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Evaluation of Biocontrol Agents and Chemical Inducers for Managing Vascular Wilt of Tomato Caused by *Fusarium oxysporum* f.sp. *lycopersici*

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Abstract

The study was conducted to evaluate the efficiency of biocontrol agents (*Bacillus mycoides* and *Trichoderma viride*) and the chemical compounds, ASM and Preservepro against *Fusarium oxysporum* f.sp. *lycopersci* the causal agent of tomato wilting disease in culture media and under greenhouse conditions .The results showed that all the control agents exhibited an inhibition rate on *F. oxysporum* growth on both culture media and under natural conditions .The addition of bio and chemical agents to the contaminated soil induced significant reduction in disease incidence and disease severity associated with increase plant dry weight .The disease incidence, severity and plant dry weights in *T.viride*, *B.mycoides*, ASM and Preservpro were found, 26.70,20.00,53.30,13.30% and 13.30,13.30,33.30,6.70% and 4.43,4.80,3.56 ,5.30 g/plants respectively compared with 86.70% ,70.00,and 0.60 g/plant in control treatment .Additional reduction in disease incidence ,disease severity and increase in plant dry weights were obtained on plant grown in soil treated with combination of control agents .The disease incidence , severity and plant dry weights were found in the combination of *T.viride* + *B.mycoides*, ASM +*T.viride* , ASM + *B.mycoides*, Preservepro + *T.viride* , Preservepro+*B.mycoides*, ASM + Preservepro 13.30, 26.70, 21.70, 8.30, 5.00, 13.30% and 6.70, 10.30, 13.30, 0.00, 0.00, 3.33% and 5.00, 4.00, 4.50, 5.80, 6.03, 5.40 g/plant respectively compared with 70%, 86.70% and 0.6 g/plant in control treatment.

Keywords:*Fusarium oxysporum*, *Bacillus mycoides*, *Trichoderma viride*, ASM, Preservpro, induce resistance.

1.Introduction

Tomato, Solanum lycopersicum L., formerly known as is one of the most important vegetable crop worldwide (Hirano & Arie 2006).It has been reported that tomato infects by many pathogens among them Fusarium oxysporum f.sp. lycopersici causal agent of tomato wilt was the most destructive and causing heavy losses in tomato yields ,in many countries worldwide including Iraq (Girhepuje &Shinde 2011). The control of the disease was restricted for long time on the use of fungicide, but the misuse of the fungicide pose many problems for human and ecosystem as well as inducing the development of resistance strains of the pathogens (Sivan et al. 1987). Therefore the efforts were oriented toward searching for compounds save and effective as alternative to fungicide able to trigger defense mechanisms in the plant refers to as induced systemic resistance (ISR) against pathogens. It has been reported that treatment of plants with variety of agents (biotic , a biotic , synthetic chemicals) can leads to the induction of resistance to subsequent pathogen attack (Hibar et al. 2006 ; Hammorschmidt 1999; Dekkers et al. 2000; Bolwerk et al . 2003; Hibar et al.2007; Karkachi et al. 2010). Benzo -(1,2,3)-thiadiazole-7-carbothioic acid (Benhamou & Belanger1998),and β –aminobutyric acid (Cohen et al. 1994), as environmentally save chemicals were widely used to induce systemic resistance in plants against variety of pathogens. This study was undertaken to evaluate the ability of some synthetic compounds and biological agents to manage tomato wilt disease caused by Fusarium oxysporum through inducing systemic resistance in the plants.

2. Materials and Methods

2.1. The Pathogen, Isolation, identification and pathogenicity

Several diseased mature tomato which showed evident symptoms of wilt disease (Agrios 2005) were collected from different greenhouses and fields in karbala city (100Km south of Baghdad ,Iraq) during April to June 2011.Affected tomato stems were cut into approximately 0.5-1cm and washed under running tap water, surface sterilized in 1% sodium hypochlorite for 1min, rinsed in sterilized water and dried in air laminar flow cabinet and cultivated on potato dextrose agar (PDA) with chloramphenicol (100 µg ml $^{-1}$)in plates of 9 cm diam, and incubated at 25± 1C° for 5 days. The isolates were purified by Single spore according to Juber (1982), and identified on the basis of its morphological and cultural characteristics (Booth 1977; Nirenberg &O'Donnell 1998; Leslie &Summerell 2006). Pathogenesity test for the fungus isolates were carried out in artificial inoculation on tomato seedlings. The pathogen re-isolated from the diseased plants to confirm Kocks' postulates.

