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Efficacy of the Arbuscular Mycorrhizal Fungi and *Bacillus subtilis* for Enhancing the Metabolic Activities and Induced Resistance in Tomato Plant when Challenged with *Fusarium oxysporum*

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THE SYNERGISTIC interaction between two biocontrol agents, an arbuscular mycorrhizal (AM) fungus (*Glomus mosseae*) and a plant growth-promoting rhizobacterium (*Bacillus subtilis*) were tested for improving metabolic activities of tomato plants infected by the pathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* (FOL). The results showed that the treatment with AM fungus, *B. subtilis* or in combination statistically increased the accumulation of the total soluble sugars, total soluble proteins and total free amino acids, especially when the mixture of two antagonistic strains was used.

Elicitation of induced systemic resistance (ISR) by plant-associated biocontrol agents, was also demonstrated in this study. Results proved that the colonization of tomato roots by *G. mosseae* and / or *B. subtilis* increased the activities of peroxidases, polyphenol oxidases and catalases. In addition, ISR by the two-biocontrol microorganisms leads to accumulation of phenolic compounds inside the plant cells. In addition, SDS-PAGE analysis of proteins, revealed that there is an induction of novel proteins as a result of AM fungi and/or bacteria. The obtained results might have contributed to restriction of invasion of *F. oxysporum* in tomato plants.

Keywords: Biological control agents, Arbuscular mycorrhizal fungi, *Bacillus subtilis, Fusarium oxysporum*, Tomato plants, Metabolic activities, Defense mechanisms, Antioxidant enzymes, Phenolic compounds, Pathogenesis proteins.

Biological suppression of plant disease has been promoted as a mean to achieve improved, sustainable crop production systems that are less reliant on chemical inputs (Adams, 1990). Successful biological control systems commonly employ naturally occurring antagonistic microorganisms that are able to reduce the activities of plant pathogens. Such antagonists (or biocontrol agents) can compete with pathogens for nutrients, inhibit pathogen growth by secreting antibiotics, or reduce pathogen populations through parasitism. In addition, some of these microorganisms induce resistance in host plants, which enhances the plant's ability to defend itself from pathogen attack (Gnanamanickam *et al.*, 2002).

Arbuscular mycorrhizae (AM) are widespread in nature and a fundamental component of agroecosystems. They are stable plant-fungus associations, with mutual benefit. AM are currently studied as agents of biological control. Their effects in plant-pathogen interactions range from mainly disease reduction (Rosendahl & Rosendahl, 1990; Liu, 1995; Mikhaeel *et al.*, 2002; Akkopru & Demir, 2005 and Abo-Ghalia, 2007), to a neutral action (Baath & Hayman, 1983, 1984 and Reddy *et al.*, 1989).

Many isolates of the *Bacillus* genus and especially strains of *Bacillus subtilis* were reported to be effective for the biocontrol of plant diseases caused by soil-born pathogens (Raupach & Kloepper, 1998 ; Harris & Adkins, 1999 ; Abo-Ghalia & Hussain, 2002; Ongena *et al.*, 2005 and Abo-Ghalia, 2007). Some of them are used in commercially available biocontrol products (Backman *et al.*, 1997 and Brannen & Kenney, 1997). These microorganisms produce variety of powerful antifungal metabolites (Jacques *et al.*, 1999 and Akpa *et al.*, 2001). It was therefore suggested that antibiotic production by *B. subtilis* play a major role in disease suppression (Yoshida *et al.*, 2001 and Yu *et al.*, 2002).

Fusarium oxysporum is a common soil-born fungus well represented in every type of soil throughout the world (Burgess, 1981). It includes a large diversity of strains, all of which are successful saprophytes, *i.e.*, able to grow and survive for long periods on organic matter in soil and in the rhizosphere of many plant species (Garrett, 1970). Many strains are parasitic but nonpathogenic, *i.e.*, able to invade to some extent plant tissues without inducing symptoms, and some of them are pathogenic and induce either root rot or tracheomycoses. The wilt-inducing strains of *F. oxysporum* cause severe damage on a wide range of economically important crops.

Most studies have focused primarily on the degree of disease reduction; however, the mechanisms of suppression in soil have not been extensively investigated.

In this work, experiments were conducted to study the synergistic interaction between one of the arbuscular mycorrhizal fungi (*Glomus mosseae*) and one strain of plant growth-promoting rhizobacteria (*B. subtilis*) upon the metabolic activities of tomato plants infected with the pathogenic fungus (*Fusarium oxysporum* f. sp. *lycopersici*). This study also wanted to further characterize the enhanced resistance observed with regards to induction of various defense enzymes and to pathogensis related protein (PR) accumulation.

