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Protection of tomato plants against fusaric acid by resistance induction

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Abstract
Objectives: The rhizobacteria *Bacillus sphaericus* B43, *Pseudomonas fluorescens* T58, and *P. putida* 53 are able to induce systemic resistance (ISR) against *Fusarium oxysporum* f.sp. *lycopersici* in tomato. This study investigated if ISR reduced damage by the toxin Fusaric Acid (FA) that is produced by *F. oxysporum*.
Methodology and Results: Bacteria were applied to the rhizosphere of tomato plants. Chlorophyll content and ion leakage were determined after placing leaf discs in FA. Active oxygen species (AOS), superoxide and hydrogen peroxide levels were determined in leaves of plants injected with FA. Activities of superoxide dismutase, ascorbate and guaiacol peroxidases involved in AOS metabolism were quantified. In untreated plants, FA led to high ion leakage and chlorophyll degradation caused by H_2O_2 accumulation. All bacteria treatments reduced chlorophyll degradation. Ion leakage was reduced by treatment with *P. fluorescens* T58 and *B. sphaericus* B43, while *P. putida* 53 was less effective. Treatment of plants with bacteria resulted in increased superoxide contents, but varying over time. Increased SOD and GPX activities in untreated plants were suppressed after bacteria treatment. Plants treated with *P. fluorescens* T58 showed only a transient increase in superoxide. *P. putida* 53-treated plants removed AOS, but high initial superoxide levels led to membrane damages. Treatment with *B. sphaericus* B43 suppressed effects of FA, but AOS metabolism showed only slight alterations.
Conclusions and potential applications of findings: The results show that ISR could also protect plant tissues from damage by pathogen toxins, which is a potential new dimension to the known mechanisms of action of biocontrol agents.

Key words: Active oxygen species, *Fusarium*, induced systemic resistance, rhizobacteria, tomato, Fusaric Acid.

Introduction
Many plant pathogens produce toxins which are non-enzymatic metabolites that damage plants at low concentrations (Kuzniak et al. 1999; Markham and Hille, 2001). Fusaric acid (FA) is produced by many fungi in the genera *Fusarium* and *Gibberella*. FA has been widely implicated in the pathogenesis of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Kuzniak et al., 1999). The toxin acts in conjunction with other substances that include dehydrofusaric acid, ethylene, lycomarasmin, vasinfuscarin, ethyl alcohol and succinic acid (Pegg, 1989; Sutherland and Pegg, 1992). Each of these compounds

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can contribute to *Fusarium* wilt symptoms in one or more ways.

The evidence for involvement of FA in disease is two-fold. FA has been detected in tomato infected with *Fusarium*, and in much higher concentrations in plants infected with virulent strains than in those infected with avirulent strains (Toyoda et al., 1988). Further, in many *Fusarium*-infected plants the spread and pattern of shoot symptoms such as acropetal foliar flaccidity, chlorosis, the very rapid development of interveinal necrosis and foliar desiccation appear to implicate toxin activity (Sutherland and Pegg, 1992). *Fusarium* toxins can indirectly promote disease by predisposing the host to injury by other fungal products that are produced simultaneously or in close sequence with the toxins (Davis, 1969; Kuzniak et al. 1999).

The most damaging effect of FA occurs through triggering of an increase in active oxygen species (AOS) in infected tissues. AOS, which result from the reduction of molecular oxygen, cause lipid peroxidation leading to increased membrane permeability and subsequent solute loss (Dhindsa et al., 1981; Remotti and Löffler, 1996; Kuzniak et al., 1999). Damage to cell membranes eventually leads to chlorosis. Although AOS are mostly detrimental to plant cells, they also play a role in plant defence. AOS have been implicated in induction of phytoalexin biosynthesis and H_2O_2 is utilised as a substrate by peroxidases in lignification (Low and Merida, 1996; Mehdy et al., 1996; Milosevic and Slusarenko, 1996). AOS, especially H_2O_2, can significantly retard microbial growth (Peng and Kuć, 1992). AOS are involved in the hypersensitive response (Doke 1983), and they also mediate protection of plants against oxidative stress (Low and Merida, 1996).

To avoid damage by FA, infected plants can inhibit synthesis of the acid by the pathogen, or accelerate its degradation or conversion into less toxic compounds (Davis, 1969). Plants could also respond by activating measures to reduce damage due to AOS. H_2O_2 is removed by catalase or peroxidase while O_2^- radicals are removed by superoxide dismutase (Dhindsa et al., 1981; Adam et al., 1995; Kuzniak et al., 1999). Whatever the strategy, plant resistance to *Fusarium* toxins has been used as an indicator of resistance to *Fusarium* in tomato (Shahin and Spivey, 1986), and several other plants (Matsumoto et al., 1994; Remotti and Löffler, 1996).

This study was based on the hypothesis that as a mechanism in biocontrol, ISR mediated by rhizobacteria may partly function by reducing the damage caused by *Fusarium* toxins in plants. The objectives were to investigate (1) the extent of chlorophyll degradation, (2) the intensity of ion leakage, (3) the content of active oxygen species (H_2O_2, O_2^-) and (4) the activity of enzymes regulating AOS metabolism, after exposing rhizobacteria-treated plants expressing ISR to Fusaric acid.

MATERIALS AND METHODS

Plant material: Tomato cv. Rheinlands Ruhm seeds were sown in a low-nutrient Klasmann seedling substrate (Klasmann-Deilmann, Gross-Hesepe, Germany) which was maintained constantly moist in the greenhouse at 23 ± 5°C until germination. Seedlings were transplanted into Klasmann organic growth substrate after 2 to 3 weeks.

Bacteria application: Three rhizobacteria were used in this study. *Pseudomonas fluorescens* T58 was isolated from sugarbeet in Germany (Oostendorp and Sikora, 1989), *Pseudomonas putida* 53 from tomato in Thailand (Terhardt, 1998) and *Bacillus sphaericus* B43 from potato in Germany (Racke and Sikora, 1992). The three bacteria were selected after a previous study in which they were determined to be inducers of systemic resistance towards *Fusarium* wilt in tomato (Mwangi et al., 2002). Bacteria cultures were grown in Tryptone Soya Broth (TSB) for 4 days at 23 ± 2°C while shaking at 100 rpm. The cultures were centrifuged at 5000 x g for 20 min at 6°C and the pelleted cells re-suspended in 0.1 M $MgSO_4$. For each isolate the cell concentration in the solution was adjusted to OD_{560} = 2, representing about 5 x 10^7 cfu ml^{-1} for *P. fluorescens* T58, 2 x 10^8 cfu ml^{-1} for *P. putida* 5, and 2 x 10^8 cfu ml^{-1} for *B. sphaericus* B43. Bacteria were applied by drenching 5 ml of the cell suspension onto the soil surface near each plant four weeks after

transplanting. Bacteria treatment was repeated after three days. Plants were kept in a climatic chamber at 23 ± 2°C and 12/12 h light / darkness cycle.

