INDIAN CLOVE ESSENTIAL OIL IN THE CONTROL OF TOMATO BACTERIAL SPOT

G. Ciavareli Lucas[1], E. Alves[1], R. Borges Pereira[2], A.B. Zacaroni[1], F.J. Perina[1] and R. Magela de Souza[1]

[1] *Universidade Federal de Lavras (UFLA), Departamento de Fitopatologia, Caixa Postal 3037, CEP 37200-000 Lavras, MG, Brazil*

[2] *Empresa Brasileira de Pesquisa Agropecuária/Embrapa Hortaliças. Centro Nacional de Pesquisa de Hortaliças (CNPH), Caixa Postal 218, 70359-970 Gama, DF, Brazil*

SUMMARY

The goals of this work were to assess the potential of Indian clove essential oil in the reduction of tomato bacterial spot, and the activation of some plants biochemical defense responses. Tomato plants of cv. Santa Cruz Kada were sprayed with Indian clove essential oil (0.2 mg ml^{-1}) and inoculated with *Xanthomonas vesicatoria* seven days later. Acibenzolar S-methyl (0.2 mg ml^{-1}) was used as control. Indian clove essential oil and acibenzolar-S-methyl gave a control of 53.0% and 89.0%, respectively. The expression of resistance induced by Indian clove essential oil was made evident by an increase in β-1,3-glucanase, chitinase and peroxidase activity, which was also observed in plants sprayed with acibenzolar-S-methyl. This increase started in the first hours after spraying and continued for up to 12 days, while the lignin content increase was observed 12 days after spraying. Indian clove essential oil is a potential resistance inducer of tomato bacterial spot.

Key words: antibiogram, induced resistance, lignin, PR protein, *Xanthomonas vesicatoria*.

INTRODUCTION

Bacterial spot is one of the most important diseases of tomato (*Solanum lycopersicum*) worldwide. In Brazil it occurs mainly in warm, humid environmental conditions where it limits the productivity of commercial tomato crops. The etiological agent is a bacterium of the genus *Xanthomonas* (Dowson) (Jones *et al*., 2000, 2004). Strategies to control bacterial spot are based on a combination of practices such as the use of pathogen-free seeds or seedlings, elimination of alternative hosts, use of resistant cultivars, chemical control (Obradovic *et al*., 2004) and activation of natural plant defenses.

Corresponding author: G. Ciavareli Lucas
Fax: +55.35.3829.1283
E-mail: gilciavareli@yahoo.com.br

Activation of natural plant defenses through systemic acquired resistance (SAR) has been used as a strategy for leaf spot control (Louws *et al*., 2001), as it elicits molecules that activate systemic resistance, thus protecting tissues against subsequent attack from a wide range of pathogens (Hammond-Kosack and Parker, 2003). SAR is expressed both locally and systemically in response to pathogens that cause tissue necrosis (TN), or by endogenous salicylic acid (SA) or synthetic compounds such as S-methyl ester of benzo [1, 2, 3] thiadiazole-7-carbothioic (ASM) and 2,6-dichloroisonicotinic acid (INA). The resistance expressed is associated with increased activity of pathogenesis-related proteins (PRP). According to Hammerschmidt and Smith-Becker (1999), these are excellent molecular markers for the response of resistance to pathogens.

Among the chemical products used for inducing SAR, acibenzolar-S-methyl (ASM) is the most studied for the control of tomato bacterial spot (Werner *et al*., 2002; Buonaurio *et al*., 2002) and the first commercial product used under the names Bion, Actigard and Boost (Venâncio *et al*., 2000).

