Research

Natamycin, a New Biofungicide for Managing Crown Rot of Strawberry Caused by QoI-Resistant *Colletotrichum acutatum*

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Abstract

Anthraxnose crown rot of strawberry, caused by *Colletotrichum acutatum*, is an important disease affecting California nursery and fruit production. Preplant dip treatments of transplants with fludioxonil-cyprodinil or azoxystrobin are industry standards for managing the disease and have been used extensively. Following reports of reduced efficacy of azoxystrobin in the field, high levels of quinone outside inhibitor (QoI) resistance were detected in California isolates of the pathogen. Resistance was associated with the G143A mutation in the cytochrome *b* gene, similar to a previous report from Florida, and there were no detected fitness penalties in pathogenicity or virulence. Therefore, several alternative fungicides were investigated in laboratory and field studies. Subsequently, the new biofungicide natamycin was identified. Baseline sensitivities of 74 isolates of *C. acutatum* to natamycin were determined to be unimodal, with a range from 0.526 to 1.996 µg/ml (mean 0.973 µg/ml).

Although this toxicity was considerably lower than that of azoxystrobin (using sensitive isolates), fludioxonil, or cyprodinil, dip treatments of transplants with natamycin (at 500 or 1000 mg/liter) were highly effective. Disease severity and plant mortality in field studies with inoculated transplants were reduced to similarly low levels as treatments containing fludioxonil, whereas azoxystrobin failed in inoculations with QoI-resistant isolates of *C. acutatum*. Fruit yield was also significantly increased by natamycin as compared with the inoculated control. Differences in disease susceptibility were observed among cultivars evaluated, with Monterey and Portola more susceptible than Fronteras. Natamycin has a unique mode of action that is different from other fungicides registered on strawberry and, based on this research, was registered in the United States as a preplant, biofungicide dip treatment of strawberry transplants for management of anthracnose crown rot.

Anthraxnose is a major disease of strawberry (*Fragaria × ananassa* Duch.) in California nursery and fruit production (Gunnell and Gubler 1992; Smith 1998), an industry that represents 83% of fresh market fruit production in the United States and is estimated to have a value of over \$1.8 billion annually (CDFA 2015-2016). All parts of the plant are susceptible to infection, resulting in significant yield loss from any of the different phases of the disease, including crown rot, root rot, flower blight, fruit rot (i.e., black spot), petiole lesions, and leaf spots (Daugovish et al. 2009; Howard et al. 1992; Smith 2008). Members of the fungal genus *Colletotrichum* are associated with strawberry anthracnose worldwide, with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and occasionally *C. fragariae* A. N. Brooks most commonly causing crown rot and root rot, and *C. acutatum* J. H. Simmonds most commonly causing foliar and fruit rot (Freeman and Rodriguez 1995; Howard et al. 1992; Smith and Black 1990). In California, however, *C. acutatum* is the most common causal agent for all above- and belowground phases of the disease (Daugovish et al. 2009; Koike et al. 2008). More recently, *C. gloeosporioides* and *C. acutatum* have been considered to be species complexes (Damm et al. 2012; Weir et al. 2012).

Salmon- to orange-colored conidial masses are produced by *C. acutatum* in acervuli on infected tissues at high humidity. They are spread short distances by water splash from overhead sprinkler irrigation or rainfall, and long distances by infected plant material, soil associated with transplants, or contaminated equipment (Debode

et al. 2015; Eastburn and Gubler 1990; Howard et al. 1992). The pathogen can survive in infected plant debris and soil for at least 9 months in the absence of a host (Eastburn and Gubler 1990; Freeman et al. 2002). In fields that have been fumigated, disease outbreaks are attributed to planting of infected nursery stock. Transplants that appear healthy may have quiescent infections that are activated once conditions are favorable (Debode et al. 2015; Horn and Carver 1968; Leandro et al. 2001), typically under warm, wet environments (King et al. 1997). Crown infections can cause the entire plant to wilt and die during early establishment of fruit production fields where overhead irrigation is common (Eastburn and Gubler 1990). Major anthracnose crown rot outbreaks occurred in California nurseries during the 2000–01 (Daugovish et al. 2009) and 2015–16 (K. Ivors, unpublished) seasons, resulting in high economic losses.

For anthracnose crown rot management, water wash or fungicide treatments of bare-root transplants have been used in the nursery or immediately before planting in production fields (Daugovish et al. 2009; Freeman et al. 1997). Running water removes soil from root and crown tissues, potentially removing inoculum, but does not control any infections already present (Koike et al. 2008). Preplant fungicide dips have been shown to decrease anthracnose crown rot but only two products are currently registered for this use in the United States. The single-site mode of action quinone outside inhibitor (QoI) azoxystrobin has been used extensively as a preplant dip and as a foliar treatment for management of anthracnose and other fungal diseases (Bartlett et al. 2002; Koike et al. 2008; Turechek et al. 2006). Resistance, however, has been reported against this fungicide group in *C. acutatum* populations from strawberry in Florida (Forcelini et al. 2016). During the 2015–16 California nursery crown rot outbreak, growers reported reduced efficacy of azoxystrobin dips, suggesting that resistance may also be present in California (K. Ivors, unpublished). A premixture product of the anilino-pyrimidine cyprodinil and the phenylpyrrole fludioxonil is also registered but phytotoxicity and stunting of plants have been reported under some environmental conditions or following off-label plant exposure

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durations (G. Holmes, unpublished). In addition to chemical treatments, heat treatment of transplants may reduce mortality from anthracnose but this practice is not widely used because it can reduce plant vigor (Freeman et al. 1997). In-season fungicide spray applications are common to prevent foliar and fruit infections (Koike et al. 2008; MacKenzie and Peres 2012) but these are ineffective against crown rot and root necrosis (Daugovish et al. 2009).

