Rhizobacteria induces resistance against Fusarium wilt of tomato by increasing the activity of defense enzymes

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Abstract

Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*), is one of the most important diseases that affect tomato yield worldwide. This study investigated the potential of three antagonists, *Streptomyces setonii* (UFV 618), *Bacillus cereus* (UFV 592) and *Serratia* marcescens (UFV 252), and as positive control the hormone jasmonic acid (JA), to reduce Fusarium wilt symptoms and to potentiate the defense enzymes in the stem tissues of tomato plants infected by *Fol*. The seeds were microbiolized with each antagonist, and the soil was also drenched with them. The plants were sprayed with JA 48 h before *Fol* inoculation. The area under the Fusarium wilt index progress curve was reduced by 54, 48, 47 and 45% for the UFV 618, JA, UFV 592 and UFV 252 treatments, respectively. The three antagonists, and even the JA spray, efficiently reduced the Fusarium wilt symptoms on the tomato plant stems, which can be explained by the lower malondialdehyde concentration (an indication of oxidative damage to lipids in the plasma membranes) and the greater activities of peroxidases, polyphenoloxidases, glucanases, chitinases, phenylalanine ammonia-lyases and lipoxygenases, which are commonly involved in host resistance against fungal diseases. These results present a novel alternative that can be used in the integrated management of Fusarium wilt on tomatoes.

Key words: *Fusarium oxysporum* f.sp. *lycopersici*, *Solanum lycopersicum*, biological control, host defense mechanisms, induced resistance, vascular pathogen.

Rizobactérias induzem resistência contra a murcha-de-fusário do tomateiro pelo aumento das atividades de enzimas de defesa

Resumo

A murcha-de-fusário, causada por *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*), é uma das mais importantes doenças que afetam a produção de tomate no mundo. Este estudo investigou o potencial de três antagonistas, *Streptomyces setonii* (UFV 618), *Bacillus cereus* (UFV 592) e *Serratia marcescens* (UFV 252), e como controle positivo foi utilizado o hormônio ácido jasmônico (AJ), na redução dos sintomas da murcha-de-fusário e potencialização das enzimas de defesa nos tecidos do caule de plantas de tomateiro infectadas por *Fol*. Sementes foram microbiolizadas com cada antagonista e o solo também foi encharcado com eles. As plantas foram pulverizadas com AJ 48 horas antes da inoculação *Fol*. A área abaixo da curva do progresso do índice da murcha-de-fusário (AACPIMF) foi reduzida em 54%, 48%, 47% e 45% para os tratamentos UFV 618, AJ, UFV 592 e UFV 252, respectivamente. Os três antagonistas, e até mesmo a pulverização AJ, foram eficientes na redução dos sintomas da murcha-de-fusário em caules de tomateiro, o que pode ser explicado pela menor concentração de aldeído malônico (uma indicação de danos oxidativos em lípideos nas membranas plasmáticas) e maior atividade de peroxidases, polifenoloxidases, glucanases, quitinases, fenilalanina amônia liases e lipoxigenases, comumente envolvidas na resistência do hospedeiro a doenças fúngicas. Esses resultados apresentam uma nova alternativa que pode ser usada no manejo integrado da murcha-de-fusário em tomateiro.

Palavras-chave: *Fusarium oxysporum* f.sp. *lycopersici*, *Solanum lycopersicum*, controle biológico, mecanismos de defesa do hospedeiro, resistência induzida, patógeno vascular.

1. INTRODUCTION

Fusarium wilt, caused by the fungus *Fusarium oxysporum* Schl. f.sp. *lycopersici* (*Fol*) (Sacc.) Snyder and Hansen, has significantly reduced tomato yield due to the premature death of plants and fruits (Lopes and Santos, 1994). There is only one product that is used to control Fusarium wilt on tomatoes by treating the soil before planting; its active ingredient is metam-sodium (AGROFIT, 2012). Currently, the disease is controlled by the use of resistant varieties (Kurozawa and Pavan, 2005). Productive varieties widely grown in Brazil are no longer cultivated by being susceptible to Fusarium wilt (Lopes and Santos, 1994; Tokeshi et al., 1966).

Some studies have demonstrated the efficiency of bacterial isolates for protecting tomato plants from a *Fol* infection (Ardebili et al., 2011; Shanmugam and Kanoujia, 2011). Disease severity is reduced by rhizobacteria that colonize the root system mainly through hyperparasitism, direct antibiosis, competition for space and nutrients and by induced systemic resistance (Baker et al., 1985; Romeiro, 2007). However, to exert a beneficial effect on the root environment, bacteria must colonize the rhizosphere and compete effectively with other microorganisms for nutrient exudates (Lugtenberg and Kamilova, 2009).

The importance of the jasmonic acid (JA) in the host's defense against pathogens has emerged from its application in solanaceous plants, which became locally and systemically protected from *Phytophthora infestans* (Cohen et al., 1993). For the pear-*Xanthomonas campestris* pv. *Pruni* interaction, a JA spray induced the expression of defense genes more than a salicylic acid spray (Sherif et al., 2012). The use of JA in melon cell cultures increased the endogenous level of this hormone and the activities of some defense enzymes (Nafie et al., 2011).

