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ORIGINAL PAPER

Effectiveness of Some Biotic and Abiotic Agents to Control Tomato Early Blight Disease Caused by *Alternaria solani*.

Khalil, M.E.K.I. and Adbelghany, R.E.A.

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ABSTRACT

Bacillus megaterium, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Trichoderma album* and *Trichoderma viride* isolates, as well as different concentrations of chemical inducers, such as chitosan and salicylic acid were evaluated against Tomato early blight, under laboratory and greenhouse conditions. Under laboratory conditions, all tested bio agents, as well as the different concentrations of the tested chemical inducers, decreased the linear growth of *A. solani*, as the inhibition of fungal growth was increased by increasing the concentration of both chemical inducers in the growing medium. The chemical inducer chitosan at a concentration of 4.0 mg/mL PDA medium, caused the highest inhibition of linear growth of the fungus, followed by anti-fungus *T. harzianum*. When, different concentrations of chitosan and salicylic acid were tested on spore germination of *A. solani*, a concentration of 4.0 (mg/mL), for chitosan and a concentration of 25.0 (mM), for salicylic acid were the best concentrations used to reduce the spore germination of *A. solani*. Under greenhouse conditions, spraying tomato plants (cv. Super Strain B hybrid), with any of the tested biological agents as well as different concentrations of both the chemical inducers before infection of tomato plants by *A. solani* reduced the severity of early blight, in addition, to increase the fresh and dry weight of tomato plants. The chemical inducer chitosan at a concentration of 4.0 (mg/mL), caused the highest decrease of the disease severity, and the highest increase in fresh and dry weight of tomato plants, followed by the bio agent *T. harzianum*. The activities of defense-related enzymes *i.e.*, polyphenoloxidase, peroxidase and chitinase were significantly increased in all treated plants with the tested biotic and abiotic agents. Chitosan at a concentration of 4.0 (mg/mL) resulted in the highest activity of oxidative enzymes, followed by *T. harzianum.* Meanwhile, the total content of phenols was higher in treated plants than in the untreated ones. The tested bio agents and chemical inducers might be playing an important role in management of tomato early blight through induction of induced systemic resistance.

Key words: Tomato, Early blight, Alternaria *solani*, Biological control, Chemical inducer.

***Correspondence:** Khalil, M.E.K.I.
E-mail: mohamedeffat631@gmail.com

Mohamed E.K.I. Khalil

Rady E.A. Adbelghany

Plant Pathology Research Institute, Agricultural Research Center, 12619, Giza, Egypt.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widespread and popular vegetables in the world. It ranks second in economic importance after the potato in many countries (Prajapati *et al.*, 2014). It is one of the crops that gives a high financial return to many farmers in most parts of the world, as it can be marketed fresh or manufactured (Anonymous, 2016). The tomato early blight caused by the fungal *Alternaria solani* is the most economically important tomato disease in most parts of the world, including the Caribbean (Ali *et al.*, 2016). Infection of tomato plants by the fungus that causes early blight can lead to great losses in crop yield due to damaging leaves and fruits (Chaerani *et al.*, 2007 and EL-Tanany *et al.*, 2018). Weather conditions are an important factor in causing this disease and it can spread in a wide range of climatic conditions, but it is widely spread in areas of rain and high relative humidity (Moustafa *et al*., 2018a). Generally, the use of fungicides in controlling plant diseases is still one of the most effective processes used, but the uncontrolled use of these substances in agriculture in many regions of the world raised serious concerns about health issues, and environmental pollution in particular, after it was published (Carson 1962). In addition, many fungi can develop resistance races for most of the fungicides used, (Chaerani *et al.*, 2007 and Ramkissoon, *et al.*, 2016). Under the aforementioned conditions, it becomes imperative to develop plant-derived plant-based pesticides or microbial pesticides, whether biological, environmentally friendly or biodegradable, in order to control plant pathogens. Biotic and abiotic inducers have potential in agriculture with regard to control plant diseases (Anand *et al.*, 2009a and Simonetti *et al.*, 2012). Currently, biological control using antimicrobials, resistance

promoters and growth promoters offers an excellent practical and economical alternative in controlling plant pathogens (Chandrashekara *et al.,* 2012 and EL-Tanany *et al.,* 2018). Epiphytic microbes have been documented for numerous phyllosphere and rhizosphere inhabiting organisms and/or stimulating the induction of systemic resistance mechanisms within the plant (Bargabus *et al.,* 2002). Recently, the induction of plant resistance by application of several microorganisms or organic materials has emerged as a new strategy in the management of plant diseases (Rais *et al.,* 2017). Biotic inducers are known to have eliciting activities leading to a variety of defense reactions in host plants in response to microbial infection, including the defense related enzymes and accumulation of phenolic compounds as well as specific flavonoids (Govindappa *et al.,* 2010; Esh *et al.,* 2011; Abd El-Rahman *et al.,* 2012; Hussein *et al.,* 2018 and Sarhan *et al*., 2018). Recently, researchers have turned to activate the systemic induced resistance (SIR) of plants using some chemical inducers, for example, chitosan and salicylic acid, as these substances enhance the same physiological and biochemical changes in plants as do systemic active biological resistance (El-mohamedy *et al.,* 2015; Ramkissoon *et al.,* 2016 and Moustafa *et al*., 2018b). Moreover, (ChunYan *et al.,* 2003 and Atia 2005), found that treating tomato plants with chitosan at a rate of (1 mg/mL) a week before the plant was infected with the fungi *Phytophthora infestans* and *Alternaria solani* led to plant disease resistance to those fungi where the (SIR) activity of tomato was stimulated in four leaves of the seedlings. The activity of defence related enzyme β-1, 3 glucanase is known to be as an inducer of systemic resistance of many infected plants with fungal pathogens (Saikia, *et al.,* 2005 and Govindappa, *et al.,* 2010). Also, this enzyme acts synergistically in the partial degradation of fungal cell walls. Moreover, a parallel increase in the activities of these enzymes is important for optimal function in plant defense (Saikia, *et al.,* 2005). Also, peroxidase (PO), phenylalanine ammonia-lyase (PAL) and polyphenoloxidase (PPO) enzymes were mentioned as elicitors of the induced systemic resistance (ISR) in plant disease control (Yasmin, *et al.,* 2016). These enzymes act as elicitors of phenylpropanoid pathway, resulting in the biosynthesis of a diverse array of plant metabolites such as, phenolic compounds, flavonoids, tannins and lignin. These products can provide defense in plants against pathogenic attack (Hahlbrock and Scheel, 1989). Many studies indicated to greater accumulation of phenolics as a result of increasing the activities of these oxidative enzymes which could offer the protection against plant diseases (Singh *et al.,* 2003; Abd El-Rahman *et al.,* 2012 and Hussein *et al.,* 2018).

