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ORIGINAL PAPER

Identification of the antifungal compound, *trans*-cinnamic acid, produced by *Photorhabdus luminescens*, a potential biopesticide against pecan scab

Clive H. Bock · David I. Shapiro-Ilan · David E. Wedge · Charles L. Cantrell

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Abstract Pecan scab (caused by *Fusicladium effusum*) limits the productivity of pecan in the southeastern USA. Alternatives to conventional fungicides should be biorational, of low environmental risk with a lower risk of fungicide resistance. Research showed that metabolites from the nematode symbiont *Photorhabdus luminescens* suppress pecan scab, but the bioactive molecules had not been identified. Extracts from *P. luminescens* were investigated using a bioactivity-directed fractionation approach to identify the constituent(s) responsible for the activity. High throughput antifungal bioautography assays against *Colletotrichum gloeosporioides*, *C. fragariae*, and *C. acutatum* were used to guide the fractionation. One of the metabolites was purified and identified as *trans*-cinnamic acid (TCA) using silica gel chromatography followed by semi-preparative high-performance liquid chromatography. In vitro tests confirmed toxicity of TCA to *C. gloeosporioides*, *C. fragariae*, and *C. acutatum* at 10 and 100 μg mL^{-1} using fungal bioautography inhibition screening plates. The antimycotic activity of TCA was tested in vitro against *F. effusum*. Zone of inhibition tests, and tests with TCA incorporated into agar showed TCA toxicity to *F. effusum* at concentration 148–200+ μg mL^{-1}. Further tests incorporating TCA into liquid media demonstrated that TCA arrested all growth of *F. effusum* at a concentration even as low as 64 μg mL^{-1}. Naturally occurring antimicrobial products might offer an alternative to disease control in crops, helping in minimizing the risk of fungicide resistance, while also minimizing any negative impact on the environment. Additional research is warranted to determine the potential to use TCA as a suppressive agent for pecan scab and other diseases.

Keywords Pecan scab · Biorational control · Integrated pest management · Bacterial metabolites

Introduction

Plant pathogens are responsible for yield loss in many crops, including nut trees such as pecan (*Carya illinoinensis* (Wangenh.) K. Koch). Pecan scab, caused by the fungus *Fusicladium effusum* G. Winter, is the major disease that reduces the productivity and quality of pecan in the southeastern USA, although several other pathogens can also cause yield loss (Goff et al. 1996; Teviotdale et al. 2002). Some host resistance is available to reduce the impact of pecan scab, but there is a history of the resistance succumbing to new strains of the pathogen (Cole and Gossard 1956; Goff et al. 1996). Thus, conventional chemical fungicides have been widely used to control scab, often with more than ten sprays being applied to some crops in some seasons to ensure adequate control of the disease (Brenneman et al. 1998). As a result, the risk of fungicide resistance developing in these pathogens has been realized, and *F. effusum* is now resistant to at least two classes of fungicide (Reynolds

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C. H. Bock (✉) · D. I. Shapiro-Ilan
Southeastern Fruit and Tree Nut Research Laboratory, USDA-ARS, Byron, GA 31008, USA
e-mail: clive.bock@ars.usda.gov

D. E. Wedge · C. L. Cantrell
Natural Products Utilization Research Unit, USDA-ARS, University of Mississippi, University, MS 38677, USA

et al. 1997; Stevenson 1999; Seyran et al. 2010). Furthermore, there is an increasing awareness of the environmental impact of conventional pesticides (National Research Council 1989).

Thus, there are incentives to try and develop alternatives to conventional fungicides, alternatives that are biorational, of low environmental risk, and that present a lower risk of fungicide resistance developing in the pathogen; a characteristic that will enhance durability of the chemistry. One option that has recently been explored is the use of naturally occurring antimicrobial compounds produced by *Xenorhabdus* spp. and *Photorhabdus* spp. of bacteria; these bacteria are symbionts of entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis*, respectively (Kaya and Gaugler 1993; Gaugler 2002; Grewal et al. 2005). The bacteria can be cultured in media, and extracts of the cultures contain the bioactive, antimicrobial metabolites, which are active against a wide range of microbial pathogens of animals and plants including bacteria, fungi, and oomycetes (Akhurst 1982; McInerney et al. 1991; Chen et al. 1994; Li et al. 1995; Webster et al. 1995, 2002; Isaacson and Webster 2002; Shapiro-Ilan et al. 2009; Boeszoermenyi et al. 2009; Fang et al. 2011; San-Blas et al. 2012).

