JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of 1,8-Dihydroxynaphthalene Melanin in *Monosporascus cannonballus* and the Analysis of Hexaketide and Pentaketide Compounds Produced by Wild-Type and Pigmented Isolates of the Fungus

MICHAEL H. WHEELER,*,† BENNY D. BRUTON,‡ LORRAINE S. PUCKHABER,† JIUXU ZHANG,‡,§ AND ROBERT D. STIPANOVIC†

Southern Plains Agricultural Research Center, USDA, ARS, 2765 F&B Road, College Station, Texas 77845, and South Central Agricultural Research Laboratory, USDA, ARS, P.O. Box 159, Lane, Oklahoma 74555

Monosporascus cannonballus causes root rot and vine decline in muskmelons and watermelons. Wild types of this fungus often undergo degenerative changes that have been associated with yellow to brown pigmentation, hypovirulence, dsRNA infection, and decreased production of perithecia. In this study, degenerate isolates that produced yellow to brown pigments and no perithecia were obtained from wild-type cultures that had been stored for extended periods of time. Cultures of the degenerate isolates were found to accumulate five related hexaketides when grown on potato-dextrose agar (PDA). In contrast, these hexaketides were present only in minute amounts in wild-type cultures unless grown on NaCl-amended PDA. 1,8-Dihydroxynaphthalene melanin was established to be present in wild-type *M. cannonballus* and absent in the degenerate isolates. Various melanin-related metabolites, however, were produced by the variants. Tricyclazole in PDA cultures blocked melanin biosynthesis by the wild types but had little effect on hexaketide production by the degenerate isolates.

KEYWORDS: 1,8-Dihydroxynaphthalene; dsRNA; fungal melanin; *Cucumis melo* L.; muskmelon; hexaketides; hypovirulence; *Monosporascus cannonballus*; pentaketides; plant pathogen; virulence; *Citrullus lanatus* (Thunb.) Matsum. and Nakai; watermelon

INTRODUCTION

Monosporascus root rot/vine decline (MRR/VD) caused by *Monosporascus cannonballus* is a destructive disease of muskmelon (*Cucumis melo* L.) and watermelon (*Citrullus lanatus* (Thunb.) Matsum. and Nakai) in several countries (*1*, *2*). MRR/VD has only been recognized in recent years although it has evidently been present in the environment for years (*1*). The genus *Monosporascus* and its holotype *M. cannonballus* were named and described by Pollack and Uecker in 1974 (*3*). *M. cannonballus* is a fast growing ascomycete that rapidly colonizes certain types of culture media and infected plant tissue with colorless mycelia, hereafter referred to as hyaline mycelia, or a mixture of hyaline and black mycelia. The fungus produces perithecia that contain mainly single (rarely two) spored asci. Large numbers of brown to black (i.e., melanized) ascospores develop in the cortex of colonized host roots late in the season. This is the only spore stage produced by this pathogen and is the primary survival structure and source of inoculum for the soilborne pathogen (*4*). Isolates of *M. cannonballus* vary considerably in their degree of virulence, ranging from weakly virulent to highly virulent (*5*, *6*). Additionally, it has been shown that temperature (*6*) and soil inoculum levels (*7*) are important determinants of disease severity.

Many wild-type isolates of the fungus undergo degenerative changes in production of perithecia and in virulence during long-term storage. Eventually, variants of the wild types that have undergone severe degeneration may even die. Martyn and Miller (*1*) have noted that the first noticeable change in colony appearance associated with degeneration is usually the development of a yellow pigment. This discoloration darkens with successive generations of the fungus on media, until it is a dark brown. In addition to changes that occur to isolates that have been maintained in the laboratory, it has been reported (*2*) that characteristics of degeneration (e.g, production of yellow pigments) are occasionally observed in isolates freshly obtained from nature.

Recently, the five colored hexaketides shown in **Figure 1** (monosporascone (**1**), dehydroxyarthrinone (**2**), demethylcerdarin (**3**), monosporascol A (**4**) and azamonosporascone (**5**)) were isolated and identified for the first time from a typical yellow/brown colored degenerate isolate of *M. cannonballus* (*8*),

* To whom correspondence should be addressed. Tel.: (979) 260-9516. Fax: (979) 260-9470. E-mail: mwheeler@cpru.usda.gov.
† Southern Plains Agricultural Research Center.
‡ South Central Agricultural Research Laboratory.
§ Current Address: Florida Department of Citrus, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850.

10.1021/jf0308298
Published on Web 05/28/2004

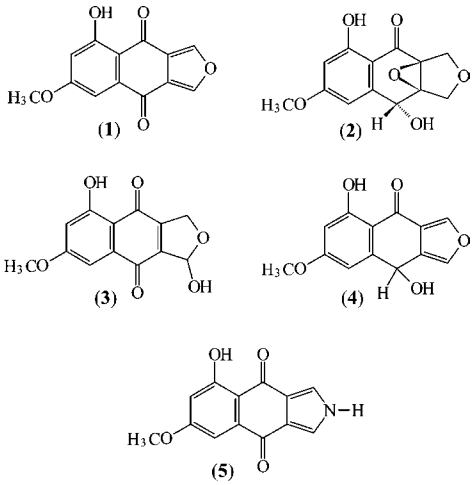

Figure 1. Structures of the hexaketides monosporascone (**1**), dehydroxyarthrinone (**2**), demethylcerdarin (**3**), monosporascol A (**4**), and azamonosporascone (**5**).

designated as TX923038. Another pigmented variant that produces the hexaketides, SP934945, also produced two unknown reddish-colored compounds. These two compounds were similar in appearance to known melanin metabolites and may have accumulated because of a block in the melanin pathway. Whereas the yellow/brown isolates do not produce appreciable amounts of melanin, the wild types do make melanin (probably, 1,8-dihydroxynaphthalene melanin), which is similar to that produced by a number of other ascomycetous and related imperfect fungi (*9*−*17*).

The present investigation with *M. cannonballus* was conducted with four main objectives: (1) to determine if 1,8-dihydroxynaphthalene melanin is responsible for the black pigmentation in the mycelium and ascospores of wild-type *M. cannonballus* via inhibition studies with tricyclazole (5-methyl-1,2,4-triazole[3,4-*b*]benzothiazole); (2) to identify the two unknown reddish compounds produced by SP934945; (3) to use quantitative methods to determine if the five hexaketides identified by Stipanovic et al. (*8*) from TX923038 and the two proposed melanin metabolites from SP934945 exist in cultures of other GYB or GWT isolates; and (4) to investigate the effect of NaCl on melanization, yellow to brown pigmentation, and perithecial production by *M. cannonballus* isolates.

MATERIALS AND METHODS

Isolates. The isolates of *M. cannonballus* used in this investigation (**Table 1**) came from five countries. Hereafter, we will designate isolates in the wild-type group that produce no yellow or brown pigment as GWT isolates. In contrast, we will designate the isolates in the group that secreted large amounts of yellow to brown pigment in cultures as GYB isolates. The GYB isolates also lost the ability to produce perithecia, which characteristically were made in large numbers by the GWT isolates. The GWT isolates consisted of 15 characteristic wild types obtained from diseased plants in the field. These isolates were from several geographical locations and had been maintained in soil culture for periods of 1 month to 13 years, depending on when they were first isolated from roots of the cucurbits listed in **Table 1**. The GYB group consisted of 11 members that were originally characterized as wild types but that changed phenotypically, while being maintained in soil-hull mix culture in the laboratory.

