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Research Article

STUDY BIOCONTROL EFFICACY OF *PSEUDOMONAS FLUORESCENS* AND *BACILLUS SUBTILIS* AGAINST *RHIZOCTONIA SOLANI* AND *FUSARIUM OXYSPORUM* CAUSING DISEASE IN TOMATO (*LYCOPERSICON ESCULENTUM* L.)

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ABSTRACT
The present research deals with the study of antagonistic effect of microorganism against tomato disease caused by *Rhioctonia solani and Fusarium oxysporum*. four bacterial strains of, *Bacillus subtilis* and fife strain of *Pseudomonas fluorescens* were isolated from tomato field soil The antagonistic microorganisms against the pathogens were observed by Dual Culture Technique. *P. fluorescens* 5 isolate was found to show 81.3% and 77.4 of growth inhibition against *R.solani and F. oxysporum* respectively while *B. subtilis*177.4% and 73.2 of growth inhibition against test pathogens respectively. In greenhouse and field experiments, soil treatment with a suspension of biocontrol agents before planting reduced significantly the incidence of diseases on tomato plants , Under greenhouse conditions, *B. subtilis* and\ or *P. fluorescens* were shown to be effective reduction of the damping off of tomato seedlings , disease severity and survival rate on tomato plants, also under field experiments enhanced values of growth parameters (number of branches, plant height, fresh and dry weight)in tomato plants, and also were gave higher records of yield and yield components, (number of fruits, fruits yield of plants) compared to infected control. In general, *P. fluorescens*5 isolate were more effective than *Bacillus subtilis*1 isolates. This study suggests that bacterial antagonists might be potential biological control agents of tomato plants in Iraqi soil.

Keywords: *Tomato, Bacillus subtilis, Pseudomonas fluorescens, Rhioctonia solani Fusarium oxysporum*

INTRODUCTION
Tomato (*Lycopersicon esculentum* L.) is considered one of the most important economic vegetable crops in Iraq. However, there are many constraints that come in the way of tomato production. Often, it is affected by many diseases leading to substantial losses in yield however; it is susceptible to over 200 pathogens that cause severe destruction for this plant and consequent great reduction in the yield. *Fusarium oxysporum* is the main casual of the tomato root rot disease in tomato plants. Moreover, *Rhizoctonia solani* are highly destructive pathogens of both greenhouse and field grown tomatoes causing damping-off diseases (Curtis *et al*., 2010). Various methods for controlling such diseases have been investigated including the use of resistant varieties (Brisa *et al*., 2007), chemical control, plant volatile compounds (El-Mougy *et al*., 2007), plant extracts (Kumar and Tripathi,1991) and biological control, (Dubey *et al*., 2007; Rojo *et al*., 2007; Hafez *et al*., 2013). Limited information is available on its sustainable management and is generally treated by chemical applications. Overuse of the chemical may result in environmental, human health and pest resistance problem. The increasing awareness of fungicide-related hazards has emphasized the need for adopting biological methods as an alternative disease control method, which is also ecofriendly (Khare *et al*., 2010). Biological control is an efficient and environmentally friendly way to prevent damping-off disease. Many microbial species such as *Trichoderma viride* (Hafez *et al*., 2013), Pseudomonas fluorescens and *Bacillus subtilis* have been shown to effectively control plants pathogens (Sivasakthi *et al*., 2014). The development of formulations and delivery systems for biocontrol by using antagonistic microorganisms to suppress the incidence of diseases caused by soil borne pathogens is a great importance (Çiğdemand Merih, 2005). Ideal formulation additives should improve the biocontrol efficacy of the antagonist but should not support the

growth of the pathogen or cause any damage to the host plant (Wiyono *et al*., 2008). The objective of the present study was to evaluate some antagonistic bacterial agents against *F. solani in vitro* and *in vivo.* Preparation of different carrier formulations of antagonistic bacteria, its effect on root rot of cantaloupe in greenhouse and field conditions, and the effect of the storage period on biological activity of formulating antagonistic bacteria were also investigated.