The induced resistance triggered by biological and chemicals inducers is due to an increase in the activities of several enzymes (Shanmugam and Kanoujia, 2011; Van Loon, 2007), such as the lipoxygenases, which contribute to the production of jasmonic acid during lipid peroxidation in the plasma membrane (Schaller and Stintzi, 2009), peroxidases and polyphenoloxidases, which together mediate lignin production (Campbell and Sederoff, 1996), and phenylalanine ammonia-lyases,which participate in the biosynthesis of phenolic compounds in the phenylpropanoid pathway (Podile and Laxmi 1998). The hydrolytic enzymes chitinases and β-1,3-glucanases degrade fungal cell walls, and the released oligosaccharides elicit defense responses against the pathogen attack (Roberts and Selitrennikoff, 1988).

The present study aimed to investigate the potential of three antagonists, previously selected from a universe of 635 isolates to promote biological control of tomato diseases, to reduce Fusarium wilt symptoms on the stem tissues of tomato plants infected by *Fol* as well as to potentiate defense enzyme activities.

2. MATERIAL AND METHODS

Source of *Fol* and the antagonists, cultivation and preservation

The race 2 of *Fol* was obtained from the Fungal Culture Collection of the Plant Disease Clinic of the Department of Plant Pathology at Viçosa Federal University. The antagonists UFV 618, UFV 592, and UFV 2522, were selected from a universe of 635 bacterial isolates, for promoting biocontrol of bacterial spot, early blight and Fusarium wilt in tomato plants. The antagonists UFV 592, UFV 618 and UFV 252 were identified, respectively, as *Bacillus cereus*, *Streptomyces setonii* and *Serratia marcescens* by sequencing the 16S rDNA fragment. The antagonists UFV 592 and UFV 252 were cultured in 523 medium (Kado and Heskett, 1970), the actinomycete UFV 618 in soil-extract agar (Pramer and Schmidt, 1964) and *Fol* on potato-dextrose agar (Dhingra and Sinclair, 1985). Bacterial and actinomycete cultures were preserved in glycerol (30%) and stored in an ultrafreezer at –80 °C (Romeiro, 2007). The *Fol* isolate was maintained on filter paper (Dhingra and Sinclair, 1985).

Plant growth, antagonist application and jasmonic acid spraying

The plants were grown in plastic pots containing 5 kg of a substrate composed of a mixture of sterilized soil, manure and sand in a 3:1:1 ratio (v:v:v). Monobasic calcium phosphate (1.63 g) was added per each kg of substrate. Fifteen seeds were sown per plastic pot, and after their emergence, six plants were left per pot. Fifteen days after sowing, the plants in each pot received 100 mL of a nutrient solution containing, in mg/l, 192 KCl, 104.42 K_2SO_4, 150.35 $MgSO_4 \cdot 7H_2O$, 61 urea, 0.27 $NH_4MO_7O_{24} \cdot 4H_2O$, 1.61 H_3BO_3, 6.67 $ZnSO_4$, 1.74 $CuSO_4 \cdot 5H_2O$, 4.10 $MnCl_2 \cdot 4H_2O$, 4.08 $FeSO_4 \cdot 7H_2O$ and 5.58 bisodium EDTA. The nutrient solution was also applied at the second and third weeks after sowing. The plants were kept in a greenhouse at a temperature of 20 ± 5 °C and relative humidity of 75% ± 2% and irrigated daily. The tomato seeds (cv. "Santa Clara") were microbiolized with three antagonist suspensions for 12 h and then sown in plastic pots. Five days before inoculation with *Fol*, 50 mL of the suspension of each antagonist was added to each pot. To obtain the suspensions, the optical density at 540 nm was adjusted to 0.5 (≈ 5×10^{10} CFU/mL) by adding saline (0.85%) to the bacterial culture. At 35 days after sowing, the plants JA treatment were sprayed with a JA solution (0.5 mM) (Sigma-Aldrich, São Paulo) (25 mL per plant) at 48 hours before inoculation with *Fol*. After JA spraying, plants were kept in closed chambers for 12 hours. The plants from non-microbiolized seeds that were sprayed with distilled water served as the control treatment. The plants

sprayed with JA were kept in a growth chamber separate from the plants of the other treatments.

Inoculation of tomato plants with *Fol* and evaluation of Fusarium wilt severity

The plants with the fully expanded fifth pair of trifoliolate leaves (37 days after sowing), from the base to the apex, were inoculated with a conidial *Fol* suspension at a concentration of 1.3×10^7 conidia/mL. The plants were removed from the pots, and the root system was carefully washed in running tap water and sectioned 1 cm from the apex. Then, the roots were immersed in a conidial suspension for 3 min and replanted into their respective pots (Reis et al., 2005). The Fusarium wilt severity was evaluated at 10, 15 and 25 days after inoculation (dai) using the scale proposed by De Cal et al. (1995), where 1 = plant without symptoms, 2 = lower leaves yellowing, 3 = lower leaves dead and some upper leaves yellowing, 4 = lower leaves dead and some upper leaves wilting, and 5 = plant completely dead. The obtained scores were used to calculate the Fusarium wilt index (FWI) according to Mckinney (1923), consisting of

the following: $\text{FWI} = \dfrac{\sum_{k=1}^{k} F_k X_k}{n.X_k}$, where F_k represents the

number of plants of the treatment withscore xk, k = 1, ...,

K, in the repetition; n is the total number of plants in the repetition; xK is the maximum score value of the scale used. FWI is proportional to the weighted average of the scores, as it corresponds to the ratio between it and the top score of the scale adopted, and can be expressed in percentage. Data from the FWI were used to calculate the area under the Fusarium wilt index progress curve (AUFWIPC) according to the formula proposed by Shaner and Finney (1997).

