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Activity of Quinones on *Colletotrichum* Species

GIOVANNI MEAZZA,† FRANCK E. DAYAN,‡ AND DAVID E. WEDGE*,‡

Isagro Ricerca Srl, via G. Fauser, 4, 28100 Novara, Italy, and The National Center for Natural Products Research, Natural Products Utilization Research Unit, Agricultural Research Service, United States Department of Agriculture, University, Mississippi 38677

The antifungal activity of 1,4-naphthoquinones, 1,2-naphthoquinones, 1,4-benzoquinones, and anthraquinones from our natural products collection was tested by direct bioautography to identify natural products with potential use in agricultural fungal pathogen control. Quinones demonstrated good to moderate antifungal activity against *Colletotrichum* spp. *Colletotrichum fragariae* was the most sensitive species to quinone-based chemistry, *Colletotrichum gloeosporioides* had intermediate sensitivity, while *Colletotrichum acutatum* was the species least sensitive to these compounds.

KEYWORDS: Fungicide; natural products; triketone; naphthoquinone; benzoquinone; antifungal activity

INTRODUCTION

Increasing incidence of resistance to fungicides by plant pathogens and loss of available agents for disease control are two factors that drive the need to search for new plant protectants. In addition, the desire for safer agrochemicals with lower environmental and mammalian toxicity is a major concern. Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by different modes of action than existing antifungal agents, consequently avoiding problems of cross-resistance to chemicals currently used (*1*−*3*).

The functions of most antimicrobial bioactive plant secondary metabolites are not known, but these chemicals are thought to be important for survival and fitness of plants by protecting against microorganisms or by targeting cell proliferation of invading pathogens (*4*). Benzo-, naphtho-, and anthraquinones are common in nature (*5*). Many naphtho- and anthraquinones occur in higher plants, and naphthoquinones are often responsible for the pigmentation of colored heartwood and bark. Shikonin, a quinone from the root bark of *Lithospermum erythrorhizon*, demonstrated good antifungal activity in our assays. Because the commercial agrochemical fungicides chloranil (2,3,5,6-tetrachloro-*p*-benzoquinone), dichlone (2,3-dichloro-1,4-naphthoquinone), dithianon (2,3-dicyano-1,4-dithiaanthraquinone), oxine (8-hydroxyquinoline), oxalinic acid, and pyroquilon (1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one) are based on quinone chemistry (*6*, *7*), the following study was undertaken to evaluate naturally occurring phenolics, especially quinones, and other related compounds for their antifungal effects on plant pathogenic fungi.

Filamentous fungi of the genus *Colletotrichum* and its teleomorph *Glomerella* are major plant pathogens worldwide. *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin (*8*, *9*) and are serious problems for strawberry (*Fragaria* x *ananassa* Duch.) fruit and plant production in many areas of the world (*10*, *11*). The pathogens *Colletotrichum acutatum* J. H. Simmonds, *Colletotrichum gloeosporioides* (Penz.) Penz., and *Colletotrichum fragariae* A. N. Brooks can occur singly or in combination and can infect flowers, fruit, leaves, petioles, stolons, and crowns (*12*−*15*). New approaches to anthracnose disease control are necessary as the effectiveness and availability of commercial fungicides decrease.

As part of a program to discover natural product-based fungicides with low environmental and mammalian toxicity, several sensitive detection systems were developed for the rapid evaluation of antifungal agents. Bioautography assays using *Colletotrichum* species as the indicator species are used routinely to identify antifungal components from plant extracts, marine organisms, and other natural sources (*16*, *17*). In this paper, we report the antifungal activity of several classes of quinones and related compounds from our collection of natural products. Their activity and selectivity toward three agronomically important *Colletotrichum* species are reported.

MATERIALS AND METHODS

Pathogen Production. *C. fragariae* (isolate CF63), *C. acutatum* (isolate CAGoff), and *C. gloeosporioides* (isolate CG162) obtained from Dr. B. J. Smith (USDA, ARS, Small Fruit Research Station, Poplarville, MS) were used for all pathogen and bioautography studies. Isolate CF63 is one of the most virulent isolates infecting strawberry plants and inducing both crown and fruit rot (*18*). CF63, CAGoff, and CG162 were used as standard test isolates because of our extensive knowledge of these isolates and their fungicide sensitivity profiles in both bioautography and microtiter formats.

