# ANTAGONISTIC ACTIVITY AND PLANT GROWTH PROMOTING RHIZOBACTERIA ISOLATED FROM FOREST PLANT RHIZOSPHERE AGAINST *FUSARIUM SOLANI* ON THUJA SEEDLINGS

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**ABSTRACT:**
**Root diseases are one of the main forest nursery problems that have a significant impact on forest production which are caused by *Fusarium solani*. Rhizobacteria from healthy forest soils were isolated and screened in streak method to select antagonistic strains against *F. solani*. Two isolates showed high antagonistic activity and molecularly identified as *Paenibacillus sp.* and *Pseudomonas sp.* The capability of the *Paenibacillus sp.* and *Pseudomonas sp.* were tested in greenhouse plastic containers experiments against *F. solani*. Soil bacterization with *Paenibacillus sp.* and *Pseudomonas sp.* significantly protected thuja seedlings from *F. solani* compared to the untreated control seedlings. The containers added by *Paenibacillus sp.* and *pseudomonas sp.* are also showed plant growth promotion including shoot length, root length, dry and wet weights of the seedlings as well as the chlorophyll contents of the thuja seedlings compared to the untreated control plants. In this research it has been showing that the rhizobacterial treatments have potential to decrease the effect of fungal disease severity, promoting the plant growth and also helps plants to maintain a good health.**

**Keywords: *Paenibacillus sp.*, *Pseudomonas sp.*, forest, root rot and antifungal.**

مجلة العلوم الزراعية العراقية -2021 :52 (6):1508-1515 سعيد وآخرون

## النشاط التضادي و تعزيز نمو النبات بواسطة البكتيريا الجذرية المعزولة من جذور نباتات الغابات ضد *Fusarium solani* على نبات الثويا

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**المستخلص:**
تعتبر امراض الجذور من اهم امرض مشاتل الغابات التي لها تاثير كبير على انتاج شتلات الغابات والتي يسببها فطر *Fusaruim solani*. تم عزل البكتريا الموجودة في المنطقة الجذرية Rhizosphere من تربة الغابات السليمة و باستخدم طريقة الخطوط لتحديد العزلة النشطة بعملية تضادية للمسبب *F. solani*. اظهرت اثنين من العزلات نشاطا مضادا عاليا وقد تم تشخيصهم جزئيا باسم *Paenibacillus sp.* و*Pseudomonas sp.* . واختبر قدرتها علي نباتات تجريبية ثبتت قدرتها علي حماية الشتلات الثويا Thuja من *F. solani* في البيوت البلاستيكية بشكل كبير مقارنة بالنباتات غير المعاملة. كما تبين تعزيز نمو النباتات المضافة اليها *Paenibacillus sp.* و *Pseudomonas sp.* بما فى ذلك طول الساق وطول الجذر والاوزان الجافه و الرطبه للشتلات و كذلك محتوى الكلورفيل فى شتلات Thuja مقارنة بالناباتات غير المعاملة. واظهرت نتائج هذا البحث ان البكتريا الجذرية لها القدرة على تقليل تاثير شدة الامراض الفطرية, وتعزيز نمو النباتات, وكذلك تساعد النباتات على الحفاظ على الصحة الجيدة في ان واحد.

كلمات مفتاحية : *Paenibacillus sp., Pseudomonas sp*, تعفن الجذور, غابات, التضاد الفطري

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## INTRODUCTION

*Thuja sp.* is a coniferous small genus that has many existing species, including *Thuja occidentalis* which is also known as white ceda. There are many pathogens attacking *Thuja sp.* including root rot disease which it causes by various fungi that affects many plants and particularly in sites with poor drainage (6). Nearly most of the pathogens that are causing damping-off and root rot are fungal such as Alternaria, Cylindrocarpon, Cylindrocladium, Fusarium, Rhizoctonia, Trichothecium and many others (13). This disease can be identified in nurseries and the impact of this disease on seedling production can cause mortality and infected seedlings have a lower survival rate after out planting to reforestation sites (18). *Fusarium solani* cause root rot disease on forest and shade seedlings (12). There are many methods for controlling root rot disease in nurseries while the seedlings are young. Chemical products, fertilizers and even fungicides, can damage forest beneficial soil microorganisms and it may be harmful to humans and the environment (22). Recently, an interest biological control has increased which concerns over the use of chemicals by using beneficial microorganisms including some soil bacterial strains that have many beneficial effects on plant health (14). Many Plant bacterial interactions are determining in the rhizosphere within the plant health and soil fertility. Some beneficial bacteria such as *Bacillus sp.* and *Pseudomonas sp.* produce different biological active compounds and are easily isolated and cultured in laboratory, it has received a great attention as a biological control agents which can control different disease among them fungal pathogens inside the greenhouse and also in the field (3, 19). Until now, there is no study on the effect of indigenous rhizospheric bacteria against *Fusarium solani* on thuja have been documented in Kurdistan Region Erbil/Iraq. Therefore, this study was designed to isolate antagonistic bacteria from forest soil and to evaluate the efficacy of selected bacterial isolates against root rot and thuja growth.