2.2. The biocontrol agents

Bacillus mycoides isolate was obtained from the Molecular diversity lab, department of environment and water of the Ministry of science and technology. *Trichoderma viride* and Bio root care (mixture of *T. viride* , *B.subtilis* , *Pseudomonas fluorescens* and *Paecilomyces lilacinus*) were obtained from Dr. Rajan,India.

2.2.1. Evaluation the antagonistic ability of *Trichoderma viride* and *Bacillus mycoides* against *Fusarium oxysporum* f.sp. *lycopersici* on culture media.

Dual culture technique on PDA was adopted to test the antagonistic of *B. mycoides* and *T. viride* against *F. oxysporum* f.sp. *lycopersici* (FOL).Plugs of 5 mm diam taken from FOL and *T. viride* colonies (7 days old) were transferred onto PDA in plates of 9 cm diam , apart from each other and maintained at 25 ± 1 C° for 7 days .To assess *B. mycoides* antagonism against *F. oxysporum*,5 mm agar discs from 7 days old mycelium of FOL were placed in the centre of plates with PDA and *B.mycoides* growth were placed equidistant sites 1 cm from plate periphery as spots around the center .After 5 days of incubation at 28 ± 1 C° ,the radial growth of FOL was recorded and the inhibition percentage was calculated by the formula I= C- T/C × 100, where I= percent growth inhibition, C = radial growth of pathogen without antagonistic agent, and T= radial growth of pathogen with antagonistic agent. Four replicates were used for each fungal isolate.

2.3. Effect of Acibenzolar – S - methyl and Preservpro on growth of *Fusarium oxysporum* f.sp. *lycopersici* on culture media

ASM (,Actigard 50 WG) was provided by syngenta crop (Green sboro ,NC,USA). Preservepro product was provided by Arysta Life Science (2% Ascorbic acid). An agar plug (5 mm diam.), taken from the margin of FOL colony (7 days old) was placed in the centre of PDA plate amended with each product at 0,100,200.400, 600, 800, and 1000µg ml^{-1}. The plates were incubated at 25 ±1C and the percent of growth inhibition was calculated as before. Four replication of each concentration were used.

2.4. Evaluation the efficiency of biocontrol agents and chemical compounds in the reduction of tomato plants infection by the fungus *Fusarium oxysporum* f.sp. *lycopersici* under greenhouse conditions:

Tomato seed (*Solanum lycopersicum L. Cv. Wejdan* , susceptible to FOL) were surface sterilized by immersion in 1% sodium hypochlorite for two min , followed by extensive rinsing in sterile distilled water (Hibar *et al.* 2006) . Ten seeds were sown in each pot of 10 cm diam at 3 cm below soil surface .The pots were distributed in the greenhouse in complete randomized design in Plant Protection Dept, College of Agriculture, University of Baghdad, Iraq during 2010-2012.

Inoculums Preparation:

Disks taken from 1 week old culture of *F. oxysporum* were added to100 ml of Czapek's Dox broth medium in 250ml conical flask and incubated at 25C°for 7days. The fungal spores suspension was filtered through three layers of cheese cloth to remove mycelia fragments .The spore concentration was determined and adjusted to 5× 10^6 spores per ml by using a haemocytometer .The suspension was used for soil inoculation 7 day after application of the inducing factors .

Treatments:

The experiments include 18 treatments with 4 replicates as detailed below:

T1= Healthy control (uninoculated) ,T2 = Diseased control (inoculated with *F. oxysporum*) , T3= *B. mycoides* added to uninoculated soil ,T4 = *T. viride* added to un inoculated soil ,T5= ASM added to uninoculated soil ,T6 = Biorootcare added to uninoculated soil,T7= Preservepro added to uninoculated soil ,T8 = *B. mycoides* added to