Effect of Fusaric acid on chlorophyll degradation: The method described by Barna et al. (1985) was used. Three days after the first bacteria application leaf disks of 10 mm diameter were cut from the third oldest leaf (counting from below). Disks from five different plants were mixed to obtain a homogenous sample. The leaf disks were floated on 10 ml water containing 1 mM synthetic Fusaric acid (Sigma, Irvine, UK) in plastic Petri dishes with the lower leaf side facing upwards to allow gas exchange through stomates. The control consisted of leaf disks from untreated plants that were floated on water with FA or on water without FA, but adjusted to the same pH (4.2) as the water with FA using HCl. Leaf discs were incubated at 23±2°C in daylight. After 24, 43 and 67 h six leaf disks were removed from the Petri plates and their chlorophyll content determined. The leaf disks were crushed in 5 ml methanol, and this mixture was centrifuged for 10 min at 5000 x g. The absorbance of the supernatant was determined at 652 nm. Chlorophyll content was calculated as $OD_{652}/36$ = mg ml^{-1} chlorophyll, and then calculated into chlorophyll content per leaf area (MacKinney, 1941).

Effect of Fusaric acid on ion leakage: The method described by Barna et al. (1983) was used. 12 h after the second bacterial treatment leaf disks were cut exactly as described above. Five leaf disks were immersed in 15 ml solution containing 1 mM synthetic fusaric acid in cylinder glass tubes of 3 cm in diameter and 8 cm in height. Three replicate tubes were used for each treatment. The glass tubes with FA solutions were kept uncovered on a laboratory bench at 23 ± 2°C in daylight. Conductivity of the FA solutions was assessed after 12, 24, 43, 56 and 67 h using an LF 539 conductivity meter equipped with a Tetracon 96 sensor (WTV, Weilheim, Germany). Data were recorded as change in conductivity in µS cm^{-1} using the initial conductivity (checked at the beginning) as the reference point. Leaf disks from untreated plants dipped in water adjusted to the same pH (4.2) as described above were used as a control.

The role of AOS in resistance against fusaric acid: After the second bacteria application 0.5 ml of 1 mM synthetic fusaric acid was injected into the stem of each plant between the 1st and 2nd leaf (counting from below) using a Sterican hypodermic needle (0.8 x 40 mm; Braun, Germany) attached to a 5 ml syringe (Kuzniak et al. 1999). Untreated plants were injected with water or FA. At 6, 9, 29, 48 and 56 h after injection leaf disks were sampled exactly as described above. Some of the leaf disks were used fresh while others for protein extraction were frozen in liquid nitrogen and stored at –20°C for later use.

Superoxide anion (O_2^-) content:The nitro blue tetrazolium (NBT) reduction test (Doke, 1983) was used. Seven fresh leaf disks were immersed in 3 ml solution containing 10 mM potassium phosphate buffer (pH 7.8), 0.05 % NBT and 10 mM NaN_3. After 1 h reaction time the mixture was placed in a water bath at 85°C for 15 min, and then cooled rapidly. The optical density of the reaction solution was checked at 580 nm. NBT reducing activity was expressed as the mean absorbance of three samples for each sampling time.

H_2O_2 content: H_2O_2 production was assayed using the method described by von Tiedemann (1997). Ten fresh leaf disks were kept for 2 h in the dark in 2 ml solution containing 50 mM Phosphate buffer (pH 7), 0.05 % guaiacol and 12.3 units ml^{-1} peroxidase (Sigma). After 2 h the absorbance of the reagent mixture was checked at 450 nm. Results were expressed as 'mean optical density x 1000' and compared statistically between treatments.

Activity of enzymes involved in AOS metabolism: Enzymes were extracted from the leaf disks previously frozen in liquid nitrogen as described by Kuzniak et al. (1999). Eight leaf disks were weighed and homogenised in 3 ml solution containing 50 mM potassium phosphate buffer (pH 7), 1 M NaCl, 1 % polyvinylpyrolidone and 1 mM EDTA. For assay of ascorbate peroxidase extraction was done with the same mixture, but additionally containing 1 mM sodium ascorbate. After extraction the homogenate was centrifuged for 20 min at 26,000 x g at 4°C (Avanti J25, Beckman, US). The supernatant ("plant extract") was used for determination of enzyme activity as described below.

Superoxide dismutase (SOD) activity: The method of Dhindsa et al. (1981) was used with some modification to measure the inhibition of the photochemical reduction of NBT. A reaction mixture of 3 ml was used containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM NBT, 0.1 mM EDTA, 50 µl enzyme extract and 2 µM riboflavin added last.

The tubes with the reaction mixture were shaken and placed 20 cm below an 18 W fluorescent tube. Reaction was started by switching on the lamps and stopped after 10 min by removing the mixture from under the light. The optical density of the reaction solution was checked at 560 nm. A non-irradiated reaction mixture did not develop colour and served as reference. The reaction mixture lacking enzyme developed maximum colour whereas normal daylight did not have a measurable effect on the reaction. SOD activity which is known to be linear and indirectly proportional to the absorbance of the reaction solution was calculated as 1/OD and expressed as units min^{-1} g^{-1} fresh weight.

Catalase activity: The initial rate of H_2O_2 consumption at 25°C was measured at 240 nm according to Dhindsa et al. (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 25 µl plant extract and 15 mM H_2O_2 (added last) in 1.5 ml. The solution was then mixed thoroughly and absorbance determined after 15 seconds at 240 nm. Catalase activity units were calculated as 1/OD s^{-1} g^{-1} fresh weight.

Ascorbate peroxidase activity (APX): The method of Nakano and Asada (1981), which measures the oxidation of ascorbate to dehydroascorbate at 265 nm, was used. 2 ml assay mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM sodium ascorbate, 25 µl plant extract and 25 µM H_2O_2 (added last to start the reaction) was used. The absorbance of the reaction mixture was checked at 265 nm after 30 seconds reaction time. APX activity units were calculated as a change of 0.001 units of absorbance min^{-1} g^{-1} fresh weight.