MATERIALS AND METHODS

Isolation of Pathogenic Fungi and Inoculum Preparation

Pathogenic isolates of *R. solani* and *F. oxysporum* were isolated from naturally infected roots of diseased tomato plants, showing damping off of seedlings, root rotted and wilted plants symptoms grown in Iraq Governorate, Baghdad. It was microscopically identified on the basis of cultural and microscopic characteristics. The isolate was maintained on PDA medium at 4°C. Inocula were prepared by growing isolates of the tested fungi in 250 ml conical flasks each containing 100 ml of Czapek's broth medium. They were inoculated separately with 5 mm agar disc obtained from 7-days old cultures of *R.solani* or *F. oxysporum*. The flasks were incubated at 25oC for 10 days. Resulting mycelial growth of the tested fungi was decanted, washed with distilled water, suspended in 100 ml of distilled water and blended for 5 minutes using a Warring blender. For soil infestation, 30 ml of fungal suspensions containing 5x106 cfu/ml were added to 30 cm diameter pots, filled with steam sterilized sandy loamy soil 7 days before planting (Abdel-Kader, 1997). Pots containing non-infested soil were used as control.

Biocontrol Agents

Fife *Pseudomonas fluorescens* and four *Bacillus subtilis* isoletes taken from laboratories in Department of biology, College of Science University of AL- Mustansarya, Baghdad, Iraq. *P. fluorescens* and *B. subtilis* inoculum was prepared and counted by plate count technique (108 CFU / ml) as maintained by (Mosa *et al*., 1997).

Efficacy of Antagonistic Bioagents against R. solani and F. oxysporum In Vitro

The antagonistic effect of the tested biocontrol agents against *R. solani* and *F. oxysporum* was examined. a streak of the bacterial strain *P. fluorescens* and *B. subtilis* was placed on PDA plates at 28°C for 24 h., then a mycelial disc (0.5cm) of the test fungi was placed onto PDA plates at 0.5 cm distant from the bacterial colony. Three replicates were prepared in each experiment. Inoculated plates were incubated at 28°C until the fungal growth of the control plates reached the edge of the plate. The growth and reduction in mycelial growth of the pathogenic fungus was calculated according to (Fokemma, 1973). Linear growth of R.solani and F.*oxysporum* was recorded. Inhibition percent of growth was calculated using the following formula: Growth reduction (%) = (Growth in control - Growth in treatment / Growth in control) X 100.

Pot Experiment

A pot experiment was designed under greenhouse conditions using plastic pots (15 cm, diameter) containing reasonable weight of sterilized soil. Soil was infested with30 ml of *R. solani* and\or *F. oxysporum* suspensions containing 5x106 cfu/ml before sowing. Infested pots were irrigated for 5 days before sowing. Ten tomato seeds (*Lycopersicon esculentum)* were sown in each pot; three replicate pots were specified for each treatment in completely randomized experimental design. The experiment included the following treatments: 1) non infested soil (control), 2) soil treated with *Rhizoctonia solani* only, *3) F. oxysporum* only 4) *Rhizoctonia solani+ B. subtilis*, 5) *Rhizoctonia solani+ P. fluorescens,* 6) *Rhizoctonia solani* +combination of two bioagents 7) 8) *F. oxysporum + B. subtilis* 8) *F. oxysporum + P. fluorescens* and 9) *F. oxysporum* +combination of two bioagents. Pots were kept under greenhouse conditions till the end of the experiment. Disease assessment for incidence of pre and post emergence damping-off, of seedlings were determined after three weeks of sowing.

Field Experiment

Field experiments were conducted at the Experimental field of ministry of technology and science department of Agriculture research, Baghdad, Iraq, in 2012 and 2013 growing seasons. The chosen field test area was (3.5x3.5 m) field plots each comprised of 7 rows and 15 holes/ row were used in split-plot

design. Three plots were used as replicates for each treatment as well as for untreated control treatment. Tomato seeds were sown at the rate of 3 seeds/hole. Infection and survival percentage were recorded after 45 days from transplanting. Numbers of branches, plant height, fresh and dry weight of plants were determined. Plant yield were calculated for all applied treatments and control as well.

Statistical Analysis

Split-plot design was used for the experiments in greenhouse and field condition. Data were subjected to statistical analysis using analysis of variance using the Statistical Analysis System (SAS, 2001), and means were compared using L.S.D. test.

