STUDIES ON TOMATO WILT CAUSED BY *FUSARIUM OXYSPORUM* F .SP. *LYCOPERSICI* IN KAZACHESTAN. 2: EFFECT OF EXOGENOUS APPLICATION OF PLANT EXTRACTS AND SAFE CHEMICALS AS RESISTANCE INDUCER TREATMENTS ON THE ACTIVETY OF THE OXIDATIVE ENZYMES

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ABSTRACT

In this study, tomato (Carolina Gold cv.) transplants were treated before planting in pots with garlic (G), black pepper (BP) extracts (at 0.5 & 4.0% conc.), salicylic acid (SA) or riboflavin (R) at 0.1 & 10.0mM conc. using different application methods (immersing roots "IR", spraying shoots "SS" and IR+SS) then inoculated with the tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopercici* (FOL). The activities of polyphenoloxidase (PPO) and peroxidase (POD) enzymes were determined in leaves of treated and untreated (control) tomato plants after two months from inoculation. The increase in POD activity was higher and more pronounced than activity of PPO. Comparing to the control treatment, the increase in PPO activity, in most treatments, was ranged between 4.4% (IR/SS/BP at 4%) to 94.2% (SS/R at 0.1mM) although few treatments slightly decreased it. However, all tested treatments increased activity of POD enzyme. The rate of increase was ranged between 60.7 to 1000.0%.

INTRODUCTION

Infection of tomato with *Fusarium oxysporum* f. sp. *lycopercici* (FOL) wilt significantly reduced the crop yield and quality **(Aba AlKhail, 2005)**. The use of resistant varieties is the best strategy for disease control **(Silva and Bettiol, 2005)** and **(Sheu and Wang, 2006).** Controlling of plant diseases mainly depend on fungicides treatments (**Rauf, 2000**; **El-Mougy *et al.*, 2004**). However, fungicidal applications cause hazards to human health and increase environmental pollution. Therefore, alternatives, eco-friendly approach treatments for control of plant diseases are needed (**Abd-El-Kareem, 2007**; **Rojo *et al.*, 2007**; **Mandal *et al.*, 2009**). Systemic acquired resistance (SAR) or induction of resistance to pathogen is a promising approach for controlling plant diseases. Exogenous or endogenous factors could substantially affect host physiology, leading to rapid and coordinated defense-gene activation in plants normally expressing susceptibility to pathogen infection (**Mandal *et al.*, 2009**). This phenomenon, that resistance of plant to pathogens can be enhanced by the application of various biotic and a biotic agent, called induce systemic resistance in plants (**Yedidia *et al.*, 2000**; **Brimner and Boland, 2003**; **Sarwar *et al.*, 2005**; **Abd-El-Kareem, 2007**). Use of chemical inducer, salicylic acid (SA) represents an interesting new opportunity in controlling fungal and bacterial diseases within an environmental friendly integrated crop protection system through enhancing the resistance of the plant to pathogen (**Oostendorp *et al.*, 2001**; **Ellis *et al.*, 2002**; **Balciuniene *et al.*, 2005**; **El-Khallal, 2007a**; **El-Khallal, 2007b**; **Khaosaad *et al.*, 2007**; **Ali *et al.*, 2009**; **Mandal *et. al.*, 2009**). The signal molecule SA is involved in some signal transduction system, which induce particular enzymes catalyzing biosynthetic reactions to form defense compounds such as polyphenols, pathogenesis- related (PR) proteins (**Métraux, 2001**; **Shah, 2003**; **Vimala and Suriachandraselvan, 2009**). In plants the positive correlation between levels of polyphenol oxidase (PPO) and peroxidase (POD) and the resistance to pathogens and herbivores is frequently observed. There are some evidences indicating that the activation of peroxidase, polyphenol oxidase plays a crucial role in the biological control and resistance of plant to pathogenic attack (**Mohammadi and Karr, 2002**; **Thipyapong *et al.*, 2004**;

Chérif *et al., 2007*; **She-ze *et al.*, 2008**). Enhancement of PPO and POD activity was reported in response to pathogen inoculation in plants pretreated with SA (**El- Khallal, 2007b**; **Chandra *et al.*, 2007**). It was reported that POD may be some of the elements of the defense systems that are stimulated in plants in response to pathogen infection especially *Fusarium oxysporum* (**Morkunas and Gemerek, 2007**).

The present study was conducted mainly to investigate the changes in activities of the oxidative enzymes (PPO and POD) in leaves of tomato plants pre-treated with different natural and chemical inducers under stress of infection with the tomato wilt fungus (FOL).

