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Efficiency of *Trichoderma viride* and *Bacillus subtilis* as Biocontrol Agents gainst *Fusarium solani* on Tomato Plants

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Tomato is one of the important economic vegetable crops which is attacked by several serious diseases such as root rot. *Trichoderma* and *Bacillus* genera are most feasible biocontrol microorganisms suppress several pathogens like *Fusarium solani*. The efficiency of these antagonistic' treated plant by strains was evaluated using an *in vitro* assay. In pot experiment, the *T. viride* and/or *B. subtilis* suppressed F. *solani* as indexed by survival rate. Field experiment was carried out at El-Fayoum Farm Research Station during 2007 and 2008 seasons. The obtained results showed that, these treatments favoured greater proliferation of rhizosphere microflora and higher dehydrogenase activity in the rhizosphere. The dual treatment by *T. viride* + *B. subtilis* decreased the percentage of infection and increased survival rate than individual one. Moreover, the dual inoculation gave the highest records of growth parameters, fruit yields and plant nutrient content than individual one. Thus, it is recommended to use these strains as a common biocontrol practice in agriculture.

Keywords: *Bacillus subtilis,* biological control, *Fusarium solani,* Tomato and *Trichoderma viride.*

Tomato (*Lycopersicon esculentum* L.) is considered one of the most important economic vegetable crops in Egypt. Tomato plants are subjected to attack by several soil born fungal pathogens, which cause serious diseases as root rot and wilt (El-Mougy, 1995; Ghonim, 1999; Montealegre *et al.*, 2003; Morsy, 2005; Montealegre *et al.*, 2005 and Srinon *et al.*, 2006). The natural control of several phytopathogens is based on the presence of suppressive soils where several biocontrol microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera are detected (Weller *et al.*, 2002; Guo *et al.*, 2004 and Huang *et al.*, 2005). *Trichoderma* spp. has proved to be useful in the control of phytopathogens affecting different crops (Benitez *et al.*, 2004 and Soytong *et al.*, 2005). Also, tomato plants treated by *Bacillus subtilis* only and/or *Trichoderma harzianum* have shown biocontrol activity against damping off and root rot disease and gave high yield of tomato (Morsy, 2005 and Zaghloul *et al.*, 2007).

Fusarium root rot has considerably increased in Egyptian soils. It causes severe high damage in tomato plants especially those grown in old soils (El-Fahham, 1993). The main objective of the current study was to evaluate *Trichoderma viride* and *Bacillus subtilis* as biocontrol agents against root rot disease of tomato.

Materials and Methods

Isolation of pathogenic fungi:

Fusarium solani (*F. solani*) was isolated from root rotted and wilted tomato plants cultivated in El- Fayoum governorate. It was microscopically identified on the basis of cultural and microscopic characteristics. Pathogencity of the isolate toward tomato plant was estimated (Sneh *et al.,* 1991). The isolate was maintained on PDA medium at 4°C. Artificial soil infestation by *F. solani* grown on barley grain medium was carried out as described by Singleton *et al.* (1992).

Microbial isolates:

Trichoderma viride was isolated from tomato plant rhizosphere located in El-Fayoum governorate and identified at the Plant Pathology Research Institute, ARC, Giza, Egypt. *Bacillus subtilis* strain was isolated from the rhizospheric area of cucumber plants grown in Ismaellia governorate and identified by Bio-log Technique at Plant Pathology Research Institute, ARC, Giza, Egypt.

Efficacy of antagonistic bioagents against F. solani:

The antagonistic effect of the tested two biocontrol agents against *F. solani* was examined. *T. viride* and *F. solani* were cultured on PDA medium for 7 days at 28-30°C. Then, a disc (0.5 cm diameter) of the antagonistic fungal colony was cut and placed opposite to the colony of the pathogen. On the other hand, a streak of the bacterial strain 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. Four 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).

