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Cynodontin: A Fungal Metabolite with Antifungal Properties

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A red pigment that accumulates in cultures of a *Drechslera avenae* pathotype with specificity for *Avena sterilis* was isolated and identified as the anthraquinone cynodontin (3-methyl-1,4,5,8-tetrahydroxyanthraquinone). Satisfactory yield of the compound was obtained with 20−60 day incubations at temperatures between 20 and 27 °C. Cynodontin was tested in vitro for fungitoxicity and was found to be a potent inhibitor of the growth of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* and, to a lesser extent, of *Verticillium dahliae*. The ED_{50} values obtained with these fungi were of the same order of magnitude as those of the commercial fungicides dicloran and carbendazim, which were used as reference chemicals. In contrast, the growth of a number of other fungi was not significantly inhibited by cynodontin. Anthraquinone and two other anthraquinone derivatives, emodin and chrysophanol, which were also included in the tests, did not affect the growth of the cynodontin-sensitive fungi. It thus appears that the type and position of the substitutions at the C-ring play a role in the expression of antifungal activity.

KEYWORDS: ***Drechslera avenae*; cynodontin; anthraquinones; fungal metabolites; natural products**

INTRODUCTION

Fungi produce a variety of substances of interesting chemistry, selectivity, and mode of action, many of which can be used as structural templates for the development of new pesticides (*1*−*7*). In a research project screening fungal metabolites as lead compounds for new herbicides, we have isolated and studied fungal pathogens of weeds. In the course of this work, an isolate of *Drechslera avenae* (Eidam) Scharif with host specificity toward wild oats (*Avena sterilis* L.) was obtained and cultured on a suitable substrate (*8*). In the process of isolation of phytotoxic metabolites produced by this pathotype, we also came across a number of non-phytotoxic compounds, which appeared of interest in other respects. A red pigment produced by this pathotype was extractable with organic solvents and was found to be selectively toxic to some plant pathogenic fungi. The isolation and chemical characterization as well as the evaluation of the bioactivity of this colored metabolite were the objectives of this work. The influence of some culture conditions on the *in vitro* production of the metabolite was also investigated.

MATERIALS AND METHODS

Fungi and Culture Media. The isolate of *D. avenae* used (strain L1) was obtained from naturally infected leaves of *A. sterilis* (*8*). It was grown on a medium containing oatmeal (30 g), agar (20 g), and deionized water (1 L). Isolates of *Botrytis cinerea* Pers. Fr (imperfect state of *Botryotinia fuckeliana* deBary, Whetzel), *Gibberella fujikuroi*, *Penicillium* sp., *Phoma* sp., *Rhizoctonia solani* Kuhn, *Rhizopus* sp., *Saccharomyces cerevisiae*, *Sclerotinia minor* Jagger, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Sclerotium rolfsii*, *Ustilago maydis* (DC.) Corda, and *Verticillium dahliae* Kleb., which were used in the fungitoxicity tests, were taken from the collection of the Pesticide Science Laboratory of the Agricultural University of Athens. The test fungi were grown on potato dextrose−agar medium. A minimal medium containing agar (20 g) and deionized water (1 L) was used for the conidial germination tests.

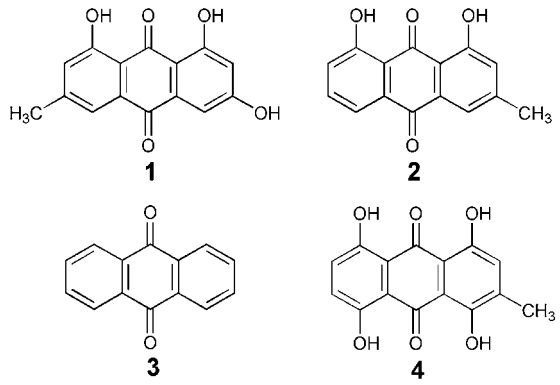

Figure 1. Structures of emodin, **1**; chrysophanol, **2**; anthraquinone, **3**; and cynodontin, **4**.

