THE LEVELS OF NITRITE AND NITRATE, PROLINE AND PROTEIN PROFILES IN TOMATO PLANTS INFECTED WITH *PSEUDOMONAS SYRINGAE*

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Abstract

In this study, the contents of nitrite-nitrate and free L-proline, and pathogenesis-related (PR) proteins in tomato plants following inoculation with *Pseudomonas syringae* pv. *tomato* strain were examined. The results of the nitrite and nitrate indicated that there was a reduction in the levels of nitrate in the infected tomato plants through 1-8 study days, compared with the healthy plants. On the other hands, when the nitrite amounts increased in the first and second days, the nitrite concentrations reduced in infected plants at subsequent time periods, compared with uninfected plants. The accumulation of free proline increased in the infected plants, according to control plants. The whole-cell protein profiles displayed that the levels of the protein bands of molecular masses 204.6 kDa and 69.9 kDa significantly increased in infected and uninfected plants during 2-10 study days. In additionally, in the quantities of the protein bands of molecular weights 90.3 and 79.4 kDa were observed an increase in the infected and healthy plants after the fourth day. However, the protein band of molecular weight 54.3 kDa was visible only in uninfected plants for the fourth and eighth days.

Finally, the study suggest that there were the sophisticate relationships among the proline accumulation, the conversion of nitrate to nitrite and the induction of PR protein genes in the regulation of defense mechanisms toward microbial invaders. Our results also indicated that the increases in nitrite and proline contents might be useful indicator for the response toward pathogen attacks.

Introduction

Plants are continuously challenged against several biotic stress factors such as fungi, bacteria and viruses (Agrios, 2005; Berber *et al.*, 2010a, Berber *et al.*, 2010b, Ashfaq *et al.*, 2010). They are improved a number of defensive response strategy to overcome from attacks of the pathogens. In plant cells nitric oxide (NO) is produced by different enzymatic and non-enzymatic pathways from the conversion of nitrate to nitrite and plant metabolites (Yamasaki & Sakihama, 2000; Jones & Dangl, 2006). NO plays a vital role as an anti-stress agent toward attempts of the invaders (Cevahir *et al.*, 2007). Although NO and its functions related studies in plants were limited, there are some valuable investigations examining the role of NO related to plant defense mechanisms (Wang & Higgins, 2006; Modolo *et al.*, 2006). NO is an essential signal for the stimulation of the hypersensitivity response and the development of resistance against the pathogen attacks (Hancock *et al.*, 2002; Wang & Higgins, 2006; Asada *et al.*, 2011). Delledonne *et al.*, (2002) demonstrated that in soybean suspension cell infected with the bacterial pathogen *P. syringae*, NO and H_2O_2 synergistically induced hypersensitive cell death. NO also react with iron and iron-bounding proteins, thus forming ironnitrosyl complexes. In additionally, free L-proline accumulation in plants is a significant stress response against microbial pathogen attacks (Grote & Claussen, 2001; Fabro *et al.*, 2004; Claussen, 2005; Grote *et al.*, 2006; Arie *et al.*, 2007). Fabro *et al.*, (2004) determined that proline accumulated in leaf tissues treated with *P. syringae* pv. *tomato* avirulent strains, whereas unchanged in leaves infected with isogenic virulent bacteria.

Several studies reported that plants produced a class of proteins, known as pathogenesis-related (PR) proteins, to protection from the microbial invaders (Edreva, 2005; Desender *et al.*, 2007; Khallal, 2007; Popescu *et al.*, 2009; Wang *et al.*, 2010). Conventery & Dubery (2001) demonstrated that there was a relationship between the prevention of fungal infections and induction of defense pathways in tobacco plants by purified lipopolysaccharide (LPS) from the endophytic bacterium *Burkholderia cepacia*. Same workers suggested that the accumulation of high level PR proteins were determined following 4 days in non-treated than in treated leaves of plants challenged with LPS.

