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Investigation of Some Physiological and Biochemical Parameters in *Pseudomonas syringae*-Infected Tomato Plants

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In the present study, the levels of manganese, magnesium, plant hormones (indole-3-acetic acid and abscisic acid), sugars (fructose and maltose), total chlorophyll and carotenoid contents in tomato plants following inoculation with *Pseudomonas syringae* pv. *tomato* were investigated. The trace element analysis showed that the levels of Mn significantly decreased in bacterium-infected tomato plants, comparing to the healthy plants. In contrary, the content of Mg in the bacterium-infected plants was higher than the uninfected plants. Manganese deficient in the bacterium-infected plants might be an important indicator against attempted bacterial infection. The results of phytohormone analysis showed that there may be an opposite relationship between the concentrations of endogenous indole-3-acetic acid and abscisic acid and the enhance disease resistance in bacterium-infected tomato plants. Therefore, it seems that indole-3-acetic acid, like abscisic acid, acts as a negative regulator of plant defense. Fructose concentrations increased in second, fourth, eight days after treatment with pathogen, but there was a decrease in tenth days. However, maltose levels decreased in all the periods after inoculation with the pathogen bacterium comparing to the control healthy plants. These results were also affirmed to the reduction in the total contents of chlorophyll and an increase of total carotenoids in the bacterium infected-plants. The investigation confirmed that there are complex relationships among trace element levels, endogenous plant hormone and sugar in the regulation of defense mechanisms against attacks by bacterial pathogens.

Key Words: Trace element, Plant hormone, Sugar, Tomato and *Pseudomonas syringae*.

INTRODUCTION

Higher plants are constantly subjected to biotic factors such as viral, fungal and bacterial pathogens and they are improved a broad range of defense mechanisms to challenge against the microbial pathogens[1]. Recently, various research groups have been conducted a number of studies dealing with the regulation of defensive

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interactions between host plant and pathogen[2-4]. The interaction between plants and microbial pathogens are specific, complex and dynamic, frequently resulting in either disease (compatible interaction) or resistance (incompatible interaction)[5].

Trace elements are known to be essential for plants, several of them are proved necessary for a few species only and others are known to have stimulating effects on plant growth, but their functions are not well known. Despite the fact that some specific functions of various trace elements (Fe, Al, Cu, Co, Mo, Mn and Zn) which seem to be participate in defense mechanisms of frost-hardy and drought-resistance plant species are reported[6]. There is very limited number of publications on the relationship of trace elements and pathogens as well as the effect on the host-plant. A negative correlation established between Cd and *Phytophthora infestans* in two potato varieties[7]. Miteva *et al*.[8] determined a relationship between the biotic and abiotic stress factors in tomato plant infected with cucumber mosaic virus (CMV) and tomato mosaic virus (ToMV). They expressed that infection affected the accumulation rate of heavy metals such as Cu, Zn and Fe in tomatoes. Berber *et al*.[1] also suggested that accumulation of Cu, Zn and Fe in *Pseudomonas*-infected tomato plants might be an important indicator against pathogen attack. However, interaction between heavy metals and microbial pathogens and their effect on plants has not been well understood.

Several studies showed that during plant-pathogen interactions, different signal molecules such as, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and gibberellic acid (GA) have significant signaling and regulatory roles to understanding the specificity of plant defense mechanisms against pathogen attack[2,8,9]. These signal molecules involved in some signal transduction system, including particular enzymes catalyzing biosynthetic reactions to form protective compounds for example polyphenols, alkaloids and pathogenesis-related (PR) proteins[4,5,10]. Nevertheless, changes in endogenous abscisic acid level following the inoculation of plants with pathogens were rarely measured in experimental studies[2,11].