## MATERIALS AND METHODS

**Plant sample collection:**All the samples of infected thuja plants that have symptoms of root rot were collected from different nursery growing including Erbil forest nursery. The plants were put in paper bags after collecting and transported to the Plant Pathology Laboratory, Department of Plant Protection at College of Agricultural Engineering Sciences/Salahaddin University.

**Bacterial isolation**

The samples of rhizosphere soil were collected from natural forests at different locations across Erbil for bacterial isolation. From each soil sample, 1 gm of dried soil has been suspended in 9 mL dH2O, and successive serial dilutions were made by transferring 1mL of aliquots to 2nd test tube containing 9 mL of sterile water, and dilutions up to $10^{-8}$ were prepared. An aliquot of 0.1 mL of each dilution has been taken and spread evenly over the surface of Nutrient agar (Difco$^{TM}$) medium supplemented with cycloheximide (100 µg/mL) on 9 cm petri dishes. Plates were incubated at 28±2°C and monitored for 3 days. The pure culture has maintained in Nutrient Agar (NA) slants in a refrigerator at 4°C for further use.

**Screening of bacteria isolates for antagonism against pathogen**

The bacteria that isolated have been used against the *F. solani*. Four millimeter diameter plugs from 5 day old fungi cultures were inoculated in the side of PDA plates. All the bacteria isolates grown on NA were inoculated on to the other side of plates with the help of a sterile loop, using streak method. The plates have been incubated for 7days at (28±2°C) and the growth of mycelium was observed. Control petri dishes were maintained by inoculating with pathogen only. The bacterial isolates showed antagonistic potential in inhibiting the mycelia growth of fungus were selected. Isolates showed no inhibition against the pathogen that discarded indicates negative test. The appearance of inhibition zone between the pathogen and the bacterial isolates has been measured. The experiments run following a completely randomized design (CRD) with 4 replications. Percent growth inhibition calculated using the following equation:

$$\mathbf{I\,\% = \frac{R1 - R2}{R1} X100}$$

Where,

I = Percent inhibition of radial growth

R1 = Maximum radius of mycelial growth on the control plate

R2 = Maximum radius of mycelial growth towards the bacteria in treated plate

**In vivo experiment:**

Thuja seeds were disinfected with 50% Benomyl fungicide and streptomycin for 2 hours prior to sowing. Disinfected seeds were hand sown in plastic bags and placed in the greenhouse under natural daylight. They were also watered daily. The experiment carried out with five replications for each treatment. Four-week old thuja plants were inoculated with bacteria isolates ($1\times10^8$ cfu/ml) were added to the soil (15ml/bag). After 24hours plants were sprayed with a conidia suspension of *F. solani* containing $3\times10^5$ conidia/mL (15 mL/plastic bag) in distilled water. To increase infection, roots of test plants were wounded with a sharp scalpel by stabbing the soil several times and then pouring of 15 ml of conidia suspension onto surface of the soil. Plants that were not inoculated were sprayed with distilled water as control. The plants were covered with black plastic bags for 24 hours after inoculation. The plastic bags were removed after 24 h and plants watered daily. To elucidate the role of application of bacterial isolates on disease severity in thuja plants treated with bacteria (the most promising rates based on the results of in vitro experiments), evaluation was done by visually assessing the lesions caused by *F. solani*. Typical symptoms of disease were observed on the thuja plants after 30 days. Disease severity based on scale by Latha et al. (2009) was measured.

