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Affectivity evaluation of *Bacillus subtilis* in controlling eggplant root rot caused by *Rhizoctonia solani* and *Fusarium solani*

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Abstract

This study included an evaluation of the efficacy of bacteria *Bacillus subtilis* obtained from Biohealth fertilizer to control the disease of eggplant root rot, caused by *Rhizoctonia solani* and *Fusarium solani*. The survey results showed that the disease was observed in all the subjected sites, which were Btera, Maymouna, Saad River and Greta in Misan province. The disease incidence ranged in the four sites between (15-44%). Several pathogenic fungi were isolated and diagnosed, with the most notable and most frequent appearance *(R. solani* and *F. solani)* with (35.29 and 29.41%) and (16.91 and 14.18%), respectively. Four isolates were obtained from the fungus *R .solani* and three isolates from *F. solani*. All tested fungal isolates caused significant decrease in the percentage of germination seeds in varying proportions, as well as the infection of eggplant root rot with varying proportions. The use of the bacteria (*B. subtilis)* led to a decrease in the percent severity of disease, and an increase in the soft and dry weight of the vegetative and root groups, as well as a clear increase in the height of plant, leaf number and leaf area of the eggplant. The use of *B. subtilis* increased the total phenol content (18.4 and 17.8 mg/ml) compared to the control treatment (16.4mg/ml).

1.Introduction

Solanum melongena, known as eggplant, pringal and eggplant, is an important vegetable crop throughout Asia and the Mediterranean where its fruits are a major ingredient in national and regional cuisine. Meanwhile, Asia is the main center of eggplant production. Iraq's production in 2009 is 396,000 tons, 21,000 hectares [1]

Eggplant crops are affected in the fields by many diseases and pests that cause severe damages during the growing season. One of these diseases is the root rot disease, which is one of the most common diseases in nurseries and greenhouses, caused by Rhizoctonia solani, Fusarium solani and Macrophomina phaseolina [2 ,3].

The use of biological agents in controlling pathogens is due to their ability to stimulate self-resistance in plants. Some bacterial species, such as Basillus, Rhizobium, Pseudomonas, Azospirillum and Azatobacter are known agents that stimulate the growth of plants [4].Bacillus subtilis has been reported as a growth promoter and antagonistic to a variety of pathogens in vitro and vivo in numerous studies [5].The disease resistance by, B. subtilis is due to the fact that these bacteria possess multiple mechanisms such as promoting plant growth, contrast, site and nutrient competition, pathogen degradation and stimulating systemic induced resistance (ISR).The aim of research was to conduct a survey of pathogens that cause eggplant root rot and to evaluate the efficiency of bacteria B. subtilis in controlling eggplant root disease.

2.Materials and Methods

2.1 Field survey and sample collection

Symptomatic Plants that showed root rot infection were collected from four areas of Misan province in the 2017-2018 agricultural season, which were Btera, Maymouna, Saad River and Greta. Disease incidence was calculated according to, the following formula:

$$\text{Disease incidence} = \text{Number of infected plants} / \text{Total number of plants tested} \times 100$$

2.2 Isolation and identification of fungi from eggplant roots

Samples were taken parts of the roots that showed symptoms of rot. The root zone was separated from the rest of plants, the infected areas then washed with running water for 30 minutes and left a period to dry on whatman-n 0.4 filter papers. These washed parts were cut into small pieces of 0.5 cm in length and sterilized with a solution of sodium hypochlorite 2% of the commercial preparation for 3 minutes, then washed with sterile distilled water for a minute to remove remaining sterile solution and, dried on filter papers. After that, four pieces were transferred to each petri dish containing a medium of Potato Dextose Agar (PDA) , (200 g potatoes, 20 g Dextrose and one liter of sterilized distilled water). An antibiotic (chloramphenicol) was added at a rate of 250 mg /L. The dishes were incubated at a temperature of 27° C for three days, after which testing was performed to investigate the presence of the pathogens. Pathogenic fungi were identified as causes of eggplant root rot, based on the morphological characteristics (fungal colonies, mycelium, spores and structures made up by these fungi) using approved classification keys [6] [7]. (The ratio (frequency and appearance) of fungal isolates was calculated according to following equations.

$$\% \text{ appearance} = (\text{Number of apparent fungi} / \text{total number of samples}) \times 100$$

$$\% \text{ frequency} = (\text{number of fungal colonies} / \text{total colonies of all fungal} \times 100$$