This work aims to evaluate the effectiveness of some biotic and abiotic agents to control early blight of tomatoes under greenhouse conditions, in addition to estimating some of the biochemical response in treated plants, which is related to activating the systemic induced resistance.

MATERIALS AND METHODS

1- Source of the fungal pathogen, the antagonistic agents and the tomato seeds:

The fungus *Alternaria solani* was isolated from tomato plants (cv. Super Strain B), with obvious early blight symptoms, collected from Qaliobia Governorate. The fungus was then purified and identified according to its morphological features using the descriptions of Singh (1982) and Barnett and Hunter (1987). Pure cultures of the pathogen were maintained on PDA slants and stored at 4°C till used.

2- Biotic and Abiotic agents used in Induction of Systemic Induced Resistance in Tomato:

Biotic agents included pure isolates of six antagonists *i.e.*, three bacterial isolates namely *Bacillus megaterium*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and three fungal isolates namely *Trichoderma harzianum, Trichoderma album* and *Trichoderma viride*, were obtained from Integrated Pest Management Department (IPMD), Plant Pathology Institute, Agricultural Research Center, Giza, Egypt. The Pure cultures of the fungal bio agents were maintained on PDA slants whereas, cultures of bacterial biogents tested were maintained on nutrient agar slants. Both fungal and bacterial cultures were Kept at 4°C until their use.

Abiotic materials used in the experiment were Chitosan with different concentrations (1.0, 2.0, 3.0 and 4.0 mg/mL) Salicylic acid of 1.0, 5.0, 10.0, and 25.0 mM concentrations to clarify their effects as inducers of systemic resistance in tomato plants against early blight infection.

3- Tomato seeds tested:

Tomato seeds (cv. Super Strain B hybrid) were kindly provided from Horticulture Research Institute., Agricultural Research Center, Giza, Egypt.

4- Laboratory experiments:

4.1- Efficacy of some antagonistic bacterial and fungal agents on linear growth of *Alternaria solani in vitro*:

The anti-bacterial effect of the tested bacterial isolates *i.e., Bacillus megaterium*, *Bacillus subtilis*, *Pseudomonas fluorescens* and the antifungal effect of each of *Trichoderma harzianum*, *Trichoderma album* and *Trichoderma viride* on the growth of the tested fungal pathogen *A. solani* were studied under laboratory conditions. Petri dishes (9 cm in diameter) containing PDA medium were inoculated by streaking a line of each antibacterial separately at a distance of 2 cm, from the edge of the dish, all dishes were incubated at 28±1°C for 24 h. Then a fungal disc (5 mm) of *A. solani* taken from the edges of a seven-day-old culture was placed at the same distance from the edge of the plate against the bacterial line. As for antagonistic fungi, the plates were inoculated with disc (5 mm in diameter) of each fungal isolate at a distance of 2 cm from the edge of the plates against the pathogen, which was placed at the same distance from the edge of the plate. Petri dishes inoculated with *A. solani* alone as a comparison treatment. All dishes were incubated at 27±1°C. Three replicate dishes were used for each treatment. The percentage of fungal growth reduction in the different treatments was calculated when the pathogen growth was completed in one of the comparison treatments plates. Inhibition ratio was calculated in all treatments according to Fokkema, (1973).

4.2-Efficacy of different concentrations of chitosan and salicylic acid on linear growth of *Alternaria solani in vitro*:

This experiment was designed to investigate the inhibitory effect of the tested chemical inducers for example chitosan ($C_{12}H_{24}N_2O_9$) and salicylic acid ($C_7H_6O_3$) on the linear growth of *A. solani* on PDA medium with streptomycin sulfate (300 mg/L). The chemical catalysts used were tested at four concentrations as follows:

Stock solutions (10 mg/mL), 2 g of high molecular weight chitosan (viscosity = 800-2000 cps and >75% deacetylation) were prepared by dissolving them in 100 mL of distilled water with 2 mL of acetic acid (stirred for 24 h), and the volume was taken up to 200 mL with distilled water. The pH was adjusted to 5.6 with the addition of sodium hydroxide 1.0 N (El- Ghaouth *et al.*,1991). The chitosan solution was sterilized for 15 minutes in an autoclave at1.5 lb. Corresponding aliquots were taken to obtain different concentrations of chitosan (1.0, 2.0, 3.0 and 4.0 mg/mL).

Salicylic acid was tested at concentrations of 1.0, 5.0, 10.0, and 25.0 mM. The corresponding aliquots were taken from a 1 M stock solution in sterile distilled water.

The desired quantities of tested promoters were added to sterile and molten PDA medium to achieve the tested target concentrations. For each compound, a 10 mL aliquots of modified PDA medium were aseptically poured into a Petri dish (9 cm in diameter), with an unmodified PDA dish used as a comparison treatment. A 5 mm agar disc bearing the growth of the pathogen *A. solani* was transferred to the test medium. Three duplicate dishes were used for each treatment. Then all dishes were incubated at 27±1°C. Colony diameters were measured when the comparison treatment surfaces in Petri dishes were covered with fungal growth.

