Optimizing Production and Evaluating Biosynthesis *in Situ* of a Herbicidal Compound, Mevalocidin, from *Coniolariella* Sp.

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Vincent P. Sica, Mario Figueroa, Huzefa A. Raja, Tamam El-Elimat, Blaise A. Darveaux, Cedric J. Pearce, and Nicholas H. Oberlies. (2016). Optimizing Production and Evaluating Biosynthesis *in Situ* of a Herbicidal Compound, Mevalocidin, from *Coniolariella* Sp. *Journal of Industrial Microbiology & Biotechnology,* 43(8): 1149-1157.

This is a post-peer-review, pre-copyedit version of an article published in *Journal of Industrial Microbiology & Biotechnology*. The final authenticated version is available online at: http://dx.doi.org/10.1007/s10295-016-1782-2

Abstract:

Mevalocidin is a fungal secondary metabolite produced by *Coniolariella* sp. It is a unique phytotoxin that demonstrates broad spectrum post-emergent herbicidal properties. With limited options for weed control, the commercialization of a natural product pesticide would be beneficial to organic farming. In this study, two mevalocidin-producing fungal strains, coded MSX56446 and MSX92917, were explored under a variety of growth conditions, including time, temperature, and media. The concentration of mevalocidin was quantitatively measured via LC–MS to determine the optimal setting for each condition. Maximum production was achieved for each condition at 20 days, at 30 °C, with YESD + agar, and with a media containing 2.5 % dextrose. Furthermore, an advanced surface sampling technique was incorporated to gain a better understanding of the fungal culture's natural ability to biosynthesize and distribute this herbicide into its environment. It was shown that both fungi actively exude mevalocidin into their environment via liquid droplet formations known as guttates.

Keywords: Fungal secondary metabolites | Herbicide | Droplet-based liquid extraction | Spatial distribution

Article:

Introduction

Organic farming has seen exponential growth over the past decade. According to the USDA, certified organic acreage in 2008 was over 2.6 million in the US alone [25], and global organic

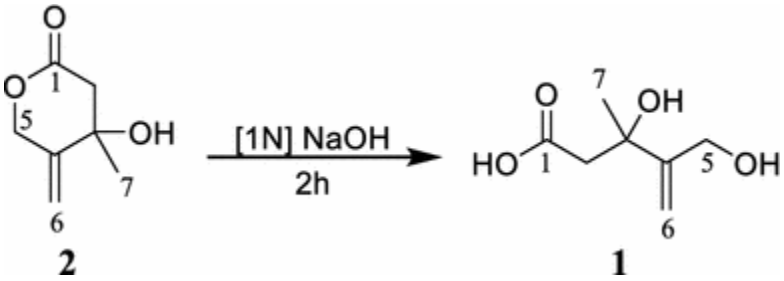
Abbreviations: *ITS*, Internal transcribed spacer; *Droplet–LMJ–SSP*, Droplet liquid microjunction surface sampling probe

sales reached $54.9 billion in 2009 [37]. With these numbers steadily increasing, the need for a natural product herbicide has grown as well. The use of herbicides for organic farming is extremely restricted [25], leaving few options available for weed control, other than manual labor. No new, unaltered natural product herbicides or farming bioherbicides have been introduced to the market in over 15 years [3].

Natural products have yielded some herbicides, but they have typically required synthetic modifications to create an effective product. Phytotoxic allelochemicals produced by microorganisms make up one class of commercialized natural product herbicides [17]. The tripeptide, bialaphos, was isolated originally from *Streptomyces hygroscopicus* [3]. Introduced to the market (Japan) in 1984 as a post-emergent herbicide, bialaphos is not phytotoxic itself, but rather becomes phytotoxic when the targeted plant converts it metabolically to phosphinothricin [5]. Synthetically, phosphinothricin is made into an ammonium salt, termed glufosinate. While bialaphos is marketed as an herbicide in Japan (as Herbiace), only glufosinate is commercially available globally (i.e., Basta, Ignite, and Liberty). Triketones, another class of naturally derived herbicides [3], were discovered in 1977 via the isolation of leptospermone from *Callistermon citrinus*. Various derivatives were synthesized to optimize the herbicidal properties, yielding the commercialized products mesotrione (Callisto), topramezone (Armezon), and tembotrione (Laudis). However, only the derivatives were found to be viable herbicides, thus eliminating the potential application towards organic farming.

From an organic farming perspective, the post-emergent natural product herbicides that are available lack in many areas. Aqueous acetic acid (20 % v/v), sold as horticultural vinegar, often requires continual treatment to be effective and is typically used on non-cropland areas due to its burn down, non-selective method of weed management [38]. Fatty acids have been used, as well, but they also require repeat treatment, since there is no residual activity after the initial burn down effect [6]. Other natural products that are similarly ineffective include cornmeal with clove oil [19], lemongrass oil, and d-limonene [30].