2.3.Pathogenicity test of fungal isolated from eggplant roots

Pathogenicity test for fungi isolated from infected roots on culture medium (water agar) in Petri dishes, where the center of each dish was pollinated with a 0.5 cm disk from the edge of the pure colonies at the age of 7 days at a rate of three repeated for each isolation. All dishes were incubated at a temperature of 25 ± 2 ° C for 48 hours, and sterile cabbage seeds were planted at a rate of 20 seeds for each dish around the fungi colony one cm from the edge of the dish after sterilizing these seeds with sodium hypochlorite solution (2%), then was washed twice with sterile distilled water to remove the remaining sterile solution and then incubated all dishes at 25 ± 2 ° C and after three days the germination percentage was calculated according to the following equation: -

$$\text{Germination \%} = (\text{Number of germinating seeds} / \text{Total number of seeds}) \times 100$$

2.4. Source of Bacillus subtilis

Bacterial source of Biohealth fertilizer produced by the German company Humin Tech Gmbh, consisting of humic acid 75%, seaweed extracts 5%, Trichderma harzianum and Bacilis strains 10% ,diagnostic tests were carried out as mentioned in [8].

2.5. Efficacy evaluation of B. subtilis in inhibition growth of R. solani and F. solani

Bacterial mixing method with the culture media was used to test the effect of *B. subtilis* against pathogenic fungi. PDA culture medium was prepared and distributed in 500 ml glass flasks at a rate of 250 ml per flask. The flasks were sterilized in an autoclave device for 20 minutes and then left to cool, then 1 ml of stuck bacteria growing was added on the liquid medium broth nutrient with the age of three days for each beaker, then I shake the beaker well, then poured the contents of each beaker into Petri dishes and incubated all the dishes at a temperature of 30 ° C. for 24 hours [9]. Then pollinate each dish with a 5 mm disk of each of the fungi colonies that are pathogenic for three replicates and leave a comparison treatment without adding bacteria, then incubate all the dishes in the incubator at 25 ± 2 ° C. for a week . Dimensions of growth in fungal colonies and the rate of inhibition were calculated according to the following formula:

$$\text{Inhibition \%} = \text{Fungal growth rate of treatment control} / \text{Fungal growth rate in dishes containing bacteria}$$

2.6. Effect isolates of fungi R. solani and F. solani on eggplant plants in a greenhouse

The experiment was conducted in the greenhouse of the College of Agriculture, University of Misan . The most pathogenic isolates were selected, then added as a vaccine, which was loaded onto millet seeds by 2% w/w [10] to sterilized soil with an autoclave device distributed in 5 kg plastic pots / pots with a diameter of 25 cm and a height of 25 cm. Eggplant seeds of Barcelona variety were planted in corks and after the emergence of real leaves, five seedlings / pots were transferred to three replicates per treatment, including the comparison treatment without adding a pathogen. The pots were watered to field capacity. Seven weeks later, when the symptoms of root infection were appeared, the seedlings were extracted and the severity of infection was estimated for the root system according to the pathological scale consisting of 5 degrees as follows: 0 = uninfected roots, 1 = secondary root rot, 2 = secondary root rot and part of the main root, 3 = primary root rot without root base rot, 4 = main root rot and base stem rot, 5 = plant death. The percent severity of infection was calculated according

to the 3 = primary root rot without root base rot, 4 = main root rot and base stem rot, 5 = plant death. The percent severity of infection was calculated according to the [11].

2.7 Effect of Bacillus subtilis in controlling R. solani and F. solani in the greenhouse

Pathogenic fungi loaded onto local millet seeds at 2% (w / w) ,were added to 5 kg plastic pots and mixed well with sterile soil. The stuck of Bacillus bacteria was added at 10 ml / pot (6.5×10^7) colony forming unit (cfu) / ml. five plants were transported for each pot as in the previous paragraph. The experiment was followed up and the pots were watered according to the field capacity of soil. The results were taken 70 days after planting and the following measurements were taken:

1. The severity of root system infectio, as in the previous paragraph
2. The soft weight of root and vegetative groups (gm): the fresh weight was calculated in each experimental unit after separation by a Sartorius sensor scale
3. The dry weight of root and vegetative groups (gm): after calculating the fresh weight of each of them. The root and vegetable parts were, oven dried at, 60 °C for 48 hours.
4. Number of leaves: calculated in each experimental unit.
5. Plant height: calculated in each experimental unit from the plant's place of contact with the soil to the growing top
6. The plant leaf area (1-2 cm). Using the Bio-Science device, five leaves were taken from each plant for each treatment and the total leaf area was calculated through the following formula:

