Induced resistance in tomato to the gray mold *Botrytis cinerea* by *Trichoderma harzianum* and *Azotobacter chroococcum*

Ahmed A.A. Alshimaysawe and Sabah L. Alwan.

1master Candidate, University of Kufa, Najaf, Iraq. E-mail: ahmed2007.2011@gmail.com

2Associate Professor, University of Kufa, Najaf, Iraq. E-mail: Sabah.alhasnawi@uokufa.ed.iq.

ABSTRACT

The study was conducted to control the gray mold disease caused by *Botrytis cinerea* on tomato grown in plastic houses for the 2017-2018 growing season in the province of Najaf. Two bio-control agents, *Trichoderma harzianum* and *Azotobacter chroococcum*, were used in the aim of inducing plant resistance by increasing the plant defense enzymes including super-desimutase, catalase and ascorbate peroxidase. Treatments were incorporating the two bio-agents individually with the pre-planting soil which was or not fertilized with organic compost while the pathogenic fungus was used as spray treatment at plant blossom

plants sprayed with the pathogenic *B. cinerea* in the absence of any bio-agent had significantly much higher percent infection (70%) than those grown in soil incorporated with either *T. h* (33%) or *A. chro* (36%). Both bio-agents resulted in significantly lower infection rates in both fertilized and non-fertilized soils. The enzymes' activities under study were also affected by treatments. The effectiveness

of all the tested enzymes was significantly higher in plants grown in soil incorporated with any of the bio-agents under study. These defense plant enzymes were not affected by the pathogen presence, but they significantly increased in the presence of *T. harzianum* followed by *A. chroococcum*. However, the amount of all the three defense enzymes was even much higher in interaction treatments between the pathogenic *B. cinerea* and *T. harzianum* or *A. chroococcum*,Bio-agents.

Plant defense enzymes were increased and resistance to *B.cinerea* in tomato was induced where *T. harzianum* or *A.chroococcum* was used as pre-planting soil treatment. Both bio-agents might be efficiently used in an IPM program to control the gray mold disease in plastic house of tomato plants.

Introduction

Tomato *Solanum lycopersicon* belongs to the Solanaceae plant family is an important vegetable crop. It is continuous crop grown in the two main seasons. Winter tomatoes are mostly grown in plastic houses and tunnels. While most summer tomatoes are grown under field condition or open farming (Shukur, 2015). According to the Iraqi central statistical organization for year 2015, the area cultivated with tomato was estimated 23000 hectare with a total production of 388,700 metric ton.

The production of vegetables including tomatoes in the recent years substantially expanded in all the areas of the country. This expansion was associated with the emergence of problems and diseases that often of little importance in open farming but have become important in controlled farming (plastic and glass houses). This cropping practice provides appropriate environmental conditions that favorable to those diseases including gray mold disease on tomato caused by the fungus *Botrytis cinerea* (Richard, 2006).

B.cinerea is one of the most important air born mitosporic fungi that affects more than 200 annual and perennial crop plants worldwide. It infects important vegetable crops such as tomato, eggplant and pepper and affects fruit plants such as strawberries (Vagelas et al., 2009*). B. cinerea* in the controlled farming system causes problematic diseases and yield losses that may reach 60% (Schoonbeek et al., 2001). Percent infection with this disease in Iraq ranged from 35 to 43%, depending on the favorable environmental conditions, crop intensity and crop susceptibility to the disease (Al-Esawy and AL-Taae, 2016). In addition to infecting tomato stems and leaves causing chlorosis and wilting, the economic damage is due to blossom blight and consequently rotting of young fruits leading to crop damage or death of the entire plant (Daughtrey, 1995 and Zitter, 2011).

Induced resistance in plants is one of the safest, inexpensive, and environmentally friendly methods to control plant pathogens (Hinampas, 2013). In 1983, it was first noticed to cause local lesions at the site of infection (metrau, 2001). In general, induced resistance is based on factors that appear after the host exposure to a specific pathogen or an external effector (chemical, biological) or to some certain secondary metabolites (Hassan, 2010). *Trichoderma harzianum* was used to induce resistance in bitter orange against *F.solani* in the presence of *P. fluorescens* (Al-Esawy, 2015). Some other examples included the use of *Azotobacter chroococcum* in combination with some growth promoting to enhance plant resistance showed efficacy that did not significantly differed from using chemical pesticides in controlling fungal pathogens (Hammami *et al.*, 2015; Chauhan *et al.*, 2015), especially in controlled farming or plastic house conditions (Al-Jabri, 2011 and Saeed, 2015). This study, therefor, aimed to control *B.cinerea*, the causal of gray rot in green-house growing tomato by plant resistance induction mechanism using bio-agents *Trichoderma harzianum* and *Agrobacterium chroococcum*.