Preparation of antagonists inocula:

Inoculum of *T. viride* was prepared in the form of a conidial suspension by heamocytometer slide (10^6 spores / ml) as described by Sivan *et al.* (1984). Meanwhile, *B. subtilis* inoculum was prepared and counted by plate count technique (10^8 CFU / ml) as maintained by Mosa *et al.* (1997).

Tomato seeds were mixed thoroughly with 2 ml of the bacterial suspension (10^8cfu/ml) or fungal spore suspension (10^6conidia/ml) in 0.1 % carboxy methyl cellulose in Petri dish. Seeds were air dried for 30 min and planted directly.

Pot experiment:

A pot experiment was designed under greenhouse conditions using plastic pots (15 cm, diameter) containing reasonable weight of sterilized loamy clay soil. Soil was infested with *F. solani* grown on barely grain at the rate of 5g/kg soil before sowing. Infested pots were irrigated for 5 days before sowing. Ten tomato seeds (*Lycopersicon esculentum)* var. Castle rock were sown in each pot; five 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 *F. solani* only, 3) *F. solani* + *B. subtilis*, 4) *F. solani*+ *T. viride*, 5) *F. solani*+combination of two bioagents and 6) *F. solani* + Vitavax T (2g/kg seeds).

Compound triple NPK mineral fertilizer was added with irrigation water at the rate of 2 g/l into two doses. Pots were kept under greenhouse conditions till the end of the experiment. Disease assessment for incidence of pre and post emergence damping-off and survival rate of seedlings were determined after 30 days of sowing as described by Phillips and Hayman (1970).

Field experiment:

Field experiment was carried out at El-Fayoum Research Station Farm during 2007 and 2008 seasons, to evaluate the efficiency of the tested strains (*T. viride* and *B. subtilis*) for controlling seedling damping-off and root rotting of tomato plants. The chosen field test area was naturally infested with *F. solani*. The experimental design was a complete randomized block with three replicates. The experimental unit area was 10.5m^2 (3.5x3m). Each unit included four rows; each row was 3.5m in length and 75cm width. Tomato transplants were at a rate of 10 seedlings within each row. Tomato seedlings were treated by drenching 10 ml of conidial suspension (10^6/ml) of *T. viride* or 10 ml bacterial suspension (10^{8}cfu/ml) of *B. subtilis* to each seedling in the seedling trays, 24h before transplanting. Seedlings transplanted into the field according to the following: 1) soil naturally infested, 2) *B. subtilis*, 3) *T. viride*, 4) *B. subtilis* and *T. viride*, 5) fungicide Vitavax T (0.15%). The NPK mineral fertilizers were applied at the recommended dose of Ministry of Agriculture and Land Reclamation. Disease incidence of root rot and percentage of healthy survival plants were recorded after 45 days from transplanting. Dehydrogenase enzyme activity was determined according to Thalmann (1967) based on the use of Triphenyeltetrazolium chloride (TTC) as an artificial electron acceptor to show the microbial activity in tomatoes rhizosphere through microbial respiration.

Total microbial counts on modified medium of Bunt and Rovira (1955) were estimated by the decimal dilution plate count technique. Numbers of branches, plant height, fresh and dry weight of plants were determined. Plant yield and estimated fedden yield were calculated. Nitrogen, phosphorus and potassium contents of tomato plants were estimated according to (Jackson, 1973).

Statistical analysis:

The significance of various treatments was evaluated by Duncan's multiple range tests ($p<0.05$) (Duncan, 1955). Statistical analysis was made using a software package "Costat," a product of Cohort Software Inc. Berkley, California, USA.

Results and Discussion

1. Evaluation of fungal and bacterial strains for antagonistic activities against F. solani in vitro:

Trichoderma viride and *Bacillus subtilis* strains were evaluated for antagonistic effect against *Fusarium solani* on Petri dishes containing PDA medium. Table (1) show that the bioagent strains succeeded in reducing the radial growth of *F. solani*. *T. viride* was more active than *B. subtilis* for reducing the radial growth of *F. solani* being 5.2 and 3.1 cm respectively. Moreover, *T. viride* inhibited the over growth of *F. solani*, comparing with *B. subtilis*. Both of *T. viride* and *B. subtilis* strains reduced growth percentage by 57.8 and 34.4%, respectively, comparing with

Table 1: Effect of *T. viride* and *B. subtilis* on the radial growth of *F. solani*

Microbial strain	Redial growth (cm)	Reduction (%)
T. viride	5.2	57.8
B. subtilis	3.1	34.4
Control	9.0	0.0

the control (Table 1). This behaviour represents an important approach for controlling a root rot disease of tomato plants. The potentialities of the used strains could be attributed to their effect to secrete hydrolytic enzymes or antifungal metabolites.