MATERIALS AND METHODS

In this study, salicylic acid (SA) and riboflavin (R) each @ 0.1mM and 10.0mM, and garlic extract (G) and black pepper extract (BP) each at 0.5% and 4.0% concentrations were used as resistance inducer treatments for treating 4 weeks-old tomato (*Solanum lycopersicum*) seedlings (Carolina Gold cv.) immediately before transplanting into plastic pots (30cm. in diameter) each containing 11 Kg of natural soil mixture consisted of clay and sand at rate of 2:1 (by weight). Each inducer treatments was performed by immersing roots (IR) for 10 min., spraying shoots (SS) until dropping or combination between IR and SS application methods (IR+SS). The plain water was used instead of inducer treatments for treating tomato seedling in the control treatment. Spore suspension of an aggressive isolate of *Fusarium oxysporum* f.sp. *lycopersici*, which was isolated from wilted tomato plants grown under glasshouse conditions in Kazakhstan, was prepared and adjusted according to **Beshir, 1991 and Amini, 2009** and immediately used for inoculating 4-weeks old tomato seedlings by pouring 20 ml of spore suspension (10^6 spores/ml) over stem base of each seedling one week after transplanting. All pots were irrigated and maintained at 25-30ºC and 70% relative humidity under glasshouse conditions. After two months from inoculation, the fifth leaf of treated and non-treated plants was harvested by cutting them at the leaf base level, weighed 1.0 g. then homogenized with 3.0 ml. of Na phosphate buffer pH 6.8 (0.1 M) and centrifuged at 2ºC for 15 min at 17.000g in a refrigerator centrifuge. The clear supernatant was taken as the enzymic source for the assay of peroxidase (POD) and polyphenoloxidase (PPO) activities after proper dilution **(Patra and Mishra, 1979)**. The supernatant was collected and stored at -20ºC until use. Peroxidase, polyphenoloxidase enzymes were carried out using a (SPECTRONIC 20-D) spectrophotometer at 27±2°C according to **Chance and Maehly (1955),** and **Taneja and Sachar (1974)**, respectively. Readings of the spectrophotometer were recorded every 30 Sec, for 5 min for the two enzymes. The reference cuvette for the spectrophotometer always contained the same concentrations of components as the sample cuvette, except that the substrate solution was replaced by extraction buffer.

RESULTS

Activity of PPO enzyme:

The data in **Table (1)** indicated that, the activity of polyphenoloxidase (PPO) enzyme (O.D at 430nm/g FW/Min) was affected differently by tested application methods, inducer treatments as well as by the interaction in between. All tested inducer treatment increased PPO activity comparing to the untreated control. Using G@0.5% recorded the highest increase in the PPO activity (27.6%) followed by BP@4.0% (13.5%), BP@0.5% (11.1%) and G@4.0% (5.0%), respectively. Most interactions, however, increased PPO activity but few decreased it. The SS method recorded the highest average of PPO activity (44.3) followed by the IR+SS method (38.5) and the IR method (38.2), respectively. In this respect, SS/BP@0.5% recorded the highest increase (46.7%) followed by IR+SS/BP@0.5% (31.5%), IR/G@0.5% (30.4%), respectively. Using G@0.5% recorded the highest increase in the PPO activity (27.6%) followed by BP@4.0% (13.5%), BP@0.5% (11.1%)

and G@4.0% (5.0%), respectively. Most interactions, however, increased PPO activity but few decreased it. In this respect, SS/BP@0.5% recorded the highest increase (46.7%) followed by IR+SS/BP@0.5% (31.5%), IR/G@0.5% (30.4%), respectively. The activity of PPO enzyme (O.D at 430nm/g FW/Min) was affected differently by tested application methods, inducer treatments as well as by the interaction in between. The SS method recorded the highest average of PPO activity (52.1) followed by the IR method (51.2) and the IR+SS method (39.6), respectively. As for inducer treatments, the highest PPO activity was recorded by R@10.0mM (55.5%) followed by SA@10.0mM (36.8%), R@0.1mM (35.2%) and SA@0.1mM (29.9%), respectively. As for interactions, SS/R@0.1mM recorded the highest increase (94.2%) followed by IR/SA@10.0mM (87.0%), SS/R@10.0mM (82.3%), IR/R@10.0mM (71.5%) and IR+SS/SA@0.1mM (48.6%), respectively. However, only IR+SS/R@0.1mM, and IR+SS/SA@10.0mM decreased PPO activity by 11.0 and 3.0%, respectively compared to the untreated control.

