Biocontrol of Fusarium oxysporum f. sp. lycopersici by plant growth promoting bacteria on Tomato plant .

Fusarium oxysporum f. sp. lycopersici المكافحة الاحيائية للفطر بأستخدام البكتريا المحفزة للنمو على نبات الطماطة .

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Abstract

This study was carried out to study the efficiencyof six strains of plant growth promoting bacteria(Bacillus subtilis, Bacillus pumilus, Pseudomonas fluorescence, Azotobacter chroococcum1, Azotobacterchroococcum2and Bacillus sp.)as biocontrol agents against the pathogenic fungus Fusarium oxysporum the causal pathogen of wilt disease of tomato plants under greenhouse conditions .

The results of antagonistic activity of the six bacterial strains against F.oxysporum showed that the tested strains varied in their abilities in reducing growth rate of the pathogen and increasing the percentage of pathogen inhibition as compared to control treatment.B.subtilis was the superior in reducing the radial growth rate of pathogen20.33mm as compared to control treatment34.66 mm .

The results revealed that most the tested bacterial strains significantly increased the percentage of seeds germination and the 1st germination .B. subtilis , P. fluorescence and B. pumilus were the best in increasing seeds germination which recorded(97.33,95.33 and 93.66)% respectively as compared to control treatment (77.40 %) .

The greenhouse experiment revealed that the plants treated with B.subtilis recorded maximum (shoot length , root length , branch no./ plant ,fresh and dry weight of plant ,branch no. / plant , fruit no/plant and plant productivity) . All these parameters were increased by 317.6 cm , 29.4 cm , 5.1 branch / plant ,(1456.6 , 298.6) g / plant ,(5.1) branch / plant ,11.63 fruit / plant , 1361.6 g / plant respectively,also the results showed B.subtilis significantly decreased disease incidence and severity of tomato plants infected by F. oxysporum which recorded(20.6 %,0.14) respectively as compared to both positive (without pathogen)and negative(with pathogen) control treatment(6.90 % , 0.05) , (82.70 % , 0.69) respectively .

الملخص

أجريت هذه الدراسة لدراسة كفاءة ست سلالات من البكتريا المحفزة لنمو النبات(Bacillus subtilis ، Bacillus pumilus ، Pseudomonas fluorescence ،Azotobacter chroococcum1 ،Azotobacter chroococcum2 و.Bacillus sp) كعوامل للمكافحة الاحيائية ضد الفطر الممرض Fusarium oxysporum المسبب المرضي لذبول نباتات الطماطة تحت ظروف البيت المحمي .

أظهرت نتائج دراسة الفعالية التضادية للسلالات البكتيرية الست ضد الفطر Fusarium oxysporum بأن السلالات المختبرة تباينت في قابليتها على خفض معدل النمو للفطر الممرض وفي زيادة النسبة المئوية لتثبيط نمو الفطر الممرض بالمقارنة مع معاملة السيطرة ، كانت البكتريا Bacillus subtilis هي الافضل في خفض معدل النمو للفطر الممرض 20.33 ملم بالمقارنة مع معاملة السيطرة 34.66 ملم .

بينت النتائج ان معظم السلالات المختبرة زادت معنويا" النسبة المئوية لانبات البذور واليوم الاول للانبات،كانت السلالات البكتيرية Bacillus subtilis ، Pseudomonas fluorescence و Bacillus pumilus هي الافضل في زيادة نسبة انبات البذور حيث سجلت 97.33، 95.33 ، 93.66 % وعلى التوالي بالمقارنة مع معاملة السيطرة 77.40 % .

أظهرت نتائج تجربة البيت المحمي بأن النباتات المعاملة بالبكتريا Bacillus subtilis سجلت اعلى طول المجموع الخضري و طول المجموع الجذري و الوزن الطري والجاف للنبات و عدد الافرع / نبات و عدد الثمار وانتاجية النبات حيث زادت جميع هذه المعايير الى317.60 ،29.40 سم ، 456.69 ، 298.60 غم/ نبات،5.1 فرع / نبات ،11.63ثمرة / نبات ، 1361.66غم / نبات وعلى التوالي،اظهرت النتائج ايضا"بان Bacillus subtilisخفضت معنويا"نسبة وشدة اصابة نبات الطماطة بالفطرFusarium oxysporum حيث سجلت 20.60%،0.14 على التوالي بالمقارنة مع معاملتي السيطرة الموجبة (بدون وجود المسبب المرضي) والسالبة (بوجود المسبب المرضي) حيث حققتا % 6.90 ، 0.05 ، 82.70% ، 0.69 وعلى التوالي .