Population dynamics of the antagonists UFV 252, UFV 592 and UFV 618 in the rhizosphere and rhizoplane of tomato plants

Semi-selective media were prepared based on the constitutive multidrug resistance of the antagonists UFV252, UFV592 and UFV618 to the antibiotics according to Ferraz et al. (2010). Specific antibiotics (ampicillin, cefadroxil and tetracycline (30 mg/mL) for UFV 252; cefepime and trimethoprim (60 mg/mL) for UFV 592 and trimethoprim and oxacillin (30 mg/mL) for UFV 618) were added to the 523 medium. To prevent fungal growth, cycloheximide (150 mg/mL) was added to the semi-selective media. The antagonists were quantified in the semi-selective media at the following times: before the second antagonist application to the soil, immediately after the second antagonist application and at 8, 15, 22 and 37 days after the second antagonist application. For quantifying the antagonists at each evaluation time, a soil sample was collected around the stem of each plant per plastic pot at approximately 1 cm from the stem and 7 cm deep with the aid of a spatula. Then, the samples from each replication were homogenized, weighed and transferred to an Erlenmeyer flask containing 200 mL of sterilized saline (0.85%) and Tween 80 (0.05%) and subjected to ultrasonic radiation for 25 min to extract the antagonists.

After obtaining the suspension, it was serially diluted and placed into test tubes containing sterile saline (0.85%) and Tween 80 (0.05%). Petri dishes containing semi-selective media specific for the antagonists (solid 523 medium with specific antibiotics) received 100 μL of the suspension dilutions (10^{-1} to 10^{-8}). Then, the suspension was spread with a Drigalski handle, and the plates were transferred to a growth chamber at 28 °C for 24 h. After this period, the number of colonies per plate was counted. As a control treatment, the suspension obtained from the tomato plants that were not treated with any antagonist was plated in each of the semi-selective media.

Determination of the activities of peroxidases (POX, EC1.11.1.7), polyphenoloxidases (PPO, EC 1.10.3.1), β-1,3-glucanases (GLU, EC 3.2.1.39), chitinases (CHI, 3.2.1.14), phenylalanine ammonia-lyases (PAL, EC 4.3.1.5) and lipoxygenases (LOX, EC 1.13.11.12)

The plant stem samples from each replication and treatment were collected at 2, 7 and 20 dai. The stem samples from non-inoculated plants were collected immediately after inoculation (0 dai). After sampling, the stem samples were stored individually in aluminum foil, rapidly frozen in liquid nitrogen and then stored in an ultrafreezer at –80 °C until further analysis.

To obtain the extracts used to determine the activities of POX, PPO, GLU,CHI and PAL, 0.3 g of stem tissues was macerated with liquid nitrogen in a mortar with the addition of polyvinylpyrrolidone (PVP) 1% (w/v) to obtain a fine powder. The powder was homogenized in 2 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 1 mM phenylmethylsulfonicfluoride (PMSF). The homogenized material was centrifuged at 20,000 × *g* for 25 min at 4 °C and the supernatant was used to determine the enzymes activities. The POX and PPO activities were determined by the oxidation of pyrogallol according to the method of Kar and Mishra (1976). For POX activity, a mixture of 300 μL of distilled water, 280 μL of 100 mM potassium phosphate buffer (pH 6.8), 200 μL of 100 mM pyrogallol and 200 μL of 100 mM hydrogen peroxide was added to 20 μL of the extract. For PPO activity, the mixture was composed of 300 μL of distilled water, 280 μL of 100 mM potassium phosphate buffer (pH 6.8) and 200 μL of 100 mM

pyrogallol, which was added to 20 μL of the extract. The absorbance was measured in a spectrophotometer (Evolution 60, Thermo Scientific, Waltham, MA, USA) at 420 nm every 10 seconds for 1 min after addition of the extract to the mixture in a total of five readings. The molar extinction coefficient of 2.47 mM⁻¹ cm⁻¹ was used to calculate POX and PPO activities (Chance and Maehly, 1955), which were expressed in mM and μM of purpurogallin produced min⁻¹ mg⁻¹ of protein, respectively. Throughout the process, the microcentrifuge tubes were covered with aluminum foil to protect the mixture from oxidation by light. The GLU activity was determined as described by Lever (1972). The reaction was initiated by the addition of 20 μL aliquots of the supernatant to a mixture of 230 μL of 100 mM sodium acetate buffer (pH 5.0) and 250 μL of the substrate laminarin (Sigma-Aldrich, São Paulo) in a concentration of 4 mg/mL. The reaction mixture was incubated in a water bath for 30 min at 45 °C. After the incubation period, the amount of reducing sugars was determined by adding 250 μL of dinitrosalicylic acid to the mixture and then incubating the resulting mixture in a water bath for 15 min at 100 °C. The reaction was interrupted by cooling the samples in an ice bath. In the control samples, the reaction mixture was the same, except that the dinitrosalicylic acid was add at the same time as the extract. The absorbance of the product released by GLU was measured at 540 nm and the activity of GLU was expressed in absorbance units min⁻¹ mg⁻¹ of protein. The CHI activity was determined by the method of Roberts and Selitrennikoff (1988) as modified by Harman et al. (1993). The reaction was initiated by the addition of 20 μL aliquots of the supernatant to a mixture of 470 μL of 50 mM sodium acetate buffer (pH 5.0) and 10 μL of the substrate *p*-nitrophenyl-β-DN-N'-diacetilquitobiose (Sigma-Aldrich, São Paulo) at a concentration of 2 mg/mL. The reaction mixture was incubated in a water bath at 37 °C for 2 h. The reaction was interrupted by adding 500 μL of 0.2 M sodium carbonate. In the control samples, only the sodium carbonate was used after adding the extract to the reaction mixture and the samples were incubated in a water bath at 37 °C for 2 h. The absorbance of the final product released by the chitinase was determined at 410 nm. The molar extinction coefficient of 7×10^4 mM cm⁻¹ was used to calculate CHI activity, which was expressed in nmol min⁻¹ mg⁻¹ of protein. PAL activity was determined by adding 100 μL of the extract to a mixture containing 400 μL of 25 mM Tris-HCl buffer (pH 8.8) and 500 μL of 100 mM *L*-phenylalanine. The reaction mixture was incubated in a water bath at 30 °C for 4 h. In the control samples, the *L*-phenylalanine was replaced with 500 μL of Tris-HCl buffer. The reaction was finalized by adding 60 μL of 6 N HCl. The absorbance of the *trans*-cinnamic acid derivatives was measured in a spectrophotometer at 290 nm and the molar extinction coefficient of 10^4 mM⁻¹ cm⁻¹ (Zucker, 1965) was used to calculate PAL activity, which was expressed in nM min⁻¹ mg⁻¹ of protein.