Material and Methods

Isolation and identification of *Botrytis cinerea*

Samples were collected from highly (89%) infested fields with gray mold on tomato plants. Plants of these fields showed sever infection represented by gray to

brown of infected part with dense fungal growth on the vegetative upper parts having gray to brown color of the area from which the fungus was isolated. Isolated fungus was cultured and purified then was diagnosed based on the taxonomic key by Ellis (1971). The isolates of the fungus were kept on a slope culture media at 4 C° until the use of the fungus.

Treatment factors used in the experiment

Bio-controlling agents, the fungus *Trichoderma harzianum* (the Australian isolate) and the bacterium *A. chroococcum* were obtained from the graduate microbiology laboratory in the Dept. of Plant Protection at the Faculty of Agriculture/University of Kufa. The natural fertilizer palm fronds compost was obtained from the Center of Organic Fertilizer and Mushroom Farming in Najaf belongs to the National Center for Organic Agriculture/The Iraqi Ministry of Agriculture.

Propagation of the bio-agents inoculums

A local variety of millet (*Panicum miliaceum* L.) seeds were used for preparation of *T. harzianum* inoculum according to Dewan (1989) method. As for *A.chroococcum*, N.B medium was used to prepare the bacterial suspension following the manufactured company (LBS Marg, Mumbai-400086, India). The bacterial final number of live units in one ml was 96x 10^7 CFU.

Number of live spores of *B.cinerea* on solid PDA

B. cinerea was cultured on PDA culture medium for 7 days. 10 ml of distilled water was added to the culture plate dish with a brush. The surface of the culture contained the fungal spores was scraped with a fine brush. The fungal suspension then was filtered, collected in 100 ml flask and subjected to a series of dilutions up to fourth dilution(10^4). (Dewan, 1989). The haemocytometer was used to calculate the final number of spores was produced by placing 100 µml of the 4th dilution with 100 µml of 10% of Lacto phenol Cotton Blue, and then the number of live spores was calculated according to Jason Fan (2016) equation. The number of live spores was $2.9*10^4$/100 µ ml.

The experiment was carried out on 1/9/2017 in a greenhouses belongs to the Nursery of the Horticulture and Forestry sector at the Plant Production Department/ Agriculture Directorate in the province of Najaf, for the spring growing season of 2017- 2018. The soil was sterilized with ethylene at rate of 2ml/kg soil, covered with polyethylene for 48h and aerated for the next 48h before use. At rate of 1:3, the organic fertilizer (palm fronds compost) was added to one part of the sterile soil while the other was left without fertilizer. The biological controlling agents were added to both types of soil at rate of 5g/kg soil for the fungus *T. harzianum* and 5ml/gk soil for the bacterial suspension of *A. chroococcum* and soil was moisturized for 3 days. Tomato seedlings were transplanted 50 cm between plants and 75 cm between treatments. Plants were irrigated regularly or as needed.

Treatments were spraying the pathogenic *B.cinerea* at plants blossom (flowering time) to both types of soil (fertilized and non-fertilized) with and without either of the bio-agent or their combinations, while the soil without any adding served as control. The experiment was complete randomized block design with six replicates for each treatment.

Infection severity

The severity of infection on tomato leaves was calculated two weeks after spraying with the pathogenic fungus (Mansoury and Salih, 2015) based on rating scale that ranged from 0 to 5. Where 0= No injury, 1= Spot area of 1-20% of leaf size, 2= 21-40%, 3=41-60%, 4= 61-80% and 5= Spot area ⩾81% of leaf area.

According to Mckinney (1923), the severity of injury for each treatment was

$$\text{Severity of injury} = \frac{\text{Number of leaf at rate (0*0)+ ...+ (number of leaf at rate (5*5)}}{\text{Total number of leaf x 5}} \times 100\%$$

Estimation of plant defense enzymes activities

The plant defense enzymes activities were estimated in the fifth leaf of each plant sample using the UV-Visible spectrophoto meter in the graduate's laboratory of Agri. College/Univ. of Kufa.

