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ANTIFUNGAL HYDROXYMETHYL-PHENOLS FROM THE MYCOPARASITE *VERTICILLIUM BIGUTTATUM*

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Key Word Index—*Verticillium biguttatum*; Moniliales; Hyphomycete; mycoparasitism; vertinoids; 1-(2′,3′-dihydroxymethylphenyl)-3-methyl-but-2-ene; bigutol; 1-(2′-hydroxy-3′-methoxy-5′-hydroxymethylphenyl)-3-methyl-but-2-ene; methylbigutol.

Abstract—Secondary metabolites were extracted from culture filtrate of three isolates of the mycoparasitic biocontrol agent *Verticillium biguttatum* and two antifungal metabolites isolated using a standard TLC procedure. The structures of 1-[2′,3′-dihydroxy-5′-(hydroxymethyl)phenyl]-3-methyl-but-2-ene and 1-[2′-hydroxy-3′-methoxy-5′-(hydroxymethyl)phenyl]-3-methyl-but-2-ene were elucidated by spectroscopic analysis. These new metabolites were named bigutol and methylbigutol, respectively. Low concentrations of each metabolite inhibited mycelial extension of *Rhizoctonia solani* (minimum inhibitory concentration 138 $\mu g\,ml^{-1}$) and other plant pathogenic fungi on potato dextrose agar. Production of these antifungals by *V. biguttatum* suggests that antibiosis may play a role during biocontrol by this mycoparasite, particularly of plant diseases caused by *R. solani*.

INTRODUCTION

Biocontrol of the plant pathogen *Rhizoctonia solani* Kühn by *Verticillium biguttatum* Gams has been argued to be effected by mycoparasitic activity [1, 2]. No effective *in vitro* inhibition of the plant pathogen by metabolites of the mycoparasite has been reported in the literature [3]. However, germination of sclerotia of *R. solani* colonized by the mycoparasite can be inhibited even though many of the sclerotial monilioid cells may still be viable. This evidence suggests that, under the relatively high nutrient conditions within sclerotia, the mycoparasite produces metabolites that inhibit the growth of *R. solani* [4].

Many different types of inhibitory metabolites have been isolated from species within the genus *Verticillium*, including antifungal peptides, siderophores and insecticidal pregnadicarboxylic acids [5–7]. Studies of melanin synthesis via the polyketide pathway by pigmented species of *Verticillium* have led to the isolation of low molecular weight melanin precursors such as juglone and vermelone which exhibit antifungal activity [8]. Such low molecular weight polyketide antibiotics from *Verticillium* are collectively known as vertinoids [9].

In a preliminary study, antibiosis by *V. biguttatum* against a range of plant pathogenic fungi was demonstrated in agar overlay tests [10]. Production of heat stable antifungal secondary metabolites was demonstrated subsequently in a defined liquid medium containing high nutrient levels (Czapek–Dox) and a TLC-based procedure resulted in the isolation of two antifungals. Three *V. biguttatum* isolates tested each produced two new antifungal hydroxymethyl-phenols (**1**, **2**) which were identified by spectrometry. Low concentrations of each metabolite were assessed for inhibition of mycelial extension of *R. solani* and some other plant pathogenic fungi on potato dextrose agar.

RESULTS AND DISCUSSION

In static liquid culture, the filtrate of *V. biguttatum* isolate M73 became significantly inhibitory to *R. solani* between days 14 and 17 and of isolates vb1 and vb2 between days 17 and 21 (Fig. 1). Concomitantly, numbers of conidia and biomass produced by each *V. biguttatum* isolate increased to maxima of around 8.0×10^7 conidia ml^{-1} culture and 3.5 $mg\,ml^{-1}$ culture, respectively (Figs 2 and 3) indicating that production of antifungal secondary metabolites was not linked to morphological mycelial differentiation, as can be seen in some fungi [11]. Culture medium pH declined from the initial 5.1 to 3.5 by day 17 and then remained stable until day 24 (Fig. 4) confirming previous observations with *V. biguttatum* [12]. This low final pH could have restricted primary metabolism even if a medium component had not become limiting [13, 14].

