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Biological control of tomato bacterial spot by saprobe fungi from semi-arid areas of northeastern Brazil

Controle biológico da mancha bacteriana do tomateiro usando fungos sapróbios do semiárido nordestino do Brasil

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Abstract

Tomato bacterial spot caused by *Xanthomonas* spp., is a common disease in tomato fields that causes significant economic losses. Due to the difficulty with control of bacterial spot by conventional methods, new techniques such as biological control and induction of resistance are gaining prominence. This study aimed to select saprobe fungi from semi-arid regions of the Brazilian Northeast for the biological control of bacterial spot of tomato. To select the best isolates to control bacterial spot, a greenhouse experiment was initially conducted. Tomato plants ('Santa Cruz Kada') were treated with filtrates of 25 saprobe fungi and inoculated three days later with *Xanthomonas euvesicatoria*. Filtrates of *Memnoniella levispora*, *Periconia hispidula*, *Zygosporium echinosporum,* and *Chloridium virescens* var. *virescens* were selected as the most effective. Filtrates and volatile compounds from these four isolates were tested for their antibacterial activity in cultures of *X. euvesicatoria* and in tomato plants ('Santa Cruz Kada') inoculated with *X. euvesicatoria*. *In vitro,* the addition of nonvolatile fungal metabolites into the culture medium at 5% and 50% (v/v) inhibited bacterial growth by 28.9% and 53.8%, respectively. The volatile compounds produced by *C. virescens* var. *virescens* reduced the number of colony-forming units of *X. euvesicatoria* by 25.9%. *In vivo*, all treatments reduced from 62.4 to 71.3% the area under bacterial spot progress curve, showing the same control efficacy as the commercial resistance inducer used as a positive control (acibenzolar-*S*-methyl). Systemicity of the fungal filtrates was confirmed in a separate experiment, where application of the treatments exclusively to the third leaf decreased the severity of the disease on the fourth leaf (except for *C. virescens* var. *virescens*). These results show that *M. levispora*, *P. hispidula*, *Z. echinosporum,* and *C. virescens* var. *virescens* are potential biocontrol agents against tomato bacterial spot. Further studies are necessary to elucidate the disease control mechanisms of these saprobe fungi.

Key words: Acibenzolar-*S*-methyl. Antibiosis. Fungal metabolite. *Xanthomonas euvesicatoria*.

Resumo

A mancha bacteriana do tomateiro causada por *Xanthomonas* spp. é uma doença frequente nos campos de cultivo, provocando importantes perdas econômicas. Devido à dificuldade de controle com os métodos tradicionais, novas ferramentas de manejo para esta doença, como o controle biológico e a indução de resistência vêm ganhando destaque. O objetivo desse trabalho foi a seleção de fungos sapróbios do semiárido nordestino brasileiro para o controle biológico da mancha bacteriana do tomateiro.

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Inicialmente, com o objetivo de selecionar os isolados que apresentassem melhor controle da mancha bacteriana, foi montado um ensaio em casa de vegetação com tomateiros ('Santa Cruz Kada') tratados com os filtrados de 25 fungos sapróbios e inoculados com a bactéria *Xanthomonas euvesicatoria* três dias depois. Foram selecionados os isolados de *Memnoniella levispora, Periconia hispidula, Zygosporium echinosporum* e *Chloridium virescens* var. *virescens*. Filtrados e voláteis fúngicos desses quatro isolados foram testados *in vitro,* para se determinar a sensibilidade da bactéria fitopatogênica, e *in vivo* em tomateiros ('Santa Cruz Kada') tratados e inoculados com *X. euvesicatoria.* A incorporação de metabólitos não voláteis dos fungos no meio de cultura a 5% e 50% (v/v) controlou o crescimento da bactéria em até 28,9% e 53,8%, respectivamente. Os compostos voláteis produzidos por *C. virescens* var. *virescens* inibiram até 25,9% das unidades formadoras de colônias. Com relação ao experimento *in vivo*, todos os tratamentos testados reduziram de 62,4 a 71,3% a área abaixo da curva de progresso da mancha bacteriana, mostrando nível de controle igual ao indutor de resistência comercial utilizado como controle positivo (acibenzolar-*S*-metílico). A sistemicidade deste controle foi confirmada em outro experimento, onde a aplicação dos tratamentos somente na terceira folha determinou menor severidade da doença na quarta folha (exceto para *C. virescens* var. *virescens*). Conclui-se que *M. levispora, P. hispidula, Z. echinosporum* e *C. virescens* var. *virescens* apresentaram potencial de biocontrole da mancha bacteriana do tomateiro. Estudos futuros são necessários para elucidar a natureza do controle da doença exercido por estes fungos sapróbios.
Palavras-chave: Acibenzolar-*S*-metílico. Antibiose. Metabólitos fúngicos. *Xanthomonas euvesicatoria.*