Carbohydrates play important roles in several developmental processes in plants[4]. These metabolites often accumulate in senescing leave during abiotic and biotic stresses and induce leaf senescence. Infection by bacterial and fungal pathogens and attack by herbivores and gall-forming insects may influence leaf senescence *via* modulation of sugar status, either by directly affecting primary carbon metabolism or by regulating steady state levels of plant hormones[4]. Several studies revealed that during plant-pathogen interactions caused by the development of chlorotic and necrotic areas and thus to a decrease in photosynthetic assimilate production, either the direct effect of the pathogen on the expression of photosynthetic genes or the indirect effect because of induction of sink metabolism mediated *via* sugar repression of photosynthetic genes[12]. Although the repression of photosynthesis and the induction of sink metabolism seem to be a general response to pathogen attack, the effect on sugar levels varies considerably between different plant-pathogen interactions. There are complex relationships among levels of sugar, endogenous plant hormone, trace

element in the regulation of defense mechanisms against microbial pathogen attacks[1]. However, the molecular basis of these cross-interactions is less known.

The aim of the present study was to determine the levels of trace element (Mn), mineral (Mg), plant hormones (indole-3-acetic acid, IAA and abscisic acid, ABA), sugars (maltose and fructose) and total chlorophyll and carotenoid contents in tomato plants following inoculation with *P. syringae* pv. *tomato* and to establish the possible interactions among them.

EXPERIMENTAL

Tomato seeds (falcon cultivar) were obtained from Department of Horticulture, Faculty of Agriculture, Yuzuncu Yil University, Van (Turkey). The surface of seeds was sterilized with 2.5 % (w/v) sodium hypochlorite (NaOCl) for 3 min. Then the seeds rinsed 4 times with distilled water and dried using sterile filter paper. The seeds were soaked in sterile distilled water for 2 h and 20-30 seeds were put in sterile petri dishes on 4 layers of sterile Whatman No.1 filter paper for germination. The germinated seeds were sown in 10 cm diameters plastic pots containing sphagnum peat substrate (Kronen Mix) and were grown in a greenhouse for 10 days at 25 °C and a relative humidity of 60-70 %. After 10 days, the soil was gently washed off the roots of seedling with tap water and the seedlings were transferred into 30 cm diameters plastic pots (5 seedlings/pot) containing sterilized sandy loamy soil (2:1 w/w) and were grown in a greenhouse for 15 days at 25 °C and a relative humidity of 60-70 %. Plants were used for experiments *ca.* 25 days after seeds were germinated and had 5 to 7 leaves including the newly emerged leaf.

Bacterium growth condition and plant inoculation: The bacterium used in this study *P. syringae* pv. *tomato* DSM 60407 (*Pst* DSM 60407) was supplied by Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ, Germany). The bacterium was grown in King's medium B broth for 24 h at 28 °C on shaker at 150 rpm and stored in 15 % glycerol at -70 °C for later use. One day prior to plant inoculation, the bacterium was cultured in King's medium B broth as mentioned above. Overnight culture of the bacterium was inoculated onto King's medium B agar plates and incubated for 24 h at 28 °C. The cells were collected from the surface of the medium with sterile saline (0.85 % w/v) and washed 3 times. Afterwards, harvested cells were resuspended in sterile distilled water and the concentration of bacterial cell suspensions was determined as colony-forming units per milliliter (CFU/mL) using pour-plate technique and adjusted to 2×10^7 CFU/mL. Tomato plants were inoculated by applying the bacterial suspension as a fine mist with a hand sprayer until the suspension run off the leaf surfaces. After inoculation, the plants were kept under a plastic cover for 4-5 days to obtain a relative humidity of 100 %. Control plants were not treated with the suspension of *P. syringae* pv. tomato.

Plant harvest and analysis: Five plants from infected and control groups were harvested at 5 different periods (1st, 2nd, 4th, 8th and 10th study days) after inoculation

with *Pst* DSM 60407. Fresh plant leaves were used for trace elements, plant hormones and sugars analysis.