Natamycin, also known as pimaricin, is a natural polyene macro-lide derived from fermentation of *Streptomyces* spp. (i.e., *Streptomyces natalensis*, *S. chattanoogensis*, *S. gilvosporeus*, and *S. lydicus*), and has been used as an antifungal additive in the food industry since the 1960s (Aparicio et al. 2016; Lu et al. 2008). Currently, it is registered in the United States as a biopesticide on mushroom beds and, recently, as a postharvest treatment on stone fruit and citrus. Because of its unique mode of action and because resistance has never been reported in filamentous fungi during extensive use in the food industry (Aparicio et al. 2016), this fungicide could be valuable in strawberry production where QoI resistance has been reported in *C. acutatum* and new rotational products are needed for dip treatments.

The purpose of this research was to evaluate new fungicides with registration potential for use as preplant dip treatments of bareroot strawberry transplants for the management of anthracnose crown rot. The specific objectives were to (i) determine whether QoI

resistance is present in *C. acutatum* populations in California and confirm with molecular tools; (ii) establish in vitro sensitivity ranges of effective concentration to inhibit mycelial growth or conidial germination by 50% (EC₅₀) values for selected new fungicides for QoI-sensitive and -resistant isolates of *C. acutatum*; (iii) evaluate the efficacy of fungicide dip treatments on disease severity, plant mortality, and fruit yield; and (iv) evaluate susceptibility of selected cultivars to disease.

Materials and Methods

Fungal isolates and culture methods. Seventy-four isolates of *C. acutatum* were obtained in 2015 and 2016 from commercial strawberry fields in Santa Barbara, San Luis Obispo, Ventura, Santa Cruz, Stanislaus, Tehama, San Joaquin, and Merced Counties of California (Table 1). The transplant materials used to establish these fields were originally sourced from five independent nursery companies that have operations throughout the state (Table 1). Crowns and petioles of symptomatic plants were sectioned into small pieces (10 mm³) using a scalpel, surface disinfested in 1% sodium hypochlorite for 2 min, triple-rinsed with sterile distilled water, and plated onto potato dextrose agar (PDA) acidified with 1 ml of 85% lactic acid per liter (APDA). Isolates were obtained from fruit lesions by touching a sterile cotton swab onto the lesion and then streaking the swab onto the surface of APDA plates.

Table 1. Isolates of *Colletotrichum acutatum* used in this study

Number	Isolate	Date of collection	Tissue	Origin ^v		Sensitivity ^w
				County	Nursery	
1	Ca117 ^x	25 May 2016	Petiole	Merced	D	S
2	Ca103	26 April 2016	Petiole	San Joaquin	B	R
3	Ca104	26 April 2016	Petiole	San Joaquin	B	R
4	Ca105	26 April 2016	Petiole	San Joaquin	B	R
5	Ca128	5 August 2016	Petiole	San Joaquin	B	R
6	Ca3 ^{xyz}	18 September 2015	Crown	San Luis Obispo	A	S
7	Ca11a ^{xyz}	18 September 2015	Crown	San Luis Obispo	A	S
8	Ca12a	18 September 2015	Crown	San Luis Obispo	A	S
9	Ca67 ^x	7 April 2016	Crown	San Luis Obispo	A	S
10	Ca75	10 April 2016	Crown	San Luis Obispo	A	S
11	Ca76 ^x	10 April 2016	Crown	San Luis Obispo	A	S
12	Ca1	9 September 2015	Crown	Santa Barbara	A	S
13	Ca5	22 September 2015	Crown	Santa Barbara	A	S
14	Ca6 ^z	22 September 2015	Crown	Santa Barbara	A	S
15	Ca7 ^x	22 September 2015	Crown	Santa Barbara	A	S
16	Ca9 ^x	22 September 2015	Crown	Santa Barbara	A	S
17	Ca10	9 September 2015	Crown	Santa Barbara	A	S
18	Ca130	7 August 2016	Crown	Santa Barbara	A	S
19	Ca134 ^x	6 September 2016	Petiole	Santa Barbara	B	S
20	Ca131 ^z	22 August 2016	Crown	Santa Barbara	E	R
21	Ca132 ^z	22 August 2016	Crown	Santa Barbara	E	R
22	Ca137 ^{xz}	22 August 2016	Crown	Santa Barbara	E	R
23	Ca21 ^{xy}	30 October 2015	Crown	Santa Cruz	D	S
24	Ca24 ^x	30 October 2015	Crown	Santa Cruz	D	S
25	Ca25	30 October 2015	Crown	Santa Cruz	D	S
26	Ca26 ^x	30 October 2015	Crown	Santa Cruz	D	S
27	Ca48	30 October 2015	Crown	Santa Cruz	D	S
28	Ca59	7 January 2016	Petiole	Stanislaus	A	S
29	Ca81	18 April 2016	Petiole	Stanislaus	A	S
30	Ca92	18 April 2016	Petiole	Stanislaus	A	S
31	Ca94	18 April 2016	Petiole	Stanislaus	A	S
32	Ca126 ^x	8 July 2016	Petiole	Stanislaus	A	S
33	Ca127	8 July 2016	Petiole	Stanislaus	A	S
34	Ca129 ^x	5 August 2016	Petiole	Stanislaus	A	S

(Continued on next page)

^v Nursery companies in California counties are coded A to E and describe the origin of plants in each field.

