 3-5 days, millet seeds that were inside beaker after cooling,inoculated with Fs4 isolation vaccine at a rate of 5 tablets with a diameter of 5 mm / beaker and at three replicates. Beakers are incubated at a temperature of 25c ^{0} ±2for 14 days, we shaken the beakers once every 2-3 days to ensure ventilation and distribution of the fungus vaccine to all seeds.

Table 3. Concentrations and quantities of treatments used to test the effect of biological agents and chemical pesticide used in the study on the percentage of soybean germination, the percentage of infection and severity of infection for soybean under the protected conditions of the fungus under study

Serial	Experience treatment	Concentration	Add quantity
1	Fungal vaccine of Fs4 disease	Loaded on millet seeds	10 g / pot
2	Bacteria stuck *Bacillus subtilis*	1 * 10^{-6}	20 ml/pot
3	Bacteria stuck *Azotobacter spp*	1 * 10^{-6}	20 ml/pot
4	Chemical pesticide beltanol	0.1%	20 ml/pot

Test the effect of *Bacillus subtilis*, *Azotobacter* sp and Beltanol on soybean germination percentage

This experiment was carried out in the germination room at a temperature 27 c° and humidity 70-75% to reveal the effect of these factors on soybean seeds germination and according to the factors and concentrations shown in **Tables 2** and **3**, the experiment treatment were added when planting seeds in pre-prepared pots with a capacity of 1 kg it is packed with a mixture soil and peats with a ratio of 2: 1 and sterilized with autoclave at a temperature of 121 c^0 and a pressure of 5 kg / cm^2 for an hour, repeat the sterilization process after 24 hours and the fungal vaccine loaded on millet seeds was prepared in advance according to the method mentioned in paragraph (2-5) mixed with the soil by 10 g / pot. Planted with five pots of sterilized soybean seeds surface with a solution of sodium hypochlorides and left 4 replicates without adding the pathogenic fungus vaccine, but add 10 grams for each sterile millet seed free of pathogenic fungus as a comparison and the seeds inside the pots were regularly watered, the percentage of seed germination was calculated after 10 days from planting after the germination of the seeds of the comparison treatment.

The effect of *Bacillus subtilis*, *Azotobacter* and Bentanol in reducing the infection percentage and severity and its effect on the length of vegetative and root groups

This experiment was carried out in plastic pots to reveal the effect of these factors on the rate of infection of the fungus under study and the severity of the infection and the length of vegetative and root groups, the fungal vaccine was added mixing with the soil which was previously prepared in paragraph 2-5 by 10 g / pot used in the experiment. As a mixture of mixture soil and peatmos was used in a ratio of 2: 1 and sterilized with an autoclave at a temperature of 121c^0 and pressure 5 kg / cm^2 for an hour, the sterilization process was repeated after 24 hours and then distributed in plastic pots of 1 kg capacity. We planted with each pot of five soybean seeds, and with three replicates, the fungal vaccine was added to it and three replicates were left without adding the fungus vaccine, but 10 g of each

Table 4. Pathological ability test for isolates of *F. solani* on red cress seeds

Serials	Isolation symbol	% seed germination	% for inhibition
1	Fs1	60%	36.5%
2	Fs2	59%	36.7%
3	Fs3	72%	23.8%
4	Fs4	29.3%	69.02%
5	Fs5	61%	36.3%
6	Fs6	71%	23.9%
7	Control	94.6%	0%

C.V.: 16.8 L.S.D.: 10.821

sterile millet seed was added free from the pathogen as a comparison and watered regularly and the plants were followed up and watered whenever needed, and the results were taken by calculating the infection percentage and severity of the disease, as calculated according to the pathological evidence mentioned at the end of this paragraph, the percentage of injury was calculated for each of the treatment shown in **Table 2** and according to the concentrations mentioned for each transaction in **Table 3** and according to the following formula:

$$\%\ of\ infection = \frac{total\ number\ of\ plants\ -\ the\ number\ of\ affected\ plnts}{the\ total\ number\ of\ plants} \times 100$$

The length of the vegetative and root system of plants was measured after five weeks after the fungal vaccine was added to the pot (Gray and Achenbach,1996) The severity of the disease was estimated according to the following pathological evidence: (Sing,1984)

0 = No injury

1 = rot and death 1-25% of root space

2 = rot and death 26-50% of the root space

3 = Rot and death 51-75% of the root space

4 = death of the entire root

Where the percentage of the severity of the injury was calculated according to the following formula developed by (McKinney,1923).