All antifungal activity was extracted from acid culture filtrate of *V. biguttatum* by the procedure used. Approx-

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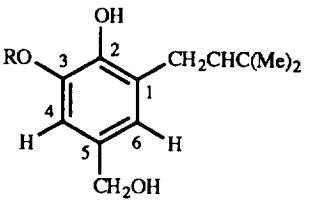

1, R = H

2, R = Me

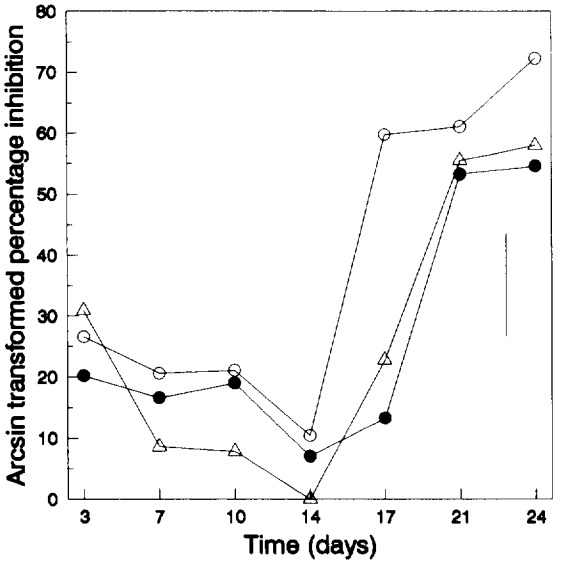

Fig. 1. The effect of culture filtrates of *V. biguttatum* isolates M73 (○), vb1 (●) and vb2 (△) produced under static culture conditions in modified Czapek–Dox liquid medium at 21° over the time range of 3–24 days on the mycelial growth of *R. solani* AG-3 when incorporated (2 ml) into potato dextrose agar (20 ml). The product of two perpendicular colony diameters, represented as percentage inhibition of control products was calculated using the formula:

$$\frac{(\text{control product} - \text{treatment product})}{\text{control product}} \times 100.$$

The bar represents the LSD ($P < 0.05$).

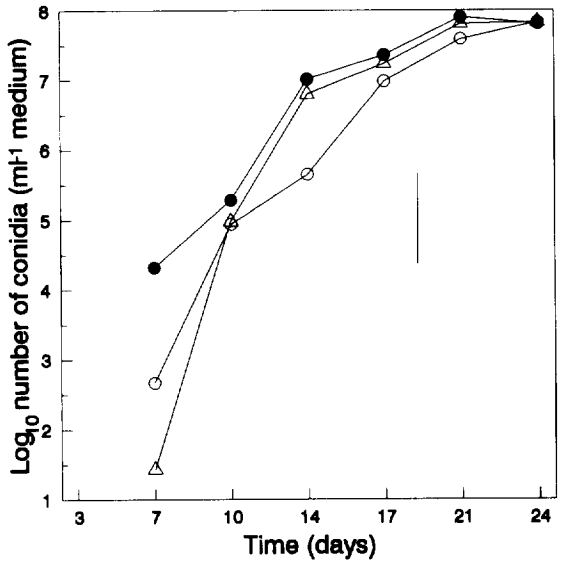

Fig. 2. The number of conidia produced by *V. biguttatum* isolates M73 (○), vb1 (●) and vb2 (△) under static culture conditions in modified Czapek–Dox liquid medium at 21° over the time range 7–24 days. The bar represents the LSD ($P < 0.05$).