Chemicals. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), chrysophanol (1,8-dihydroxy-3-methylanthraquinone), and anthraquinone (9,10-anthracenedione) (**Figure 1**) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Dicloran (technical grade 95%) and carbendazim (technical grade 95%) were kindly provided by Upjohn and Du Pont De Nemours, respectively. The carrier liquid used for the seed dressing applications was the product Disco Color Blue, Coat L-79, Incotec, which was kindly given by Spirou Co., Athens, Greece.

Isolation of the Pigment. The metabolite was isolated from cultures of the *D. avenae* isolate L1 grown at 20 °C in the dark for 25 days. Agar cultures were chopped, and the fragments (100 g) were suspended in ethyl acetate (400 mL) for 24 h. After filtration, the solvent extract

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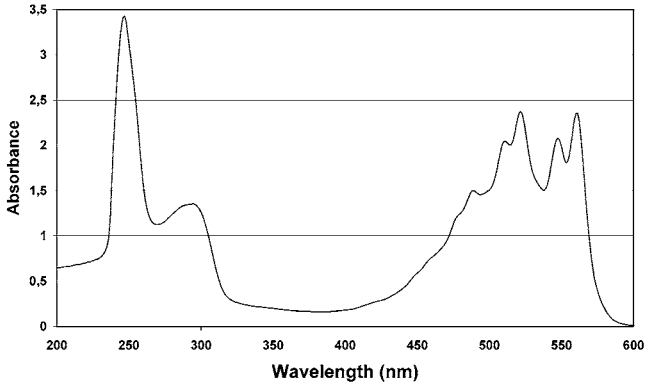

Figure 2. UV–vis spectrum of cynodontin.

was dried over anhydrous sodium sulfate (40 g) and reduced to a volume of 10 mL under vacuum at 36 °C. The concentrated preparation was transferred to a centrifuge tube (12 × 1 cm), kept at 4 °C for 24 h, and then centrifuged at 2000*g* for 15 min. The supernatant was removed, and the precipitate was taken up in ethyl acetate (0.5 mL) and subjected to thin-layer chromatography in acetone/chloroform (4:6) on preparative TLC plates coated with silica gel 60G (Merck). The visible red band (R_f 0.4) was scraped off, suspended in 10 mL of ethyl acetate, and centrifuged, and the supernatant was brought to a volume of 0.1 mL at 36 °C under vacuum. After the addition of 10 mL of hexane and incubation at 4 °C in the dark for 12 h, the red pigment was isolated in a crystalline form.

Identification of the Pigment. The melting point (259−260 °C) of the purified compound was obtained with a Buchi melting point apparatus and was not corrected. Mass spectra were acquired with a Hewlett-Packard (HP) 5890 series II gas chromatograph (GC) equipped with a Hewlett-Packard 5971A mass selective detector (MSD) interfaced with a personal computer and analyzed with the use of HP G1034B software for MS Chemstation. The compound had a molecular weight of 286, and the main peaks of the mass spectrum were at *m*/*z* 129, 143, 184, 229, 257, 286, and 287. UV−visible spectra of the compound, with λ_{max} $CHCl_3$ (log ϵ) 490 (6.497), 507 (6.619), 518 (6.667), 544 (6.614), and 557 nm (6.643), were taken with a Kontron Unicon 922 spectrophotometer interfaced with a personal computer (**Figure 2**).

Estimation of Pigment Production. The production of the red metabolite was monitored in agar cultures incubated in the dark at 20 °C for 90 days or determined at various temperatures 30 days after inoculation. The quantitative analysis was based on the characteristic absorbance of the metabolite at 518 nm. All calculations were made within the linearity limits (1−500 μg/mL) as defined from the standard absorbance curve.