Table (1): Activity of polyphenoloxidase (PPO) enzyme (Δ430/min/g fresh weight) in tomato leaves as affected by inducer treatments (garlic (G) and black pepper (BP) extracts at 0.5 & 4.0% conc. and salicylic acid (SA) and riboflavin (R) at 0.1 and 10.0mM conc.) using application methods* after two months from inoculation with *F. oxysporum lycopersici*

Inducer treatments	Activity (Δ430/min/g fresh weight)			Efficiency**		
	IR	SS	IR+SS	IR	SS	IR+SS
G@0.5%	47.2	47.0	44.4	30.4	29.8	22.7
G@4.0%	47.0	40.7	26.3	29.8	12.4	-27.3
BP@0.5%	20.0	53.1	47.6	-44.8	46.7	31.5
BP@4.0%	40.8	44.7	37.8	12.7	23.5	4.4
SA@0.1mM	45.3	42.0	53.8	25.1	16.0	48.6
SA@10.0mM	67.7	45.8	35.1	87.0	26.5	-3.0
R@0.1mM	44.6	70.3	31.9	23.2	94.2	-11.9
R@10.0mM	62.1	66.0	40.8	71.5	82.3	12.7
Control (untreated)	36.2	36.2	36.2	0.0	0.0	0.0

* **IR=** root immersion; **SS=** shoot spraying.

** **Efficiency=** (Treatment-Control)/Control x 100

Activity of POD enzyme:

The data in **Tables (2)** indicated that, the activity of peroxidase (POD) enzyme (Δ_{420}/min/g fresh weight) was affected differently by tested application methods, inducer treatments as well as by the interaction in between. The SS method recorded the highest average of POD activity (61.5) followed by the IR+SS method (55.2) and the IR method (27.0), respectively. Using BP@0.5% recorded the highest POD activity (339.0%) followed by BP@4.0% (325.9%), G@0.5% (311.1%) and G@4.0% (299.3%), respectively. All tested interactions increase POD activity comparing to the untreated control which recorded 13.5 (Δ_{420}/min/g fresh weight). In this respect, SS/G@4.0% recorded the highest increase (640.7%) followed by IR+SS/BP@4.0% (595.6%), SS/G@0.5% (411.1%), IR+SS/BP@0.5% (401.5%), SS/BP@0.5% (397.8%), IR+SS/G@0.5% (352.6%) and SS/BP@4.0% (328.9%) whereas the remained interactions increased it by 53.3-217.8% comparing to the untreated control. The activity of POD enzyme (Δ_{420}/min/g fresh weight) was affected differently by tested application methods, inducer treatments as well as by the interaction in between. The SS method recorded the highest average of POD activity (79.5) followed by the IR+SS method (53.5) and the IR method (50.4), respectively. Regardless application methods, SA@0.1mM recorded the highest increase (636.5%) followed by R@10.0mM (432.8%), R@0.1mM (363.2%) and SA@10.0mM (331.4%), respectively comparing to the untreated control. The POD activity was increased by all tested interactions. In this respect, SS/SA@0.1mM increased POD activity by 10 folds

over than the untreated control followed by IR+SS/SA@0.1mM (6.69 times), SS/R@0.1mM (5.71 folds) and SS/R@10.0mM (5.41 folds) while the remained interactions increased the POD activity by 2.23 to 4.57 times over that recorded by the untreated control.

Table (1): Activity of peroxidase (POD) enzyme (Δ420/min/g fresh weight) in tomato leaves as affected by inducer treatments (garlic (G) and black pepper (BP) extracts at 0.5 & 4.0% conc. and salicylic acid (SA) and riboflavin (R) at 0.1 and 10.0mM conc.) using application methods* after two months from inoculation with *F. oxysporum lycopersici*

Inducer treatments	Activity (Δ430/min/g fresh weight)			Efficiency*		
	IR	SS	IR+SS	IR	SS	IR+SS
Garlic@0.5%	36.4	69.0	61.1	169.6	411.1	352.6
Garlic@4.0%	21.7	100.0	40.0	60.7	640.7	196.3
BP@0.5%	42.9	67.2	67.7	217.8	397.8	401.5
BP@4.0%	20.7	57.9	93.9	53.3	328.9	595.6
SA@0.1mM	45.9	148.5	103.9	240.0	1000.0	669.6
SA@10.0mM	63.9	58.2	52.6	373.3	331.1	289.6
R@0.1mM	53.4	90.6	43.6	295.6	571.1	223.0
R@10.0mM	75.2	86.6	54.0	457.0	541.5	300.0
Control (untreated)	13.5	13.5	13.5	0.0	0.0	0.0

* **IR=** root immersion; **SS=** shoot spraying.

