Baysal, Ö.[1*], Gürsoy, Y.Z.[2], Örnek, H.[2], Duru, A.[2], Soylu, S.[3], Soylu, M.[3], Zeller, W.[4]
[1]West Medit. Agricult. Research Inst. (BATEM), P.B. 35, 07100 Antalya Turkey, [*] E-mail: obaysal@ttnet.net.tr
[2]Bornova Plant Protection Research Institute (BZMAE) İzmir, Turkey
[3]Mustafa Kemal University Faculty of Agriculture Plant Pathology, Department Hatay, Turkey
[4]Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für biologischen Pflanzenschutz, Heinrichstraße 243, 64287 Darmstadt, Germany

Induction of oxidative burst in tomato leaves treated with unsaturated fatty acids of turtle oil (*Caretta caretta*) against *Pseudomonas syringae* pv. *tomato*

Abstract

Tomato bacterial speck disease caused by the bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) is effectively controlled on tomato (*Lycopersicon esculentum* Mill.) by foliar spray of unsaturated fatty acids. Effecting induced resistance by different unsaturated fatty acids (UFAs) was investigated with three-week old plants. The plants were inoculated with 10^8 cfu/ml bacterial suspension and bacterial growth was evaluated 48 h post inoculation. Resistance induced by UFAs showed a suppressive effect against *Pst*, and was correlated with rapid resistance response of treated leaves challenged with pathogens by day 1 after treatment. The bacterial multiplication significantly decreased in decosohexaenoic acid and eicosapentaenoic acid treated plants by 2 days post inoculation. In order to understand the cause of the suppressive effect of UFAs, H_2O_2 generation and NADPH oxidase activity were investigated in the early phase of inoculation. In these studies, the plants, sprayed with linoleic acid, decosohexaenoic acid and eicosapentaenoic acid, accumulated H_2O_2 and had elevated activity of NADP(H) oxidase. However, oleic acid failed to show higher NADP(H) oxidase activity, while it increased by linoleic acid, decosohexaenoic acid and eicosapentaenoic acid treatment. These findings indicate the lack of suppressive effect of exogenous oleic acid application in plant defence pathways. No considerable changes were observed in water-treated plants. The findings suggest that linoleic acid, decosohexaenoic acid and eicosapentaenoic acid activate plant defence mechanisms, and the treatments with UFAs lead to induction of active oxygen species, acting as mediators of plant immunity against the bacterial pathogen.

Introduction

Pst is the causal agent of bacterial speck disease in tomato. This disease is of moderate economic importance to tomato production under greenhouse or field conditions and is disseminated primarily by water. It has the potential to rapidly move through plug greenhouses; thus, infested seedlings could become an important inoculum source for field epiphytes. The frequent use of copper compounds leads to resistant bacterial strains making the control of the disease more difficult. In addition, copper contributes to environmental and water pollution. Therefore alternative and efficient control methods should be improved, particularly in greenhouse crops in southern Turkey.

Recent studies on defence signalling pathways revealed that induced plant defence against microbial pathogens is regulated by a network of interacting signalling pathways in which plant signal molecules, such as salicylic acid, jasmonic acid (JA), and ethylene, play a dominant role (Feys and Parker 2000). However, fatty acids and oligosaccharides (Kobayashi et al. 1993; Sticher et al. 1997) are also known to be effective inducers of plant resistance to various diseases.

Plants have developed a complex protection system to cope with pathogen attack. Active oxygen species (AOS) and enzymatic systems govern their metabolism. H_2O_2 plays an important role in pathogenesis (Baker et al. 1995). Rapid generation of AOS is considered to be an important component of the resistance response of plants to pathogen challenge. AOS intermediates can serve as direct protective agents by their toxicity or by their ability to prevent pathogen ingress by enhancing the cross-linking of the cell wall (Baker and Orlandi 1995). A burst of AOS is known to be involved in local cell death as part of plant defence against pathogens. The hypersensitive response is characterised by the rapid production of active oxygen species referred to as the oxidative burst, which prevents further spread of

the pathogen, and by programmed cell death. NADPH oxidase, which catalyses the generation of O_2^-, is responsible for the oxidative burst (Slusarenko 1996).