$$\%\ of\ infection\ severity = \frac{total\ number\ of\ leaves\ per\ grade\ \times grade\ score}{total\ number\ of\ cards\ \times the\ highest\ score} \times 100$$

RESULTS AND DISCUSSION

Pathological ability test for isolates of *F. solani* on germination of cress seeds:

The results shown in **Table 4** that all the tested isolates caused a reduction “in the percentage of germination of cress seeds and a significant difference compared to the comparison treatment in which the germination percentage reached 94.6, and most isolates differed significantly” among themselves in reducing the germination percentage. Fs4 reduced the germination rate over the remaining five other isolates, as the percentage of germination percentage reached 29.3, this means that the percentage of inhibition amounted to 69.02% for this isolation, while the percentage of germination in the rest of the isolates was between 59-72% and this is due to the variation in the ability pathogenic isolates, so we select Fs4 isolate.

Table 5. Antibacterial test of the two bacteria under study on the radial growth and inhibition against the isolation of the pathogen Fs 4

Serial	Isolate	Dilution	Average/9cm	% for inhibition
1	*Bacillussubtilis*	$1*10^6$	1.2	83.33%
2	*Bacillussubtilis*	$1*10^7$	1.4	80.77%
3	*Azotobacter spp*	$1*10^6$	2.2	64.52%
4	*Azotobacter spp*	$1*10^7$	2.6	60.64%
5	Control	--------	7	0

C.V.: 18 L.S.D.: 1.261

Test the effect of bacteria under study on the radial growth of the isolate of pathogenic fungi Fs4

The results of this test showed that the use of *Bacillus subtilis* and *Azotobacter spp* as a biological control agent inhibited the growth of the pathogenic fungus *fusarium solani* on the PSA culture, compared to the comparison treatment within the two concentrations $1*10^6$ and $1*10^7$ using the fungus under study as it reached radial growth rate for fungus and the percentage of inhibition are 1.2,1.4,2.2, 2,6 and 80.77,83.33, 64.52, 60.64, respectively, This results shown in **Table 5**. This results is agreement whit the results that found by (AL-Ani et al,2012) in our research about effect of another genus of *Rhizobacteria* on radial growth and percentage of inhibition of infection of Fusarium fungus on soy bean on culture media PDA,reaches1.8,58 in concentration 100. The effect by use of these bacteria in inhibiting and radial growth of pathogenic fungi may be due to the ability of these bacteria to produce metabolites, organic compounds, some enzymes and antibiotic.

Test the effect of *Bacillus subtilis*, *Azotobacter spp* and Beltanol on soybean seed germination rate under germination conditions.

The results of this study showed the effect of the biological factors used in the study on soybean germination in the soil and peats in the seedlings treated with isolation of Fs 4,as shown in **Table 6**, there was a variation in the seed germination rates after a week of planting.We will showing a significantly “and increase in the percentage of seed germination compared to the comparison treatment adding the fungus only, where the percentage of germination was 70%, while the percentage of germination of seeds treated with *Bacillus subtilis*, *Azotobacter spp* and Beltanol are 95, 95 and 90%, comparison without pathogenic fungi 95%. The results of this study confirm that these bacteria have a high competitive ability against pathogenic fungi, which gives scope for expansion using them in integrated disease management programs. One of the characteristics that enabled these bacteria to control pathogenic fungi is their rapid growth property in the medium in which it lives and her high competitive ability

Table 6. Test the effect of *Bacillus subtilis*, *Azotobacter spp* and Bentanol on soybean seed germination rate under germination conditions

Serial	Experiment factor	% for seed germination	% for inhibition
1	comparison 1 without adding	95%	0%
2	Add the pathogen with bacteria Bacillus	95%	0%
3	Add the pathogen with bacteria Azotobacter	95%	0%
4	Addition of pathogen with Bentanol	90%	5.26%
5	Comparison 2 addition pathogen only	%70	%26.3

C.V.: 14.8 L.S.D.: 2.18

Table 7. Test the effect of *Bacillus subtilis*, *Azotobacter spp* and Beltanol in reducing percentage infection and severity infection on soybean root rot disease

Serial	Experiment factor	% percentage infection	% severity infection
1	Comparison 1 without adding	0%	0%
2	Add the pathogen with bacteria Bacillus	15%	6.6%
3	Add the pathogen with bacteria Azotobacter	10%	5%
4	Add Beltanol only	0%	0%
5	Addition of pathogen with Beltanol	5%	2.5%
6	Comparison 2 addition pathogen only	85%	42.5%

C.V.: 12.4 L.S.D.: 3.152

that enables her to settle in the Rhizospher root zone and to exploit the available nutrients, thus it showed an effective role “in increasing the percentage of germination. another results that found by(Al- Ani et al,2012) in our research about effect of another genus of Rhizobacteria (*Rhizobium japanicum*)on seed germination, they showed a significant effect to this bacteria reaches 50.93 measured with the control treatment reach 60%.