Natural substances derived from medicinal plants have also shown promising results for controlling diseases of different crops (Carneiro, 2003; Pinto *et al*., 2010; Pereira *et al*., 2011). As to essential oils, the studies aimed at elucidating the mechanisms of disease suppression, suggest that the active compounds of the oils act directly on the pathogens or induce host resistance through the production of phytoalexins, increased PRP activity, synthesis of structural compounds and biochemical plant defense (Schwan-Estrada, 2003; Kagade *et al*. 2004; Balbi-Peña *et al*., 2006; Guleria and Kumar, 2006; Pereira *et al*., 2008), resulting in disease reduction (Schneider and Ullrich, 1994; Paul and Sharma, 2002). Ponce *et al.* (2003) found that essential oils inhibit bacterial growth at a concentration of 500 µl l^{-1}, whereas Ventura *et al.* (2011) found complete inhibition of mycelial growth of pathogenic *Aspergillus* sp., *Penicillium* sp., *Cercospora kikuchii*, *Colletotrichum* sp., *Fusarium solani* and *Phomopsis* sp.

The successful exploitation of the biological activity of the compounds present in essential oils as a potential way to control plant diseases prompted the present

study aimed at verifying the effect of Indian clove essential oil on the control of tomato bacterial spot and characterizing the biochemical responses of induced resistance against *Xanthomonas vesicatoria*

MATERIAL AND METHODS

Preparation of the inoculum. The *X. vesicatoria* inoculum used in this study was strain 89T kindly provided by Embrapa Hortaliças (Brazil) and maintained in suspension of 15% glycerol peptone in a deep-freezer (-80°C). Leaves of tomato plants inoculated to check the inoculum virulence were used for the isolation of the bacterium in medium 523 of Kado and Heskett (1970; MB1) through the parallel grooves technique. Bacterial cultures were then grown in the same medium at 28°C for 24 h for the preparation of the suspensions to be inoculated. The concentration of bacterial suspension used in all experiments was adjusted with a spectrophotometer to A_{600} = 0.30, corresponding to approximately $5x10^8$ CFU ml^{-1}.

***In vitro* trial.** The Indian clove essential oil was purchased from Brasil Portrait (2009). The potential inhibitory effect of Indian clove essential oil (OECR) on the growth of *X. vesicatoria* was studied at concentrations of 0.1, 1.0, 10.0 and 100% in 1.0% powdered milk. ASM (0.2 mg ml^{-1}), streptomycin sulfate (25 mg ml^{-1}) and sterile water were used as control. The experiment was conducted in a completely randomized design with six replicates. Autoclaved filter-paper discs, 6 mm in diameter, were soaked in 20 µl of each test substance, dried at room temperature and placed in Petri dishes containing medium 523 of Kado and Heskett (1970) and 100 ml of the suspension ($5x10^8$ CFU ml^{-1}) of *X. vesicatoria*. The presence and diameter of inhibition zones were determined after 48 h incubation in a growth chamber at 28ºC.

***In vivo* trial.** To evaluate the effect of Indian clove essential oil in controlling tomato bacterial spot in greenhouses, an aliquot of 0.1% was added to 1.0% powdered milk. Three additional treatments were used, acibenzolar-S-methyl (0.2 mg ml^{-1}) as an induced resistance standard, Recop (84% of cooper oxychloride) at the dose of 2.0 mg ml^{-1}, as copper standard, and 1.0% powdered milk. Tomato plants were treated 23 days after sowing (DAS) and inoculation occurred on the 30^{th} DAS. Evaluations began seven days later. Tomato plants previously kept in a moist chamber for 24 h, were inoculated by foliar sprays to the point of run-off, then placed again in a moist chamber for 24 h. The experiments were conducted in a randomized block design with five replications and plots consisting of six plants. The experiment involved five assessments of the severity of bacterial spot using the Mello *et al*. (1997) scale. The area under the disease-progress curve (AUDPC) of each treatment was later calculated according to Shaner and Finney (1977), and the percentage of disease reduction yielded by the treatments compared to the control.