## RESULTS AND DISCUSSION

**Pathogen isolation and pathogenicity test**

Twenty samples were collected from symptomatic thuja plants in different nursery at Erbil provenance in this study. From twenty samples 9 different Fusarium isolates were isolated and purified and named F1 to F9. All the isolates were tested for pathogenicity on thuja seedlings. The most pathogenic isolate was chosen for the later experiments. *Fusarium solani* from thuja root samples were isolated, characterized and identified. The pathogenicity tests on thuja seedlings indicated that *F. solani* was highly pathogenic. Fusarium fungi are a common pathogen and it attacks a wide range of plants and causes economically important diseases and this pathogen also affects coniferous seedling in bare root nurseries. According to Robles et al. (14) showed in the research that all tests has proved that *F. solani* is the fungal species that cause symptoms of root rot and wilting of conifers and many types of Conifers can suffer significant damage due to this disease in the nursery, primarily root rot and wilting of the seedlings. This fungus can effect on tree growth and according to Gordon et al. (9) coniferous trees can suffer significant damage from seedling diseases caused by Fusarium and this fungus can be responsible for both pre- and post-emergence damping-off.

**Screening for antagonism against pathogen**

Bacterial isolates were selected based on the pattern of their ability to fungal growth inhibition in streak method. The minimum percentage of inhibition against the pathogen was B18 (59.37%) and B13 (60.62%) which showed clear inhibition during interaction with the fungus of more than 50% respectively and was statistically similar. Also, the percentage inhibition was followed by B14 (73.75%) and B9 (76.66%). The highest reduction (81.25% and 80.75%) over the control was obtained with isolates B4 and B6 respectively (Figures 1 and 2). Isolates B4 and B6 were selected for further experiments because of their highly percentage of inhibition.

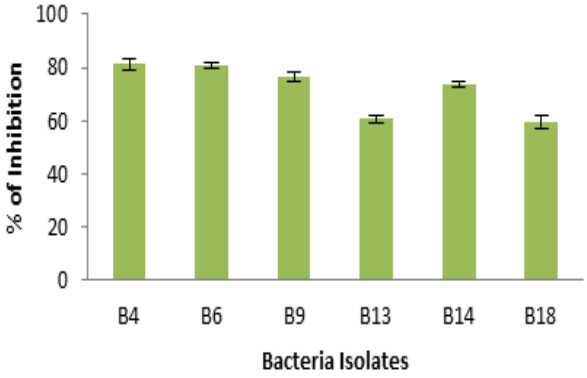


**Figure 1. Effect of bacterial isolates on mycelial growth of F. solani after seven days of incubation**

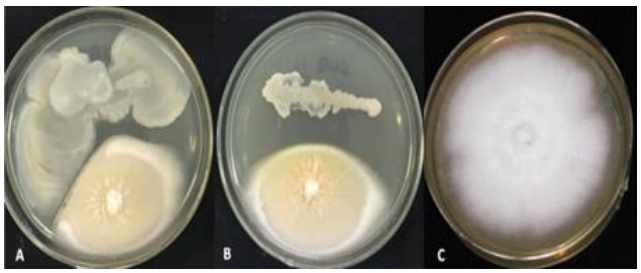


**Figure 2. Growth inhibition of *F. solani* caused by bacteria isolates in dual culture test on PDA after seven days of incubation at 28˚C. a) B4; b) B6 and c) Control**

**Molecular identification**

Running of the PCR products in gel showed that DNA fragments of two studied bacteria isolates were amplified at 1500 bp (Figure 3). The isolates were identified as *Paenibacillus sp.* (B4) and *Pseudomonas sp.* (B6). On the basis of the results derived from the BLAST of the NCBI (http://blast.ncbi.nlm.nih.gov/), partial 16S rDNA sequences of the B4 revealed the closest matches with the sequences of *Paenibacillus sp.* up to 100%, and isolate B6 with the sequence of *Pseudomonas sp.* up to 100%. The phylogenetic analysis of 16S rDNA sequences of isolates B4 revealed that it was similar to the sequence of *Paenibacillus sp.* strain NA11031 (AB921273). Isolate B6 showed 100% similarity with *Pseudomonas sp.* strain AA363 (MN540125). The partial 16S rDNA nucleotide sequence of the test bacteria isolates was submitted to the NCBI database. The accession numbers for the sequences of phyllosphere bacterial isolates B4 and B6 were MT416113 and MT416115, respectively.