Guaiacol peroxidase activity (GPX): GPX was assayed according to Kuzniak et al. (1999) with modification. A mixture containing 50 mM sodium acetate (pH 5.6), 5 mM guaiacol, 15 mM H_2O_2 and 25 µl plant extract in 3 ml was used. The reaction mixture was placed in a waterbath at 30°C and the linear increase in absorbance checked at 470 nm over 6 min.

Data analysis: Data were analysed by One Way ANOVA using Statgraphics Plus 3.1 software (Manugistics Inc., Rockville, USA) and means separated using Fisher's LSD procedure at $P \leq 0.05$.

RESULTS

Chlorophyll content: In the leaf disks that were sampled from untreated plants and floated on solution without FA, chlorophyll content remained fairly constant after an initial decline in the first 24 h (Fig.1A). When leaf disks from non-induced plants were floated on FA chlorophyll loss started immediately and continued steadily at a significantly faster rate when compared to loss in leves of induced plants. In the leaf disks from plants treated with *P. fluorescens* T58 chlorophyll loss started about 40 h after placing them on FA solution. Leaf disks from plants treated with *B. sphaericus* B43 had increased chlorophyll contents at 24 h after treatment, which subsequently decreased to reach a level comparable to that of the leaf discs from untreated plants floated on in water. In the leaf disks from plants treated with *P. putida* 53 chlorophyll loss started about 24 h after placing disks on FA solutions. Generally, chlorophyll loss in leaf disks from bacteria-treated plants occurred at a slower rate and chlorophyll contents were higher than in the leaf disks from non-induced plants exposed to FA

Ion leakage: Ion leakage from leaf disks was assessed by measuring increase in conductivity of the FA solutions. When leaf disks from non-induced plants were floated on water, there was only a small change in conductivity, reaching a maximum of about 40 µS cm^{-1} after 90 h (Fig. 1B). Conductivity of the FA solution with leaf disks from untreated plants increased significantly to stabilise at 160 µS cm^{-1} after about 70 h. Conductivity of the FA solution with leaf disks from plants treated with *P. fluorescens* T58 was significantly lower in the first 60 – 70 h when compared to that of untreated plants. Treatment of plants with *P. putida* 53, however, did not reduce the extent of ion leakage (Fig. 1B).Treating plants with *B. sphaericus* B43 reduced also rduced ion leakage due to FA, but to a lesser extent than *P. fluorescens* T58.

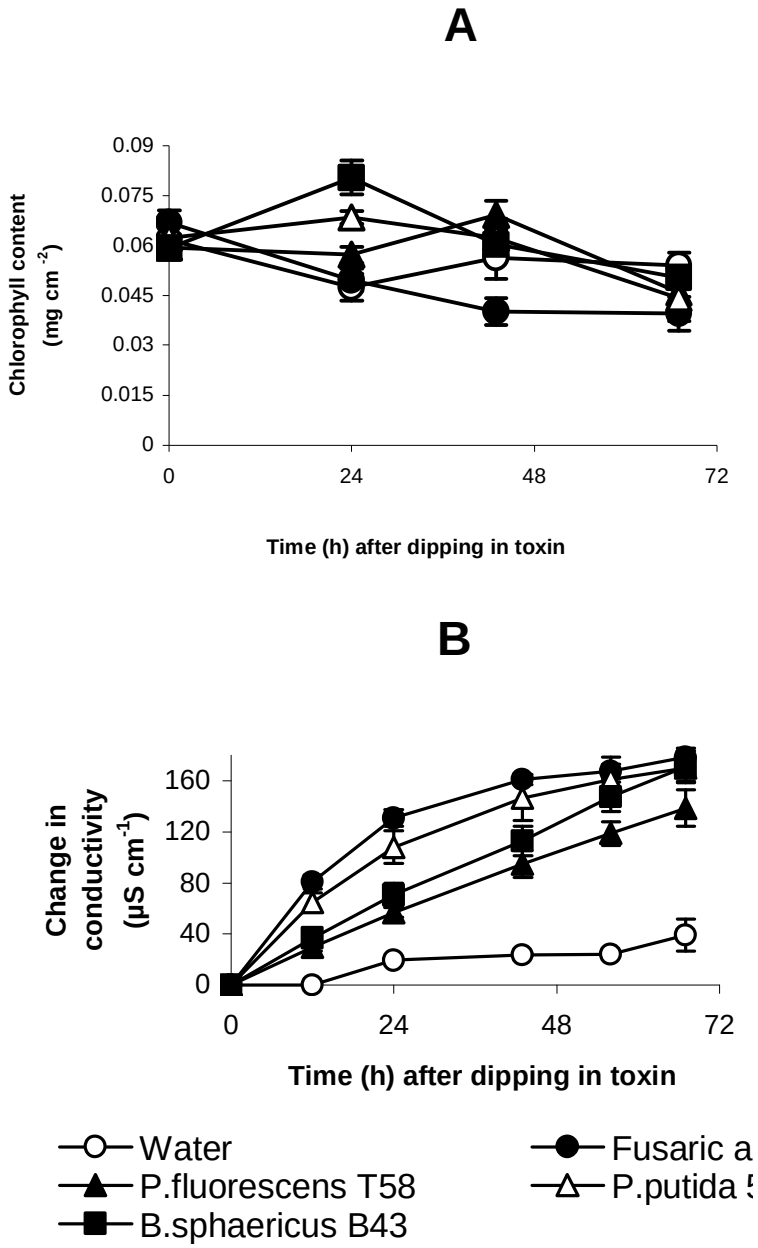
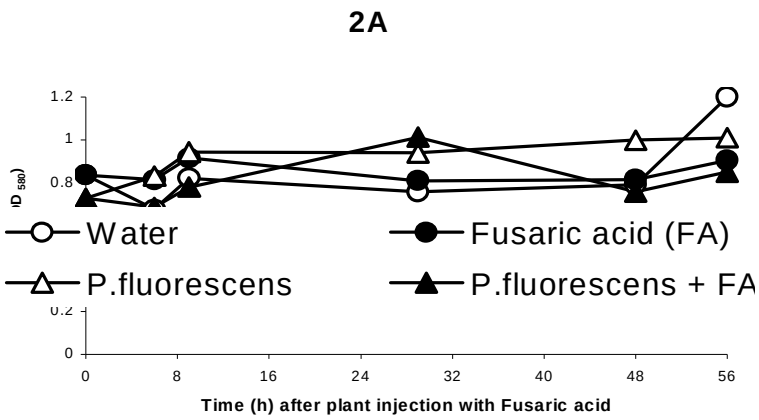
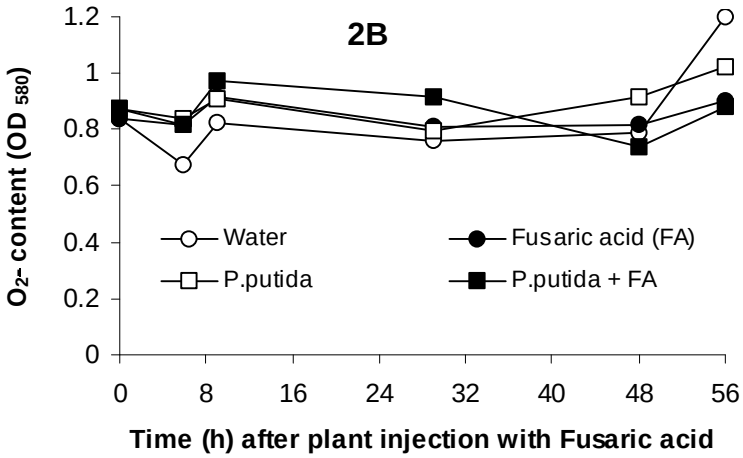