Fungitoxicity Tests. The anthraquinones and the reference fungicides were added to the agar medium after sterilization as solutions in acetone. The amount of solvent did not exceed 0.8% in the treated and control samples. Solidified medium was inoculated with mycelial blocks (0.5 cm in diameter). All cultures were incubated in the dark at 25 °C for 4−8 days, depending on the fungal species, and linear growth was estimated by colony diameter measurements. The effect on the germination of *B. cinerea* conidia was examined by applying 0.2 mL droplets of spore suspension (10^6/mL water) on water−agar medium with or without the test substance, in a Petri dish. The percentage of germination was estimated by counting the number of germinated conidia in a total of 200 per Petri dish after 5 h of incubation in the dark at 25 °C. The observations were made under a light adverse phase microscope (Olympus CK40).

Seed Germination Tests. The effect of cynodontin on seed germination of various vegetable species was tested by applying the metabolite as a seed dressing on tomato (*Lycopersicon esculentum* Miller), pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.), lettuce (*Lactuca sativa* L.), cabbage (*Brassica oleracea* L.), broccoli (*Brassica oleracea* L. var. *botrytis*), cauliflower (*Brassica oleracea* L. var. *italica*), cucumber (*Cucumis sativus* L.), and melon (*Cucumis melo* L.) seeds. The purified pigment and anthraquinone, which is commercially used in seed dressings as a bird repellent, were mixed with the carrier liquid (250 mg/mL), and the seeds were immersed in the mixture for 5 min. Then they were air-dried on a glass plate and placed on moistened filter paper to germinate at 25 °C, in the dark. The germinated seeds were removed and discarded at 24 h intervals, and the germination percentage was estimated 8−14 days after treatment, depending on the plant species and according to guidelines for seed testing of the International Seed Testing Association (*9*). Untreated seeds and seeds covered only with the film coat liquid were used as reference.

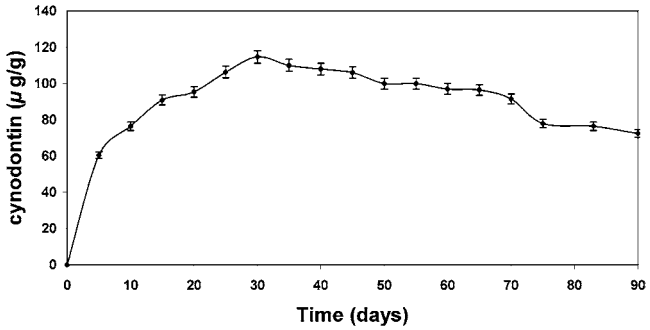

Figure 3. Production of cynodontin in oatmeal agar cultures of *D. avenae* at 20 °C, in the dark. Yield is expressed as micrograms per gram of culture medium, and values are means of five replications. Standard deviations are plotted as vertical bars.

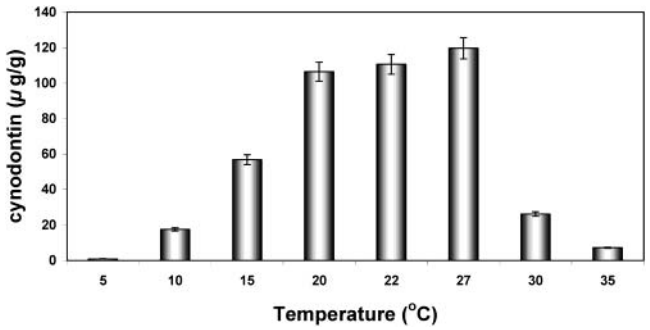

Figure 4. Production of cynodontin in oatmeal agar cultures of *D. avenae*, following 30 days of incubation at various temperatures, in the dark. Yield is expressed as micrograms per gram of culture medium. Results are means of three replications. Vertical bars represent standard deviations.