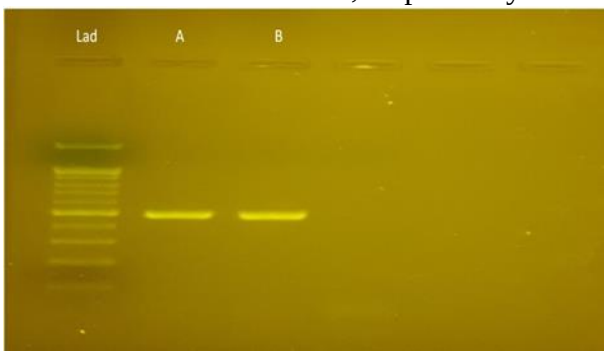


**Figure 3. Amplification of 16S rDNA of bacteria isolates in 1% TAE buffer fragmented at 1500 base pairs (Lane A = B4; Lane B = B6; and Lad = Molecular weight marker (1kb ladder).**

**1.1 Phylogenetic analysis**

A phylogenetic tree based on 16S rDNA sequences of both active bacteria isolates and related bacteria species demonstrated that isolate B4 had 100% with the same sequence as *Paenibacillus sp.* and B6 had 100% with the same sequence as *Pseudomonas sp.* (Figure 4).

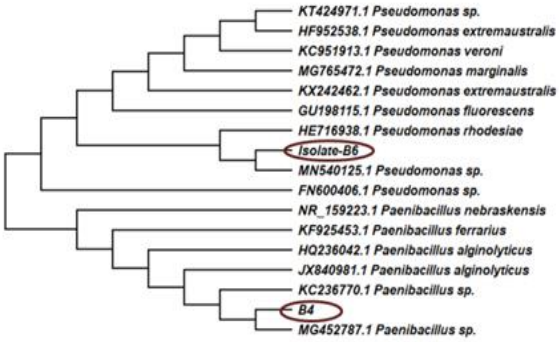


**Figure 4. Phylogenetic tree constructed by the neighbor-joining method showing the phylogenetic relationships of both active isolated bacteria compared with the reference sequences from gene bank**

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.24897053 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 1366 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

**In vivo experiment:**

**Efficacy of active bacteria isolates on disease suppression**

The results in Figure 5 shows that both applied bacteria isolates significantly reduced the total AUDPC in all the studied disease. The total AUDPC from the treatment ranged from 379.5 to 1171.2. Untreated control plants inoculated with F. solani had significantly high (1171.2) AUDPC followed by 588.6 and 416.4 plants inoculated with pathogen and treated with B4 and B6 respectively.

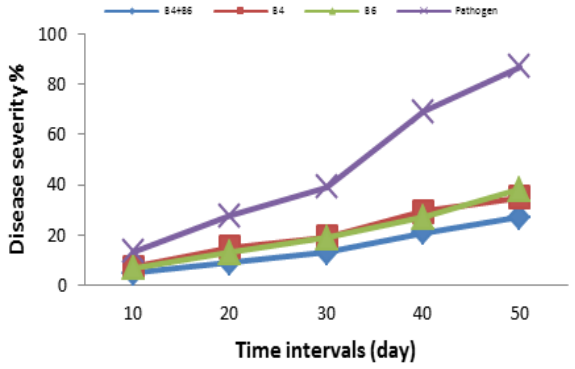


**Figure 5. Progression of thuja wilt (as measured by % disease severity) over time after application of treatments**

Treated plants with combining both bacteria provided the highest reduction of disease severity (Table 1). The highest reduction of disease severity was seen at B4+B6 with plants inoculated with F. solani (70.38%) and B6 alone (67.50%). The lowest reduction was seen in thuja plants inoculated with F. solani and treated with B4 (54.07%). In general, plants inoculated with pathogen with both bacteria had the highest reductions and this was followed by treating plants treatments with separate bacteria isolates.

**Table 1. Effect of different bacterial isolates on AUDPC and % of disease reduction**

| Treatments | AUDCP | Disease Reduction% |
|---|---|---|
| **Pathogen** | **1171.2** | **0** |
| **B4** | **588.6** | **54.07** |
| **B6** | **416.4** | **67.50** |
| **MIX** | **379.5** | **70.38** |