Figure 1: Effect of fusaric acid on chlorophyll degradation (A) and ion leakage (B) in leaves of tomato plants treated with the systemic resistance inducing rhizosphere bacteria *Pseudomonas fluorescens* T58, *Pseudomonas putida* 53 and *Bacillus sphaericus* B43. Bars indicate standard deviation (Fig.1A, n = 3; Fig. 1B, n = 5).

Superoxide anion (O_2^-) content: The content of superoxide anions in tomato leaves was determined after injecting fusaric acid into the stem of plants previously treated with bacteria in the rhizosphere. O_2^- content was lowest and remained fairly constant in the untreated plants injected with water, but reached a maximum at 56 h (Fig. 2A). There was only a slight non-significant increase of the O_2^- content when the untreated plants were injected with FA. In the plants treated with *P. fluorescens* T58 O_2^- content increased to reach a maximum at 29 h before decreasing. In the plants treated with *P. putida* 53 O_2^- content increased substantially in the first 9 h, then decreased thereafter (Fig. 2B). Plants treated with *B. sphaericus* B43 had a significant increase in O_2^- content peaking at 9 h, followed by a decrease and a second peak at 56 h, which was comparable to the trend observed for plants that were bacteria-free but injected with water (Fig. 2C).





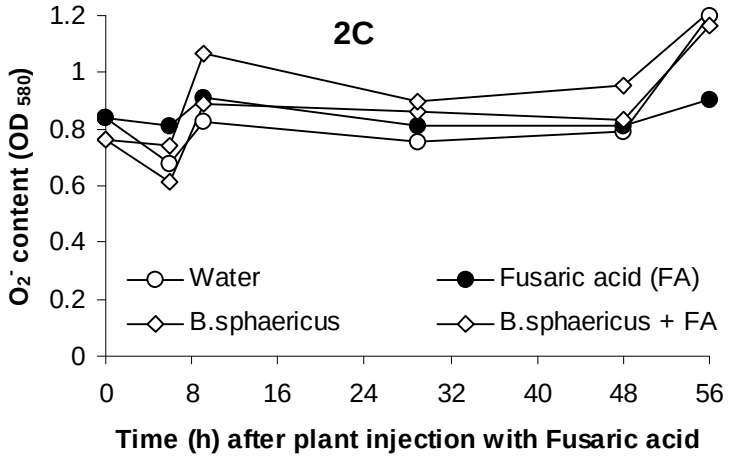
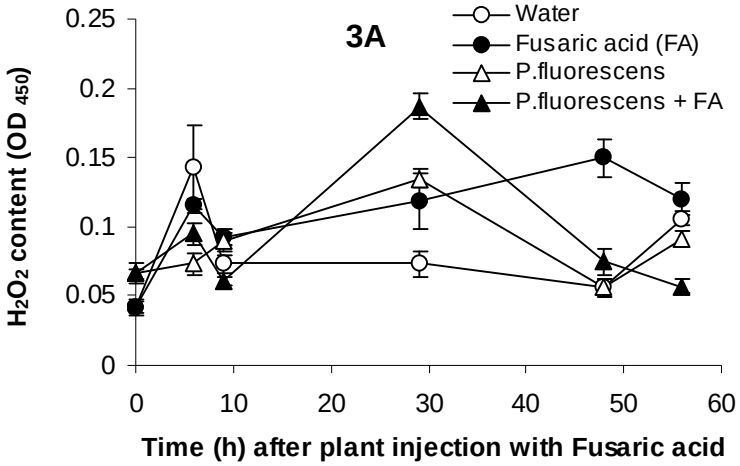
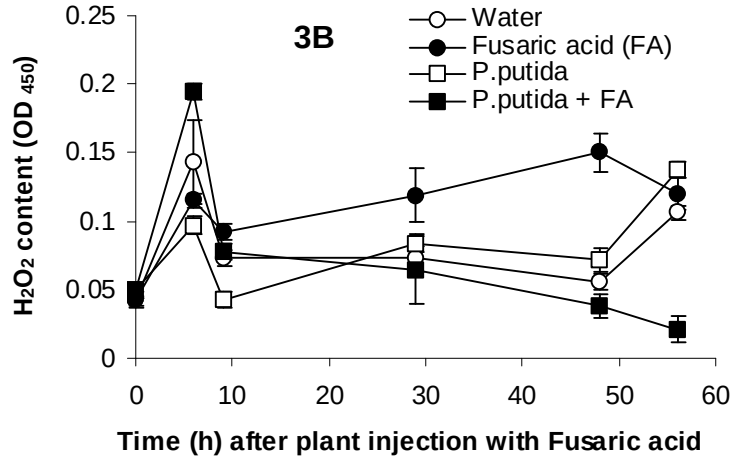


Figure 2: Effect of fusaric acid on O_2^- content in leaves of tomato plants treated with the systemic resistance inducing rhizosphere bacteria *Pseudomonas fluorescens* T58 (Fig. 2A), *Pseudomonas putida* 53 (Fig. 2B) and *Bacillus sphaericus* B43 (Fig. 2C), (n = 3).