These findings are in harmony with those obtained by Montealegre *et al.* (2005) who reported that *Trichoderma* spp. secreted chitinase and B 1,3 glucanase in supernatants. 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, Alippi and Monaco (1994) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin which have an inhibitory effect on fungal pathogens.

2. Efficiency of two antagonistic biocontrol agents under greenhouse conditions:

A pot experiment was carried out to examine the *in- vivo* efficiency of bioagents to antagonize *F. solani* under greenhouse conditions.

Data presented in Table (2) reveal that soil infested with *F. solani* has significantly increased damping off of tomato seedlings and severely reduced survival rate (35%) than untreated control (90%) or Vitavax T. treatment (85%).

Table 2. Influence of antagonistic strains applied individually or in dual application against *F. solani* and their effect on seedling dry weight under greenhouse conditions

Treatment	Damping off (%)		Survival plants (%)
	Pre-emergence	Post-emergence	
Control	4.0	6.0	90.0
Fusarium solani	45.0	20.0	35.0
F. solani + *B. subtilis*	19.0	11.0	70.0
F. solani + *T. viride*	16.0	11.0	73.0
F. solani + *B. subtilis*+*T. viride*	12.0	8.0	80.0
F. solani + Vitavax T.	9.0	6.0	85.0
LSD at 0.05 %	3.8	5.14	5.15

Inoculation with *T. viride or B. subtilis,* significantly increased survival rate compared with the *F. solani* infested soil, ranging between 73-70%, respectively (Table 2). However, higher percentage of survival rates of tomato seedlings were attained in response to treatment with dual bioagents (80%) than the individual one. Similar results were reported by Getha *et al.* (2005) who observed that *T. harzianum* and *B. subtilis* were effective antagonists against *F. oxysporum*.

3. Efficiency of two antagonistic biocontrol agents against F. solani under field conditions

3.1. Infection and survival percentage:

Data in Table (3) revealed that the high infection percentage of tomato plants with the pathogen recorded with control whereas, low infection percentage was observed in the treated seedlings with Vitavax T. In addition, high percentage of survival rate was recorded. The dual treatment using *B. subtilis* + *T. viride* has significantly decreased the percentage of infection and increased percentage of survival plants comparing with the individual application.

Table 3. Influence of two biological control agents individually or in dual application on infection and survival percentage of tomato plants

Treatment	Season 1		Season 2	
	Infection (%)	Survival (%)	Infection (%)	Survival (%)
Control	35.8	64.2	43.2	65.8
B. subtilis	21.5	78.5	17.5	82.5
T. viride	20.0	80.0	14.2	85.8
B. subtilis+ T. viride	13.3	86.7	10.0	90.0
Vitavax T. (0.15%)	9.2	90.8	7.5	92.5
LSD at 0.05	9.1		6.3	

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 Cal *et al.*, (2004) and Zaghloul *et al.*, (2007) who revealed that the combination of *B. subtilis* and *T. harzianum* have significantly decreased disease severity in comparison with the individual ones. The mechanism of *Trichoderma* and *Bacillus* action on pathogens may be by attacking and binding the pathogenic organisms by sugar linkage and begins to secrete extracellular protease and lipase (Cal *et al.*, 2004).

3.2. Dehydrogenase activity and microbial population:

Dehydrogenase activity (DHA) was recorded after 45 and 75 days of planting (Table 4). Low DHA and total microbial flora values were observed with treatment of Vitavax T, indicating the antimicrobial activity of this fungicide against the rhizosphere microbial flora and the pathogenic fungi.