Introduction

Tomato (*Solanum lycopersicum* L.) is the most commonly cultivated vegetable in Brazil (IBGE, 2013). The national production in 2013 was 4.19 million tons in a total area of 66.8 thousand hectares (FAO, 2016). The cultivation is affected by several diseases including bacterial spot, which can be caused by *Xanthomonas vesicatoria*, *X. euvesicatoria*, *X. perforans,* or *X. gardneri* (JONES et al., 2004). In Brazil, the most frequent causative agent of bacterial spot in tomato is *X. perforans* (ARAÚJO et al., 2017), whereas *X. euvesicatoria* is the predominant species in bell pepper crops (AREAS et al., 2015). Bacterial spot is a severe disease that can affect the leaves, fruits, flowers, and stems of tomato plants and ultimately cause the loss of these organs (BYRNE et al., 2005; LOPES; ÁVILA, 2005). In addition to production losses, the disease also causes fruit depreciation due to disease lesions and sun scalding associated with defoliation caused by the disease (BYRNE et al., 2005).

Conventional control methods for bacterial spot include crop rotation, the use of certified healthy seeds, uncontaminated transplants, adequate disposal of crop residues, and application of cupric bactericides (BYRNE et al., 2005). Although the use of resistant varieties is an efficient practice for disease control, tomato bacterial spot resistance is difficult to obtain and can be overcome by the pathogenic bacteria over time (JONES et al., 1998; RITCHIE, 2007). The chemical control of bacterial spot by the application of cupric bactericides alone or in combination with the fungicide mancozeb, is currently the most popular method among the growers of commercial tomato crops. However, under environmental conditions favorable to the development of the disease (high temperatures, rainfall, and especially rainfall with wind), chemical control methods have been shown to be ineffective (PERNEZNY et al., 2012). In addition, cupric bactericides have lost efficacy for the control of bacterial spot because of the emergence of copper-resistant isolates (MARTIN et al., 2004). These chemicals may persist at sites beyond the target, such as the soil and water, with residence time varying according to chemical properties (soil adsorption capacity and degradation susceptibility) and environmental factors (soil type, rainfall, topography, and agricultural practices). Furthermore, they may have adverse effects, including toxicity to beneficial soil microorganisms, fish, and aquatic invertebrates (WIGHTWICK et al., 2010). There is a need for new bactericidal products for plant

disease control with low toxicity to humans and to the environment.

Chemical compounds that activate plant defenses, which are termed systemic acquired resistance (SAR) inducers, such as acibenzolar-*S*-methyl (ASM), have shown bacterial spot control efficacies comparable to those of cupric bactericide and mancozeb treatments (PERNEZNY et al., 2012). Biological control methods using bacteriophages specific for *X. vesicatoria* strains (FLAHERTY et al., 2000; OBRADOVIC et al., 2004, 2005), antagonistic bacteria applied to the phylloplane (BYRNE et al., 2005; EL-HENDAWY et al., 2005; ROBERTS et al., 2008), SAR-inducing rhizobacteria (FERRAZ et al., 2015), SAR-inducing endophytic bacteria (LANNA-FILHO et al., 2013), aqueous extracts containing various organic compounds (AL-DAHMANI et al., 2003), and avirulent strains that antagonize natural virulent strains (MOSS et al., 2007) have shown varying efficacies for bacterial spot control.

Saprobe fungi have desirable characteristics for use in biological control (BARROS et al., 2015) and a resistance-inducing potential (RESENDE et al., 2015; YEDIDIA et al., 2003). They can secrete pectinases, induce oligogalacturonides release; and activate the plant defense responses (BARROS et al., 2015). The use of saprobe fungi from semi-arid Brazilian caatinga ecoregion was favored due to their potential as biocontrol agents, including their ability to remove nutrients from dead tissue, resistance to water restriction, and competitiveness with other microorganisms (PINTO, 2013; RESENDE et al., 2015).

The aim of this study was to select saprobe fungi from semi-arid regions of the Brazilian Northeast for the biological control of bacterial spot of tomato.