Characterization of the mode of action. To characterize the biochemical mechanisms of tomato resistance to *X. vesicatoria* induced by stimulation of Indian clove essential oil tomato seeds of cv. Santa Cruz Kada were sown in pots containing 3.0 litres of substrate Plantmax HT and 15-day-old plants were treated with 0.1% Indian clove essential oil (with and without inoculation), acibenzolar-S-methyl (AS - 0.2 mg ml^{-1}) (with and without inoculation) and the control (with and without inoculation). A randomized block design was used with three replications and experimental units consisting of one pot containing three plants. Inoculation with *X. vesicatoria* were done four days after the treatment, leaves were collected 1/2, 1, 2, 4, 5, 6, 9 and 12 days after treatment and were immediately frozen in liquid nitrogen for further analysis.

To prepare the protein extract, fresh leaf material (1.0 g) from treated and untreated tomato plants was homogenized for 5 min with pestle and mortar in 3 ml of ice-cold 50 mM sodium acetate buffer pH 5.2, containing 0.1 mM EDTA. After filtration in cheesecloth, the homogenate was centrifuged at 13,000 *g* for 15 min and the supernatant (crude extract) used as the source of enzymes. All steps were carried out at 0-4°C. The protein content of crude extracts was determined using the Bradford (1976) protein assay, with bovine serum albumin (BSA) as a standard.

The activity of chitinase (CHI; EC 3.2.1.14) was determined by adding 70 µl of enzymatic extract to the solution with 130 µl of sodium acetate 50 mM, pH 5.2, and 60 µl of CM-Chitin-RBV (2.0 mg ml^{-1}), a specific substrate for chitinase supplied by Loewe Biochemica (Germany), in a 96-well microplate, with a capacity of 350 µl. After incubation at 35ºC for 80 min, samples were acidified with 50 ml of HCl 0.5 N, cooled in an ice bath for 10 min and centrifuged (1,450 *g* for 10 min). An aliquot of 210 µl of the supernatant of each sample was transferred to a new microplate to take readings at 492 nm in an EIA-compatible player (Wirth and Wolf, 1990).

The activity of β-1,3-glucanase (GLU; EC 3.2.1.6) was measured using a method similar to that used to measure chitinase, replacing the substrate with CM-Curdlan-RBB (4 mg ml^{-1}) and adjusting the aliquot of enzyme extract to 100 µl (reducing the volume of acetate buffer to adjust the final volume to 310 µl per well). To promote the hydrolytic action of β-1,3-glucanase, an incubation time of 35ºC for 100 min was adopted. The samples were then photometrically measured with a 620 nm filter in an EIA reader (Wirth and Wolf, 1990).

The activity of guaiacol peroxidase POX (EC 1.11.1.7) was determined by adding 25 µl of enzyme extract adjusted to 2.0 ml of a solution containing sodium acetate 50 mM, pH 5.2, guaiacol 20 mM and hydrogen peroxide 20 mM. After incubation at 30ºC for 10 min, absorbance was measured at 480 nm (Urbanek *et al*., 1991). One unit of POX was expressed as a variation of OD_{480} per 1.0 milligram of soluble protein per minute (D_{480nm} mg $P^{-1}min^{-1}$).

Lignin content was determined by testing with thioglycolic acid (TGA) (Montie, 1989) 0.2 g of meristematic tissues incubated in acetone (85%) for 48 h and centrifuged at 7500 *g* for 15 min. The precipitate was dried and incubated with 5.0 ml of thioglycolic acid in HCl 2 N 1:10 (v/v) for 4 h. The samples were centrifuged at 7,500 *g* for 15 min and the supernatants transferred to new tubes where they received 200 µl of HCl 10 N. After an ice bath for 4 h and a centrifugation at 7,500 *g* for 30 min, the precipitate was homogenized in 5.0 ml of NaOH 0.5 N and the absorbance was measured at 280 nm. The amount of TGA derivatives (acid-soluble lignin) formed was measured by comparison with a standard curve (0.01-0.1 mg ether 2-hydroxypropyl ml^{-1}) and the values expressed in micrograms of lignin per milligram fresh material (µg mg^{-1} MF). All determinations were performed in triplicate.