Material and Methods

Plant material

Seeds of tomato plants (*Lycopersicon esculentum* cv Castel Rock) obtained from the Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt, were surface sterilized for five minutes in 2% (w/v) NaOCl and rinsed twice with

sterilized distilled water then dried up between two sterilized filter paper. The sterilized seeds were allowed to germinate for two weeks in autoclaved sandy soil to produce healthy seedlings. Five uniform seedlings were transplanted into clay pots (30 cm diameter) filled with 10 kg of sterilized sandy clay soil [1:1 (v/v)].

Test organisms

1- The AM fungus, which is used, was *Glomus mosseae*. It was produced by Biorize R and D, France and propagated on *Sorghum bicolor* in sterilized pot culture for three months prior to the start of the experiment. Twenty grams of soil-root mixture containing 40-50 *Glomus* spores/g dry soil were placed 2-4 cm below the planting holes in each pot before seedling transplanting. Non-mycorrhizal plants received 20 g of soil identical to that used for growing the pot cultures.

2- *Bacillus subtilis* strain was isolated from an Egyptian soil cultivated with tomato plants and confirmed by the Agricultural Microbial Research Department, Agriculture Research Centre, Giza, Egypt. It was maintained on nutrient agar medium at approximately 6°C. The culture was grown on fresh medium composed of peptone, beef extract, yeast extract and glucose in a ratio of 5:3:1:5 (g/l) in distilled water and incubated on rotary shaker (120 r.p.m) at 28°C for 3 days. The growth was collected by centrifugation (2000 r.p.m) and it was resuspended in sterile distilled water. Twenty ml aliquots of the bacterial suspension (1×10^6 cells/ml) were added using a sterile syringe to each pot at transplanting time.

3- Phytopathogenic strain, *Fusarium oxysporum* f. sp. *lycopersici* (FOL) was isolated from naturally infected tomato plants and identified at the Regional Center for Mycology and Biotechnology, Al-Azhar Univ., Cairo, Egypt. The fungus was allowed to grow on potato dextrose agar (PDA) plates for 5-7 days at 27 ± 1°C, and then the spores were collected by scraping the surface of the Petri dish with sterile distilled water. The suspension was completed to 20 ml. Four ml of the spore suspension (10^7 CFU/ml) were added to the rhizosphere of each seedling (20 ml /pot), two weeks after transplanting.

The treatments were laid out in a factorial experiment as follows:

1- Plants left without any treatments (Control, C).
2- Plants inoculated with the pathogenic fungus only (F).
3- Plants inoculated with AM fungus and the pathogen (AM + F).
4- Plants inoculated with the antagonistic bacteria and the pathogen (BS + F).
5- Plants inoculated with the AM fungus, the bacteria and the pathogen (AM +BS + F).

Five pots were used for each treatment. All pots were randomized in a greenhouse at 25-28°C and watered regularly to near field capacity with tap water.

Harvest

After eight weeks of transplanting, three plant replicates of each treatment were washed carefully, and weighted. Weighed samples from the seedlings were stored in ice and used as soon as possible for the determination of the following:

Determination of total soluble sugars

The total of soluble sugars were measured in an ethanolic extraction of each tomato plant roots and leaves, using the phenolsulphuric method according to Dubios *et al.* (1966). Pure glucose was used as a standard.

Determination of total free amino acids

Total free amino acids in each of the roots and leaves of tomato plants were determined using ninhidrin reagent according to Moore & Stein (1954). Pure glycine was used as standard for amino acids.

Determination of phenolic compounds

Extraction of phenolic compounds was carried out according to Sarma *et al.* (2002). Total phenolic compounds were estimated spectrophotometrically using the Folin Denis method of Swain & Hillis (1959). Absorbancy of samples was measured at 700 nm, using a VIS Spectrophotometer Spectronic 21D. Salicylic acid was used as standard.

Extraction, estimation and characterization of proteins

Quantitative estimation of total soluble proteins in each of the roots and leaves of tomato plants was determined according to the method of Bradford (1976) with Bovine serum albumin as standard.

Protein fractions were characterized and identified using one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12.5%, w/v) was prepared according to Laemmli (1970). The destained gel was photographed, and then scanned for quantitative determination using a laser densitometer.

Estimation of enzymes activity

After harvest, each of leaves and roots of tomato plants were immediately frozen in liquid nitrogen. The plant material was extracted following the method of Kar & Mishra (1976) with slight modifications. One gram of fresh sample was homogenized in cold phosphate buffer (0.05M at pH 6.5). The homogenate was centrifuged at 10,000g for 10 min. The pigments were removed from the supernatant by absorbing with activated charcoal and filtered. The filtrate was completed to a known volume and used as an enzyme source.

Assay of peroxidase activity: Peroxidase activity was determined following the method of Kar & Mishra (1976). The colour developed was read at 430 nm and the enzyme activity was expressed as the change in the optical density. g^{-1} fresh weight. min^{-1}.

