EFFICIENCY OF DUAL INOCULATION WITH MYCORRHIZAL FUNGI AND *Azotobacter chroococcum* IN STIMULATING TOMATO PLANT RESISTANCE to root-ROT DISEASE CAUSED BY *Rhizoctonia solani*

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ABSTRACT

The results of antagonistic activity of five isolates of *Azotobaetar chroococcum* showed that the isolates varied in their ability in reducing radial growth rate of *R. solani* . Azo.4 and Azo. 5 isolates showed significant reduction in radial growth rate (3.16, 3.03) cm respectively compared to control treatment (5.16) cm .

Results of the effect of *Azotobaetar* suspension dilution on radial growth of *R. solani* revealed that the antagonistic activity was depend upon the isolate type and inoculum level .Azo. 4 and Azo. 5 isolates were the superior in all tested inoculum levels (10, 15 and 20) % which recorded (3.20, 3.13) cm (3.06, 3.03) cm and (3.0, 2.90) cm respectively compared to control treatment (5.0) cm.

Results of greenhouse experiment showed that most treatments significantly increased tomato growth parameters (shoot and root length ,dry and soft weight of shoot and root , no. of leaves and flowers /plant), and decreased the percentage of infected tomato plants by *R. solani* (pre and post emergency).

INTRODUCTION

Tomatoes are the most popular vegetable crop in Iraq . Many diseases can effect tomatoes during the growing season . Soil borne fungi like *Rhizoctonia solani* that causes root rot disease of tomato plant, and causes losses on all vegetables, flowers and several field crops (1). Control methods have traditionally involved cultural practices and the application of chemical fungicides. Alternative measures such as biological control tactics utilizing antagonistic microorganisms are attractive for plant diseases such as root rot disease. Biological control of plant pathogens is currently accepted as a key practice in sustainable agriculture because it is based on the management of a natural resource (7). Nature is bestowed with many biocontrol agents including plant growth promoting microorganisms (PGPM) could regulate plant growth by inducing defense responses in plants via an systematic resistance and suppression plant disease (4). Among the PGPM, arbuscular mycorrhizal fungi which is involved in the most universal intimate and important symbiosis (18).

The rhizosphere is a heterogeneous, continues and natural habitat in which different types of interactions occur between soil microbes and plants (6). Recently the term has been broaded to include both the volume of soil influenced by the root and the root tissues colonized by microorganisms (17). Microorganisms in the rhizosphere react to many metabolites released by plant roots (11). The microorganisms and their products, also interact with plant roots in a variety of positive, negative, and neutral interactions (10),

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such interactions can influence plant growth and development, change nutrient dynamics, and alter a plant's susceptibility to disease and abiotic stress (17).

The beneficial plant–microorganisms interactions in the rhizosphere are the primary determinants of plant health and soil fertility (13). Arbuscular mycorrhizal fungi (AMF) are one of the most beneficial plant – microorganisms interaction for the majority of plants (5, 8). Under phosphate – limited conditions, AMF can influence plant community development , nutrient uptake , water relations, and aboveground productivity , they also act as bioprotectants against pathogens and stress factors (3, 12, 20).

Nitrogen–fixing bacteria (*Azotobacter*) are known to improve the bioavailability of nitrogen to plants (9). *Azotobacter* is also of interest because it has the highest respiratory rate of any living organism (19). *Azotobacter* not only provides N_2 but produced a variety of growth promoting substances such as indole acetic acid (IAA), gibberellins , vitamin-B (15, 22, 2, 21), antimicrobial and antifungal substances (23, 14) .

The aim of this study was conducted to evaluate the efficiency of five isolates of *Azotobaetar chroococcum* and one isolates from each of mycorrhizal fungi (*Glomus mosseae* and *Giqaspora sp.*) as bioagents for stimulating tomato plant resistance to root rot disease caused by *Rhizoctonia solani* .

MATERIAL AND METHODS

Tomato seeds *(Lycopersicon escolentum* Mill.) class hybrid production by TAKII and Co., LTD , Kyoto , Japan were used in this study. *R. solani* isolate was provided from the Integral Management of Agricultural Pests Center / Ministry of Sciences & Technology .