RESULTS AND DISCUSSION

Antagonistic Activities of Bioagentes against R.solani and F.oxysporum

The bioagentes *(P. fluorescens* and *Bacillus subtilis*) isolates were tested for antagonistic effect against *R.solani* and *F.oxysporum* on Petri dishes, all the investigated bioagentes isolates showed individual inhibition patterns towards at tow fungus, and even but weakly in some isolates, this shows that antagonism is fungicidal in nature. On this basis, we selected the most efficient antagonistic isolates based on the criteria showing capacity of mycelia inhibition of at two fungi, Table 1 showed that among four isolates from *B. subtilis* isolates that *B. subtilis* 1 had greatly inhebition on the linear growth of both *R.solani*and *F. oxysporum*. Results obtained in table 1 indicate that the isolate of *R. solani* its growth was inhibited by 77.4%. While, the growth of *F. oxysporum* was inhibited by *B. subtilis*1, by 73.2.the isolate *P. fluorescens*5 had a significant antagonistic from fife isolates on thelinear growth of *R solani* and *F. oxysporum*it was81.3 and 77.4%, respectively comparing with the control. This behavior represents an important approach for controlling a root rot disease and damping off of tomato seedlings. The potentialities of the used strains could be attributed to their effect to secrete hydrolytic enzymes or antifungal metabolites. Moreover (Montealegre *et al*., 2003) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens.

Table 1: Effect of *P. fluorescens* and *B. subtilis*on the radial growth of *R. solani*

Antagonists	**Inhibition (%) of**	
	R. solani	***F. oxysporum***
P. fluorescens1	69.9	64.8
P. fluorescens2	78	69.6
P. fluorescens3	43.5	45.4
P. fluorescens4	23.9	13.7
P. fluorescens5	81.3	77.4
B. subtilis1	77.4	73.2
B. subtilis2	63.7	59.3
B. subtilis3	46.1	34.9
B. subtilis4	66.4	68.5
L.S.D (0.05) .	1.54	1.98

The potentialities of the used strains could be attributed to their effect to secrete hydrolytic enzymes or antifungal metabolites. Also, (Sarhan *et al*., 2001) and (Montealegre *et al*., 2005) pointed that the cell free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, spore germination and germ-

tubes length of *F. oxysporum*. Moreover, *P. fluorescens* secrete antifungal metabolites such as Lipopeptide cyclic, Amphisin in addition it can secrete several hydrolytic enzymes such as Endochitinase, Chitinase, β-1, 4 glucanase, β-1,3 glucanase, Protease and Lipase which have an inhibitory effect on fungal pathogens (Saad, 2006).

Effect of Biocontrol Agents of Percentage of Damping off of Seedlings under Greenhouse Conditions

Under greenhouse conditions, *B. subtilis* and\ or *P. fluorescens* were shown to be effective in reduction of damping off of tomato seedlings, when applied in mix with *F. oxysporum* or *R. solani*, compared to soil infested only with the pathogens. Data presented in table 2 reveal that soil infested with *R. solani*and *P. fluorescens*has significantly decreased damping off of tomato seedlings and rate (10.8%) compared to soil infested only with the *R. solani* only (54%), while treated soil only with *F. oxysporum* reduction of damping off of seedlings were more higher (66.5%). Compared with the treatment *F. oxysporum* + *P. fluorescens* the reduction was (Saad, 2006; El-Mougy *et al*., 2007). Table 2 show that the efficiency of *B. subtilis* to antagonize *R. solani* or *F. oxysporum* under greenhouse conditions were (17.1%, 14.1 %) respectively. However, higher percentage in reduction of damping off of tomato seedlings was attained in response to treatment with dual bioagents (*B. subtilis*and *P. fluorescens*) than the individual one. It were (9.4%, 7.2%) in soil infested with *R. solani*, *F. oxysporum* respectively

Table 2: Effect of biocontrol agents in reduction of damping off of tomato seedlings

Treatment	Damping off (%)	
	Pre-emergence	Post-emergence
Control	22.3	22.9
Rhizoctonia solani only	54	42.3
Rhizoctonia solani+ *P. fluorescens*	10.8	7.6
B. subtilis Rhizoctonia solani+	17.1	15.9
Rhizoctonia solani +combination of two bioagents	9.4	6.5
F. oxysporum only	66.5	52.1
F. oxysporum+ *P. fluorescens*	19.3	12.4
B. subtilis F. oxysporum+	14.1	10.3
F. oxysporum +combination of two bioagents	7.2	6.8
L.S.D. (0.05)	2.54	1.43