Statistical Analysis. Data were submitted to variance analysis and measurements compared according to Tukey's test ($p \leq 0.05$). For statistical analysis the SAS (2001) programme was used.

RESULTS

***In vitro* and *in vivo* trials.** Indian clove essential oil (OECR) at concentrations of 1.0%, 10.0% and 100% as well as streptomycin sulfate promoted the appearance of inhibition zones in the radial growth of *X. vesicatoria* (Fig. 1), whereas Acibenzolar-S-methyl (ASM) and OECR at a concentration of 0.1% did not.

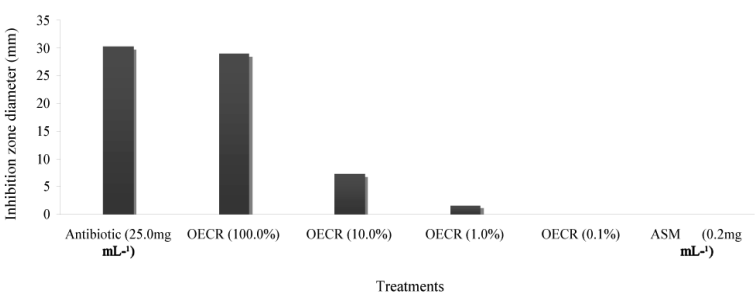

Fig. 1. Growth inhibition *in vitro* of *Xanthomonas vesicatoria* subjected to different concentrations of Indian clove essential oil (OECR), streptomycin sulfate (antibiotic) and acibenzolar-S-methyl (ASM).

Under greenhouse conditions, all treatments controlled tomato leaf spot by reducing disease progress (Fig. 2). ASM was the most effective in reducing disease severity by 89.0%, followed by 53.0% afforded by OECR. Recop was much less efficient when compared with other treatments, as it promoted only 26.0% reduction of disease severity.

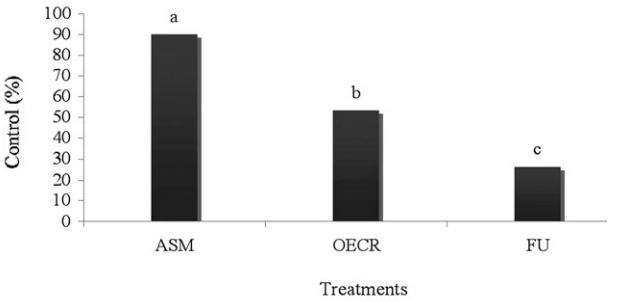

Fig. 2. Control of tomato bacterial spot through the application of acibenzolar-S-methyl (ASM) 0.2 mg ml^{-1}, Indian clove essential oil (OECR) 0.1% and Recop (FU) 2.0 mg ml^{-1} at 7 days before inoculation. Means followed by same letter do not differ among themselves through the Tukey test ($p < 0.05$).

Biochemical analyses. As to the characterization of the biochemical mechanisms of defense evaluated, the activity of β-1,3-glucanase, chitinase and peroxidase increased (Fig. 3), while lignin content differed significantly from the control in all treatments (Fig. 4). It was observed that inoculation with *X. vesicatoria* increased the activity of these enzymes and lignin content in tomato leaves for all treatments, including the control. However, the lignin content did not differ significantly from plants sprayed only with OECR and ASM.