^w Sensitivity to the quinone outside inhibitor (QoI) azoxystrobin was determined using the spiral gradient dilution method: S = sensitive and R = resistant (threshold of effective concentrations of fungicides to inhibit mycelial growth or conidial germination by 50% $\geq$ 1 μ g/ml).

^x Isolate molecularly confirmed as *C. acutatum* using CaInt-1 and CaInt-2 primers (Adaskaveg and Hartin 1997).

^y Isolate used in a mixture of four QoI-sensitive or -resistant isolates for inoculation of transplants.

^z Isolate used for molecular characterization of QoI resistance.

Colonies with morphological characteristics of *Colletotrichum* spp. were subcultured onto PDA and examined microscopically. Colonies of *C. acutatum* were differentiated from other species of *Colletotrichum* by conidial morphology (Adaskaveg and Hartin 1997; Smith and Black 1990), and a subset of 26 isolates (Table 1) was confirmed to belong to the *C. acutatum* species complex using CaInt-1 and CaInt-2 primers targeting the internal transcribed spacer region of ribosomal DNA (Adaskaveg and Hartin 1997; Sreenivasaprasad et al. 1996). Cultures were stored in liquid nitrogen, and working stocks were kept as PDA plugs in sterile water at 12°C. For conidial production, a mycelial suspension was prepared from 4- to 7-day-old PDA cultures, spread onto new PDA plates, and allowed to air dry; then, 5 to 10 1.5-cm-long autoclaved strawberry petiole pieces were randomly placed onto the agar surface. Orange conidial sporulation was present after 3 to 4 days of incubation at 25°C and was used for inoculations or in vitro assays within 4 weeks.

Fungicides. Formulated products of natamycin (DelvoCid Instant; DSM, Heerlen, The Netherlands), azoxystrobin (Abound Flowable; Syngenta Crop Protection Inc., Greensboro, NC), chlorothalonil (Bravo Weather Stick; Syngenta Crop Protection Inc.), cyprodinil (Vangard 75WG; Syngenta Crop Protection Inc.), fludioxonil (Scholar 50WP; Syngenta Crop Protection Inc.), and pydiflumetofen (experimental product A19649B; Syngenta Crop Protection Inc.) were used for in vitro sensitivity studies (Fig. 1). For treatment of

plants, these products were used by themselves or in premixtures of fludioxonil and cyprodinil (Switch WG; Syngenta Crop Protection Inc.) or of pydiflumetofen and fludioxonil (experimental product A20560C; Syngenta Crop Protection Inc.) (Fig. 2). Additionally, the biological control *S. lydicus* WYEC 108 (minimum of 10³ CFU/g of formulated product; Actinovate AG; Valent U.S.A. LLC, Walnut Creek, CA) was included in field trials. Aqueous solutions were used in all studies.

Evaluation of in vitro sensitivity of fungicides. Fungicide sensitivities for all isolates of *C. acutatum* were determined using the spiral gradient dilution method (Förster et al. 2004). Briefly, an exponential concentration gradient for each fungicide was obtained by depositing appropriate stock concentrations radially onto 15-cm PDA plates using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood, MA). For azoxystrobin, PDA was amended with salicylhydroxamic acid at 100 µg/ml. Stock concentrations were 1,000 µg/ml for natamycin, 100 or 5,000 µg/ml for azoxystrobin, 200 µg/ml for chlorothalonil, 50 µg/ml for cyprodinil, 20 µg/ml for fludioxonil, and 10,000 µg/ml for pydiflumetofen. Sterile water was applied to control plates. After 2 to 4 h, 10 µl of each conidial suspension (5 × 10⁵ spores/ml) was streaked radially from the outer edge toward the center of the plate using a sterile pestle. Each isolate was replicated on opposite sides of the plate, and four isolates were evaluated on each plate. Plates were incubated in the dark for 20 h at 20°C for evaluation of conidial germination (azoxystrobin only) or

Table 1. (Continued from previous page)

