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Kenneth J. Curry
University of Southern Mississippi, Kenneth.Curry@usm.edu

Maritza Abril
University of Southern Mississippi

Anthony J. DeLucca
USDA-ARS, anthony.delucca@ars.usda.gov

Stephen M. Boue
United States Department of Agriculture, steven.boue@ars.usda.gov

Barbara J. Smith
USDA-ARS Southern Horticultural Laboratory, barbara.smith@ars.usda.gov

See next page for additional authors

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M. Abril and K.J. Curry
Department of Biological Sciences
The University of Southern Mississippi
Hattiesburg, MS 39406
USA

A.J. DeLucca and S. Boue
United States Department of Agriculture
ARS, Southern Regional Research Center
New Orleans, LA 70124
USA

B.J. Smith
USDA-Agricultural Research Service
Southern Horticultural Laboratory
Small Fruit Research Unit
Poplarville, MS 39470
USA

D.E. Wedge
USDA-Agricultural Research Service
Thad Cochran National Center
for Natural Products Research
Oxford, MS 38677
USA

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Abstract

CAY-1 is an experimental, natural product being tested as a potential fungicide. This saponin isolated from *Capsicum frutescens* interacts with membrane sterols causing leakage of cell components and ultimately cell death in a variety of fungi. CAY-1 and the commercial fungicide captan were tested in an in vitro dose-response dilution-broth assay. They caused at least 85% growth inhibition of the fungal pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* when tested at 3.0 µM. Even though CAY-1 strongly reduced the growth of these fungal pathogens in laboratory assays and prevented anthracnose development in detached leaf assays, it did not control foliar or fruit rot diseases of strawberry in field trials.

INTRODUCTION

The strawberry industry in the USA accounts for over a quarter of the total world production of strawberries (*Fragaria* × *ananassa* Duch.). Fruit rot diseases cause significant losses to this industry, especially in the southeastern USA where temperature and humidity often favor fungal pathogens. Prevalent diseases in this region include anthracnose fruit rot and crown rot (*Colletotrichum* spp.) (Smith, 1998), Botrytis gray mold (*Botrytis cinerea*), leather rot (*Phytophthora cactorum*), and stem end rot (*Gnomonia comari*) (Mass, 1998). Controlling these diseases has become challenging as the efficacy of some fungicides has diminished due to development of resistant strains of fungi (Smith and Black, 1993a, b; Peres et al., 2002, 2004), and other fungicides have lost their registration for use on strawberries (Wedge et al., 2007). Natural product-based fungicides are being developed as alternatives to traditional fungicides for use on strawberries. The purpose of this study was to evaluate the efficacy of CAY-1 as a natural product-based fungicide (De Lucca et al., 2002; Renault et al., 2003) in laboratory trials.

MATERIALS AND METHODS

CAY-1 is a fungicidal steroidal saponin isolated from the ground fruit of cayenne pepper (*Capsicum frutescens*). The commercial fungicides, benomyl (a methyl benzimidazole carbamate fungicide that is no longer commercially available in the USA), captan (a multisite phthalamide fungicide), and iprodione (a dicarboxymide fungicide) were used as industry standards for efficacy comparison against CAY-1 in laboratory trials. Preparation of pure CAY-1 was performed as previously reported (De Lucca et al., 2002, 2006). Crude, dry, ground cayenne pepper was aqueously extracted overnight. The slurry was centrifuged, the supernatant filtered, and the pelleted pepper was resuspended in water, allowed to equilibrate for 10 min, and centrifuged as before. The resulting

supernatant was passed through cheesecloth and the filtered supernatants combined. This extract was then centrifuged at 10,000 RPM for 10 min at 4ºC, and the supernatant was filtered and freeze-dried. The crude, freeze-dried material was dissolved in water and eluted through a 400 gram C_{18} (125Å, 55-105 µm, Waters Corp, Milford, MA, USA) gravity column using a step gradient of methanol: water. The semi-pure CAY-1 eluted in the 75% MeOH eluate. The methanol was removed under vacuum and the remaining liquid freeze-dried. Pure CAY-1 was prepared using high pressure liquid chromatography (HPLC) and confirmed by mass spectrometry.

Fungal Isolates and Inoculum Preparation

Four plant pathogenic fungal species (*Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides* and *Fusarium oxysporum*) were grown on potato dextrose agar (PDA) in 9-cm petri dishes and incubated under cool-white fluorescent lights with a 12h photoperiod at ~24°C as previously described (Abril et al., 2008). Conidia were harvested from 7- to 10-day-old cultures, and the aqueous conidial suspensions were filtered to remove hyphae. The conidial matrix was removed from the conidia by washing each suspension three times as previously described (Abril et al., 2008). The conidia were suspended in mycological liquid medium (Roswell Park Memorial Institute 1640 [RPMI], Life Technologies, Grand Island, NY, USA). Conidial concentrations of the suspensions were determined photometrically (Espinel-Ingrof and Kerkering, 1991; Wedge and Kuhajek, 1998) and adjusted to a final concentration of approximately10^6 conidia/ml.