Shapiro-Ilan et al. (2009) demonstrated that soluble organic metabolites from *X. bovienii* Akhurst, *X. nematophila* Poinar and Thomas, and *Photorhabdus luminescens* Thomas and Poinar, and unidentified species of both *Xenorhabdus* and *Photorhabdus* suppressed growth of *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk, *Phomopsis* sp., *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. and *F. effusum*, fungal pathogens on pecan, and *Monilinia fructicola* Winter on peach, showing that pathogens of scab were sensitive to extracts from several species both in vitro and in vivo.

Although extracts of *P. luminescens* had good antimycotic activity against *F. effusum*, there has been no study to identify the major compounds responsible for the antimycotic activity. Identification of these bioactive compounds may facilitate optimization of potency in bacteria/metabolite production. Or, conceivably, these bioactive compounds could themselves be used as alternatives to standard fungicides. Thus, there is a need to both identify the bioactive compounds responsible, and to test these in a purified form to confirm efficacy and ensure that they might have a practical application to disease management, i.e., have low environmental risk, be sufficiently durable in the environment, and present a low risk of pathogen resistance developing.

The main objective of this study was to identify the major bioactive antimycotic compound produced by *P. luminescens* that is responsible for suppression of pecan scab.

Materials and methods

Culture of bacteria and extraction of metabolites

Bacteria of *P. luminescens* were isolated from the nematode host, *Heterorhabditis bacteriophora* Poinar, which was cultured in the last instar of *Galleria mellonella* L., as described by Kaya and Stock (1997). The bacteria were subsequently cultured on nutrient agar (NA) by streaking hemolymph from insects inoculated with the nematode (Kaya and Stock 1997).

The extraction of metabolites was performed following the procedures of Ng and Webster (1997). For purposes of metabolite extraction, a loopful of bacteria from the NA colony was transferred to 50 mL Tryptic Soy broth (DIFCO, Detroit, MI) in an Erlenmeyer flask. Cultures were incubated at 25 °C on a rotary shaker for 18–24 h when they were harvested by centrifuging at 10,000 rpm for 20 min. The supernatant from each flask was extracted thrice with ethyl acetate (Fisher Scientific, Fair Lawn, NJ) and the organic fractions dried with anhydrous ammonium sulfate (Sigma, St. Louis, MO), and concentrated using a rotary evaporator. The dried fraction was dissolved in acetone (Sigma, St. Louis, MO) and stored at 4 °C.

Culture of fungal plant pathogens

Pathogen production

Colonies of *F. effusum* were grown on potato dextrose agar (PDA) in Petri-plates placed in an incubator maintained at 27 °C for 3 weeks, from which conidia were harvested, and a suspension of 10^6 conidia mL^{-1} was prepared in sterile, distilled water for in vitro testing of the metabolite.

Isolates of *Colletotrichum acutatum* J.H. Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. and Sacc. were obtained from B. J. Smith, USDA, ARS, Small Fruit Research Station, Poplarville, MS. The three *Colletotrichum* species were isolated from strawberry (*Fragaria* × *ananassa* Duchesne).

Direct bioautography

Standardization of Colletotrichum inoculum

Standardizing the inoculum allows for meaningful comparison of growth inhibition between the different *Colletotrichum* species, test compounds, and replication of experiments in time. Conidia of each fungal species were harvested from 7–10-day-old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through sterile

Miracloth (Calbiochem-Novabiochem Corp., La Jolla CA) to remove mycelia. Conidia concentrations were determined photometrically (Espinel-Ingroff and Kerkering 1991; Wedge and Kuhajek 1998) from a standard curve based on the percent of transmittance (*%T*) at 625 nm and using manual hemocytometer counts. Conidial stock suspensions were adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia mL^{-1}.

Bioautography technique

Bioautography provides a simple technique to visually follow antifungal components through the separation process. Matrix, one-dimensional, and two-dimensional bioautography protocols on silica gel thin-layer chromatography (TLC) plates using *Colletotrichum* spp. as the test organisms were used to identify the antifungal activity according to published bioautography methods (Homans and Fuchs 1970; Moore 1996; Wedge and Nagle 2000). Matrix bioautography was used to screen total amounts of the crude extract (10 and 100 μg).