Number	Isolate	Date of collection	Tissue	Origin ^v		Sensitivity ^w
				County	Nursery	
35	Ca83 ^{xz}	17 April 2016	Petiole	Tehama	C	S
36	Ca84	17 April 2016	Petiole	Tehama	C	S
37	Ca85	17 April 2016	Petiole	Tehama	C	S
38	Ca88	17 April 2016	Petiole	Tehama	C	S
39	Ca89	17 April 2016	Petiole	Tehama	C	S
40	Ca90 ^x	17 April 2016	Petiole	Tehama	C	S
41	Ca91	17 April 2016	Petiole	Tehama	C	S
42	Ca12b ^{xyz}	26 October 2015	Crown	Ventura	B	R
43	Ca13 ^{xyz}	26 October 2015	Crown	Ventura	B	R
44	Ca14 ^{xyz}	26 October 2015	Crown	Ventura	B	R
45	Ca17 ^z	6 October 2015	Crown	Ventura	B	R
46	Ca28	30 October 2015	Crown	Ventura	B	R
47	Ca29	30 October 2015	Crown	Ventura	B	R
48	Ca30 ^x	30 October 2015	Crown	Ventura	B	R
49	Ca31	30 October 2015	Crown	Ventura	B	R
50	Ca32	30 October 2015	Crown	Ventura	B	R
51	Ca33	30 October 2015	Crown	Ventura	B	R
52	Ca49 ^{xyz}	6 October 2015	Crown	Ventura	B	R
53	Ca51	23 December 2015	Crown	Ventura	B	R
54	Ca52 ^z	23 December 2015	Crown	Ventura	B	R
55	Ca55 ^x	29 January 2016	Fruit	Ventura	B	R
56	Ca60	9 March 2016	Crown	Ventura	B	R
57	Ca61	9 March 2016	Crown	Ventura	B	R
58	Ca62 ^z	9 March 2016	Crown	Ventura	B	R
59	Ca65 ^x	5 April 2016	Crown	Ventura	B	R
60	Ca120 ^x	29 June 2016	Crown	Ventura	B	R
61	Ca121	29 June 2016	Crown	Ventura	B	R
62	Ca123 ^z	29 June 2016	Crown	Ventura	B	R
63	Ca124	29 June 2016	Crown	Ventura	B	R
64	Ca15 ^{xyz}	6 October 2015	Crown	Ventura	B	S
65	Ca16	4 October 2015	Crown	Ventura	B	S
66	Ca18	4 October 2015	Crown	Ventura	B	S
67	Ca19	4 October 2015	Crown	Ventura	B	S
68	Ca53	23 December 2015	Crown	Ventura	B	S
69	Ca57 ^x	29 January 2016	Fruit	Ventura	B	S
70	Ca69	5 April 2016	Crown	Ventura	B	S
71	Ca70	5 April 2016	Crown	Ventura	B	S
72	Ca82	2 June 2016	Petiole	Ventura	B	S
73	Ca122	29 June 2016	Crown	Ventura	B	S
74	Ca125	29 June 2016	Crown	Ventura	B	S

for 72 h at 25°C for evaluation of mycelial growth. EC₅₀ values were determined as described previously (Förster et al. 2004). Each isolate was evaluated twice for each fungicide.

Molecular characterization of QoI resistance in *C. acutatum*. A subset of 5 QoI-sensitive and 11 QoI-resistant *C. acutatum* isolates (Table 1) were grown for 7 to 10 days on PDA. DNA was extracted from mycelia using the MPBio FastDNA kit (MPBio, Santa Ana, CA). Polymerase chain reaction amplification was done using primers C.gramcytb-bf1 and C.gramcytb-br1 targeting the cytochrome *b* gene (*cytb*), according to Forcelini et al. (2016). Product amplification was confirmed by agarose (1%) gel electrophoresis in 0.5× Tris-borate-EDTA buffer. Amplicons were purified using ExoSap-IT (Affymetrix Inc., Santa Clara, CA) and sequenced at the Core Instrumentation Facility of the University of California Riverside Institute for Integrative Genome Biology. Sequences were aligned using the BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html).

Effect of preplant dip treatments on disease severity and plant mortality. Two trials were conducted using Portola and Fronteras (trial 1) or Monterey and Fronteras (trial 2) strawberry cultivars.

Bareroot transplants were obtained from two nurseries and dipped for 4 min with agitation in aqueous conidial suspensions (10⁶ conidia/ml) of a mixture of four each of QoI-sensitive or -resistant isolates of *C. acutatum* (Table 1). Plants were stored at 20°C for 8 h to allow conidia to germinate and infect, and then up to 3 days at 5°C. Plants were then dipped in aqueous fungicide solutions for 4 min with agitation. Inoculated control plants were dipped in water or were left untreated. Excess solution was allowed to drain for approximately 2 min, and transplants were stored in plastic bags at 5°C overnight. Planting in the field was done on 4 March 2016 (trial 1), and 29 October 2016 (trial 2), at the University of California Riverside Agricultural Operations facility. The field area had been fallow for over 1 year, and had not been planted with strawberry previously. No fumigation treatment was applied prior to planting. Plants were spaced 30 cm apart in double-row beds in a randomized complete block design. Each bed represented a block (replication) of plots, and there were four blocks each for plants inoculated with QoI-sensitive or -resistant isolates in each study. Each replication of treatments consisted of a plot of 10 plants of each cultivar.

Fig. 1. Frequency distribution of effective concentrations to inhibit **A** and **C** to **F**, mycelial growth or **B**, spore germination of 74 isolates of *Colletotrichum acutatum* by 50% (EC₅₀ values) as determined by the spiral gradient dilution method. Bar height represents the number of isolates within each bin, and bin widths were calculated using Scott's method (Scott 1979). For determination of bin width for azoxystrobin evaluations, only sensitive isolate values were considered.

Overhead irrigation was applied 3 days each week for 30 min each in the morning and afternoon for the first month, and drip irrigation was applied for 2 days each week for 2 h throughout the study. Fertilizer was applied through drip irrigation weekly or biweekly. No foliar fungicide or insecticide applications were made.