Media and Culture Conditions. The GWT and GYB isolates of *M. cannonballus* were stored in 16-mL glass screw cap vials that contained 12 mL of a mixture of 99.5% artificial soil (Terra-Lite, Scotts-Sierra Hort. Products Co., Marysville, OH) and 0.5% ground oat hulls (Quaker Oats Company, Chicago, IL), and 5 mL of deionized water.

Table 1. Isolate, Isolate Type, Cucurbit Group, Source, and Year Isolated and Placed in Soil-Hull Mix Culture

isolate	type[a]	group	source location	year
CA941029	GWT	cantaloupensis	Kern County, California, USA	1994
CA941030	GWT	cantaloupensis	Kern County, California, USA	1994
CA941032	GYB	cantaloupensis	Kern County, California, USA	1994
CA941040	GWT	cantaloupensis	Kern County, California, USA	1994
CA941042	GWT	cantaloupensis	Kern County, California, USA	1994
CA921457	GYB	watermelon	Riverside County, California, USA	1994
GT960051	GWT	cantaloupensis	Zacapa, Guatamala	1996
GT960052	GWT	cantaloupensis	Zacapa, Guatamala	1996
HO980076	GYB	cantaloupensis	Choloteca, Honduras	1998
HO980085	GWT	cantaloupensis	Choloteca, Honduras	1998
IS980126	GYB	muskmelon	Israel	1998
SP935039	GYB	inodorus	Valencia, Spain	1993
SP935043	GYB	inodorus	Valencia, Spain	1993
SP934945	GYB	cantaloupensis	Valencia, Spain	1993
SP935051	GWT	inodorus	Castellon, Spain	1993
SP960189	GWT	piel de sapo	Castellon, Spain	1996
SP960199	GYB	piel de sapo	Castellon, Spain	1996
TX902020	GYB	cantaloupensis	Starr County, Texas, USA	1990
TX912035	GWT	cantaloupensis	Starr County, Texas, USA	1991
TX923035	GYB	muskmelon	Hildago County, Texas, USA	1992
TX970062	GWT	cantaloupensis	Hildago County, Texas, USA	1997
TX970084	GWT	cantaloupensis	Starr County, Texas, USA	1997
TX923038	GYB	inodorus	Starr County, Texas, USA	1992
TX020157	GWT	watermelon	Hildago County, Texas, USA	2002
TX020162	GWT	watermelon	Hildago County, Texas, USA	2002
TX020167	GWT	watermelon	Hildago County, Texas, USA	2002

[a] The isolates used in this study were obtained as wild types but many later became yellow/brown isolates as mentioned in the text. The type column shows whether an isolate belongs to the wild-type group (GWT) or the yellow/brown group (GYB).

The tubes containing soil-hull mix were autoclaved for 1 h, kept for 2 days at room temperature, and then autoclaved for an additional 1 h. The cooled soil-mix in each tube was inoculated with a 5-mm plug (#3 cork borer) of fungal colonized agar taken from the leading edge of a 4 day-old Potato Dextrose Agar (PDA; Difco Laboratories, Sparks, MD) culture. The soil-hull cultures were kept at room temperature in the dark. Before each study, a small aliquot of the fungal-colonized soil-hull mixture was transferred to PDA and allowed to grow for 4 days at 30 °C. Afterward, 7-mm hyphal plugs (#5 cork borer) of the GWT and GYB isolates were removed from the mycelial perimeter and single plugs were placed at the centers of 100 × 15-mm dishes containing 20 mL of PDA. The dishes of inoculated PDA were then incubated for 10, 20, or 30 days at 30 °C in the dark before isolating and analyzing pigments that were present. At the end of 30 days, perithecia/cm^2 in three sub-sample locations of each Petri dish were enumerated with the aid of a stereoscope, and mean values for the plate were determined.

Tricyclazole was used to evaluate its effects on melanin biosynthesis by the GWT isolates and on pigment synthesis by the GYB isolates. Several reviews of the 1,8-dihydroxynaphthalene melanin literature (*9*−*17*) describe the 1,8-dihydroxynaphthalene melanin pathway, the use of melanin deficient-mutants and inhibitors (i.e., tricyclazole) in studies of the pathway and/or the role of fungal melanins as virulence factors in a number of fungi, including the plant pathogens *M. grisea* and *Colletotrichum miyabeanus* and the animal pathogens *W. dermatitidis*, *Aspergillus fumigatus*, and *Sporothrix schenckii*. In the present investigation, tricyclazole was added in ethanol to PDA, so that the final tricyclazole concentration in the medium, designated as PDAT, was 15 μg/mL, and the ethanol concentration was 0.8% (v/v). One set of controls was amended with 0.8% ethanol without tricyclazole; a second set of controls lacked both tricyclazole and ethanol. In addition, the different GWT and GYB isolates were grown on PDA, containing 2.5% NaCl (w/v), hereafter referred to as PDAS, for 10, 20, or 30 days at 30 °C in the dark, to determine the effects of NaCl on pigmentation and perithecial production. GWT isolates TX020157, TX020162, and TX020167, which were obtained from watermelon in 2002 (**Table 1**), also were grown on a series of PDA media containing 0.0, 0.25, 0.5,