P. syringae pv. *tomato* induced by bacterial speck disease in tomato plants. The infection is characterized by plant leaf and fruit dark brown spotting with yellow necrosis. The disease causes by economic loss of millions of dollars in many countries around the world (Agrios, 2005; Berber *et al.*, 2010a). Many considerable investigations presented that during plant-pathogen interactions, different signal molecules such as, plant hormones, trace elements, carbohydrates, and PR proteins had significantly roles in the regulation of plant defense mechanisms, but there are still unknown (Miteva *et al.*, 2001; Zhao *et al.*, 2003; Robert-Seilaniantz *et al.*, 20007; Khallal, 2007; Wingler & Roitsch, 2008; Santner & Estelle, 2009; Berber *et al.*, 2010a, Sarwar *et al.*, 2011). The propose of the present study was to the accumulation of nitrite-nitrate and free L-proline, and PR proteins in tomato plants following inoculation with *P. syringae* pv. *tomato*.

Materials and Methods

Plant material: Tomato seeds (Falcon cultivar) were supplied 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 were rinsed four 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 plates on four sheets 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 carefully washed off the roots of seedling with distilled water and they were put into 30-cm diameters plastic pots (five plants per 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 about 25 days after seeds were germinated and had five to seven leaves including the new emerged leaf.

Bacterium culture condition and pathogen inoculation: The bacterium used in the study *P. syringae* pv. *tomato* DSM 60407 (*Pst* DSM 60407) was obtained by Deutsche Sammlung Von Mikroorganismen und Zellkulturen (DSMZ, Germany). The bacterial strain was cultivated in King's medium B broth for 24 h at 28°C on orbital shaker at 150 rpm. Then the culture of the bacterium was inoculated onto King's medium B agar plates and cultivated for 24 h at 28°C. The bacterium cells were harvested from the medium surface with sterile isotonic water (0.85% w/v) and washed 3 times. Subsequently, obtained cells were resuspended in sterile distilled isotonic solution and the viable cell number in the suspension was calculated as colony-forming units per milliliter (CFU/ml) and adjusted to 2×10^7 CFU/ml. Finally, plants were sprayed by the bacterium suspension by using a hand sprayer. After inoculation, the plants were hold onto a transparent nylon cover for 4-5 days to obtain a relative humidity of 100%. Control groups were not sprayed by the suspension of pathogen bacterium (Fig. 1A, B).

Fig. 1. Greenhouse experiments and the systemic symptoms of the infection: (A) control plants, (B) infected plants, (C and D) the necrosis and the shoot apex death.

Plant harvest and applying procedures: Five plants from infected and control groups were gathered at 5 various time periods (first, second, fourth, eighth, and tenth study days) following sprayed by pathogen bacterium. The fresh leaf samples were used for nitrite, nitrate, proline and PR proteins analysis.

Preparation of whole-cell proteins: Five grams of fresh leaf sample was ground into powder in liquid nitrogen and transferred into the eppendorf tube. After adding 25µl of sample buffer containing 0.06 M Tris-HCl, 2.5% glycerol, 0.5% SDS and 1.25% β-mercaptoethanol (pH 6.8), the cell were stirred, and the proteins were denatured in boiling water for 5 min. Then centrifugation for 5 min at 10.000g, the supernatant was place into the eppendorf tube and this supernatant was used electrophoresis.

SDS-PAGE technique: The total cell proteins were subjected to SDS-PAGE in gel slabs 1 mm thick (3.5 cm, 4% stacking and 15.5 cm, 12% resolving gels) as mentioned by Laemmli (1970). Electrophoresis was carried out with a discontinuous buffer system in a UVP vertical electrophoresis chamber (Cambridge, UK). The gel was run at 30 mA until the bromophenol blue marker had reached the bottom of the gel. The molecular weight of proteins was calculated on the basis comparison with the following standards (PageRuler[TM] Protein Ladder SDS-PAGE Standards, Fermentas, molecular weight range 10-200 kDa). After electrophoresis the gels were stained for 6 h in 0.01% (w/v) Coomassie Brilliant Blue R-250. Finally, the gels were destained in a methanol-acetic acid-water (3:1:6) mixture until protein bands became clearly visible.