Inoculum Preparation. Fungal cultures were initiated on potato−dextrose agar (PDA, Difco, Detroit MI) from spores stored on silica gel. Conidia harvested from PDA cultures were subsequently transferred

* To whom correspondence should be addressed. Tel: 662-915-1137. Fax: 662-915-1035. E-mail: dwedge@olemiss.edu.
† Isagro Ricerca Srl.
‡ United States Department of Agriculture.

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to a strawberry agar (SA) sporulation medium comprised of 125 mL of pureed strawberry fruit and 20 g of agar (Bacto-agar, Difco) per liter and incubated at 24 ± 2 °C under cool-white fluorescent lights (55 ± 5 μmols/m^2/s^1) with a 12 h photoperiod. *Colletotrichum* cultures were subcultured or harvested from SA every 7−10 days. Conidia were harvested from 7 to 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 from a standard curve based on the percent of transmittance (%T) at 625 nm, and suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^6 conidia/mL (*19, 20*).

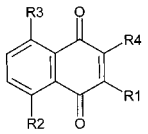
Conidial suspensions of each *Colletotrichum* species (CF63, CAGoff, and CG162) were adjusted to a concentration of 3.0×10^5 conidia/mL with liquid potato−dextrose broth (PDB, Difco) and 0.1% Tween-80. Using a chromatographic sprayer, each 250 μm silica Gel GF Uniplate (Analtech, Inc. Newark, DE) thin-layer chromatography (TLC) plate was sprayed lightly (to a damp appearance) three times with the conidial suspension.

Bioautography. Antifungal compounds were indicated by inhibition of fungal growth on chromatographic plates using modifications of TLC bioautographic assays (*16, 21, 22*). Quinone and commercial fungicide standards were dissolved in 95% MeOH at 2 mM concentrations. Using a disposable glass micropipet for each sample, 4 μL of each test compound was placed on the TLC plate in a grid format to achieve a final amount of 8 nmol (ca. 2.5 μg) of active ingredient (*17*). To detect biological activity directly on the TLC plate, silica gel plates were sprayed with either of the three spore suspensions. Inoculated plates were placed in a 30 cm × 13 cm × 7.5 cm 398-C moisture chamber (Pioneer Plastics, Inc. Dixon, KY) and incubated in a growth chamber at 24 ± 1 °C and 12 h photoperiod under 60 ± 5 μmols/m^2/s^1 light. Inhibition of fungal growth was measured 4 days after treatment. Sensitivity of each fungal species to each test compound was determined by comparing the size of inhibitory zones. Existing methods for detecting antifungal activity are nearly as numerous as the compounds being evaluated. Most methods evaluate the inhibition of radial growth on various substrate or in liquid media. In this paper, we report on compounds evaluated in our primary screen using direct bioautography where compounds are applied in a matrix format. Many factors including molecular weight, functional groups, and thickness of the substrate govern concentration gradients, which influence the diameter of growth inhibition. Thus, interpretation of the data is limited by the fact that the diameter of growth inhibition is dependent on the combined effect of the potency and the physicochemical properties of the compounds being tested (*23*).

Benomyl, chlorothalonil, and captan (Chem Service, Inc. West Chester, PA) were used as fungicide standards. Benomyl (a benzimidazole inhibitor of microtubule formation) and captan (a phthalimid with multisite inhibition) are the commercial fungicides labeled for control on strawberry anthracnose. Chlorothalonil (a substituted benzene with multisite inhibition) is used for control of anthracnose disease on turf and ornamentals.

Sources of Natural Products and Chemicals. Menadione, emodin, and leucoquinizarin were purchased from Sigma/Aldrich; 1,8- and 1,4-dihydroxyanthraquinone and 1,2-naphthoquinone were from Fluka (Buchs, Switzerland); shikonin, 5,8-dihydroxy-*p*-naphthoquinone, and alizarin were from TCI (Portland, OR); lupulone was from APIN chemical (Abingdon, U.K.); and 2,5-dihydroxy-*p*-naphthoquinone was from Maybridge Chemical Company (Tintagel, U.K.). Other quinones were purchased from the specialty library of Aldrich (Milwaukee, WI). The structures of the compounds used in this study are found in **Figures 1** and **2**.

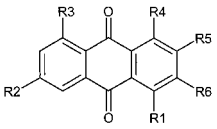
Experimental Design. Antifungal activity of the compounds was evaluated using a direct bioautography protocol in a matrix format. Antifungal activity of each quinone is determined by comparing the dimensions of the antifungal zone for each of three *Colletotrichum* spp. Inhibition means and standard deviations were pooled and reported for two separate experiments. Three technical grade fungicides were used as internal standards.