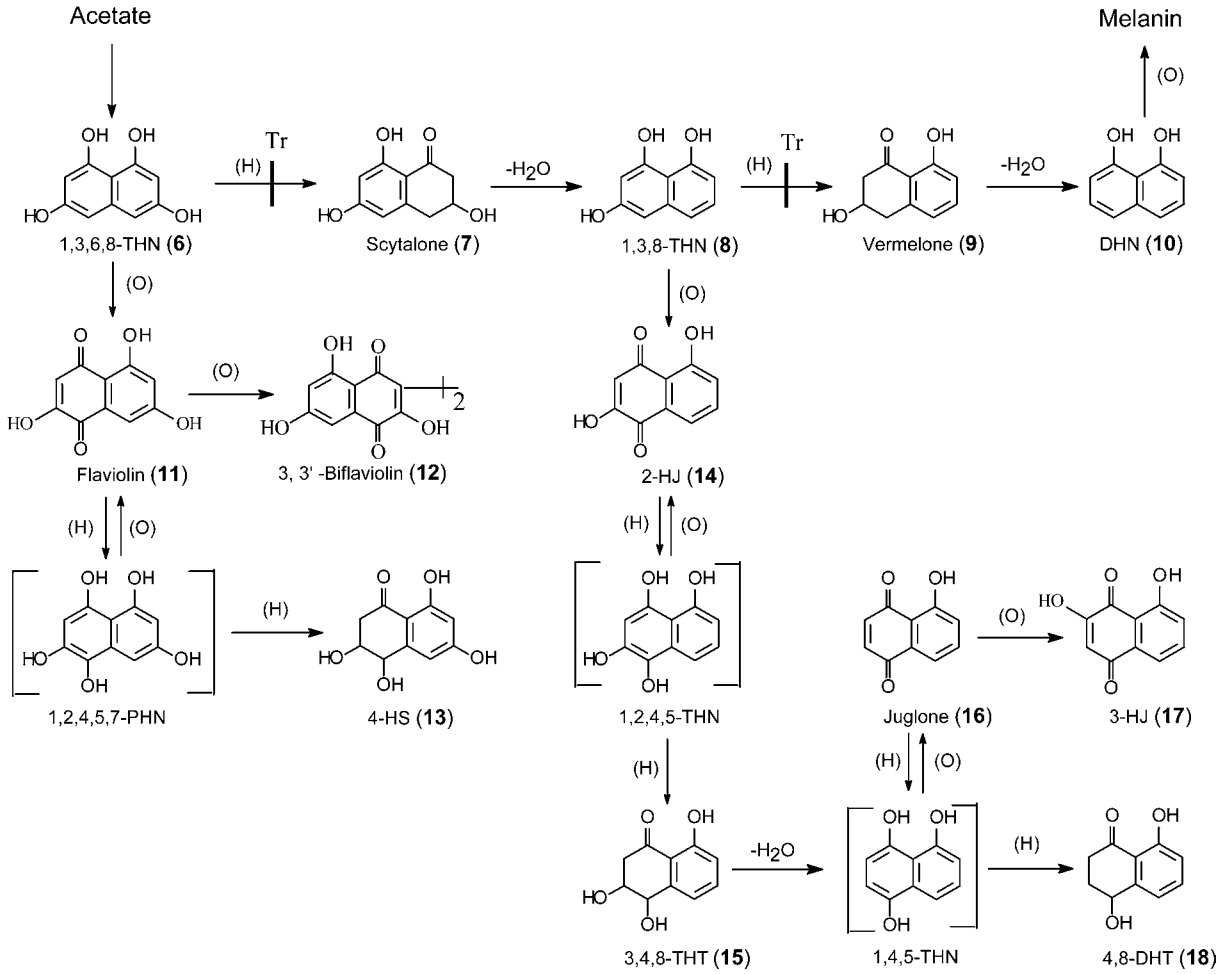

Figure 2. Schematic representation of the 1,8-dihydroxynaphthalene melanin biosynthetic pathway and related pentaketide shunt pathways (adapted from refs *9*, *10*, and *11*). Reaction types are indicated: (O) = oxidation; (H) = reduction; and $-H_2O$ = dehydration. Tricyclazole (T) specifically inhibits the pathway at the two sites indicated. Brackets around 1,2,4,5,7-pentahydroxynaphthalene (1,2,4,5,7-PHN), 1,2,4,5-tetrahydroxynaphthalene (1,2,4,5-THN) and 1,4,5-trihydroxynaphthalene (1,4,5-THN) indicate these compounds are extremely unstable and have not been isolated from fungi. Other abbreviations: 1,3,6,8-THN = 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN = trihydroxynaphthalene; DHN = 1,8-dihydroxynaphthalene; 4-HS = 4-hydroxyscytalone; 2-HJ = 2-hydroxyjuglone; 3-HJ = 3-hydroxyjuglone; 4,8-DHT = 4,8-dihydroxytetralone; and 3,4,8-THT = 3,4,8-trihydroxytetralone.

and 1.5% NaCl, in addition to PDAS with its 2.5% NaCl, for 20 days at 30 °C to compare the effects of different NaCl concentrations on newly collected GWT isolates.

Identification of Two Unknown Compounds in Cultures of GYB Isolate SP934945. The five hexaketides (**1**−**5**) in **Figure 1**, were identified in PDA cultures of GYB isolate TX923038 by Stipanovic et al. (*8*). In the present study, two unknown reddish colored compounds from *M. cannonballus* were isolated and identified from cultures of GYB isolate SP934945. The growth medium, hereafter referred to as FLM, used in this study to grow SP934945 was Medium B as described elsewhere (*18*) for growing *Fusarium* species, except in our studies it was modified to contain sorbitol as a carbon source, 1 g/L of yeast extract (Difco Laboratories, Sparks, MD), and a pH of 7.0. SP934945 was grown in 100 mL of FLM in 250-mL Erlenmeyer flasks after inoculation with two 7-mm diameter plugs of the fungus from 4-day-old PDA cultures. The fungus was cultured at 24 °C for 20 days on a rotary shaker at 100 rpm. The liquid culture was filtered through Miracloth (Calbiochem-Bering, La Jolla, CA), and the supernatant was collected. The supernatant was then extracted three times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined, evaporated to a small volume (15−50 mL) and stored at 4 °C until needed.

The extracts were subjected to single-dimension thin-layer chromatography (TLC) to isolate the two reddish colored compounds. To accomplish this, 20 cm^2, 500-μm thick, 150 Å silica gel TLC plates with fluorescent indicator (Whatman International Ltd., Maidstone, England) were developed successively until the compounds were pure, using two solvent systems: system 1, acetone−chloroform−formic acid (20:79:1); or system 2, methanol−toluene−formic acid (20:79:1). During this procedure, a number of compounds were observed on the TLC plates based on visible and/or fluorescent colors at 365 or 254 nm. The two reddish colored compounds were apparent even under natural light. Individual bands were scraped from the TLC plates with a razor blade, eluted from silica gel with ethyl acetate, filtered through Whatman filter paper no. 5, and concentrated with an evaporator.

Solid compound was obtained by precipitation through reducing the volume of the solvent. Chemical structures of the unknown compounds were then determined by using NMR and mass spectroscopy as described previously (*19*). Once the two compounds were identified as flaviolin (**11**) and 3,3′-biflaviolin (**12**) (**Figure 2**), their spectra were compared with those of authentic standards of **11** and **12**.

Analysis of Polyketides in Cultures of the GWT and GYB Isolates. Following identification of pentaketides **11** and **12** in cultures of GYB isolate SP934945 and identification of hexaketides, **1**−**5** in cultures of GYB isolate TX923038 (*8*), additional chemical studies were conducted to screen a larger number of *M. cannonballus* isolates for these compounds. The GWT and GYB isolates described in **Table 1** were analyzed for the hexaketide and pentaketide metabolites shown in **Figures 1** and **2**, respectively. PDA, PDAT, and PDAS cultures of the GWT and GYB isolates were chopped into 1-cm^2 fragments and extracted twice with acetone (2 mL/g of agar) over a 48-h period. The acetone was removed under vacuum at 30 °C. The remaining aqueous

phase was then adjusted to pH 5 with 1 N H_3PO_4, saturated with NaCl, and extracted twice with equal volumes of ethyl acetate. The extracts were concentrated under vacuum and analyzed by TLC, dried, dissolved in a small amount of acetone, and analyzed by HPLC to identify and quantify the compounds shown in **Figures 1** and **2**. Authentic pentaketide standards were obtained as described previously (*20*, *21*). Standards of the hexaketides were obtained from PDA cultures of GYB isolate TX923038, as described by Stipanovic et al. (*8*).

For TLC analysis of pentaketide melanin metabolites, approximately six TLC plates were made from a suspension of 40 g Baker G/HR silica gel and 1.2 g zinc orthosilicate fluorescent indicator in 100 mL deionized water. The ethyl acetate concentrate from 4 PDA, PDAT, or PDAS culture dishes was brought up to a final volume of 1.2 mL with ethyl acetate, then 50 μL of the solution was spotted near the bottom of a TLC plate. The TLC plates were developed in one dimension with diethyl ether−hexane−formic acid (60:39:1) and examined under 254 and 365-nm light.