Analysis of trace element and mineral: All samples were washed in fresh running water to eliminate dust, dirt, possible parasites, or their eggs and were subsequently washed with double distilled water, cut in slices and oven-dried at 90 °C for 24 h before grinding[13]. They were ground with a porcelain mortar and sieved (200 mesh). One gram of dry matter (as two parallel) was weighed in a porcelain crucible, followed by the addition of 2 mL of a mixture of ethyl alcohol and sulfuric acid (95/5, v/v) and burned. After burning, ash was obtained at 500-550 °C in a muffle furnace. Then the ash was dissolved with 4 mL 3 N HCl and the solution was transferred to a 100 mL calibrated flask and diluted to 100 mL with double distilled water and filtered after 5-6 h with blue-band filter paper and again regulated to 100 mL. Concentration of heavy elements (Mg and Mn) were measured by Solaar flame atomic absorption spectrophotometer (Thermo Electron Corporation, Solaar House, Cambridge England).

Extraction and quantitative analysis of endogenous indole-3-acetic acid (IAA) and abscisic acid (ABA): The analysis of indole-3-acetic-acid (IAA) and abscisic acid (ABA) were performed as described by Cakmak *et al.*[14]. One g of frozen sample was powdered in liquid nitrogen. Then, cold methanol was added and stored at 4 °C for 24 h in dark. The samples were homogenized in an ultra tissue lysis (Ultrasonic Processor, Jenway Ltd. Essex, UK) and filtered trough a filter paper (Whatman No. 1). Then, the filtrates were collected. The residue was reprocessed in the same way as mentioned above and combined with the former one in order to minimize the loss of phytohormone. The filtrates were passed through polytetrafluoroethylene filters with a pore diameter of 0.45 μm. Methanol phase was discarded at 35 °C under reduced pressure. The obtained extracts were redissolved in K_2PO_4 buffer (pH 8.5) and centrifuged at 10.000 × g for 1 h at 4 °C. Afterwards, the supernatant was put in a flask (25 cm^3, each containing 1 g polyvinylpolypyrolidone (PVPP)); Sigma Chemical, UK), well mixed and filtered through Whatman No. 1 filter paper. The filtrates were introduced to Sep-Pak C_{18} cartridges (Waters, Hichrom Ltd. UK). The hormone was collected from cartridge with 80 % methanol and collected in vials. The hormone extract was injected into HPLC to detected IAA and ABA.

The isocratic system was used for HPLC analysis. The extracts in the vials were injected into HPLC equipped with waters 1525 binary pump (Waters, Hicrom,. UK); ultraviolet detector (Unicam Analytical Systems, Cambridge, UK) and μ Bondapak C_{18} column (Waters, Hicrom, UK) using acetonitrile (11.00 %; pH 4.92) as the mobile phase. The flow rate, pressure and wavelength were selected to be 2 mL min^{-1}, 6.000 psi and 245 nm, respectively. Under these conditions, the retention time of IAA and ABA were determined as 9.22 and 26.59 min for standards, respectively.

Determination of sugars: The analysis of sugars (fructose and maltose) was carried out according to the modified methods of Karkacier *et al.*[15]. Two g of sample

was ground into power in liquid nitrogen and 40 mL of methanol was added. The mixture was incubated on a magnetic stirrer at 65 °C for 0.5 h. It was centrifuged at 4 °C, 1.300 rpm for 40 min. The supernatant was transferred in clean tube and made up to 50 mL with methanol. Methanol was removed by rotary evaporator and the residue was dissolved in 25 mL double distilled water. The extract was passed through Sep-Pak C_{18} cartridge and 2.5 mL of filtrate was mixed with 7.5 mL acetonitrile. Then it was filtrated by 0.45 μm membrane filter and injected into high performance liquid chromatography (HPLC). The column was calibrated by fructose and maltose standards. Sugar contents were expressed as g/100 g.

Determination of chlorophyll and carotenoid content: Fresh plant leaves were homogenized with a pestle in a mortar. Chlorophyll and carotenoids were extracted from the leaves of plants both bacterium-infected and control groups with 80 % acetone and absorbance values were measured at 663 and 645 nm wavelengths for chlorophyll a and b and at 450 nm for carotenoids in a UV-160 Shimadzu spectrophotometer. The amount of chlorophyll a, b and total chlorophyll and carotenoids were calculated according to Witham *et al.*[16].

Statistical analysis: All data were expressed as means ± standard error of means $(\overline{X} \pm SE)$ of duplicates and was performed using SPSS software version 9.0 for Windows.