There are many studies on the antifungal activity of Paenibacillus isolates had have reported their effects on controlling plant pathogenic fungi. *Paenibacillus sp.* showed a significant effect on the growth of Fusarium which it decreased the growth range toward the bacteria in all replications that has been done in laboratory. Dijksterhuis et al. (8) showed that paenibacillus culture had an inhibitory effect on the development of the fungus, antagonism was clearly enhanced when the bacteria clustered around the hyphae. According to Dunlap et al. (9) Paenibacillus has an important role of controlling fungal disease such Fusarium specially in greenhouses and the importance of these antibiotics in biological control systems is well established and studied. Paenibacillus has produced the greatest antifungal activity against Fusarium can be used to inhabit Fusarium root colonization (7). It is possible that the Paenibacillus under our experimental conditions altered the substrate enhancing growth of Fusarium and its ability to grow more. As Song et al. (16) mentioned in their research that the bacterial Paenibacillus displayed an enhanced inhibitory activity against the fungal pathogen mycelial growth. In pot experiments, pretreatment with the bacterial isolate in the presumed optimal time for disease control reduced disease severity significantly with a higher control efficacy and they also showed that all of the results suggest that the bacterial isolate has good potential as a microbial agent for the biocontrol of the ginseng root rot caused by *Fusarium.*

**Bacterial isolate effects on thuja growth**

The effects of bacterial isolates on the shoot height, root length, plant fresh and dry weight are shown in Table 2. The variance analysis indicated that plant height and plant dry weight were influenced by bacteria treatments, pathogen inoculation and control treatments. Plant heights improved significantly at combining both bacterial isolates and decreased when plants inoculated with pathogens and untreated with the bacterial isolates. Plant height improved with the addition of B4 isolate in comparison to un-inoculated plants with pathogens that were not treated with the bacteria. The lowest value, 15.1 cm, came from thuja plants inoculated with the *F. solani* and not treated with bacterial isolates, followed by control plants (15.36 cm) and plants inoculated with the *F. solani* and treated with B4 (15.66 cm). Root length of thuja plants treated with B6 were the longest compared to the control and other treatments. Thuja plants inoculated with *F. solani* and treated with B6, had the longest root length as compared to thuja plants inoculated with pathogens only. The shortest root lengths of thuja plants were those inoculated with *F. solani* and untreated with the any bacteria isolates as compared with thuja plants in the other treatments. The highest plant fresh weight (3.41 g) were recorded in the plants treated with both bacteria isolates and the lowest fresh weight (1.58 g) were recorded in the plants inoculated with the pathogen and not treated with

bacteria. As compared to the treatments, the plant dry weight in all treatments increased in thuja plants treated with bacteria isolates. Furthermore, plant dry weight of thuja plants inoculated with *F. solani* and untreated with bacteria were the lowest as compared to thuja plants inoculated with pathogen and treated with one of the isolates or in combination. Intensification of bacterial isolates in all treatments significantly increased plant biomass. Plant biomass in all treatments benefited greatly when treated with bacteria but decreased drastically in pathogen inoculated plants not treated with bacteria.

**Table 2. Effect of bacterial isolates on selected disease and plant growth**

| Treatments | Shoot height | Root length | Plant fresh weight | Plant dry weight |
|---|---|---|---|---|
| B4 | 19.33±1.45 $^{ab}$ | 34±2.52 $^{ab}$ | 2.9±0.11$^{ab}$ | 1.65±0.26$^{ab}$ |
| B6 | 18.13±0.33 $^{bc}$ | 40.33±1.67 $^{a}$ | 2.95±0.28$^{ab}$ | 1.51±0.06$^{ab}$ |
| B4+B6 | 21±2.69 $^{a}$ | 37.66±3.38 $^{ab}$ | 3.41±0.39$^{a}$ | 1.66±0.08$^{a}$ |
| F+B4 | 15.66±0.57 $^{de}$ | 29.66±4.81 $^{ab}$ | 2.81±0.02$^{a}$ | 1.54±0.15$^{ab}$ |
| F+B6 | 16.33±0.78 $^{cd}$ | 35.83±3.72 $^{ab}$ | 2.72±0.32$^{ab}$ | 1.47±0.21$^{ab}$ |
| F+B4+B6 | 19±0.65 $^{abc}$ | 35.66±2.4 $^{ab}$ | 2.95±0.02$^{a}$ | 1.65±0.19$^{ab}$ |
| Pathogen | 13.1±0.2 | 31.33±3.93$^{b}$ | 1.58±0.11$^{a}$ | 1.31±0.05$^{b}$ |
| Control | 15.36±0.16 $^{e}$ | 34.66±3.71$^{b}$ | 2.84±0.21$^{ab}$ | 1.73±0.19$^{ab}$ |

## Chlorophyll content

The absorbency of chlorophyll changed by escalations in B4, B6 and in combination and significantly went down in all treatments that were inoculated with pathogen and not treated (Figure 6). The lowest chlorophyll content came from plants inoculated with *F. solani*, followed by control plants then plants inoculated with pathogen and treated with B4. Thuja plants treated with B6 had significantly higher chlorophyll absorbency than plants treated with other bacterium and in combination.