Plants sprayed only with the treatments tended to increase β-1,3-glucanase activity from 12 h after spraying (*has*) to 12 days after spraying (*das*) of the plants inoculated with the bacterium, only those sprayed with ASM tended to increase the activity of the enzyme. Plants sprayed with ASM, inoculated and non inoculated, showed maximum activity of β-1,3-glucanase at 12 *das,* with an 8.92% and a 35.33% increase in enzyme activity in relation to inoculated and non-inoculated control, respectively (Fig. 3A). On the other hand, plants sprayed with OECR, and then inoculated or non-inoculated, showed maximum activity of β-1,3-glucanase at 9 *das,* with a 52.29% and a 58.29% increase in enzyme activity in relation to the inoculated and non-inoculated control, respectively (Fig. 3B), indicating increases in enzyme activity higher than those obtained by ASM. It was also observed that plants treated with ASM and OECR, inoculated or non-inoculated, tended to increase the activity of β-1,3-glucanase during the harvest period, at times non-significantly.

Plants sprayed with ASM and inoculated with the

bacterium had two periods of increased activity of chitinase, from 5 to 6 *das* and 9 to 12 *das* (Fig. 3C). The maximum activity was observed at 6 *das*, but did not differ significantly from the inoculated control. At 12 *das*, the plants showed an increase of 41.93% in enzyme activity compared with the inoculated control. Plants sprayed with ASM only showed maximum enzyme activity at 4 *das* with an 85.94% increase in activity compared with the control. Tomato plants sprayed with OECR and inoculated with *X. vesicatoria* showed a decrease in chitinase activity until the last harvest. The maximum activity of this enzyme occurred at 5 *das* with a 62.72% increase compared with the inoculated control (Fig. 3D). Plants sprayed only with OECR had two periods of increase in chitinase activity: from 12 *has* to 4 *das* and 9 to 12 *das*. Like the plants treated only with ASM, plants sprayed only with OECR showed maximum chitinase activity at 4 *das*, with a 69.79% increase compared with the control. There was a reduction in chitinase activity at 12 *das*, however, with a 56.70% increase compared to the control.