Assay of catalase activity: Catalase activity was determined by monitoring the decomposition of H_2O_2 at 240 nm following the method of Aebi (1995).

Mohamedin (2000) and Abo-Ghalia, & Hussain (2002). The reduction in protein content in plants in response to pathogen attack might result in an idleness of protein synthesis machinery and blocking the formation of protein units having functional roles in the cells. In addition, Srinivasan *et al.* (1996) stated that the indol-acetic acid producing *Bacillus* isolates increased nodule number, nodule fresh weight, nitrogenase activity and total soluble protein content in the root nodules of *Phaseolus vulgaris*. El-Sayed *et al.* (2002), also showed that the presence of *Streptomyces albovinaceae* or *Bacillus* sp. is apparently necessary to increase nodulation and to enhance mycorrhizal infection of *Vicia faba* plants, indicating that the stimulation effect of microorganisms on the arbuscular mycorrhizal fungus was due to release of biologically active molecules from the microorganisms.

Free amino acids

Results presented in Fig. 1 show that the free amino acids content in tomato plants inoculated with *G. mosseae* and/or *B. subtilis* was significantly higher than those of non-inoculated control plants or plants infested with the pathogen. These results accorded with those reported by Baltruschat & Schonbeck (1975), who found that VA mycorrhizae inhibited chlamydospore formation by *Thielaviopsis basicola* on tobacco roots, probably because it increased root concentration of arginine and citrulline. These amino acids, when added to extracts of non-mycorrhizal roots, could inhibit chlamydospore formation of *T. basicola*. Moreover, it was reported by Young *et al.* (1972) that the content of free amino acids was 3-10 times greater in roots of corn plants infected with VA-mycorrhizae than in non-infected plants. It was suggested that the growth of mycorrhizal plants is primarily maintained by an increase in the amount of solutes (amino acids and reducing sugars) in the cell and consequent turgor regulation. Aboul-Nasr (1997), also recorded an increase in phenylalanine, proline and arginine.

The direct effect of *B. subtilis* application on free amino acids content is probably due to the effect of this bacterial strain on the stimulation of AM colonization.

Induced systemic resistance of tomato plants by G. mosseae and / or B. subtilis

Plants have endogenous defense mechanisms that can be induced in response to attack by pathogens and insects. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be novel plant protection strategy (Ramamoorthy *et al.*, 2002). The use of AM fungi and *B. subtilis* for controlling soil-born diseases has been well-documented (Cordier *et al.*, 1996; Abo-Ghalia & Hussain, 2002; Sid Ahmed *et al.*, 2003; Akköprü & Demir, 2005 and Idoia *et al.*, 2006). In the present study, prior application of *G. mosseae* and / or *B. subtilis* to induce systemic resistance of tomato plants against FOL was through induction of some defense enzymes and accumulation of phenolic compounds and PR-proteins.

Changes in the activity of peroxidase, polyphenol oxidase and catalase

It is clear from Fig. 2, that the plants inoculated with *G. mosseae* and *B. subtilis*

showed an increase in the activities of peroxidase (PO), polyphenol oxidase (PPO) and catalase (Cat). However, the infection by *F. oxysporum* strongly decreased the activity of these enzymes in plant leaves and roots. In higher plants, a variety of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen ($^1O_2$) are continuously produced in chloroplasts, mitochondria and peroxisomes (Apel & Hirt, 2004). Production and removal of ROS are strictly controlled under natural conditions. When the higher plants are subjected to environmental stresses such as water stress, salinity or pathogen infection, the ROS may initiate destructive oxidative processes such as chlorophyll bleaching, lipid peroxidation, protein oxidation and damage to nucleic acids (Herbinger *et al.*, 2002 and Wu *et al.*, 2006).

