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Study of the effectiveness of some enzymatic antioxidants of tomato plant (*Lycopersiconesculentum* L.) infected byfusarium wilt disease caused by *Fusariumoxysporum* with biological control by *Pseudomonas fluorescence* and *Bacillus subtilis* bacteria

*Kareem U Hasan
Dept. of Desertification Combat, College of Agriculture / University of Baghdad, Iraq
Corresponding Author: Kareem U Hasan

Abstract:*Pseudomonas fluorescens and Bacillus subtilis bacteria were isolated from different agricultural soil samples and Fusariumoxysporum fungus was isolated from the infected roots of tomato plant.The isolated fungus pathogenicity was tested, with the presence of the biological control, affecting the growth of fungal pathogen in the laboratory and greenhouse. The results of fungus isolation and microscopic tests showed presence of F. oxysporum fungus in most of plant samples, and fungus pathogenicity, using millet seeds (Panicummiliaceum), had reducedgermination ratio of 5%compared with the control treatment (without fungus of 90%). Also, the antagonistic capability of Pseudomonas fluorescens and Bacillus subtilis bacteria against the fungal pathogen, grown on Kings B Agar medium, showed an inhibition ratio of 76% and 55.05%, respectively, compared with the control treatment of 0%. The results showed significant differences between the activities of enzymatic antioxidants SOD, POD, CAT, and GPX (absorbing unit.ml^{-1}) where it has taken the same behavior to increase this effectiveness with the presence of bacteria (B. sub. and P. flu.) compared with the absence of bacteria after 30 days of planting, and the activity of SOD enzyme decreased at 30 days compared with the activity of other enzymes. Inoculation by P. flu.led to increasing the activity of studied enzymes compared with inoculation by B. sub.*

Keywords: *Bacillus subtilis, biological control, enzymatic antioxidants, Fusariumoxysporum, Pseudomonas*

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I. Introduction

Tomato plant is infected with various fungal diseases including fusarium wilt disease caused by *Fusariumoxysporum* causing big loss in production [1], and despite the use of fungicides that are hazardous to human health and the environment, fungal strains resistant to these pesticides have emerged [2].In many studies, researchers have sought to find alternatives to these hazardous substances. Many strategies were used, including biological control agents such as Bacillus spp, Pseudomonas spp., and some fungi such as genus Trichoderma and Mycorrhiza which have a symbiotic relationship with plant roots [3]. Matta [4] stated that there were two kinds of stress: biotic and abiotic. The two kinds of stress effects on plant led to induced resistance which defined as the interaction between two beings: inducer and challenger. The induced resistance representing a physiological status in which the defensive ability of plant was induced through the specific stimulation of the different plant defensive means [5]. There are two kinds of induced resistances in the plant: local acquired resistance, formed in the infection place, and systemic acquired resistance, found far of infection place [6].

One of the examples of different responses is the production of plant defensive hormones such as salicylic and Jasmonic acids and others [7] as well as increasing production of Reactive oxygen species (ROS) which also called Free Radicals, they are uncontrolled intermediate compounds produced by oxidation processes with the presence of oxygen [8].

The mechanism of enzymatic antioxidants started at the first defensive line which representative by Superoxide Dismutase (SOD) which acting to remove superoxide (O_2) led to accumulating H_2O_2 which reduced by Catalase (CAT) converting hydrogen peroxide into oxidized GPX and water, thus CAT, APX, and GPX work to assist in preventing oxidative damage [9]. About Peroxidase enzyme, Stroble et al. [10] explained that there was a positive relationship between this enzyme and the induced resistance against disease in the host plant, they confirmed that the enzyme had an important role to create ethylene, resisting diseasing, wound healing, and forming lignin, and had an important role in building cell wall by converting Polymerizing hydroxyl and Methoxycinnamic alcohols into lignin [11].