4.3-Efficacy of different concentrations of chitosan and salicylic acid on spore germination of *Alternaria solani in vitro*:

The effect of different concentrations of chitosan and salicylic acid was studied using the slide technique described by Nair and Ellingboe, (1962). *A. solani* inoculum was prepared by growing on V8 juice agar-CaCO3 media 6.5 pH, at 25±1°C, with alternating 12-hour periods of light and darkness for seven days (Rodrigues *et al.* 2010). Then 10 mL of sterile distilled water were added to each dish and the colonies were carefully scraped with a sterile needle. The resulting conidial suspension was adjusted to (1×10^5 spore/mL). The different concentrations of the chemical inducers were prepared as previously mentioned. A drop of each concentration was placed individually on a clean, sterile dried glass slide as a film, and then 0.1 mL of the suspended spore of *A. solani* was placed on top of this film using a sterile pipette. A drop of sterile distilled water was placed on another sterile glass slide and mixed with the fungus spores suspension, where it was used as a comparison treatment. Three replicates were used for each treatment. Each slide was placed on a U-shaped glass rod in a wet chamber composed of a sterile Petri dish lined with filter paper impregnated with sterile distilled water. All Petri dishes were incubated at 27±1°C for 24 hours prior to assay. Spore germination percentage was calculated.

5- Greenhouse experiments:

5.1- Effect of treating tomato plants with biotic agents and different concentrations of abiotic inducers on systemic induced resistance of tomato against Tomato early blight (disease severity) as well as on plant fresh and dry weight (g/plant) under artificial infection with *Alternaria solani in vivo*:

The two potted experiments were designed under greenhouse conditions, in Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt to study the effect of antagonistic bacterial isolates (*B. megaterium*, *B. subtilis* and *Ps. fluorescens*) and antagonistic fungal isolates (*T. harzianum*, *T. album* and *T. viride*), also to investigate the efficacy of different concentrations of two chemical inducers (chitosan and salicylic acid) on the severity of tomato early blight caused by *A. solani*, as well as fresh and dry weight (g/plant) of tomato plants. In this experiment, 25 cm of plastic pots were used, each contained 5 kg of sandy loam soil. Five tomato transplants (cv. Super Strain B hybrid), 4-5 weeks old containing 4-5 whole real leaves, were transplanted in each pot, and five pots were used as replicates per each treatment in addition to the untreated control of each experiments. The two experiments were designed as complete randomized block design. Antagonistic bacterial suspensions were prepared at a rate of (2.8×10^8cfu/mL) for each of treatment. Meanwhile, spore suspensions of fungal bioagents were prepared at a rate (2.5×10^5 spore/mL) for each one, according to EL-Tanany *et. al*. (2018). Tomato plants were sprayed with spore suspensions of each of the six tested bioagents individually once every two weeks. On the other experiment, tomato plants were sprayed every two weeks individually with either chitosan at a concentration of (1, 2, 3 and 4 mg/mL), or salicylic acid at concentrations (1, 5, 10, and 25 mM). After 48 hr. all tomato plants treated either by bio agents or abiotic chemicals were sprayed once with spore suspension (1×10^5 spore/mL) of *A. solani*, using a manual spraying machine for all treatments. Tomato plants received all the recommended normal agricultural practices except that the greenhouse floor was sprayed with water early in the morning to provide adequate air humidity. Disease severity% was recorded two weeks after artificial inoculation with the tested pathogen, using a scale consisted of six categories ranging from 0 to five (0 = no infection, 1 = scattered spots of infection less than 10% of the leaf area, 2 = more than 10% >20%, 3= 20% >30%, 4 = 30% >40% and 5 = < 40% of the leaf area, then disease severity was calculated using the formula developed by Townsend & Heuberger, (1943).

$$\mathbf{DS\ (\%) = \Sigma\ (nV) / NV \times 100}$$

Where, n - degree of infection according to the scale; v - number of samples per each category; V - total number of samples examined; N - the highest score of the categories.

Then efficacy of biological control agents and different concentrations of chemical inducers was calculated using Abbott's formula (Abbott, 1925) as follows:

$$\mathbf{Efficacy\ (\%) = (X - Y) / X \times 100}$$

Where, X = disease severity in untreated control. Y = disease severity in each treatment

Also, some growth parameters *i.e.*, fresh and dry weight g/plant of shoot and root of tomato plants were recorded.

5.2- Estimation of enzyme activity and total phenols:

Treated and untreated, tomato leaf samples (cv. Super Strain B hybrid), were collected 48 hr. after each treatment every two weeks and at the end of the experiments. Leaf samples were ground using a 0.2 M TrisHCl buffer (pH 7.8) containing 14 mM-mercaptoethanol at the rate 1/3 w/v. The extracts were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used to estimate the activity of polyphenoloxidase, peroxidase and hydrolytic enzymes (chitinase), according to the method described by Tuzun *et al.* (1989).

5.2.1- Determination of polyphenol oxidase activity:

Polyphenol oxidase activity was determined according to the method described by Maxwell and Bateman (1967). In the beginning, an amount of crude enzyme (0.5 mL) was mixed with sodium phosphate buffer (pH 7) and then 0.5 mL of catechol was placed and mixed together and finally completed the total volume to be 3 mL using sterile distilled water. Polyphenoloxidase activity was expressed by the change in absorbance at the optical density of 495 nm min-1 g-1 on a fresh weight, using a spectrophotometer.

5.2.2- Estimate peroxidase activity:

Peroxidase activity was estimated according to Allam and Hollis (1972). In this method, the oxidation of pyrogallol was calculated and converted to pyrogalline in the presence of H_2O_2 at a wavelength of 425 nm. The peroxidase enzyme activity was differentiated as the change

in absorption at 425 nm/ min-1 g-1 on a fresh weight using a spectrophotometer.

5.2.3- Estimation of chitinase activity:

Chitinase activity was determined according to the method described by Ried and Ogryd-Ziak (1981). Enzyme activity was characterized as µmoles N-acetylglucosamine (NAG) g/mL at 575 nm^{-1} g^{-1} fresh weight of tissue using a spectrophotometer.