Each was sprayed with a spore suspension (10^5 spores mL^{-1}) of the fungus of interest and incubated in a moisture chamber at 26 °C with a 12-h photoperiod. Inhibition of fungal growth was evaluated at 3–4 days after treatment. Clear zones, devoid of fungal growth on the TLC plate, indicate the presence of the constituents of antifungal metabolites in each TLC plate (Vincent et al. 1999; Tellez et al. 2000).

Bioassay-directed purification and identification of TCA from the ethyl acetate extract of P. luminescens culture media

A 680-mg aliquot of the ethyl acetate extract was dissolved in chloroform. The chloroform extractables were separated using a Biotage Silica, 100 g, 40 + M cartridge (40–63 μm, 60 Å, 40 × 150 mm) running at 32 mL min^{-1} using a hexane:ethyl acetate (EtOAc) gradient beginning with 100:0 to 50:50 over 1,728 mL followed by 50:50 to 0:100 over 576 mL, and finishing with a 500 mL methanol wash. Portions of 22-mL volume were collected in 16 × 150 mm test tubes. Seven fractions (A to G) were generated on the basis of TLC similarities, and re-evaluation of activity was determined using bioautography. The most active fraction E was subjected to further purification using a reversed-phase C-8 HPLC column (Zorbax, 9.4 mm × 250 mm, 5 μm) running a linear gradient from 50/50 (H_2O/methanol both containing 0.25 % trifluoroacetic acid) to 28/72 (H_2O/methanol both containing 0.25 % trifluoroacetic acid) providing a pure extract of the metabolite of interest. The metabolite of interest was identified as *trans*-cinnamic acid (TCA; Fig. 1). As described in the previous section, matrix bioautography was used to screen toxicity of total amounts of TCA (10 and 100 μg).

Analytic instrumentation used in identification

Identification of TCA was confirmed using ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and a comparison of chemical shift data with commercially available TCA (Acros, Sigma-Aldrich, St. Louis, MO). ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova AS600 spectrometer (Varian, Palo Alto, CA). Electron impact mass spectrometry spectra were recorded on a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 mass spectrometer (Varian, Inc., Palo Alto, CA). Column chromatography was performed using a Biotage Isolera One Flash Purification System (Biotage, Uppsala, Sweden). High-performance liquid chromatography (HPLC) was performed using an Agilent 1200 HPLC system (Agilent, Palo Alto, CA).

In vitro tests of TCA toxicity against *F. effusum*

Agar-based media tests

Two different Petri plate tests were undertaken. The first was a zone of inhibition test (Shapiro-Ilan et al. 2009). The conidial suspension of *F. effusum* was uniformly spread over the surface of PDA on a Petri plate, and a 1-cm-diameter filter paper disk, into which 20 μL acetone containing TCA (99 % purity, Sigma, St. Louis, MO) with 20, 200, and 2000 μg had been pipetted, was placed in the center of the plate. In addition, there was a water control and a treatment of crude metabolite (2,000 μg) dissolved in acetone. Control plates received filter paper disks with only the acetone. The treatments and control were replicated four times, and the experiment repeated twice. After 6 and 10 days, the zone of inhibition was measured taking the average of the two diameters perpendicular to each other.

In the second series of experiments, the TCA was incorporated into the PDA at 0, 74, 148, 741, and 1482 μg mL^{-1}. The TCA was dissolved in ethanol, with

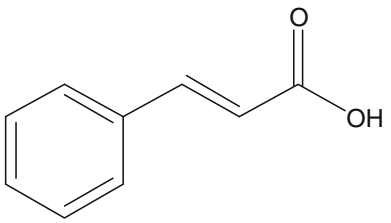

Fig. 1 A bioactive constituent from *P. luminescens: trans*-cinnamic acid, chemical formula $C_9H_8O_2$, mass 148.05, mol wt 148.05

5.8 mL of ethanol added per 500 mL of PDA media. Control treatments included PDA with no amendment and PDA amended with ethanol alone. A well in the center of the agar in the plate was made using a transfer tube to remove a plug of agar. The conidia suspension (0.1 mL) of *F. effusum* was added to the well in the center of the agar. The diameter of the resulting colony was measured (mm) at 7, 14, and 21 days after inoculating the plate. The experiment was repeated once. There were three replicate plates of each treatment in each experiment.