Plants were evaluated periodically for disease. Fungal isolations were done from diseased tissues of 15 randomly selected, symptomatic plants each that were inoculated with QoI-sensitive or -resistant isolates to validate that *C. acutatum* was the causal agent. Isolations from crown, leaf, and fruit tissues were done as described above, except that tissues were plated onto PDA amended with ampicillin (130 µg/ml) and rifampicin (20 µg/ml). Plants in the field were evaluated after 5 (trial 1) or 14 (trial 2) weeks for disease severity on aboveground growth using the following scale: 0 = healthy, vigorous plant; 1 = growth reduced; 2 = growth and vigor reduced, less than 50% the size of healthy plants; 3 = growth and vigor extremely reduced, less than 25% the size of healthy plants, and

4 = plant dead. Severity ratings were averaged for each 10-plant replication. The incidence of plant mortality was based on the number of dead plants (rating = 4) of the 10 plants of each cultivar in each replication.

Effect of preplant dip treatments on fruit yield. Crop yield was determined using Fronteras. In trial 1, a single harvest of all mature red, commercially acceptable fruit for each treatment replication was conducted 10 weeks after planting, and fruit were weighed. In trial 2, flowers and immature fruit were removed 7 and 12 weeks after planting, similar to commercial practices to encourage vegetative growth during the winter period. All mature red, commercially acceptable fruit were harvested 20 and 23 weeks after planting, and fruit weights were combined for the two harvests for each treatment replication.

Statistical analysis. For in vitro fungicide toxicity experiments, variances were homogeneous for the two experiments according to Bartlett's test, and data were combined for each isolate–fungicide combination. This data set was used to determine mean values and

Fig. 2. Effect of fungicide dip treatments of **A** and **B**, Portola; **C** and **D**, Monterey; or **E** and **F**, Fronteras strawberry transplants inoculated with a mixture of four isolates each of **A**, **C**, and **E**, quinone outside inhibitor (QoI)-sensitive or **B**, **D**, and **F**, QoI-resistant isolates of *Colletotrichum acutatum* on disease severity. Plants were evaluated after 5 or 14 weeks for aboveground vegetative growth based on a rating scale of 0 = healthy, vigorous plant; 1 = growth reduced; 2 = growth and vigor reduced; 3 = growth and vigor extremely reduced; and 4 = dead. Average disease severity was determined based on 10 plants for each of four replications in each experiment. Bars followed by the same letter within a graph are not significantly different based on least significant difference mean separation ($P > 0.05$) procedures. Rates are based on active ingredient, except for *Streptomyces lydicus*, where the rate is based on formulated product.

the range of sensitivity. Mean EC₅₀ values for mycelial growth or spore germination were plotted for each fungicide in frequency distributions according to Scott's method (Scott 1979).

Data for incidence of mortality were arcsin-transformed, whereas yield data were transformed using $y = \log_{10}(x + 1)$ prior to statistical analysis for both trials. All data for disease severity, incidence of mortality, and yield were normally distributed according to Shapiro-Wilk's test. Data were combined from different experiments when variances were homogeneous according to Bartlett's test. Analyses of variance or generalized linear model procedures were performed following a factorial design with cultivar, inoculum type, and fungicide treatment as the main factors. When interactions between cultivar and inoculum type were significant, data were analyzed separately for each combination, with treatment as the single factor. Multiple comparisons for treatment means were conducted using Fisher's least significant difference test. All statistical procedures were done using SAS (version 9.4; SAS Institute, Inc., Cary, NC), with a significance value of $\alpha = 0.05$.

Results

In vitro sensitivity of isolates of *C. acutatum* to fungicides. Of the 74 isolates tested, 29 were determined to be QoI resistant, with EC₅₀ values for mycelial growth and conidial germination higher than 40 µg/ml (Table 1). The remaining 45 isolates were QoI sensitive, with mean EC₅₀ values for mycelial growth and germination inhibition of 0.034 µg/ml (range 0.015 to 0.064 µg/ml) and 0.129 µg/ml (range 0.060 to 0.267 µg/ml), respectively. Mean EC₅₀ values for mycelial growth for all 74 isolates for chlorothalonil, cyprodinil, fludioxonil, and natamycin were 0.267 µg/ml (range 0.115 to 0.549 µg/ml), 0.025 µg/ml (range 0.012 to 0.390 µg/ml), 0.001 µg/ml (range 0.0006 to 0.0014 µg/ml), and 0.973 µg/ml (range 0.526 to 1.996 µg/ml), respectively. Sensitivity data are presented separately in Table 2 for QoI-sensitive and -resistant isolates. For azoxystrobin, a >1,000× difference in sensitivity between sensitive and resistant groups was observed. No significant differences in sensitivity to cyprodinil ($P = 0.3468$), fludioxonil ($P = 0.2556$), and natamycin ($P = 0.1010$) were found for QoI-sensitive and -resistant isolates. For chlorothalonil, QoI-sensitive isolates were significantly ($P = 0.0375$) less sensitive (mean EC₅₀ value of 0.276 µg/ml) than QoI-resistant isolates (mean EC₅₀ value of 0.250 µg/ml) (Table 2). Pydiflumetofen was not inhibitory to mycelial growth at concentrations of up to 80 µg/ml tested.

Frequency distributions of EC₅₀ categories for each fungicide are shown in the histograms of Figure 1A to F. Distributions for all fungicides were unimodal, except for azoxystrobin, where a bimodal distribution was observed.

Molecular characterization of QoI resistance in *C. acutatum*. All 11 QoI-resistant isolates selected for *cytb* sequencing were found to contain a single amino acid substitution from glycine (G) to alanine (A) at codon 143 (G143A mutation), resulting from a base pair substitution from guanine to cytosine. None of the QoI-sensitive isolates sequenced showed this mutation.