To obtain the extract for LOX activity, 0.2 g of stem tissues was macerated with liquid nitrogen in a mortar to obtain a fine powder. The powder was homogenized in 2 mL of 20 mM sodium phosphate buffer (pH 6.8) containing Triton X-100 1% (v/v) and PVP 1% (w/v). The homogenized material was centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was used as the extract for the determination of LOX activity. The reaction was started by adding 7 μL of the extract to a mixture containing 790 μL of buffer, 50 mM sodium phosphate buffer (pH 6.5) and 5 μL of a 10 mM sodium linoleate substrate. The LOX activity was determined according to the method described by Axelrod et al. (1981). The absorbance of the product released by LOX was measured in a spectrophotometer at 234 nm. The molar extinction coefficient of 25,000 M⁻¹ cm⁻¹ was used to determine LOX activity, which was expressed as μmol min⁻¹ mg⁻¹ of protein.

The protein concentration in each sample was determined according to the method of Bradford (1976).

Determination of the malonic aldehyde (MDA) concentration

The plant stem samples were collected as described above. The oxidative damage to lipids was estimated as the total content of the 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA) according to Cakmak and Host (1991) with a few modifications. Briefly, stem tissues (0.2 g) were homogenized in 2 mL 0.1% (w/v) of a trichloroacetic acid (TCA) solution at 4 °C. After centrifugation at 10,000 × *g* for 15 min, 250 μL of the supernatant was incubated with 750 μL TBA (0.5% in 20% TCA) for 20 min in a boiling water bath. After this period, the reaction was stopped by immersion in an ice bath. The samples were centrifuged at 13,000 × *g* for 4 min, and the supernatant absorbance was recorded at 532 nm. The MDA concentration formed in each sample was calculated by using the extinction coefficient of 155 mM/cm and expressed as nmol of MDA per g of fresh weight (FW).

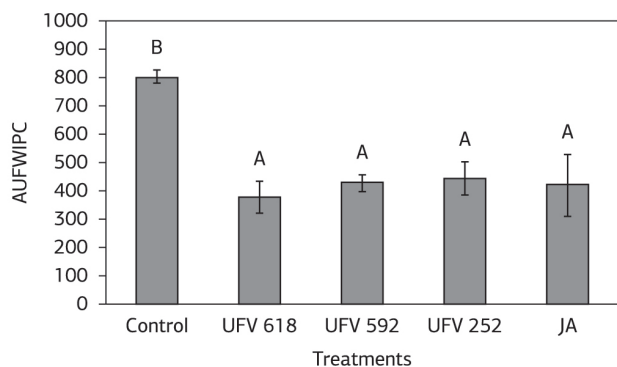## Experimental design and statistical analysis

Three experiments were performed. In Exp. 1, the Fusarium wilt severity was evaluated. In Exp. 2, the antagonist populations in the phylloplane were quantified. In Exp. 3, stem tissues samples of non-inoculated or inoculated plants were obtained for biochemical analyses. Exp. 1 was performed in a completely randomized design with five treatments and five replications. Exp. 2 was performed in a completely randomized design with four treatments (three antagonists UFV 252, UFV 592 and UFV 618 and the control treatment) and five replications. Data from CFU g⁻¹ of soil were transformed to log₁₀ before plotting the antagonist growth curves over time. The five replicates