Total leaf area of plant = average area of one leaf × average number of leaves per plan

2.8. Estimation of total concentration of phenolic compounds in eggplant leaves

Phenolic compounds were estimated according to the Folin –Ciocalten method cited by [12]. A 5 g of dry eggplant leaves was taken from each treatment, mixed with 400 ml of distilled water and then put the mixture in a bath aqueous at a temperature of 60 °C for an hour. The boiled mixture was filtered for extracting the phenolic compounds, 1 ml of the filtrate was taken, (1 ml) of Folin Ciocalten Indicator reagent was added to be placed in a test tube and left for 3 mins for the purpose of balancing and then 3 ml Na2Co3 concentration 2% was added to the solution, the mixture was left for two hours at, room temperature, absorbance was read at 760 nm wavelength by Spectrophotometer and samples were estimated based on gallic acid as standard solution. The experiments included the following treatments and three iterations per treatment: 1. Comparative treatment 2. *R. solani* 3. *F. solani* 4. *R. solani* + *B. subtilis* 5. *F. solani* + *B. subtilis*

2.9.Statistical Analysis

The experiment was designed using Complete Randomization Design (CRD) and the Least Significant Difference (LSD) test was used to compare the averages at the 0.05 probability level. The Statistical Package for Social Science (S.P.S.S.) version (23) in data analysis was used. Results represented an average of three replicates per treatment.

3. Results and Discussion

3.1. Field survey of disease:

Results of the field survey of eggplant root rot disease in some sites of Misan province showed the presence and spread of the disease. The cause of disease spread may be attributed to the repeated crop cultivation and the failure to follow agricultural cycles**.**

Table 1. The disease incidence in the four sites of Misan province

Sites	Disease incidence %
Petera	25
Maymouna	44
Saad River	15
Greta	30

3.2. Isolation and identification of pathogens caused eggplant root rot

Results of isolation and identification of symptomatic plant samples showed the appearance of four isolates from *R. solani* and three isolates from *F. solani* Table (2). The percent appearance and frequency of *R.* solani and *F. solani* (35.29 & 16.91 %) and (29.41 & 14.18 %) respectively. The fungus *R. solani* remains on organic wastes in the form of mycelium or sclerotia and also can continue in the soil for several years as a source of initial infection that grows when appropriate conditions are available [13]. As for *F. solani*, it is found in many types in soil and is considered to be widespread species. Both fungi have the ability to resist difficult environmental conditions by producing resistant vaccine units. Chlamydospores in the case of *F. solani* have ability to remain in the soil for at least five years and sometimes ten years [14]. *R. solani* was classified morphologically according to the brown fungal colony on the PDA medium, the branched mycelium with a right angle, which is contained specialties at the branch origin and barrier formation in the branches near development areas and non-configuration of sexual spores as some isolates showed the formation of barrel cells called Monilioid cells. As for the fungus *F. solani*, it is characterized by the production of fungal white to cream color on the cultivated PDA medium, colony's strength is lumbar and its edges are regular, large conidian spores are relatively wide, basal cell is old or cylindrical in shape either the top of cell is not sharp and round, small conidian spores are oval and may be undivided or divided by 1-2 barriers Figure 1.