Introduction

Tomato(*Lycopersicon esculentum Mill.*) is one of the most popular and important commercial vegetable crops grown throughout the world (1) .Tomato is affected by a number of fungi diseases causing substantial losses in yields including root rot and vascular wilt and damping – off diseases , which inflict heavy losses in its production (9) . Tomato wilt disease caused by *F. oxysporum f. sp. lycopersici* and caused reduction in weight of tomato fruits and productivity (5) . *F. oxysporum*is economically important wilt pathogen of tomato in the world (11) . Management of this pathogen is difficult due to their endophytic growth and persistence in soil (3) . Several disease management strategies are available e.g. resistant cultivars , biological control , crop rotation and chemical fungicides . A promising strategy for replacement of chemicals has been the implementation of biocontrol technology(4) .

Microorganisms such as plant growth promoting bacteria (PGPB)are a group of root associated bacteria which intimately interest with plant roots and consequently influence plant health and soil fertility (10).These PGPB strains are known to reduce fungal diseases in a number of crops by producing secondary metabolites and antimicrobial substances which have antagonistic activity against many of phytopathogenic fungi and bacteria that causing diseases in plants (4,12, 18,20,21) . These microorganisms promote the circulation of plant nutrients and reduce the need of chemical fertilizers, which are costly and create environmental problems for warranting high yield and quality (5) . Hence, there has recently been a resurgence of interest in environmentally friendly, sustainable and organic agricultural practices (6) .

They have been a number of inoculated bacterial species called plant growth promoting bacteria(PGPB),including the strains in the genera *Azotobacter, Bacillus , Azosperilium , Rhizobium , Pseudomonas* (7, 15, 16,17) . These bacteria were previously reported as plant growth promoting bacteria and had potential biocontrol agents against a wide range of fungal pathogens (2 , 11 , 14) .

The objective of this study was to determine the effects of inoculation bacteria (*Bacillus subtilis , Bacillus pumilus , Pseudomonas fluorescence , Azotobacter chroococcum*1 *, Azotobacter chroococcum* 2 , *Bacillus sp.*) on control wilt disease caused by *Fusarium oxysporum* and on yield and growth of tomato vegetable crop in greenhouse conditions .

Materials and methods

Bacterial strains and culture conditions :

Strains of plant growth promoting bacteria ,*Bacillus subtilis , Bacillus pumilus , Pseudomonas fluorescence* were obtained from Organic Culture Center/ Ministry of Agriculture, whereas, bacterial strains, *Azotobacter chroococcum* 1 ,*Bacillus sp., Azotobacter chroococcum* 2were isolated from bean , okra rhizosphere and garden soil(table1) , identified according to characteristic feathers of colony and bacterial cells. Bacterial strains were grown on Nutrient Agar (3 g / l beet extract , 5 g / l peptone and 15 g / l agar) for routine use , a single colony was transferred to 250 ml flasks containing Nutrient Broth (3 g / l beet extract , 5 g / l peptone) and grown aerobically in flasks on a rotations shaker (95 rpm) for 48 hr at 28˚C .The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10^8 CFU/ml , and the resulting suspensions were used to treat seeds and seedlings tomato plants .

Fungal pathogen (*Fusarium oxysporum*) :

One isolate of fungal pathogen(*Fusarium oxysporum*)was obtained from Department of Biocontrol for Plant Diseases at Agricultural Researcher Office/Ministry of Science and Technology was used in this study .