Test the effect of *Bacillus subtilis*, *Azotobacter spp* and Beltanol in reducing percentage and severity of infection in the soybean plant

The results of the study of the effect of the experiment factors showed a variation “in reducing the percentage and severity of infection by fusarium in the soybean plant as shown in **Table 7**. All the treatments resulted in reducing the severity of the infection with the pathogen by a significant difference from the comparison treatment, as the treatment of soybean seedlings by bacteria *Bacillus subtilis* and *Azotobacter spp* led to reduce the percentage of infection of the disease after 40 days of cultivation, which ranged between 15 and 10%, respectively, compared to the comparison treatment in which the pathogen was used only, as the percentage of infection rate reached 85%, while the severity of the infection in these two treatments

Table 8. Test the effect of *Bacillus subtilis*, *Azotobacter spp*, and Bentanol on length of vegetative and root groups of soybean plants

Serial	Experiment factor	length of vegetative	root groups
1	comparison 1 without adding	57	6.6
2	Add the pathogen with bacteria Bacillus	65	7
3	Add the pathogen with bacteria Azotobacter	62	7.66
4	Add Beltanol only	56	6.4
5	Addition of pathogen with Beltanol	51.33	7
6	Comparison 2 addition pathogen only	29.66	4

C.V.: 12.6 L.S.D.: 1.24

was 6.6 and 5%, respectively, compared to the control treatment in which the disease severity reached 42.5%.These results are corroborated with (Hassoun et al. 2013) regarding the possibility of using Bacillus bacteria in the biological resistance of soil fungi to control the Rhizoctonia fungus on okra to reduce the severity of infection to 13.10% compared to a comparison treatment of 94.75%.It also agrees with what was mentioned by (Abdel et al. 2018) regarding the use of *Bacillus subtilis* and *Azotobacter chroococcum* bacteria to control the *fusarium solani* that causes sorghum root rot, as well as (Al-Ani et al. 2012 and Bakker et al. 2003) stated that Rhizobacteria have an important role in the biological resistance to pathogens. These bacteria enables to settle in the Rhizospher root zone and take advantage of the available food sources and thus these bacteria have a role in increasing the percentage of germination by affecting pathogens (Sapna et al. 2012).

Test the effect of *Bacillus subtilis*, *Azotobacter spp* and Beltanol on length of vegetative and root groups of soybean plants

The results showed that there were significant differences in the height of the plant, i.e. the length of the vegetative group, as well as the length of the root group of soybean plants whose seedlings were treated when planting with bacteria *Bacillus subtilis*, *Azotobacter spp*, and Bentanol, compared to the comparison treatment with the presence of pathogenic fungi only *F. solani*, as well as the comparison treatment without addition as shown in **Table 8** where the treatment of the two genus of bacteria under study improved the vegetative and root growth, as the length of both the vegetative and root groups reached at this time, due to the increased readiness of mineral nutrients, as the bacterium Azotobacter spp is one of the bacterial species that stimulate the growth of the plant and this supports what it found (Hassoun et al. 2013) that the treatment of *Bacillus subtilis* plants to protect them against the pathogenic fungus Rhizoctonia had an effect on

increasing the length of the vegetative and root groups as well as what (Chen and Villani, 2002; Al-Ani et al, 2012) mentioned regarding the role of Rizobium bacteria stimulating plant growth in the activity of the vegetative and root groups. As for the role of the chemical pesticide Bentanol in increasing growth criteria are due to its effect on pathogenic fungi, as it provides adequate protection for seedlings.

CONCLUSION

The presence and spread of Fusarium wilt disease in soybean fields in Wasit Governorate.

The efficiency of the plant growth promoting Rhizobacteria (PGPR) *Azotobacter spp* and *Bacillus subtilus* isolates, in increasing of percentage germination of soybean seeds and reducing percentage of infection and severity of disease under study.

The effect of bacteria under study in increasing the length of vegetative and root groups of soy bean plant In addition to their role in the biological control of disease.

The use of bacteria *Azotobacter spp* and *Bacillus subtilus* to control the disease under study gave the same results as the use of beltanol pesticides in controlling the disease.

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