Trans-cinnamic acid and *Xenorhabdus szentirmai* metabolites synergize the potency of some commercial fungicides

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ABSTRACT

Development of novel approaches for the control of fungal phytopathogens is desirable. In this study we hypothesized that the combination of commercial fungicides with certain enhancing agents could result in synergistic levels of control. Prior research has indicated that *trans*-cinnamic-acid (TCA), a metabolite of the bacteria *Photorhabdus luminescens* and metabolites of *Xenorhabdus szentirmai* are particularly toxic to various phytopathogenic fungi when compared to metabolites of other *Xenorhabdus* or *Photorhabdus* spp. In this study we explored the efficacy of commercial fungicide interactions when combined with either TCA or *X. szentirmai*. Fungicides (active ingredient) included Abound® (Azoxytrobilin), Serenade® (*Bacillus subtilis*), Elast® (dodine), Regalia® (extract of *Reynoutria sachalinensis*), Prophyt® (potassium phosphite) and PropiMax® (propiconazole). In laboratory experiments, singly-applied or combined agents were assessed for fungicidal activity against four plant-pathogenic fungi, *Monilinia fructicola*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Fungicidal activity was measured by the phytopathogen's growth on potato dextrose agar with and without fungicide. The interactions between fungicidal agents were determined as antagonistic, additive or synergistic. For suppression of *M. fructicola*, synergy was observed between TCA when combined with certain concentrations of Elast®, PropiMax®, Regalia®, Prophyte® or Serenade®, and for combinations of *X. szentirmai* with Abound®. For suppression of *R. solani*, synergy was observed between TCA combined with Regalia® or Serenade®. Additionally, when TCA was combined with *X. szentirmai* synergistic levels of suppression to *M. fructicola* were observed. Other combinations of TCA or *X. szentirmai* with the fungicides or using alternate concentrations were either additive or occasionally antagonistic in nature. Our results indicate that TCA and *X. szentirmai* can each act as strong synergists to enhance fungicidal efficacy. These results may be used to reduce negative environmental impacts of pesticide use while improving control of plant diseases. Additional research is needed to explore the diversity of the synergistic effects and confirm our observations under field conditions.

1. Introduction

Xenorhabdus spp. and *Photorhabdus* spp. bacteria are mutualistically associated with entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis*, respectively (Lacey et al., 2015; Shapiro-Ilan et al., 2016). In nature, these nematodes are obligate lethal parasites of insects and the nematode/bacterium complex works together as a biological unit to kill insect hosts (Boemare et al., 1997; Fischer-Le Saux et al., 1999). In the infective juvenile, the bacterial cells are carried in a vesicle in the anterior part of the intestine for steinernematids and in the intestinal tract for heterorhabditids (Hazir et al., 2003; Stock, 2015).

Both nematode and bacteria contribute to the mutualistic relation-

ship (Forst and Clarke, 2002; Shapiro-Ilan et al., 2016). The symbiotic bacteria cannot survive outside the host and therefore, require the nematodes for dispersal from one insect to another. The bacteria also benefit from nematode-induced inhibition of the host's antibacterial proteins. On the other hand, inside the insect cadaver, the bacteria serve as a direct food source to the nematodes, or supply nutrients through degradation of the insect cadaver (Hazir et al., 2003).

For successful nematode reproduction, the insect cadaver has to be protected from secondary invasion by contaminating organisms and scavenging predators. Thus, another important function of the symbiotic bacteria is producing a wide variety of antibiotic or antagonistic compounds which adversely affect opportunist bacteria, fungi, viruses, protozoans (Kaya, 2002), and scavengers (Gulcu et al., 2012). To date

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several antimicrobial compounds such as xenorhabdins, xenocoumains, cabanillasin and a range of indole derivatives have been isolated from *Xenorhabdus* species (Webster et al., 2002; Houard et al., 2013). Likewise, antifungal and/or antibacterial products such as hydroxystilbenes, *trans*-stilbenes, *trans*-cinnamic acid (TCA), anthraquinone pigments and the toxin complex (Tc) have been isolated from *Photobacterium* species (Webster et al., 2002; Boemare and Akhurst, 2006; Bode, 2009; Bock et al., 2014).