Analysis of nitrate and nitrite: The extraction of nitrite and nitrate was performed by using some alteration to the procedure described by Stopes *et al*., (1998). Ten grams of leaf sample were homogenized by drying and grinding was mixed by 200ml hot distilled water. The extract was kept in a refrigerator for 12 h then filtered by way of a filter paper (Whatman No.1). Then the filtrate was used for nitrate and nitrite determinations.

Nitrate and nitrite determination: The nitrate amounts of the filtrates were detected by the phenoldisulphonic acid procedure as cited by Taras (1950). The content of the nitrite in the filtrates was determined by the diazotization method of the American Public Health Association (Anon., 1995).

Analysis of proline content: The proline content of leaf samples was determined by using the procedure described by Bates (1973). Measured values were referred to L-proline (Sigma) calibration curves. Six leaves were used for each measurement. Values were represented the average of at least three independent experiments.

All data were expressed as means ± standard error of means ($\overline{X}$ ± SE) of triplicate and was applied by using SPSS software version 9.0 for Windows.

Results

In this present study, the accumulation of nitrite-nitrate and free L-proline, and PR proteins in tomato plants after inoculation with *P. syringae* pv. *tomato* were examined. When virulent *Pst* DSM 60407 strain was inoculated into sensitive plants, the first typical disease symptoms such as, small diameter necrotic spots and chlorotic areas could be seen within 3-4 days. Then, we observed the systemic symptoms include the large necrosis spread extensively within the leaves, foliar dark brown spotting and shoot apex death within 8-10 days post-inoculation (Fig. 1C, D).

Nitrate and nitrite contents of uninfected and infected tomato plants after the inoculation with pathogen bacterium are shown in Table 1. There was a reduction at the nitrate concentrations of infected plants compared with the healthy plants, except for the tenth day. On the contrary, while the contents of nitrite were high in infected plants on the first and second days, the nitrite concentrations were low in infected plants at subsequent time periods, compared with healthy plants. The results obtained from the proline analysis of infected and uninfected tomato plants are given in Table 2. The levels of free proline increased in bacterium infected plants compared with the healthy control plants.

Table 1. The contents of nitrite and nitrate in infected and healthy tomato plants.

Groups	1. Day $\overline{X}$ ± SE	2. Day $\overline{X}$ ± SE	4. Day $\overline{X}$ ± SE	8. Day $\overline{X}$ ± SE	10. Day $\overline{X}$ ± SE
Nitrite (µg/g)					
Control	378,30 ± 2,33	329,00 ± 6,72	239,95 ± 1,65	208,30 ± 2,67	477,95 ± 2,05
Infected	488,30 ± 2,33	465,95 ± 5,16	189,95 ± 1,65	198,30 ± 2,10	266,60 ± 1,51
Nitrate (µg/g)					
Control	483,0 ± 0,005	484,5 ± 0,015	484,0 ± 0,015	486,5 ± 0,02	342,0 ± 0,02
Infected	348,0 ± 0,055	468,0 ± 0,030	465,5 ± 0,020	462,5 ± 0,315	484,5 ± 0,03

The SDS-PAGE of whole-cell protein profiles of infected and healthy control tomato plants following inoculation with virulent *Pst* DSM 60400 strain are shown in Fig. 2. The protein patterns of plants infected with pathogen bacterium were compared with healthy plants. In the Fig. 2 showed that the whole-cell protein profiles of infected and uninfected plants had 5 major protein bands. We also observed some characteristic changes in the concentration of the polypeptides. Such as, the amounts of 2 protein bands of molecular masses 204.6 kDa and 69.9 kDa (marked as 1 and 4, respectively) continuously increased in all the applications and the accumulation of these proteins peaked infected plants on the tenth day. In additionally, we observed an increase in the quantities of the protein bands of molecular weights 90.3 and 79.4 kDa (numbered as 2 and 3, respectively) in infected and uninfected plants after the fourth day of the experiment. The polypeptide in molecular weight 54.3 kDa, numbered as 5, was visible only in the healthy plants on the fourth and eighth days.

Table 2. The contents of free L-proline in infected and uninfected tomato plants (mg/g).