inoculated soil , T9=*T. viride* added to inoculated soil ,T10= ASM added to inoculated soil,T11 = Preservepro added to inoculated soil,T12 = *B. mycoides* + *T. viride* added to inoculated soil, T13 = ASM + *B. mycoides* added to inoculated soil, T14= ASM + *T. viride* added to inoculated soil,T15= Preservepro + *B.mycoides* added to inoculated soil,T16 = Preservepro + *T.viride* added to inoculated soil, T17 = Presrevepro + ASM added to inoculated soil, T18= Biorootcare added to inoculated soil. The seedlings were treated with 25ml of solution at 50 µg ml^{-1} into each pot- as a soil drench for the ASM, 50µg ml^{-1} into each pot as a soil drench for the Preservepro, 50 ml of bacterial suspension (7×10^{5}CFU ml^{-1}) and 1g each (1×10^{9} spores / ml) of either the *T. viride* or Bio root care . Percentage of disease incidence was recorded 30 days after inoculation with pathogen, and after 3 months the disease severity was determined according to Souza *et al*. (2010). Using a rating scale of 1to 5 on the basis of leaf yellowing 1 = plant free of symptoms; 2 =plant without wilt symptoms but present conspicuous vascular browning; 3 = plant showing vascular browning with wilting symptoms or with chlorosis; 4 = severe wilting associated with the presence of foliar necrosis and chlorosis, and 5 = dead plant, Then Plants were carefully uprooted and roots were washed under tap water to remove adhering soil. To determine shoots and roots dry weight, shoots and roots were separately weight and dried at 70C until weigh fixed.

2.5. Enzyme studies:

The activity of oxidative enzymes, peroxidase (PO) and phenyl alanine ammonia –layase (PAL) were determined in the 7 days old leaves ,as well as the total protein was determined in the 15 days old leaves after inoculation with pathogen of treated and untreated tomato plants.

Assay of Peroxidase activity was carried out as described by Hassan (2013). One gram of leaves was ground in 1 ml of 0.1 M phosphate buffer (pH7) using a cold pestle and mortar (4 C^{o}). The homogenate was transferred to a 1.5 ml centrifuge tube and centrifuged at 15.000 g at 4 C^{o} for 15 min. and the supernatant was immediately used for the assay. The Phenylalanine ammonia layase activity was determined as described by Narwal *et al*. (2009). The reaction mixture contained 0.1 ml phenyl alanine and 0.2 ml enzyme extract in a total 2.5 ml of sodium borate buffer (pH 8.7) .The mixture was placed in a water bath at 37 C^{o} for 1 h and 0.5 ml of 1 M trichloro acetic acid (TCA) was added. The amount of trans- cinnamic acid formed from L- phenylalanine was measured spectro photometerically at 290 nm. Enzyme activity was expressed as µg of Trans –cinnamic acid h^{-1} g^{-1} protein.The Total protein content was determined as described by Scheffelen *et al*. (1961).

3. Results

3.1. Fungal isolation, identification and pathogenicity:

The cultural and morphological characteristics on culture media indicated that the isolates from tomato plants showing wilt symptoms belong to *Fusarum oxysporum f.sp. lycopersc*i. White, floccose mycelium was formed from the infected pieces of tomato stem on potato dextrose agar, that turned to grayish white with age. The microscopic observation showed straight to slightly curved, relatively cylinder and thin walled Macro conidia with curved apical cell and foot shaped basal cell varied in size 28-45µm x 3-5µm with three septa. Abundant, oval, ellipsoidal or kidney shaped and usually 0- septet Micro conidia borne on short monophialides in the aerial mycelium clustered into so cold false heads where observed also under microscope. Chlamydospores formed abundantly after 2-3 weeks by all the isolates, usually formed singly or in pairs but also found in clusters or in short chains, either terminal or intercalary in aerial mycelium.

The Pathogenicity test revealed that all the isolates where pathogenic to tomato seedlings; it gave 33-73% and 16.33-36.33% disease incidence and severity respectively. The more virulence isolate was selected for further study.

3.2. Antagonistic activity of bioagents against *F. oxysporum* f.sp. *lycopersici*on culture media:

Results of the antagonistic ability of *T. viride* and *B. mycoides* against FOL on PDA showed that both of the biocontrol agents gave significant inhibition in FOL mycelia growth. *T. viride* gave 100% inhibition after 5 days of inoculation while *B. micoides* revealed 86.52% growth inhibition in the same period. (Fig.1)

3.3. Effect of Preservepro and ASM on FOL mycelia growth on PDA:

Results showed that both of chemical agents Preservepro and ASM gave significant reduction in FOL mycelial growth in all used concentrations (fig.2). The reduction of mycelia growth was found to be correlated with increasing concentrations. Preservepro gave 100% growth inhibition in 600 µg ml^{-1} while ASM gave the same result at 1000 µg ml^{-1} .