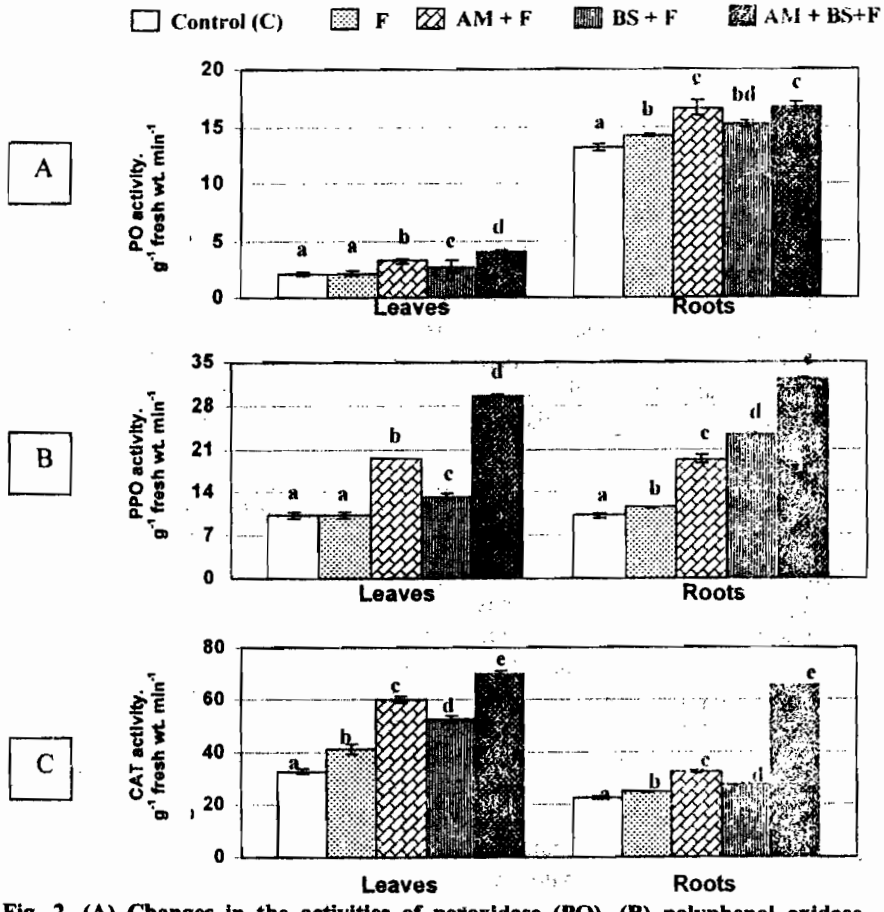

Fig. 2. (A) Changes in the activities of peroxidase (PO), (B) polyphenol oxidase (PPO) and (C) Catalase (CAT) in the leaves and roots of tomato plants infected with *F. oxysporum* f. sp. *lycopersici* (F) and treated with the biocontrol agents [(*G. mosseae* (AM) and/or *B. subtilis* (BS).
(Different letters besides columns indicate statistically different means at $p \leq 0.05$ according to Duncan's multiple range test).

Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce & West, 1989). Increased activity of cell wall bound peroxidases has been elicited in different plants such as cucumber (Chen *et al.*, 2000), tomato (Mohan *et al.*, 1993) and pepper (Abo-Ghalia & Hussain, 2002 and Idoia *et al.*, 2006) due to pathogen infection. In our study, increased peroxidase activity has been recorded in AM fungus and / or *B. subtilis* treated plants challenged with the pathogen (Fig. 2A). However, the increase in the PO activity in the roots was higher than that in the leaves. The increase in PO activity may be contributed to induced defense in tomato plant tissues against *F. oxysporum*. The rapid increase of enzyme activity in infected tomato plants with *F. oxysporum* is associated with parallel increase in total soluble phenols (Ferraris *et al.*, 1987). PO and PPO may oxidize phenolics of the plant to quinines derivatives that are more fungitoxic and inhibitory to pectolytic enzymes of the fungi (Byrde, 1963). PO may also contribute to increased resistance by increase of the concentration of free phenoxyradicals and their polymerization products (Hammerschmidt & Kuc, 1982). In addition, there is a good relation between ethylene and PO in stressed tomato plants. PO is involved in ethylene biosynthesis (Lieberman, 1979) and ethylene induces resistance to *F. oxysporum* (Retig, 1974), and at the same time stimulates PO and PPO activity in tomato plants (Retig, 1974).

Polyphenol oxidase (PPO) activity was also induced by both *G.mosseae* and *B. subtilis* against the challenged pathogen (Fig. 2B). The induced PPO has also been implicated in induced defense response against the pathogen invasion (Abo-Ghalia & Hussain, 2002 and Ramamoorthy *et al., 2002).*

Chen *et al.* (2000) reported that various plant growth promoting rhizobacteria induced the PPO activity in cucumber root tissues. Kavitha *et al.* (2005) also noticed that several isolates of PGPR belonging to the species of *B. subtilis* and *Pseudomonas* sp. were found to trigger the defense resulted enzymes involved in phenylpropanoid pathway and phenolics. Higher activities of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and beta 1,3-glucanase were observed in *B. subtilis* and *Pseudomonas* sp. pre-treated chilli plants challenged with *Pythium aphanidenatum*.

Mycorrhizal cucumber seedlings were found to have higher concentrations of proline and polyphenol oxidase activities, but lower malondialdehyde than non-mycorrhizal seedlings, indicating that AM inoculation may have protected membrane permeability and reduced the extent of the damage caused by *F. oxysporum*, also the mycorrhizal fungus may influence plant secondary metabolites and increase resistance to wilt disease in cucumber seedlings indicating, some potential as a biological control agent (ZhiPeng *et al.*, 2005) . Increase in PO and PPO in mycorrhizal pepper plants infected by *Phytophthora capsici* was also reported by Huzhe *et al.* (2005), in addition the inoculation of the antagonistic *Glomus intraradices* alleviated root mortality, activated changes of lignification-related enzymes (peroxidase, polyphenol oxidase and phenylalanine ammonia- lyase) and induced some of the isozymes in pepper plants infected by *P. capsici*. Finally, they suggested that the AM fungus is a potentially effective protection agent against the fungal pathogen.