Materials and Methods

Collection and preservation of saprobe fungi and Xanthomonas euvesicatoria isolates

Saprobe fungi were collected in the semi-arid region of the Brazilian Northeast and belong to the Collection of Cultures of Microorganisms of Bahia (CCMB), State University of Feira de Santana, BA, Brazil (CNPq process number 010044/2014-0). Twenty-five fungal isolates (Table 1) were tested. The isolates were kept at 25 °C in Petri dishes containing potato dextrose agar (PDA) culture medium (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2 at 25 °C) with a photoperiod of 12 h.

We used *X. euvesicatoria* isolate 81-23 (STALL et al., 1986). Phytobacteria was kept in nutrient agar culture medium (NA; 3 g L^{-1} meat extract, 5 g L^{-1} peptone, 15 g L^{-1} agar, pH 6.8 ± 0.2) at 28 °C in darkness. The isolate was characterized by polymerase chain reaction (PCR) using primers specific for the four pathogenic bacterial species that cause bacterial spot in tomato. The bacterial DNA was extracted according to the protocol of Dellaporta et al. (1983), and the primers were designed by Koenraadt et al. (2007). The reaction conditions and thermal cycler programs matched those used by Areas et al. (2015).

Preparation of fungal filtrates

To obtain the fungal filtrates, saprobe fungi were cultured in flasks containing 100 mL of potato dextrose broth (PD) (200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, pH 5.6 ± 0.2), incubated at 25 ± 2 °C with a photoperiod of 12 h for 10 days, and then incubated at 5 ± 2 °C for a further 48 h. The supernatant was separated with filter paper (Whatman No. 2; Whatman, Maidstone, UK) and centrifuged twice for 15 min at 5000 rpm to remove the remaining fungal structures. The supernatant was stored at 5 °C.

Table 1. Severity (percentage of affected leaf area) and area under disease progress curve (AUDPC) of bacterial spot of tomato at 7, 14, and 21 days after inoculation (DAI) with *Xanthomonas euvesicatoria* in plants treated with saprobe fungi filtrates. Londrina, 2015.

Treatments	7 DAI	14 DAI	21 DAI	AUDPC	% reduction AUDPC
Negative control	15.0 a[1]	23.0 a	52.0 a	22.03 a	---
Phialomyces macrosporus	6.0 c	21.0 a	50.0 a	18.05 a	18.05
Pseudobotrytis terrestris	13.0 a	19.0 a	39.0 c	17.70 a	19.64
Clonostachys rosea	11.0 b	18.0 a	42.0 c	17.23 a	21.80
Myrothecium sp. isolate 2	8.0 c	19.0 a	45.0 b	17.13 a	22.26
Dictyosporium tetraseriale	9.0 b	19.0 a	36.0 c	15.88 b	27.93
Beltrania rhombica	6.0 c	14.0 a	40.0 c	13.85 b	37.11
Sarcopodium circinatum	7.0 c	18.0 a	29.0 d	13.65 b	38.04
Thozetella submersa	5.0 c	16.0 a	35.0 c	13.35 b	39.38
Myrothecium sp. isolate 1	10.0 b	18.0 a	21.0 e	13.23 b	39.96
Memnoniella echinata	5.0 c	14.0 a	38.0 c	13.18 b	40.18
Dictyochaeta heteroderae	7.0 c	17.0 a	28.0 d	13.13 b	40.41
Volutella minima	8.0 c	16.0 a	28.0 d	13.10 b	40.53
Beltrania copaifera	5.0 c	18.0 a	29.0 d	13.00 b	40.97
Curvularia inaequalis	8.0 c	18.0 a	19.0 e	12.23 b	44.50
Stachybotrys chartarum	7.0 c	15.0 a	23.0 d	11.55 c	47.56
Gonytrichum chlamydosporium	6.0 c	16.0 a	21.0 e	11.23 c	49.03
Stachybotrys globosa	4.0 c	12.0 b	28.0 d	10.40 c	52.78
Lappodochium lageniforme	5.0 c	10.0 b	27.0 d	9.85 c	55.28
Curvularia eragrostidis	5.0 c	8.0 b	26.0 d	8.98 c	59.25
Beltraniella portoricensis	6.0 c	12.0 b	16.0 f	8.95 c	59.37
Gonytrichum macrocladum	6.0 c	12.0 b	15.0 f	8.78 c	60.16
ASM*	5.0 c	12.0 b	14.0 f	8.28 c	62.43
Periconia hispidula	5.0 c	7.0 b	20.0 e	7.58 c	65.60
Zygosporium echinosporum	5.0 c	6.0 b	21.0 e	7.40 c	66.39
CChloridium virescens var. *virescens*	5.0 c	8.0 b	15.0 f	7.05 c	68.00
Memnoniella levispora	5.0 c	8.0 b	13.0 f	6.70 c	69.58
C.V. (%)	40.3	19.8	13.6	11.8	

[1] Mean values followed by the same letter are not significantly different from each other according to the Scott-Knott test at 5% probability. *Acibenzolar-*S*-methyl.