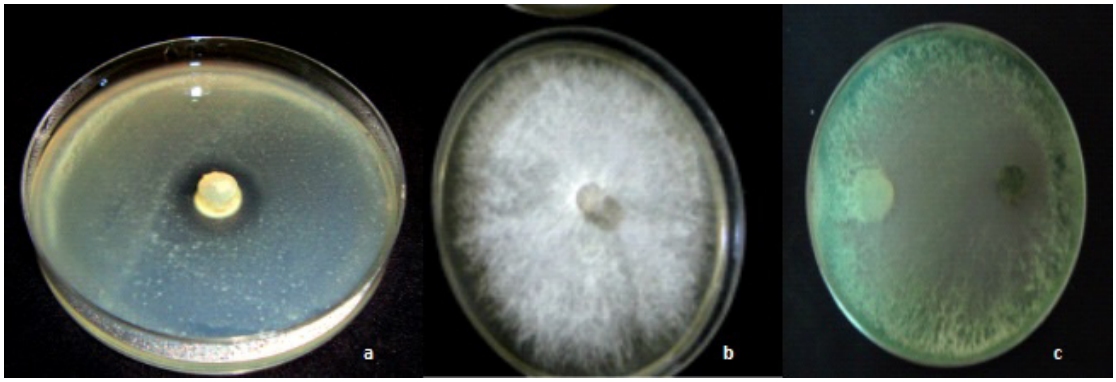

Figure 1. The inhibition activity of B. *mycoides* and T. *viride* againist *F. oxyporum* on PDA. a: *B. mycides* + FOL, b:FOL only, c: *T. viride*+ FOL.

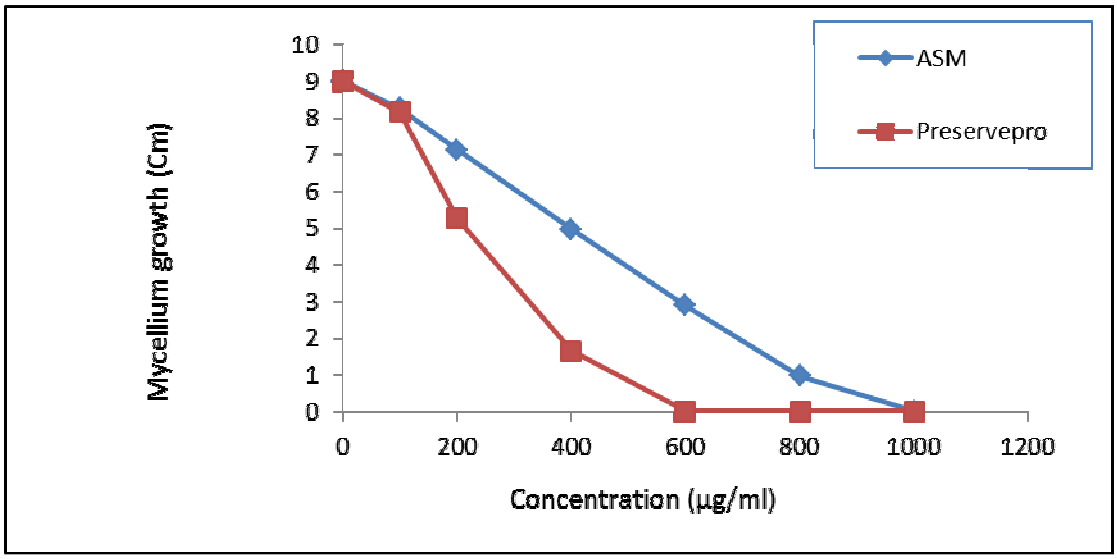

Figure2. Effect of Preservepro and ASM concentrations on *F. oxysporum* growth on PDA after 7 days of incubation
LSD (P=0.05)for ASM=0.53,LSD(P=0.05)for Preservepro=0.75.
Four replicates for each treatment.