Plants sprayed with ASM and inoculated with bacte-

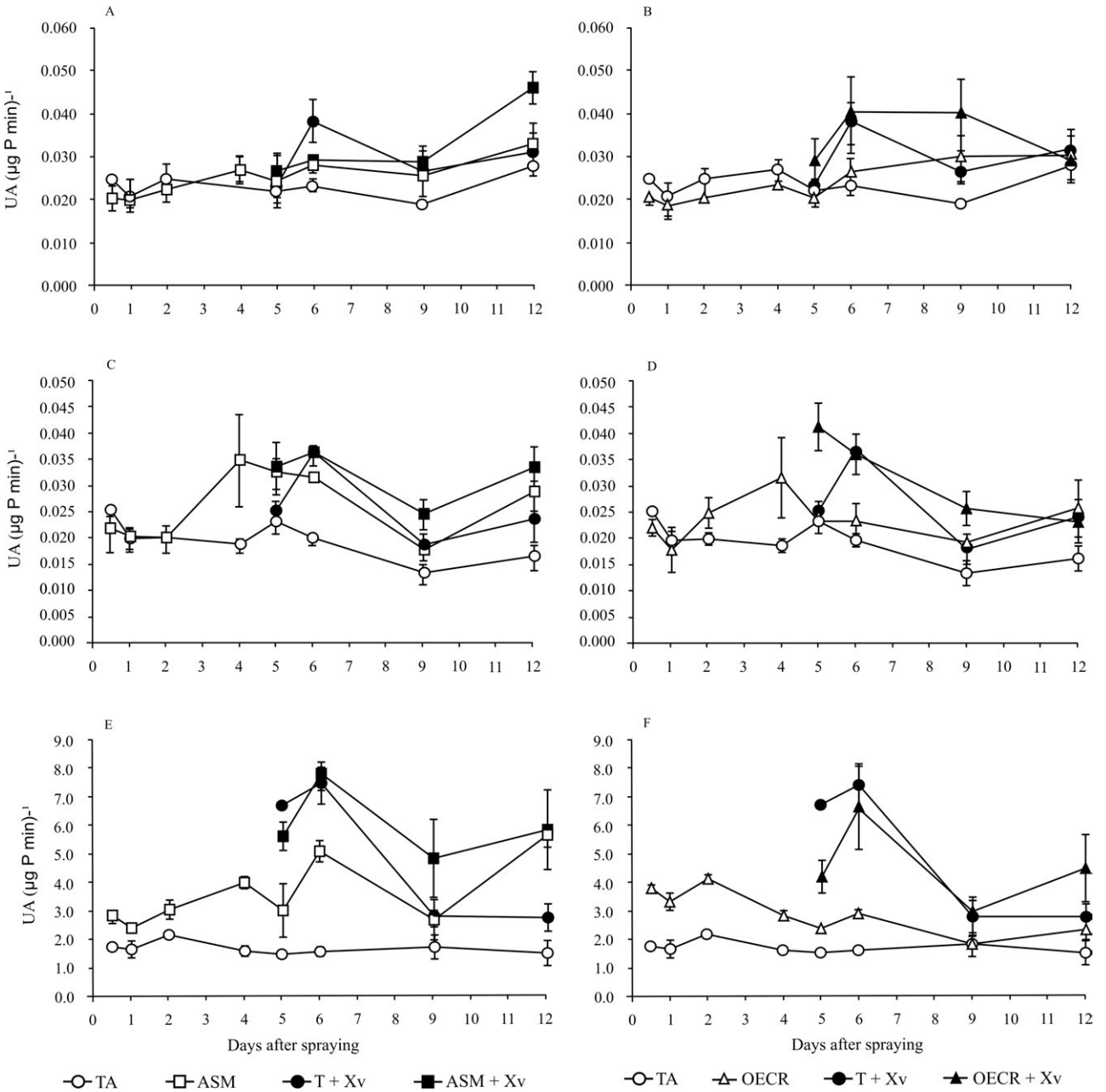

Fig. 3. Activity of the enzymes β-1,3-glucanase (A and B), chitinase (C and D) and peroxidase (E and F) on tomato plants cv. Santa Cruz Kada. Absolute control (TA), acibenzolar-S-methyl (ASM), Indian clove essential oil (OECR), followed by control inoculation (T+Xv), acibenzolar-S-methyl followed by inoculation (ASM+Xv) and clove essential oil followed by inoculation (OECR+Xv). Plants inoculated with *Xanthomonas vesicatoria* four days after spraying. Bars indicate standard error of mean.

ria showed two periods of increase in peroxidase activity: 5 to 6 *das* and 9 to 12 *das* (Fig. 3E). The maximum enzyme activity occurred at 6 *das*, but did not differ significantly from the inoculated control. At 12 *das* there was an increase of 110.5% in the activity of the enzyme compared with the inoculated control. Plants sprayed only with ASM obtained maximum enzyme activity at 12 *das* with a 266.03% increase compared with the control. In tomato plants sprayed with OECR and inoculated with *X. vesicatoria* two periods of increased activity of the enzyme were noted: 5 to 6 *das* and 9 to 12 *das* (Fig. 3F). The maximum enzyme activity occurred at 6 *das*, but this was non-significant for the inoculated treatment. At 12 *das* enzyme activity showed a 62.14% increase compared with the inoculated control. Plants sprayed with only OECR obtained maximum enzyme activity at 2 *das* with a 93.28% increase compared with the control. Nevertheless, it was observed that the peroxidase activity of plants sprayed with oil remained higher throughout the harvest period compared to the control, a fact at times not as significant as at 9 *das*.

Levels of soluble lignin concentrations were higher in plants sprayed with OECR and ASM, with and without inoculation of *X. vesicatoria*, than in the control. Treated and inoculated plants showed small increments of lignin in relation to the non-inoculated ones.