1a-v

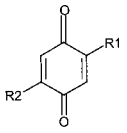
	Naphthoquinones	R_1	R_2	R_3	R_4
1a	2,3-dichloro-1,4-naphthoquinone	Cl	H	H	Cl
1b	1,4-naphthoquinone	H	H	H	H
1c	plumbagine	H	OH	H	CH_3
1d	5,8-dihydroxy-1,4-naphthoquinone	H	OH	OH	H
1e	2-hydroxy-3-(2,3-dimethylbutyl) -1,4-naphthoquinone	$CH_2CH(CH_3)CH(CH_3)_2$	H	H	OH
1f	2-hydroxy-3-isobutyl-1,4-naphthoquinone	$CH_2CH(CH_3)_2$	H	H	OH
1g	2-hydroxy-3-(5-methylhexyl) -1,4-naphthoquinone	$(CH_2)_4CH(CH_3)_2$	H	H	OH
1h	shikonin	H	OH	OH	$CH(OH)CH_2CHC(CH_3)_2$
1i	2-hydroxy-3-(2-methylbutyl) -1,4-naphthoquinone	$CH_2CH(CH_3)CH_2CH_3$	H	H	OH
1j	lapachol	$CH_2CHC(CH_3)_2$	H	H	OH
1k	2-hydroxy-3-chloro-1,4-naphthoquinone	Cl	H	H	OH
1l	2-hydroxy-3-(3-chloro-3-methylbutyl) -1,4-naphthoquinone	$(CH_2)_2C(CH_3)_2Cl$	H	H	OH
1m	juglone	H	OH	H	H
1n	2-hydroxy-1,4-naphthoquinone	H	H	H	OH
1o	2,5-dihydroxy-1,4-naphthoquinone	H	OH	H	OH
1p	menadione	H	H	H	CH_3
1q	2-hydroxy-3-methyl-1,4-naphthoquinone	CH_3	H	H	OH
1r	2-hydroxy-3-dodecylaminomethyl -1,4-naphthoquinone	$CH_2NH(CH_2)_{11}CH_3$	H	H	OH
1s	2-hydroxy-3-bromo-1,4-naphthoquinone	Br	H	H	OH
1t	2-hydroxy-3-(3-(4-tolyl)propyl) -1,4-naphthoquinone	$(CH_2)_3$ (4-$CH_3C_6H_5$)	H	H	OH
1u	2-hydroxy-3-(2-phenylbutyl) -1,4-naphthoquinone	$CH_2CH(C_6H_5)CH_2CH_3$	H	H	OH
1v	2-hydroxy-3-(3-methylbutyl) -1,4-naphthoquinone	$(CH_2)_2CH(CH_3)_2$	H	H	OH

Figure 1. Structures of the naphthoquinones **1a**–**v** used in this study.



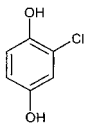
2a-d

	Anthraquinones	R_1	R_2	R_3	R_4	R_5	R_6
2a	1,8-dihydroxyanthraquinone	H	H	OH	OH	H	H
2b	emodin	H	CH_3	OH	OH	H	OH
2c	1,4-dihydroxyanthraquinone	OH	H	H	OH	H	H
2d	alizarin	H	H	H	OH	OH	H



3a-d

	Benzoquinones	R_1	R_2
3a	*p*-tolyl-1,4-benzoquinone	$CH_3C_6H_5$	H
3b	2,5-dichloro-1,4-benzoquinone	Cl	Cl
3c	2-*t*-butyl-1,4-benzoquinone	$C(CH_3)_3$	H
3d	2-*t*-butyl-5-methyl-1,4-benzoquinone	$C(CH_3)_3$	CH_3



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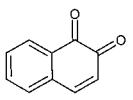

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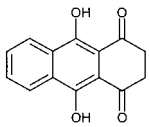

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	Other quinones
4	chlorohydroquinone
5	1,2-naphthoquinone
6	leucoquinizarin

Figure 2. Structures of the anthraquinones **2a**–**d**, benzoquinones **3a**–**d**, and other compounds **4**–**6** used in this study.