Bacterial antagonistic activity evaluation:

The method described by (14) was used to determine antagonistic activity of the six strains of plant growth promoting bacteria (*Azotobacter chroococcum*1 , *Azotobacter chroococcum* 2 , *Bacillus sp., Bacillus subtilis* , *Bacillus pumilus* and *Pseudomonas fluorescence*) against fungal pathogen (*Fusarium oxysporum*) . One 5mm disk of a pure culture of the pathogen was placed at the center of a Petri dish (10 cm diameter) containing PSA(potato sucrose agar media). A circular line , made of a 5cm diameter Petri dish dipped in a bacterial suspension of one of the six strains of bacteria was placed surrounding the fungal pathogen .plates were inoculated for 72 has at 25˚c and growth diameter of the pathogen was measured and compared to control growth ,where the bacterial suspension was replaced by sterile distilled water .

Results are expressed as the means of the percentage of the growth inhibition in the presence of any of the bacterial strains.

Inhibition percentage was calculated using the following formula:

%Inhibition =[1-fungal growth / control growth] × 100 (13) .

Greenhouse experiment;

The trial was carried out in the Greenhouse of Plant Pathology Department,/Agricultural and Food Technology Researches Center in Al-Zafarania City from 22/3/2011 to4/7/ 2011 to evaluated the interaction between six strains of plant growth promoting bacteria and the pathogen (*F oxysporum*)for their potential to stimulate tomato plants resistance against *Fusarium* tomato wilt disease.One isolate from the pathogen was used as causal agent of tomato wilt disease at rate of 1 ml of fungal suspension(10^5spore /ml)of the7days old culture on PSA(Potato sucrose agar medium) per 1 ml of soil before planting . Tomato seeds were soaked in bacterial suspension(10^8 CFU/ml) for 10 min and air dried before seeded at rate of 5 seeds / pot .Tomato seeds were sown in trays (30 cm , 50 cm , 10 cm deep) containing autoclave coarse clay sand (1:1 v/v) and watered twice a week. after 45 days, similar healthy seedlings(10 cm length)were uprooted and treated with bacterial suspension of the six strains (10^6 CFU / ml) and planted in holes at rate(2 seedlings / hole) on 26 / 5 / 2011 and remained till 4/7/2011. Treatments were distributed in greenhouse according to Randomized Complete Blocks Design (RCBD) in three replicates (each replicate 10 plants) to evaluated the following treatments :

1- Control .
2- Pathogen only .
3- Azotobacter chroococcum 1+ pathogen .
4- Azotobacter chroococcum 2 + pathogen .
5- Bacillus sp. + pathogen .
6- Bacillus subtilis + pathogen .
7- Bacillus pumilus + pathogen .
8- Pseudomonas fluorescence + pathogen .

Plants were harvested at the end of the experiment , growth parameters were recorded beside disease incidence and severity as following :

1-Mean length of shoot and root of plants .
2-Fresh and dry weight of plants .
3-Seeds germination and the first day of germination .
4-Fruits weight and plant productivity .
5-Disease incidence and severity .

Diseases severity assessments.

Disease severity (DS) was estimated after 8 weeks from transplanting, as a wilting percent using the rating scale in which infected plants were classified according to a numerical grades ranging from 0 to 4 as follows:
0=healthy, 1=1-25 of plant leaflets are yellow and of vascular root bundles are dark brown, 2=26-50 of plant leaflets are yellow and of vascular root bundles are dark brown, 3=51-75 of plant leaflets are yellow and of vascular root bundles are dark brown, 4=76-100 of plant leaflets are yellow and of vascular root bundles are dark brown.
DS%=E(1A+2B+3C+4D)/4T x 100 where, A, B, C and D are the number of plants corresponding to the numerical grade, 1, 2,3 and 4 respectively and 4T is the total number of plants (T) multiplied by the maximum discoloration grade 4, where T=A+B+C+D.
For each treatment 10 plants were used (two plants per pot) to determine DS. The experiment was repeated twice. Reduction % was calculated using the formula of (8).

