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Study of some antioxidant enzymes of Cucumber *(Cucumis sativus* L.) infected by *Fusarium solani* fungus with biological control by *Pseudomonas fluorescens bacteria.*

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ABSTRACT

Pseudomonas fluorescens bacteria was isolated from rhizosphere different soil samples and Fusarium solani fungus was isolated from the infected roots of cucumber plant. The isolated fungus pathogenicity was tested, with the presence of the biological control, affecting the growth of fungal pathogen in the laboratory and pots experiment . The results of fungus isolation and microscopic tests showed presence of F. solani fungus in most of plant samples, and fungus pathogenicity, using cucumber seeds (Cucumis sativus L.), had reduced germination ratio of 5% compared with the control treatment (without fungus of 90%). Also, the antagonistic capability of Pseudomonas fluorescens (P.flu.) bacteria against the fungal pathogen, grown on Kings B Agar medium, showed an inhibition ratio of 82.5%, compared with the control treatment of 0%. The results showed significant differences between the activities of enzymatic antioxidants: SOD and CAT (absorbing unit.ml^{-1}) where it has taken the same behavior to increase this effectiveness with the presence of bacteria (P. flu.) compared with the absence of bacteria after 60 days of planting. Inoculation by(P. flu.) led to increasing the activity of studied enzymes,and some parameters of plant growth.

Keywords: Pseudomonas, fluorescence, enzymatic, antioxidants, Fusarium solani, Biological control.

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INTRODUCTION

Cucumber plant is infected with various fungal diseases including fusarium wilt disease caused by *Fusarium* solani causing big loss in production [1]. 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 [2]. Matta [3] 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 [4]. 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 [5].

One of the examples of different responses is the production of plant defensive hormones such as salicylic and Jasmonic acids and others [6] 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 [7].

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 [8]. About Peroxidase enzyme, Stroble et al. [9] 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 Methoxy cinnamic alcohols into lignin [10].

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 by biochemical reactions occurred inside plant cells and stimulated Hypersensitive Resistance (HR) or systemic acquired resistance [11]. 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 [12].

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 [13]. 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 [14]. 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 cucumber plant at the infection by fusarium disease caused by *Fusarium solani,* and study the effect of the biological control of *P. fluorescence* bacteria against *F. solani* pathogen causing cucumber plant wilting.

MATERIALS AND METHODS

SOIL SAMPLING

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

ANALYSIS OF POTS SOIL

pH and EC were measured in 1:1 (soil: water)[15], organic matter was evaluated according to Walkley-Black [16], calcium carbonate was evaluated by Calcimeter method, available phosphorus was evaluated using the extraction by sodium bicarbonate method [17], exchanged potassium was evaluated using the extraction by ammonium acetate method [18], Evaluating of Cation Exchange Capacity (CEC) [19], soil texture evaluated using hydrometer method [20], and aerobic microflora were evaluated by CFU g^{-1}dry soil.

BACTERIA ISOLATION

P. fluorescence bacteria were isolated using King B medium (KBM) culture medium [21] by taking 1 g of rhizosphere soil putting in 9 ml of desterilized water, then taking 0.5 ml of the suspension and spreading on the KBM in the dishes. After 48 h of incubation at 28 ˚C.

BACTERIA IDENTFICATION

Cultural, microscopic, and biochemical tests (bacteria shape, Gram stain, movement, oxidase, citrate decomposition, indole, catalase, nitrate reduction, and H_2S production) of isolated bacteria were conducted (Bergeys Manual) [22].

***Fusarium solani* FUNGUS ISOLATION**

The fungus was isolated from infected roots of the Cucumber 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 were incubated at 25 ˚C for 5 days. After the appearance of growing fungal colonies, they were transfered to new dishes to identify the type of fungal isolate based on the morphological properties, mentioned by Parmeter and Whiteny [23], then transfered to the PDA slant medium to be used later.