RESULTS AND DISCUSSION

In this present work, the levels of trace element (Mn), mineral (Mg), two plant hormones (IAA and ABA), sugars (maltose and fructose) and the contents of total chlorophyll and carotenoid in tomato seedlings after inoculation with *P. syringae* pv. *tomato* were examined. When virulent *Pst* DSM 60407 bacterium was inoculated into sensitive tomato seedling, the first typical disease symptoms such as, small diameter necrotic spots and chlorotic areas were seen within 3-4 days. The systemic symptoms including the large necrosis spread extensively within the leaves, foliar dark brown spotting and shoot apex death within 8-10 days post-inoculation were observed.

The contents of Mg and Mn in bacterium-infected and healthy tomato plant after the 1st, 2nd, 4th, 8th and 10th days from pathogen inoculation are given in Table-1. The manganese concentrations significantly decreased in bacterium-infected plants comparing to the uninfected tomato plants. On the other hands, Mg level was higher than the controls at the 1st day.

The levels of endogenous IAA and ABA in infected and healthy tomato plants after infected with virulent bacterium are shown in Table-2. Comparing with the non-infected plants, there was a significant decrease tendency in all the periods for endogenous IAA levels in the tomato plants infected with pathogen bacterium. Additionally, ABA levels in the bacterium-infected plants were lower than the healthy tomato plants, except for 2nd days.

The levels of fructose and maltose in uninfected and infected plants after the 1st, 2nd, 4th, 8th and 10th days from pathogen inoculation are shown in Table-3.

Fructose concentrations increased in the 2nd, 4th, 8th day after treatment with pathogen; however there was a decrease for tenth day. The maltose contents in the bacterium-injured tomato plants drastically decreased in the all periods comparing with the control plants as well as this reduction tendency prolonged in the subsequent study days.

TABLE-1
Mg AND Mn LEVELS IN THE INFECTED AND CONTROL TOMATO PLANTS

Groups	1 Day $\bar{X}$ ± SE	2 Day $\bar{X}$ ± SE	4 Day $\bar{X}$ ± SE	8 Day $\bar{X}$ ± SE	10 Day $\bar{X}$ ± SE
			Mg (µg/g)		
Control	416.82±19.07	415.77±22.19	412.61±20.97	418.42±18.25	414.57±20.31
Infected Plant	419.51±34.45	417.55±18.05	420.27±25.39	421.00±25.63	426.15±9.33
			Mn (µg/g)		
Control	66.41±34.52	63.44±39.34	64.98±30.26	51.41±27.96	57.32±32.52
Infected Plant	65.40±33.38	45.42±41.21	46.25±31.06	30.49±0.57	32.69±15.61

TABLE-2
PHYTOHORMONES (IAA AND ABA) LEVELS IN
THE INFECTED AND CONTROL TOMATO PLANTS

Groups	1 Day $\bar{X}$ ± SEM	2 Day $\bar{X}$ ± SE	4 Day $\bar{X}$ ± SE	8 Day $\bar{X}$ ± SE	10 Day $\bar{X}$ ± SE
			IAA (µg/g FW)		
Control	0.0050 ± 0.006	0.0050 ± 0.007	0.0076 ± 0.001	0.0079 ± 0.006	0.0098 ± 0.001
Infected Plant	0.0030 ± 0.003	0.0035 ± 0.001	0.0036± 0.001	0.0053± 0.004	0.0023 ± 0.003
			ABA (µg/g FW)		
Control	0.104 ± 0.020	0.102 ± 0.001	0.119 ± 0.016	0.088 ± 0.007	0.117 ± 0.002
Infected Plant	0.090 ± 0.001	0.135 ± 0.002	0.055 ± 0.006	0.061 ± 0.009	0.104 ± 0.024