1- Superoxide dismutase (SOD) activities were estimated according to Marklund (1974) which relies on the SOD susceptibility to inhibit pyroclal oxidation. It was measured at a wavelength of 420 nanometers and calculated based on the following equation by (Frary et al., 2010)

$$\text{SOD activity (units)} = \frac{\%\,inhibition / 50\% \times reaction\,volume}{total\,test\,period}$$

2- Catalase activities

The method implied by Aebi (1984) was followed to estimate Catalase efficacy at 240 nanometer wavelength at three running times (0, 30 and 60 second) µmol/min with three replicates, based on the below equation:

$$\text{Catalase activity (units)} = \frac{\Delta \text{Abs} / T \times R}{0.01}$$

3- Ascorbate Peroxidase

The effectiveness of the ascorbate peroxidase (APX) was determined (Nakana and Asada, 1981) and then the difference in absorbance was measured using spectrophotometer at 290 nm wavelength.

$$\text{APX activity (unit)} = \frac{\Delta Abs. \min \times reaction\ volume}{2.8\ mM - 1}$$

Data analysis

All experimental data were subjected to analysis of variance ANOVA using Genstat Discovery 2012 Edition. Means were separated at $P \leq 0.05$ based on the Least Significant Difference LSD.

Results and discussion

Efficacy of biological control agents against *B.cinerea*: The results of percent infection by *B.cinerea* and severity are shown in Table 1. Infection severity rates were highly reduced in *B.cinerea* infected plants treated with *T.harzianum* (31.83%) or with *A. chroococcum* (33.58%) compared to significantly ($P \leq 0.05$) the highest infection rate (68.79%) from untreated plants infected with *B.cinerea*.

Generally, treatments with compost fertilized soil resulted in total lower infection (27.69%) rate compared to non-fertilized soil (30.32%) infection rate. The control treatment had relatively high infection rate (31.62%) that similar to infection rate from interaction treatment between *B.cinerea* and *T. harzianum*. Both bio-agents reduced infection with *B.cinerea* on tomato plants especially those grown in fertilized soil.

These results agreed with Hard et al. (2014) that *T. harzianum* applied to planting soil enhanced systemic resistance in tomato plants against plant pathogens. Herter et al. 2011 also reported that *A. chroococcum* stimulated host plant resistance to several pathogens and the synergistic effect with other bacteria was much effective in reducing infection severity.

Table1. Effect of different treatments on infection severity (%) on tomato plants two weeks after spraying with *B.cinerea*

Treatments	Planting soil		
	Fertilized	Non-fertilized	Average*
Control	33.63	29.61	31.62

B.cinerea	70.48	67.10	68.79
T.harzianum	4.47	3.44	3.96
A.chroococcum	4.52	3.99	4.26
T.harzianum+ B.cinerea	33.50	30.15	31.83
A. chroococcum+ B.cinerea	35.33	31.83	33.58
Average	30.32	27.69	

* Values are average of six replicates. L.S.D. values are 0.891 and 3.088 for treatments and interaction, respectively (P≤0.05).

Estimation of plant defense enzymes activities

SOD levels

Results showed that Superoxide dismutase levels were highly affected by treatments. The highest level of this enzyme (93.99 µmol/min) was detected in the interaction treatment between *T. harzianum* and *B. cinerea* while the lowest level (30.36 µmol/min) was in the control followed by the 31.37 µmol/min from plants treated with the pathogenic fungus alone. This enzyme was also higher in Fertilized soil treatments compared with non-fertilized ones. Interaction of pathogenic fungus with the bio-agent *T. harzianum* in fertilized soil had significantly the highest SOD

level (102.57 µmol/min) compared to the lowest value of this enzyme (29.45 µmol/min) from the control treatment in non-fertilized soil plants.

Catalase levels

Results of estimation the catalase levels are shown in Table (3). With significant difference, *T. harzianum* treatment had the highest amount of this enzyme that was 92.66 µmol/min when treated with *B.cinerea*, while the lowest amount of the enzyme was in the control treatment with 31.79 µmol/min. Again, all the fertilized treatments resulted in higher levels of catalase than non-fertilized treatments. For the effect of interaction, the treatment of *T. harzianum* interacted with *B.cinerea* in fertilized soil resulted in the highest amount of the enzyme (104.72 µmol/min), while the lowest amount of the catalase was 31.14 µmol/min in the control without fertilizer.