TESTING PATHAGENICITY OF FANGAL PATHOGENE

Fungal pathogenicity was tested using the seeds of cucumbr plant where 10 cm in diameter Petri dishes, containing PDA Agar medium, were prepared. Local cucumbr seeds, which surface sterilized by 3.5% of sodium hypochlorite, were planted and distributed on the dish margin as 10 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:

$$\% \text{ Germination} = (\text{number of germinated seeds} / \text{total seeds}) \times 100$$

EXTERNALLY TESTING ANTAGONISTIC CAPABILITY OF BACTERIA AND FUNGUS

An experiment was conducted to know the antagonistic relationship between *F. solani* fungus and *P. fluorescens* bacteria. This experiment was carried out based on Dual Culture Technique [24] by taking 8 mm, in diameter, disc of the fungal pathogen from the culture margin of *F. solani* 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, was prepared. All dishes were incubated at 28 ˚C for 5 days. After the incubation, the fungus growth diameter, with presence 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 [25]:

$$\% \text{ FGI} = [1 - (\text{FG in bacteria treatment} / \text{FG in control}) \times 100 \quad (2)$$

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PREPARATION OF THE FUNGAL INOCULUM

Fungal inoculum was prepared by grown on PDA for 7 days at 25C fungal colony were used to inoculate 500 ml-capacity flasks containing 100 gm seeds of (*Panicum miliaceum L.*)plus 40 ml D.water, incubated at 25C for 15 days with the mixing. [26].

PREPARATION OF BACTERIAL INOCULUM

P. fluorescense bacteria were grown in an N.B nutrient medium into test tubes, the tubes were incubated at 28 ˚C for 48 h then in the centrifuge, and the suspension of bacteria was re-prepared into sterilized and distilled water, then the bacterial inoculum density was calculated [27], which was 10^6 – 10^8 cell ml^{-1} then the bacterial inoculum transphered to the petmose as carrier(part1) and the(part 2) bacterial inoculum immobilized [28].preeti

POTS EXPERIMENT

Five seeds were planted in each pot with three replicates per treatment. The fungal inoculum, *F. solani* of 10 gm, was added [29]and 10 gm of immobilized inoculum and (95×109^7) cell ml^{-1}bacterial inoculums with petmose. Pots without fungus *F. solani* and antagonistic bacteria were used as a control. Chemical fertilizers (NPK), basis on the fertilizers recommendation of the cucumbr plant, were added. carbohydrats (%) dry weight of shoot system (g $plant^{-1}$), and the plant length (cm) and carotin (mg g^{-1}) were evaluated after 60 days and %50 from available water.

THE EVALUATION OF ENZYMATIC ANTIOXIDANTS ACTIVITY

The activity of enzymatic antioxidants (SOD and CAT) was evaluated. After 60 days planting, 1 g of the fresh plant sample (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 [30]. SOD activity was evaluated by Nitro Blue Tetrazolium (NBT) according to [31]. CAT activity was evaluated according to [32] using Spectrophotometer which depended on calculating the absorption change value at wavelength of 240 nm.

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 (< 0.05).

RESULTS AND DISSCUSION

SOIL ANALYSIS

Soil properties confirmed that Electrical Conductivity (EC) was moderate, and Cation Exchange Capacity (CEC) was 19.53 $cmol.Kg^{-1}$. The soil was high calcareous and silt loam texture (Table-1).

Table 1: Some soil properties

O.M %	K $mg.Kg^{-1}$	P $mg.Kg^{-1}$	N $mg.Kg^{-1}$	CaCO3 %	CEC $Cmol.Kg^{-1}$	EC dsm^{-1}	pH
0.71	64.17	5.26	28	33.5	19. 53	2.11	7.15

Total Bacteria Count Cfu g^{-1}dry soil	Total Fungi Cfu g^{-1}dry soil	P.fluorescns Bacteria Cfu g^{-1}dry soil
4.6×10^6	1.5×10^4	2.3×10^6

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BACTERIA ISOLATION

The results of isolation showed different shapes and morphology of 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:

MORPHOLOGICAL AND MICROSCOPIC TESTS

A difference in morphological properties of bacteria was observed under microscope test after staining with Gram. There were differences in responding to biochemical tests [33]. Based on identification results, isolated colonies referred to *P. fluorescens* bacteria one isolate was chosen for used as antagonist for pathogen fungus.