Screening of saprobe fungi for their ability to control bacterial spot at greenhouse conditions

The experiment was designed in randomized blocks of 27 treatments and five replicates. Tomato seedlings were grown in 180-cell trays using a commercial substrate (Tropstrato HT Hortaliças®, Vida Verde, Mogi Mirim, SP, Brazil) and 'Santa Cruz Kada' cultivar. Transplanting was performed 21 days after sowing in 1-L pots containing a mixture of soil (eutroferric red latosol) and sand in the ratio 1:2 (v/v).

The 25 isolates of saprobe fungi were individually cultured in 100 mL of PD medium and incubated at 25 ± 2 °C with a photoperiod of 12 h for 10 days. Next, 100 mL of sterilized distilled water was added and the isolates were homogenized in a blender. The treatments were applied on both sides of the tomato leaves using cotton swabs. The commercial resistance inducer ASM (Bion®; Syngenta Crop Protection, Inc., Greensboro, NC) was used as a positive control at a dose of 30 g ha^{-1}. Plants treated only with distilled sterilized water served as a negative control.

Bacterial inoculation was performed three days after application of the treatments. Colonies grown for 18 h in NA were used. The bacterial suspension was adjusted to a concentration of 10^8 colony-forming units (CFU) mL^{-1} in saline (0.85% NaCl) and inoculated with sprayer. The plants were kept in a humid chamber for 48 h before and after the inoculation. The percentage of leaf area affected was evaluated weekly on the same leaf, according to the scale proposed by Mello et al. (1997). The area under disease progress curve (AUDPC) was calculated using the data from the three assessments. The AUDPC was calculated according to Shaner and Finney's formula (1977) and normalized by dividing the AUDPC value by the total time duration (number of days from the first occurrence of the disease until the end of the observation period) (FRY, 1978). The formula used to obtain this normalized AUDPC was as follows:

$$\sum_{i=1}^{n} \frac{[(X_i + X_{i+1})/2][t_{i+1} - t_i]}{(T_n - T_1)}$$

where X_i is the proportion of diseased host tissue at the i^{th} evaluation, t_i is the time in days at the i^{th} evaluation, and n represents the total number of observations.

The four fungal isolates with the lowest AUDPC values were selected.

In vitro effects of saprobe fungi filtrates on Xanthomonas euvesicatoria

The experimental design was completely randomized, with five replicates per treatment. Filtrates from the four selected fungi were incorporated into liquid NA medium at 5% and 50% (v/v) and plated. Bacterial suspension was obtained from colonies grown for 18 h in NA culture medium at 30 °C. Next, 100-µL aliquots of the bacterial suspension at the concentration of 10^8 CFU mL^{-1} were spread with a Drigalski spatula on the prepared NA plates and incubated at 25 ± 2 °C for three days, after which the number of colonies was evaluated. A negative control was prepared by the addition of PD medium.

In vitro effects of volatile compounds on Xanthomonas euvesicatoria

The experimental design was completely randomized, with five replicates per treatment. Two-sections polystyrene Petri dishes were used. PDA culture medium was placed on one side of the plate, and 5-mm diameter discs of the saprobe fungi were transferred and incubated at 25 ± 2 °C with a photoperiod of 12 h for seven days. Next, NA culture medium was placed on the opposite side of the plate, and a 100-µL aliquot of the bacterial suspension (10^8 CFU mL^{-1}) was dispersed with a Drigalski spatula, and incubated under the conditions described above. The bacterial colonies were evaluated after three days.

Control of tomato bacterial spot by saprobe fungi in tomato plants

The experiment was designed in randomized blocks of six treatments and five replicates. Seedlings were grown in 180-cell trays using a commercial substrate and 'Santa Cruz Kada' tomato cultivar. Transplanting was performed 21 days after sowing in 1-L pots containing a mixture of soil (eutroferric red latossol) and sand in the ratio 1:2 (v/v).

The isolates of the four selected saprobe fungi were cultured in 100 mL of PD medium and incubated at 25 ± 2 °C with a photoperiod of 12 h for 10 days. Next, 100 mL of distilled sterilized water were added and the suspensions were homogenized in a blender. The treatments were applied on both sides of the tomato leaves using cotton swabs. The commercial resistance inducer ASM (30 g ha^{-1}) was used as a positive control and plants treated with distilled sterilized water represented the negative control.