Microtiter Assay

Differences in mycelial growth in vitro have been used to demonstrate the sensitivity of fungal plant pathogens to natural product-based and commercially available fungicide standards with known modes of action (Wedge et al., 2001). A 96-well culture cluster was prepared for each replicate of each fungal isolate. Two hundred microliters of the buffered fungicide solution containing the conidial suspension were placed in each well of the 96-well culture plates (Corning, Inc., Corning, NY, USA) at fungicide concentrations of 0.3, 3.0, and 30 µM. Each compound was evaluated in triplicate against a non-inoculated well (negative control) containing the fungicide solution and RPMI at each concentration. The 96-well culture plates were incubated in a growth chamber for 72h to allow time for mycelial development past the initial germination times. Growth was evaluated by measuring absorbency of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL, USA).

The 96-well cell culture plates were set up so that eight wells containing the conidial suspension and RPMI served as unamended positive controls and four wells containing RPMI without conidial suspension were used as negative controls. Each test fungicide was run in duplicate at each concentration, and the experiment was repeated three times. Treatments were arranged as a split-plot design where whole plots were the four fungal isolates and sub-plots were the four fungicide solutions at the three doses. Mean absorbency values were used to evaluate fungal growth of three *Colletotrichum* spp. and *F. oxysporum* at each concentration of the fungicide solution at 48 and 72h. Mean absorbency values were expressed as percent inhibition/stimulation of fungal growth (% growth) using the formula:

$$\% \text{ growth} = \frac{[(\text{mean sample absorbency}) - (\text{mean unamended control absorbency})]}{(\text{mean unamended control absorbency})} \times 100$$

Positive numbers indicate stimulation of growth and negative numbers indicate inhibition compared to growth of the fungus on unamended medium.

Data Analysis

Statistical comparisons were made of fungal growth within each concentration of fungicide for each isolate. Each dose level and response time was analyzed separately. In

all trials data were evaluated by analysis of variance (ANOVA) using the general linear model procedure (GLM) of Statistical Analysis Systems (SAS) software (Version 9.1). Significant factors were separated and tested using Fisher's protected least significant difference (LSD) tests ($P<0.05$).

RESULTS AND DISCUSSION

None of the fungicides in the in vitro dose-response assays greatly inhibited growth of the four fungal species at 0.3 μM (Table 1). CAY-1 and captan inhibited the growth of all four species at 3 and 30 μM while benomyl inhibited the growth of three of the species, *C. fragariae*, *C. gloeosporioides* and *F. oxysporum*. Benomyl stimulated the growth of *C. acutatum* which supports previous reports that isolates of *C. acutatum* are resistant to benomyl (Smith and Black, 1993 a, b). Iprodione was ineffective against all four fungi at the concentrations tested. These initial tests with CAY-1 suggested that it might perform similarly to captan and benomyl against *Colletotrichum* spp. and *F. oxysporum*.

Results of follow-up field studies (not shown) were disappointing because plants treated with CAY-1 had as much disease as untreated control plants due to injury of fruit and foliage. This damage by CAY-1 (a saponin) could have been due to (1) its detergent-like properties affecting the epidermis of the receptacle of the fruit and the cuticle of the leaves of the strawberry or (2) other compounds present in the semi-purified CAY-1 used in the field trials that were not present in the purified CAY-1 used in the in vitro trials. Improvements in the formulation of CAY-1 might prevent this injury and result in better disease control.

ACKNOWLEDGMENTS

Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Dept. of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

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Tables

Table 1. Efficacy of CAY-1 and three commercial fungicides against four plant pathogenic fungi. Fungal growth is reported as mean percent inhibitions (-) or stimulation (+) for each fungicide at three concentrations after 72 hours growth[z].

	Fungicide rate and Fungal pathogen											
	0.3 µM				3.0 µM				30 µM			
Fungicide	Ca[y]	Cf	Cg	Fo	Ca	Cf	Cg	Fo	Ca	Cf	Cg	Fo
CAY-1	27	15	18	1	-99	-85	-100	-3	-99	-100	-100	-29
Benomyl	20	-29	-20	-3	30	-95	-83	-6	25	-96	-71	-83
Captan	12	18	16	0	-99	-98	-98	-23	-100	-99	-100	-100
Iprodione	11	5	6	-8	7	11	6	-9	41	52	22	5

[z] Compared to the growth of each fungal species in unamended potato dextrose broth.
[y] Ca = *Colletotrichum acutatum*; Cf = *Colletotrichum fragariae*; Cg = *Colletotrichum gloeosporioides*; Fo = *Fusarium oxysporum.*