H_2O_2 content: In all treatments there was a rapid increase in H_2O_2 content in the first 6 h after injection, followed by a decrease up to about the 9th hour (Fig. 3A). Untreated plants injected with water showed an increase in H_2O_2 content at 56 h. In the bacteria free plants injected with FA, a second peak occurred at 48 h followed by a decline. Plants treated with *P. fluorescens* T58 showed a sharp increase in H_2O_2 with an intial peak at 9 h and an early second peak at 29 h after plant injection (Fig. 3A). Notably, the peak reached after 29 h in plants treated with *P. fluorescens* T58 was significantly higher and occurred earlier when

compared to the peak at 48 h in the untreated plants injected with FA. Plants treated with *P. putida* 53 did not exhibit significant increases in H_2O_2 content. Plants treated with *B. sphaericus* B43 had two H_2O_2 peaks, at 29 and 56 h after injection, repsectively(Fig. 3C).





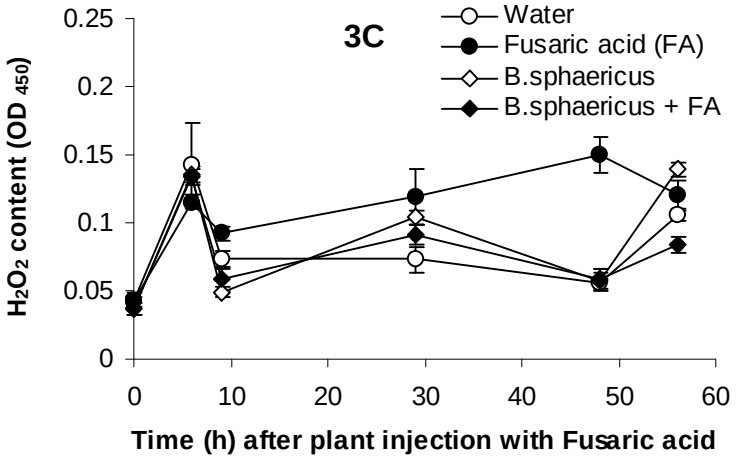


Figure 3: Effect of fusaric acid on H_2O_2 content in leaves of tomato plants treated with the systemic resistance inducing rhizosphere bacteria *Pseudomonas fluorescens* T58 (Fig. 3A), *Pseudomonas putida* 53 (Fig. 3B) and *Bacillus sphaericus* B43 (Fig. 3C), (n = 3). Bars show standard deviation.

Superoxide dismutase (SOD) activity: In all treatments SOD activity generally decreased in the first 6 h following plant injection (Fig. 4A). In the non-induced plants injected with water SOD activity declined continuously after 9 h, while in the non-induced plants injected with FA, activity increased to peak after 48 h. Plants treated with *P. fluorescens* T58 showed decrease in SOD activity after 9 h, to levels that were comparable to the untreated plants that were injected with water. Except for an initial burst, plants treated with *P. putida* 53 or *B. sphaericus* B43 had generally lower levels SOD activity levels than the untreated plants injected with FA (Fig. 4B & C).

Catalase activity: Catalase activity did not change significantly after injecting fusaric acid into plants treated with bacteria when compared to untreated plants and over time (data not shown).

Ascorbate peroxidase (APX) activity: In bacteria-free plants injected with water, APX activity increased at 6 h, then decreased until a minimum at 29 h before increasing again (Fig. 5A). In the bacteria free plants injected with FA, APX activity increased steadily to a maximum at 9 h, then declined. In the plants treated with *P. fluorescens* T58 APX activity resembled that in untreated plants injected with FA, but showed a second peak in APX-activity at 48 h. This '2nd phase' of increased APX activity was not observed in the untreated plants injected with FA, and thus was attributed to the bacterial treatment (Fig. 5A). APX activity in plants treated with *P. putida* 53 and *B. sphaericus* B43 varied as shown in Fig 5B and Fig. 5C.

Guajakol peroxidase (GPX) activity: Bacteria free plants injected with water had increased GPX activity in the first 6 h, followed by a decline to a minimum after 29 h (Fig. 6A). In the bacteria free plants injected with FA, GPX activity increased to reach a peak after 48 h. *P. fluorescens* T58 caused a rise in GPX activity to a sharp peak at 29 h, followed by a decrease to levels significantly lower than in the bacteria free plants injected with FA. GPX activity levels in plants treated with *P. putida* 53 and *B. sphaericus* B43 are shown in Figures 6B & 6C.

DISCUSSION

In many crops a part of the resistance to *Fusarium* is related to fusaric acid (Remotti and Löffler, 1996). Ouchi et al. (1989) concluded that fusaric acid might play a role in pathogenesis and that insensitivity of the plant to this toxin may enhance the resistance against the pathogen. In an associated study (Mwangi et al., *unpublished*) *Fusarium* colonisation of plants treated with the rhizobacteria used in this study had been found to be generally high but did not correlate with wilt symptoms. Plant insensitivity to toxins could be one of the reasons to explain the reduced severity of symptoms in plant tissues that are colonised by the pathogen.

The extent of solute leakage from leaves is used as an indicator of membrane damage by biotic or abiotic factors (Dhindsa et al. 1981). It is already well known that changes in membrane permeability due to toxin damage or normal senescence are closely followed by a decrease in chlorophyll content (Sutherland and Pegg, 1992; Wu and Tiedemann, 2002). Considering that incubation of leaf disks without FA did not cause significant changes in chlorophyll and ion contents, it can be concluded that the damage observed when leaves were treated with FA was a direct effect of the acid. Lower ion loss could partly explain the lower rate of chlorophyll loss in the plants treated with either isolate T58 or B43. Plants treated with *P. putida* 53 lost ions more rapidly but chlorophyll loss was comparable to that of plants treated with other bacteria. This observation suggests the likelihood of another mechanism by which *P. putida* 53 protects chlorophyll from degradation, which might warrant further investigation. Bochow and Dolej (1998) have also reported increased tolerance to fusaric acid after treating tomato plants with *Bacillus subtilis* FZB 24. Such increased tolerance could be due to induced changes in membrane lipids associated with leaf juvenility, which can confer insensitivity to fusaric acid (Barna et al., 1985). Phospholipids are high, while free sterols are low, in membranes of juvenile tissues (Borochov et al., 1982). Treatments that increase leaf juvenility such as high doses of Nitrate-N have been observed to also reduce sensitivity to toxins (Barna et al., 1983).