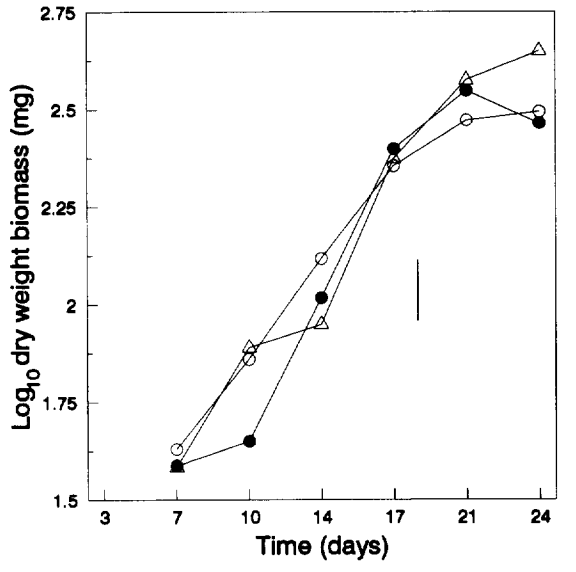

Fig. 3. The production of dry weight biomass by *V. biguttatum* isolates M73 (○), vb1 (●) and vb2 (△) under static culture conditions in modified Czapek–Dox liquid medium at 21° over the time range of 7–24 days. The bar represents the LSD ($P < 0.05$).

imately 102 mg l^{-1} of a dark brown aromatic gum was recovered from the extract of 24-day-old culture filtrate. TLC bioautography of extract of each *V. biguttatum* isolate demonstrated the presence of two antifungal activities, metabolite A (R_f 0.32) and metabolite B (R_f 0.44). Low level activity remained at the origin. Semi-quantitative GC of the extract showed metabolite B (R_t 8.34 min) accumulated between days 17 and 21 while the amounts of all other metabolites changed very little. So, this antifungal was a secondary metabolite. Metabolite A was not detected under the GC conditions used. Approximately 127 mg l^{-1} of the gum was recovered from the extract of 24-day-old culture filtrate when 0.4 μM biotin was added to cultures on day 18. GC showed the amount of metabolite B in the extract had increased by 267%. Greater production of metabolite B is of interest as biotin can act as a cofactor of acetyl CoA carboxylase, an enzyme which catalyses the addition of carbon dioxide to acetyl-CoA to produce malonyl-CoA. This is the first step in a pathway that leads to production of fatty acids from acetate during primary metabolism or to

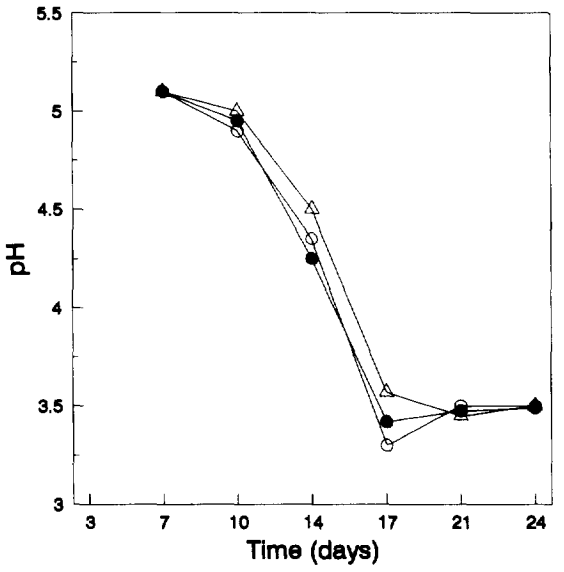

Fig. 4. The effect of *V. biguttatum* isolates M73 (○), vb1 (●) and vb2 (△) on the pH of modified Czapek–Dox liquid medium under static culture conditions at 21° over the time range 7–24 days. Values are the mean of three replicates.

polyketide-derived aromatic secondary metabolites [14]. This biochemical evidence suggested metabolite B was possibly of polyketide origin.

