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Trans-cinnamic acid and *Xenorhabdus szentirmaii* 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 szentirmaii* are particularly toxic to various phytpathogenic 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. szentirmaii*. Fungicides (active ingredient) included Abound® (Azoxystrobin), 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. szentirmaii* with Abound®. For suppression of *R. solani*, synergy was observed between TCA combined with Regalia® or Serenade®. Additionally, when TCA was combined with *X. szentirmaii* synergistic levels of suppression to *M. fructicola* were observed. Other combinations of TCA or *X. szentirmaii* with the fungicides or using alternate concentrations were either additive or occasionally antagonistic in nature. Our results indicate that TCA and *X. szentirmaii* 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 relationship (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, xenocoumacins, 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 *Photorhabdus* 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 *Photorhabdus* 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 *Photorhabdus* 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 *Photorhabdus* 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 *Photorhabdus* 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 *Photorhabdus* 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 rarum*) and TCA were tested alone and in combination with commercially available fungicides. Mutualistic bacteria were isolated from the hemolymph of *S. rarum* strain 17C & E infected *Galleria mellonella* (Lepidoptera: Pyralidae) larvae according to Kaya and Stock (1997). The nematode isolate (*S. rarum* 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 suspensions 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 Phospohorous, 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