Isolation of *A. chroococcum* from soil

Samples from (10–25) cm layer of 23 soils from rhizospheric soils of different crops were collected in February to March 2008 for the isolation of *A. chroococcum*. The samples were transported to the laboratory for microbial analysis within 24 hrs. Grad dilutions preparation of soil solution (10^{-3} , 10^{-5}) for each sample .One ml from each dilution was placed in 250 ml flask containing 50 ml of N- free Jensen's broth and incubated at 30º C for 2-5 days .The flasks were examined for a film of surface growth formation , and prepare a wet mount preferably of the surface film and observe with compound microscope .Plates of N – free Jensen's agar were streaked and incubated at 30 º C for 1-2 days .The plates were examined for colonies presence, the colonies wet mounted and gram stain examined .The pure colonies were examined and used as inoculums for a slant of N - free Jensen's agar medium . All the isolates of *A. chroococcum* were subjected to biochemical characterizations: Gram stain reaction, Growth on N- free medium containing 1 % (sucrose, mannitol, and rhamnose) as a sole carbon sources (2) .

Preparation a bacterial suspension of *A. chroococcum*

Five isolates of *A. chroococcum* were grown on 100 ml of N- Free Jensen's broth medium using 250 ml flasks, after sterilizing, the flasks were inoculated with one loop full of bacterial cells transferred from N - free Jensen's agar medium. The flasks were incubated at 30˚C in a shaker with gentle agitation 150 rpm for 7, 10, 14 days. The bacterial suspension of each of the five isolates of

A. Chroococcum was extracted by Buckner funnel supplied with filter paper Whatman No.1 and was stored at 4˚C .

Bacterial antagonistic activity evaluation

The method described by Montealegre *et al*. (16) was used to determine antagonistic activity of *A. chroococcum* isolates against fungal pathogen. One 5mm disk of a pure culture of the phytopathogen (*R. solani*) was placed at the center of a Petri dish 10cm diameter containing PSA. A circular line, made of a 5cm diameter Petri dish dipped in a bacterial suspension of one of the five isolates of *A. chroococcum* was placed surrounding the fungal inoculums. Plates were inoculated for 72 hrs. at 25˚C and growth diameter of the pathogen (fungal growth) was measured and compared to control growth ,where the bacterial suspension was replaced by sterile distilled water . Each experiment using a single pathogen isolate was run in triplicate. Results are expressed as the means of the percentage of growth inhibition of the pathogen in the presence of any of the isolates of *A. chroococcum*.

Inhibition percentage was calculated using the following formula :

$$\% \text{ Inhibition} = [\, 1 - \text{control growth} / \text{treatment growth} \,] \times 100 .$$

Effect of bacterial suspension dilution of *A. chroococcum* on growth of *R. solani*

Three dilutions of the bacterial suspension of *A. chroococcum* (7 days culture old) 10, 15 or 20 % from the medium (N – free Jensen's agar medium) were used to determine the effect of bacterial suspension of *A. chroococcum* on radial growth of the tested pathogenic fungus(*R. solani*) . Petri dishes (10 cm diameter) were used , after sterilization , the medium poured in Petri dishes , and the bacterial dilutions of the five isolates of *A. chroococcum* were added to the plates , after the medium hardening, the plates inoculated with disk(5mm diameter) of *R. solani*, and incubation at 25˚C for 3 days . The growth diameter of the pathogen was measured and compared to control growth where the bacterial suspension of *A. chroococcum* was replaced by sterile distilled water .

Interaction between *A. chroococcum* and AM fungi (*G. mosseae* and *Gigaspora sp.*) for their potential to stimulate tomato plants resistance to root rot disease .

One experiment was carried out in greenhouse at Al-Zafarania field south east of Baghdad city from 2 April to 15 June 2008 to evaluated the interaction between two isolates of *A. chroococcum* (Azo. 4 and Azo. 5) with and without pathogen (*R. solani*), and one isolate from each of the AM fungi (*G. mosseae* and *Gigaspora sp*.) for their potential to stimulate tomato plants resistance against root rot disease . One isolate of *R. solani* was used as causal agent of tomato root rot disease at rate of 1ml of fungal suspension (1×10^{5} CFU / ml) of their 10days old culture on PSA per 1 kg sterilized soil. Plastic pots supplemented with 5 kg of formalin sterilized soil were inoculated with mycorrhizal inoculate (spores + hyphae + mycorrhizal roots) at rate of 5 g / pot before 5 days from seeded . Tomato seeds were soaked in bacterial suspension of *A. chroococcum* (1×10^{6} CFU/ ml) were supplemented with 3 % Arabic gum as adhesive agent for 30 min and air dried before seeded at rate of 10 seeds/ pot. Pots distributed in greenhouse (25 ±5˚ C) according to Randomized Complete Blocks Design (RCBD) in three replicates to tested the following treatments :

a-Control , b- *R. solani* only , c-*Glomus* + *R. solani* ,d-*Gigaspora* + *R. solani*, e-*Azotobacter* isolate 4 + *R. solani*, f-*Azotobacter* isolate 5 + *R. solani*, g-*Glomus* +