The production of antifungal metabolites proposed as a main mechanism of antifungal activity of *Pseudomonas fluorescens* against *Fusarium oxysporum* (Karkachi *et al*., 2010). (Manikandan *et al*., 2010) reported that *Pseudomonas fluorescens*as an effective biocontrol agent reduced several plant diseases. Biocontrol *P. fluorescens* implicated in the suppression of fungal root diseases by various mechanisms including production of antibiotics, toxins, bio-surfactants, or lytic enzymes, competition for colonization sites, nutrients and minerals, and induction of systemic resistance (Erdogan and Benlioglu, 2010). Although Bacillus has shown some of the above-mentioned new mechanisms such as antibiotic production and spore formation, they do not show others, including siderophore production (Nielson *et al*., 1998). (Kannahi *et al*., 2013) show that ability of *Bacillus subtilis and Pseudomonas fluroscens* on control tomato plant diseases against *Fusariumoxysporum, Rhizoctoniasolani*. Similar results were reported by (Getha *et al*., 2005) who observed that *B. subtilis* were effective antagonists against *F. oxysporum*. Also, *Pseudomonas fluorescens* applied to pea seeds acted as a biological control agent against *Pythium* damping-off able to reduce disease incidence (Debode *et al*., 2007).

Effect of Biocontrol Agents against Pathogenic Fungi under Field Conditions

Infection and Survival Percentage

Under greenhouse conditions, *B. subtilis* and\ or *P. fluorescens* were shown to be effective reduction of the disease severity and survival rate on tomato plants, when applied in mix with *F. oxysporum*or *R. solani*, compared to soil infested only with the pathogens. Data presented in table 3 shown that soil

infested with *R. solani* and *P. fluorescens* has significantly decreased in disease severity it was 16.5% and survival rate 83.5% compared to soil infested only with the *R. solani* only disease severity was 34.3%, while treated soil only with *F. oxysporum* of disease severity were 50.3 compared with the treatment *F. oxysporum* + *P. fluorescens* the it decreased to 20.1. table 3 show that the efficiency of *B. subtilis* to decreased in disease severity agnaist *R. solani*to 7.9% with survival rate was 92.1%, while reduce the effect of *F. oxysporum* on plant decreased by to 20.9% by *B. subtilis*. However, higher effective of the disease severity and survival percentage of tomato plants were attained in response to treatment with dual bioagents (*B. subtilis*and *P. fluorescens*) than the individual one. It were 4.2%, 10.5% in soil infested with *R. solani, F. oxysporum* respectively under greenhouse conditions.

Table 3: Effect of biological control agents on infection and survival percentage of tomato plants

Treatment	**Disease severity (%)**	**Survival (%)**
Control	1.7	99.3
*Rhizoctoniasolani*only	34.3	66.7
Rhizoctonia solani+ *P. fluorescens*	16.5	83.5
B. subtilis Rhizoctonia solani+	7.9	92.1
Rhizoctonia solani +combination of two bioagents	4.2	95.8
F. oxysporum only	50.3	49.7
F. oxysporum+ *P. fluorescens*	20.1	79.9
B. subtilis F. oxysporum+	20.9	79.1
F. oxysporum +combination of two bioagents	10.5	89.5
L.S.D. (0.05)	3.98	4.87

These results could be attributed to the synergistic effect between the combinations of the two microorganisms in this treatment. These results were in harmony with those reported by (Sallam *et al*., 2013) who revealed that the combination of *B. subtilis* and *P. fluorescens* have significantly decreased disease severity in comparison with the individual ones. The treatment with *B. subtilis* significantly decreased the disease severity of tomato plants infected, with either *R. solani*or *F. oxysporum* (7). The inhibitory effect of antagonistic bacteria such as *B. subtilis* and *P. fluorescen*s against growth reduction of phytopathogenic fungi may be due to the production of hydrolytic enzymes that can degrade cell walls, iron-chelating siderophores, and several cyclic lipodepsipeptides (LDP) (Kim *et al*., 2008). Varieties of *Bacillus* help to promote the health of crops and control diseases by producing antibiotic metabolites, suppressing plant pathogens, others antagonise plant pathogens by competing for nutrients like iron and phosphate, others indirectly fix nitrogen which they make available to the plants and help stimulate plant nutrient uptake (Gardener, 2004). (Mansoori *et al.,* 2013) reported that, *P. fluorescens* isolates performed relatively more effectively than Bacillus isolates in reducing wilt disease of cotton. This could be due to the various antagonistic mechanisms of *P. fluorescens* bacteria such as antibiosis, siderophore production, hormone production, and inducing systemic resistance in host plants.