Table 4. Influence of two biological control agents individually or in dual application on dehydrogenase activity and total microbial count

Treatment	Dehydrogenase activity (μg TPF*/g dry soil/day)				Microbial total count (CFU x10^6) g^{-1} dry soil			
	1st season (days)		2nd season (days)		1st season (days)		2nd season (days)	
	45	75	45	75	45	75	45	75
Control	35.7	140.8	39.4	152.4	22.0	55.1	27.3	66.7
B. subtilis	54.2	175.1	60.8	186.9	27.0	46.5	35.1	62.8
T. viride	63.9	190.4	70.4	202.5	12.8	43.9	15.4	57.5
B. subtilis+T. viride	84.1	291.5	101.5	317.3	32.9	70.1	36.1	82.3
Vitavax T. .(0.15%)	28.4	137.7	34.7	142.6	19.9	34.2	21.3	42.7
LSD at 0.05	5.39	7.70	5.43	6.39				

*TPF =2,3,5 triphenyl- formazan

Dual inoculation (*T. viride* + *B. subtilis*) of tomato plants increased DHA activity and microbial flora in tomato rhizosphere than individual ones. Similar results were reported by Morsy (2005) and Zaghloul *et al.* (2007) who estimated high DHA activity and total microbial flora in case of co-inoculation with *B. subtilis* + *T. harzianum* in root rot disease of tomato.

In general, the values of DHA activity and counts of microbial flora of various treatments were higher at the flowering stage than the vegetative one. This difference could be attributed to the qualitative and quantitative changes in the nature of root exudates during different growth stages (Abdel-Jawad, 1998).

3.3. Growth parameters:

Data presented in Table (5) revealed low values of growth parameters, (number of branches, plant height, fresh and dry weight of plants) with the control treatment in comparison with other treatment. The growth parameters of tomato plants were significantly increased with the dual inoculation of *B. subtilis* and *T. viride* compared with the individual one.

The increase of plant growth could be attributed to the aforementioned role of both microorganisms present in dual inoculum.

The promotion of tomato growth parameters by *B. subtilis* and *T. viride* strains may be due to their abilities to produce phytohormones, vitamins and solublizing minerals besides, their role in direct inhibition of pathogen growth (Morsy, 2005 and Zaghloul *et al.*, 2007).

3.4. Fruit yield:

Data in Table (6) revealed a decrease in the yield of tomato plants, *i.e.* number of fruits, fruits yield / plant and the estimated fruits yield / fed of control treatment. However, significant increase was determined with the fungicide treatment.

Table 5. Influence of two biological control agent individually or in dual application on growth characters of tomato plants

Treatment	Av. number of branches /plant		Av. plant height (cm)		Fresh weight (g/plant)		Dry weight (g/plant)	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
	Cultivation season							
Control	3.9	4.2	37.8	42.5	95.2	138.7	13.9	17.2
B. subtilis	4.9	5.2	52.0	57.6	205.9	337.4	35.8	44.9
T. viride	4.5	4.9	49.7	53.1	167.5	297.2	26.1	35.5
B. subtilis+ T. viride	5.6	6.0	68.5	76.4	411.8	495.1	73.4	82.7
Vitavax T. (0.15%)	5.0	5.4	60.2	64.9	356.7	420.9	58.2	64.9
LSD at 0.05	1.7	1.8	15.6	10.3	9.2	13.9	7.3	4.3

Soil inoculation with *B. subtilis* only gave higher records of yield and yield components than that treated by *T. viride*. Moreover, dual treatment by *B. subtilis* + *T. viride* has significantly increased yield and yield components of tomato plants compared to the individual ones.

This could be attributed to the synergistic effect between the microbial rhizosphere and biocontrol agents. In this respect, Niknejad *et al.* (2000) and Zaghloul *et al.* (2007) reported that application of selected antagonists (*B. subtilis*, *T. harzianum*) either individually or in combination has significantly increased the number of fruits / plant, weight of fruits and the total yield of tomato fruits.