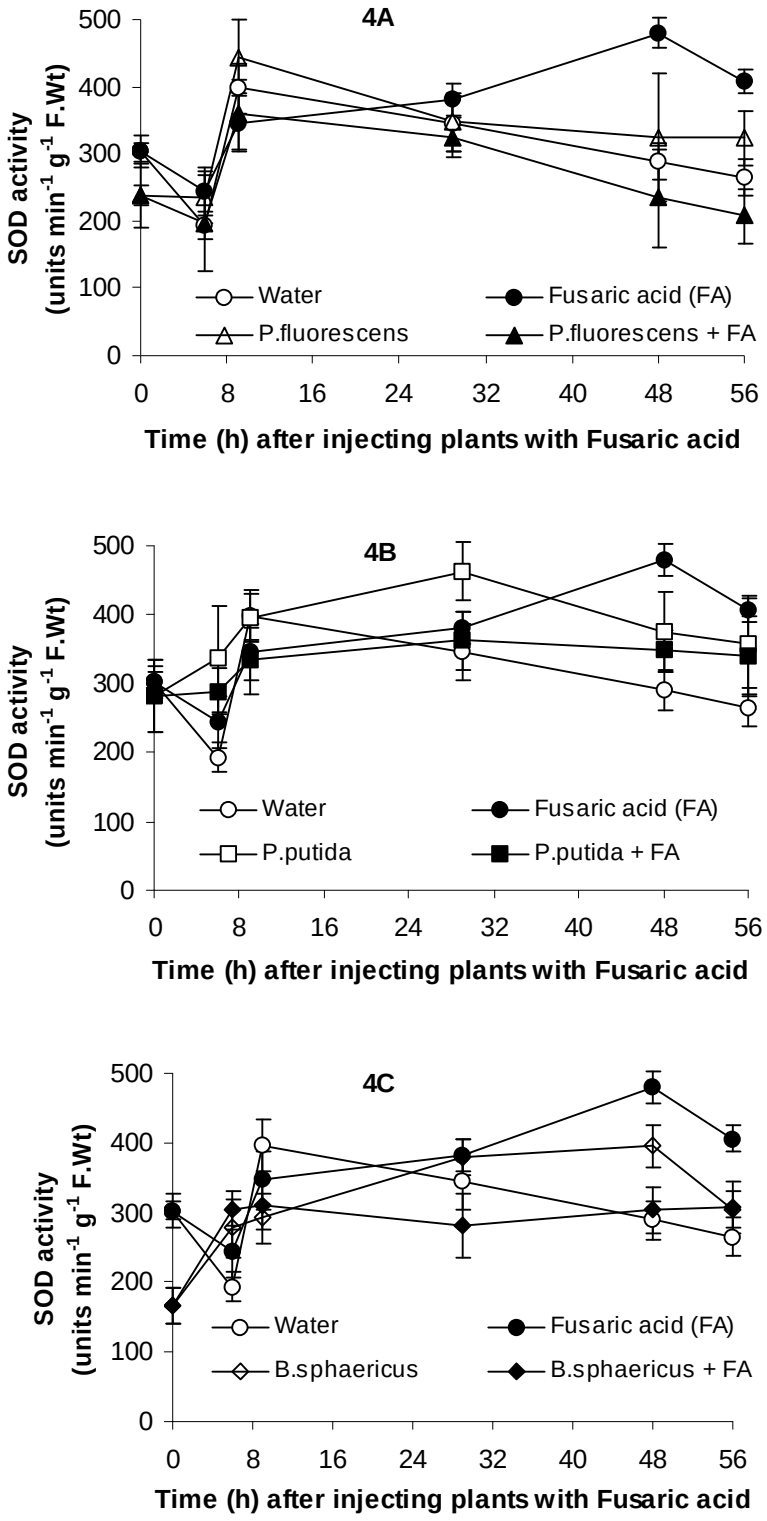


Figure 4: Effect of fusaric acid on superoxide dismutase (SOD) activity in leaves of tomato plants treated in the rhizosphere with *Pseudomonas fluorescens* T58 (Fig.4A), *Pseudomonas putida* 53 (Fig. 4B) and *Bacillus sphaericus* B43 (Fig.4C). Bars show standard deviation (n = 4). FW is fresh weight.

Another possibility is that bacteria induce production of compounds that inhibit fusaric acid activity. Tomatin and solanin, which are found in the vascular sap of tomato plants, have been previously studied as possible inhibitors of *Fusarium* toxins (Gäumann, 1957). When FA is supplied in small doses plants are able to degrade or inactivate it sufficiently to reduce its effect. Tomato plants have been reported to have the capacity to break down up

to 93 % of fusaric acid introduced artificially within 48 h (Gäumann, 1957). However, in this study degradation of FA could not have played a role since the leaf disks were continuously immersed in the FA solution. Detoxification of fusaric acid by bacteria has also been reported previously (Toyoda et al. 1988). A previous study (Mwangi et al. 2002) showed that only isolates *P. fluorescens* T58 and *P. putida* 53 have endophytic capacities, but none of the two isolates could be detected in the stem as early as three days after application. Data obtained in this study do not support the possibility of bacteria degrading fusaric acid because bacteria would have had to be in the leaves by the third day after application.

Chlorosis associated with lipid peroxidation due to toxin damage has previously been associated with the effect of AOS (Dhindsa et al. 1981; Wu and Tiedemann, 2002). Therefore, this study also sought to determine the contents of superoxide ions and hydrogen peroxide and the activities for superoxide-dismutase, ascorbate peroxidase and guaiacol peroxidase. According to Kuzniak et al. (1999), tomato leaves exposed to fusaric acid had an early production of superoxide anions, which could be sustained for two days. However, similar observations were not made in our experiments, though there was significant early increase in the O_2^- content between 6 and 9 h after fusaric acid injection.

To avoid formation of the highly toxic and extremely reactive hydroxyl radical ($OH\cdot^-$) in the "Haber-Weiß-Reaction" (Babbs et al. 1989), the superoxide radical is converted spontaneously or enzymatically by Superoxide-Dismutase (SOD) into H_2O_2 (Allen, 1995). In this study SOD activity varied significantly between treatments. However, taken together, and under the conditions of this study, the data show that SOD activity is not limiting the conversion from O_2^- to H_2O_2. This conclusion is supported by the fact that the changes in O_2^- and H_2O_2 content were closely related in plants treated with *P. fluorescens* T58 even when SOD activity did not increase. Apparently, SOD activity was sufficient, or alternatively, dismutation from O_2^- to H_2O_2 occurred spontaneously. It is also possible that hydrogen peroxide may be derived from other sources than O_2^- as several other enzyme activities, other than SOD, can lead to production of H_2O_2 in cells (Doke, 1983; Milosevic and Slusarenko, 1996). In the plants treated with *P. putida* 53 O_2^- content increased substantially in the first 24 h after FA injection without a change in SOD activity or H_2O_2 contents. In this case, H_2O_2 was obviously consumed through the high activities for APX and GPX. Accumulation of H_2O_2 was observed in two phases, similar to previous observations by Low and Merida (1996). The first phase occurred in all treatments 6 h after injection but timing of the second phase was influenced by FA and bacteria treatment. The first phase is most likely a reaction of plants to the mechanical disturbance during injection rather than due to any specific treatment, as has also been reported by Legendre et al. (1993).