***Azotobacter* isolate 4 + *R. solani*, h-*Glomus* + *Azotobacter* isolate 5 + *R. solani*, i-*Gigaspora* + *Azotobacter* isolate 4 + *R. solani* j-*Gigaspora* + *Azotobacter* isolate 5 + *R. solani*, k-*Glomus* + *Gigaspora* + *R. solani*.**

Pots daily were observed, and the following growth parameters were recorded at the end of the experiment :

1-The percentage of diseased plants for survival plants (No. of infected plants / Total of observed plants) × 100 .

2-Dry weight of shoot and root system for survival plants .

3-Mean number of leaves and flowers / plant.

3-3-12 Statistical analysis All experiments were conducted at least twice and analyzed by analysis of variance (ANOVA), and followed by least significant difference (LSD) was calculated at $p \leq 0.05$.

RESULTS AND DISCUSSIONS

The results showed that the tested isolates varied in their ability in reducing radial growth rate (RGR) of the tested pathogen and the percentage of pathogen growth inhibition (PPGI) (table 1). Azo.4 and Azo.5 isolates showed significant reduction in RGR of *R. solani* 3.16 , 3.03 cm respectively compared to other three isolates which recorded 4.80, 4.50 and 4.60 cm respectively. Also, Azo.4 and Azo.5 appeared increment in PPGI (38.56 and 41.16 %) compared to Azo.1, Azo.2 and Azo.3 which recorded 6.90,12.79 and 10.85 % respectively .

Table 1: Antagonistic activity between *Aztobacter chroococcum* isolates and pathogenic fungus (*R. solani*)

Treatment	*Rhizoctonia solani*	
	Radial growth rate (cm)	Inhibition*(%)
Azo. 1	4.80	6.90
Azo. 2	4.50	12.79
Azo. 3	4.60	10.85
Azo. 4	3.16	38.56
Azo. 5	3.03	41.16
Control	5.16	0.0
LSD(P = 0.05)	1.19	1.28

*** % Inhibition = (1- control growth / treatment growth) × 100 .Values are an average of three replicates.**

Effect of *A. chroococcum* suspension dilution on growth of *R. solani*

The results revealed that the antagonistic activity was depend upon the isolate type and inoculum level (table 2). Although inoculum level of all tested isolates induced significant reduction in fungal growth , but isolates Azo.4 and Azo.5 were the superior when used in all tested inoculum levels 10 , 15, and 20 % which showed significant reduction in radial growth of *R. solani* 3.20 , 3.13 cm, 3.06, 3.03 cm and 3.0, 2.9 cm respectively compared to control treatment (5.0 cm) . these results is due to the antagonistic metabolites secreted by *Azotobacter* isolates, and suggests that the mode of action exerted and the type of antifungal metabolites produced by *Azotobacter* isolates was varied. Reduction of fungal growth of *R. solani* and formation of inhibition zones were presumably due to the material (antifungal substances and/or cell wall degrading enzymes) released by *Azotobacter* into the culture medium (14, 23).

Table 2. Effect of *A. chroococcum* suspension dilution (7days culture age) on radial growth of *R. solani*

Treatment	Bacterial suspension dilution					
	10 %		15 %		20 %	
	Radial growth cm	Inhibition *%	Radial growth cm	Inhibition *%	Radial growth cm	Inhibition *%
Azo. 1	4.50	10.0	4.43	11.33	4.30	14.0
Azo. 2	4.06	18.66	4.0	20.0	3.93	21.33
Azo. 3	4.20	16.0	4.03	19.33	3.86	22.66
Azo. 4	3.20	36.0	3.06	38.66	3.00	40.0
Azo. 5	3.13	37.33	3.03	39.33	2.90	42.0
Control	5.00	0.0	5.00	0.0	5.00	0.0
LSD(P= 0.05)	0.06	1.31	0.19	1.85	0.07	1.39

***% Inhibition = (1-control growth / treatment growth) ×100 .**

Values are an average of three replicates

Efficiency of *Azotobacter* isolates and AM fungi and their combination in protecting tomato plants from root rot disease caused by *R. solani* under greenhouse conditions.