On the basis of mass spectra and ^1H and ^{13}C NMR spectra, each *V. biguttatum* isolate produced the same pair of metabolites. Thus, metabolite A from each isolate had a mass spectrum with a putative molecular ion at *m/z* 208 and metabolite B a corresponding molecular ion at *m/z* 222. Comparison of these low resolution spectra with library spectra suggested metabolite A, $C_{12}H_{16}O_2$, was a phenolic and metabolite B, $C_{13}H_{18}O_3$, was a related methylated compound. The major peak (100%) in the mass spectrum of metabolite B had *m/z* 167 corresponding to the loss of $CH{=}CMe_2$ with a peak 166 (38%) $[M - H - CH{=}CMe_2]^+$. In metabolite A the corresponding peaks were at *m/z* 153 (75%) and 152 (100%).

The ^1H and ^{13}C NMR spectra of both metabolites A and B were determined in chloroform-*d* methanol-d_4 (Table 1). Inspection of ^1H NMR spectra confirmed that the only significant difference between A and B was the replacement of a hydroxy group (in A) by a methoxy group (in B). The ^1H NMR spectra of metabolites A and B in chloroform-*d* and methanol-d_4 suggested both species were tetrasubstituted benzene derivatives with the remaining aryl protons in a meta orientation. The substituent groups CH_2OH and $CH_2CH{=}CMe_2$ were readily identified in both cases, the only slightly unusual feature being the coincidence of the methyl groups in both metabolites. For metabolite A the presence of two phenolic hydroxy groups was inferred from the presence of a very broad peak (100 Hz) at *ca* δ5.5. In metabolite B only one phenolic hydroxy peak was detected which sharpened with increasing temperature. This proton is probably hydrogen bonded to the adjacent methoxy group (see below).

With the nature of the four substituents in the aryl ring of both A and B established, the structure analysis reduced to the determination of the substituent pattern. There are 12 different arrangements of substituent pattern which have the remaining two protons in a *meta* disposition but the majority of these can be eliminated on the basis of the benzene ring chemical shifts. Using standard substituent chemical shift values [15] it was readily established that the oxy substituents (OH, OH in metabolite A and OH, OMe in metabolite B) must be mutually *ortho*. Thus, the substituent arrangement shown in **1** has a mean deviation between observed and calculated benzene ring carbon chemical shifts of $\pm$ 2 ppm. Although the relative positions of the hydroxyl and methoxyl groups is reasonably certain on this basis the two possible arrangements of the methylene groups cannot be distinguished.

The best high resolution spectral data were obtained from metabolite B in methanol-d_4. The allyic CH_2 mul-

Table 1. ^1H and ^{13}C NMR spectral data for metabolites A and B produced by *V. biguttatum* isolates M73, vb1 and vb2

		Species A		Species B			Species A		Species B	
	C	$CDCl_3$	CD_3OD	$CDCl_3$	CD_3OD	C	$CDCl_3$	CD_3OD	$CDCl_3$	CD_3OD
m (6H)	2 Me	1.78	1.71	1.73	1.71	Me	18.0	18.7	17.9	18.6
d (2H)	$-CH_2-C=$	3.35	3.30	3.36	3.30	Me	25.9	27.8	25.9	26.7
s (3H)	OMe	—	—	3.89	3.85	$CH_2C=$	29.8	30.0	28.2	29.9
s (2H)	OCH_2 aryl	4.54	4.46	4.58	4.46	OMe			56.1	57.3
m (1H)	$-CH=$	5.31	5.29	5.31	5.29	CH_2	65.4	66.3	65.8	66.4
d (1H)	aryl H-6	6.67	6.67	6.73	6.67	aryl C-4	112.5	113.8	107.7	110.1
d (1H)	aryl H-4	6.78	6.78	6.78	6.78	aryl C-6	120.5	121.3	120.9	122.5
s (1H)	OH			1.8		CH=	121.7	124.9	122.2	124.8
s (1H)	aryl OH			5.7		aryl C-1	127.5	130.2	127.5	129.7
br s	OH	5.5				C=	133.2	133.4	132.2	133.5
						aryl C-5	135.1	134.1	132.9	133.9
						aryl C-2	141.8	144.2	143.0	145.2
						aryl C-3	144.1	146.7	144.0	149.3