The question arises as to whether the observations reflect underlying differences in the way treated plants respond to FA, and by extension to *Fusarium* infection. Given that H_2O_2 plays an important role in plant defence it would appear that some amount of H_2O_2 is necessary in cells, and that too much or too little is not appropriate when a cell is under infection. An abvious conclusion seems to be that bacteria-free plants exposed to FA accumulated too much H_2O_2, or the accumulation was too late, leading to cell damage and the observed high ion leakage and chlorophyll loss. It is unlikely that the plants injected with FA benefited from protection by APX. Guaiacol peroxidase activity has been observed to increase in tomato plants infiltrated with fusaric acid (Kuzniak et al. 1999). Generally, GPX and other peroxidases may increase when a plant is mobilising defence towards a pathogen. Such peroxidases play a role in lignin synthesis (Milosevic and Slusarenko, 1996), and protein cross linking (Adam et al. 1995) among other roles. Some of the peroxidase activity could have been utilizing some of the H_2O_2 for defence purposes. It is interesting that the highest tissue damage (as measured by ion leakage) was in plants treated only with FA, although GPX activity was highest in the same tissues. Most likely GPX and APX activities together were not sufficient to prevent tissue damage by the high H_2O_2 content. Similar observations have been made in barley physiological leaf spot (Wu and Tiedemann, 2002) and in beans undergoing infection by *Pseudomonas syringae* pv. *phaseolicola* (Adam et al. 1995).

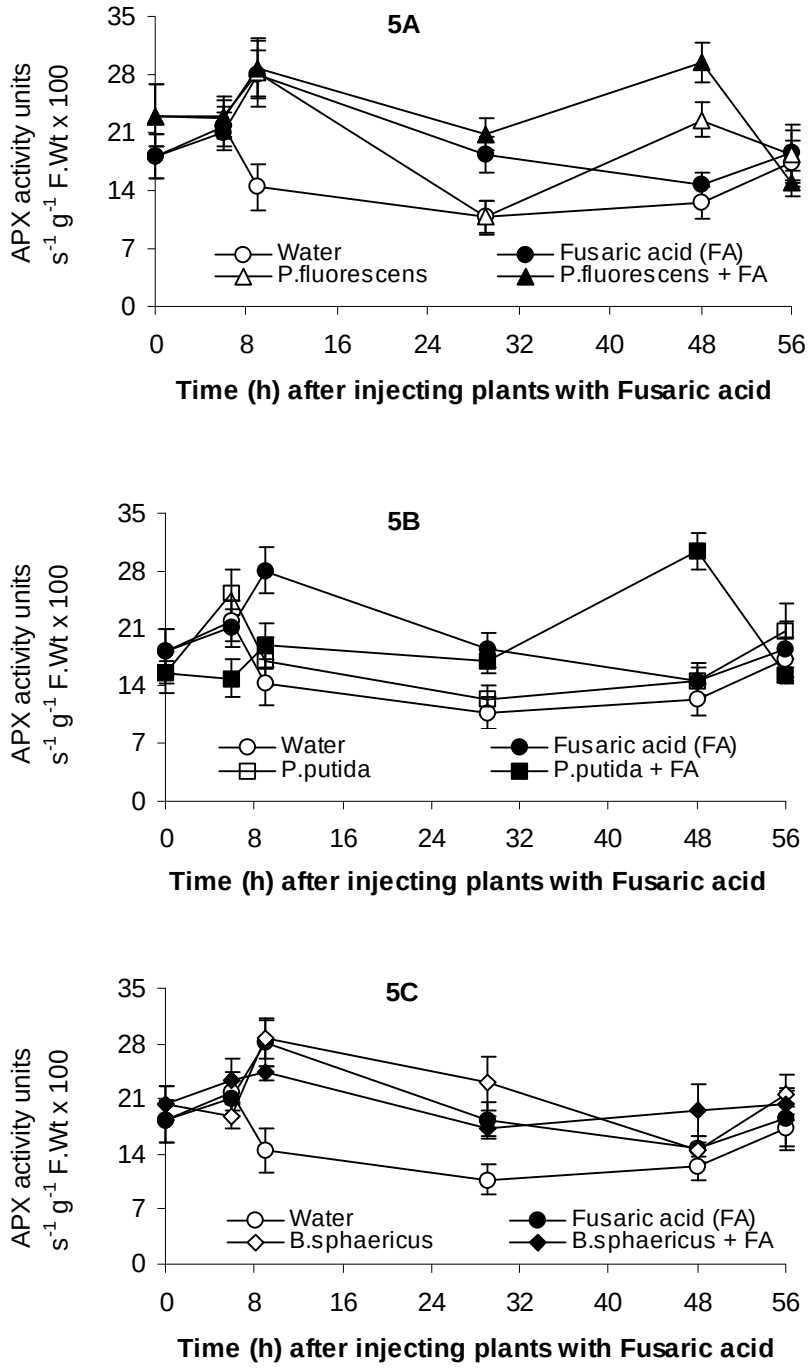


Figure 5: Effect of fusaric acid on ascorbate peroxidase (APX) activity in leaves of tomato plants treated in the rhizosphere with *Pseudomonas fluorescens* T58 (Fig.5A), *Pseudomonas putida* 53 (Fig.5B) and *Bacillus sphaericus* B43 (Fig.5C). Bars show standard deviation (n = 4). FW is fresh weight.