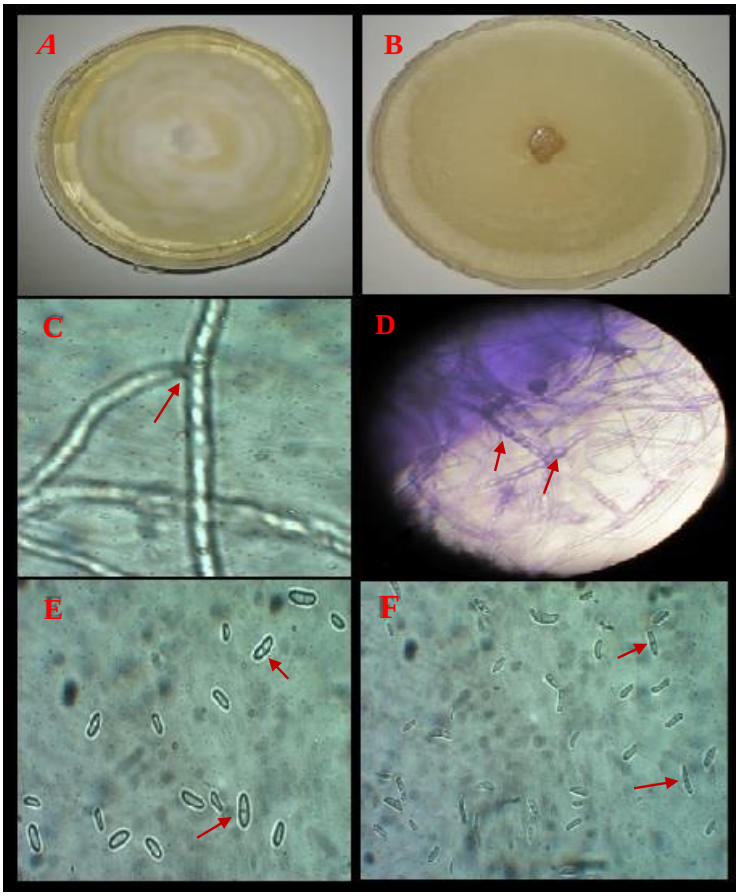

Figure 1. Characteristics of fungal and microscopic cultures of *R.solani* and *F.solani*

A. Colonies of *F. solani* on PDA B. Colonies of *R. solani* on PDA

C. The divided fungal thread, the existing branching angle, and the excretion in the branching area and the barrier near the branching area of *R.solani*

D. Monilioid cells of *R.solani* E.Chlamydiospores of *F.solani*

Table 2. Fungi associated with eggplant root rot

Fungus	Frequency %	Appearance %
R.solani	16.91	35.29
F.solani	14.18	29.41
F. oxysporum	12.13	23.53
Aspergillus fumigatus	9.93	17.65
Rhizopus stolonifer	15	12.1
Pencillium spp.	5	17
Alternaria alternata	2.94	5.88
Phoma sp.	1.6	18.5
Fusarium culmorum	10	14.6
Aspergillus niger	2.5	7.4

3.3. Pathogenic susceptibility to isolates of R. solani and F. solani

Pathogenic results showed, four isolates of *R. solani* and three isolates of *F. solani* on germination of Seeds of cress (Table 3). All fungal isolates caused a decrease in the germination rate of seeds ranging from 41.6 - 60.0% for *R. solani* and 68.3 - 76.6% for *F. solani*. The results of this experiment are consistent with several studies that indicated that the pathogenic ability of different isolates of *R. solani* differs between highly pathogenic isolates to weak or non-pathogenic isolates[15].

Table 3 . Pathogenicity of *R. solani* and *F. solani* isolates

Fungus	Germination percentage
control	100
R.s. 1	41.6
R.s. 2	46.6
R.s. 3	48.3
R.s. 4	60.0
F.s. 1	68.3
F.s. 2	68.3
F.s. 3	76.6
L.S.D	8.137

3.4. Effect of isolates of fungi R. solani and F. solani on eggplant in a greenhouse

Results of Table (4) indicated that all tested isolates of pathogenic fungi showed a significant difference in the infection severity and the injuries were evident on the eggplant plants in relation to the fungus *R. solani*, as the isolate R1 exceeded the severity of injury by 59.3 with high significant differences than the rest of isolates. As for the fungus *F. solani*, the F1 isolate was significantly outperformed by a 37.7% difference, with significant differences from rest of isolates. *R,solani* has the ability to produce enzymes, such as Cellulase and Pectinase, which break down the cell wall, particularly the middle lamella, due to the pectin and cellulose content, and thus lead to infection [16]. The fungus *F. solani* has the ability to secrete enzymes that break down cell walls that help penetrate cells, including the enzymes Chitinase, Cellulase and Protease,

and pectin, in addition to the ability of producing a wide range of toxins (phytotoxin) that have a role in injury events such as fusaric acid and javanicine [17]. Based on the results, isolation R1 and F1 were chosen in subsequent trials.