Bacterial inoculation was performed three days after the treatments using colonies cultured for 18 h in NA medium. The bacterial suspension was adjusted to a concentration of 10^8 CFU mL^{-1} in saline (0.85% NaCl) and inoculated onto the plants using a sprayer. The plants were kept in a humid chamber for 48 h before and after inoculation. The percentage of leaf area affected was evaluated weekly on the third leaf according to the scale proposed by Mello et al. (1997). The normalized AUDPC was calculated using the data from the three evaluations.

Systemicity of bacterial spot control by saprobe fungi in tomato plants

The experiment was designed in randomized blocks of six treatments and five replicates. Filtrates of the four selected saprobe fungi isolates were used, according to the methodology described above, except that the treatment was applied exclusively on the third leaf. The severity of the disease was evaluated on the third and fourth leaves. The AUDPC was calculated using severity assessment data.

Statistical analysis

Data were subjected to analysis of variance at the 0.05 significance level. When treatments effects were significant, the means were separated by comparison tests. The Scott-Knott clustering test ($p \leq 0.05$) was used to compare means among treatment groups in the experiment with 25 fungi isolates, and Tukey's test ($p \leq 0.05$) was used to compare means among treatment groups in the experiments with four saprobe fungi.

Results and Discussion

The screening of saprobe fungi for their ability to control bacterial spot at greenhouse conditions revealed that *Phialomyces macrosporus*, *Pseudobotrytis terrestris*, *Clonostachys rosea*, and *Myrothecium* sp. isolate 2 did not significantly alter disease progress compared with the negative control (water), with AUDPC values between 17.13 and 22.03 (Table 1). Treatment with *Dictyosporium tetraseriale*, *Beltrania rhombica*, *Sarcopodium circinatum*, *Thozetella submersa*, *Myrothecium* sp. isolate 1, *Memnoniella echinata*, *Dictyochaeta heteroderae*, *Volutella minima*, *B. copaifera*, and *Curvularia inaequalis* showed statistically significant intermediate control, with reductions of AUDPC ranging from 27.93 to 44.50%. The most effective isolates to control bacterial spot were *Stachybotrys chartarum*, *Gonytrichum chlamydosporium*, *Stachybotrys globosa*, *Lappodochium lageniforme*, *Curvularia eragrostidis*, *Beltraniella portoricensis*, *G. macrocladum*, ASM, *Periconia hispidula*, *Zygosporium echinosporum*, *Chloridium virescens* var. *virescens*, and *Memnoniella levispora*, with reductions of AUDPC ranging from 47.56 to 69.58% (Table 1).

ASM is a commercial systemic acquired resistance (SAR) inducer, that provides the plant with protection against pathogens and causes the expression of molecular and biochemical markers in the plant, but exerts no direct action against the pathogen (KESSMANN et al., 1994). The induction of SAR to bacterial spot by ASM has been demonstrated in tomato and pepper (OBRADOVIC et al., 2005; ROBERTS et al., 2008; ROMERO et al., 2001).

P. hispidula, *Z. echinosporum*, *C. virescens* var. *virescens*, and *M. levispora* showed the

highest efficacies for the control of bacterial spot in greenhouse tomato plants and they were therefore selected for additional experiments. These saprobe fungi isolates were then tested for their growth inhibitory effects on *X. euvesicatoria*. The addition of *Z. echinosporum* and *C. virescens* var. *virescens* filtrates to the culture medium at 5% (v/v) produced the highest inhibition of bacterial growth, with inhibition of colony-forming units of 21.9% and 28.9% (assay 1) and 16.0% and 27.8% (assay 2), respectively (Table 2). *P. hispidula* did not significantly inhibit bacterial growth compared with the negative control. The *M. levispora* filtrate inhibited bacterial growth by 7.0% in assay 1, which was significantly higher than that observed for the negative control and *P. hispidula*, although this growth inhibitory effects were lower than those of the *Z. echinosporum* and *C. virescens* var. *virescens* treatments (Table 2).

Table 2. Inhibition of colony-forming units (CFUs per plate) of *Xanthomonas euvesicatoria* by addition of saprobe fungi filtrates to the bacterial culture medium at two concentrations (v/v). Londrina, 2015.