Analytical HPLC of pentaketide and hexaketide metabolites was carried out using a Hewlett-Packard 1090-LC system (Agilent Technologies, Palo Alto, CA), equipped with a diode array detector and a 250- × 4.6-mm i.d. Hypersil 5-C18 column (Phenomenex, Torrance, CA). The mobile phase used was a gradient of acetonitrile (MeCN) and water (both with 0.07% H_3PO_4) run at 1.25 mL/ min. Set points for the linear gradient were as follows: 10% MeCN (0 min), 40% MeCN (24 min), 40% MeCN (28 min), 100% MeCN (31 min), 100% MeCN (33 min), and 10% MeCN (34 min). The equilibration time between runs was 4 min, and the chromatographic signal was obtained at 254 nm (bandwidth 20 nm) with reference to 550 nm (bandwidth 100 nm), while spectra were collected over 210−600 nm. Retention times for the various pentaketide and hexaketide compounds that were studied were as follows (common names of the compounds are given in **Figures 1** and **2**): **1**, 28.9 min; **2**, 15.3 min; **3**, 17.9 min; **4**, 20.1 min; **5**, 23.0 min; **6**, 8.9 min; **7**, 7.8 min; **8**, 14.8 min; **9**, 12.5 min; **10**, 22.2 min; **11**, 13.4 min; **12**, 20.2 min; **13**, 5.3 min; **14**, 17.1 min; **15**, 7.0 min; **16**, 20.1 min; **17**, 14.5 min; and **18**, 11.6 min. To quantify polyketides produced by GWT and GYB isolates, standard curves were developed for the compounds.

Statistical Analysis. Means and standard errors for hexaketides **1**−**5** were obtained for the 30 day-old isolates in group GWT and for those in GYB, as reported electronically under Supporting Information. Data were subjected to analysis of variance (SAS-ANOVA-GLM; SAS Institute Inc., Cary, NC). These tests were applied to the data to determine if significant differences occurred when GWT and GYB and PDA, PDAT, and PDAS were considered as treatment groups. The experiment with 30 day-old isolates was repeated, and data from 10 and 20 day-old cultures also were analyzed.

RESULTS

Morphology, growth, and pigment production on PDA. The isolates used in this investigation were easy to distinguish as either GWT or GYB isolates, based on differences in colony appearance. Typical 30 day-old GWT and GYB isolates on PDA are shown in **Figure 3**. Between isolates in the GWT and GYB groups, there were striking differences in pigmentation of the solid media and in mycelial appearance. Furthermore, the mean perithecial production on PDA for 30 day-old GWT isolates was 113 perithecia/cm^2, as compared to a mean count of 0 perithecia/cm^2 for GYB isolates. In addition to GYB isolates, hyaline variants also occasionally developed from the wild types. These variants failed to produce melanin or perithecia and did not produce yellow or brown pigments in agar. The hyaline variants were easy to grow on PDA but were not studied in further detail. The GWT isolates listed in **Table 1** generally exhibited hyaline to light gray mycelia by 4−7 days growth, and in certain isolates, parts of the mycelia evolved into black hyphae, arranged in solid or weblike patterns. By 20−30 days, these wild types produced characteristic perithecia, containing dark ascospores (**Figure 3**, parts **A** and **B**). The ascospores and

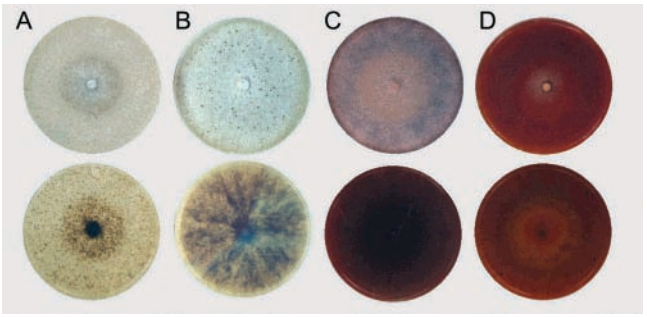

Figure 3. Upper (top row) and reverse (bottom row) sides of 30 day-old wild-type (GWT) and yellow/brown (GYB) isolates of *M. cannonballus* grown on PDA. (**A**) GWT isolate CA941040, showing a large number of perithecia with melanized ascospores on the reverse side of the dish. (**B**) GWT isolate TX912035, showing perithecia with melaninzed ascospores on the upper side of the dish and melanized hyphae and ascospores on the reverse side of the dish. (**C**) GYB isolate TX902020, showing heavy accumulation of brown colored pigments. Perithecia and ascospores are absent. (**D**) GYB isolate CA921457, showing reddish brown colored pigments. Perithecia and ascospores are absent.

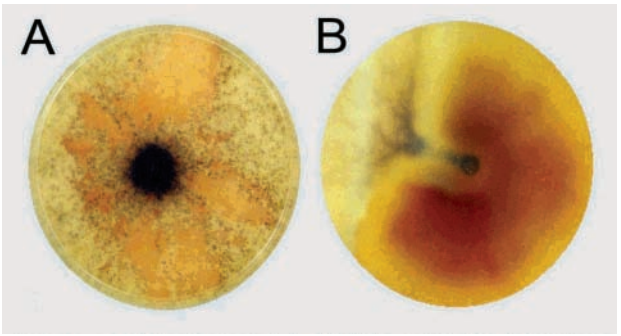

Figure 4. Reverse side of 30 day-old GWT isolates on PDA showing yellow to orange-brown colored sectors. (**A**) Left: CA941040 with sectors, perithecia, and medium containing yellow pigment. (**B**) Right: large sector of CA941032 from colony with black hyphae.

black hyphae were found distributed throughout the colonies and often were most visible from the reverse side of the Petri dish. During the course of the experiments, some of the wild-type isolates were observed to produce yellow to brown sectors. When plugs from the colored sectors were transferred to PDA, they usually grew out as fairly uniform yellow, yellow-brown, or orange-brown GYB colonies, with a greatly diminished capacity for producing perithecia. **Figure 4** shows two 30 day-old wild types of the fungus producing colored sectors on PDA.

The GYB isolates in **Table 1** secreted yellow pigments into PDA medium during their first 3 to 10 days of growth, and by 30 days, the agar medium was moderate to dark yellowish brown, or dark brown in color as shown in **Figure 3**, parts **C** and **D**. The mycelia in most GYB isolates did not appear to be melanized and ranged from hyaline or light yellow to brown in color. GYB isolates that were less than two years old often still produced small numbers of perithecia and ascospores; however, older GYB isolates no longer produced these structures.

Discovery of Melanin-Related Metabolites in Cultures of SP934945. The two unknown red-colored compounds from FLM cultures of SP934945 were identified as **11** and **12** based on their ^{1}H NMR and mass-spectrometric values. In addition, their spectra were similar to those reported previously (*19*) for **11** and **12** from other fungi. The compounds also had identical HPLC retention times and UV−vis spectra as authentic standards of **11** and **12**.