Liquid culture tests

Erlenmeyer flasks containing 50 mL potato dextrose broth were amended with TCA at 0, 64, 127, 635, and 1270 μg mL^{-1}, and 0.1 μL of *F. effusum* conidia suspension was added to each flask. The TCA was dissolved in ethanol, with 0.58 mL ethanol being added to each flask to obtain the required concentrations. In addition to the TCA treatments, there was a nontreated control and a control with only ethanol. After 21 days, the experiment was terminated, and the cultures were vacuum filtered through No. 4 filter paper (Whatman, Maidstone, UK). The filter paper was dried in an oven at 70 °C for 24 h before vacuum filtering the culture broth. After vacuum filtering, the filter paper was again dried in an oven for 24 h at 70 °C and re-weighed.

Data analysis

All data from the zone of inhibition tests and the concentration tests in solid and liquid media were analyzed to determine treatment effects using an analysis of variance. Where a significant effect ($\alpha = 0.05$) was found, the differences between the means were explored using the Student–Newman–Keul's test ($P = 0.05$). All data were analyzed using SAS software (SAS Institute Inc., Cary, NC).

Results

The ethyl acetate crude extract of *P. luminescens* culture medium was fungistatic to two of the three species of *Colletotrichum* using the fungal bioautography inhibition screening plate technique (Table 1). Only *C. acutatum* showed no zone of inhibition at either amount tested. At the lower amount (10 μg), the extract was fungistatic to only *C. gloeosporioides*, but at the higher amount (100 μg), it was fungistatic to both *C. gloeosporioides* and *C. fragariae*.

One of the biologically active compounds was identified as TCA. The fungal bioautography inhibition screening plates test showed that TCA prevented growth of all three species of *Colletotrichum* at both 10 and 100 μg (Table 2). The greater the quantity of TCA on the plate, the larger the zone of inhibition, with a 4–5-fold increase in the diameter of the inhibition zone at 100 μg as compared to 10 μg.

There was no effect of the solvent (acetone) or water alone on *F. effusum*, but there was a significant effect (F value = 166, df = 5, 64, $P < 0.0001$ for the 6-day assessment and F value = 139, df = 5, 63, $P < 0.0001$ for the 10-day assessment) on growth of *F. effusum* on Petri-plates of PDA when filter paper disks infused with TCA were placed in the center of the plate (Fig. 2a, b). The characteristics of the zone of inhibition were similar when measured at 6 or 10 days after plate inoculation, and the Student–Newman–Keul's means separation confirmed that concentrations of TCA ≥0.2 mg were toxic to *F. effusum* in these zone of inhibition tests. At day 10, the crude extract (2,000 μg) produced a zone of inhibition, a third that of the TCA at 2,000 μg, and threefold that of the TCA at 200 μg.

On PDA amended with different concentrations of TCA, there was inhibition of growth of *F. effusum* (Fig. 3a–c). At 7 days after seeding the plate, no growth of *F. effusum* could be seen on the amended plates, although there was appreciable growth on the control plates and on those amended with the solvent, ethanol (F value = 706, df = 5, 24, P value = <0.0001). By 14 days after seeding, growth was measurable on both the 74 and 148 μg mL^{-1} concentrations of TCA, but less than the controls, and none on plates amended with TCA at concentrations >148 μg mL^{-1} (F value = 240, df = 5, 24, P value = <0.0001). By 21 days after seeding, the growth on the 74 and 148 μg mL^{-1} concentrations of TCA had increased, but there was no evidence of growth on any plates with concentrations of TCA >148 μg mL^{-1} (F value = 358, df = 5, 24, P value = <0.0001). Nonetheless, the TCA at

Table 1 Diameter of the zone of inhibition due to metabolites in crude extracts from cultures of *P. luminescens* on three plant-pathogenic species of *Colletotrichum* on fungal bioautography inhibition screening plates

Ethyl acetate metabolite extract of *P. luminescens* (μg)	Diameter of zone of inhibition (mm)		
	C. acutatum	*C. fragariae*	*C. gloeosporioides*
10	NA	NA	FS
100	NA	8.5 FS	8.5 FS

NA not active, *FS* fungistatic

concentrations of 74 and 148 μg mL^{-1} were significantly less compared with the control on all days.

In PDB liquid culture, there was no growth of *F. effusum* at any of the concentrations of TCA tested (Fig. 4). There was no difference between the control and the ethanol solvent used to dissolve the TCA (F value = 64, df = 5, 18, P value = <0.0001), and the lowest concentration of TCA (64 μg mL^{-1}) was completely inhibitory to growth of the fungus.