Results and Discussion

The results of the antagonistic activity of the six bacterial strains against the phytopathogenic fungus (*F. oxysporum*) showed that the tested bacteria varied in their abilities in reducing radial growth rate of the pathogen and increasing percentage of pathogen inhibition as compared to control treatment (table 2) . Results appeared that *B. subtilis* was the superior in reducing radial growth rate of pathogen which recorded (20.33mm) as compared to control treatment(34.66 mm) , also *B. subtilis* significantly increased the percentage of pathogen inhibition (41.33 %) while , *Bacillus sp*. recoded the lowest in percentage of pathogen inhibition (6.73 %) . The varied in their bacterial strains abilities may be due to the strain type and the type of metabolic materials that produced by the strains in culture media (2) .

The effect of tomato seeds treatment with bacterial suspension of the six strains on seed germination showed that most the tested strains significantly increased the percentage of seeds germination and the 1st germination of tomato seeds (table 3) . The bacterial strains , *B. subtilis* ,*P. fluorescence* and *B. pumilus* were the best in increasing the seeds germination which recorded (97.33 , 95.33 and 93.66) % as compared to control treatment (77.40) % . These results were due to the plant growth promoting materials that produced from this strains (21) .

Results of greenhouse experiment revealed that tomato seeds and seedlings treatment with the bacterial suspension of strains significantly improved most the plant growth parameters (shoot length , root length , no. of branches / plant , fresh and dry weights of plant) as compared to both negative and positive control treatments (table 4) .*B. subtilis* was the superior in increasing the plant growth parameters which recorded (317.6 , 29.4) cm , (5.1) branch / plant ,(1456.6 , 298.6) g /plant respectively as compared to positive and negative control treatments (225.0 , 110.6) cm , (16.7 , 6.3) cm , (3.5 , 1.2) branch / plant , (638.3 , 218.6) g / plant , (174.0 , 57.6) g / plant respectively (table 4) . *B. subtilis* + pathogen treatment recorded (308.3 , 22.0) cm , (3.7) branch / plant , (937.3 , 237.6) g / plant respectively . Results appeared that growth parameters of treatments did not affected by pathogen (*F. oxysporum*) , these results are related to the plant growth promoting substances such as IAA and gibberellins and secondary metabolites that produced by these bacteria (12 , 19 , 20) .

The results of table 5 revealed that most the tested treatments significantly increased no. of fruits / plant and plant productivity as compared to control treatment,*B.subtilis* was the best in increasing no. of fruits / plant and plant productivity which recorded(11.63) fruit / plant , 1361.6 g / plant respectively while , *P. fluorescence* plus pathogen recorded 9.80 fruit / plant , 1078.0 g / plant respectively as compared to control treatment (6.16) fruit / plant , 697.6 g / plant respectively , also the results revealed that *B. subtilis* treatment increased the percentage of plant product (95.30 %) , while P. fluorescence recorded (80.20 %) .

Application of tomato plants with plant growth promoting bacteria greatly reduced percentage of disease incidence of tomato plants as compared with infected control (table 6) .thus ,these treatments improved plant health through reducing wilt symptoms . our results are in harmony with other researchers (8 , 11) .

Results also showed that inoculation of tomato plant with these bacteria are more effective in reducing disease incidence and severity than control treatment (pathogen alone) . treatment of *B. subtilis* plus pathogen had a higher efficacy in reducing disease incidence and severity (20.6 % , 0.14) as compared to pathogen alone treatment (82.7% , 0.69) . this efficiency might be related to the elimination of pathogen by the production of cyanide and siderophores by plant growth promoting bacteria especially the genus *Bacillus* and *Pseudomonas* (12 ,16 ,21) . Our results showed that *B. subtilis* and *P. fluorescence* , were able to reduce disease incidence and severity of *F. oxyproum* in tomatoes by stimulating vegetable growth and root development of the treated plants . From these results it may be concluded that application of PGPB provide a reasonable level of protection against *F. oxysporum* in tomatoes under greenhouse conditions .

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Table 1 : Source of bacterial isolates used in this study .