There are issues with the use of commercial fungicides, especially due to negative environmental impacts and the development of resistance in many phytopathogens to the various classes of fungicide (Brent and Holloman, 2007; De Costa and Bezerra, 2009). Therefore, development of new fungicidal materials or approaches that are more benign to the environment or possess novel modes of action is desirable. *Xenorhabdus* or *Photobacterium* metabolites that possess anti-fungal properties can potentially be used as natural bio-fungicides against fungal pathogens of plants. The crude bacterial exudates and metabolites of *Photobacterium* spp. and *Xenorhabdus* spp. have been examined for toxicity against important fungal pathogens and all of these studies offered encouraging results (San-Blas et al., 2012; Bock et al., 2014; Shapiro-Ilan et al., 2009, 2014; Fang et al., 2014; Hazir et al., 2016). In a recent study, Hazir et al. (2016) discovered that TCA and metabolites from *X. szentirmaii* possess higher antifungal activity compared to metabolites derived from a variety of other *Xenorhabdus* or *Photobacterium* species; this finding was consistent even when comparing potency against a variety of phytopathogens including major pathogens of peach, *Armillaria tabescens* (the causal agent of root rot), *Venturia carpophila* (causal agent of peach scab) and *Monilinia fructicola* (causal agent of brown rot), and pecan pathogens *V. effusa* (causal agent of pecan scab), and *Glomerella cingulata* (causal agent of anthracnose = *Colletotrichum gloeosporioides*). Some of these pathogen species or genera cause disease in a variety of other crops as well (Strand, 1999; Anon, 2016). Therefore, there is strong justification to explore further the potential role of *Xenorhabdus* and *Photobacterium* metabolites in the control of plant diseases.

Combined application of different anti-fungal compounds can result in synergism resulting in improved efficacy and reduced potential for development of resistance (Moreno-Martinez et al., 2015). However, combination of pesticidal agents can also result in additive or antagonistic interactions (Shapiro-Ilan et al., 2004, 2016). Fungicidal interactions between *Xenorhabdus* or *Photobacterium* metabolites and existing commercial fungicides have not been explored. Our hypothesis was that certain combined applications would result in synergy and enhanced suppression of vegetative growth of fungal phytopathogens. Therefore we evaluated interactions in vitro between TCA or metabolites from *X. szentirmaii* when combined with commercial fungicides. Phytopathogens included *M. fructicola*, *Rhizoctonia solani*, *C. gloeosporioides* and *Fusarium oxysporum*. These fungi were targeted because they represent diverse genera that are of high economic importance to many crops and regions of the world.

2. Materials and methods

2.1. Bacterial extracts and fungicides

The bacterial exudates of *X. szentirmaii* (associated with the nematode, *Steinernema rarium*) and TCA were tested alone and in combination with commercially available fungicides. Mutualistic bacteria were isolated from the hemolymph of *S. rarium* strain 17C & E infected *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to Kaya and Stock (1997). The nematode isolate (*S. rarium* 17C & E) was identified based on molecular and morphological characteristics (Nguyen et al., 2006) and maintained in the USDA-ARS culture collection (Byron, GA). The bacteria were identified by their cell and colony morphology on NBTA plates and by using the catalase test (Akhurst, 1980; Boemare and Akhurst, 2006). Bacterial stock suspen-

sions were kept in Tryptic Soy Broth with yeast extract [TSBY, (Difco, Detroit, MI) + 0.5% yeast extract Sigma, St. Louis, MO)] with 20% glycerol at -80 °C until they were used in experiments (Boemare and Akhurst, 2006). As needed, the bacterial cells were taken from the frozen stock cultures and transferred directly to NBTA medium (nutrient agar with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue). The growth of the bacteria and colony morphology were checked after 48 h incubation to ensure that there was no contamination. After the phase and purity of the cultures were checked (as in Kaya and Stock, 1997 and Boemare and Akhurst, 2006), a loopful of bacteria from NBTA medium was transferred to 100 ml TSBY in an Erlenmeyer flask (300 ml capacity). The liquid cultures were incubated on a rotary shaker at 130 or 200 rpm for 24 h in the dark at 25 °C (Shapiro-Ilan et al., 2014; Hazir et al., 2016). Subsequently, the bacterial broth was centrifuged at 10,000 rpm for 20 min at 4 °C (Sorvall RC6 Plus, ThermoScientific, Ashville, NC). The supernatant was filtered through a 0.22 µm Millipore filter (ThermoScientific, NY) (Houard et al., 2013). The bacterial exudates were poured into 50 ml sterile centrifuge tubes (Corning, NY) and kept at 4 °C for up to two weeks prior to use. The supernatant thus prepared was considered the working metabolite solution and was used directly in the experiments.