5.2.4- Estimation of total phenols content:

Total phenols extract was obtained from treated tomato seedlings growing under greenhouse conditions as described by Hsu *et al*. (2003). A 5gm extract sample was taken from every treatment, and it was mixed up with 80 mL methanol and kept overnight. The solution was filtered through four layers of cheesecloth and the product was diluted to 100 mL (Slinkard and Singleton), (1997). A volume of the prepared solution was taken and mixed with 1.4 mL of distilled water, and 0.1 mL of 50% (1N) Folin-Ciocalteu phenol reagent. After of time period at least 30 seconds and not exceeding 8 minutes, the chemical substance (sodium carbonate 20%) was added at 0.3 mL w/v. Then, the mixture was left for 2 hours until it could complete the reaction and after vortexing for a short time, the color and the change in absorbance was estimated at 765 nm. The total dissolved phenol content was standardized against tannic acid and the values of the change in absorbance were converted to mg of phenols per 100 grams of fresh weight tissue.

6- Statistical analyzes:

The results obtained for appropriate statistical analysis were set using the MSTAT-C program (MSTAT-C, 1991), while the means were compared using ANOVA where LSD ($P \leq 0.05$).

RESULTS

1- Laboratory experiments:

1.1- Efficacy of some antagonistic bacterial and fungal agents on linear growth of *Alternaria solani in vitro*:

The inhibitory effect of the tested six bio agents *i.e.*, *B. megaterium*, *B. subtilis*, *Ps. fluorescens*, *T. harzianum*, *T. album* and *T. viride* against *A. solani* fungal growth is shown in Table (1). The tested bio agents were classified into three groups according to their efficacy against the fungal growth of *A. solani*. The first group contained the fungal bio agents *T. harzianum* and *T. viride*, which gave the highest inhibition efficacy against *A. solani* growth, being, 97.06 and 85.30%, respectively, while the second group contained the bacterial bio agents *B. megaterium* and *B. subtilis*, which gave a moderate decrease in the growth of the fungus, being 67.53 and 60.30%, respectively. On the other hand, the lowest inhibition was given by the bio agents *T. album* and *Ps. fluorescens*. The corresponding mean values were 53.01 and 39.19%, respectively, which ranked in the third group. The obtained results (Table,1) indicate that *T. harzianum* was the best bio agent which inhibited the fungal growth of *A. solani*, while *T. album* was the least effective one in this respect.

Table (1): Efficacy of some antagonistic bacterial and fungal agents on growth inhibition of *Alternaria solani*.

Bio agents	Inhibition %
Bacillus megaterium	67.53
Bacillus subtilis	60.30
Pseudomonas fluorescens	53.01
Trichoderma harzianum	97.06
Trichoderma album	39.19
Trichoderma viride	85.30
Control	0.00
L.S.D at 0.05	3.09

1.2-Efficacy of different concentrations of chitosan and salicylic acid on linear growth and spore germination of *Alternaria solani*:

Data in Table (2) indicate that all the tested concentrations of chemical inducers, *i.e.*, chitosan and salicylic acid reduced both linear growth and spore germination of *A. solani* compared to the untreated control. Inhibition rates were increased with increasing the concentration of both chemical inducers. Concentrations of 3 and 4 mg/mL of chitosan, as well as 25 mM of salicylic acid, were the most effective treatments in inhibiting the linear growth of *A. solani*, which gave complete inhibition of *A. solani* growth. Whereas the concentrations of 1 mg/mL of chitosan and 5 & 1 mM of salicylic acid were the least efficient ones against *A. solani* growth giving 2.3, 2.7 and 6.5 cm, respectively. Moreover, the chemical inducers were tested on spore germination rate of *A. solani*. The obtained results (Table, 2) indicate that the concentrations 4 mg/mL and 25 mM of chitosan and salicylic acid, respectively, gave the same trend of the results that were mentioned when applying these concentrations to the linear growth of the tested fungus.

Concentrations of 3 & 4 mg/mL of chitosan gave complete inhibition of spore germination (0.0%). The lowest percentage of spore germination (16.6%) was obtained when chitosan was used at 3mg/whereas, the concentration of 25 mM of salicylic acid gave the lowest percentage of spore germination (1.0%). Moreover, the first concentration (1.0) of chitosan and salicylic acid was the least effective in reducing spore germination (23.3 and 50.0%, respectively). Generally, chitosan was more efficient in reducing mycelia growth and spore germination rate of *A. solani* than salicylic acid.

Table (2): Efficacy of different concentrations of chitosan and salicylic acid on linear growth (cm) and spore germination % of *Alternaria solani*.

Chemical inducers	Concentration	Linear growth (cm)	Spore germination (%)
Chitosan (mg/mL)	1	2.3	23.3
	2	1.4	16.6
	3	0	0
	4	0	0
	Mean	0.93	9.98
Salicylic acid (mM)	1	6.5	50
	5	2.7	33.3
	10	1.2	26.6
	25	0	1
	Mean	2.60	27.73
Control	-	9	80
LSD at 0.05		Inducers (I) = 0.7	
		Conc. (C) = 2.4	
		I × C =4.2	

2- Greenhouse experiments:

2.1- Effect of treating tomato plants with biological control agents and different concentrations of some chemical inducers on severity of tomato early blight as well as fresh and dry weight (g/plant):

2.1.1- The effectiveness of some antagonistic bacteria and fungi on the severity of tomato early blight as well as fresh and dry weight (g/plant):