Treatments	**5%**			**50%**		
Assay 1	**CFU plate^{-1}**		**% inhibition**	**CFU plate^{-1}**		**% inhibition**
Negative control	201	Aa[1]	-	201	Aa	-
Memnoniella levispora	187	Ab	7.0	154	Bb	23.4
Periconia hispidula	198	Aa	1.5	164	Bb	18.4
Zygosporium echinosporum	157	Bc	21.9	103	Bc	48.8
Chloridium virescens var. *virescens*	143	Bc	28.9	98	Bc	51.2
C.V. (%)	3.7			4.1		
Assay 2						
Negative control	212	Aa	-	212	Aa	-
Memnoniella levispora	198	Aa	6.6	162	Bb	23.6
Periconia hispidula	200	Aa	5.7	178	Bb	16.0
Zygosporium echinosporum	178	Ab	16.0	102	Bc	51.9
Chloridium virescens var. *virescens*	153	Ac	27.8	98	Bc	53.8
C.V. (%)	5.6			5.3		

[1]Mean values followed by the same lowercase letters vertically and capital letters horizontally are not significantly different from each other according to Tukey's test at 5% probability.

All the treatments produced significant bacterial growth inhibition in comparison to the negative control upon the addition of the filtrates to the culture medium at the 50% (v/v). The bacterial growth inhibitory effects of *M. levispora* (23.4 to 23.6%) and *P. hispidula* (16.0 to 18.4%) did not significantly differ from each other. The bacterial growth inhibitory effects of *Z. echinosporum* (48.8 to 51.9%) and *C. virescens var. virescens* (51.25 to 53.8%) were significantly greater than those of the other isolates. Significant difference was observed between the tested concentrations of the fungal filtrates (Table 2); notably, bacterial growth inhibition was higher at 50% than at 5%.

Other authors have observed that saprobe fungi have antagonistic and fungitoxic effects on some other fungi. Silva et al. (2008) evaluated the *in vitro* antagonistic activity of *Trichoderma* spp. on *Phytophthora citrophthora* using the paired culture method. Those authors found that *T. stromaticum* had the highest antagonistic effect. Balbi-Peña et al. (2012) and Barros et al. (2015) studied *in vitro* antagonistic effect of saprobe fungi *S. globosa* and *M. levispora* on *Sclerotinia sclerotiorum*. Also, *C. virescens* var. *virescens* had an *in vitro* inhibitory effect on the mycelial growth of *Colletotrichum gloeosporioides*, the causal agent of anthracnose of guava trees, which was associated with competition for space, nutrients, and/or antiobiosis (ALVES,

2013). According to Sid Ahmed et al. (2003) the presence of diffusible antifungal metabolites in the culture medium inhibits mycelial growth by promoting cell disruption and causing the lysis of hyphae.

The incubation of *X. euvesicatoria* with saprobe fungi colonies in 2-sections divided dishes indicated that only *C. virescens* var. *virescens* produced volatile compounds capable of reducing the number of bacterial CFUs (~25%) (Table 3). Volatile compounds produced by *P. hispidula* and *Z. echinosporum* led to an increase in the number of bacterial CFUs per plate relative to the negative control. Volatile compounds of *M. levispora* did not alter the number of *X. euvesicatoria* colonies.

Table 3. Effects of volatile compounds of saprobe fungi on the colony-forming units (CFUs per plate) of *Xanthomonas euvesicatoria*. Londrina, 2015.

Treatments	CFU plate^{-1}		% inhibition
Assay 1			
Negative control	245	b[1]	----
Memnoniella levispora	266	b	-8.6
Periconia hispidula	385.6	a	-57.4
Zygosporium echinosporum	299.6	a	-22.3
Chloridium virescens var. *virescens*	185.2	c	24.4
C.V. (%)	3.7		
Assay 2			
Negative control	283	b	----
Memnoniella levispora	299	b	-22.0
Periconia hispidula	401.3	a	-63.8
Zygosporium echinosporum	399.6	a	-63.1
Chloridium virescens var. *virescens*	181.6	c	25.9
C.V. (%)	4.2		

[1]Mean values followed by the same letter in the column are not significantly different according to Tukey's test at 5% probability.

Volatile organic compounds are low-molecular-weight molecules. Because they are gaseous compounds, their diffusion into biological plasma membranes is favored, and they may represent a good strategy for phytopathogen control (DUDAREVA et al., 2006). The fungus *Muscodor albus* produces volatile organic compounds (alcohols, esters, ketones, acids, and lipids) that inhibit phytopathogenic fungi and bacteria (STROBEL et al., 2001). Similarly, *Muscodor crispans* produces organic compounds capable of inhibiting certain phytopathogens *in vitro*, including *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker (MITCHELL et al., 2010).