The exposure of the plant to stress (both biotic and abiotic) causes a defect in the chain of electron transmission to the living cells leading to increment in the formation of ROS. There are two main forms of ROS:

the molecular form such as hydrogen peroxide (H_2O_2) and single oxygen (O_2), and the free radical form such as superoxide (O_2), hydroxide (OH) and Perhydroxyl (HO_2), and Alkoxy radicals (RO) [9]. Some ROS kinds, such as Superoxide Anion and Hydrogen peroxide, were naturally and commonly formed bybiochemical reactions occurred inside plant cells and stimulated Hypersensitive Resistance (HR) or systemic acquired resistance [12]. Hydrogen peroxide had an anti-spores germination effect of many fungal pathogens and contributed to form Phenoxyl radicals through Phenol polymerization within plant cell wall [13].

ROS is a strong oxidant with a harmful and toxic effect on living cells because it attacks the components of the cell (protein) and causes damage and deterioration. In addition, causes oxidizing unsaturated fatty acids of cellular membranes, carbohydrates, and photosynthesis, as well as genetic changes in DNA Leading to membrane damage and metabolic dysfunction [14]. The development of plant antioxidant defense system, to protect itself against the damage of oxidative stress, is either by reducing ROS production or removing Scavenging produced by ROS [15]. There is a balance between what is constituted of reactive oxygen and what is destroyed in the normal status. Reactive oxygen species had great importance by acting as a messenger in defensive response against pathogens. They also play a key role in the formation of lignin through two kinds of oxygen, hydrogen peroxide and superoxide.

The current study aimed to determine the activity of some enzymatic antioxidants of tomato plant at the infection by fusarium disease caused by *Fusariumoxysporum f. sp. lycopersici,* and study the effect of the biological control of *Bacillus subtilus*and *P. fluorescens* bacteria against *F. oxysporum* pathogen causing tomato plant wilting.

II. Materials And Methods

1. SOIL SAMPLING

30 soil samples were obtained from the rhizosphere of different agricultural areas, placed in sterile containers, brought to the laboratory for bacterial isolation, and placed at a temperature of 4 ˚C until the isolation.

2. ANALYSIS OF GREENHOUSE SOIL

Soil samples were randomly chosen, pH and EC were measured in 1:5 (soil: water) [16], organic matter was evaluated according to Walkley-Black [17], calcium carbonate was evaluated by Calcimeter method, available phosphorus was evaluated using the extraction by sodium bicarbonate method [18], exchanged potassium was evaluated using the extraction by ammonium acetate method [19], Evaluating of Cation Exchange Capacity (CEC) [20], soil texture evaluated using hydrometer method [21], and aerobic microflora were evaluated by CFU g^{-1} soil.

3. BACTERIA ISOLATION

P. fluorescens bacteria were isolated using King B Medium (KBM) culture medium [22] by taking 1 g of rhizosphere soil putting in 9 ml of desterilized water, then taking 0.1 ml of the suspension and spreading on the KBM in the dishes. After 48 h of incubation at 28 ˚C, dishes were exposed to ultra violet rays (365 nm) for many seconds. The colony appeared as fluorescence, and a part of it was taken and lined on the slant medium to be kept for long-term use.

B. subtilis bacteria were isolated where 1 g, from each soil sample, was taken and placed into test tube containing 5 ml of the liquid nutrient medium (broth), and the test tubes were incubated at 30 ˚C for 48 h, then 0.1 ml was transferred from the culture to the Petri dishes containing the hard nutrient medium (N. Agar) and incubated at 30 ˚C for 48 h [23].

4. BACTERIA IDENTFICATION

Cultural, microscopic, and biochemical tests (bacteria shape, Gram stain, spores formation, movement, oxidase, citrate decomposition, indole, catalase, nitrate reduction, and H_2S production) of isolated bacteria were conducted (Bergeys Manual) [24].