In recent years, a new approach using oils for controlling destructive plant pathogens has been developed and an emulsion of fish oil stimulated the host defence against some bacterial diseases (Abbasi et al. 2003). In other studies fatty acids were suggested as signal molecules involved in activation of defence responses in plants and its relation with other signalling compounds such as jasmonic acid were described (Itoh et al. 2002). On the other hand, sea turtle oil is well known among Mediterranean fishermen as an effective cure for wounds and our previous study indicated that turtle oil treatment resulted in lower bacterial growth, related to the increase of LPOX and peroxidase activity in tomato against *Clavibacter michiganensis* ssp. *michiganensis*. UFA ingredients of turtle oil were investigated with GC analysis, and docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA) and oleic acid (OA) were also determined (Baysal et al. 2005). In higher plants, lipoxygenase reacts with either linoleic acid or linolenic acid and forms hydroperoxides. These products are involved in plant defence against pathogens and insects (Rusterucci et al. 1999), wound response, senescence, and development (Hildebrand 1989).

Up to now there is no information regarding the role of active oxygen species in plants treated with UFA components of turtle oil against bacterial pathogens (Pst). In addition to our previous study, in this paper H_2O_2 generation and changes in NADPH oxidase activity were followed in treated plants, compared to uninoculated and Pst inoculated plants.

Material and methods

Plant material: Greenhouse-grown ten-week-old tomato seedlings (Ikram) with four fully expanded leaves were used for all experiments. Plants were grown in pots in a soil mix containing sand, perlite, and peat compost, in the greenhouse at 25 ± 5°C with 68 - 80% RH. The soil mix also contained a slow-release fertilizer (14-12-14, N-P-K). Natural light was supplemented by a single 1000-watt sodium vapour lamp during a 16 h photoperiod.

Bacterial strain and inoculation: The two youngest leaves of the seedlings were sprayed and inoculated with a *Pst* suspension of 10^8 cfu/ml as described by Scarponi et al. (2001). Before 24 h inoculation the two reciprocal plant leaves were uniformly sprayed with an aqueous solution of turtle oil and five unsaturated fatty acids. Turtle oil (1 ml turtle oil dissolved in chloroform at 4:1v/v) was diluted in 100 ml of water and sprayed (ca. 200 µl turtle oil per leaf). Control plants were sprayed with the solution water/chloroform (4:1 v/v, ca. 200 µl per leaf). The seedlings were covered with plastic bags after the treatments. The treated leaves were inoculated with the bacterial suspension according to Scarponi et al. (2001) one day after treatments.

Effect of turtle oil and UFAs on bacterial growth in planta: Bacterial colony forming units (cfu) were recovered from *Pst*-inoculated tissues, treated with either turtle oil or water 24 h before inoculation, by removing 5 mm-diameter leaf discs aseptically from the region of inoculation. Excised discs were homogenized in 1 ml of sterile 0.06 % NaCl solution, diluted serially in 10-fold dilutions. Aliquots of alternate dilutions were plated on NYA agar plates, containing appropriate antibiotics. Plates were incubated at 26°C for 48 h, and emerging colonies were counted on all dilution plates showing bacterial growth. Young leaves were sampled from seedlings for physiological assays.

Physiological studies: The first group consisted of uninoculated plants and seedlings were sprayed with four unsaturated fatty acids: docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA) and oleic acid (OA), obtained from Sigma-Aldrich chemicals. 5 mg/ml fatty acid were sonicated in 100 ml of distilled water and sprayed on plants. In a second group, the plants were treated with fatty acids or water and inoculated with the pathogens 24 h post treatment. From inoculated leaves, tissues were taken at the actual site of inoculation with Pst. From control plants, tissues were taken from sites similar to inoculated leaves. Preparation of samples for the physiological assays and samples for enzyme extraction from all treatments (inoculated or not) were separately taken 4, 8, 12, 24, 48 and 72 h after treatment. Thereafter, the supernatant (crude enzyme extract) was collected into 1.5 ml portions. Protein concentrations were determined by the method of Bradford (1976) using BSA as standard. The extract

was obtained from two different lots of leaf samples (1 g fresh weight each) for each treatment. All assays were spectrophotometrically performed at 25°C.

Assay of hydrogen peroxide concentration: The concentration of H_2O_2 in the leaves was determined according to a modified method of Baker et al. (1995). Leaves were ground in 5% TCA (2.5 ml per 0.5 g leaf tissue) with 50 mg active charcoal at 0°C and centrifuged for 10 min at 15000g. Supernatant was collected, neutralised with 4 N KOH to pH 3.6 and used for H_2O_2 assay. The reaction mixture contained 200 µl of leaf extract, 100 µl of 3.4 mM 3-methylbenzothiazoline hydrazone. The reaction was initiated by adding 500 µl of horseradish peroxidase solution (90 U 100 ml^{-1}) in 0.2 M sodium acetate (pH 3.6). Two minutes later 1400 µl of 1 N HCl was added. The extinction at 630 nm was read after 15 min.