In the present study, the preventive treatment in the greenhouse tomato plants with the four selected saprobe fungi filtrates (Table 4) reduced AUDPC by approximately 70% in both assays (not significantly different from ASM) seven days after inoculation (DAI). Treatments with the four selected saprobe fungi filtrates and ASM in the second assay reduced the disease severity compared with the negative control. Plants treated with *Z. echinosporum* with 7% of the leaf area affected by bacterial spot were statistically different from plants treated with *P. hispidula* with 3% of the leaf area affected but neither of these fungal treatments was different from the positive control (ASM; Table 4).

Table 4. Severity (percentage of affected leaf area) and area under disease progress curve (AUDPC) of bacterial spot of tomato plants at 7, 14, and 21 days after inoculation (DAI) with *Xanthomonas euvesicatoria* in plants previously treated with saprobe fungi filtrates. Londrina, 2015.

Treatments	Affected leaf area (%)			AUDPC	% reduction AUDPC
Assay 1	**7 DAI**	**14 DAI**	**21 DAI**		
Negative control	15.0 a[1]	23.0 a	52.0 a	22.1 a	
Positive control (ASM)[2]	5.0 b	12.0 b	14.0 b	8.3 b	62.4
Periconia hispidula	5.0 b	7.0 b	20.0 b	7.6 b	65.6
Zygosporium echinosporum	5.0 b	6.0 c	21.0 b	7.4 b	66.5
Chloridium virescens var. *virescens*	5.0 b	8.0 b	15.0 b	7.2 b	67.4
Memnoniella levispora	5.0 b	8.0 b	13.0 c	6.7 b	69.9
C.V. (%)	40.3	19.8	13.6	11.8	
Assay 2					
Negative control	15.0 a	23.0 a	54 a	23.4 a	
Positive control (ASM)	5.0 bc	12.0 b	14.0 d	8.2 b	64.9
Periconia hispidula	3.0 c	7.0 b	20.0 bc	7.5 b	67.9
Zygosporium echinosporum	7.0 b	7.0 b	21.0 b	7.4 b	68.3
Chloridium virescens var. *virescens*	5.0 bc	8.0 b	15.0 cd	7.1 b	69.6
Memnoniella levispora	5.0 bc	8.0 b	13.0 d	6.7 b	71.3
C.V. (%)	41.3	22.0	15.8	10.9	

[1]Mean values followed by the same letter in a column are not significantly different according to Tukey's test at 5% probability.
[2]Acibenzolar-*S*-methyl.

At the second evaluation (14 DAI) of the first assay all the treatments with the fungal filtrates and ASM reduced bacterial spot severity. Treatment with *Z. echinosporum* resulted in a significantly smaller affected leaf area than ASM, demonstrating higher disease control. In the second assay, all the treatments also reduced bacterial spot severity but there were no significant differences between control efficacies of the fungal filtrates and ASM.

In the third evaluation (21 DAI), all the treatments with the four fungal filtrates and ASM again reduced disease severity. The treatment with *M. levispora* in the first assay resulted in a significantly smaller affected leaf area compared to the other fungal isolates and the ASM positive control. In the second assay, all the saprobe filtrates and ASM significantly controlled the disease, with the *M. levispora*, ASM, and *C. virescens* var. *virescens* treatments resulting in the lowest affected leaf areas (13%, 14%, and 15%, respectively).

With respect to the AUDPC, both assays showed that all the treatments with saprobe fungi filtrates provided efficient control of bacterial spot in tomato plants, similar to that of ASM.

According to Pernezny et al. (2012), the most promising biological control agents for bacterial spot of tomato are bacteriophages specific for *X. vesicatoria*, which should be applied preventively. Other biocontrol agents and methods, such the application to the phylloplane of *Cellulomonas turbata*, *Pseudomonas syringae*, *Pseudomonas putida*, hrp (*hypersensitive response and pathogenicity*) mutant isolates of *X. campestris* pv. *vesicatoria*, *Rahnella aquatilis*, or *Bacillus subtilis* (BYRNE et al., 2005; MOSS et al., 2007; EL-HENDAWY et al., 2005; ROBERTS et al., 2008), have been previously tested for the control of bacterial spot. However, studies on the effects of fungal metabolites on plant bacteria control are scarce in the literature. Botrel (2013) tested saprobe

fungi cultures for the control of coffee tree spot (caused by *Pseudomonas syringae* pv. *garcae*) in Brazil. That author found that the fungus *M. levispora* reduced the severity of the disease in curative applications, showing the same AUDPC values as the ASM positive control. *M. levispora* also induced rooting of eucalyptus cuttings and inhibited the germination of urediniospores of *Puccinia psidii* (PIEROZZI, 2013).