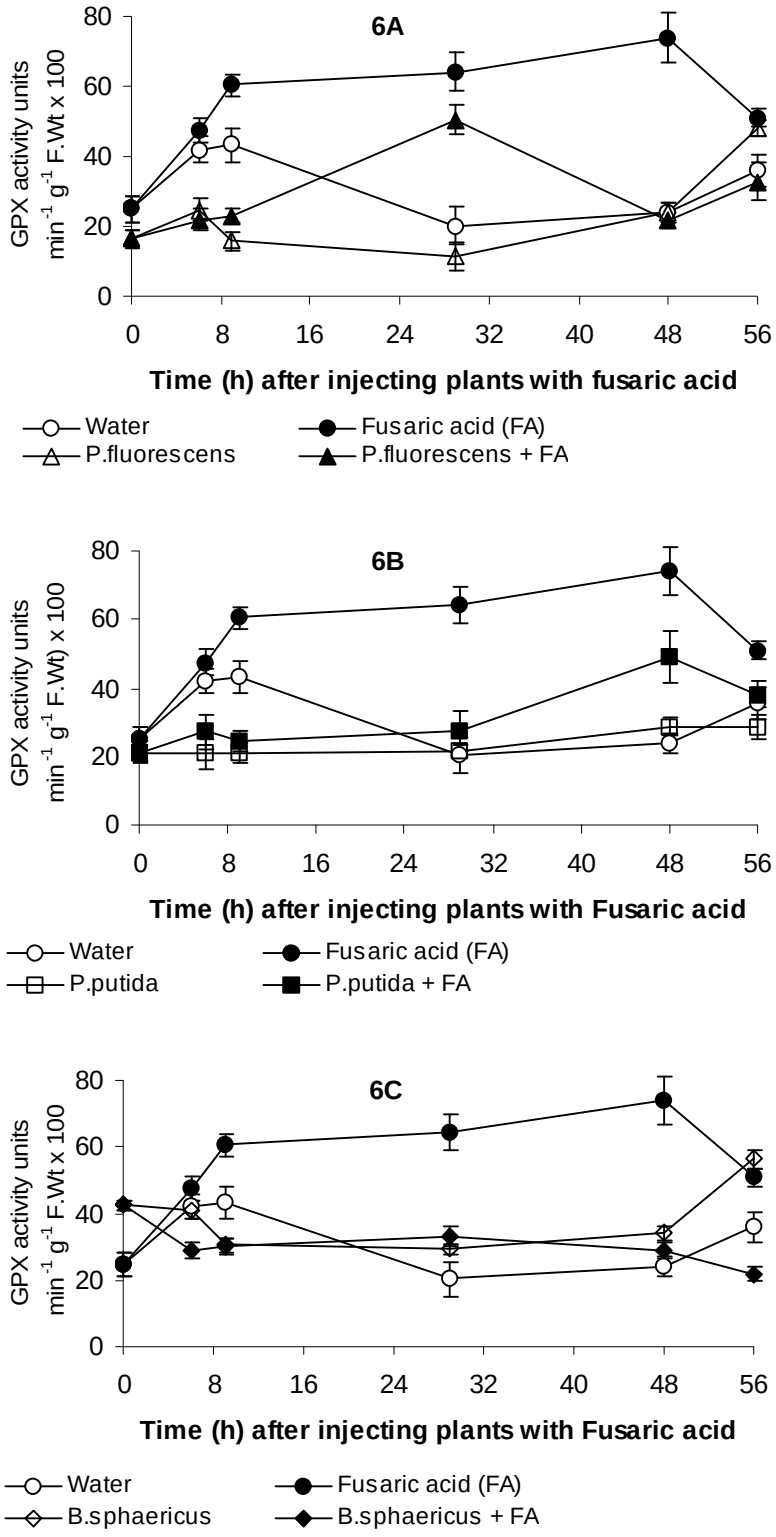


Figure 6: Effect of fusaric acid on guaijacol peroxidase (GPX) activity in leaves of tomato plants treated in the rhizosphere with *Pseudomonas fluorescens* T58 (Fig.6A), *Pseudomonas putida* 53 (Fig.6B) and *Bacillus sphaericus* B43 (Fig.6C). Bars show standard deviation, n = 4. FW is fresh weight.

In plants treated with *P. fluorescens* T58, it would appear that APX and GPX utilise or scavenge H_2O_2 thus stopping its accumulation from cells where it would cause damage to membranes. This could partly explain the positive effect of isolate T58. Considering the results of plants treated with *P. putida* 53 it can be safely concluded that these

enzymes play a role in scavenging and consumption of hydrogen peroxide and help to maintain constant and considerably low H_2O_2 contents.

No correlation was observed between H_2O_2 accumulation and activities of APX and GPX, and where parallel increases were observed (as for *P. fluorescens* T58), it would seem to be a coincidence. The relationship between H_2O_2 content and peroxidase activity is unclear. It has been stated that an increase in peroxidase activity can also trigger increased H_2O_2 production (Adam et al. 1995; Baker et al. 1995).

In AOS metabolism catalase plays a role in the removal of H_2O_2 (Baker et al. 1995). In this study catalase activity did not vary significantly over time regardless of the treatment applied. Kuzniak et al. (1999) reported a decrease in catalase activity after infiltration of fusaric acid into tomato leaves. In our study, catalase activity did not vary significantly between differently treated plants. Enzyme inhibition by Fusaric acid can be excluded because catalase is not inhibited even at high concentrations of fusaric acid (Kuzniak et al. 1999). We thus conclude that catalase does not play a role in the changes observed in the plant responses to different treatments. Rather, it is possible that catalase activity accounted for a part of the H_2O_2-scavenging, even if activities were not altered in response to treatment with bacteria or fusaric acid. Systemic induction of catalase was reported to occur after infection of susceptible potato plants with the bacterium *Erwinia* and root-knot and cyst nematodes (*Meloidogyne* and *Globodera)* (Niebel et al., 1995). However, the results of this study suggest that catalase activity is not regulated by the plant in response to fusaric acid or to bacteria treatments leading to induction of ISR.

We have concluded that the critical factors to avoid plant damage by FA are conversion of O_2^- (if formed) to H_2O_2, and a mechanism to remove the H_2O_2 to avoid its accumulation. Treatment of plants with the three bacteria used in this study led to induction of changes in plant metabolism that had clear positive effects on plant health, reflected by reduced losses in chlorophyll and reduced membrane permeability following injection of fusaric acid. Thus, FA may act as an elicitor in bacteria-induced plants to trigger mechanisms linked to ISR against *F. oxysporum* f.sp. *lycopersici*. We suppose that APX and GPX activities were mostly responsible for AOS scavenging, although catalase activity cannot be entirely excluded. The data further show that susceptible tomato plants respond to the presence of fusaric acid at the biochemical level, and this reaction may be partly mediated through metabolism of active oxygen species. We recommend that further studies be carried out to determine whether ISR is related to changes in leaf juvenility, and to FA degradation or inactivation.

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