of each treatment were used to calculate the standard error of the mean. Exp. 3 was a 5 × 4 factorial experiment arranged in a completely randomized design with five treatments (antagonists UFV618, UFV592 and UFV252, JA and the control treatment [no antagonist and no JA spray]), four evaluation times (0, 2, 7 and 20 dai) and four replications. Exps. 1, 2 and 3 were repeated once. Data from all variables evaluated at the three experiments were subjected to an analysis of variance. For the AUFWIPC, the means were compared using Tukey's test (p≤0.05). The means from non-inoculated and inoculated plants at each evaluation time were compared using the *t*-test (p≤0.05). For all experiments, each experimental unit corresponded to a plastic pot containing six plants. The statistical analyses were performed using SAS (version 6.12; SAS Institute, Inc., Cary, NC, U.S.A.).
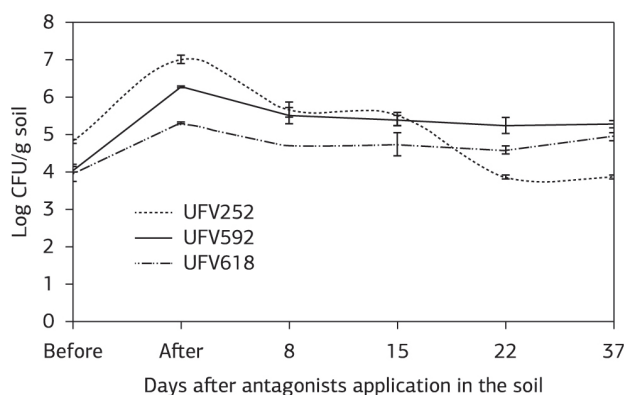
3. RESULTS AND DISCUSSION

The AUFWIPC was significantly reduced with the use of the three antagonists (UFV 618, UFV592 and UFV 252) and JA in comparison to the control treatment (Figure 1). The AUFWIPC was reduced by 54, 47, 45 and 48%, respectively, for the UFV618, UFV592, UFV252 and JA treatments in comparison to the control treatment (Figure 1). In the control, the average severity was 66.66%, which corresponds to severity score was 3.33. The severity scores ranged 2-5 for the control treatment, in contrast to plants sprayed with JA or receiving antagonists varied 1-3. For example, UFV 618 treatment had average severity of 30.66%, which corresponds to an average severity score of 1.53.

Two main factors make the use of biocontrol agents an alternative for the management of Fusarium wilt of tomato. Firstly, in spite of Fusarium wilt of tomato be efficiently controlled by the use of genetic resistance, many cultivars with superior agronomic characteristics no longer grown by farmers due to susceptibility to Fusarium wilt. Second, the lack of registered products other than one based metam-sodium, for controlling Fusarium wilt on tomatoes makes the biological control and the induction of resistance promising alternatives for use in an integrated management program of this vascular disease. The antagonists UFV 252, UFV 592 and UFV 618 effectively reduced the Fusarium wilt severity at the same level of hormone JA. JA and its derivatives have been considered as signaling molecules for inducing systemic resistance, which is effective against pathogens with different lifestyles (Hase et al., 2008). In a study using tomato plants incapable of producing JA (*def1*), plants inoculated with five of eight pathogens were more susceptible to two bacteria (*Pseudomonas syringae* and *X. campestris*), two fungi (*Verticillium dahliae* and *Fol*) and an oomycete (*Phytophthora infestans*) but were not altered in their resistance to *Cladosporium fulvum*, *Oidium neolycopersici* and *Septoria lycopersici*. When the *def1* plants were sprayed with JA, the susceptibility was re-established and was similar to the wild type plants (Thaler et al., 2004). The results obtained in the present study are consistent with the findings of Thaler et al. (2004) and highlight the importance of JA to tomato resistance against Fusarium wilt.

The population dynamics of the antagonists UFV252, UFV592 and UFV618 tended to equilibrate over time (Figure 2). However, there was a sharper decline in the population of the antagonist UFV252 from 15 to 37 days after its second application. The population of the antagonist UFV252 was 3.5×10^5 and 7.26×10^3 CFU g^{-1} soil at 15

Figure 1. Area under the Fusarium wilt index progress curve (AUFWIPC) on tomato plants that received the following treatments, antagonists (UFV 618, UFV 592 and UFV252), jasmonic acid (JA) and control (absence of antagonist and without JA spraying), were inoculated with *Fusarium oxysporum* f.sp. *lycopersici*. The bars represent the standard error of the means. The means followed by the same letter are not significantly different (p≤0.05) as assayed by Tukey's test. Two experiments were conducted with consistent results; the data represent the best experiment. *n* = 5.

Figure 2. Population dynamics of the three antagonists (UFV 252, UFV 592 and UFV618) before their second application (Before), after their second application (After) and from 8 to 37 days after their application in the soil. The bars represent the standard error of the means. Two experiments were conducted with consistent results; the data represent the best experiment. *n* = 5.