Table 5. Severity (percentage of affected leaf area) and area under disease progress curve (AUDPC) of bacterial spot of tomato plants on their third and fourth leaves at 7, 14, and 21 days after inoculation (DAI) with *Xanthomonas euvesicatoria* in plants previously treated with saprobe fungi filtrates only on the third leaf. Londrina, 2015.

Treatments	3rd Leaf			
	Affected leaf area (%)			AUDPC
	7 DAI	14 DAI	21 DAI	
Negative control	15.0 a[1]	23.0 a	52.0 a	22.1 a
Positive control (ASM)[2]	5.0 b	12.0 b	14.0 b	8.3 b
Periconia hispidula	5.0 b	7.0 b	20.0 b	7.6 b
Zygosporium echinosporum	5.0 b	6.0 c	21.0 b	7.4 b
Chloridium virescens var. *virescens*	5.0 b	8.0 b	15.0 b	7.2 b
Memnoniella levispora	5.0 b	8.0 b	13.0 c	6.7 b
C.V. (%)	40.3	19.8	13.6	11.8
			4th Leaf	
Negative control	15.0 a	25.0 a	58.0 a	21.3 a
Positive control (ASM)	5.0 c	12.0 b	14.0 d	8.1 b
Periconia hispidula	5.0 c	7.0 b	20.0 cd	7.3 b
Zygosporium echinosporum	5.0 c	6.0 b	21.0 c	7.1 b
Chloridium virescens var. *virescens*	10.0 b	20.0 a	50.0 b	18.3 a
Memnoniella levispora	4.8 c	6.0 b	12.0 d	6.9 b
C.V. (%)	24.5	23.1	8.9	11.7

[1]Mean values followed by the same letter in a column are not significantly different according to Tukey's test at 5% probability.
[2]Acibenzolar-*S*-methyl.

The assay intended to verify systemicity of the control of bacterial spot by the saprobe fungi filtrates revealed that all the treatments with the four selected saprobe filtrates and ASM presented a lower disease severity in the third leaf (7 DAI) than the negative control did (Table 5). As for the fourth leaf, it was found that the filtrates of *P. hispidula*, *Z. echinosporum*, *M. levispora*, as well as the ASM positive control, all presented a lower affected leaf area (~5%) than the negative control did. In addition, all the treatments resulted in lower disease severity than the negative control did in the third leaf at 14 DAI. The treatment with *Z. echinosporum* filtrates resulted in a significantly smaller affected leaf area than the ASM positive control did. Except for the *C. virescens* var. *virescens* treatment, all treatments resulted in a lower disease severity in the fourth leaf. At 21 DAI, all the treatments exerted significant bacterial control in the third leaf; in particular, the treatment with *M. levispora* showed a significantly higher control efficacy than the other treatments. Lower percentages of diseased area on the fourth leaf were observed after treatment with the filtrates of *P. hispidula*, *M. levispora*, and ASM in comparison to the negative control.

With respect to the AUDPC for the third leaf, all the treatments significantly controlled bacterial spot compared with the negative control, without significant differences with ASM. As for the fourth leaf, treatment with *C. virescens* var. *virescens* did

not show significant bacterial control compared to the negative control, while the other treatments with fungal filtrates reduced disease severity. These results indicate that the preventive treatment of tomato plants with the filtrates of *Z. echinosporum*, *M. levispora*, or *P. hispidula*, or with ASM, induced a systemic control of tomato bacterial spot and the filtrate of *C. virescens* var. *virescens* exerted a localized disease control.

Conclusions

The selected saprobe fungi (*Periconia hispidula*, *Zygosporium echinosporum*, *Chloridium virescens* var. *virescens*, and *Memnoniella levispora*) showed potential for the biological control of bacterial spot of tomato. This study revealed that these fungi produce nonvolatile compounds (all the fungi selected) and volatile ones (in the case of *C. virescens* var. *virescens*) that are toxic towards *X. euvesicatoria*. Although the activity of defense-related enzymes and other plant defense responses were not evaluated, systemicity of tomato bacterial spot control by preventive treatment with *P. hispidula*, *Z. echinosporum*, and *M. levispora* filtrates was verified, suggesting a possible induction of resistance to bacterial spot by compounds produced by these fungi. Biochemical and molecular defense responses of the plant should be addressed in future studies to elucidate the disease control mechanism of these saprobe fungi.

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