5.*Fusariumoxysporum*FUNGUS ISOLATION

The fungus was isolated from infected roots of the tomato plant, cut into small parts of 0.5 cm, washed and sterilized with 5% sodium hypochlorite and then washed with sterilized distilled water to remove the sterilizer effect, the infected roots were transferred to Petri dishes which contained Potato Dextrose Agar (PDA) which 250 mg of streptomycin was added. The Petri dishes wereincubated at 25 ˚C for 5 days. After the appearance of growing fungal colonies, they were transferred to new dishes to identify the type of fungal isolate based on the morphological properties, mentioned byParmeter and Whiteny [25], then transferred to the PDA slant medium to be used later.

6. TESTING PATHAGENICITY OF FANGAL PATHOGENE

Fungal pathogenicity was tested using the seeds of millet (*Panicummiliacium*), where 9 cm in diameterPetri dishes, containing water – Agar medium, were prepared. Local millet seeds, which surface

sterilized by 5% of sodium hypochlorite, were planted and distributed on the dish margin as 20 seeds for each dish. Three replicates were used as well as the control treatment (without pathogen), and the dishes were incubated at 25 ˚C for one week, then the germination ratio was calculated as the following equation:

% Germination = (number of germinated seeds / total seeds) × 100 (1)

7. EXTERNALLY TESTING ANTAGONISTIC CAPABILITY OF BACTERIA AND FUNGUS

An experiment was conducted to know the antagonistic relationship between *F. oxysporum*fungus and *P. fluorescence* bacteria. This experiment was carried out based on Dual Culture Technique [26] by taking 8 mm, in diameter, disc of the fungal pathogen from the culture margin of *F. oxysporum* fungus (5 days age) and putting in the center of dish that containing KBA medium then being lined by the loop on 3 cm from *P. flu.* Bacteria, and another dish of *B. sub.*was prepared. All dishes were incubated at 28 ˚C for 5 days. After the incubation, the fungus growth diameter, with presence of both genus of bacteria, was recorded and compared with the fungus growth in the control (without bacteria) for the three replicates. The percentage of Fungal Growth Inhibition (FGI) was calculated based on the following equation [26]:

% FGI = [1 – (FG in bacteria treatment / FG in control) × 100 (2)

8. PREPARATION OF THE FUNGAL INOCULUM

Fungal inoculum was prepared by obtaining fungus conidia by a sterilized tool from the fungal colony surface and put in sterilized and distilled water and filtered by using Nylon Mesh. The density of suspended spores was 2.1×10^5 spore m^{-1} and calculated based on El-Mougy and Abdel-Kader [28].

9. PREPARATION OF BACTERIAL INOCULUM

P. fluorescence and *B. subtilis* bacteria were grown in an N.B nutrient medium into test tubes, each bacteria was separated from the other. The tubes were incubated at 28 ˚C for 48 h then in the centrifuge, and the suspension of both bacteria were re-prepared into sterilized and distilled water, then the bacterial inoculum density was calculated [28], which was $10^9 – 10^{10}$ cell ml^{-1}.

10. PREPARATION OF TOMATO SEEDS WITH BACTERIAL INOCULUM

The previous suspension of the both bacteria was used and added 1% pectin to *P. flu.*and*B. sub.*bacteria, separately, in conical flasks. The suspension was mixed for 15 min by Magnetic Stirrer Plate. The seeds of tomato were added to the suspension of 500 g. L^{-1} and left to imbibe for 12 h [29]. The seeds were dried by filtration paper for 2 h, then stored at 4 ˚C to be used later.

11. GREENHOUSE EXPERIMENT

The greenhouse soil was sterilized by the sun for three months June, July, and August by moistening the soil and covering with polyethylene plastic for the purpose of sterilizing the soil by pasteurization. The seeds of the tomato treated with the bacterial inoculum were planted in the greenhouse soil in plots (1.5 x 2 m) containing 2 rows, each row contains 2 holes. Five seeds were planted in each hole with three replicates per treatment. The fungal inoculum, *F.oxysporum* of 20 ml, was added [30] per hole. Plots containing the fungus *F.oxysporum* without antagonistic bacteria were used as a control. Chemical fertilizers (NPK), basis on the fertilizers recommendation of the tomato plant, were added. Total chlorophyll (mg. g^{-1}) according to Goodwin [31]. Fresh and dryweight of shoot system (g $plant^{-1}$), and the plant length (cm) were evaluated.