RESULTS AND DISCUSSION

Identification of the Purified Metabolite. The physical and spectroscopic data of the isolated metabolite were consistent with those reported in the literature (*10*−*16*) for the octaketide 3-methyl-1,4,5,8-tetrahydroxyanthraquinone, **4** (**Figure 1**), which was named cynodontin by Raistrick et al. (*17*). Anthraquinones are known (*18*−*21*) secondary metabolites of several ascomycete, basidiomycete, and imperfect fungi. In quite a few instances such metabolites distinctively characterize the cultures of the respective fungi and are utilized for species identification, as is the case with members of the genus *Drechslera* (*22*, *23*), the imperfect stage of *Pyrenophora* (*24*).

Production in Culture. Cynodontin could be detected in cultures of *D. avenae* as soon as after 3 days of incubation at 20 °C. Cynodontin production was increased drastically with incubation time, reaching a maximal concentration of 120 μg/g in 30 days, and the metabolite was present at high levels in the medium for 30 more days (**Figure 3**). A slight decrease in concentration was observed for a prolonged incubation of >60 days. Cynodontin production by *D. avenae* was also greatly influenced by the incubation temperature (**Figure 4**). The production was favored at temperatures between 20 and 27 °C, with a maximal yield of 110−120 μg/g of fresh weight of agar

Table 1. Effect of Cynodontin, Anthraquinone, Emodin, and Chrysophanol on the in Vitro Growth of *B. cinerea*, *S. minor*, *S. sclerotiorum*, and *V. dahliae*[a]

	ED_{50}[b] ± SD (μg/mL)				
fungus	cynodontin	anthraquinone	emodin	chrysophanol	reference fungicide
B. cinerea	5.25 ± 0.05	>100	41.24 ± 0.07	>100	5.51 ± 0.04
S. minor	4.31 ± 0.04	>100	38.86 ± 0.09	74.12 ± 0.11	7.13 ± 0.06
S. sclerotiorum	5.52 ± 0.05	>100	66.47 ± 0.08	>100	3.24 ± 0.02
V. dahliae	11.54 ± 0.09	>100	>100	>100	0.68 ± 0.01

[a] Dicloran was used as reference fungicide in the tests with the first three fungi and carbendazim with the latter. [b] Estimation of median effective doses (ED_{50}) was based on colony diameter measurements.

Table 2. Seed Germination of Vegetable Crops in the Presence of Cynodontin

	seed germination ± SD (%)								
treatment[a]	tomato	pepper	eggplant	cucumber	melon	cabbage	broccoli	cauliflower	lettuce
control	94.0 ± 2.0	84.7 ± 4.6	84.7 ± 2.3	94.7 ± 1.2	90.0 ± 2.0	90.0 ± 5.3	90.7 ± 4.2	97.3 ± 1.2	90.0 ± 5.3
carrier	95.3 ± 2.3	84.7 ± 1.2	83.3 ± 1.2	94.0 ± 4.0	89.3 ± 2.3	88.7 ± 1.2	90.0 ± 2.0	96.0 ± 0.0	88.7 ± 1.2
cynodontin	95.3 ± 1.2	84.7 ± 3.1	82.7 ± 3.1	94.0 ± 3.5	90.7 ± 1.2	89.3 ± 4.2	88.0 ± 2.0	96.7 ± 2.3	96.7 ± 2.3
anthraquinone	94.7 ± 2.3	85.3 ± 5.0	82.7 ± 1.2	93.3 ± 3.1	90.7 ± 5.0	88.0 ± 3.5	87.3 ± 3.1	96.7 ± 1.2	88.0 ± 3.5

[a] Cynodontin and anthraquinone were applied as seed dressings at 250 mg/mL of the carrier.

culture after 30 days at 27 °C. This observation is in agreement with the findings of White and Johnson (*11*) on the in vitro production of cynodontin by *Helminthosporium cynodontis.* Other factors that have been known to affect production of the metabolite include nutrient availability (*10*, *11*, *25*) and light periodicity (*13*). The fact that cynodontin is produced at a rather wide range of temperatures and remains at relatively high concentrations for about a month is of practical importance because isolation experiments can be easily planned and performed.