TCA (98 + % purity) was obtained from Sigma (St. Louis, MO). TCA solutions were prepared by dissolving in acetone. The solvent (acetone) in dilute concentrations such as used in this study, does not have any fungicidal properties (Bock et al., 2014, see Results section). The concentrations of TCA used varied by target pathogen (Table 1). The concentrations were chosen based on preliminary data (unpublished) and were expected to provide a low or intermediate level of suppression (thereby providing a basis on which to quantify a synergistic effect).

The following fungicides were used in experiments: Abound® (azoxystrobin, Syngenta, Greensboro, NC USA), Elast® (dodine, Greensboro Port Washington, NY), ProPhyt® (potassium phosphite, Helena Chemical Company, Collierville, TN), PropiMax® (propiconazole, Dow, Indianapolis IN), Regalia® (a plant extract from *Reynoutria sachalinensis*, Marrone Bio Innovations, Davis CA), Serenade®, (based on the bacterium *Bacillus subtilis*, Bayer CropScience LP Research Triangle Park, NC) and Supertin 4L (tri-phenyltin hydroxide, United Phosphorous, Inc., King of Prussia, PA). To simulate applications and combinations that growers would apply, we used formulated products in all experiments (rather than using technical grade active ingredient alone).

2.2. Phytopathogen cultures

M. fructicola was isolated from peach and pecan trees located at the USDA-ARS research station in Byron, GA. *R. solani* was isolated from the roots of cotton seedlings in Aydin/Turkey. *C. gloeosporioides* was isolated from diseased pecan leaves in Albany, GA and *F. oxysporum* was isolated from the roots of melon in Izmir/Turkey. All fungi were cultured on potato dextrose agar (PDA) in Petri dishes at 25 °C; as needed, fungi were stored up to one week at 4 °C prior to experimentation.

2.3. Effects of fungicidal agents applied alone or in combination

Treatment effects were compared by measuring vegetative growth of the phytopathogens in vitro when they were exposed to the fungicidal agents applied alone or in combination; the approach was based on methods described by Hazir et al. (2016). Solutions of the fungicidal treatments were incorporated into PDA to achieve the desired concentrations, and autoclaved. Prior to autoclaving the PDA, the prescribed volume of distilled water was reduced to allow addition of the fungicide solutions. Controls consisted of PDA without any amendment or PDA with acetone only (the solvent used for TCA). The media were gently stirred while cooling to 40–42 °C and poured into

Table 1

Experiments and treatments used to assess suppression of vegetative growth of various fungal phytopathogens.^a