and 20 days after its second application. There was an increase in the population of the antagonists after their second application in comparison to the population levels prior to their second application. The populations of the antagonists UFV 252, UFV 592 and UFV 618 were 163, 168 and 21 times higher, respectively, after their second application. Throughout the experiment, the average populations of the antagonists UFV252, UFV592 and UFV618 were 1.85×10^6, 4.77×10^5 and 1.52×10^5 CFU g^{-1} soil, respectively (Figure 2). For the control treatment, the antagonists UFV 252, UFV 592 and UFV 618 did not grow in their respective semi-selective media. The second antagonist application efficiently increased the population levels in both the rhizoplane and the rhizosphere of tomato plants, especially the antagonists UFV 252 and UFV 592. Perhaps only a single antagonist application through microbiolizing the seeds did not effectively reduce the Fusarium wilt severity because a minimum biocontrol agent population is required for effective biocontrol. The *Cryptococcus laurentii* concentration of 10^9 CFU mL^{-1} reduced the incidence of gray rot on peach fruits by 100%; however, a concentration of 10^6 CFU mL^{-1} reduced this disease incidence by only 40% (Zhang et al., 2007). According to Raaijmakers et al. (1995), the minimum rhizobacterial population needed to induce systemic resistance in plants was 10^5 CFU mL^{-1}. Thus, a quorum-sensing signal is necessary to produce the rhizobacterial inducer in a concentration sufficient to be recognized by the plant. The maintenance of the antagonist populations UFV 592 and UFV 618 at high levels (on average greater than 10^5 CFU g^{-1} of soil) allows them to colonize and multiply in the rhizosphere and rhizoplane of tomato plants and qualifies them as good biocontrol agents (Romeiro, 2007). With the exception of the antagonist UFV 252, the antagonist population levels in this study remained almost constant after their second application. The antagonist UFV 252 did not produce resistant structures possibly because it was not able to maintain a high population level throughout the evaluation times.

It is hypothesized that the antagonists UFV 252, UFV 592 and UFV 618 could induce resistance in tomato plants because they were previously selected for their ability to reduce the severities of bacterial spots, early blight and Fusarium wilt on tomatoes. The induced systemic resistance promoted by rhizobacteria can increase the synthesis of defense compounds

in response to a pathogen attack (Van Loon, 2007), which was observed for the greater defense enzyme activities in the present study. Even for the non-inoculated plants (0 dai) treated with the antagonists UFV 252, UFV 592 and UFV 618, the enzyme activities increased. In some interactions between plants and rhizobacteria, certain genes related to defense are known to be over expressed even in the absence of a pathogen (Van Loon, 2007). The high activities of some defense enzymes observed for the non-inoculated plants suggest that even beneficial rhizobacteria can be perceived by the plant as a potential threat and may involve the production of defensive compounds that respond similarly to elicitors produced by fungi and bacteria pathogenic to plants (Van Loon, 2007).

Activity the all defense enzyme was significantly affected by the treatments (UFV 618, UFV 592, UFV 252, JA and control) and the effect of the evaluation time was not significant for the PAL enzyme (Table 1). Some double interactions among treatments and evaluation times were significant (Table 1). All antagonists increased the activities of defense enzymes in at least one evaluation times, but only the antagonist UFV 592 and JA increased the activity of GLU. The results of this study reveal that UFV 252, UFV 592 and UFV 618 antagonists are able to induce resistance in tomato plants against Fusarium wilt.

The POX activity was significantly higher for the all treatments compared to the control treatment at 0, 2 and 7 dai (Figure 3a). At 20 dai, only for the UFV 252 and JA treatments resulted in a higher POX activity compared to the control treatment (Figure 3a). All treatments had the POX activity at least doubled compared to the control treatment at 2 dai. POX is important for the lignification of plant tissues, and the lignin precursors exhibit antimicrobial activity (Ride, 1975). The application of *Bacillus subtilis* to soil increased POX activity on tomato plants at a level comparable to spraying acibenzolar-S-methyl (Araujo and Menezes, 2009). According to these authors, although POX activity increased in the presence of ASM in the same pattern as *B. subtilis*, ASM alone could not reduce the tomato diseases intensities. Under the experimental conditions of the present study, all antagonists increased the POX activity.

PPO activity was significantly higher for the all treatments compared to the control treatment at 0 and 2 dai (Figure 3b). The PPO activity was significantly higher for the UFV

Table 1. Analysis of variance of the effects of the treatments (antagonists UFV 618, UFV 592 and UFV 252; jasmonic acid and control) and evaluation times for chitinases (CHI), β-1,3-glucanases (GLU), peroxidases (POX), polyphenoloxidases (PPO), lipoxygenases (LOX) and phenylalanine ammonia-lyases (PAL) activities and for the malonic aldehyde (MDA) concentration

Sources of variation	df	F values						
		POX	PPO	GLU	CHI	PAL	LOX	MDA
Treatments (T)	4	247.06**	303.35**	5.72**	247.94**	166.04**	1031.80**	21.46**
Evaluation times (ET)	3	4.90*	6.01*	271.47**	4.91*	3.29ns	3429.68**	141.36**
T × ET	12	0.09ns	0.11ns	7.11**	0.09ns	0.06ns	1098.03**	8.69**