RESULTS AND DISCUSSION

Standardization of the Fungicidal Assay. Fungicide standards used as internal positive and negative controls demon-

Table 1. Antifungal Activity of Compounds Tested as Measured by Bioautography

	mean zone inhibition diameter (mm ± standard deviation)		
	C. fragariae	*C. acutatum*	*C. gloeosporioides*
benomyl	24.2 ± 4.2	0	0
captan	18.2 ± 2.1	17.8 ± 2.2	19.0 ± 2.3
chlorothalonil	12.2 ± 1.0	12.0 ± 1.7	14.3 ± 2.9
1a	18.0 ± 1.4	16.0 ± 0.0	15.5 ± 2.1
1b	14.5 ± 0.7	11.0 ± 0.0	12.0 ± 0.0
1c	10.5 ± 0.7	11.0 ± 1.4	9.5 ± 2.1
1d	9.5 ± 0.7	9.5 ± 0.7	9.5 ± 0.7
1e	9.0 ± 1.4	5.5 ± 0.7	7.5 ± 2.1
1f	8.7 ± 0.4	0	0
1g	8.0 ± 0.0	0	2.5 ± 3.5
1h	5.7 ± 1.1	0	0
1i	5.0 ± 7.1	3.5 ± 4.9	9.0 ± 1.4
1j	5.5 ± 0.7	0	0
1k	0	7.0 ± 0.0	4.5 ± 5.7
1l	7.0 ± 0.0	3.0 ± 4.2	3.0 ± 4.2
1m	14.5 ± 0.7	11.0 ± 1.4	11.5 ± 0.7
1n	0	0	0
1o	0	0	0
1p	0	0	0
1q	0	0	0
1r	0	0	0
1s	0	0	0
1t	0	0	0
1u	0	0	0
1v	0	0	0
2a	7.5 ± 0.7	0	0
2b	4.5 ± 0.7	0	0
2c	0	0	0
2d	0	0	0
3a	10.2 ± 0.4	10.0 ± 1.4	10.0 ± 0.0
3b	8.5 ± 2.1	7.5 ± 0.7	9.0 ± 0.0
3c	0	0	0
3d	0	0	0
4	15.5 ± 2.1	13.5 ± 0.7	14.0 ± 1.4
5	8.0 ± 0.0	7.0 ± 0.0	7.5 ± 0.7
6	5.7 ± 1.1	0	6.5 ± 2.1

strated the appropriate growth inhibition in all three *Colletotrichum* test species (**Table 1**). The systemic fungicide, benomyl, is an effective growth inhibitor of *C. fragariae* (CF63) and is not effective against *C. acutatum* (CAGoff). Benomyl activity as a spore germination and mycelial growth inhibitor is test format- and concentration-dependent and shows low activity to *C. gloeosporioides* (CG162) in the bioautography assay but high activity in the microwell plate assay (*24*). The contact fungicides captan and chlorothalonil are effective growth inhibitors of CF63, GAGoff, and CG163. CF63 is sensitive to the benzimidazole class of fungicides (benomyl, thiophananate-methyl, and thiabendazole) and shows ca. 90% growth inhibition when challenged with these fungicides at 3 μM rates in 96 well microtiter assays. Benzimidazole resistance in *C. acutatum* is used as a taxonomic trait to identify this species (*8*, *25*, *26*). *C. acutatum* (CAGoff) is highly resistant to benzimidazole fungicides and shows no growth inhibition when challenged with this fungicide class at 250 μM rates (Smith, personal communication).

Fungicidal Activity of the Naphthoquinones Tested. Naphthoquinones are known to possess, among other biological properties, fungicidal activity (*27*−*29*). Several of the naphthoquinones tested had antifungal activity. The most active antifungal compound tested, 2,3-dichloro-1,4-naphthoquinone (**1a**), was as inhibitory as captan against *C. fragariae*. The substitution of the two hydrogen atoms in the 2- and 3-position with two chlorine atoms **1a** enhanced the antifungal activity of **1b** by 24% in *C. fragariae*, 45% in *C. acutatum*, and 29% in *C. gloeosporioides*. Not surprisingly, this dichloro-substituted naphthoquinone is registered as a fungicide under the name dichlone (Hopkins Agricultural Chemical Co., Madison, WI). This naphthoquinone fungicide is particularly effective for control of brown rot of stone fruit and scab on apples and pears, as well as blossom blights (*30*).

The 1,4-naphthoquinones **1b** and its analogues with a hydroxyl group on 5-position plumbagine (**1c**) and juglone (**1m**) or those with hydroxyl groups at 5- and 8-positions displayed good antifungal activity on all three *Colletotrichum* species (**Table 1**), suggesting that adding hydroxyl groups on 5- and 8-positions was tolerated. The inhibitory activity of juglone (**1m**) was 28% greater to CF63 than plumbagine (**1c**). No difference in fungicide activity was demonstrated between **1m** and **1c** in CAGoff or CF162.