Tomato plants (cv. Super Strain B hybrid) were treated with two groups of antagonistic microorganisms under artificial infection with *A. solani* under greenhouse conditions. The first group included the antagonistic bacteria (*B. megaterium*, *B. subtilis* and *Ps. fluorescens*), while the second group contained the antagonistic fungi (*T. harzianum*, *T. album* and *T. viride*), Tomato plants were sprayed with these biological agents once each two weeks before infection with the pathogen (*A. solani*) and assessing tomato early blight disease severity and disease control efficiency, as well as measuring fresh and dry weight of tomato plants two weeks after infection by *A. solani*. The obtained results (Table, 3) show that when the plants were treated with *T. harzianum* and *T. viride*, this procedure recorded the lowest disease severity, being 15.42 and 19.56% with efficacy in controlling the disease reached to 71.60 and 63.97%, respectively. The highest fresh and dry weight of tomato plants were also accompanied with the previously mentioned treatments by the two bio agents tested being, (4.05 and 0.95 g/plant) and (3.00 and 0.78 g/plant), respectively. Meanwhile, the antagonistic bacteria *B. megaterium* and *B. subtilis*, came next in controlling the disease and recorded 26.14 and 29.86% disease severity, and (51.86 and 54.00% efficacy), respectively. Also, these treatments recorded moderate weights for both fresh and dry weight of the tomato plants (2.84 and 0.74 & 2.14 and 0.66 g/plant), respectively. Treatment with either the anti-fungus *T. album* and the anti- bacterium *Ps. fluorescens* showed the highest disease severity (34.56 and 31.30%) and the least disease control efficacy (36.35 and 42.53%) respectively, as well as the lowest fresh and dry weight of the tomato plants with (1.95 and 0.33 & 2.00 and 0.25 (g/plant), respectively. In general, the results indicated that the fungal bio agent *T. harzianum* was the best one of all tested treatments in reducing the severity of tomato early blight, as well as increased fresh and dry weight of tomato plants. On the contrary, *T. album* was the least effective one in this regard.

2.1.2- The effectiveness of different concentrations of chitosan and salicylic acid on the severity of tomato early blight as well as fresh and dry weight (g/plant):

Results in Table (4) show that treatment of tomato plants (cv. Super Strain B hybrid), with different concentrations of the chemical inducers chitosan and salicylic acid, once every two weeks before infection with the pathogen *A. solani*, reduced the severity of tomato early blight in addition, increasing the fresh and dry weight of tomato plants under greenhouse conditions compared to untreated control. Chitosan at 4 mg/mL and salicylic acid at 25 mM were the superior inducer treatments which gave the lowest disease severity (12.16 and 17.18%, respectively) and the highest disease control efficacy reached to 78.86 and 70.13%, respectively. As well as the treatment recorded

the highest fresh and dry weights of tomato plants that were increased and reached 6.60 and 0.99 & 3.75 and 0.66 g/plant, respectively. On the other hand, using both chemical inducers at the first concentration (1 mg/mL and 1 mM, respectively) was the least effective inducer treatments in reducing disease severity and increasing fresh and dry weights of the tomato plants which recorded (30.52 and 35.18%) disease severity and (46.94 and 38.84%) disease control efficacy, respectively, as well as (2.43 and 0.45 & 1.67 and 0.40 g/plant respectively) for fresh and dry weights of the tomato plants. Generally, the chemical inducer chitosan was more superior in reducing disease severity of early blight and increasing the fresh and dry weights of tomato than salicylic acid.

Table (3): Effect of treating tomato plants with some antagonistic bacteria and fungi on the severity of tomato early blight as well as fresh and dry weight (g / plant) under greenhouse conditions.

Bio-agents	Disease severity %	Efficiency %	Fresh weight (g/plant)	Dry weight (g/plant)
Bacillus megaterium	26.14	51.86	2.84	0.74
Bacillus subtilis	29.86	45.00	2.14	0.66
Pseudomonas fluorescens	31.30	42.35	2.00	0.52
Trichoderma harzianum	15.42	71.60	4.05	0.95
Trichoderma album	34.56	36.35	1.95	0.33
Trichoderma viride	19.56	63.97	3.00	0.78
Control	54.30	0.00	1.19	0.12
LSD at 0.05	2.69		1.01	0.25

Table (4): Efficacy of different concentrations of chitosan and salicylic acid on the severity of tomato early blight as well as fresh and dry weights (g/plant) under greenhouse conditions.

Chemical inducers	Concentration	Disease severity %	Efficiency %	Fresh weight (g/plant)	Dry weight (g/plant)
Chitosan (mg/mL)	1.0	30.52	46.94	2.43	0.45
	2.0	22.09	61.60	3.35	0.65
	3.0	19.83	65.53	4.55	0.86
	4.0	12.16	78.86	6.60	0.99
Salicylic acid (mM)	1.0	35.18	38.84	1.67	0.40
	5.0	27.92	51.46	2.35	0.45
	10.0	24.02	58.24	3.00	0.57
	25.0	17.18	70.13	3.75	0.66
Control		57.53	0.00	1.11	0.25
LSD at 0.05		2.61		0.17	0.11

2.2- Estimation of enzyme activity and total phenols:

2.2.1-The effectiveness of the antagonistic bacteria and fungi on the oxidative enzyme's activity, hydrolysis enzyme and total phenols:

Results in Table (5) show that noticeable increases of oxidative enzymes activity, i.e., polyphenoloxidase (PPO) and peroxidase (PR) and hydrolysis enzyme chitinase as well as total dissolved phenols determined from leaves of tomato plants (cv. Super Strain B hybrid), artificially inoculated with *A. solani*, and treated with the antagonistic bacterial and fungal isolates (*B. megaterium*, *B. subtilis* and *Ps. fluorescens*), and (*T. harzianum*, *T. album* and *T. viride*) before infection with *A. solani*, *in vivo.*

Concerning activity of oxidative enzyme, the highest increase in the activity of the three tested oxidative enzymes was observed in the leaves of tomato plants treated with *T. harzianum* and *T. viride*. Meanwhile, the lowest increase in the activity was obtained when tomato plants were treated with *Ps. fluorescens* and *T. album*.

The same trend *i.e.*, increase the activity of chitinase enzyme in the leaves of tomato plants treated with both antagonistic bacterial and fungal isolates under the same conditions was

also true (Table, 5). *T. harzianum* recorded the highest activity of chitinase, followed by *T. viride*. On the other hand, *Ps. fluorescens* recorded the lowest chitinase activity followed by *T. album*.