Bioactivity of Cynodontin. The growth of the majority of the fungi used in the fungitoxicity tests was not affected by cynodontin at concentrations of up to 100 μg/mL. Antifungal activity would probably have not been detected if the three members of the family Sclerotiniaceae of the cup fungi (Discomycetes) had not been included in these tests. Cynodontin was recognized as a potent inhibitor of the mycelial growth of *B. cinerea*, *S. minor*, and *S. sclerotiorum* (**Table 1**). The ED_{50} values of 5.25, 4.31, and 5.52, respectively, were comparable to those obtained with dicloran, which is used commercially to control diseases caused by these pathogens. Some cynodontin sensitivity was also exhibited by one unrelated species, namely, *V. dahliae* (**Table 1**). It is interesting that the in vitro growth of the cynodontin-sensitive fungi was affected by neither unsubstituted anthraquinone nor the derivatives, emodin and chrysophanol, which differ from cynodontin in the C-ring substitutions (**Figure 1**). Some bioactivity has been reported for emodin (*26*−*29*), chrysophanol (*29*), and anthraquinone (*30*−*34*), but none of these compounds has been reported to possess antifungal activity. The possibility of fungal spore germination inhibition was considered using conidia of *B. cinerea*. All four anthraquinones used appear to have little effect on conidia germination of this species (**Figure 5**). The fact that cynodontin is a more potent inhibitor of mycelial growth but not of conidia germination in the same fungus may suggest an involvement of the compound in metabolic processes required for mycelial growth but not for conidia germination.

Although several anthraquinones produced by *Drechslera* species have been isolated and chemically identified (*18*−*20*), little is known about their bioactivity and role in nature. A possible involvement of anthraquinones in disease development has been proposed for plant pathogenic fungi. The biosynthesis

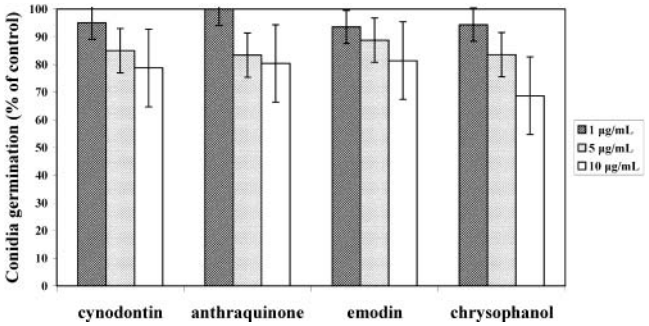

Figure 5. Effect of cynodontin, anthraquinone, emodin, and chrysophanol on the conidia germination of *B. cinerea.* Results are means of three experiments. Vertical bars represent standard deviations.

of the anthraquinone chrysophanol, for example, has been associated with the symptoms of the "mal secco" disease of citrus caused by the fungus *Phoma tracheiphila* (*35*). A correlation has also been found between the production of anthraquinone pigments by *Pyrenophora avenae* and resistance to phenylmercuric acetate (*25*).

This appears to be the first report of fungitoxic activity of an anthraquinone, although some other synthetic quinones, namely, chloranil (*33*, *36*) and dichlone (*33*, *36*), have been developed as commercial fungicides. Further evaluation of the in vivo activity of cynodontin and related anthraquinones against plant pathogenic fungi and persistence studies are required for the exploration of such compounds as potential disease control chemicals. Another requirement is, of course, the lack of toxicity to important crop plants. Encouraging in this regard is the information presented in **Table 2**. Although this was only a preliminary test, it is shown that cynodontin, used as a seed dressing, did not affect the germination of seeds of nine different cultivated species. Considering that some of the cynodontin-sensitive fungi are soilborne, the compound may thus be a good candidate for the control of seedling diseases, although its spectrum of activity appears to be rather narrow.

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