Levels of probability: ns,* and **= non significative, significative at 0.05 and 0.01, respectively.
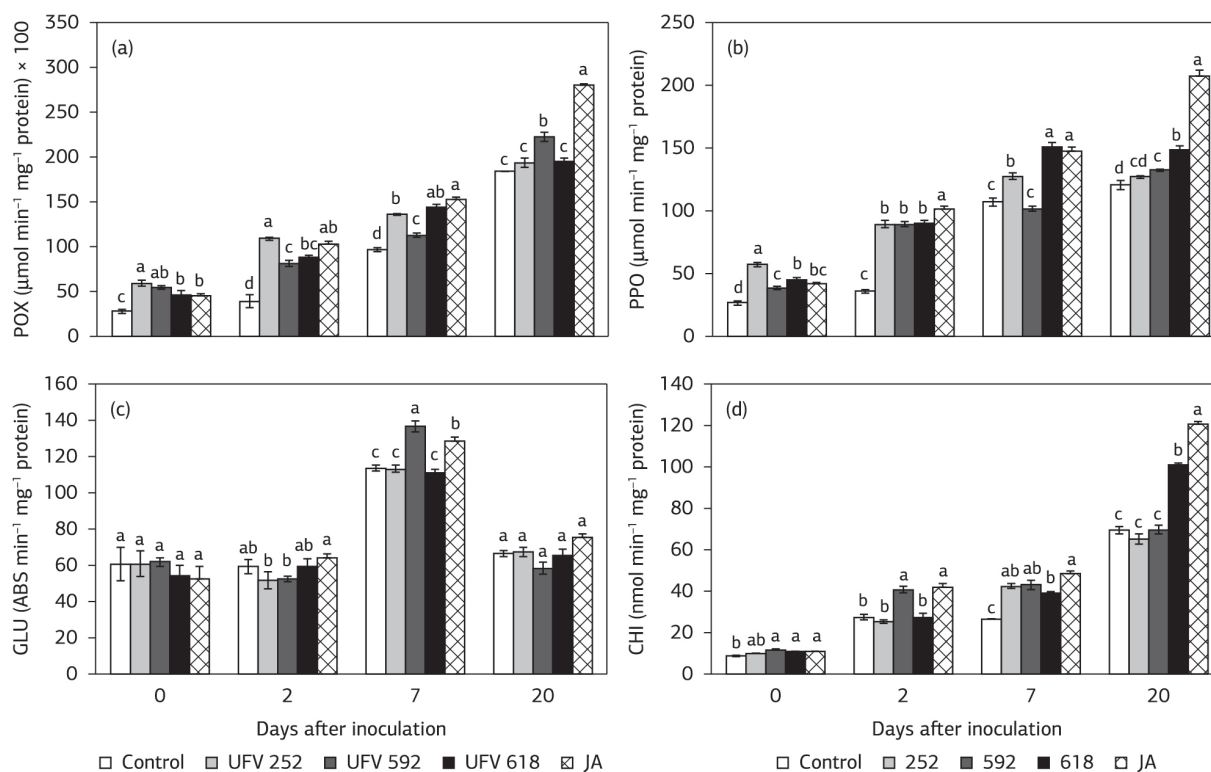
Figure 3. Activities of peroxidases (POX) (a), polyphenoloxidases (PPO) (b), β-1,3-glucanase (GLU) (c) and chitinases (CHI) (d) on the stem tissues of tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* from the following treatments: control (absence of antagonist and without jasmonic acid spray), the antagonists UFV 618, UFV 592 and UFV 252 and jasmonic acid. The bars represent the standard error of the means. The means followed by the same letter are not significantly different (p≤ 0.05) as assayed by Tukey's test. Two experiments were conducted with consistent results; the data represent the best experiment. *n* = 4.

252, UFV 618 and JA treatments at 7 dai and for the UFV 592, UFV 618 and JA treatments at 20 dai compared to the control treatment (Figure 3b).The PPO is important in the initial phase of plant defense, where damage to the membranes induces the release of phenolic compounds (Campbell and Sederoff, 1996). This enzyme participates in the oxidation of many phenolic compounds that lead to the production of quinones, which are toxic to many pathogens (Campbell and Sederoff, 1996). In the present study, it was also observed that PPO activity was at least two times greater for the plants receiving antagonists or sprayed with JA at the earlier stage of the *Fol* infection process (2 dai).

There was no significant difference in the GLU activity between for the all treatments and the control treatment at 0, 2 and 20 dai (Figure 3c). The GLU activity was significantly higher for the UFV 592 and JA treatments compared to the control treatment at 20 dai (Figure 3c). β-1,3-glucanase is a member of the pathogenesis-related protein (PR) family, known to directly destroy pathogen cell walls; its degradation products are oligosaccharides that may induce disease resistance-related enzymes such as PAL (Keen and Yoshikawa, 1983).

The CHI activity was significantly higher for the UFV 592, UFV 618 and JA treatments at 0 dai, for the UFV 592 and JA treatments at 2 dai, for the all treatments at

7 dai and for the UFV 618 and JA at 20 dai compared to the control treatment (Figure 3d). The pattern of CHI gene expression was compared between melon genotypes susceptible and resistant to *Fusarium oxysporum* f.sp. *melonis*, and the transcript quantity was higher for the resistant genotype, which demonstrated the importance of this hydrolytic enzyme for the host's resistance against Fusarium wilt (Zvirin et al., 2010).

There was no significant difference in the PAL activity between for the all treatments and the control treatment at 20 dai (Figure 4a). PAL activity was significantly higher for the UFV 252 and UFV 592 treatments at 0 dai, for the UFV 252, UFV 592 and UFV592 treatments at 2 dai and only for the UFV 252 treatment at 7 dai compared to the control treatment (Figure 4a). PAL is important for phenolic compound synthesis in the phenylpropanoid pathway, and also plays a crucial role in lignin and flavonoid biosynthesis (Podile and Laxmi, 1998). For at least one evaluation time, PAL activity was higher for the plants treated with the antagonists UFV 618, UFV 592 and UFV 252.