Table 2 The effect of *trans*-cinnamic acid on the zone of inhibition on three plant-pathogenic species of *Colletotrichum* on fungal bioautography inhibition screening plates

Trans-cinnamic acid (μg)	Diameter of zone of inhibition (mm)		
	C. acutatum	*C. fragariae*	*C. gloeosporioides*
10	3	2.5	3
100	13	14	14.5

Discussion

A bioactive ingredient of extracts of *P. luminescens* was found to be TCA, a small molecule compound which is known to have antibiotic properties (Si et al. 2006; Wong et al. 2008; Chen et al. 2011; Hakkim et al. 2012), hence its use as a possible preservative in food (for which patents exists [e.g., US patent doc numbers 6036986; 6042861]). Furthermore, TCA has previously been identified as a necessary precursor in the biosynthesis of the antibiotic stilbene in *Photorhabdus* (Williams et al. 2005; Eleftherianos et al. 2007; Chalabaev et al. 2008). The results here demonstrate antimycotic activity of TCA against at least two genera of important fungal plant pathogens (*Colletotrichum* and *Fusicladium*), the latter being the major disease of pecan in the southeastern USA.

The antimicrobial activity of metabolites produced by *Photorhabdus* spp. has been demonstrated before (Paul et al. 1981; Isaacson and Webster 2002; Webster et al.

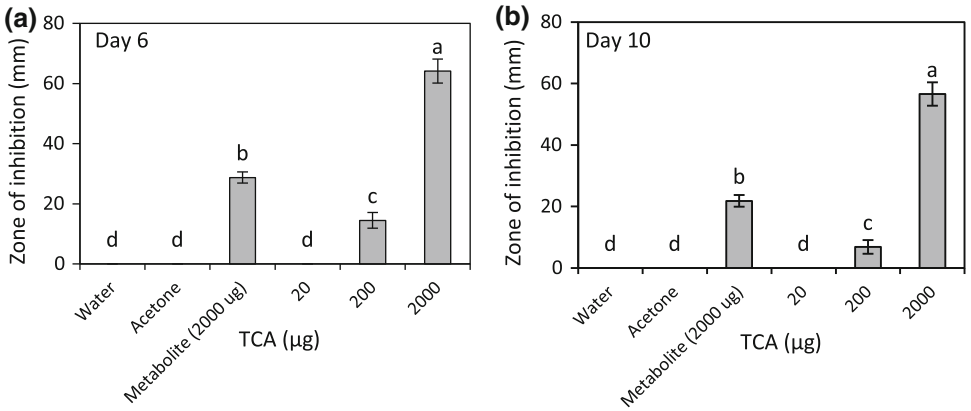

Fig. 2 The inhibition of growth of *Fusicladium effusum* on potato dextrose agar due to *trans*-cinnamic acid (TCA) at different concentrations. Filter paper disks infused with the TCA solution were placed in the center of a Petri plate on which a spore suspension of conidia of *F. effusum* was spread. The zone of inhibition was measured after 6 (**a**) and 10 (**b**) days. Standard *error bars* are indicated. Means with *different letters* are significantly different according to Student–Newman–Keul's means separation ($P = 0.05$)

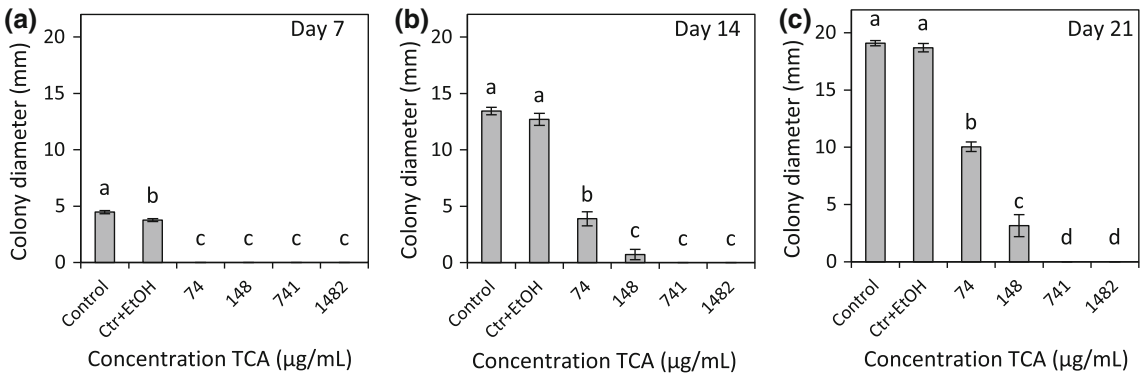

Fig. 3 Effect of *trans*-cinnamic acid (TCA) on growth of *Fusicladium effusum* on potato dextrose agar after incubation for 7 (**a**), 14 (**b**) and 21 (**c**) days. Standard *error bars* are indicated. Means with *different letters* are significantly different according to Student–Newman–Keul's means separation ($P = 0.05$)