tiplet was obscured by the solvent signal but the following coupling interactions were observed. The methoxy protons show a doublet splitting of 0.21 Hz corresponding to a five bond coupling to one *ortho* proton (cf. a value of 0.29 Hz in 2-hydroxy anisole) [16]. This confirms the methoxy group is at position 3 (using the numbering indicated in structure **1**). The aromatic proton at δ6.78 had a *meta* coupling of 1.98 Hz with a further poorly resolved multiplet splitting of *ca* 0.2 Hz. This suggests that this proton is H-4, coupled to the methoxy group and this was confirmed by a two-fold decrease in the overall line width when the methoxy group was decoupled. Thus, the proton at δ6.78 is unequivocably established as *ortho* to the methoxy group. Decoupling of the OCH_2 group leaves a poorly resolved multiplet for H-4, the combination of the quartet splitting to the methoxy and triplet splitting to the $CH_2C=$group (^6J = 0.3 Hz). In contrast, the other aromatic proton H-6 is a sharp doublet of quintets (line width *ca* 0.2 Hz) indicating equal coupling to both ortho methylene groups, with a ^4J value of 0.61 Hz. This confirms the general substituent pattern.

The OCH_2 group is a triplet consistent with couplings of 0.5 and 0.6 Hz to the aromatic protons. The other methylene multiplet was obscured (in CD_3OD) but a suitable decoupling experiment confirmed its presence and showed that this group was coupled to H-6 (^4J = 0.6 Hz) and to the olefinic proton (^3J = 7.38 Hz). The olefinic proton had the multiplicity expected of equal (degenerate) coupling (^4J = 1.44 Hz) to two methyl groups with coincident chemical shifts.

Thus, the metabolites of *V. biguttatum* are identified as 1- [2′,3′-dihydroxy-5′-(hydroxymethyl)phenyl]-3-methyl-but-2-ene (metabolite A, **1**) and 1-[2′-hydroxy-3′-methoxy-5′-(hydroxymethyl)phenyl]-3-methyl-but-2-ene (metabolite B, **2**). Searches of the literature showed the metabolites to be new, so they were named bigutol and methylbigutol, respectively. The structures (**1**, **2**) suggest these are polyketide derived vertinoids similar to those previously isolated from *Verticillium* species [9].

Bigutol and methylbigutol inhibited mycelial extension of a range of plant pathogenic fungi at low concentration (Table 2). Bigutol was approximately twice as inhibitory as methylbigutol to all test fungi except the most susceptible fungus, *R. solani* (MIC 138 μg ml^{-1}), which was affected equally by both metabolites. Inhibition of *F. fulva* growth was lower on blocks of potato dextrose agar (MIC > 500 μg ml^{-1}) than suggested by TLC bioautography (MIC < 200 μg ml^{-1}). This difference could be explained if conidia used for TLC bioautography were more susceptible than mycelium used in the agar block test. Such effects undoubtedly contribute to differences reported for activities of antifungal metabolites [17, 18].

In relation to biocontrol of *R. solani* by the mycoparasite *V. biguttatum*, these metabolites may be produced in high enough concentrations during colonization of *R. solani* sclerotia to be responsible for the inability of such sclerotia to germinate when many of the constituent monilioid cells are still viable [4]. So, antibiosis may have a role in biocontrol of *R. solani* using the mycoparasite *V. biguttatum*.

Table 2. Antifungal activities of bigutol and methylbigutol against five plant pathogens and *V. biguttatum* isolate vb1 assessed by application of 10–200 μg (28–550 μg metabolite ml^{-1} agar) each metabolite in HPLC grade MeOH to potato dextrose agar blocks of known volume*

Fungal isolate	Antifungal activity (MIC†)	
	Bigutol	Methylbigutol
Pyrenochaeta lycopersici	275	550
Fusarium oxysporum f. sp. *narcissi*	> 550	> 550
Botrytis cinerea	275	550
Rhizoctonia solani AG-3	138	138
Fulvia fulva	> 550	> 550
Verticillium biguttatum isolate vb1	275	550

*Mean block volume ± s.d. = 0.363 ± 0.011 ml.
†MIC = minimum inhibitory concentration.