Arbuscular mycorrhizal fungi and / or *B. subtilis* significantly increased catalase activity in the leaves and roots of the infected tomato plants (Fig. 2C). Catalase played a crucial role in the eliminating poisonous accumulation of H_2O_2, which increased during stress from plant cells. Bassham & Calvin (1992), reported that catalase activity converts harmful by products in non-toxic substances. Induction of catalases by AM fungi under heavy metal stress conditions was noticed by El-Khallal & Abo-Ghalia (2005). However, no information was available on the effect of AM fungi or the rhizobacteria on the activity of CAT under stress caused by pathogen invasion.

Changes in the total phenols

The results of quantitative spectrophotometric determination of the total phenolic compounds in the leaves and roots of tomato plants infested by *F. oxysporum*, showed phenolics stimulation in the plants treated with the two biocontrol agents. However, the accumulation of phenolic compounds was greater in the plant leaves than those in the roots (Fig. 3), suggesting that phenolic compounds are implicated in disease resistance and although they are found in healthy as well as diseased plants, their synthesis or accumulation seems to be accelerated after infection . The same observation was decided in pea plants infested by *F. oxysporum* (Hussain *et al.*, 2003) or by *F. solani* (Ali, 2005). The phenolic compounds may enhance the mechanical strength of host cell wall and may inhibit the fungal growth, as phenolics are fungitoxic in nature (Ramamoorthy *et al.*, 2002).

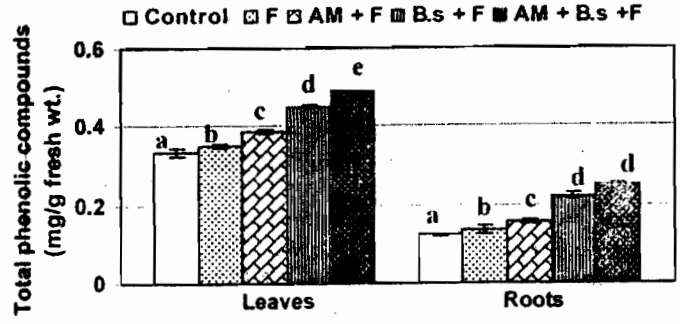

Fig. 3. Total phenolic compounds in the leaves and roots of tomato plants grown in infested soil with *F. oxysporum* (F) and treated with the biocontrol agents [*G. mosseae* (AM) and *B. subtilis* (BS)].

Different letters besides columns indicate statistically different means at $p \leq 0.05$ according to Duncan's multiple range test).

Phenolic compounds have been known to occur in all plants (Nicholson & Hammerschmidt, 1992). Some of them occur constitutively, while others are formed in response to pathogen ingress and were considered to play role in active defense response in the host (Steijl *et al.*, 1999 and Sarma *et al.*, 2002). Benhamou & Lafontaine (1995) noticed that pectin and phenolic compounds play an important role in the infected tomato plant defense mechanism. Benhamou *et al.* (1996) observed when the hyphae of the pathogen, surrounded by phenolic substances, considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content would occur.

Production of inhibitory phenolic compounds by mycorrhizal fungi or their host plays a role in the plant protection mechanism against pathogenic microbes (Morandi, 1989). In addition, some authors (Morandi *et al.*, 1992 ; Metraux & Raskin, 1993 and Hassan, 2007) emphasized that phenolic phytoalexin are involved in the resistance of plant to pathogenic microbial infection. Two successive steps were carried out during the effect of symbiotic association on phenolic metabolism, where in the beginning the phenolic metabolism is under the control during the establishment of the mycorrhizae and then later phenolics increase strongly, thus preventing pathogenic interaction.

Phenolics were also observed to accumulate in bacterized tomato root tissues challenged with *F. oxysporum* (Ramarnoorthy *et al.*, 2002). Higher accumulation of phenolic compounds was also observed in the shoot system than in the root of pea plants infected by *F. oxysporum* and treated by *Bacillus circulance* (Hussain *et al.*, 2003). Beimen *et al.* (1992) and Steijl *et al.* (1999), also demonstrated the accumulation in the shoot system, which indicated that post infection activate phenol metabolism in xylem vessels.