** **Efficiency=** (Treatment-Control)/Control x 100

DISCUSSION

The oxidative enzymes i.e. polyphenoloxidase (PPO) and peroxidase (POD) are important in the defense mechanism against pathogens, through their role in the oxidation of phenolic compounds to quinines, causing increasing in antimicrobial activity. Therefore, they may be directly involved in stopping pathogen development **(Quiroga *et al.*, 2000; Shimzu *et al.*, 2006; Melo *et al.*, 2006).** The present results showed that, the garlic (G) and black pepper (BP) which tested at 0.5 and 4.0% concentrations as resistance inducer treatments increased activities of PPO and POD enzymes to different extents in leaves of treated tomato plants comparing to the untreated control. Using G@0.5% recorded the highest increase in the PPO activity (27.6%) followed by BP@4.0% (13.5%), BP@0.5% (11.1%) and G@4.0% (5.0%), respectively. Most interactions, however, increased PPO activity but few decreased it. In this respect, SS/BP@0.5% recorded the highest increase (46.7%) followed by IR+SS/BP@0.5% (31.5%), IR/G@0.5% (30.4%), respectively. The remained interactions increased the PPO activity by 4.4-29.8% except IR/BP@0.5% and IR+SS/G@4.0% which decreased it by 44.8 and 27.3%, respectively compared to the untreated control. Also, As for inducer treatments, the highest PPO activity was recorded by R@10.0mM (55.5%) followed by SA@10.0mM (36.8%), R@0.1mM (35.2%) and SA@0.1mM (29.9%), respectively. As for interactions, SS/R@0.1mM recorded the highest increase (94.2%) followed by IR/SA@10.0mM (87.0%), SS/R@10.0mM (82.3%), IR/R@10.0mM (71.5%) and IR+SS/SA@0.1mM (48.6%), respectively. However, only IR+SS/R@0.1mM, and IR+SS/SA@10.0mM decreased PPO activity by 11.0 and 3.0%, respectively compared to the untreated control. Using BP@0.5% recorded the highest % increase in POD activity (339.0%) followed by BP@4.0% (325.9%), G@0.5% (311.1%) and G@4.0% (299.3%), respectively. As for method/extract interactions, SS/G@4.0% recorded the highest increase (640.7%) followed by IR+SS/BP@4.0% (595.6%), SS/G@0.5% (411.1%), IR+SS/BP@0.5% (401.5%), SS/BP@0.5% (397.8%), IR+SS/G@0.5% (352.6%) and SS/BP@4.0% (328.9%) whereas the remained interactions increased it by 53.3-217.8% comparing to the untreated control. Regarding safe chemical treatments, SA@0.1mM recorded the highest % increase in POD activity (636.5%) followed by R@10.0mM (432.8%), R@0.1mM (363.2%) and SA@10.0mM

(331.4%), respectively comparing to the untreated control. The POD activity was increased by all tested interactions. In this respect, SS/SA@0.1mM increased POD activity by 10 folds over than the untreated control followed by IR+SS/SA@0.1mM (6.69 times), SS/R@0.1mM (5.71 folds) and SS/R@10.0mM (5.41 folds) while the remained interactions increased the POD activity by 2.23 to 4.57 times over that recorded by the untreated control. In fact, the activity of POD was sharply increased in response to foliar spray of salicylic acid **(El-Khallal, 2007b; Mandal *et. Al.*, 2009).** The present results were in agreement with **(Gail *et al.*, 2007; Jayalakshmi *et al.*, 2009)**, since they reported that the activity of both polyphenoloxidase and peroxidase increased after the treatment with *Trichoderma harzianum*, and also with **(El-Khallal, 2007b; Mandal *et al.*, 2009)**, since they showed that the activity of POD was sharply increased in response to foliar spray of salicylic acid. Spraying of salicylic acid enhanced the activity of the enzyme in trichoderma seedling root dipping treatment not in soil applied treatment. The last obtained result was supported by **(Godt and Roitsch, 1997)**. **Houssien *et al.* (2010)** reported that all of their resistance inducer treatments increased the activity of PPO and POD relative to the infected control. T2 treatment was more effective in increasing the activity of the enzymes. Combination of salicylic acid with trichoderma as seedling root dipping (T2) and thiophanate methyl with its half recommended rate recorded a higher PPO and POD activity since it reached to 305% for the first one and 315% for the second relative to the infected control. **Houssien *et al.* (2010)** concluded that combinations of biocontrol agents and resistance inducer could provide promising integrated alternatives in suppression of Fusarium wilt disease of tomato plants due to number of mechanisms involved. So, a new approach for the management of biological control of Fusarium wilt disease of tomato plants depends on the activation of the defense of the plant against pathogen (Systemic resistance) by salicylic acid as inducer and suppression of the fungal pathogenicity by applying *Trichoderma harzianum* as bicontrol agent.

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