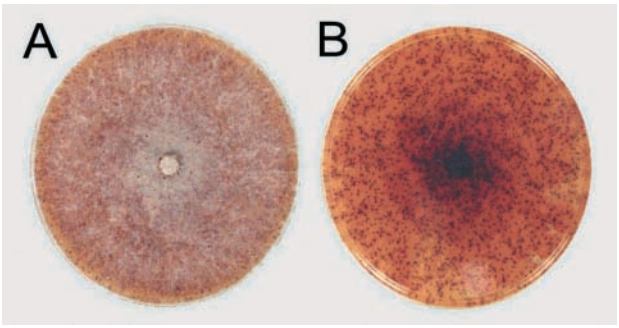

Figure 5. 30-day-old CA941040 on PDA containing 15 μg/mL tricyclazole. (**A**) Top: upper side of isolate, showing reddish brown color due to inhibition of melanin biosynthesis by tricyclazole. (**B**). Bottom: reverse side of isolate, showing reddish brown ascospores and the color of reddish brown metabolites in the agar medium.

Inhibition Studies with Tricyclazole and Identification of Melanin-Related Pentaketides. The 1,8-dihydroxynaphthlene melanin inhibitor tricyclazole at 15 μg/mL in PDA inhibited melanin biosynthesis in hyphae and ascospores of the GWT isolates, causing hyphae and ascospores of the test isolates to be reddish brown in color instead of gray or black (compare **Figures 3A** and **5**, parts **A** and **B**). GWT isolates usually were grown on PDA for 30 days to evaluate melanin biosynthesis, as it takes 20 to 30 days for mature ascospores of the fungus to develop under the test conditions. HPLC results showed that the 30 day-old PDAT cultures of wild-type isolates often contained the 1,8-dihydroxynaphthalene melanin metabolites 2-hydroxyjuglone (**14**), 3-hydroxyjuglone (**17**), **11,** and **12**, which were not found in untreated GWT cultures. Without exception, the GWT isolates that produced the most pentaketide metabolites in the presence of tricyclazole were the most heavily melanized isolates used in a study.

PDA cultures of the GYB isolates often accumulated detectable levels of 4-hydroxyscytalone (**13**), scytalone (**7**), **11**, **12,** and **17** in the absence of tricyclazole. In contrast, SP934945 differed from the other GYB isolates in that it produced larger amounts of 1,8-dihydroxynaphthalene melanin metabolites in PDA culture than the other GYB isolates. As shown in **Table 2**, SP934945 was the weakest GYB producer of hexaketide compounds. The GYB isolates produced melanin metabolites in the presence of tricyclazole. However, tricyclazole did not appear to have a significant effect on pentaketide production by SP934945 or the other GYB isolates, compared to controls without tricyclazole. A table showing the presence or absence of melanin metabolites in cultures of the GWT and GYB isolates is shown electronically under Supporting Information.

The data for pentaketide and hexaketide metabolites were obtained from PDA and PDAT cultures that contained approximately 0.8% EtOH at the time of inoculation. Data from controls without EtOH are not shown, since 0.8% EtOH did not significantly affect melanization, growth rate, morphology, or pentaketide and hexaketide accumulation in the GWT or GYB cultures.

Hexaketides. In a typical experiment, 30-day-old PDA cultures of the 15 GWT isolates contained concentrations of compound **1** from undetectable levels to 0.9 μg/mL (**Table 2**). The GWT cultures seldom produced hexaketides **2**−**5**. In contrast, cultures of the 11 GYB isolates contained much higher levels of compound **1** (4.6 to139.5 μg/mL), and all produced at least two of the other hexaketides (**2**−**5**) compared to cultures of the GWT isolates (**Table 2**). The concentrations of compound **1** in the GYB cultures were significantly higher than those for the other four compounds. Similar hexaketide and pentaketide results were obtained with PDA and PDAT cultures obtained directly from yellow to brown sectors such as those shown in **Figure 4** (data not shown)**.** Also, on a group basis, tricyclazole did not significantly affect hexaketide concentrations in 30 day-old PDAT cultures of the GWT or GYB isolates, when the group values were compared with those in unamended cultures.

Hexaketides were usually analyzed in 30-day-old GWT and GYB cultures; however, we also analyzed levels of hexaketides in 10 and 20 day-old PDA cultures of the two groups of isolates. The concentrations of hexaketides obtained in 10 and 20-day-old cultures of the two groups were similar to the concentrations obtained in 30-day-old cultures. This indicated that synthesis of the hexaketides probably was nearly complete by 10 days. As in 30-day-old cultures, hexaketide **1** was the major metabolite present in the younger GWT and GYB cultures (data not shown).

Effect of NaCl on GWT and GYB isolates. PDAS medium contained 2.5% NaCl and was used in our studies to investigate the effect of NaCl on the GWT and GYB isolates. PDAS cultures of the GWT isolates accumulated hexaketide pigments identical to those found in PDA cultures of the GYB isolates (**Table 2**). Also, GWT isolates grown for 10, 20, or 30 days on PDAS produced higher levels of hexaketide **1** than the other hexaketides **2**−**5** (data for 10 and 20 days not shown). None of the wild types grown on PDAS produced perithecia or melanized hyphae. In general, a NaCl concentration of 0.5% completely inhibited perithecial production in GWT isolates. However, an occasional isolate did produce some perithecia at 2.0% NaCl. Unlike the GYB isolates, which were permanently altered in the absence of NaCl, the GWT isolates that had been affected by NaCl produced normal looking wild-type colonies after being transferred to regular PDA.

Additional studies with GWT isolates TX020157, TX020162, and TX020167 were carried out to determine the effect of lesser concentrations of NaCl on polyketide production and morphology. These isolates were less than one year old at the time of the investigation. Cultures of these three isolates contained hexaketides when grown for 20 days on PDA media containing 0.0, 0.25, 0.5, 1.5, and 2.5% NaCl. The control cultures (0.0% NaCl) and cultures with 0.25% NaCl contained small or undetectable amounts of **1** and the other hexaketides. However, cultures containing 0.5, 1.5, and 2.5% NaCl usually contained progressively higher levels of the hexaketides, with **1** being the most abundant (data not shown). Also, melanin and perithecial production were suppressed but not eliminated in cultures of all three isolates at 0.25% NaCl, whereas none of the wild types produced melanin or perithecia at NaCl concentrations of 0.5% or above.

Melanin metabolites were found in 10, 20, and 30 day-old PDAS cultures of the wild-type and GYB isolates, but these compounds often were present only in trace amounts and therefore were not present in comparable concentrations to the hexaketides. Compound **13**, an indirect product of **6** (**Figure 2**), was the most common pentaketide present in 30 day-old GWT cultures, and was observed in 6 of the 14 GWT isolates. PDAS cultures of the GYB isolates accumulated pentaketides **7**, **11**, **12**, **13**, **14**, and **17**.