Changes in protein pattern

The electrophoretic profile of different protein bands in the shoots and roots of tomato plants infected with FOL and treated with the biocontrol agents, *G. mosseae* (AM) and *B. subtilis* (BS) separately or in a mixture of them are shown in Fig. 4 and 5. However, the relative molecular weights and percentage of polypeptide bands are recorded in Tables 1 and 2. The present results revealed that pathogen stress evokes changes in the number and concentrations of protein bands as compared with non-treated plants (control). Induction of new bands may play important role in plant defense (Ramamoorthy *et al.*, 2002; Hussain *et al.*, 2003 and Jayaraj *et al.*, 2004).

Scanning of SDS-PAGE gels in the shoot of tomato plants, indicated the occurrence of about 35 protein bands with molecular weights ranging from 142.9 to 4.4 kD in proteins extracted from control and different treatments. In the roots, however only 23 protein bands with molecular weights ranging from 4.5 to 49.4 kD were obtained.

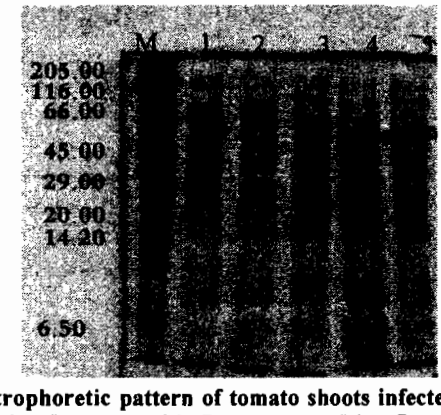

Fig. 4. Protein electrophoretic pattern of tomato shoots infected by *F. oxysporum* f. sp. *lycopersici* and treated with *G. mosseae* and / or *B. subtilis*.

Lane 1: Control (C). Lane 2: *F. oxysporum*.
lane 3: *F. oxysporum* + *G. mosseae*. Lane 4: *F. oxysporum* +*B. subtilis*.
Lane 5: *F. oxysporum* + *G. mosseae* +*B. subtilis*.

TABLE 1. Relative concentration (band %) and molecular weight (M wt) of different types of polypeptide bands in the shoots of tomato plants grown under the pathogen stress (*F. oxysporum* f. sp. *lycopersici*) and treated with biocontrol agents (*G. mosseae* and / or *B. subtilis*).

No. of bands	Control, C	F	AM + F	BS + F	AM + BS + F	M wt (kD)
1	—	0.92	—	—	—	142.9
2	1.28	1.52	0.93	0.15	0.95	120.5
3	1.75	1.51	1.55	1.16	1.20	112.9
4	1.77	1.13	1.27	0.47	2.23	88.7
5	2.14	3.28	2.92	2.10	2.48	81.8
6	2.85	4.20	2.82	2.60	3.72	69.6
7	3.63	3.01	2.67	—	—	64.9
8	2.29	2.50	2.15	3.45	1.43	53.7
9	—	—	4.20	—	—	49.7
10	—	1.73	—	—	—	49.4
11	—	—	—	—	3.55	47.8
12	3.31	2.17	3.60	3.91	—	46.4
13	2.27	2.41	2.58	4.22	7.31	37.7
14	2.10	2.01	5.05	3.71	5.31	35.5
15	3.50	4.20	5.00	5.00	4.10	26.6
16	4.70	5.50	4.50	3.50	4.80	21.5
17	—	—	3.90	—	—	18.4
18	—	—	—	4.19	—	18.1
19	—	—	—	—	3.28	17.7
20	2.00	—	—	—	—	16.4
21	1.82	3.82	4.27	4.19	2.78	15.2
22	—	—	0.43	—	—	12.8
23	0.33	—	—	—	—	12.6
24	0.61	1.40	0.43	0.34	0.86	11.5
25	0.48	—	—	—	—	10.0
26	0.91	1.20	1.70	—	—	9.5
27	1.46	1.97	1.95	2.34	2.22	8.2
28	2.88	3.14	2.28	2.46	2.59	7.7
29	1.73	2.00	2.93	2.12	2.48	6.1
30	3.50	4.62	1.79	2.48	2.14	5.3
31	—	—	—	2.52	—	4.9
32	—	—	—	—	1.15	4.7
33	—	—	1.35	—	—	4.6
34	—	2.36	—	—	—	4.5
35	—	—	—	2.75	1.48	4.4
Total no of bands	**22**	**22**	**23**	**20**	**20**	