In regard to determination total dissolved phenols in leaves of treated tomato plants with the bio agents tested logical organisms, data shown in Table (5) illustrate that treated tomato plants with the antagonistic bacteria and fungi led to an increase in the total dissolved phenols compared to the untreated control. The highest significant increase in the total dissolved phenols in the tomato leaves was obtained from treated tomato plants with *T. harzianum*, by a greater amount than using any of the other bio agents, followed by *T. viride* (174.27 and 169.42 mg/ 100g fresh weight, respectively). However, the lowest value for total soluble phenols was obtained from leaves of tomato plants treated with *Ps. fluorescens* and *T. album* (95.52 and 84.46 mg / 100g fresh weight, respectively).

Table (5): Effectiveness of some antagonistic bacteria and fungi on polyphenol oxidase, peroxidase, hydrolysis enzyme (chitinase) and total soluble phenol content in tomato leaves artificially inoculated with *Alternaria solani* under greenhouse conditions.

Bio agents	Polyphenol oxidase activity (m/g f. w.)	Peroxidase activity (m/g f. w.)	Chitinase activity (m/g f. w.)	Total phenol (mg/100g f. w.)
Bacillus megaterium	2.14	3.52	3.53	148.72
Bacillus subtilis	1.79	2.87	2.49	136.54
Pseudomonas fluorescens	1.30	2.44	1.38	95.52
Trichoderma harzianum	4.38	6.43	5.93	174.27
Trichoderma album	0.83	1.98	1.02	84.46
Trichoderma viride	3.10	5.33	4.87	169.42
Control	0.39	0.69	0.52	62.83
Correlation coefficient	-0.75	-0.76	-0.84	-0.89

*f. w. = fresh weight

2.2.2- The effectiveness of different concentrations of chitosan and salicylic acid on the enzyme activity of oxidative and hydrolytic enzymes and total phenols:

Data illustrated in Table (6) show that treated tomato plants with different concentrations of chitosan and salicylic acid under greenhouse conditions before artificially inoculated by *A. solani*, exhibited an increase of oxidative enzyme activity *i.e.*, polyphenoloxidase (PPO) and peroxidase (PR) and hydrolysis enzyme chitinase as well as total dissolved phenols in leaves of treated tomato plants compared to untreated control.

As for activity of oxidative enzymes, the highest increase in the activity of the two tested oxidative enzymes PPO and PR was observed in the leaves of tomato plants treated with chitosan at a concentration of 4 mg/mL, followed by treatment of plants with salicylic acid at a concentration of 25 mM (5.60 and 3.90 m/g fresh weight, respectively) compared to the rest of the other used concentrations for both of inducers. On the other hand, the lowest activity of both oxidative enzymes was obtained when plants were treated with 1 mg/mL and 1 mM concentration of both chitosan and salicylic acid, 1.32 and 1.08 m/g fresh weight, respectively.

The effectiveness of different concentrations of chitosan and salicylic acid on chitinase activity and total content of dissolved phenols observed in tomato plant leaves artificially inoculated by *A. solani* was studied under greenhouse conditions. The results in Table (6) also, show that when plants were treated with these different concentrations of both tested chemical inducers, this led to a significant increase in the activity of the enzyme chitinase as well as an increase in the total dissolved phenols compared to the untreated treatment. The highest increase in activity of chitinase, as well as the total dissolved phenols in the leaves of plants was observed by chitosan 4 mg/mL (6.29 m/g fresh weight), and (125.85 mg/100g fresh weight) respectively. Salicylic acid 25 mM came the next one in increasing chitinase enzyme activity and total dissolved phenols in tomato leaves (4.72 m / g fresh weight) and (108.92 mg / 100g fresh weight) respectively. On the other side, the lowest value of chitinase enzyme activity as well as the total dissolved phenols was obtained in tomato leaves treated with the first concentration for each inducer, which recorded 1.29 mM / g fresh weight, and 74.97 mg/100g fresh weight respectively.

Table (6): Effectiveness of different concentrations of chitosan and salicylic acid on activity of polyphenoloxidase, peroxidase, hydrolysis enzyme (chitinase) and total soluble phenol content in tomato leaves artificially inoculated with *Alternaria solani* under greenhouse conditions.

Biochemical changes	Chitosan Concentration (mg/mL)				Salicylic acid Concentration (mM)				Control
	1.0	2.0	3.0	4.0	1.0	5.0	10.0	25.0	
Polyphenoloxidase (m/g f. w.)	1.32	2.14	3.51	5.60	1.08	1.89	2.78	3.90	0.39
Peroxidase (m/g f. w.)	2.80	3.07	5.47	6.43	1.44	1.87	3.64	4.40	0.69
Chitinase (m/g f. w.)	2.39	3.52	4.87	6.29	1.29	2.46	3.61	4.72	0.52
Total phenol (mg/ 100g f. w.)	87.75	97.87	101.84	125.85	74.97	89.76	92.64	108.92	62.83