12. THE EVALUATION OF ENZYMATIC ANTIOXIDANTS ACTIVITY

The activity of enzymatic antioxidants (SOD, POD, CAT, and GPX) was evaluated. After 10 weeks planting, 1 g of the fresh plant sample (roots and leaves) was mashed,after cutting into small pieces, with 0.1 M of potassium phosphate at pH 7.8. After filtration using a piece of gauze, the filtered liquid centrifuged at 1000 rpm for 30 min, then taken to evaluate the enzymatic activity [32].SOD activity was evaluated by Nitro Blue Tetrazolium (NBT) according to [33]. POD activity was evaluated according to [34] which depended on light absorption change within 30 sec for 3 min at wavelength of 420 nm. CAT activity was evaluated according to [35] using Spectrophotometer which depended on calculating the absorption change value at wavelength of 240 nm. GPX activity was evaluated according to [36] using Spectrophotometer at 420 nm.

13. STATISTICAL ANALYSIS

Statistically, data was analyzed using Statistical Analysis System (SAS) program for a factorial experiment according to Complete Random Design (CRD). Significant differences, among treatments means, were compared by choosing least difference using Turkey-Karmer test (< 0.05).

III. Results And Disscusion

1. SOIL ANALYSIS

Soil properties confirmed that Electrical Conductivity (EC) was moderate, and Cation Exchange Capacity (CEC) was almost high of 31 – 35 cmol.Kg^{-1}. The soil was high calcareous and loamy texture (Table 1).

Table 1: Some soil properties

Total Bacteria Count Cfu g^{-1}soil	O.M %	K $mg.Kg^{-1}$	P $mg.Kg^{-1}$	N $mg.Kg^{-1}$	CaCO3 %	CEC $Cmol.Kg^{-1}$	EC dsm^{-1}	pH
2.3×10^{6}	0.7	33.0	12.0	15.6	23.5	33.0	3.2	7.2

2. BACTERIA ISOLATION

The results of isolation showed different shapes and morphologyof bacterial colonies. Some of colonies had fluorescence brilliance on KBM. To identify other isolated colonies on liquid (N.B) and solid (N.A), the following tests were conducted:

2.1 MORPHOLOGICAL AND MICROSCOPIC TESTS

A difference in morphological properties of bacteria was observed under microscope test after staining with Gram and spores stains. There were differences in responding tobiochemical tests [37]. Based on identification results, isolated colonies were put into two groups: one referred to *P. fluorescence* bacteria and the other referred to *B. subtilis*, one isolate was chosen for each other to be used as antagonist for pathogen fungus.

Table 2: Identification tests of the two chosenisolates as antagonist for pathogen fungus

Bacteria Isolate	Gram Stain	Bacillary	Stain product	Methyl Red	Indole	Catalase	Starch decomposition	Oxidase	Citrate
P.flu.	-	+	+			+	-	+	+
B.sub.	+	+	-			-	+	-	-

3. ISOLATION OF PATHOGEN FUNGUS

Pathogen fungus got 10 fungal isolates identified as it referred to *Fusariumoxysporum*. One isolate was chosen based on the results of pathogenicity experiment test of the fungus for millet seeds. The results showed that *F. oxysporum* led to a significant decrease the germination ratio of millet seeds of 5%, compared to the control treatment of 90%. This test confirmed that the isolate was a pathogenic due to its excretions of toxic metabolic compounds which had a role in killing embryos of millet seeds as well as the fungus ability to produce pectin and cellulose analyzer enzymes that responsible on the seeds rot then preventing germination. Asperlin and isoasperlin are toxins assisting the fungus to cause the infection.