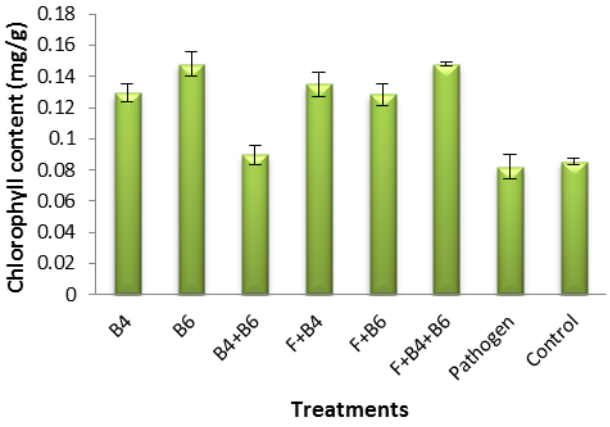


**Figure 6. Effect of bacteria isolates on total chlorophyll contents in thuja leaves at 3 months of plant growth**

Biocontrol agent *Pseudomonas sp.* has received attention among researchers and this bio agent can be used as plant growth promoting rhizobacteria and as well as controlling fungal pathogens specially Fusarium (1, 4). *Pseudomonas sp.* has been used in many countries and many research mentioned its importance as a biological control for decades. In which it have ability to reduce the effect of fungal pathogens including Fusarium on plants and controls many diseases such as blight, welting, damping of, root rot and many others Plants inoculated with pathogen and both bacterial isolates had the highest disease reductions and this was followed by treating plants with each isolates alone. Zhao in (23) showed a high level of antifungal activity and Hyphal growth of *Fusarium* while the mycelial growth was analyzed by inhabiting 87.9% with different concentration of *Paenibacillus and* it showed that the mycelium diameter of *Fusarium* was significantly decreased with increase in concentration of *paenibacillus* in PDA plate. Al-Jedabi in (2) also mentioned that the maximum inhibition achieved by Paenibacillus was 67.7% against mycelial growth of F. solani, and the control plates in his results were not treated with the bacterial isolates were completely covered by the phyto-pathogens showing no inhibition. which that means the mean mycelial growth inhibition of the most effective bacterial and fungal isolates revealed that the inhibition was highly significant.Another biocontrol agent bacteria that is mentioned in this research in which it is also had a significant effect of reducing the effect of Fusarium was *Pseudomonas sp.*. It has also a great effect on controlling the spread of fungi during the growth of plants. As we showed the results after using this bacterium, the growth of plants has promoted intelligibly and most of the plants survived from damping off or root rots disease. According to Bano and Musarrat (5) many pseudomonas strains has

been used in their research, and all showed significant effects on the infected plants which has been affected by fungi, and they showed that the pseudomonas is biocontrol agent bacteria that works as biological control of diseases as well as it helps plant growth promoting. Pseudomonas works on the plant growth promoting for many agriculture plants and recently has been used as biological control. As Gull and Hafeez (11) showed by the research, after using many Pseudomonas strains, most of them worked as a biological control and with its multi-mechanisms of defense has excellent potential to be used as successful biocontrol agent against root rot disease. Is also mentioned that Pseudomonas have drawn attention worldwide because it stimulate plant growth by producing secondary metabolites, plant hormones and hydrolytic enzymes. Weller in (21) mentioned that Pseudomonas spp. are ubiquitous bacteria in agricultural soils and have many traits that make them well suited as biocontrol agents of soil borne pathogens. Stockwell and Stack (16) Notified that many biological productions has been manufactured lately in which Pseudomonas spp. has been used inside the production which it is easy to use for plant disease for many agriculture crops and trees. Also Wahyudi and Astuti in (19) showed in their results that pseudomonas is on the basis of excellent growth promoter and biocontrol activities and in the tests of hypersensitivity that was used to screen for Pseudomonas sp. Also Pseudomonas sp. classified as non-pathogenic rhizobacteria and most of pseudomonas strains uses as a biological control against fungi diseases including Fusarium. In conclusion, the results indicate that Pseudomonas sp. and Bacillus sp. can be utilized as a restrictive and curative natural product for the control of *Fusarium solani* on Thuja seedlings. Furthermore, treatment with both bacteria isolates is a promising new technology, offering an alternative for fungicidal control as well as plant growth promoting.

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