*f. w. = fresh weight

DISCUSSION

Tomato early blight, caused by *Alternaria solani*, is one of the most common leaf diseases in tomato plants, which causes damage to leaves, stems and fruits, causing severe damage to the plants, especially the aerial part, and reducing the size and number of fruits, which leads to significant economic losses in the yield, up to 79% (Sherf and MacNab, 1986 and Gwary and Nahunnaro, 1998). Agricultural pesticides have a clear and specific purpose that everyone knows. This purpose is to control insects and plant pathogens, including fungi and bacteria, as well as harmful weeds that harm agricultural crops, however, pesticides can negatively effect of some beneficial organisms. Therefore, it becomes necessary to develop environmentally friendly and biodegradable agricultural pesticides in order to control plant pathogens. Currently, biological control with antimicrobial organisms and resistance promoters offers a practical and economical alternative to control plant pathogens (Chandrashekara *et al.*, 2012 and Sarhan, 2018). In this study, the effectiveness of bacterial agents (*Bacillus megaterium*, *Bacillus subtilis* and *Pseudomonas fluorescens*) and fungal agents (*Trichoderma harzianum*, *Trichoderma album* and *Trichoderma viride*), as well as the efficacy of different concentrations of the chemical inducers chitosan and salicylic acid, on *Alternaria solani* the causal pathogen of early blight of tomato, were evaluated under laboratory and greenhouse conditions. The results obtained in this study showed that all tested fungal and bacterial bio agents reduced linear growth of *A. solani in vitro*. The two antagonistic fungi *T. harzianum* followed by *T. viride* were the best fungal bio agents in reducing the linear growth of *A. solani*. However, the two antagonistic bacteria, *B. megaterium* and *B. subtilis* were the superior bacterial bio agents in this respect. These results are in agreement with the findings of EL-Tanany *et al.*, (2018) who evaluated different isolates of *Trichoderma* and some bacterial bio agents on the linear growth of *A. solani* and stated that *T. viride* and *B. subtilis* were the best bio agents in reducing linear growth of *A. solani*. The obtained results showed that spraying of the aforementioned biological organisms on tomato plants before being infected with *A. solani* under greenhouse conditions, led to decrease the severity of tomato early blight, as well as increased fresh and dry weights of tomatoes compared to untreated treatment. These results are in agreement with the findings of EL-Tanany *et al.*, (2018) and Moustafa *et al.* (2018a), they stated that using different isolates of *Trichoderma* and bacteria such as *B. subtilis* and *Ps. fluorescens*, as well as, biocides, Bio Zeid 2.5% powder (*Trichoderma album* 10×10^6 spore/g), and Bio Arc 6% WP (*Bacillus megaterium* 25×10^6 cells/g), gave significant reduction in disease severity of early blight on tomatoes under greenhouse conditions. Antagonistic microorganisms play an important role in fighting plant diseases. They generally have a wide spectrum of action. Protective effect of the biological organisms includes different mechanisms like producing toxic substances that directly oppose the growth of pathogens (Leelasuphakul *et al.*, 2008). It is known that fungi of the genus *Trichoderma* are effective in controlling pathogenic fungi through an antagonistic effect against the pathogen, by producing metabolites derived from cyclopentenone (George *et al.*, 1977 and Strunz

et al., 1977). Also, several authors explained the antifungal affectivity of fungi belonging to genus *Trichoderma*, this may be due to stimulation the anti-pathogen compounds or can wrap around the pathogen and form aspirin on its surface. Also, most of the antagonistic fungi produce many enzymes that degrade the pathogen's cell wall, and it may also produce antibiotics. (Handelsman and Parke, 1989; Schirmböck *et al.*, 1994, Chet *et al.*, 1998 and Inbar *et al.*, 1998). While the bacterial bio agents *B. megaterium*, *B. subtilis* and *Ps. fluorescens* have more mode of action in controlling plant pathogens, the method of plant disease resistance relies on the production of the "megacin" metabolite that causes a radical change in the osmotic barrier of sensitive organisms by attacking the cytoplasmic membrane (Ivanovics *et al.*, 1959 and Khalil *et al.*, 2009). In addition, it acts as a catalyst for resistance, as it leads to an increase in polyphenols, as well as increasing the activity of defence-related enzymes peroxidase, chitinase, β-1,3-glucanase, and phenyl alanine ammonia lyase. On the other hand, they act as a growth promoter, able to dissolve phosphates, produce indole acetic acid (IAA), and siderophore (Chakraborty *et al.*, 2006).

In our investigation, all the different concentrations of two the tested chemical inducers, *i.e.*, chitosan and salicylic acid resulted a significant decrease in linear growth and spore germination of *A. solani*, *in vitro*. The chemical inducer chitosan at a 4 mg/mL followed by salicylic acid at 25 mM were the most effective ones in reducing linear growth and spore germination of *A. solani*. Eid (2017) indicated that the use of sodium bicarbonate, potassium hydrogen carbonate, ascorbic acid, and salicylic acid, under laboratory conditions, reduced the linear growth of *A. solani* on PDA compared to the control treatment. In addition, when spraying tomato plants with different concentrations of chemical inducers chitosan and salicylic acid before the artificial infection with *A. solani* under greenhouse conditions reduced disease severity of early blight and increasing fresh and dry weight of tomato compared to control. In general chitosan was better than salicylic acid of the different tested concentrations in reducing disease severity and increasing the fresh and dry weight of the tomato plants. These results agree with the findings of Eid (2017) who found that treating tomato leaves with chitosan prior to inoculation with *A. solani* reduced leaf infection rates and disease index. Also, EL-Tanany *et al.* (2018) and Moustafa *et al*. (2018b), reported that spraying tomato plants with tested chemical inducers, chitosan, and salicylic acid, prior to infection of tomato plants with the pathogen *A. solani*, was highly effective in controlling tomato early blight, under greenhouse conditions. The results obtained can be interpreted considering the findings reported by Katiyar *et al.* (2015), who stated that chitosan affects various physiological responses such as plant immunity and defense mechanisms involving different enzymes such as polyphenoloxidase, tyrosine ammonia lyase and antioxidant enzymes. It was also indicated that there is a link between stimulating plant resistance by chemical inducers and some biochemical changes in tomato leaves. In this regard treatment of tomato plants with chitosan led to an increase in the activity of peroxidase, polyphenoloxidase (catechol oxidase), phenylalanine ammonia-lyase, chitinase and 1,3-glucanase in the leaves, but to different degrees depending on the cultivar. Also, a high percentage of phenols, which are indicative of the first stage of the defense mechanism, were recorded in the treated plant. Ahmed (2015) recorded a significant decrease in the incidence and severity of chocolate spot disease (%) caused by the fungus *Botrytis fabae* in faba bean plants treated with bio-stimulants, and resulted an increase of chlorophyll, phenols, and flavonoids contents in faba bean leaves.