3.4. Effect of bioagents and chemical compound on fungal growth in pot trial:

The addition of bio and chemical agents to the contaminated soil with *F. oxysporum* separately or in combination have induced significant reduction in disease incidence(DI) ,disease severity index(DSI) , and increased plant dry weight compared with untreated plants (control) Table(1). The disease incidences , were found to be 26.70,20.00,53.30,13.30 and 55.00% on the plants in soil treated with *T.viride*+ FOL, *B.mycoides*+ FOL, ASM+ FOL, Preservepro + FOL, and Biorootcare + FOL respectively compared with 86.70% of plants in soil treated with the pathogen only (control). The disease severity was attained to 13.30, 13.30, 33.30, 6.70, and26.70% for the same treatment respectively compared with 70.00 % in control. It has been found that *T. viride*, *B.mycoides*, and Preservepro induced similar results concerning the plant dry weights 4.43, 4.80, 3.56, 5.30, and 2.96 g / plant, for the above treatments respectively compared with 0.60 g /plant in control. Additional increase in DI , DSI and plant dry weights were manifested, on plants in soil treated with combination of control agents. The DI values were found to be 13.30, 26.70, 21.70, 8.30, 5.00, 13.30% for the treatments, *T. viride*+*B.mycoides*+FOL, ASM+*T.viride*+FOL, ASM+*B.mycoides*+FOL, Preservepro+*T.viride*+FOL, Preservepro+*B.mycoides* + FOL , ASM+Preservepro+FOL respectively compared with 86.70% in control. The DSI were 6.70, 10.30, 13.30, 0.00, 0.00 and 3.330% for the treatments before respectively compared with 70.00% in control. The plant dry weights were 5.00, 4.00, 4.50, 5.80, 6.03, 5.40 g/plant for the same treatments respectively compared with 0.6g/plant in control.

Table 1. Activity of bioagents and chemical inducers against *F. oxysporum* on tomato under greenhouse conditions.

Treatments	Disease incidence (%) DI	Disease severity index(%)DSI	Dry weight g/plant
Control	0.00	0.00	4.0
Pathogen only(FOL)	86.70	70.00	0.60
T.viride only(Tr.)	0.00	0.00	5.00
*B.mycoides*only (Ba.)	0.00	0.00	5.26
ASM only	0.00	0.00	4.00
Preservepro only	0.00	0.00	4.73
Biorootcare only	0.00	0.00	4.43
Tr.+ FOL	26.70	13.30	4.43
Ba. + FOL	20.00	13.30	4.80
Tr. +Ba. + FOL	13.30	6.70	5.00
ASM + FOL	53.30	33.30	3.56
ASM + Tr. FOL	26.70	10.30	4.00
ASM + Ba. + FOL	21.70	13.30	4.50
Preservepro + FOL	13.30	6.70	5.30
Preservepro + Tr. + FOL	8.30	0.00	5.80
Preservpro + Ba.+ FOL	5.00	0.00	6.03
ASM + Preservepro+ FOL	13.30	3.33	5.40
Biorootcare+ FOL	55.00	26.70	2.96
LSD=0.05	16.56	12.55	0.27

***Four replicates for each treatment.**

3.5. Effect of bioagents and chemical inducers on Peroxidase (PO) and Phenyl alanine ammonia-layase(PAL) activities in leaves (7 day old):

Data in Figure (3) showed that all tested bioagents and chemical inducers increased the activity of oxidative enzymes i.e. Peroxidase and Phenyl alanine ammonia-layase in tomato leaves compared to those grown from untreated seeds (control).In this respect, activity of PO showed the highest increase when acombination of *B.mycoides* + Preservepro was used followed by the combination of Preservepro+*T. viride* and*T. viride* with ASM .The same trend was recorded for the activity of PAL. Combination of Preservepro +*B.mycoides* and Preservepro+*T. viride* induced the higher level of PAL activity followed by combination of *B.mycoides*+ ASM , *T. viride* +ASM , and ASM.