4. EXTERNALLY ANTAGONISM BETWEEN PATHOGEN FUNGUS AND BACTERIA

Results showed that *P. fluorescence* bacteria had a high antagonism against *F. oxysporum* fungus. There was no link between bacteria and fungus, but a huge aura between each other, thought that it was found due to excreting toxins excreted into the nutrient medium to kill the pathogen fungus [38]. Those toxins (pyoverdin or pseudopectin) had an ability to affect ferric ion (Fe^{+3}) which had the ability to form a complex compound from Fe^{+3} which reacting with the outer shell of the fungus by sidrophore [39]. There was a change in Mycelia color (orange) at the zone between bacteria and fungus resulted from cytoplasmic leakage of fungal spinning and in some cases this color is surrounded by dark green and excreting some volatile components (HCN) [40].Inhibition (fungus growth) ratio of *B. subtilis* bacteria was calculated which was 93.5%, while inhibition ratio of *P. fluorescence*bacteria was 50.5% after 4 days in the culture medium, that can be attributed to the ability of *P. fluorescence*bacteria to excrete five antibiotics: subtiline, subterolin, bacitracin, bacillin, and bacilomycin which act to inhibit pathogen fungus growth [41], as shown in Table 3.

Table 3: The inhibition activity of *B. sub.*and*P. fluor.*bacteria to *F. oxysp.* fungus growth

Treatment	Fungus Growth cm	Inhibition Ratio %
F. oxysporum × *P.fluorescens*	2.20^{b}	93.5^{b}
F. oxysporum × *B.subtilis*	4.00^{c}	55.05^{a}
Control	9.00^{a}	0.00^{c}
LSD.0.05	0.52	2.98

5. EVALUATION OF SOME PLANT GROWTH CRITERIA

Results (Table 4) showed that biological stress, resulted by fungal infection, affected plant growth criteria, in which total chlorophyll of only fungus treatment decreased compared the control. Other criteria had

the same decreased behavior compared to the control, while an increment was observed in all criteria of bacterial (*P. fluor.* and *B. sub.*) inoculum treatment with pathogen fungus compared with the other treatments. The decrement in leaves chlorophyll content was due to increasing chlorophyllase activity, increasing products of active oxygen, and ionic equilibrium destabilization due to the biological stress and plant fungal infection [42]. The bacterial inoculum modified the plant growth, for all growth criteria, compared with only fungus treatment.

Table 4: Results of analyzing and calculating some plant growth criteria

Treatment	Total Chlorophyll Mg g $^{-1}$	Fresh Weight g Plant $^{-1}$	Dry Weight g plant $^{-1}$	Length cm Plant $^{-1}$
Control	1.921^{c}	0.195^{c}	0.043^{c}	110.30^{c}
F. oxysporum	1.222^{d}	0.172^{c}	0.039^{c}	100.03^{d}
P.fluorescens +F.	2.103^{a}	0.281^{a}	0.050^{a}	150.23^{a}
B.subtilis + F.	1.831^{b}	0.211^{b}	0.049^{b}	120.70^{b}

6. ENZYMATIC ANTIOXIDANTS ACTIVITY

Results (Table 5) showed that the enzymatic antioxidants (SOD, CAT, POD, and GPX) increased with the plant fungal infection (*F. oxysporum*) within 15 – 30 days after planting compared with the control (without fungus).