NADP(H) oxidase assay: NADPH oxidase activity in plants was evaluated by measuring the superoxide dismutase-inhibited reduction of NBT (van Gestelen et al. 1997). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, and 0.1 mM NBT. After 5 min pre-incubation at 25 °C, the reaction was initiated by the addition of 0.1 mM NADPH. The reduction of NBT was measured as the change of extinction at 560 nm. The activity was expressed as nmol min^{-1} mg^{-1} protein using extinction coefficient of 12.8 $mM^{-1}cm^{-1}$.

Results and discussion

Turtle oil and its UFAs induced substantial levels of oxidative burst and enhanced disease resistance in tomato seedlings. Interestingly, this effect of turtle oil and its UFAs have not been shown in tomato plants against any plant pathogens, except for our previous studies (Baysal et al. 2005). The oxidative burst in plants was induced against *Pst* when turtle oil and its UFAs were applied 1 day before inoculation. In the present study, turtle oil and fatty acids induced substantial levels of resistance in tomato plants against *Pst*, and suppressed bacterial growth *in planta* (Figure 1). Significant changes were found in the enzymatic activities related to oxidative burst in plants expressing induced resistance following treatment with turtle oil and UFAs. The signal released by the pathogen may play an important role in activation of resistance if unsaturated fatty acids are applied as an inducer. A remarkable increase of H_2O_2 occurred in inoculated UFA-treated plants, compared to inoculated water-treated plants, which can be compared with the findings of Milosevic and Slusarenko (1996) on disease resistance reactions. The results indicate that the activation of resistance is stronger if plants are challenged with a pathogen (Figures 2, 3). AOS, produced via an oxidative burst, are under the control of enzymes such as NADPH oxidase and peroxidases (POXs) (Wojtaszek 1997). The here obtained results show that *Pst* are sensitive to H_2O_2 and they apparently confirm the role of hydrogen peroxide in inhibition of bacterial multiplication in plant tissue. The higher level of hydrogen peroxide generation in tomato leaves (Figure 3) of UFA treated plants is likely to be much more harmful to the bacteria than to the plant tissues; they restrict pathogen multiplication. Therefore the increase of peroxidase activity, which was shown in our previous study (Baysal et al. 2005) on tomato plants, treated with turtle oil, and NADPH oxidase activity (Figure 2) indicate that these activities are involved in the regulation of the level of H_2O_2 in plant tissues (Wojtaszek 1997). Peroxidases can decompose and produce hydrogen peroxide. The locally accumulated H_2O_2 may directly damage the bacteria (Brown et al. 1998). In our study, the increase in H_2O_2 concentration was found to be significantly higher in the leaves of UFA-treated plants and showed a close relationship with the induction of resistance in UFA-treated plants. Therefore, the lower number of bacteria on UFA-treated plants can be assumed to result from the effect of UFA on tomato plants. However, further molecular studies are also necessary and are carried out in our lab.

In conclusion, the present study indicates that the linoleic acids EPA and DHA are able to trigger active oxygen species that can act as mediators of plant immunity, thus new non-pesticidal plant protection strategies could be developed. Therefore, the protective effect of the linoleic acids, EPA, and DHA could be used as means of controlling the severity of bacterial speck on tomato.

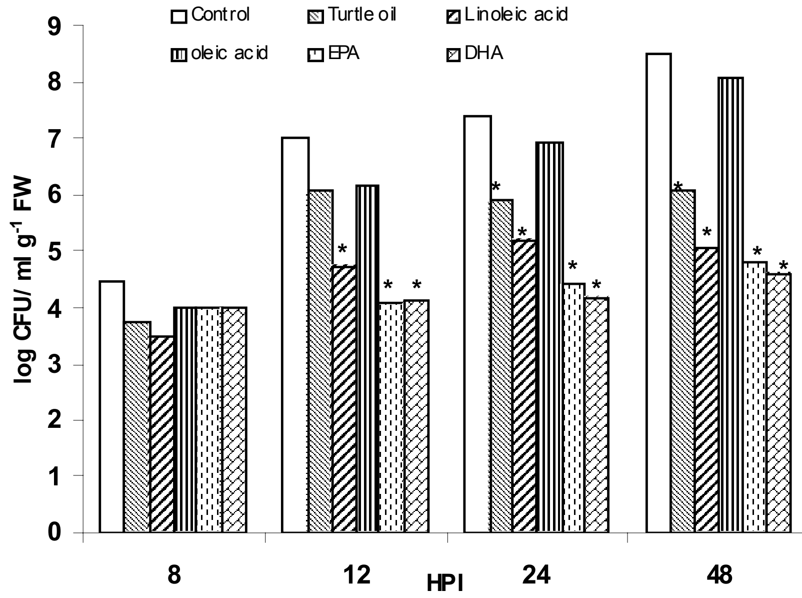

Fig. 1 The effect of UFAs on bacterial growth of *Pst*. One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst*. The asterisks on bars represent significantly different values, compared to the control (P < 0.05).