DISCUSSION

In this study, degenerative changes, including changes in pentaketide and hexaketide metabolism, occurred in wild-type isolates of *M. cannonballus* that were maintained on soil-hull medium for prolonged periods of time or when the fungus was

Table 2. Concentrations of Hexaketides 1–5 in Cultures of 14 GWT and 11 GYB Isolates (μg/mL)[a]

culture		PDA					PDAT					PDAS				
type	isolate	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
GWT																
	CA941029	0.1	n.d.	n.d.	n.d.	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	8.6	1.3	n.d.	2.1	n.d.
	CA941030	0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.
	CA941040	0.1	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	n.d.	1n.d.	1.3	n.d.	1.8	n.d.
	CA941042	n.d.	n.d.	n.d.	n.d.	n.d.	0.3	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.
	GT960051	0.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	44.4	2.0	0.8	3.2	0.8
	GT960052	0.1	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	0.5	n.d.	34.3	2.5	0.6	1.5	1.9
	HO980085	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	0.6	n.d.	14.6	0.3	n.d.	0.4	0.3
	SP935051	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3	n.d.	n.d.	12.8	2.8	n.d.	1.8	0.9
	SP960189	n.d.	n.d.	n.d.	n.d.	n.d.	0.5	n.d.	n.d.	n.d.	n.d.	12.2	2.0	n.d.	5.7	0.3
	TX912035	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	n.d.	n.d.	n.d.	n.d.	17.2	5.2	n.d.	2.9	0.4
	TX970062	0.2	n.d.	n.d.	n.d.	n.d.	0.8	n.d.	n.d.	n.d.	n.d.	25.4	1.0	n.d.	5.4	0.5
	TX970084	0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	28.3	4.6	n.d.	6.1	0.4
	TX020162	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	16.7	0.4	n.d.	5.8	n.d.
	TX020167	0.1	n.d.	n.d.	n.d.	n.d.	0.1	n.d.	n.d.	n.d.	n.d.	13.2	1.1	0.3	18.0	0.2
GYB																
	CA921457	101.1	n.d.	0.8	3.5	n.d.	45.9	6.2	n.d.	1.6	n.d.	98.9	17.5	94.5	n.d.	n.d.
	SP935043	13.3	0.7	n.d.	4.9	n.d.	5.9	0.6	n.d.	3.6	n.d.	37.9	9.2	n.d.	3.3	1.7
	SP960199	38.2	n.d.	2.7	3.2	n.d.	57.2	3.2	7.8	1.7	n.d.	10.7	4.0	9.2	n.d.	n.d.
	CA941032	13.6	1.2	4.4	n.d.	n.d.	20.2	1.9	n.d.	4.2	0.9	45.8	2.6	2.0	n.d.	1.4
	HO980076	34.5	10.1	n.d.	9.7	7.1	74.0	9.6	n.d.	12.6	3.9	23.3	7.7	4.9	2.6	n.d.
	IS980126	53.4	13.4	n.d.	9.2	3.4	46.4	4.9	n.d.	12.1	1.7	35.8	24.8	n.d.	2.6	3.7
	SP935039	63.4	12.0	n.d.	7.8	2.6	31.6	4.6	n.d.	10.1	0.7	65.8	12.7	9.2	1.9	n.d.
	TX902020	32.6	9.2	n.d.	5.2	3.3	147.6	5.9	n.d.	5.9	2.1	8.9	1.6	n.d.	n.d.	0.3
	TX923038	139.5	7.6	n.d.	24.3	1.2	147.3	6.5	n.d.	28.9	1.6	115.6	11.6	39.4	1.9	n.d.
	TX923035	78.6	3.9	n.d.	15.4	1.4	43.6	6.2	n.d.	8.0	1.3	77.4	18.5	16.3	1.0	n.d.
	SP934945	4.6	n.d.	1.3	1.6	0.3	0.3	n.d.	1.7	n.d.	0.1	0.2	n.d.	0.8	n.d.	0.2

[a] n.d. = not detected (lower detection limits: **1**, 0.04 μg/mL; **2**, 0.10 μg/mL; **3**, 0.16 μg/mL;**4**, 0.18 μg/mL; and **5**, 0.06 μg/mL). PDAT = PDA + 15 μg /mL tricyclazole; PDAS = PDA + 2.5% NaCl. Chemical structures of **1**–**5** are shown in **Figure 1**. PDA and PDAT cultures of the GWT and GYB isolates initially contained 0.8% EtOH as it was used to dissolve the tricyclazole. Statistical results for these data are available in Supporting Information.

grown on PDA containing NaCl. Whereas prolonged storage in culture caused changes that were irreversible, the changes resulting from exposure to NaCl were temporary, as wild-type characteristics were restored after the fungus was grown without NaCl exposure on regular PDA. The GYB variants that we obtained from wild-type cultures closely resembled yellow to brown isolates described previously from cultures and the field by other workers (*1*, *2*). However, we did not obtain any of our GYB isolates directly from host plants or the field. In the present study, we did not focus on the factors that affected perithecial production and polyketide metabolism in *M. cannonballus*, although it has been reported (*1*, *2*, *22*) that double-stranded RNA (dsRNA) infection is the cause of increased pigmentation, development of hypovirulence, and a decrease or loss of perithecia and ascospore production in the degenerate isolates. Apparently, wild types of the fungus are often infected with dsRNA that can remain inactive or latent without causing visible symptoms. However, under the right conditions, the resident dsRNA becomes active and causes the fungus to lose virulence and deteriorate (*2*). The idea that dsRNA elements may be common in isolates of *M. cannonballus* was proposed by Lovic and co-workers **(***1*, *23*, *24*) who found that approximately 65% of *M. cannonballus* isolates recovered from diseased plants in two commercial muskmelon fields in south Texas harbored dsRNA. Lovic and co-workers (*23*, *24*) also reported that a diverse assortment of different sizes and numbers of dsRNAs were associated with these isolates and that isolates with markedly different dsRNA profiles were often found to coexist on the same roots. The dsRNA pattern in individual isolates in subculture was reportedly stable.

Hypovirulence in GYB isolates of *M. cannonballus* probably results because of a number of factors; however, the loss of perithecial and melanin production, along with increased hexaketide production, may all be strong contributing factors. 1,8-Dihydroxynaphthalene melanins, for example, have been shown to be virulence factors in both plant and animal pathogens and are known to operate through a variety of mechanisms (*9*−*17*). Certainly, melanin may play a role in virulence of *M. cannonballus* wild types; however, at this time, it has not been demonstrated that melanin serves such a role in this fungus.

Hexaketides **1**−**5** are structurally similar and apparently metabolically related. Although little is known about how they are made in *M. cannonballus* or about their biological function, it is possible that when combined and present in elevated concentrations, they may have a deleterious effect on *M. cannonballus* isolates. Hexaketide **1** has previously been shown to inhibit monoamine oxidase from mice (*25*), while **2** is known to have antifungal activity against *Candida albicans* and antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (*26*). Presently, we are conducting additional studies with these hexaketides to assess their toxicity to wild types and their potential role as antifungal and antibacterial compounds.