F: *F. oxysporum* f. sp. *lycopersici* AM: *G. mosseae* BS: *B. subtilis*

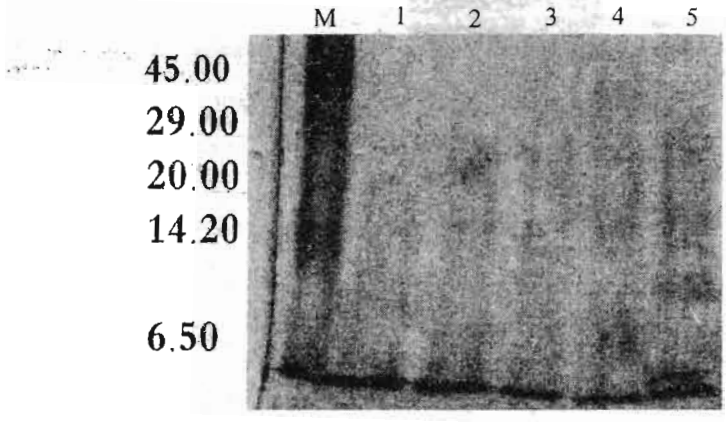

Fig. 5. Protein electrophoretic pattern of tomato roots infected by *F. oxysporum* f. sp *lycopersici* and treated with *G. mosseae* and / or *B. subtilis*.
Lane 1: Control (C). Lane 2: *F. oxysporum*
lane 3: *F. oxysporum* + *G. mosseae*. Lane 4: *F. oxysporum* +*B. subtilis*.
Lane 5: *F. oxysporum* + *G. mosseae.* + *B. subtilis*.

In the shoot system (Table 1), 16 bands were induced in the control and different treated plants. They considered as main bands with the following molecular weights: 120.5, 112.9, 88.7, 81.8, 69.6, 53.7, 37.7, 35.5, 26.6, 21.5, 15.2, 11.5, 8.2, 7.7, 6.1 and 5.3 kD. The control (untreated) plants induced the accumulation of three proteins with molecular weights of 16.4, 12.6 and 10 kD. The infested plants (F) accumulated the proteins with M.wt of 142.9, 49.4 and 4.5 kD. The same Table, showed a set of four proteins with M.wt 49.7, 18.4, 12.8 and 4.6 kD in plants treated with the AM fungus and infested by the pathogen (AM+F). In addition, *B. subtilis* treatment (BS+F) induced the accumulation of two proteins with M.wt of 18.1 and 4.9 kD. However, new structural proteins with M.wt of 47.8, 17.7 and 4.7 kD were produced in tomato plants infected by FOL and treated with *G. mosseae* and *B. subtilis* (AM +BS + F).

In the root system (Table 2), only eight bands are considered the main bands. Their molecular weight were 35.5, 24.1, 20.2, 17.9, 15.9, 13.9, 10.1 and 4.5 kD. Because of FOL infection, only one band at M.wt of 46.3 kD was observed. Also, one band at 11.7 kDa was obtained in the plants treated with *G. mosseae* and FOL (AM+F). In the plants treated with *B. subtilis*, a set of three polypeptide bands (47.2, 41.1 and 26 kD) were accumulated. However, five proteins (49.4, 48.2, 42.2, 26.2 and 4.6 kD) were excited in response to *G. mosseae* and *B. subtilis* treated plants. These data revealed that AM fungi and / or *B. subtilis* induced de-novo synthesis of specific proteins, suggesting that new genes may be transcribed or at least, the products of some genes are increased and others decreased (Tanimoto & Ishioka, 1990), which may play functional role in plant defense against soil born pathogens. This, to some extent, agreed with those reported by Dumas-Gaudot *et al.* (1994), Ramamoorthy *et al.* (2002) and Hussain *et al.* (2003).

TABLE 2. Relative concentration (band %) and molecular weight (M wt) of different types of polypeptide bands in the roots of tomato plants grown under the pathogen stress (*F. oxysporum* f. sp. *lycopersici*) and treated with biocontrol agents (*G. mosseae* and / or *B. subtilis*).

No. of bands	Control, C	F	AM + F	BS + F	AM + BS + F	M wt kD
1	—	—	—	—	1.64	49.4
2	—	—	—	—	1.43	48.2
3	—	—	—	1.00	—	47.2
4	—	—	—	—	1.10	46.8
5	—	0.50	—	—	—	46.3
6	—	—	—	—	0.94	42.2
7	—	—	—	2.17	—	41.1
8	0.71	0.28	1.55	1.41	2.20	35.5
9	—	—	0.72	1.11	0.83	28.6
10	—	—	—	—	1.74	26.2
11	—	—	—	0.68	—	26.0
12	0.43	1.98	1.91	1.22	1.27	24.1
13	1.18	5.61	2.37	1.04	1.11	20.2
14	2.10	1.88	2.57	2.05	1.90	17.9
15	1.74	2.62	2.42	2.39	2.82	15.9
16	0.70	0.89	0.98	2.00	1.67	13.9
17	—	—	1.23	—	—	11.7
18	1.03	0.51	0.88	0.67	4.92	10.1
19	2.41	—	1.41	3.75	2.36	8.2
20	—	1.53	1.58	1.48	1.82	6.6
21	—	—	3.19	—	1.67	5.2
22	—	—	—	—	6.49	4.6
23	3.63	3.73	4.99	3.08	5.85	4.5
Total no of bands	**9**	**10**	**13**	**14**	**18**	