Bacterial isolates	Source	Location
Azotobacterchroococcum1	Bean rhizosphere (Phaseolus vulgaris)	Field in Al-Tweetha town / south east of Baghdad
Azotobacterchroococcum2	Garden soil	Al-Zafarania city / Baghdad
Bacillus sp.	Okra rhizosphere (Hibiscus esculentus)	= = =
Bacillus subtilis	Ministry of Agriculture / Organic Culture Center	
Bacillus pumilus	= = =	
Pseudomonas fluorescence	= = =	

Table 2 : Antagonistic activity of bacterial strains against *F. oxysporum*

Treatment	Radial growth rate (mm)	% Inhibition
Control	34.66	0.0
B. subtilis	20.33	41.33
B. pumilus	25.33	26.93
P. fluorescence	22.33	35.56
A.Chroococcum 1	29.66	14.36
A.Chroococcum 2	28.33	18.23
Bacillus sp.	32.33	6.73
LSD(P=0.05)	1.01	2.85

Table 3 : Effect of tomato seeds with bacterial suspension on seeds germination under greenhouse conditions

Treatment	Seeds germination	1st germination after ( ) days
Control	77.40	15.0
B. subtilis	97.33	10.6
B. pumilus	93.66	13.0
P. fluorescence	95.33	11.3
A.Chroococcum 1	80.66	14.0
A.Chroococcum 2	85.33	13.6
Bacillus sp.	73.66	14.0
LSD (P= 0.05)	2.26	0.93

Table 4 : Treatment of tomato seeds with bacterial suspension and their effect on some plant growth parameters infected by*F. oxysporum* under greenhouse conditions .

Treatment	Shoot length cm	Root length cm	Branch no./plant	Fresh plant w.	Dry plant w.
Control	225	16.7	3.5	683.3	174
Pathogen	110.6	6.3	1.2	218.6	57.6
B. subtilis	317.6	29.4	5.1	1456.6	298.6
B. pumilus	282.3	21.1	4.1	994.0	245.6
P. fluorescence	292.6	21.4	4.3	1037.6	275.0
A. Chroococcum 1	241.3	18.8	3.5	857.0	229.6
A.Chroococcum 2	252.6	20.0	4.0	885.0	236.0
Bacillus sp.	224.3	16.5	3.4	711.6	177.6
B. subtilis + Path.	308.3	22.0	3.7	937.3	237.6
B. pumilus + Path.	274.0	19.9	3.5	873.0	229.0
P. fluorescence+ Path.	282.0	19.8	3.8	995.0	254.0
*A.Chroococcum*1+Path.	232.3	18.0	2.7	696.3	185.6
*A.Chroococcum*2+Path.	239.6	19.5	3.3	746.6	191.3
Bacillus sp. + Path.	212.3	16.4	2.6	648.0	174.0
LSD (P= 0.05)	5.78	0.94	0.36	26.15	28.31

Table 5 : Rate of fruits no. and plant productivity of tomato plants under greenhouse conditions .

Treatment	Fruit no.	Plant productivity	% Increased product
Control	6.16	697.6	-
Pathogen	0.0	-	-
B. subtilis	11.63	1361.6	95.30
B. pumilus	9.06	1197.6	71.73
P. fluorescence	10.08	1257.3	80.23
A. Chroococcum 1	6.40	809.3	16.01
A.Chroococcum 2	6.93	839.0	20.26
Bacillus sp.	5.90	753.3	5.40
B. subtilis + Path.	9.43	958.3	37.37
B. pumilus + Path.	8.20	951.6	36.44
P. fluorescence+ Path.	9.80	1078.0	54.52
A.Chroococcum 1+ Path.	6.20	769.0	10.23
A.Chroococcum 2+ Path.	6.33	771.0	10.52
Bacillus sp. + Path.	5.76	697.6	0.0
LSD (P= 0.05)	0.50	42.91	8.86

Table 6: Disease incidence and severity of tomato plants infected by *F. oxysporum* under greenhouse conditions .

Treatment	Disease incidence %	Disease severity
Control	6.9	0.05
Pathogen	82.7	0.69
B. subtilis + Path.	20.6	0.25
B. pumilus + Path.	29.4	0.14
P. fluorescence+ Path.	24.9	0.27
A.Chroococcum 1+ Path.	50.4	0.55
A.Chroococcum 2+ Path.	47.2	0.46
Bacillus sp. + Path.	49.3	0.49
LSD (P= 0.05)	2.40	0.04