Details of the 1,8-dihydroxynaphthalene melanin pathway have been elucidated using a number of different fungi, and much of what is known about the pathway and its enzymes has come from the use of melanin-deficient mutant strains and compounds (i.e., tricyclazole) that inhibit specific enzymes in the pathway (*9*−*11*). In the present study, tricyclazole inhibited melanin biosynthesis in wild types of *M. cannonballus* and caused the accumulation of melanin metabolites (i.e., **11**, **12**, **13**, **14** and **17**). This is consistent with the fact that tricyclazole is known to inhibit enzymes in the 1,8-dihydroxynaphthalene melanin pathway that reduce 1,3,6,8-tetrahydroxynaphthalene (**6**) to **7** and 1,3,8-trihydroxynaphthlaene (**8**) to vermelone (**9**) (**Figure 2**). Compounds **11** and **14** are known autoxidative

products of the melanin precursors **6** and **8**, respectively, and with many fungi, both these compounds have been shown to accumulate in cultures when tricyclazole is used to inhibit melanin biosynthesis (*9*, *10*). It has been reported for certain fungi (i.e., *Magnaporthe grisea* and *Verticillium dahliae*) that tricyclazole is a stronger inhibitor in vivo at the enzymic site between **8** and **9** than at the site between **6** and **7** (*27*). In the present study, this apparently explains why, in the presence of tricyclazole at 15 μg/mL, wild-type cultures of *M. cannonballus* accumulated metabolites **11**, **12**, and **13** at the same time they accumulated **14** and **17**. Since tricyclazole did not prevent the production of perithecia or ascospores by the GWT isolates or appear to inhibit hexaketide synthesis, its main target of action apparently was the melanin pathway.

The accumulation of compounds **11** and **14** and other melanin metabolites in pigmented cultures of the GYB isolates most likely occurred because of defects in the 1,8-dihydroxynaphthalene melanin pathway. Cultures of SP934945, for example, accumulated appreciable amounts of several melanin metabolites (i.e., **8**, **11**, **12**, **13**, **14**, and **17**), but the fungus did not make detectable 1,8-dihydroxynaphthalene melanin. It is possible that reactions that reduce compounds **6** and **8** were inhibited, causing a build up of upstream precursors in SP934945. However, the accumulation of **7** in cultures of this isolate suggests that the dehydratase enzyme that converts **7** to **8** also was partially blocked. It is known that **7** is easily dehydrated to **8** in fungi with a normal functioning dehydratase enzyme (*28*), and **7** does not accumulate in fungal cultures unless the dehydratase enzyme is defective or blocked by a chemical inhibitor such as carpropamid (*29*). Additional work is required to determine the exact enzymes that are affected in SP934945 and the other GYB isolates.

Calcium, magnesium, and sodium are usually the major cations in the cation-exchange complex of soils in arid regions as a consequence of saline irrigation water and inadequate rain-induced leaching. The fact that 0.5−2.5% NaCl caused GWT isolates to produce elevated levels of hexaketides in NaCl-treated PDA cultures indicates that wild types of the fungus may produce elevated levels of the compounds when grown on plant debris under high saline field conditions. The stimulatory effects of NaCl on hexaketide production by wild-type isolates is of interest because the fungus appears to be adapted to hot, semiarid soils that tend to be saline and alkaline in nature (*1*). At this time, we do not understand the mechanism by which NaCl affects polyketide metabolism and perithecial production in *M. cannonballus*, but we find it interesting that many of the changes that occurred on NaCl-treated PDA were similar to those observed when GYB isolates were grown on unamended PDA. Since dsRNA may be responsible for the production of GYB isolates, it would be interesting to learn why dsRNA and NaCl produce similar effects on polyketide metabolism and on perithecial production. We will continue to study the effects of NaCl on wild-type development and on the synthesis of hexaketide and pentaketide compounds to determine how NaCl affects the polyketide pathways and to see if NaCl plays a significant role in stimulating hexaketide production in nature.

In the present investigation, we did not attempt to identify dsRNA in the GWT or GYB isolates. However, it has been suggested previously (*1*, *2*) that hypovirulent strains of *M. cannonballus* infected with dsRNA may be of use as biocontrol agents. There are several other fungi, including *Cryphonectria parasitica* (*30*), *Ophiostoma* (formerly *Ceratocystis*) *novo-ulmi* (*31*), *Rhizoctonia solani* (*32*), and *Sclerotinia minor* (*33*) that are known to undergo morphological and biochemical changes after infection with dsRNA. As previously reported for *M. cannonballus* (*1*, *23*), these fungi are capable of transferring dsRNA elements between strains via hyphal anastomosis. In the case of chestnut blight, viruses containing dsRNA are known to cause changes in the fungus *C. parasitica* and have been used as biocontrol agents. One such virus, known as *Cryphonectria hypovirus I* (CHV1), exists in the unencapsidated form and has been successfully used to control chestnut blight in Europe (*30*). CHV1 is transferred from hypovirulent strains of the fungus to more virulent strains via hyphal anastomosis and causes them to lose virulence. The virus-infected strains of *C. parasitica* are considered the functional equivalent of strains with a mutation in a virulence controlling pathway and have been used to study various aspects of fungal virulence.

Although dsRNA has been reported to cause the cultural changes and development of hypovirulence in wild types of *M. cannonballus* (*22*), additional studies with the fungus are needed to show how dsRNA converts wild types to the GYB isolates of the fungus. Earlier reports (*1*, *2*) concerning the relationship between dsRNA and the fungus are based on a limited number of studies that should be expanded. As with *C. parasitica* (*30*), learning how dsRNA affects *M. cannonballus* should help identify factors that control fungal development and virulence. These factors may include the effects of dsRNA on the hexaketide and 1,8-dihydroxynaphthalene melanin pathways. Loss of fungal melanization in *M. cannonballus* may be associated with loss of virulence in the pigmented isolates.

ABREVIATIONS USED

CHV1, *Cryphonectria hypovirus I*; dsRNA, double-stranded RNA; GWT, wild-type group; GYB, yellow to brown group; MRR/VD, Monosporascus root rot/vine decline; PDA, potato-dextrose agar; PDAS, PDA containing 2.5% (w/v) NaCl; PDAT, PDA containing 15 μg/mL tricyclazole.

ACKNOWLEDGMENT

We thank Sara E. Duke (USDA; College Station, TX) for help with statistical analysis and Bob Ehr (Dow AgroSciences; Indianapolis, IN) for supplying tricyclazole. We thank Diann M. Baze and Lucille W. Young for excellent technical assistance.

Supporting Information Available: A table with statistical results, including means, standard errors and t-groups, for the hexaketide data displayed in **Table 2** and a table showing the presence or absence of pentaketide melanin metabolites in cultures of GWT and GYB isolates are available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Martyn, R. D.; Miller, M. E. Monosporascus root rot and vine decline: An emerging disease of melons worldwide. *Plant Dis.* **1996**, *80*, 716−725.

(2) Batten, J. S.; Scholthof, K. G.; Lovic, B. R.; Miller, M. E.; Martyn, R. D. Potential for biocontrol of Monosporascus root rot/vine decline under greenhouse conditions using hypovirulent isolates of *Monosporascus cannonballus*. *Eur. J. Plant Pathol.* **2000**, *106*, 639−649.

(3) Pollack, P. G.; Uecker, F. A. *Monosporascus cannonballus*, an unusual Ascomycete in cantaloupe roots. *Mycologia* **1974**, *66*, 346−349.

(4) Staghellini, M. E.; Kim, D. H.; Waugh, M. Microbe-mediated germination of ascospores of *Monosporascus cannonballus*. *Phytopathology* **2000**, *90*, 243−247.

(5) Bruton, B. D. Soilborne diseases in Cucurbitaceae: Pathogen virulence and host resistance. In *Cucurbitaceae '98*; McCreight, J., Ed.; Am. Soc. Hortic. Sci.: Alexandria, VA., 1998; pp 143−166.