EXPERIMENTAL

Fungal isolates. Verticillium biguttatum Gams isolates M73 from The Netherlands (CBS 228.80, supplied by P.H.J.F. van den Boogert, Institute of Soil Fertility, Haren, The Netherlands) and vb1 and vb2 from the U.K. [19] were used throughout. The range of Asco-Deuteromycete plant pathogens used as test fungi included *Pyrenochaeta lycopersici* Schneider & Gerlagh, *Fusarium oxysporum* f.sp. *narcissi* Snyder & Hansen (from Horticulture Research International, Littlehampton) and *Botrytis cinerea* Pers. and *Fulvia fulva* (Cooke) Cif. as well as the Basidiomycete *Rhizoctonia solani* Kühn AG-3 (from the Department of Applied Biology culture collection, University of Hull). Conidial suspensions containing 1×10^6 conidia ml^{-1} were prepared in distilled water from 7-day-old cultures of *V. biguttatum* and *F. fulva* on potato dextrose agar (PDA) which had been uniformly inoculated with conidial suspensions and incubated at 20° in darkness.

Static culture study. A modified Czapek–Dox liquid medium (MCD) [20] containing glucose (10 g l^{-1}) and glutamine (2 g l^{-1}) as carbon and nitrogen sources, respectively, and biotin (0.4 μM) as a growth factor, adjusted to pH 5.1 with conc HCl, was used throughout [12]. Aliquots (40 μl) of conidial suspensions of *V. biguttatum* isolates M73, vb1 or vb2 were used to inoculate MCD (100 ml in each 1 l Roux bottle). Cultures were incubated as thin layers on the flat side of Roux bottles under static conditions at 21° in darkness.

The number of conidia, dry weight of biomass produced, and antifungal activity of culture filtrate were determined twice weekly up to 24 days for three replicate cultures. The pH of the medium was determined at each sampling time. The number of conidia present (ml^{-1} medium) was calculated from counts made using an

improved Neubauer haemocytometer after vigorous shaking of the culture. Biomass was recovered by filtration under vacuum on to pre-weighed filter paper (Whatman no. 1) and dried at 80° to constant weight. Culture filtrate was sterilized by filtration (0.22 μm sieve, Millipore). Antifungal activity of culture filtrate was determined by growing *R. solani* AG-3 on dishes containing PDA (2 ml) into which filtrate (2 ml) had been incorporated at 45° just before pouring. MCD (2 ml) was incorporated into PDA for control dishes. Following central inoculation with discs containing actively growing edges of the test fungus, two perpendicular colony diameters were measured after incubation of dishes for 4 days at 25° in darkness. Percentage inhibition of growth was calculated from the products of perpendicular diameters using the formula:

$$\text{percentage inhibition} = \frac{(\text{control product} - \text{treatment product})}{\text{control product}} \times 100.$$

Dry weight of biomass ($\log_{10}$ mg), percentage inhibition (arcsin transformed) and number of conidia ($\log_{10}$ number ml^{-1} medium) were subjected to analysis of variance.

Heat stability of antifungal activity. Initially it was essential to determine whether inhibition was due to enzyme activity or a heat labile chemical. Filtrates of 24 day cultures of *V. biguttatum* isolates M73 and vb2 were heated to 60° for 5 min. Antifungal activity was then compared with that of filtrates which had not been heated. Results were subjected to analysis of variance. Antifungal activity was not reduced by heating so the investigation was continued.

Extraction and isolation. A standard antifungal activity based TLC procedure was used [21]. Filtrates from 24 day cultures of each *V. biguttatum* isolate were extracted with distilled EtOAc (3 × 1/3 vol.), the crude extracts concd under vacuum at 30°, dried on a hot-plate under nitrogen, weighed and redissolved in EtOAc (1 μl redissolved extract ≡ 0.5 ml culture filtrate). Residual antifungal activity of filtrates was tested after extraction.