Table 6. Influence of two biological control agents individually or in dual application on growth characters of tomato plants

Treatment	Av. number of fruits/plant		Fruit yield (kg/plant)		Fruit yield (ton/fed)	
	1st	2nd	1st	2nd	1st	2nd
	Cultivation season					
Control	9.00	9.70	0.79	0.85	12.64	13.60
B. subtilis	11.50	13.90	1.21	1.34	19.36	21.44
T. viride	10.60	12.40	0.98	1.12	15.68	17.92
B. subtilis+T. viride	16.70	17.30	1.43	1.51	22.88	24.16
Vitavax T. (0.15%)	14.50	16.10	1.30	1.41	20.80	22.56
LSD at 0.05	5.18	6.35	0.35	0.55	1.09	2.55

3.5. Plant nutrients content:

In general, the values of NPK concentration in tomato plants were highly significant in all treatments comparing with that of control (Table 7). The dual treatment by of *B. subtilis* + *T. viride* for tomato plants cause a significant increase in NPK content in comparison to individual ones.

Table 7. Influence of two biological control agents individually or in dual application on growth characters of tomato plants

Treatment	Macronutrient (%)					
	1st season			2nd season		
	N	P	K	N	P	K
Control	3.90	0.41	2.70	4.20	0.44	3.10
B. subtilis	4.80	0.55	3.50	5.20	0.59	4.60
T. viride	4.30	0.49	3.80	4.70	0.53	4.90
B. subtilis+ *T. viride*	5.30	0.58	4.30	5.90	0.63	5.60
Vitavax T. (0.15%)	4.90	0.56	4.50	5.10	0.59	5.20
LSD at 0.05	1.01	0.14	1.06	0.53	0.04	0.40

From the aforementioned results, it could be concluded that the dual treatment with *B. subtilis* combined with *T. viride* has a significant and more feasible to control root rot disease and increased the yield components of tomato comparing with the individual treatments, because of their potentialities to produce plant growth promoting substances (Bochow *et al.*, 2001 and Morsy, 2005), which might create favourable conditions for improving minerals uptake by plants.

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تقييم كفاءة تريكودرما فيردى وباسيلس سابتلس فى المكافحة الحيوية لفطر فيوزاريم سولانى على نباتات الطماطم

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تعتبر الطماطم أحد أهم محاصيل الخضر الأقتصادية والتى يمكن أصابتها بالعديد من الأمراض كأعفان الجذور. ومن أهم الكائنات الحية التى لها قدرة على مقاومة فطر فيوزاريم سولانى وفطر تريكودرما وبكتريا باسيلس ساتلس. وقد تم تقييم كفاءة هذه السلالات في المكافحة الحيوية تحت الظروف المعملية. فى تجربة الأصص، أضيفت التريكودرما فيردى والباسيلس سابتلس لأختبار كفاءتهما في تثبيط نمو فطر فيوزاريم سولانى وذلك بتقدير نسبة الشتلات المتحملة للأصابة. أجريت تجربة حقلية بمزرعة محطة البحوث بالفيوم خلال موسمي ٢٠٠٧ ، ٢٠٠٨ وقد أظهرت النتائج المتصل عليها أن هذه المعاملات أدت إلى زيادة فى أعداد الميكروبات وزيادة نشاط أنزيم الديهيدروجينيز بمنطقة الريزوسفير. كما أن التلقيح المزدوج بكلا الجنسين أدى إلى إنخفاض نسبة الأصابة وبالتالي زيادة نسبة النباتات المتحملة للأصابة بالمقارنة بالتلقيح الأحادى باى منهما. وأوضحت النتائج أيضاً أن التلقيح المزدوج أدى إلى زيادة فى مؤشرات النمو ومحصول ثمار الطماطم ومحتوى النبات من العناصرالكبري مقارنة بالتلقيح الأحادى. وعلى ذلك توصى هذه الدراسة باستخدام هذه السلالات معا فى المكافحة الحيوية كأحد العمليات الزراعية.