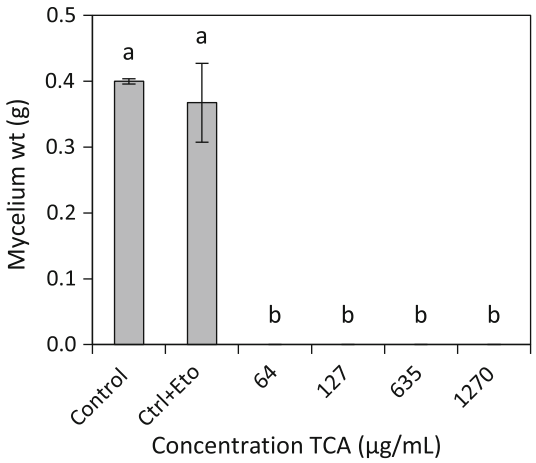

Fig. 4 Effect of *trans*-cinnamic acid (TCA) concentration on the dry weight of mycelium of *Fusicladium effusum* grown in potato dextrose broth amended with different concentrations of TCA after 21 days incubation. Means with *different letters* are significantly different according to Student–Newman–Keul's means separation ($P = 0.05$)

2002), and the antimycotic activity was characterized as being based on exo- and endochitinases, as well as other proteinaceous moieties and small molecules. In this study, we discovered that, in *P. luminescens*, one of these small molecules is TCA, and the preliminary in vitro studies using fungal bioautography inhibition screening showed that TCA produced by *P. luminescens* was active at concentrations of $\sim$100 µg mL^{-1} against both *C. fragariae* and *C. gloeosporioides*. However, *C. acutatum* was not sensitive at any of the concentrations tested, and the reason for this is unknown, but warrants further testing with more fungal pathogens.

Based on these observations, it would appear that the level of toxicity of raw extracts from *P. luminescens* was probably in large part due to the TCA component within, although other factors in the extract might also be involved in the antimicrobial activity (Paul et al. 1981; McInerney et al. 1991; Shapiro-Ilan et al. 2009). Other components of the extracts that are antimycotic remain to be identified (indeed other components were also antimycotic, but did not have as strong an antimicrobial effect and were not identified—data not presented).

The potency of TCA as an antimycotic is comparable with other observations of the effect of TCA on other microbes including human pathogens (Si et al. 2006; Hakkim et al. 2012), where concentrations in the range of 100–500 µg mL^{-1} provided a maximum zone of inhibition. TCA extracted from Serrano chili was previously found to be an antibiotic to bacterial plant pathogens at concentrations of 500 µg mL^{-1} (Acero-Ortega et al. 2003). The various solid and liquid media experiments in this study demonstrate that TCA is antimycotic over a range of concentrations similar to those previously reported, although sensitivity appeared to be a little lower, in the 50–100+ µg mL^{-1} range. The reason for the liquid culture assay resulting in greater antimycotic activity is not known, but no growth was observed in this test, even at TCA concentration of 64 µg mL^{-1}. A greater sensitivity in liquid cultures similar to that in certain antifungal molecules has been noted before (Azevedo and Cassio 2010; Shreaz et al. 2011).

Interestingly, TCA is an important compound in the induced resistance response in plants (Métraux 2002), although it is not known if it can induce resistance per se. It can also build up in soils as an allelopathic compound released from plant roots, and cause autotoxicity and increased root disease (Chen et al. 2011), yet exogenously applied TCA can also reduce the effects of drought stress in some plant species (Ye et al. 2004, 2006; Sun et al. 2012). Thus, it has diverse effects on plant hosts and pathogens, and application of TCA to protect plants from disease might have some unexpected consequences.

If TCA (or other antimycotic metabolites from these bacteria) is to be considered as a potential biorational plant protection chemical, it will be necessary to address several other issues. In particular, further in vitro and in vivo laboratory, greenhouse, and field tests on the efficacy of pathogen and disease suppression need to be established, possible phytotoxicity issues will need to be ascertained, and for any practical formulations, field longevity and environmental impact characterized. Finally, there is the cost of production and application, which would need to be considered before utilizing TCA, or any other antimycotic from bacteria as a potential biorational control agent for pecan scab or other crop diseases.

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