TABLE-3
FRUCTOSE AND MALTOSE CONTENTS IN THE
INFECTED AND HEALTHY TOMATO PLANTS

Groups	1 Day $\bar{X}$ ± SE	2 Day $\bar{X}$ ± SE	4 Day $\bar{X}$ ± SE	8 Day $\bar{X}$ ± SE	10 Day $\bar{X}$ ± SE
			Fructose (mg/g FW)		
Control	2.16 ± 0.33	1.82 ± 0.29	0.81 ± 0.13	0.96 ± 0.11	2.03 ± 0.98
Infected Plant	1.70 ± 0.288	2.07 ± 0.31	1.04 ± 0.14	4.82 ± 0.94	0.46 ± 0.37
			Maltose (mg/g FW)		
Control	1.48 ± 0.04	0.82 ± 0.10	0.99 ± 0.02	1.16 ± 0.07	2.21 ± 0.02
Infected Plant	0.17 ± 0.02	0.21 ± 0.03	0.34 ± 0.01	0.43 ± 0.03	0.47 ± 0.04

The quantity of the total chlorophyll and carotenoid of bacterium-infected and uninfected tomato seedlings are presented in Table-4. The total chlorophyll contents reduced in the 4th, 8th and 10th day of the study in bacterium-infected tomato plants, whereas, the total carotenoid contents elevated at the same time periods for the infected tomato plants, comparing to the control plants. Moreover, there was an increase in the concentration of total chlorophyll and carotenoid for the 1st day.

TABLE-4
TOTAL CHLOROPHYLL AND CAROTENOIDS LEVELS
IN THE INFECTED AND HEALTHY TOMATO PLANTS

Groups	1 Day $\bar{X}$ ± SE	2 Day $\bar{X}$ ± SE	4 Day $\bar{X}$ ± SE	8 Day $\bar{X}$ ± SE	10 Day $\bar{X}$ ± SE
Total chlorophyll (mg/g)					
Control	4.48 ± 0.07	9.05 ± 0.22	7.08 ± 0.23	13.51 ± 0.19	14.56 ± 0.44
Infected Plant	6.34 ± 0.03	11.53 ± 0.27	4.77 ± 0.11	7.23 ± 0.24	7.67 ± 0.51
Total carotenoids (mg/g)					
Control	0.69 ± 0.01	1.26 ± 0.02	0.52 ± 0.01	0.83 ± 0.01	1.10 ± 0.06
Infected Plant	0.86 ± 0.07	0.96 ± 0.055	1.99 ± 0.013	1.85 ± 0.12	1.76 ± 0.20

Bacterial speck infection caused by *Pseudomonas syringae* pv. *tomato* is one of the most important diseases in tomato. The disease is characterized by foliar and fruit dark brown spotting with a yellow chlorotic halo and, consequently, results in economic loss of millions of dollars in many countries around the world where tomatoes are grown[1].

The results of the study revealed that there was an increase in Mg level in injured tomato plants when comparing with the control plants. It is suggested that this high Mg quantity might be existed to the degradation of chlorophyll molecule in consequence of pathogen attack. Moreover, this result was compatible to the reduction of the total chlorophyll contents and the increase of total carotenoid levels in the infected plants. The Mn concentrations significantly decreased in the bacterium-infected plants, comparing to the uninfected tomato plants. Manganese is essential micronutrient for higher plants both as a cofactor of several enzymes and as a regulation of photosynthesis[17]. The presence of Mn also assist the synthesis of chlorophyll and assimilation of nitrate and activates enzymes of fat biosynthesis.

Present findings indicated that the Mn contents were very lower in the tomato plants infected with virulent *Pst* DSM 60407 bacterium than uninfected tomato plants. It is suggested that there may be a close relationship between Mn deficient and disease. Additionally, it might be said that most of the Mn in the cytosol of the infected-tomato plants may be used for synthesis of Mn-bounded antioxidant enzymes to reduce high levels of H_2O_2 and other reactive oxygen species (ROS). Furthermore, the reduction the total chlorophyll contents and the increase in the total carotenoid in the infected plants might leads to typical disease symptoms such as, necrotic spots on leaves and chlorotic areas.