Regarding the chemical inducer, salicylic acid, the results obtained can be explained, in light of the findings of Spletzer and Enyedi (1999) who stated that salicylic acid (SA) is an important signaling molecule that plays an important role in plant defense against invading pathogens. In addition, the reaction of salicylic acid can be clarified in the fight against plant diseases, as many properties of this chemical inducer have been demonstrated, as it has been widely used in medicine as an analgesic, antipyretic and anti-inflammatory agent, in addition to its bactericidal, fungicidal effects as a protective property (Madan and Levitt, 2014). More recently, salicylic acid is used in agricultural operations as an alternative to fungicides, as a resistant catalyst (Wang *et al.*, 2007) and a growth promoter (Sharma, 2013). El-Mohamedy *et al.*, (2014) reported that when tomato plants were treated with 100 mM salicylic acid, this treatment led to a significant increase in plant height as well as the branches number / plant. The stimulation of plant resistance may be explained by the chemical

inducers of chitosan and salicylic acid, based on the studies of "omics", where it was recently suggested that the priming phenomenon be divided into three different phases. In the first stage, levels of different transcription, proteins, and metabolites are altered, making the plant an alert, however, in the post-challenge state, the primary anti-stress or induced state reactions (Alexandersson *et al.,* 2016). All tested fungal and bacterial bio agents, as well as different concentrations of chemical inducers triggered some defense mechanisms in tomato plant leaves such as affecting the activity of oxidizing enzymes (peroxidase, polyphenoloxidase and hydrolytic enzymes (chitinase), as well as total phenols associated with activation the systemic resistance induction against pathogen infection *A. solani*, compared to untreated control.

From the obtained results, it was found that the tested antifungal *T. harzianum* and *T. viride* were more effective than bacterial isolates in reducing the severity of early blight on tomato plants, as well as increasing the activity of enzymes and total phenols. Regarding chemical inducers, the different concentrations of the chitosan followed by salicylic acid resulted an increase in the activity of oxidative and hydrolytic enzymes, as well as total phenols compared to control treatment, respectively. It can be concluded that the tested biological and chemical treatments stimulate the activity of (peroxidase and polyphenoloxidase) and hydrolytic enzymes (chitinase), as well as total phenols are associated with reducing the severity of early blight in tomato plants. In this study, great emphasis was placed on the interaction between disease severity and biochemical changes in leaves of tomato plants. The results showed that the tested isolates of the antagonistic organisms differed in their effect on disease severity and the activity of polyphenoloxidase, peroxidase, hydrolytic enzymes (chitinase) as well as the total dissolved phenols content in tomato leaves. In general, all the antagonistic organisms reduced the severity of early blight on tomato plants. Also, the negative relationship between the decrease of disease severity of early blight and increased activity of polyphenoloxidase, peroxidase and hydrolytic enzymes (chitinase), as well as an increase in total soluble phenolics, which is related to induction of induced systemic resistance activity in tomato plants, compared to the control treatment. The lowest disease severity as well as the highest increase in enzyme activity and total phenols content were recorded in tomato leaves when treated with *T. harzianum* and *T. viride*. These results were confirmed by the finding reported by EL-Tanany *et al.* (2018). The results obtained can be discussed in the light of the results indicated by (Ramamoorthy *et al.,* 2002; El-Khallal, 2007; Latha *et al.,* 2009; Abd-El-Khair *et al.,* 2011 and Surekha *et al.,* 2014), who reported the importance of using of *Trichoderma* spp., *Bacillus* spp., *Pseudomonas* spp. The action of these oxidative enzymes such as peroxidase and polyphenoloxidase has been explained, as they lead to enhanced formation of lignin and oxidation of phenols to more toxic quinones, while other oxidized phenols contribute to forming defence barriers to enhance cell structure as mentioned by Conti *et al.* (1974); Yamamoto and Tani (1978); Arora (1979). Gogoi *et al.* (2001); Ramamoorthy *et al.* (2002) and Anand *et al.* (2009b), found that the high enzymatic activity of oxidative enzymes (peroxidase, polyphenoloxidase) and hydrolytic enzyme (chitinase), leads to additional production and accumulation of phenols which may impede the spread of the pathogen from infected cells into healthy cells, and thus infection can be prevented or restricted. Also, Yoshida *et al.* (2003) reported that peroxidase plays an important role in ethylene biosynthesis, auxin regulation and plant cell wall components, *i.e.,* lignin, suberin and wall thickening as part of the defense response to pathogens, especially fungi. Also, peroxides participate in the formation of lignin, the polymerization of glycoproteins rich in hydroxyproline, and the regulation of cell wall elongation. Several investigators explained the action of hydrolytic enzymes (chitinase) (Schlumbaum *et al.,* 1986; Ham *et al.,* 1991; Leah *et al.,* 1991 and Velazhahan *et al.,* 2003) they reported that analysis of chitin, which is a major component of fungal cell walls, which leads to direct inhibition of the growth of many fungi. On the other hand, the reaction of total phenols can be explained according to the findings of Guleria *et al.* (2005); Ali *et al.* (2007); El- Khallal (2007) and Abo-Elyousr *et al.* (2009), who showed that the accumulation of phenolic compounds at the site of infection as a result of treatment with antimicrobial organisms and chemical inducers was related to the restriction of pathogen development, because these compounds are toxic to pathogens. As for the interaction of phenolic compounds within plants (Abo-Elyousr *et al.,* 2009), they indicated that many phenolic compounds have a function within the plant as

anti-pathogens, as precursors to structural polymers such as lignin, or as signaling molecules. Also, these compounds may prevent the pathogen from progressing by increasing the mechanical plant cell wall strength.

CONCLUSION

It was concluded that the bio agent *Trichoderma harzianum* and the chemical inducer chitosan had a good effect on *A. solani*, the causative agent of tomato early blight, and could be a promising alternative to the use of chemical fungicides for controlling early blight disease of tomato, and it is better to use them for protection before infection catches.

CONFLICTS OF INTEREST

The authors declare no conflict of interest exists.

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