Effect of preplant dip treatments on disease severity and plant mortality. The overall models for severity and mortality disease measurements, as well as for the independent variables cultivar,

type of inoculum (i.e., QoI-sensitive or -resistant isolates of *C. acutatum*), and treatment, were highly significant ($P < 0.0001$). There were significant ($P < 0.0001$) interactions between cultivar and treatment, as well as between type of inoculum and treatment, but not between cultivar and type of inoculum ($P = 0.8133$ and 0.0564 for severity and mortality, respectively). Due to these interactions, data were analyzed separately for each cultivar and inoculum type. In trial 1, Fronteras was significantly ($P < 0.0001$) less susceptible to anthracnose crown rot (mean severity 1.4, mean mortality 24.9%) than Portola (mean severity 2.3, mean mortality 52.2%). Similarly, in trial 2, Fronteras was significantly ($P < 0.0001$) less susceptible (mean severity 1.6, mean mortality 22.4%) than Monterey (mean severity 2.2, mean mortality 39.9%). Inoculation with QoI-resistant isolates resulted in significantly ($P < 0.0001$) higher disease severity (mean 2.1) and incidence of mortality (mean 40.7%) than when using QoI-sensitive isolates (mean severity 1.6, mean mortality 29.0%).

Disease severity and incidence of mortality of untreated, inoculated control plants were high at evaluation time 5 weeks after spring-time planting of Portola or 14 weeks after fall planting of Monterey strawberry. After inoculation with QoI-sensitive isolates, disease severity was 3.5 and 3.2 and incidence of mortality was 85.0 and 69.1% for untreated Portola and Monterey controls, respectively (Figs. 2 and 3A to D). After inoculation with QoI-resistant isolates, these values were 4.0 and 3.4, and 100 and 74.6%, for the two cultivars, respectively. In contrast, average disease severity and mortality for two studies with Fronteras planted in the spring or fall were 2.1 and 32.9%, respectively, for QoI-sensitive inoculum and 2.5 and 47%, respectively, for QoI-resistant inoculum (Figs. 2E and F and 3E and F). Fungal isolations from diseased tissues of randomly selected symptomatic plants confirmed *C. acutatum* as the causal agent of disease symptoms. Water dip treatments of transplants were generally ineffective in reducing disease and mortality as compared with the untreated control, and only resulted in a significant ($P < 0.05$) reduction in disease severity for Monterey inoculated with QoI-resistant isolates of *C. acutatum* (Fig. 2D).

Transplants of all three strawberry cultivars that were inoculated with QoI-sensitive or -resistant isolates and dip treated with natamycin at either rate or with the pydiflumetofen-fludioxonil or fludioxonil-cyprodinil premixtures in all cases showed significantly ($P < 0.05$) lower disease severity (Fig. 2) and plant mortality (Fig. 3) as compared with the controls. Furthermore, there were generally no significant ($P > 0.05$) differences among these four treatments, and the new fungicides provided similar control to the registered premixture of fludioxonil and cyprodinil. Among the six cultivar-inoculum combinations, the 0.5 g/liter rate of natamycin was significantly ($P > 0.05$) less effective than the 1 g/liter rate only for Portola inoculated with QoI-resistant isolates (Figs. 2B and 3B). No stunting of plants or other signs of phytotoxicity were observed in any treatment in these trials.

Treatments with azoxystrobin provided a high level of disease control when transplants were inoculated with QoI-sensitive isolates of the pathogen, and efficacy was similar to natamycin or the two premixtures containing fludioxonil (Figs. 2A, C, and E and 3A, C, and E). Azoxystrobin, however, was not effective when plants were

Table 2. Effective concentrations of fungicides to inhibit mycelial growth or conidial germination of quinone outside inhibitor (QoI)-sensitive and -resistant isolates of *Colletotrichum acutatum* by 50% (EC₅₀)

Fungicide	Stage	EC ₅₀ (µg/ml) ^a			
		QoI-sensitive, n = 45		QoI-resistant, n = 29	
		Mean	Range	Mean	Range
Azoxystrobin	Mycelium	0.034	0.015–0.064	>40	NA
Azoxystrobin	Conidia	0.129	0.060–0.267	>40	NA
Chlorothalonil	Mycelium	0.276	0.155–0.549	0.250	0.115–0.400
Cyprodinil	Mycelium	0.026	0.019–0.360	0.025	0.012–0.390
Fludioxonil	Mycelium	0.0009	0.0006–0.0013	0.0010	0.0006–0.0014
Natamycin	Mycelium	0.996	0.526–1.778	0.902	0.565–1.996
Pydiflumetofen	Mycelium	>80 ^a	NA	>80	NA

^a Inhibition was not observed using the highest concentration tested (i.e., 40 or 80 µg/ml) in the spiral gradient dilution method. NA = not applicable.

inoculated with QoI-resistant isolates, and there was no reduction in crown rot severity and plant mortality as compared with the controls (Figs. 2B, D, and F and 3B, D, and F).

Among the remaining treatments, chlorothalonil showed inconsistent performance. The fungicide significantly reduced disease severity as compared with the untreated control in five of the six cultivar–inoculum combinations (Fig. 2) and plant mortality in four of the six combinations (Fig. 3). Efficacy of chlorothalonil was often statistically lower than that of natamycin and the two premixture treatments containing fludioxonil. Pydiflumetofen was among the least effective treatments, and disease severity was significantly reduced from the untreated control only on Monterey using QoI-sensitive inoculum (Fig. 2C). The biocontrol *S. lydicus* was ineffective in all studies.