Extracts (20 μl) from filtrates of each *V. biguttatum* isolate were sepd by TLC (5 × 0.5 mm thickness 20 × 20 cm plates from 80 ml distilled water + 40 g Kieselgel (Merck)) in EtOAc–cyclohexane (1 : 1). Antifungal activity of separated extract constituents was demonstrated using bioautography with *F. fulva* as the test fungus. Air-dried plates were sprayed with double strength Czapek–Dox liquid medium (Oxoid) containing approximately 1×10^6 conidia of *F. fulva* ml^{-1} and incubated for 4 days under moist conditions at 25° in darkness. The residue of distilled EtOAc was used as the control because in preliminary investigations both the residue of EtOAc which had not been distilled and extract of MCD were shown by bioautography to contain antifungal activities different from those produced by the mycoparasite. The TLC system was modified by addition of UV fluorescent mass marker rhodamine (80 ml 0.32% solution, Rhodamine 6G, BDH) to the gel and used preparatively to purify antifungal activity. Separated extract constituents were visualized as bands which quenched fluorescence under UV. Fluorescence quenching bands that contained antifungal activity were identified by bioautography R_fs, recovered by filtration into distilled diethyl ether, dried under nitrogen and redissolved in HPLC grade MeOH.

GC. Crude extracts from 17 and 21 day filtrates of *V. biguttatum* isolates M73, vb1 and vb2 were sepd by semi-quantitative GC (OV-225 column, N_2 carrier 30 ml min^{-1} at 170°, FID, Pye Unicam PU4500, Phillips) [21]. Semi-purified and purified metabolites were also subjected to GC to determine which metabolites had been purified and to check the purity of those present. Further, a semi-quantitative GC experiment showed addition of 0.4 μM biotin to *V. biguttatum* isolate M73 cultures at 18 days increased production of one antifungal metabolite and so during batch production biotin was added at 18 days.

Identification. Samples of the antifungal metabolites from each *V. biguttatum* isolate were subjected to low resolution MS and GC-MS (GC conditions: 25 m BP1 column, helium carrier 15 psi, initial column temp. 130° for 4 min, ramped 10° min^{-1} to 200°. Probe MS, conditions: room temp. to 300° at 120° min^{-1}, 1 scan sec^{-1}, 1020 automated GC-MS, Finnigan MAT). ^1H and ^{13}C NMR ($CDCl_3$, 270 and 68 MHz, respectively, Joel JNM-GX270 FT NMR Spectrophotometer) studies were carried out using standard conditions to elucidate the chemical structures of the antifungal metabolites.

Antifungal activities. Each metabolite produced by the three *V. biguttatum* isolates was bulked and redissolved in HPLC grade MeOH to provide stock solutions (2 mg ml^{-1}). As a preliminary step, antifungal activity against *F. fulva* was estimated by TLC bioautography. The minimum inhibitory concentration (MIC) of each metabolite was then determined against mycelial inoculum of *P. lycopersici*, *F. oxysporum* f. sp. *narcissi*, *B. cinera*, *R. solani* AG-3, *F. fulva* and *V. biguttatum* isolate vb1 on PDA blocks and expressed as μg metabolite ml^{-1} PDA. PDA (32 ml) was poured into pre-weighed dishes, allowed to dry for 1 day, weighed and its average density calculated from 10 replicate dishes. Blocks (1 cm diameter) were cut from these dishes of PDA and placed in pre-weighed dishes (5 per dish), weighed and the average weight of the blocks calculated from 30 replicate dishes of blocks. The average volume of each block was then calculated. Stock solutions (5–100 μl containing 10–200 μg of metabolite) were applied to PDA blocks and allowed to dry for 30 min. HPLC grade MeOH (100 μl) was applied to control blocks. Blocks were inoculated with mycelium (1 mm^3) cut from the actively growing edge of test fungus PDA cultures and incubated at 25° in darkness in sealed plastic bags until control blocks were completely colonized.

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