Pathogen/experiment#	Treatment	Conc ^b	%Field rate ^c	Abbre ^d
<i>M. fructicola</i> + TCA/1	Acetone	0.2	NA	Ace
	ProPhyt®	0.5	100	Prohi
	ProPhyt®	0.25	50	Prolo
	Regalia®	0.5	100	Reghi
	Regalia®	1	50	Reglo
	Serenade®	0.003	0.213	Serhi
	Serenade®	0.002	0.1	Serlo
	TCA	0.20	NA	TCA
<i>M. fructicola</i> + TCA/2	Abound®	0.004	4.3	Abohi
	Abound®	0.0004	0.43	Abolo
	Acetone	0.2	NA	Ace
	Elast®	0.0002	0.05	Ehi
	Elast®	0.0001	0.005	Elo
	PropiMax®	0.032	50.8	Pxhi
	PropiMax®	0.0032	5.08	Pxlo
	TCA	0.2	NA	TCA
<i>M. fructicola</i> + <i>X. szentirmai</i> /3	Abound®	0.004	4.3	Abohi
	Abound®	0.0004	0.43	Abolo
	Elast®	0.0002	0.05	Ehi
	Elast®	0.0001	0.28	Eme
	Elast®	0.00002	0.005	Elo
	ProPhyt®	0.5	100	Prohi
	ProPhyt®	0.25	50	Prolo
	PropiMax®	0.032	50.8	Pxhi
	PropiMax®	0.0176	27.9	Pxme
	PropiMax®	0.0032	5.08	Pxlo
	Regalia®	0.5	100	Reghi
	Regalia®	1	50	Reglo
	Serenade®	0.003	0.213	Serhi
	Serenade®	0.002	0.1	Serlo
	TSYB	10	NA	Tsy
	<i>X. szentirmai</i>	10%	NA	Xs
<i>M. fructicola</i> with TCA + <i>X. szentirmai</i> /4	TCA	25.0	NA	TCAhi
	TCA	3.0	NA	TCAlo
	<i>X. szentirmai</i>	5	NA	Xshi
	<i>X. szentirmai</i>	1	NA	Xslo
<i>R. solani</i> + TCA/5	Acetone	0.2	NA	Ace
	Regalia®	1	100	Reghi
	Regalia®	0.5	50	Reglo
	Serenade®	0.003	0.213	Serhi
	Serenade®	0.002	0.1	Serlo
	TCA	20.0	NA	TCA
<i>C. gloeosporioides</i> /TCA/6	PropiMax®	0.016	25	Prop
	Supertin®	0.023	50	Supertin
	TCA	120	NA	TCA
<i>F. oxysporum</i> /TCA/7	Regalia®	0.1	100	Reg
	Serenade®	0.1	0.02	Ser
	TCA	63	NA	TCA

^a Phytopathogens included *Monilinia fructicola*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Within each experiment, *trans*-cinnamic-acid (TCA) or *Xenorhabdus szentirmai* was combined with each of the other treatments, and all fungicidal treatments were also applied alone. All treatments were incorporated into potato dextrose agar (PDA). Acetone (the solvent of TCA) was used as a control, and an untreated control (no amendment) was also included in all experiments.

^b Concentrations are expressed as percentage (v/v) per 500 ml potato dextrose agar (PDA) except TCA is expressed as µg per ml (final concentration in 500 ml PDA).

^c Percentage (v/v) of recommended field rate.

^d Abbreviation used in the text and illustrations.

Petri dishes.

A 5 mm diameter mycelia plug from a fungal culture plate was transferred to the center surface of each agar dish (Fang et al., 2011; Hazir et al., 2016) and the culture incubated at 25 °C. Vegetative growth of the fungus was measured after 7 days. Growth was assessed by measuring the diameter of the colony in two perpendicular directions, taking the average, and calculating the approximate area.

Prior to experimentation, a dose-response test was conducted for each fungicidal treatment and each phytopathogen (data not shown). A

range of rates of each fungicidal treatment (TCA, *X. szentirmai* and the commercial fungicides) were applied individually and assessed for fungicidal activity against each phytopathogen as described above. The goal was to obtain a level of fungal suppression that was intermediate with the stand-alone product (approximately 30–60%). As with the TCA, an intermediate level of suppression was desired so that synergistic or additive effects could be discerned, i.e., if a single treatment caused too high a level of suppression, there would be no basis on which to assess enhanced efficacy of the treatment combination.

Based on the dose-response tests, and selection of fungicide concentrations, a series of experiments was designed. The list of experiments, single treatments and their rates of application (and treatment abbreviations for use hereafter) is provided in Table 1. Some commercial fungicides were applied at two rates, high and low (hereafter referred to as hi and lo) and in one case a third medium rate was used; we chose to apply multiple rates because application rate can impact the type of interaction between two agents (Koppenhöfer and Grewal, 2005). Within each experiment, all of the fungicide treatments (TCA, *X. szentirmai* and the commercial fungicides) were applied individually. Moreover, TCA, or the supernatant of *X. szentirmai* was combined individually with each of the commercial fungicides at each concentration within an experiment. The experiments testing fungicide combinations against *M. fructicola* were split as follows: two experiments with TCA and the commercial fungicides (splitting the fungicidal treatments = Experiments 1 & 2), one experiment with *X. szentirmai* and commercial fungicides (Experiment 3), and one experiment combining TCA and *X. szentirmai* (Experiment 4). Additionally, combinations of TCA and commercial fungicides were also tested against *R. solani* (Experiment 5), *C. gloeosporioides* (Experiment 6) and *F. oxysporum* (Experiment 7). Within each experiment, there were nine replicate plates for each treatment, and each experiment was repeated once (two trials).