F: *F. oxysporum* f. sp. *lycopersici* AM :*G. mosseae* BS: *B. subtilis*

Pathogensis related proteins (PRs) are host-coded proteins induced by different types of pathogens under a biotic stress (van Loon, 1997). Synthesis and accumulation of PRs have been reported to play an important role in plant defense (Maurhofer *et al.*, 1994 and van Loon, 1997). It has been suggested that, in induced plants, the accumulation of intracellular PRs proteins form the first line of defense, lysing the pathogen with lytic enzymes (Lorito *et al.*, 1994 and

Ramamoorthy *et al.*, 2001). These enzymes act upon the fungal cell wall resulting in degradation and loss of inner contents of cells (Benhamou *et al.*, 1996).

Advanced investigations suggested that some genes involved in defense mechanisms are more weakly activated during a symbiotic interaction than in pathogenic infection (Gianinazzi-Pearson *et al.*, 1992). In this connection, the production of pathogensis related PR-b1 proteins has been compared in tobacco roots infected by the pathogenic fungus *Chlara elegans* or by the mycorrhizal fungus *Glomus mosseae*. The level of PR-b1 protein and amounts of RNA coding for these proteins were very low in mycorrhizal roots when compared with roots inoculated with pathogens. Moreover, the PR-b1 proteins appear to be strictly localized at the symbiotic interface in contrast with the broader localization in plant wall material during host pathogen interactions (Dumas-Gaudot *et al.*, 1994).

In addition, Jayaraj *et al.* (2004) noticed that the treatment with *B. subtilis* increased the activities of phenylalanine ammonia-lyase and peroxidase, and the accumulation of PR proteins in rice leaves infected with *R. solani*. The application of *B. subtilis* also induced the accumulation of thaumatin-like protein (17 kD) and two beta-1-3- glucanases (30 and 33 kD). Western blotting analysis for chitinase revealed the induction of two chitinases with molecular masses of 30 and 35 kD in the induced plants. These suggest that the defense responses may be involved in the restriction of *R. solani*.

In conclusion, the interaction between the two biocontrol agents (AM fungi and *B. subtilis*) led to decrease in the disease symptoms and enhancement in the growth and nutrition of tomato plants infested by *F. oxysporum* f. sp. *lycopersici* (Abo-Ghalia, 2007).On the other hand, the present study, showed that the treatment with these biocontrol agents increased some of metabolic products (total soluble sugars, total soluble proteins and free amino acids), accumulation of phenolic compounds and PR proteins and finally the induction of some defense enzymes (peroxidase, polyphenol oxidase and catalase) in the same plant, which may be collectively contributed to induced resistance in tomato plants against the fungal pathogen .

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كفاءة كل من فطريات الميكوريزا الداخلية و بكتريا *الباسيللس ستلس* كعوامل مقاومة حيوية لزيادة الأنشطة الأيضية ولاستحثاث تفاعلات المقاومة في نبات الطماطم المصاب بفطر *الفيوزاريوم اكسيسبورم*

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يهدف هذا البحث إلى دراسة التأثير التعاوني للفطرة الجذرية الداخلية (*G. mosseae*) وإحدى أنواع البكتريا الجذرية المحفزة للنمو (PGPR) وهي *B. subtilis* على بعض الأنشطة الأيضية لنبات الطماطم المصاب بمرض الذبول الناتج عن فطر الفيوزاريوم (*F. oxysporum f. sp. lycopersici*). وقد أظهرت النتائج أن النباتات المحقونة بفطر الميكوريزا أو بالبكتريا كل على حدة أو كليهما معا زيادة معنوية في تراكم السكريات والبروتينات الذائبة والأحماض الأمينية الحرة خاصة النباتات المعاملة بمخلوط السلالتين المضادتين.

أثبت الدراسة استحثاث تفاعلات مقاومة الفطر الممرض داخل أنسجة النبات نتيجة معاملتها بفطر الميكوريزا أو بالبكتريا أو كليهما معا،و تمثل ذلك في زيادة نشاط بعض الإنزيمات مثل إنزيمات البيروكسيديز والبولي فينول أوكسيديز والكتاليز، بالإضافة لتراكم المركبات الفينولية داخل النبات .

كشف الفصل الكهربي للبروتينات الذائبة على وجود استحداث لأنواع جديدة من البروتينات نتيجة لاستخدام فطر الميكوريزا والبكتريا أو كليهما معا والتي ربما يكون لها دور ايجابي في زيادة مقاومة النبات للفطر الممرض.

وبذلك يتضح أن التأثير المشترك لكل من فطرة الميكوريزا الداخلية وبكتريا *الباسيللس ستلس* قد يفسر الحد من التأثير الضار لغزو الفطر الممرض لأنسجة نبات الطماطم .