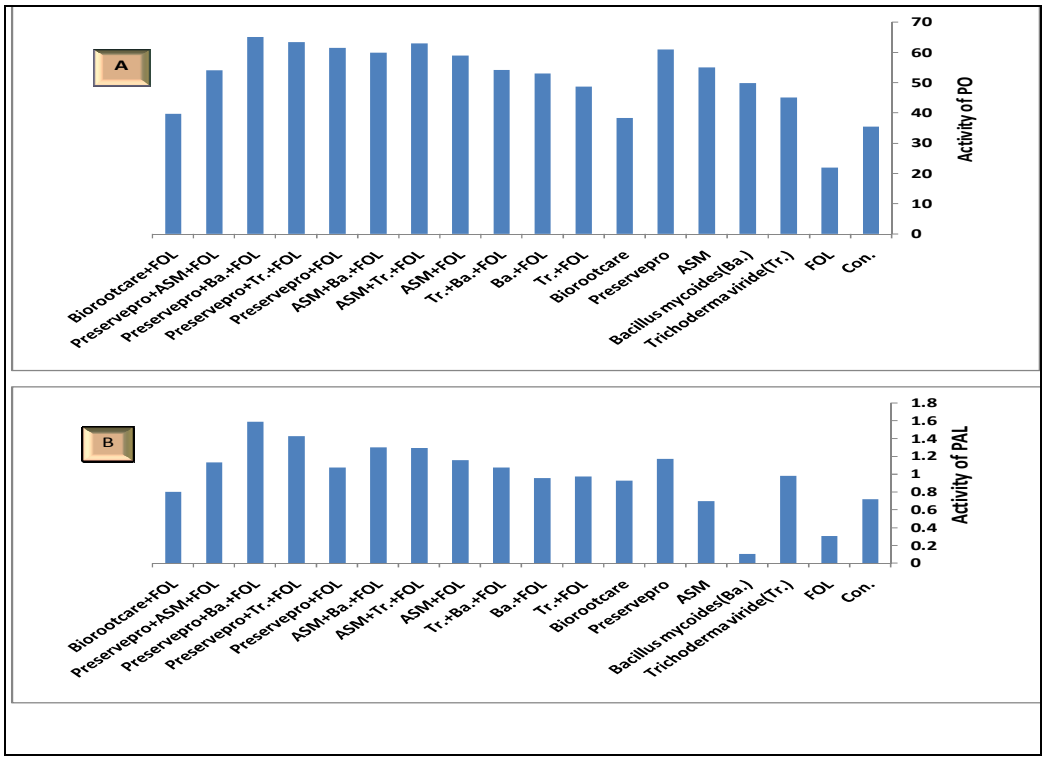

Figure 3. Effect of bioagents and chemical inducers on oxidative enzyme activities. (A): Activity of peroxidase enzyme (enzyme unit mg protein-1min-1) of tomato leaves cv.wejdan[y] as affected by bioagents and chemical inducers separately or in combination.(B): Activity of phenylalanine ammonia layase enzyme (enzyme unit mg protein-1min-1) of tomato leaves cv.wejdan[y] induced by bioagents and chemical inducers separately or in combination*Four replicates for each treatment.y) Soil in each pot was infested with *F. oxysporum* at thevrate of 5×10^6 spores per ml.LSD=0.05 of Peroxidse =0.908 and LSD=0.05 of Phenylalanine ammonia layase = 0.224.

3.6.Effect of bioagents and chemical inducers on protein contents in leaves(15-day-old):

Results presented in (Figure 4) indicated that, total proteins was obviously higher in leaves of plants grown in soil treated with bioagent and chemical inducer than the untreated control. The highest protein contents were induced by combination of Preservepro+*T.viride* , Preservepro+ASM followed by combination of Preservepro + *B.mycoides*.