2.4. Data analyses

Treatment effects for testing antimycotic activity in vitro (based on area of fungal growth) were analyzed using ANOVA (SAS V9.3, SAS Institute Inc., Cary, NC). Data from repeated trials were pooled and trial was considered as a block effect. If a significant treatment effect was detected in the ANOVA ($\alpha = 0.05$), a means separation was performed using Tukey's HSD test. The area of vegetative growth (cm²) was log transformed prior to analysis (Southwood, 1978); non-transformed means are presented in the results.

The nature of interactions (antagonistic, additive, or synergistic) between fungicidal agents was determined through a comparison of expected and observed fungal growth (Koppenhöfer and Kaya, 1997; Shapiro-Ilan et al., 2004, 2011). Corrected fungicidal suppression was calculated based on Abbott's formula, i.e., (growth in the control - growth in the treatment)/growth in the control (Abbott, 1925). Subsequently, the expected additive proportional suppression, S_E for combination treatments was calculated as $S_E = S_C + S_M (1 - S_C)$, where S_C and S_M are the proportional suppression, caused by commercial fungicide and bacterial metabolites (TCA or *X. szentirmai*) applied alone, respectively. A chi-square (χ^2) value was calculated as $(S_{CM} - S_E)^2 / S_E$, where S_{CM} is the observed suppression for the combination of commercial fungicide and bacterial metabolites. If the calculated value was > 3.84 (as specified for 1 df), a non-additive effect of the two control agents was indicated. A positive value for the difference $S_{CM} - S_E$ indicated synergism, whereas a negative value indicated antagonism.

3. Results

In the first experiment with *M. fructicola* and TCA, a differential in fungal growth and interactions was observed among the treatments ($F = 31.27$; $df = 14, 240$; $P < 0.0001$) (Fig. 1; Table 2). Based on chi-

Fig. 1. Mean ($\pm$ SEM) vegetative growth of *Monilia fructicola* on potato dextrose agar following treatments of *trans*-cinnamic-acid (TCA), Prophyt® (Pro), Regalia® (Reg), Serenade® (Ser) or combinations thereof. Hi and lo indicate higher or lower rates of the fungicides (see Table 1 for details). Controls had no amendment or Acetone only (the solvent for TCA). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). Asterisks above bars indicate the combined treatment caused synergistic levels of suppression relative to the respective treatments applied alone.

Table 2

Chi-square values and interaction type observed for the combined fungicidal treatments.^a