The results revealed high compatible performance between *A. chroococcum* isolates and each of *G. mosseae* and *Gigaspora sp.* as manifested by the significant reduction in disease incidence (pre and post emergence damping-off) (table 3). Azo.5 + *Glomus* + *R. solani* treatment showed highest decrement in disease incidence (43.33 %) compared to *R. solani* treatment (83.33 %), while *Gigaspora* + *R. solani* treatment showed lowest decrement (66.66 %). Bioagents, alone or in combination induced significant reduction in disease incidence (table3), but in the same time not all combination showed the same efficiency in reducing disease incidence which reflected different level of compatibility between the bioagents whoever Azo. 5 + *Glomus* + *R. solani* , *Glomus* + *Gigaspora* + *R. solani*, Azo. 5 + *Gigaspora* + *R. solani* treatments which recorded 43.33, 46.66 and 50.0 % respectively were the best .

Table 3: Efficiency of *A. chroococcum* isolates and AM fungi and their combination in protecting tomato plants from root rot disease caused by *R. solani* under greenhouse conditions

Treatment	% Infected tomato plants with *R. solani*		
	Pre-emergency	Post-emergency	Pre and post emergency
Control	3.33	3.33	6.66
R. solani only	76.66	6.66	83.33
Azo.4 + *R. Solani*	23.33	26.66	50.0
Azo. 5 + *R. Solani*	20.00	36.66	56.66
Gigaspora + *R. solani*	30.00	36.66	66.66
Glomus + *R. solani*	40.00	20.00	60.00
Azo 4 + *Glomus* + *R. solani*	36.66	16.66	53.32
Azo. 5 + *Glomus* + *R. solani*	33.33	10.00	43.33
Azo. 4+ *Gigaspora*+ *R. solani*	33.33	26.66	60.0
Azo. 5 +*Gigaspora*+ *R. solani*	26.66	23.33	50.0
Glomus + *Gigaspora*+ *R. solani*	30.00	16.66	46.66
LSD (P = 0.05)	7.80	8.84	10.63

Values are an average of three replicates in each replicate 10 plants

The effects of seeds treatment with two *Azotobacter* isolates (Azo.4 and Azo.5) and soil treatment with *G. mosseae* and *Gigaspora sp.* showed that all tested bioagents separately or in combination had significantly increased most tomato shoot growth parameters (shoot length , shoot fresh and dry weight , and no. of leaves and flowers / plant) (table 4) .

Azo.5 + *Gigaspora* + *R. solani* treatment showed the highest increment in mentioned shoot growth parameters of tomato plants 48.330 cm , 107.16 g/ plant, 44.33 g/plant, 51.66 leaves/plant and 34.0 flowers/plant respectively as compared to both positive control treatment 24.31cm, 65.26 g / plant , 19.83 g /plant, 23.90 leaves/plant and 12.46 flowers/plant and negative control treatment (*R. solani*) which were recorded 18.46 cm, 25.70 g / plant, 9.03 g/ plant, 6.96 leaves / plant and 1.93 flowers / plant respectively.

Table 4: Interaction between *A. chroococcum* and AM fungi and their effects on shoot growth of tomato plants infected with *R. solani* under greenhouse conditions

Treatment	Shoot length cm	Shoot fresh weight g/plant	Shoot dry weight g/plant	No. of leaves / plant	No. of flowers / plant
Control	24.31	65.26	19.83	23.90	12.46
R. solani only	18.46*	25.70*	9.03*	6.96*	1.93*
Azo.4 + *R. solani*	29.43	70.23	22.90	29.30	14.33
Azo.5 + *R. solani*	33.71	73.63	25.30	31.0	16.46
Gigaspora + *R. Solani*	37.55	75.53	28.0	34.63	16.73
Glomus + *R. Solani*	42.36	90.50	30.83	36.93	26.70
Glomus+Gig+ R. solani	42.40	102.30	34.26	42.70	32.23
Azo4 + *Glo* + *R. solani*	40.16	97.03	32.93	40.0	22.90
Azo.5 + *Glo* + *R. solani*	45.76	107.16	42.63	48.30	33.46
Azo.4 + *Gig* + *R. solani*	46.80	101.13	33.86	42.60	28.33
Azo.5 + *Giga* + *R.solani*	48.30	107.16	44.33	51.66	34.0
LSD (P= 0.05)	2.31	1.95	1.30	1.81	1.46

Values are an average of three replicates in each replicate 10 plants .