(6) Bruton, B. D.; Garcia-Jimenez, J.; Armengol, J. Analysis of the relationship between temperature and vine declines caused by *Acremonium cucurbitacearum* and *Monosporascus cannonballus* on muskmelon. *Subtrop. Plant Sci*. **1999**, *51*, 23−28.

(7) Bruton, B. D.; Garcia-Jimenez, J.; Armengol, J.; Popham, T. W. Assessment of virulence of *Acremonium cucurbitacearum* and *Monosporascus cannonballus* on *Cucumis melo*. *Plant Dis*. **2000**, *84*, 907−913.

(8) Stipanovic, R. D.; Zhang, J.; Bruton, B. D.; Wheeler, M. H. Isolation and identification of hexaketides from a pigmented *Monosporascus cannonballus* isolate. *J. Agric. Food Chem.* **2004**, *52*, 4109−4112.

(9) Bell, A. A.; Wheeler, M. H. Biosynthesis and function of fungal melanins. *Annu. Rev. Phytopathol*. **1986**, *24*, 411−451.

(10) Wheeler, M. H.; Bell, A. A. Melanins and their importance in pathogenic fungi. In *Current Topics in Medical Mycology*; McGinnis, M. R., Ed.; Springer-Verlag: New York, 1988; Vol. 2; pp 338−387.

(11) Butler, M. J.; Day, A. W. Fungal melanins: A review. *Can. J. Microbiol*. **1998**, *44*, 1115−1136.

(12) Henson, J. M.; Butler, M. J.; Day, A. W. The dark side of the mycelium: Melanins of phytopathogenic fungi. *Annu. Rev. Phytopathol*. **1999**, *37*, 447−471.

(13) Brakhage, A. A.; Jahn, B. Molecular mechanisms of pathogenicity of *Aspergillus fumigatus*. In *Molecular Biology of Fungal Development*; Osiewacz, H. D., Ed.; Marcel Dekker: Dordrecht, 2002; pp 559−582.

(14) Gomez, B. L.; Nosanchuk, J. D. Melanin and fungi. *Curr. Opin. Infect. Dis.* **2003**, *16*, 91−96.

(15) Langfelder, K.; Streibel, M.; Jahn, B.; Haase, G.; Brakhage, A. A. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Gen. Biol.* **2003**, *38*, 143−158.

(16) Morris-Jones, R.; Youngchim, S.; Gomez, B. L.; Aisen, P.; Hay, R. J.; Nosanchuk, J. D.; Casadevall, A.; Hamilton, A. J. Synthesis of melanin-like pigments by *Sporothrix schenckii in vitro* and during mammalian infection. *Infect. Immun*. **2003**, *71*, 4026−4033.

(17) Tsai, H.-F.; Fujii, I.; Watanabe, A.; Wheeler, M. H.; Chang, Y. C.; Yasuoka, Y.; Ebizuka, Y.; Kwon-Chung, K. J. Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. *J. Biol. Chem*. **2001**, *276*, 29292−29298.

(18) Esposito, R.; Fletcher, A. M. The relationship of pteridine biosynthesis to the action of copper-8-hydroxyquinolate on fungal spores. *Arch. Biochem. Biophys*. **1961**, *93*, 369−376.

(19) Stipanovic, R. D.; Wheeler, M. H. Accumulation of 3,3′-biflaviolin, a melanin shunt product, by tricyclazole-treated *Thielaviopsis basicola*. *Pestic. Biochem. Physiol.* **1980**, *13*, 198−201.

(20) Greenblatt, G. A.; Wheeler, M. H. HPLC analysis of fungal melanin intermediates and related metabolites. *J. Liq. Chromatogr*. **1986**, *9*, 971−981.

(21) Wheeler, M. H.; Stipanovic, R. D. Melanin biosynthesis and the metabolism of flaviolin and 2-hydroxyjuglone in *Wangiella dermatitidis*. *Arch. Microbiol.* **1985**, *142*, 234−241.

(22) Park, Y. J.; Martyn, R. D.; Miller, M. E. dsRNA is responsible for cultural aberrations in *Monosporascus cannonballus* and hypovirulence to muskmelon. *Phytopathology* **1996**, *86*, S107.

(23) Lovic, B. R. Molecular studies of *Monosporascus* spp: Applications in diagnostics, taxonomy, population biology, and control. Ph.D. Dissertation; Texas A&M University, College Station, TX, 1994.

(24) Lovic, B. R.; Valadez, R. D.; Martyn, R. D.; Miller, M. E. Preliminary analysis of dsRNA length polymorphisms in clonal, root, and field populations of *Monosporascus cannonballus*. In *Proc. Cucurbitaceae 94: Evaluation and enhancement of cucurbit germplasm*; Lester, G. E., Dunlap, J. R., Eds.; Gateway Printing: Edinburg, TX.; 1995; pp 201−203.

(25) Fujimoto, H.; Okuyama, H.; Motohashi, Y.; Yoshida, E.; Yamazaki, M. Some monoamine oxidase inhibitory components from Mycelia sterilia derived from an Ascomycete, *Gelasinospora pseudoreticulata*. *Mycotoxins* **1995**, *41*, 61−66.

(26) Whyte, A. C.; Gloer, K. B.; Gloer, J. B.; Koster, B.; Malloch, D. New antifungal metabolites from the coprophilous fungus *Cercophora sordarioides*. *Can. J. Chem*. **1997**, *75*, 768−772.

(27) Wheeler, M. H.; Greenblatt, G. A. The inhibition of melanin biosynthetic reactions in *Pyricularia oryzae* by compounds that prevent rice blast disease. *Exp. Mycol*. **1988**, *12*, 151−160.

(28) Wheeler, M. H. Melanin biosynthesis in *Verticillium dahliae*: dehydration and reduction reactions in cell-free homogenates. *Exp. Mycol*. **1982**, *6*, 171−179.

(29) Kurahashi, Y.; Araki, Y.; Kinbara, T.; Pontzen, R.; Yamaguchi, I. Intermediates accumulation and inhibition sites of carpropamid in melanin biosynthesis pathway of *Pyricularia oryzae*. *Pestic. Sci*. **1998**, *23*, 22−28.

(30) McCabe, P. M.; Van Alfen, N. K. Molecular basis of symptom expression by the *Cryphonectria* hypovirus. In *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry, and Medicine*; Tavantzis, S. M., Ed.; CRC Press: Boca Raton, FL, 2002; pp 125−143.

(31) Buck, K. W.; Brasier, C. M. Viruses of the Dutch Elm disease fungi. In *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry, and Medicine*; Tavantzis, S. M., Ed.; CRC Press: Boca Raton, FL, 2002; pp 165−190.

(32) Tavantzis, S. M.; Lakshman, D. K.; Liu, C. Double-stranded RNA elements modulating virulence in *Rhizoctonia solani*. In *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry, and Medicine*; Tavantzis, S. M., Ed.; CRC Press: Boca Raton, FL, 2002; pp 191−211.

(33) Savard, M. E.; Melzer, M. S.; Boland, G. J.; Bensimon, C.; Blackwell, B. A. A new 1-hydroxy-2,6-pyrazinedione associated with hypovirulent isolates of *Sclerotinia minor*. *J. Nat. Prod.* **2003**, *66*, 306−309.

Received for review December 22, 2003. Revised manuscript received April 12, 2004. Accepted April 12, 2004.

JF0308298