Table 5: The activity of SOD, CAT, POD, and GPX in the plant roots

Tretment / Days after planting	SOD U abs.ml^{-1}		CAT U abs. ml^{-1}		POD U abs. ml^{-1}		GPX U abs.ml^{-1}	
	Root		Root		Root		Root	
	15	30	15	30	15	30	15	30
Control	16.78^{d}	20.70^{c}	23.90^{d}	29.48^{d}	20.38^{c}	22.44^{d}	19.83^{d}	22.13^{c}
F.oxysporum	25.91^{a}	30.18^{b}	37.98^{c}	79.42^{c}	45.78^{b}	49.23^{c}	30.01^{c}	38.13^{b}
F.oxysporum + P.fluorescnse	29.94^{b}	34.95^{a}	54.86^{a}	90.27^{a}	52.29^{a}	61.03^{a}	38.45^{a}	41.66^{a}
F.oxysporum + B.subtilis	24.27^{c}	32.93^{a}	50.00^{b}	85.08^{b}	48. 12^{b}	56.65^{b}	32.15^{b}	39.91^{b}
L.S.D. 0.05	0.46	2.03	4.27	5.50	3.39	4.02	0.48	1.83

There were significant differences among enzymatic activity values of bacterial inoculum with the fungus compared with the only fungus treatment for all enzymes, this can be attributed to the ability of bacteria to limit producing types of active oxygen through stimulating enzymatic defense system where it possible to increasing antioxidants enzymes activity through bacteria metabolic activities which positively reflected on the plant growth [43]. The results showed that the SOD enzyme had a different behavior for all studied enzymes within 30 days after planting where the SOD values (34.95 U abs. ml^{-1}) decreased compared with the others, CAT, POD, and GPX of 90.27, 51.03, and 40.65 U abs. ml^{-1}, respectively, at adding the bacterial inoculum (*P. fluor.*), while at adding *B. sub.*the value of SOD was 32.91 U abs. ml^{-1} compared to others of 85.38, 46.56, and 35.91 U abs. ml^{-1}, respectively. This decrement in the SOD activity, compared to others, can be attributed to that it considered as the first defensive line in the plant anti-oxidative defense system had an important role in dismantlingfree hydroxide root converting it into hydrogen peroxide.

Results showed that CAT activity (90.27 U abs. ml^{-1}) was a higher than others at 30 days after the planting for the bacterial inoculum (*P. fluor.*). Willekens et al. [44] explained that the velocity and continuity of increasing CAT activity might point that the enzyme is a key enzyme for removing toxicity of hydrogen peroxide under the stress. POD is a part of enzymatic protection system and the effect by stress where the infection of tomato by pathogen fungus led to a significant increment in POD activity which was 51.03U abs. ml^{-1} of the bacterial inoculum, *P. fluor.*, after 30 days of planting, while was 46.56 U abs. ml^{-1} of *B. sub.* compared to the control treatment of 21.44 U abs. ml^{-1}.

The results showed that GPX had the same behavior of POD where its activity of *P. fluor.*was 40.65 U abs. ml^{-1} while was 35.91 U abs. ml^{-1} of *B. sub.*compared to the control of 22.13 U abs. ml^{-1}after 30 days of planting, as well as its activity of *B. sub.*had a superiority compared to the only fungus treatment. That might be attributed to that GPX had a role in converting hydrogen peroxide into oxidized glutathione and water, thus CAT, POD, and GPX assist to prevent lipid peroxidation.

IV. Conclusion

The activity of enzymatic antioxidants had stimulated at the infection of tomato plant by pathogen fungus (*F. oxysporum*) and by the effect of prompting enzymatic antibiotics at the inoculation with (*P. fluor.*and *B. sub.*), the values of enzymes activity in tomato plant inoculated with bacteria higher than those in non-inoculated plant after 30 days of planting for all enzymes compared with 15 days after planting. Bacteria can

play an important role in reducing plant bio-stress based on the ability and effectiveness of bacterial genus and the antagonism ability to inhibit pathogen. In the case of inoculation by *P. flu*., all studied enzymes higher activity than the activity of the same enzymes at the inoculation with *B.sub*. It is believed that *P.flu*. had a high antagonistic ability against pathogen fungus and its ability to produce toxic substances, such as Pseudopectin and pyoverdin, which considered as metabolites to kill the fungus as well as it excreted a metabolic substances including growth regulators such as Indole Acetic Acid (IAA) and other substances had a role to stimulate plant bio-resistance.

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