There was no significant difference in the LOX activity between for the all treatments and the control treatment at 7 dai (Figure 4b). The LOX activity was significantly higher for the UFV 592 and JA treatments at 0 dai, for the UFV 252, UFV 592 and JA treatments at 2 dai and for the UFV
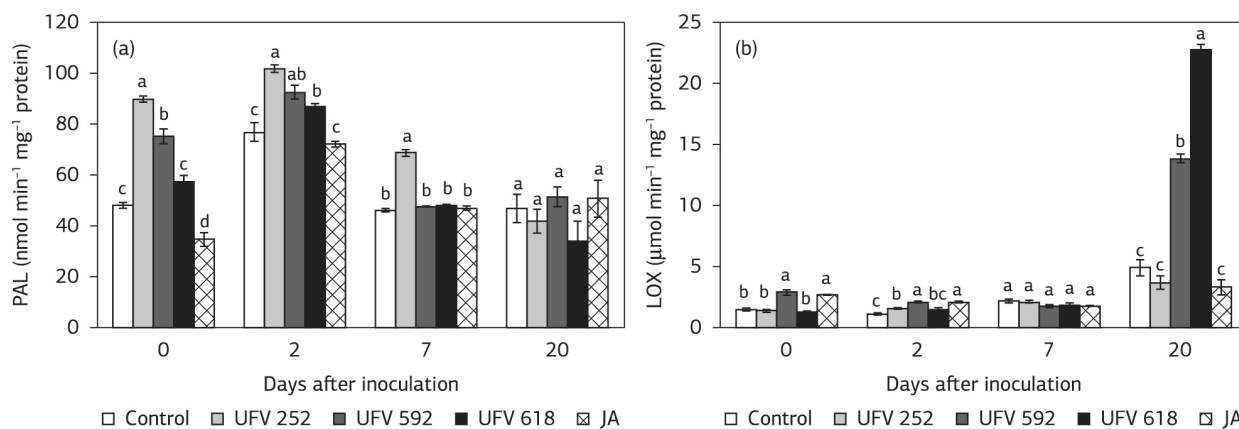
Figure 4. Activities of phenylalanine ammonia-lyases (PAL) (a) and lipoxygenases (LOX) (b) on the stem tissues of tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* from the following treatments: control (absence of antagonist and without jasmonic acid spray), the antagonists UFV 618, UFV 592 and UFV 252 and jasmonic acid. The bars represent the standard error of the means. The means followed by the same letter are not significantly different (p≤0.05) as assayed by Tukey's test. Two experiments were conducted with consistent results; the data represent the best experiment. *n* = 4.
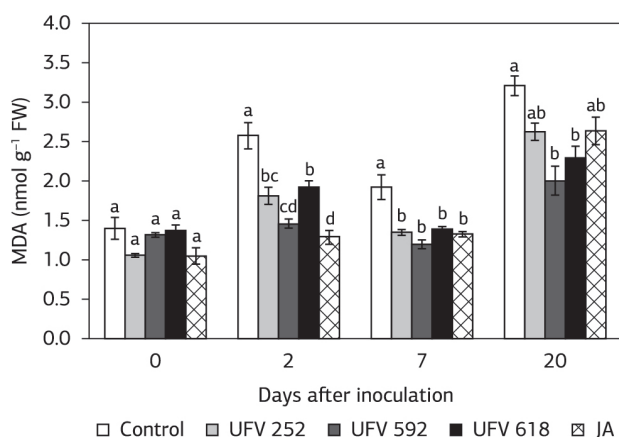
Figure 5. Concentration of malonic aldehyde (MDA)on the stem tissues of tomato plants inoculated with *Fusarium oxysporum* f.sp. *lycopersici* from the following treatments: control (absence of antagonist and without jasmonic acid spray), the antagonists UFV 618, UFV 592 and UFV 252 and jasmonic acid. The bars represent the standard error of the means. The means followed by the same letter are not significantly different (p≤0.05) as assayed by Tukey's test. Two experiments were conducted with consistent results; the data represent the best experiment. *n* = 4.

592 and UFV 618 treatments at 20 dai compared to the control treatment (Figure 4b). LOX is the first enzyme in the JA biosynthetic pathway (Schaller and Stintzi, 2009). LOX activity on the inoculated plants treated with the antagonists UFV 592 and UFV 618 was 3 and 5 times higher, respectively, compared control treatment at 20 dai. The high LOX activity on plants treated with the antagonists UFV 618 and UFV 592 indicates the importance of this enzyme for tomato resistance against Fusarium wilt. Soybean plants that originated from seeds microbiolized with plant growth-promoting rhizobacteria and inoculated with *Macrophomina phaseolina* exhibited a greater LOX activity

compared with plants obtained from non-microbiolized seeds (Choudhary, 2011).

There was no significant difference in the MDA concentration between for the all treatments and the control treatment at 0 dai (Figure 5). The MDA concentration was significantly lower for the all treatments at 2 and 7 dai and at 20 dai for the UFV 592 and UFV 618 treatments compared to the control treatment (Figure 5).The levels of MDA, which is an indirect indicator of lipid peroxidation in the cell wall membrane (Cakmak and Host, 1991), suggest that the lower concentration of this metabolite in the plant stem tissues treated with the antagonists UFV 618, UFV 252 and UFV 592 or sprayed with JA over the course of the *Fol* infection was due to less fungal colonization, which can also be indirectly confirmed by the low AUFWIPC values.

4. CONCLUSION

The results of the present study indicate that the antagonists UFV618, UFV592 and UFV252 are as effective as the hormone JA in reducing the Fusarium wilt symptoms on tomatoes with the participation of the defense enzymes, POX, PPO, GLU, CHI, PAL and LOX which are commonly involved in the host's resistance against fungal diseases.

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