Experiment	Treatment combination ^b	Chi-square	Interaction
<i>M. fructicola</i> + TCA/1	TCA + Serenade-lo	0.75	Additive
	TCA + Serenade-hi	29.96	Synergistic
	TCA + Prophyte-hi	0.49	Additive
	TCA + Prophyte-lo	7.82	Synergistic
	TCA + Regalia-hi	4.12	Synergistic
	TCA + Regalia-lo	0.02	Additive
<i>M. fructicola</i> + TCA/2	TCA + Elast-hi	1.14	Additive
	TCA + Elast-med	66.77	Synergistic
	TCA + Elast-lo	2.42	Additive
	TCA + PropiMax-hi	15.28	Antagonistic
	TCA + PropiMax-med	11.75	Antagonistic
	PropiMax-lo + TCA	9.22	Synergistic
	TCA + Abound-hi	8.87	Antagonistic
	TCA + Abound-lo	5.62	Antagonistic
<i>M. fructicola</i> + <i>X. szentirmai</i> /3	Xs + Abound-hi	1.00	Additive
	Xs + Abound-lo	3.94	Synergistic
	Xs + Elast-hi	49.10	Antagonistic
	Xs + Elast-lo	6.58	Antagonistic
	Xs + PropiMax-hi	0.02	Additive
	Xs + PropiMax lo	2.45	Additive
<i>M. fructicola</i> with TCA + <i>X. szentirmai</i> /4	Xs-hi + TCA-hi	3.16	Additive
	Xs-hi + TCA-lo	22.90	Antagonistic
	Xs-lo + TCA-hi	80.04	Synergistic
	Xs-lo + TCA-lo	21.20	Antagonistic
	TCA + Regalia-lo	83.75	Synergistic
<i>R. solani</i> + TCA/5	TCA + Regalia-hi	0.00	Additive
	TCA + Serenade-lo	0.03	Additive
	TCA + Serenade-hi	10.59	Synergistic
	TCA + PropiMax	6.175	Antagonistic
<i>C. gloeosporioides</i> + TCA/6	TCA + Supertin	0.002	Additive
	TCA + Regalia	0.72	Additive
<i>F. oxysporum</i> + TCA/7	TCA + Serenade	0.17	Additive

^a Chi-square values were calculated by comparing observed and expected fungicidal activity of treatments applied alone or in combination; chi-square > 3.84 indicates significance.

^b lo = lower rate of application, hi = higher rate of application, see text and Table 1 for details.

square results, synergistic fungal suppression (as indicated by reduced fungal growth) was observed at certain concentrations for all three commercial fungicides (Prophyt®, Regalia® and Serenade®) when

combined with TCA (Table 2). Specifically the combination treatments Pro-lo + TCA, Reg-hi + TCA, and Ser-hi + TCA, were synergistic (Table 2). Reductions in fungal growth caused by synergistic interactions ranged from approximately 28% (in Reg-hi + TCA) to 42% (with Ser-hi + TCA) relative to the more effective corresponding single-applied treatments (Fig. 1). Furthermore, according to the ANOVA, all three of these combination treatments were in the Tukey grouping with the least fungal growth, and the fungal growth in the combinations was lower than in each of the respective fungicide treatments when applied alone (Fig. 1). Fungal growth in the combination treatment Pro-hi + TCA was also in the lowest statistical grouping, but this treatment did not differ from the high rate of Prophyt® applied alone. Other combination treatments were additive (Table 2).

Similarly in the second experiment with *M. fructicola* and TCA, differences in fungal suppression and interactions among fungicidal agents were observed ($F = 1008.43$; $df = 18,321$; $P < 0.0001$) (Fig. 2; Table 2). Synergistic levels of fungal suppression were observed at certain concentrations of Elast® and PropiMax® (Table 2), and both of these synergistic interactions caused > 60% reduction in fungal growth relative to the fungicides applied alone. In contrast, TCA interacted antagonistically with both rates of Abound® (Table 2). Both combination treatments that showed synergy also had less fungal growth than the respective treatments applied alone (Fig. 2). Some of the fungicidal treatments applied alone caused complete suppression of *M. fructicola*, i.e., the high rates of Elast® and PropiMax®, and thus there was no opportunity for synergy (Fig. 2).

In Experiment 3, treatment effects were observed when cell-free *X. szentirmai* supernatant and commercial fungicides were applied alone or in combination for suppression of *M. fructicola* ($F = 304.82$; $df = 14,254$; $P < 0.0001$) (Fig. 3). Synergy was observed only in the combination of *X. szentirmai* + Abound® (low rate) (an approximate 20% reduction in fungal growth relative to the more effective single-applied treatment), whereas combinations with Elast® were antagonistic at both rates, and combinations with PropiMax® were additive at both rates (Fig. 3; Table 2).

In Experiment 4, when TCA was applied with *X. szentirmai*, only Xslo + TCAhi showed a synergistic effect with less growth compared with either fungicide alone (an approximate 50% reduction) ($F = 198.94$; $df = 8, 72$; $P < 0.0001$, Fig. 4; Table 2). The other rate combinations were antagonistic or additive (Table 2).