Table 4. The effect of pathogenic isolates on eggplant in a greenhouse

Fungus	The severity of infection
Control	0.0
R. s. 1	59.3
R. s. 2	35.5
R. s. 3	22.1
R.s. 4	20.1
F.s. 1	37.7
F.s. 2	33.4
F.s. 3	31.1
L.S.D	2.31

3.5. Efficiency of B. subtilis to growth inhibition of R. solani and F. solani

Results showed there was a high antagonistic ability of *B. subtilis* against pathogenic fungi. The radial growth of the pathogenic fungus *R. solani* was 35.5%, while for *F. solani* was 56.1% compared to the control treatment (0%), (Table 5). It may be attributed to its ability to produce many antibiotics that inhibit pathogenic fungi such as Subtilin, Subtenolin, Bacitracin, Bacillin and Bacillomycin [18], or it may be due to its ability to produce a number of decomposing enzymes such as B-1, 3 glucanase and Chitinase it breaks down the walls of pathogenic fungi cells [19]

Table 5. Effect of *B. subtilis* on growth inhibition of *R. solani* and *F. solani* in PDA

Treatments	colony diameter cm	Inhibition%
R.s.	9	0
F.s.	9	0
R.s. +B.subtilus	5.1	35.5
F.s. + B.subtilis	4.3	56.1
L.S.D	1.77	19.61

3.6. The effect of Bacillus subtilis in controlling of R. solani and F. solani in a greenhouse

The use of *B. subtilis* had notably reduced the percentage of infection severity of *R . solani* and *F. solani* to 46.5% and 55.2%, respectively, compared to fungi treatments without adding bacteria that reached (66.2 and 60.1%), respectively Table 6). The results in Table (7) showed that *B. subtilis* achieved an increase in the soft and dry weight of both root and vegetative groups of plants in presence pathogenic isolates of *R. solani* and *F.solani*, which reached 138.6 and 151.8 g for the vegetative soft weight, 36.6 and 47.8 g for the soft root weight, 22.4 and 24.5 g for the dry vegetable weight and 8.3 and 10.1 g for the root weight, respectively compared to the treatment of pathogenic fungi of *B. subtilis* that used to control the causes of eggplant root rot, which were achieved a significant increase in the total amount of phenols with (17.8 and 18.4) Mg / ml , respectively, compared to the control treatment that reached 16.4 Mg / ml. The use of *B. subtilis* increased in the plant height , number of leaves and leaf area with significant differences compared to the treatments of pathogenic fungi. The use of *B.*

subtilis may contribute in controlling plant pathogens by impeding the mechanisms possessed by pathogen. Increasing the phenolic content in plants increases the plantation of plant cells, which impedes the movement and transmission of pathogens within the plant and stimulates defense pathways and systemic resistance in the plant. Plants treated with *B. subtilis* can increase salicylic acid levels compared to untreated plants, as well as physiological changes in the treated plant such as thickening of cell walls and many blockages of intercellular spaces with osmophili and amorphous materials that work to block the entry of pathogens [20]. [8] indicated that the treatment of okra plant with *B. subtilis* increased the phenolic compounds in the okra plant infected with *R. solani* and found several compounds resulting from the separation process for the extract of okra leaves and roots treated with bacteria on thin layer chromatography (T.L.C).

Table 6. Effect of *B. subtilis* on infection severity, fresh, and dry weight of vegetative and root groups

Treatments	infection severity	fresh vegetable weight	fresh root weight	dry vegetable weight	dry root weight
Control	0	120.4	30.7	20.8	7.8
R.s.	66.2	81.4	19.7	16.4	3.4
F.s.	60.1	85.1	22.1	18.3	5.1
R.s. +B.subtilus	46.5	138.6	36.6	22.4	8.3
F.s. + B.subtilis	55.2	151.8	47.8	24.5	10.1
L.S.D	1.5	2.8	3.3	2.4	0.8

Table 7. Effect of *B. subtilis* on total phenols and some growth criteria

Treatments	Phenolic compounds	Plant height (cm)	Number of leaves	leaf area index
Control	16.4	44.1	18.3	60.6
R.s.	12.6	28.3	14.5	45.8
F.s.	13.8	36.3	15.4	53.3
R.s. +B.subtilus	17.8	47.5	22.1	65.3
F.s. + B.subtilis	18.4	54.6	25.2	67.5
L.S.D	0.320	0.22	0.28	0.41

Conclusion

That eggplant root rot is widespread in all areas covered by the field survey. Bacteria *Bacillus subtilis* have the ability to control the disease and reduce its severity.

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