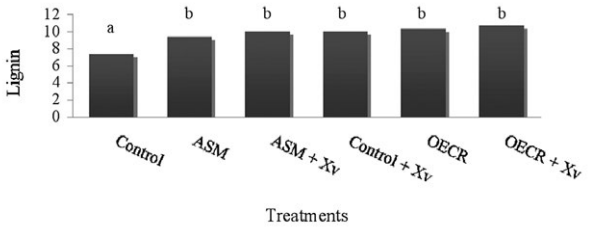

Fig. 4. Lignin content (mg g^{-1} MS^{-1}) in leaves of tomato seedlings 12 days after treatment with: ASM - acibenzolar-S-methyl and Indian clove essential oil (OECR) compared with control sample (Control). Inoculation with *Xanthomonas vesicatoria* (Xv) occurred 4 days after spraying. Means followed by same letter do not differ among themselves through the Tukey test ($p<0.05$).

DISCUSSION

Only Indian clove essential oil (OECR) 0.1% and acibenzolar-S-methyl (ASM) 0.2 mg ml^{-1} showed no direct effect on the growth of *X. vesicatoria*. Kessmann *et al.* (1994) reported that chemical or biological agents to be considered as inducers of resistance should not have direct inhibitory activity on the plant-pathogenic microorganism. However, nowadays this concept is more flexible, and various compounds considered resistance inducers exert also a direct action on the pathogens. Even when a direct effect on the *in vitro* growth of *X. vesicatoria* was not observed, greenhouse tests provided evidence that OECR 0.1% promoted progress in reducing the severity of tomato bacterial spot. This treatment was more effective than the copper-based fungicide recommended for the crop.

OECR 0.1% and ASM 0.2 mg ml^{-1} increased the activity of β-1,3-glucanase, chitinase and peroxidase, and increased lignin content in tomato plants. It was observed in this study that OECR sometimes promoted increased activity of these enzymes more effectively than ASM, which in fact underlines the promising use of oil as an inducer of resistance in tomato. Little is known about the correlation between chitinases and their hydrolytic roles in attacking bacterial infection of the plant, but there are reports that endochitinases with lysozyme activity bonds can hydrolyze β-1,4 between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell wall peptidoglycan (Majeau *et al.*, 1990). Thus, it can be said that an arsenal of defense was elicited after spraying the plants with ASM and OECR.

The participation of peroxidase in tomato - induced resistance to *X. vesicatoria* has previously been demonstrated by Cavalcanti *et al.* (2006), which was evident in the activity of these enzymes in the first few hours after spraying the plants with ASM, with greatest activity occurring on the fifth day post inoculation. A similar phenomenon was observed by Ribeiro Júnior *et al.* (2004) in tomato plants treated with ASM, which showed peaks of peroxidase in plants, both inoculated and non-inoculated with *X. vesicatoria*, at 9 *das*. According to Silva *et al.* (2007), peroxidase activity involves a variety of processes related to plant defense, including hypersensitivity reaction, lignification and curing. In tomato, peroxidase is an enzyme involved in the last step of lignification. The reinforcement of the cell wall by lignin and phenolic compounds is characterized as one of the reactions elicited by the defense system of plants, increasing resistance to enzymatic degradation by pathogens and acting as a mechanical barrier to the entry of toxins and the physical penetration and restriction of colonization of tissue by pathogens (Resende *et al.*, 2007).

Treated and inoculated plants showed small increments of lignin in relation to the non-inoculated ones. This phenomenon was also observed by Pereira *et al.* (2008) in coffee, which, according to the authors, may have occurred because of host recognition of the pathogen. ASM is recommended and marketed as an inducer of resistance in tomato, while OECR, which is 100% natural, has been shown to be a promising product, especially as a substitute for ASM and copper-based fungicides in organic farming. It should also be useful in the management of disease in conventional crops, preventing the emergence of pathogens resistant to these products.

The ultimate goal of this study was to obtain through

the use of OECR a new strategy to control diseases that can be passed on to farmers, for both conventional and organic agriculture, in order to cut costs and reduce the environmental damage caused by the indiscriminate use of pesticides.

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