Fig. 2. Mean ($\pm$ SEM) vegetative growth of *Monilinia fructicola* on potato dextrose agar following treatments of *trans*-cinnamic-acid (TCA), Elast® (E), PropiMax® (Px), Abound® (Abo) or combinations thereof. Hi, lo and me indicate higher, lower and intermediate rates of the fungicides (see Table 1 for details). Controls had no amendment or Acetone only (the solvent for TCA). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). Asterisks above bars indicate the combined treatment caused synergistic levels of suppression relative to the respective treatments applied alone.

Treatment differences and differential interactions were observed when TCA was combined with commercial fungicides against *R. solani* (Experiment 5, $F = 10752.9$; $df = 10,186$; $P < 0.0001$) (Fig. 5; Table 2). TCA and the low rate of Regalia® each caused $< 25\%$ suppression when applied alone but the combination resulted in 100% suppression of fungal growth (Table 2). Serenade® did not cause any suppression of fungal growth when applied alone at either rate yet combinations with TCA caused significant suppression, and the combination at the high rate of Serenade was synergistic (with an approximate 22% reduction in fungal growth relative to TCA alone) (Table 2). Both combination treatments that showed synergy had less fungal growth when compared with the respective fungicides or TCA applied singly (Fig. 5). The high rate of Regalia® caused 100% suppression of *R. solani* and thus there was no opportunity for synergy (Fig. 5).

In Experiment 6, suppression of *C. gloeosporioides* occurred when TCA and commercial fungicides were applied alone or in combination ($F = 245.34$; $df = 6, 208$; $P < 0.0001$) (Fig. 6). Although combined applications of TCA and fungicides significantly suppressed growth

more than other treatments, interactions were additive in one case and antagonistic in the other (based on the chi-square results) (Fig. 6; Table 2).

In Experiment 7 (targeting *F. oxysporum*), differences in fungal suppression among fungicidal agents were observed ($F = 292.89$; $df = 6, 132$; $P < 0.0001$) (Fig. 7). The combination treatments caused greater suppression than any of the single-applied treatments yet combination treatments exhibited only additive effects (Fig. 7; Table 2).

4. Discussion

Overall, our data indicated a broad spectrum of synergistic interactions among the fungicidal agents tested. In regard to synergistic effects on *M. fructicola* suppression, TCA was synergistic with concentrations of all six of the commercial fungicides tested, with the exception of Abound®. However, the cell-free supernatant of *X. szentirmai* was synergistic in combination with Abound (although it was not synergistic with the other two fungicides tested). In relation to suppression of *R.*

Fig. 3. Mean ($\pm$ SEM) vegetative growth of *Monilinia fructicola* on potato dextrose agar following treatments of *Xenorhabdus szentirmai* metabolites (Xs), Abound® (Abo), Elast® (E), PropiMax® (Px), or combinations thereof. Hi and lo indicate higher or lower rates of the fungicides (see Table 1 for details). Controls had no amendment or TSY (the culture broth for *X. szentirmai*). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). Asterisks above bars indicate the combined treatment caused synergistic levels of suppression relative to the respective treatments applied alone.

Fig. 4. Mean ($\pm$ SEM) vegetative growth of *Monilinia fructicola* on potato dextrose agar following treatments of *trans*-cinnamic-acid (T), *Xenorhabdus szentirmai* metabolites, or combinations thereof. Hi and lo indicate higher or lower rates of the fungicides (see Table 1 for details). The controls had no amendment. Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). Asterisks above bars indicate the combined treatment caused synergistic levels of suppression relative to the respective treatments applied alone.