Abscisic acid as a plant hormones plays important role in developmental processes and the plant hormone involved in plant responses against a broad range of biotic and abiotic stresses[2,18]. It was reported that basal and high ABA levels appear to have a negative effect on disease resistance[11,19]. These investigations showed that plants exogenously ABA applications were more susceptible to pathogens and amounts of some disease resistance molecules such as phytoalexins, glyceolin, were greatly reduced[19]. Conversely, positive effects of ABA on pathogen defense were reported[18].

Cahill and Ward[20] determined that there was a decrease in ABA contents in soybeans after inoculation with *Phytophthora*. By contrast, viral disease of tobacco led to an increase in ABA levels[21]. However, the roles of endogenous plant hormones

such as abscisic acid (ABA) in plant response system are still unclear and there is no experimental study regarding to the endogenous IAA contents in plants inoculation with pathogens.

This work indicated that the levels of ABA and IAA in bacterium-infected tomato plants were lower than healthy control plants. Present findings were in agreement with the previous studies as cited above. Therefore, it is speculated that a tendency reduction in endogenous ABA levels in infected plants may be related to the induction of the plant defense pathways in host-tomato plants, such as salicyclic acid (SA) and jasmonic acid (JA)-dependent. It is also suggested that as a phytohormone, IAA had an effect like-ABA and the reduced-endogenous IAA levels can be trigged SA and JA/ET-dependent defense pathways. In summary, there was a coherent relationship between lower ABA and IAA levels and enhanced the expression of defense-related genes. We proposed that low ABA and IAA levels might be repressed the expression of photosynthetic genes due to an indirect interaction between sugar and plant hormones signaling pathways, responsible for the establishment of plant resistance.

Several considerable studies emphasized that sugars play a key role not only a response to abiotic stresses, but also participate in several defense strategies against biotic stress factors[4,19]. Induction of plant defense mechanisms against pathogen infections are usually accompanied by a rapid initiation of sink metabolism, possibly to satisfy the increased demand for carbohydrates as an energy source to sustain to cascade of coast-intensive direct defense responses[22]. It was suggested that the repression of photosynthesis in response to pathogens can be originated either the direct effect of the pathogen on the expression of photosynthetic genes or the indirect effect due to induction of sink metabolism mediated *via* sugar repression of photosynthetic genes[12]. Moreover, pathogen infection frequently leads to the development of chlorotic and necrotic spots and thus to a decrease in photosynthetic assimilate production.

Present findings showed that there was significant decrease in maltose contents for the days after inoculation with pathogen bacterium, compared to healthy plants. These results were parallel with Oladiran and Iwu[23] who determined the total soluble sugars of the infected tomato fruits with fungal pathogens during the storage periods. The lowest level of maltose in bacterium-injured tomato plants for the 1st day can be related to both the proliferation of the bacterium within host-plants and the manipulation plant carbohydrate metabolism for its own needs. The present study is in good agreement with the previous studies[1,12,22]. However, fructose contents elevated following inoculation with virulent bacterium, except for 1 and 10 days. The findings were also partial agreement with Berger *et al.*[24]. The results proposed that the activity of cell wall invertases may be induced after inoculation with virulent *Pst* DSM 60407 bacterium. The study also support that there was a relationship between carbohydrate metabolism of plant and the regulation of defense mechanisms against pathogenic bacterium attack.

As a result, it has become clear that Mn deficient in the injured tomato plants might be an important indicator against to the attempted bacterial infection. Furthermore, low IAA and ABA levels in infected plants may be enhanced the induction of

the plant defense pathways in host-tomato plants, such as salicylic acid and jasmonic acid-dependent. Hence, it seems that IAA, like ABA, acts as a negative regulator of plant defense. The reduced IAA and ABA levels also can affect sugar metabolism by decreasing photosynthetic assimilate in consequence of the repression of photosynthetic genes. The results confirmed that there were complex relationships between the trace element levels, soluble sugar and plant hormones in the regulation of defense mechanisms against microbial attacks. However, understanding the molecular basis of cross-interaction in plant defense pathways by providing more quantitative fundamental data will not sufficient. It is necessary to explore how plants are able to regulate the signalization among these molecules.

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