*** Values in *R. solani* treatment are an average of three replicates in each replicate 5 plants .**

The results of treatment of tomato seeds with *Azotobacter* isolates 4 and 5, and soil treatment with *G. mosseae* and *Gigaspora sp.* showed that most of tested treatments significantly (P = 0.05) improved all the root growth parameters (root length, root fresh and dry weight) as compared to *R. solani* treatment (table 5) .The results revealed that Azo. 5 + *Gigaspora* + *R. solani* and Azo.5 + *Glomus* + *R. solani* treatments showed the highest capability as manifested by the significant increment in root length , root fresh and dry weight 30.43, 32.03 cm, 31.96, 30.96 g / plant , 9.36 , 7.96 g / plant respectively as compared to *R. solani* treatment 6.66 cm, 4.33 g/plant, 1.83 g/plant respectively . These results due to many factors includes , stimulation of host-plant disease resistance mechanisms , improvement of plant nutrition, direct interaction between AM fungal mycelium and pathogen (St-Arnaud *et al.* (20). *Azotobacter* produces an antifungal antibiotic which inhibits the growth of the pathogen , and production of phytohormones , have all been suggested to explain the interaction between AM fungi and *Azotobacter* in stimulating tomato plants resistance to root rot disease .

Table 5: Interaction between *A. chroococcum* and AM fungi and their effects on root growth of tomato plants infected with *R. solani* under greenhouse conditions .

Treatment	Root length (cm)	Root fresh weight (g / plant)	Root dry weight (g / plant)
Control	17.26	20.33	5.03
R. solani only	6.66*	4.33*	1.83*
Azo.4 + *R. solani*	17.83	21.83	5.90
Azo. 5 + *R. solani*	18.80	22.80	6.43
Gigaspora + *R. solani*	21.66	26.20	7.46
Glomus + *R. solani*	24.36	26.93	7.50
Glomus + *Gigaspora* + *R. solani*	28.03	30.46	8.76
Azo. 4 + *Glomus* + *R. solani*	26.43	28.36	7.43
Azo. 5 + *Glomus* + *R. solani*	32.03	30.96	7.96
Azo. 4 + *Gigaspora* + *R. solani*	29.50	27.36	7.66
Azo. 5 + *Gigaspora* + *R. solani*	30.43	31.96	9.36
LSD = 0.05	1.30	1.38	0.40

Values are an average of three replicates in each replicate 10 plants . * Values in *R*

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كفاءة التلقيح المزدوج بفطريات المايكورايزا وبكتريا *Azotobacter chroococcum* في تحفيز مقاومة نبات الطماطة لمرض تعفن الجذور المتسبب عن الفطر *Rhizoctonia solani*

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الملخص

بينت نتائج الفعالية التضادية للعزلات الخمس للازوتوباكتر بأن العزلات قد تباينت في قابليتها على خفض معدل النمو القطري للفطر ***R. solani*** ، أظهرت العزلتان **Azo. 4** و **Azo. 5** خفضاً معنوياً لمعدل النمو القطري للفطر ***R. solani*** (**3.16** ، **3.03**) سم وعلى التوالي بالمقارنة مع معاملة السيطرة (**5.16**) سم .أظهرت نتائج تأثير تركيز الراشح لبكتريا الازوتوباكتر في معدل النمو القطري لفطر ***R. solani*** بأن الفعالية التضادية أعتمدت على نوع العزلة ومستوى اللقاح .كانت العزلتين **Azo. 4** و **Azo. 5** هي الافضل وفي مستويات اللقاح جميعها هي (**10** ، **15** و **20**) % التي سجلت (**3.20** ، **3.13**) سم ، (**3.06** ، **3.03**) سم و (**3.0** ، **2.90**) سم وعلى التوالي بالمقارنة مع معاملة السيطرة (**5.0**) سم .

اظهرت تجربة البيت المحمي إن معاملة بذور الطماطة بالراشح البكتيري للازوتوباكتر ومعاملة التربة بفطري المايكورايزا (***Glomus mosseae*** و ***Gigaspora sp.***) قد زاد معنوياً معظم معايير النمو المدروسة (طول المجموع الخضري والجذري والوزن الطري والجاف للمجموع الخضري والجذري ،وعدد الأوراق وعدد الأزهار / نبات)، كما خفضت معظم المعاملات معنوياً النسبة المئوية لنباتات الطماطة المصابة بالفطر الممرض ***R. Solani*** (قبل وبعد البزوغ) .

جزء ن اطروحة دكتوراه للباحث الاول

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