Effect of preplant dip treatments on fruit yield. Because harvest frequency and timing were different for the two trials conducted in different growing seasons with Fronteras, data were analyzed separately for each study and inoculum type. Yield data generally reflected performance of the treatments in reducing disease severity

and plant mortality (Table 3). Water dip treatments did not result in increased yield as compared with the untreated control except in the first trial with QoI-sensitive inoculum. In both studies, natamycin treatments had yields statistically similar to those of the industry standard fludioxonil-cyprodinil or the experimental pydiflumetofen-fludioxonil premixture. Yield of azoxystrobin-treated transplants was statistically higher ($P < 0.05$) than the untreated control and statistically similar to natamycin and fludioxonil-containing treatments in inoculations with QoI-sensitive isolates (Table 3). Yield for azoxystrobin, however, was similar to the untreated control in both studies after inoculation with QoI-resistant isolates.

Fruit yields of the remaining treatments (i.e., water control, *S. lydicus*, pydiflumetofen, and chlorothalonil) were not significantly different from the untreated control in trial 1. In trial 2, results were similar but chlorothalonil and *S. lydicus* significantly ($P < 0.05$) increased yield from the untreated control for transplants inoculated with QoI-sensitive isolates. Still, natamycin at both rates, fludioxonil-containing treatments, and azoxystrobin (in inoculations with QoI-sensitive isolates) had the highest numerical yields among all treatments.

Fig. 3. Effect of fungicide dip treatments of **A** and **B**, Portola; **C** and **D**, Monterey; or **E** and **F**, Fronteras strawberry transplants inoculated with a mixture of four isolates each of **A**, **C**, and **E**, quinone outside inhibitor (QoI)-sensitive or **B**, **D**, and **F**, QoI-resistant isolates of *Colletotrichum acutatum* on plant mortality. Plants were evaluated after 5 or 14 weeks. Average plant mortality is based on the number of dead plants out of 10 plants for each of four replications in each experiment. Bars followed by the same letter within a graph are not significantly different based on least significant difference mean separation ($P > 0.05$) procedures. Rates are based on active ingredient, except for *Streptomyces lydicus*, where the rate is based on formulated product.

Discussion

Preplant dip treatments of strawberry transplants for the management of anthracnose crown rot have relied on the use of fludioxonil-cyprodinil and azoxystrobin since the early 2000s. With extensive use of these fungicides for pre- and postplant disease management, there is a high risk for selection for resistance. Thus, new preplant fungicide treatments are needed that have different modes of action from registered products. In this study, we report the distribution and genetic basis for QoI resistance in California populations of *C. acutatum*, and we identify a new, highly effective preplant treatment, the biofungicide natamycin, that has a unique mode of action from all other fungicides registered on strawberry worldwide.

QoI resistance was first described in *C. acutatum* from strawberry in Florida in 2013 (Forcelini et al. 2016). G143A and F129L mutations in *cytb* were identified conferring high and moderate resistance levels, respectively. In our study on California isolates, only the G143A mutation was detected although only 11 resistant isolates were sequenced of the total of 29 isolates that we identified. Because all of our resistant isolates had EC₅₀ values for azoxystrobin of >40 µg/ml and, thus, were highly resistant, other resistance genotypes may not be present in California at this time. Diseased plants that yielded resistant isolates originated from two of five California nursery sources and were collected in three of eight counties sampled. Therefore, resistance currently may still be limited in distribution but could spread quickly with frequent movement of plant material. Population genetic analyses of isolates may provide information if resistance developed independently in the two nurseries and in Florida. Resistance can be managed by using treatments with different modes of action. The availability of alternative fungicides is important because resistance conferred by the G143A mutation characteristically cannot be mitigated using higher, off-label application rates of QoI compounds in the field (Bartlett et al. 2002; Fernández-Ortuño et al. 2012; Kim et al. 2003; Lesniak et al. 2011). Additionally, this mutation was shown to be stable in *C. acutatum* populations and did not result in a fitness penalty in laboratory assays (Forcelini et al. 2018). In our study, QoI-resistant and -sensitive isolates were similarly pathogenic but disease severity and mortality after inoculation with resistant isolates were higher than for QoI-sensitive isolates. Several studies on other fungal pathogens have indicated that resistant isolates harboring the G143A mutation did not have reduced fitness as compared with wild-type sensitive isolates (Corio-Costet et al. 2011; Karaoglanidis et al. 2011; Rallos et al. 2014). Fitness of resistant isolates of *Magnaporthe oryzae*, however, was reduced (Ma and Uddin 2009).

In vitro toxicities of chlorothalonil, cyprodinil, fludioxonil, and natamycin all exhibited unimodal distributions of sensitivities. Although cyprodinil and fludioxonil have been used for many years in California strawberry production, no resistant isolates were detected in our samplings, and results reflect current sensitivity levels

in *C. acutatum* populations in the sampling locations. A high in vitro activity of the fludioxonil-cyprodinil premixture was previously determined for *C. truncatum* (Torres-Calzada et al. 2015), and values were in a range similar to that in our evaluation of the individual active ingredients. Because natamycin has never been used in commercial strawberry production, EC₅₀ values determined can be considered a baseline sensitivity that can be used as a reference in future resistance monitoring.