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

Bacteria Isolate	Gram Stain	Movem-ment	Stain product	Methyl Red	Indole	Catalase	Starch decomposition	Oxidase	Citrate
P.flu.	-	+	+	-	-	+	+	+	+ -

ISOLATION OF PATHOGEN FUNGUs

Pathogen fungus got 10 fungal isolates identified as it referred to *Fusarium solani*. One isolate was chosen based on the results of pathogenicity experiment test of the fungus for cucumbr seeds. The results showed that *F. solani* led to a significant decrease the germination ratio of cucumber 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 cucumber 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.

EXTERNALLY ANTAGONISM BETWEEN PATHOGEN FUNGUS AND BACTERIA

Results showed that *P. fluorescens* bacteria had a high antagonism against *F.solani* 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 [34]. 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 [35]. 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) [36]. Inhibition (fungus growth) ratio of *P. fluorescens* bacteria was calculated which was 76%, after 4 days in the culture medium, as shown in Table 3.

Table 3: The inhibition activity of *P. flu.* bacteria to *F. solani* fungus growth

Treatment	Fungus Growth cm	Inhibition Ratio %
Control	9.00	0.00
F. oxysporum × *P.fluorescens*	1.56	82.6

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EVALUATION OF SOME PLANT GROWTH CRITERIA

Results (Table 4) showed that biological stress, resulted by fungal infection, affected plant growth criteria, in which total carbohydrate 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. flu.*) 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 [37]. 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 after 60 days and 50% from available water

Treatment	Carbohydrate %	Carotine Mg g^{-1} plant	Dry Weight g $plant^{-1}$	Length cm $Plant^{-1}$
Control	3.73	0.62	6.08	82.33
P.fluorescens	4.41	0.69	8.59	127.33
P.fluorescens+ f.solani	2.83	0.65	6.59	96.67
F.solani	1.74	0.56	2.96	76.33
Mean	3.72	0.65	6.76	117.33
LSD0.05	ns	0.025	2211.	4.323

ENZYMATIC ANTIOXIDANTS ACTIVITY

Results (Table 5) showed that the enzymatic antioxidants (SOD and CAT) increased with the plant fungal infection (*F. solani*) within 60 days after planting and 50% from available water compared with the control (without fungus).

Table 5: The activity of SOD and CAT in the plant roots and leaves

Treatment	SOD U $abs.ml^{-1}$		CAT U $abs.\ ml^{-1}$	
	Leave	Root	Leave	Root
	58.49	28.30	8.77	6.39
Control	67.51	44.45	10.30	6.83
P.fluorescns +Petmose	76.94	50.27	10.36	6.85
F.solani+P.fluorescns	61.72	40.13	9.41	6.02
F.solani	68.24	44.64	10.20	6.96
Mean	2.975	3.478	1.171	NS
L.S.D. 0.05				

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 [38], at adding the bacterial inoculum (*P. fluor.*), 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 dismantling free hydroxide root converting it into hydrogen peroxide.

Results showed that CAT activity (90.27 U abs. ml^{-1}) was a higher than others at 60 days after the planting for the bacterial inoculum (*P. fluor.*). Willekens et al. [39] 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.

CONCLUSION

The activity of enzymatic antioxidants had stimulated at the infection of cucumbr plant by pathogen fungus (*F. solani*) and by the effect of prompting enzymatic antibiotics at the inoculation with (*P. fluor.*), the values of enzymes activity in cucumbr plant inoculated with bacteria higher than those in non-inoculated plant after 60 days of planting for all enzymes. 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. 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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