Growth Parameters

Results in Table 4 show low values of growth parameters (number of branches, plant height, fresh and dry weight) with the pathogens treatment in comparison with Biocontrol agent's treatment. The growth parameters of tomato plants were increased with add *B. subtilis* and \ or *P. fluorescens* to the soil with either *R. solani*or *F. oxysporum* under field condition data indicate that significantly increased of growth

parameters with the dual inoculation of *B. subtilis* and *P. fluorescens* compared with the individual treatment .

Table 4: Effect of biological control in the growth parameters of tomato plants

Treatment	Av. number of branches /plant	Av. plant height (cm)root, shoot	Fresh weight (g/plant)		Dry weight (g/plant)	
			Shoot	Root	Shoot	Root
Control	4.6	40.1	15.75	5.43	3.74	0.57
Rhizoctoniasolani only	3.8	31.02	12.43	4.56	2.4	0.41
Rhizoctonia solani+ P. fluorescens	4.1	38.4	14.98	5.01	3.69	0.53
B. subtilis Rhizoctonia solani+	4.05	35.8	13.5	4.89	3.60	0.50
Rhizoctonia solani +combination of two bioagents	5.6	40.53	16.03	6.23	3.92	0.76
F. oxysporum only	3.5	28.07	11.67	3.76	2.03	0.35
F. oxysporum+ P. fluorescens	4.4	36.8	14.04	4.89	3.44	0.57
B. subtilis F. oxysporum+	4.1	35.05	13.97	4.41	3.31	0.54
F. oxysporum +combination of two bioagents	5.5	39.3	15.7	6.12	3.9	0.70
L.S.D. (0.05)	1.75	1.43	1.98	1.07	1.21	0.21

Fruit Yield

Under felid conditions treatment tomato plants with, *B. subtilis* and\ or *P. fluorescens* were gave higher records of yield and yield components, (Fruit yield number of fruits, fruits yield of plants) , when applied in mix the biocontrol agents with *F. oxysporum* or *R. solani*, compared to soil infested only with the pathogens. Data presented in table 5 shown that soil infested with *B. subtilis* and *P. fluorescens* together has significantly increased in yield and yield components of tomato plants compared to the individual treatment.

Table 5: Effect of biological control in the fruit yield of tomato plants

Treatment	Av. number of fruits/plant	Fruit yield (kg/plant)
Control	7.9	0.65
Rhizoctoniasolani only	5.3	0.48
Rhizoctonia solani+ P. fluorescens	7.2	0.59
B. subtilis Rhizoctonia solani+	7.06	0.53
Rhizoctonia solani +combination of two bioagents	8.9	0.74
F. oxysporum only	4.7	0.36
F. oxysporum+ P. fluorescens	6.2	0.42
B. subtilis F. oxysporum+	5.9	0.40
F. oxysporum +combination of two bioagents	7.95	0.71
L.S.D.(0.05)	1.78	0.14

(Grover *et al*., 2009) research that treated Cotton plant with *Bacillus* isolates had more root hairs compared to the uninoculated control. Plant, height, root and shoot fresh weight increased significantly compared to the control. Because *B. subtilis* inhibited fungal growth and also promoted the growth of

tomato plant in screen house trial. *B. subtilis* has been shown to have a broad spectrum of antimicrobial activities over diverse fungal and bacteria pathogen (Akhtar *et al*., 2010). Over 70 % of mycelial growth of *F. solani in vitro* was inhibited by *B. pumilus*. This may be as a result of production of antibiotic, competition with pathogen for nutrients and direct antagonism (Yao *et al*., 2010). *Pseudomonads* promote plant growth by secreting auxins, gibberellins and cytokinins (Collins and Jacobsen, 2003). (Jataraf *et al*., 2005) reported that *Pseudomonas fluorescens* CHA0 bioagent can be used to promote germination and plant growth and to control *Fusarium oxysporum* f. sp. *lycopersici*. In this study, *P. fluorescens* isolates performed relatively more effectively than Bacillus isolates in reducing wilt disease of cotton. This could be due to the various antagonistic mechanisms of *P. fluorescens* bacteria such as antibiosis, siderophore production, hormone production, and inducing systemic resistance in host plants that has been shown in some previous studies (Jorjani *et al*., 2011).

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