Fig. 5. Mean ($\pm$ SEM) vegetative growth of *Rhizoctonia solani* on potato dextrose agar following treatments of *trans*-cinnamic-acid (TCA), Regalia® (Reg), Serenade® (Ser) or combinations thereof. Hi and lo indicate higher or lower rates of the fungicides (see Table 1 for details). Controls had no amendment or Acetone only (the solvent for TCA). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$). Asterisks above bars indicate the combined treatment caused synergistic levels of suppression relative to the respective treatments applied alone.

solani, TCA was synergistic with both of the fungicides tested, Serenade® and Regalia®. Also, TCA was synergistic in combination with *X. szentirmai* (in suppressing *M. fructicola*) albeit at only one of the four concentrations tested. These data demonstrated that an array of

synergistic interactions can occur among fungicides and TCA or *X. szentirmai*, yet the outcome is dependent on concentration of the fungicide. Furthermore, in two of the targeted phytopathogens (*C. gloeosporioides* and *F. oxysporum*), only additive or antagonistic effects

Fig. 6. Mean ($\pm$ SEM) vegetative growth of *Colletotrichum gloeosporioides* on potato dextrose agar following treatments of *trans*-cinnamic-acid (TCA), PropiMax®, Supertin® or combinations thereof. Controls had no amendment or Acetone only (the solvent for TCA). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$).

Fig. 7. Mean ($\pm$ SEM) vegetative growth of *Fusarium oxysporum* on potato dextrose agar following treatments of *trans*-cinnamic-acid (TCA), Regalia®, Serenade® or combinations thereof. Controls had no amendment or Acetone only (the solvent for TCA). Treatments effects were assessed 7 d after application. Different letters above bars indicate statistically significant differences (Tukey's test, $\alpha = 0.05$).

were observed. Therefore, concentrations selected to achieve maximum reduction in growth of the targeted phytopathogens will need to be optimized specifically for each combination and for each target pathogen. The impact of concentration on the interaction (synergistic, additive or antagonistic) has been observed in other systems including with combinations of entomopathogens with each other or with chemical pesticides (Koppenhöfer and Grewal, 2005).

Synergy between fungicidal agents has been observed previously (Moreno-Martinez et al., 2015). For example, Baider and Cohen (2003) reported BABA (DL-3-aminobutyric acid) and the protectant fungicide mancozeb were more effective than either agent applied alone for controlling late blight (*Phytophthora infestans*) in potato and tomato, and downy mildew (*Pseudoperonospora cubensis*) in cucumber. Also, Evenhuis et al. (1996), reported synergism between cymoxanil and mancozeb in suppressing *P. infestans*. However, there is a dearth of examples that extend to field results. Furthermore, no reports prior to the current study have examined fungicidal interactions between metabolites of *Xenorhabdus* or *Photorhabdus* bacteria and commercial fungicides.

Our findings may have application and be particularly valuable for enhancing control of key phytopathogens in an effective and environmentally sound manner. Specifically, using biorational fungicidal agents (of which the bacterial metabolites are an example) is likely to have reduced environmental impact when compared to broad spectrum, synthetic chemicals. However, it should be acknowledged that just because a chemical is biorational does not automatically result in reduced environmental impact. Some metabolites might be shown to have broad spectrum activity and have the potential to negatively impact favorable microbial communities. However, combinations using TCA or *X. szentirmai* in a synergistic combinations with other fungicides can be considered biorational, e.g., Regalia® and Serenade®, both of which are already used in organic systems, and thus could be particularly beneficial as efficacy is increased. The synergistic combinations of TCA or *X. szentirmai* with conventional synthetic chemical fungicides will also be beneficial due to greater efficacy that may be achieved while reducing the quantity of synthetic chemical fungicide required. Some of the synergistic interactions observed in our study involved lower rates of the commercial fungicide (whether organic or conventional), and therefore may have more potential for reduced environmental impact than others, e.g., TCA combined with Elast®, Prophyt® or Regalia®. Moreover, in one case an organic fungicide

(Serenade®) did not produce any suppressive effect when applied alone to *R. solani*, yet synergistic suppression was achieved when the high rate was combined with TCA; thus, a fungicide that was ineffective may be more efficacious by adding TCA or another bacterial metabolite(s) as an enhancer. We also suspect overall costs will be reduced as lower amounts of active ingredients can be used based in combinations and we expect benefits in terms of resistance management due to introduction of novel modes of action.

Additional research is needed to explore the potential for TCA or supernatants of *X. szentirmai* (or other *Xenorhabdus* spp. or *Photorhabdus* spp. metabolites) as fungicide synergists and to screen the combinations against other phytopathogens not tested in this study. Furthermore, research will be needed to confirm and optimize the synergistic interactions under field conditions.

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