Table2. Effect of different bio-control agents treatments on quantity of plant defense enzymes in tomato plants two weeks after spraying with *B.cinerea*

Treatnments	Ascorbate Peroxidase		Catalase		Superoxide dismutase*	
	Fertilized	**Unfertilized**	**Fertilized**	**Unfertilized**	**Fertilized**	**Unfertilized**
Control	29.45	31.26	31.14	32.44	0.077	0.100
B.cinerea	30.86	31.87	32.43	33.44	0.080	0.103
T.harzianum	82.16	93.34	72.96	95.45	0.374	0.477
A.chroococcum	77.23	89.27	74.11	90.37	0.373	0.450
B.cinerea+ *T.harzianum*	85.40	102.57	80.60	104.72	0.497	0.563
B.cinerea + *A. chroococcum*	79.53	99.16	79.05	94.72	0.472	0.530
Average	**64.11**	**74.58**	**61.72**	**75.19**	**0.312**	**0.371**

*Values are average of six replicates. The L.S.D. ($P \leq 0.05$) values between treatments are kkkk, llll, and hhhh; between soils are 3.312, 3.478 and 0.0314; for interactions are 11.472, 2.048 and 0.108 representing Superoxide dismutase, Catalase and Ascorbate Peroxidase, respectively.

Ascorbate Peroxidase levels

For the Ascorbate peroxidase levels, results are shown in table 4. The highest level (0.53 µmol/min) of this enzyme was recorded in the treatment of *T. harzianum* in the presence of *B. cinerea* while significantly the lowest level (0.08 µmol/min) was in the control treatment ($P \leq 0.05$). This enzyme was also higher in fertilized soil treatments compared with non-fertilized ones. Interaction between *T. harzianum* and *B. cinerea* with fertilizer resulted in the highest amount (0.563 µmol/min) of this enzyme compared to significantly the lowest amount (0.077 µmol/min) from the control treatment with no fertilizer.

The results of Table (2) showed an increase in the amount of tested defense plant enzymes where treated with *T. harzianum* especially in the presence of pathogenic fungus *B.cinerea*. This may be attributed to *T. harzianum* ability to secrete certain compounds such as fatty acids, carbohydrate and proteins that stimulated plant resistance by increasing the Peroxidase efficiency (Howell et al., 2000). This also may be due to the fact that *T.harzianum* stimulated plant enzymes production, some non-pathogenic fungi stimulated plant resistance against pathogens leading to increase resistance to pathogenic fungi (Al-Murad, 2011).

The induction of resistance against pathogens is enzyme's effectiveness related. This enzyme, pectinase for instance, works with hydrogen peroxide in breaking down the pathogenic enzymes and activating the breakage of the pathogen's cell. This process may result in the induction of phytoalxines. And this may also involve the structural defense mechanisms such as strengthen the cells walls by reconstructing and consolidating the lignin. The enzyme also reacts with cell wall proteins to form cross - links and multiple compounds, which increases the hardness of the cell wall and thus reduces the pathogen's ability to penetrate or to further development (Hiber et al., 2007).

The higher level of plant defense enzymes in the presence of the pathogen is mostly explained as plant reaction to a strange biotic factor, and this often stimulates plant resistance (Hard and others 2014). This isolation of *T.harzianum* stimulated the systemic resistance in tomato plants against the gray mold disease caused by *B.cinerea*. Moreover, the combination of *T.harzianum* and the organic fertilizer in the planting soil increased the enzyme Peroxidase, Poly phenol oxidase and other phenolic compounds. This indicates the ability of the fungus in favorable conditions to induce plant systemic resistance against several pathogens (Temur, 2009 and Matrood, 2015).

A. chroococcum showed a high ability to stimulate resistance in the plant by increasing the effectiveness of plant defense enzymes where exposed to pathogenic

B.cinerea. This is compatible with previous studies of the ability of different types of bacteria used as bio-fertilizes or as bio-control agents in their ability to enhance host resistance by increasing enzymes involved in plant defense mechanism (Mali, Bodhankar, 2009, Karthikeyan, Sakthivel, 2011, Herter and others, 2011).

This research showed that using organic fertilizers combined with one or more of biological control agents, especially those involved in plant systemic resistance, has played an important role as preventive measures in suppressing the pathogen and reducing disease severity.

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