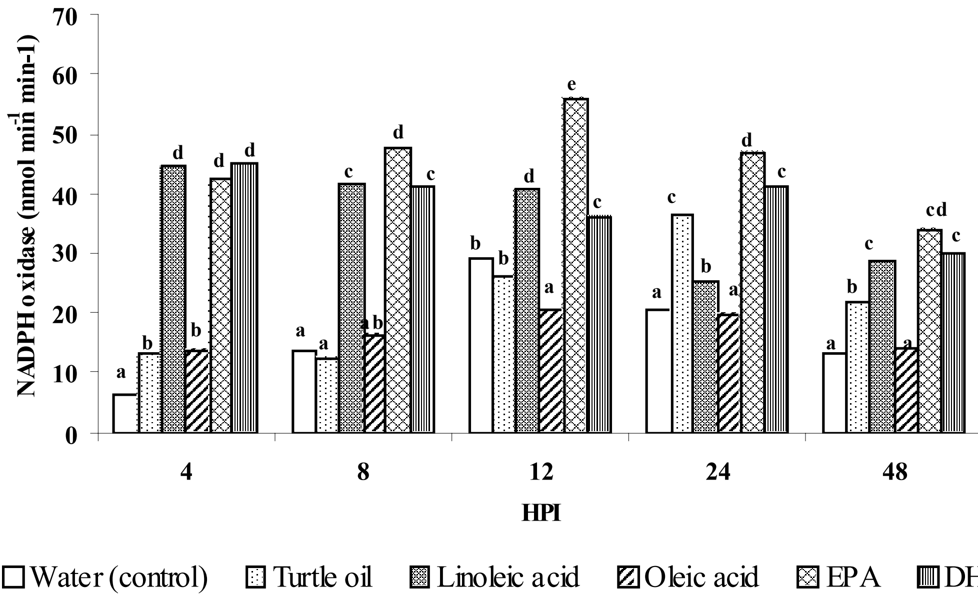

Fig. 2 The effect of UFAs on NADP(H) oxidase. One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst*. Data are mean values of three leaf discs.

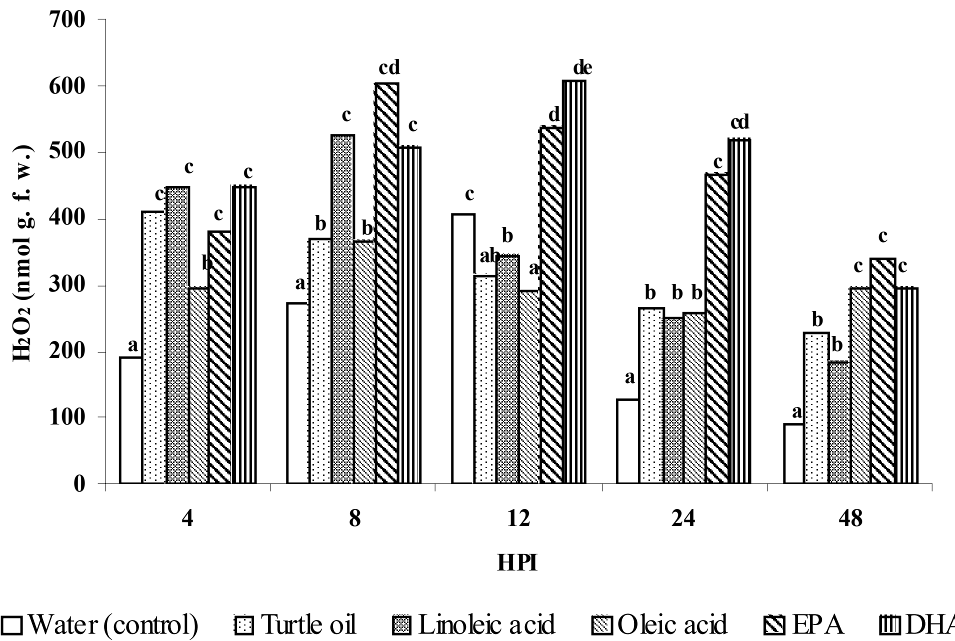

Fig. 3 The effect of UFAs on H_2O_2 generation. One day after treatment with UFAs or water, the tomato seedlings were inoculated with *Pst*. Data are mean values of three leaf discs. Inhibitory effect of BABA treatment on bacterial growth was significantly different after inoculation according to Student's two-sample *t*-test ($P < 0.05$)

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