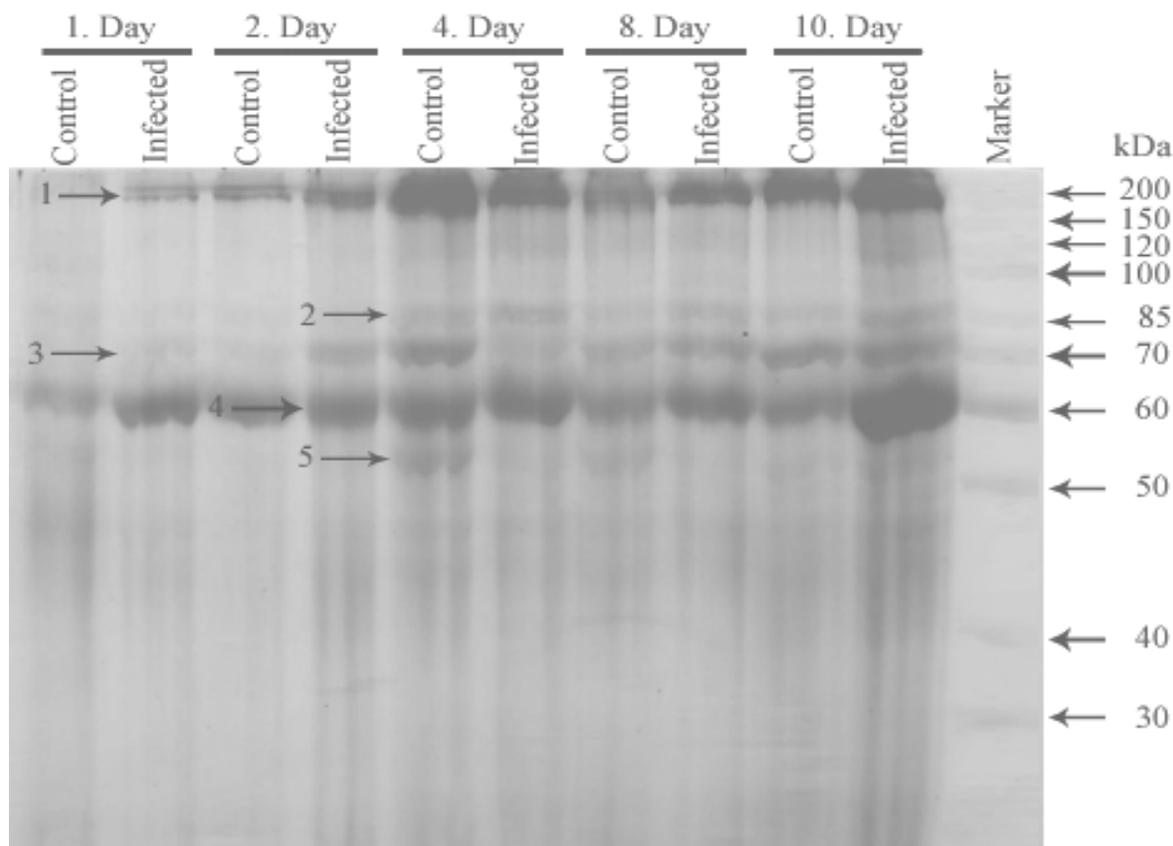| Groups	1. Day $\overline{X} \pm SE$	2. Day $\overline{X} \pm SE$	4. Day $\overline{X} \pm SE$	8. Day $\overline{X} \pm SE$	10. Day $\overline{X} \pm SE$
Control	$0,031 \pm 0,001$	$0,040 \pm 0,007$	$0,047 \pm 0,001$	$0,042 \pm 0,003$	$0,045 \pm 0,002$
Infected	$0,051 \pm 0,010$	$0,047 \pm 0,005$	$0,051 \pm 0,002$	$0,045 \pm 0,004$	$0,051 \pm 0,002$

Fig. 2. SDS-PAGE of whole-cell protein profiles in infected and control tomato plants.

Discussion

Recently years, a number of studies have been carried out the relationships between the host-plant and pathogen microorganism (Farhatullah *et al*., 2011). In this paper, we focused on the levels of some parameters related to plant response mechanisms, such as nitrite-nitrate, free L-proline and PR proteins.