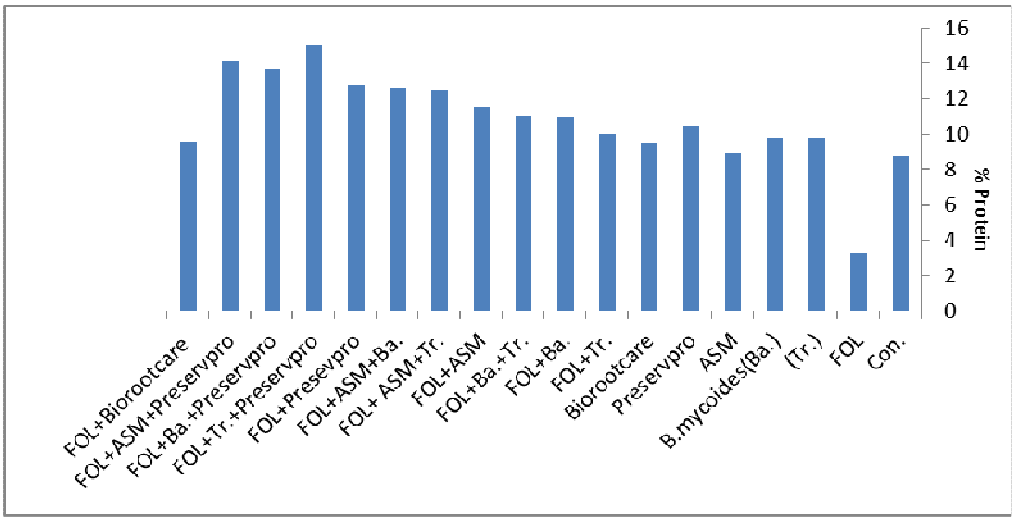

Figure 4. Effect of bioagents and chemical inducers on total protein. LSD= 0.05 = 0.726,*Four replicates for each treatment.

4. Discussion:

The results obtained in this study demonstrated clearly the high activity of bio-and chemical control agents against *F. oxysporum f.sp. lycopersc*i, the causal agent of tomato wilt disease , and improved plant growth as proved by increasing the dry weight of treated plant, compared with control. The effect of *T. viride* and *B. mycoides*, on the pathogen may be directly through competition for nutrients,antibiosis , or secretion of lytic enzymes.It was reported that many strain of rhizosphere bacteria isolated from naturally disease suppressive soil have been shown to promote plant growth by suppressing soil borne pathogens(Bargabus *etal*.2003; Jacobsen *et al.* 2007; Choudhary &Johri2009; Houssien *et al.* 2010).The restriction of pathogen growth by the biocontrol agents may be indirectly through activation of host defense mechanisms that refer to as induced systemic resistance against the pathogens. The resistance induced is characterized by a restriction of pathogen growth and suppression of disease symptoms development compared with non- induced plant infected with the same pathogen (Hammorschmidt1999; Walters *et al* 2005). The results showed that the chemical agents, ASM and Preservepro , exert significant inhibition to *F.oxysporum* growth on culture media which indicate to direct effect of these agents on the pathogen .In addition ,significant reduction of disease severity and increased plant dry weight of plants treated with these two agents that led to the conclusion that these agents are activators of plant defense system. Similar results concerning the activity of chemical agents ,including ASM ,in inducing systemic resistance in the plant were previously reported (Gorlach *et al.* 1996).Significant increases in peroxidase (PO) and phenyl alanine ammonia-layase (PAL) have been observed associated with bio-and chemical agents treatment in the treated plants which indicated to induction of systemic resistance against *F. oxysporum.* Several studies reported that systemic resistance is characterized by induction of several formation of PRs and has been taken as a marker of the induced resistance (Kessman *et al.* 1994).Some of these PRs are β – 1,3 –gluconase and chitinase capable of hydrolyzing fungal cell walls ,PO and PAL enzymes play a role in phenolic compound metabolisms. Other studies indicated to the activation of large amount of enzymes including peroxidase ,chitinase,β – 1,3 –glucanase, and phenyl alanine ammonia-layase upon treatment of plants with different agents ,biotic and abiotic .These activation were found associated with cellular alteration in the epidermal and cortical cells that inhibited further colonization and inhibited the causal pathogen to reach the vascular tissue (Hoffland *et al*. 1995). The treatment of plants with a combination of *B.mycoides* and*T. Viride* was found more effective,then each of them separately ,in reducing disease severity in tomato infected with *F. oxysporum***,** this may came from the fact that using more than one control agents with different mechanism of action give an additive effect toward the pathogen .It has been reported that treating plants with more than one control agents can lead to more increase in plant growth and reduction in plant infection with root knot nematodes (Siddiqus &Sakhator 2007).The promotion of plant growth came mainly from the suppression of the disease as well as the control agents make some element more available to be absorbed by plant roots like P(Validov *et al*, 2005 ;Quecin *et al.* 2009). The results revealed that treatment of tomato plant with PGPR and chemical agents can provide protection against *F. oxysporum* under natural conditions.PGPR are ideal to control tomato wilting disease because it can be applied to seed or mixed with soil at seedling or transplanting .In addition a major advantages of PGPR is that once systemic resistance is induced, the natural defense .Mechanisms of the plant are operative for prolonged periods even if population of PGPR decline over time (VanLoon *et al*. 1998).

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