Although in vitro toxicity of natamycin to *C. acutatum* was considerably lower than that of azoxystrobin (using sensitive isolates), fludioxonil, and cyprodinil, dip treatments of strawberry transplants with natamycin were highly effective and similar to those with azoxystrobin (when disease was caused by QoI-sensitive isolates) or fludioxonil-cyprodinil. Chlorothalonil was inconsistent in reducing anthracnose crown rot and plant mortality in our studies. This confirms previous studies on strawberry anthracnose where chlorothalonil was not very effective (Daugovish et al. 2009). Fruit yields increased as disease severity and plant mortality decreased after treatment with fungicides that were effective against anthracnose crown rot. Yields overall were much lower in the first trial that was initiated in the spring because high temperatures that sporadically occur at the inland California field site in the spring caused plants to decline. The second trial that was initiated in fall to winter and was harvested in the spring allowed time for greater plant growth. Additionally, flowers during early growth were removed to encourage vegetative growth, and two consecutive harvests were combined in the second study. For both spring- and fall-planted trials, the lack of foliar fungicide sprays that are standard in commercial field settings also likely contributed to lower overall yield values.

Treatments were evaluated as dips of transplants because planting material is a main source of introducing *C. acutatum* into a field and, currently, dip treatments of transplants are a common practice in California in the establishment of new strawberry fields. As shown in our results, plant mortality was reduced significantly by natamycin and fludioxonil-cyprodinil when transplants were inoculated with QoI-sensitive or -resistant isolates. Under highly favorable disease conditions, additional foliar treatments will have to be applied to protect plants from petiole and leaf lesions, as well as fruit rot throughout the season, and these can be integrated with those for management of gray mold caused by *Botrytis* spp. The experimental fungicide pydiflumetofen was not effective against *C. acutatum* in our in vitro and field studies. This fungicide, however, is very active against *B. cinerea* and has shown high efficacy in reducing powdery mildew of strawberry (J. E. Adaskaveg, unpublished). The pydiflumetofen-fludioxonil premixture was among the most effective of our preplant dip treatments for crown rot of strawberry but this was most likely due to the fludioxonil component. Therefore, this premixture has potential as a broad-spectrum postplant field fungicide for strawberry.

Table 3. Effect of fungicide dip treatments of Fronteras strawberry transplants on fruit yield

Treatment	Rate (g/liter) ^z	Mean fruit weight (g) per replication ^y			
		Trial 1		Trial 2	
		Sensitive	Resistant	Sensitive	Resistant
Untreated control	–	23.9 f	81.2 bc	834.6 d	640.9 c
Water control	–	125.0 cde	101.8 ab	992.8 cd	813.0 c
<i>Streptomyces lydicus</i> WYEC 108	1.8	57.4 ef	29.0 c	1220.0 bc	924.4 bc
Pydiflumetofen	0.08	63.3 def	47.8 bc	988.6 cd	747.1 c
Chlorothalonil	1.35	102.4 cdef	116.7 ab	1,294.0 bc	1,136.9 abc
Natamycin	0.5	161.8 bc	261.7 a	1,826.8 ab	1,979.7 a
Natamycin	1.0	161.1 bc	145.2 ab	1,738.4 ab	1,670.6 ab
Azoxystrobin	0.16	378.1 a	16.4 c	1,946.5 ab	682.6 c
Fludioxonil/cyprodinil	0.15/0.22	190.2 bc	254.9 a	1,998.8 ab	1,651.5 ab
Pydiflumetofen/fludioxonil	0.08/0.14	243.1 b	217.7 a	2,325.2 a	1,921.3 a

^y For each treatment, 10 plants were used for each of four replications for each inoculum mixture (isolates of *Colletotrichum acutatum* sensitive or resistant to quinone outside inhibitors). Fruit weight for trial 1 was determined in a single harvest approximately 10 weeks after planting and for trial 2 in two combined harvests approximately 20 and 23 weeks after planting. Data were transformed using the equation $y = \log_{10}(x + 1)$ and evaluated using analysis of variance and least significant difference mean separation procedures ($P < 0.05$). Mean values followed by the same letter within each column are not significantly different.

^z Rates are based on active ingredient, except for *S. lydicus*, where the rate is based on formulated product.

Natamycin is classified as a biofungicide because it is a commercial fermentation product of *S. natalensis* (Delves-Broughton 2014). The dip application of transplants developed in this study led to its registration (as the product Zivion M; DSM) on strawberry, representing the first use of natamycin in field agriculture. Natamycin previously was registered for management of dry bubble disease of mushroom caused by *Verticillium fungicola* var. *fungicola*, and as a postharvest treatment for managing citrus and stone fruit decays caused by *Monilinia*, *Penicillium*, *Geotrichum*, and *Botrytis* spp. (Chen et al. 2016). Its unique mode of action is the direct binding to ergosterol, the primary sterol in fungi, leading to disruption of membrane function and cellular transport (Aparicio et al. 2016; Hamilton-Miller 1974). It does not inhibit ergosterol production as do sterol biosynthesis- or demethylation-inhibiting fungicides. Thus, in 2017, it was assigned its own Fungicide Resistance Action Committee Code, Number 48 (FRAC 2017). Natamycin is designated as a biopesticide by the United States Environmental Protection Agency and, therefore, it is exempt from pesticide tolerances in the United States, and is “generally recognized as safe” by the U.S. Food and Drug Administration. The registration of natamycin is furthermore important for the strawberry industry because, after many years of use in the food industry, resistance in filamentous fungi has not been reported (Delves-Broughton 2014).

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