NO in plants is synthesized either the reduction of NO_2^- by nitrate reductase or from chemical reactions involving nitrogen oxides and plant metabolites (Yamasaki *et al*., 1999; Neill *et al*., 2003). NO appear to be essential second messengers for the activation of defense-related genes and programmed cell death. In additionally, Delledonne *et al*., (1998) reported that NO increased in parallel with other reactive oxygen species in suspension culture soybean (*Glycine max*) cells infected with *P. syringae*. Our findings displayed that there was a reduction in the levels of nitrate in bacterium-infected tomato plants within 1-8 days, compared with the healthy

plants. On the other hands, when the nitrite amounts increased in the first and second days, the nitrite concentrations reduced in infected plants at subsequent time periods, compared to healthy plants. In plant cell NO is produced by different enzymatic pathways from the conversion of nitrate to nitrite (Yamasaki & Sakihama, 2000; Cevahir *et al*., 2007). Thus, we speculated that the nitrate levels might be reduced in infected plants to produce NO from the conversion of nitrate to nitrite. The data obtained from this investigation supported that there was a correlation between the increase nitrite contents and the stimulation of hypersensitivity response following the pathogen invasion.

It is well known that free L-proline accumulation in higher vascular plants is a significant stress response against a number of abiotic and biotic stress agents such as pathogens (Grote and Claussen, 2001; Fabro *et al*., 2004; Claussen, 2005; Grote *et al*., 2006). Besides, proline involves in scavenging of reactive oxygen species

and in regulating of the cytosolic acidity (Smirnoff and Cumbes, 1989; Fabro *et al.*, 2004). Grote *et al.*, (2006) reported that water uptake per plant and proline content of leaves represent useful tools to assess plant health status during growth. The findings gathered from the study indicated that free proline contents increased in bacterium infected tomato plants compared with the healthy plants, except for the first day. In this sense, our findings were good agreement with previous studies as mentioned above. We suggested that there was a linear relationship between the increase proline accumulation and the infection with bacterium in tomato plants.

PR proteins are known to be expressed in response to various external stimuli, including a variety of abiotic and biotic stress factors (McKenzie *et al.*, 2002; Ellis & Dodds, 2003; Chong *et al.*, 2005; Desender *et al.*, 2006; Khallal, 2007). The findings of SDS-PAGE showed that the levels of 2 protein bands of molecular masses 204.6 kDa and 69.9 kDa gradually increased in bacterium infected and healthy tomato plants throughout all the time periods, and their concentrations reached at the highest amounts on the tenth study days in infected plants (Fig. 2). In the quantities of the protein bands of molecular weights 90.3 and 79.4 kDa also were observed an increase in infected and healthy plants after the fourth study day. The protein band of molecular weight 54.3 kDa was visible only in healthy plants on the fourth and eighth days, but it was disappeared in infected and healthy plants at other time periods. The results indicated that the accumulation of 2 protein bands (marked as 1 and 4) increased in after the first study day of the experiment, however the highest accumulation of the protein bands were observed in the infected and the uninfected healthy plants. Our findings are in partially agreement with Conventery & Dubery (2001) who suggested that the accumulation of high level PR proteins were determined following 4 days in non-treated than in treated leaves of plants challenged with LPS. We suggested that these proteins may be related with pathogenesis. In briefly, the findings of SDS-PAGE revealed that several proteins involved in plant defense response, and the gene expression levels of the proteins either enhanced or reduced in different time periods.

In conclusion, it is worth noting that high nitrite levels in infected plants during the first and second days may be trigged the stimulation of the defense pathways in host-tomato plants, such as hypersensitivity response and systemic acquired resistance. In additionally, our results indicated that there were the sophisticate relationships among the proline accumulation, the conversion of nitrate to nitrite and the induction of PR protein genes in the regulation of defense mechanisms toward microbial invaders. However, to understanding further signaling network processes related to the disease resistance and plant defense mechanisms will be necessary to supply more quantitative data focusing cross-interactions between these parameters.

Acknowledgments

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