On the other hand, introducing a hydroxy group in the 2-position of the naphthoquinone ring (**1n**) resulted in loss of antifungal activity in all three fungi, as did the presence of a methyl group in the same position **1p**. However, the presence of a group in the 3-position with lipophilic properties greater than methyl restored some of the antifungal activity in **1e**−**l**. Within the 2-hydroxy-naphthoquinone subset of compounds, the presence of a sufficiently lipophilic side chain contributes to antifungal activity, but lipophilicity alone is not a very good indicator of activity. Compounds with log *P* values lower than 2 were not active (*31*). A group of 2-hydroxy-naphthoquinones with log *P* values ranging from 2.5 to 4.1 had antifungal activity, except for those that have a phenyl ring as part of the three side chain. It is worthy to note that in the series of the halogenated 2-hydroxy-1,4-naphthoquinone, the 3-chloro derivative **1k** is still active in contrast to the more lipophilic 3-bromo derivative **1s** (*31*). The 2-hydroxy-1,4-naphthoquinones bearing a phenyl ring or a relatively large dodecylaminomethyl side chain in the 3-position were completely inactive **1r**,**t**,**u**.

While lipophilicity (as measured by log *P*) is generally an important factor contributing to the biological activity of pesticides, it is not always the case for quinones. For example, there was no correlation between toxicity of a set of naphthoquinones against *Tetrahymena pyriformis* and log *P* (*32*). Along the same line, there was a poor correlation between the herbicidal activity of 2-hydroxy-3-alkyl-naphthoquinones and their lipophilicity (*33*), whereas there was a good correlation between the insecticidal activity of the same compounds and their lipophilicity (*34*).

Fungicidal Activity of the Anthraquinones Tested. In the anthraquinones series, only **2a**,**b** showed some activity against *C. fragariae* (**Table 1**). The presence of the second phenyl ring on the anthraquinone, when compared to the naphthoquinones, seems to negatively affect their antifungal activity. To our knowledge, no commercial fungicides have been developed on the anthraquinone backbone. Dithianone (2,3-dinitrilo-1,4-dithia-9,10-anthraquinone) has been used as a fungicide, but this commercial product has an unusual sulfur-containing ring not closely related to the typical anthraquinones tested in this study.

Fungicidal Activity of the Benzoquinones Tested. In the benzoquinone class, **3a**,**b** were active on all of the *Colletotrichum* spp. (**Table 1**). Chlorohydroquinone **4** displayed much better activity as compared with the benzoquinone **3b**. This hydroquinone was the second most active compound in this set and demonstrated more growth inhibition with CF63 (**Table 1**) and was as active as the turf and ornamental fungicide chlorothalonil against CaGoff and CF162 (**Table 1**). The 1,2-naphthoquinone **5** is less active than its isomer 1,4-naphthoquinone **1b**. Leucoquinizarin showed low antifungal activity to CF63 and CG162 with no activity on CAGoff. Structurally

similar to naphthoquinone **1d**, leucoquinizarin **6** had less fungicidal activity than **1d** CF 63 and CG162 and no activity against CAGoff.

Naturally occurring quinones are known for their biological activities. They possess antitumor, antiinflammatory, antiparasitic, antimicrobial, insecticidal, herbicidal, and fungicidal activities (*33*−*40*). Quinones are known to inhibit electron transport involved in mitochondrial respiration (*41*). Quinone-based fungicides are in a general class of "organic fungicides" that are considered to be multisite inhibitors (*42*). Multisite inhibitors may be advantageous to slow or prevent fungal pathogens from developing resistance. However, the registration of compounds with multiple sites of action may be cumbersome.

Quinones demonstrated good to moderate antifungal activity against the *Colletotrichum* species tested. *C. fragariae* appeared to be the most sensitive species to quinone-based chemistry, and *C. gloeopsorioides* often displayed intermediate sensitivity. *C. acutatum* was the most resistant to these compounds, and this fungus is also highly resistant to the benzimidazole fungicides. Whether differences in antifungal activity of quinones parallel benzimidazole sensitivity or are associated with membrane transport phenomenon, influenced by the lipophilicity of the compounds or due to natural resistance, is not clear. While many of the quinone compounds tested have significant antifungal activity against the plant pathogens used in this study, the extent of their potential use as agrochemicals requires further examination.

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