Dow AgroSciences in collaboration with Mycosynthetix discovered mevalocidin [13] (Scheme 1) from two, now patented [14], fungal strains, MSX56446 and MSX92917, originally identified as *Fusarium* sp. and *Rosellinia* sp., respectively, using only the D2 region of the nuclear large subunit (nLSU/28S), which was approximately 320 bp; the identification of these organisms has been revised using both the internal transcribed spacer region of the rRNA gene (ITS) and the D1/D2 region. Mevalocidin (**1**) has shown promise in filling the need as an effective herbicide for organic farming, since it is produced naturally from fungi. It has displayed strong post-emergent activity with greater than 50 % injury to all of the broadleaf and grass species tested at 4 kg/ha after 16 days and lethality after 21 days [13]. Perhaps more importantly, mevalocidin displays the rare, yet desirable, attribute of being phloem and xylem mobile, allowing distribution throughout the plant, including the meristems. Hence, this study determined the growth conditions for optimal mevalocidin production in support of efforts to commercialize these fungal strains as organic bioherbicides for the farming industry. Furthermore, mass spectrometry mapping experiments were employed to examine the potential ecological role of mevalocidin in these fungi.



Scheme 1. Conversion of methylidene mevalonolactone (**2**) to mevalocidin (**1**)

Materials and methods

Fungal strain and fermentation

Mycosynthetix fungal strain MSX92917 (*Coniolariella* sp.; Figs. S1 and S2, Supporting Information) was isolated from twigs and wood collected in 1998, while fungal strain MSX56446 (*Coniolariella* sp.; Fig. S1 and S2) was isolated from oak and sweetgum leaf litter. The strains were deposited at the NRRL culture collection of the National Center for Agricultural Utilization Research (Peoria, IL) under the accession numbers NRRL 30882 (for DA056446/MSX56446) and NRRL 30883 (DA092917/MSX92917) [13], respectively. A fresh culture of each isolate was grown on a malt extract slant, and a piece was transferred to a medium containing 2 % soy peptone, 2 % dextrose, and 1 % yeast extract (YESD media). After incubation at 22 °C for 7 days (with agitation), the culture was used to inoculate 50 mL of rice medium [containing rice, vitamin solution, and water (twice the volume of rice)] in a 250-mL Erlenmeyer flask. The cultures were incubated at 22 °C until sufficient fungal growth (~14 days) was observed. The scaled-up cultures were grown in a 2.8-L Fernbach flask containing 150 g of rice and 300 mL of H_2O and inoculated using a seed culture grown in YESD medium followed by incubation at 22 °C for 14 days. For the time variable experiments, the growths were stopped at the indicated time points by freezing the entire culture prior to processing in parallel. For the temperature variable experiments, the cultures were grown at either 25, 30, or 35 °C for 14 days. For the comparison of the liquid vs solid YESD media experiments, the above YESD formula was used in both cases, with the addition of 1.5 % agar for the latter; in both cases, rice was not added. For the experiments where the amount of dextrose was varied, each fungal strain was inoculated in 50 mL of medium containing 1 % malt extract and 1 % agar with incremental amounts of dextrose (Table 2) and grown at 22 °C.

Molecular identification of the fungal strains

For molecular identification and phylogenetic analysis of the fungal strains employed in this study, the ITS region was sequenced using primers ITS5 and ITS4 [12, 36] and PCR amplification methods outlined previously [8, 26]. The ITS region has been proposed as a barcoding marker for members of the kingdom fungi [29]. Two portions of ribosomal genes were sequenced to get an indication of where the strains belonged using BLAST search. Since there were no other genes sequenced (e.g., beta tubulin) for *Coniolariella* in NCBI GenBank, our phylogenetic analyses relied on ribosomal genes (ITS + LSU) for identification purposes. After obtaining the complete ITS region for both the strains MSX56446 and MSX92917, the

individual ITS sequences were aligned using MUSCLE, which was implemented in the program Seaview [15]. The sequences from the two strains were then analyzed with PAUP* 4.0b10 [33] to calculate the uncorrected *p* distances. *p* distance compares the two sequences by calculating the proportion of nucleotide sites at which the sequences are different; uncorrected *p* distances can be obtained by dividing the number of nucleotides by the total number of nucleotides in the sequences being compared.

A BLAST search was implemented in GenBank with the ITS sequences obtained from the two stains. Sequences from the top BLAST search were downloaded and analyzed using PHYML [16] to obtain the closest phylogenetic disposition for the MSX strains. BLAST search was employed using nucleotide collection (nr/nt) with uncultured/environmental samples sequences excluded. The BLAST search was also performed with RefSeq database [28]. In addition, for one of the strains (MSX56446), a portion of the D1/D2 region of the large subunit, 28SrDNA (LSU) was also sequenced using primers LROR and LR3 [27]. The LSU sequences of other *Coniolariella* spp. from a recently published study on this genus [4] were downloaded and analyzed using PHYML. The sequence data obtained for both the MSX strains were deposited in the GenBank (MSX56446: KT835371; MSX92917: KT835372).

Extraction and isolation

The initial processing of fungal cultures was derived from well-established procedures (Fig. S3) [1, 34]. To each flask of the fungus grown on specific media, 80 mL of $CHCl_3$, 20 mL of MeOH, and 100 mL of deionized H_2O, adjusted to pH ~ 3 with formic acid were added. The samples were mixed/chopped with a large spatula before being shaken overnight (~16 h) at room temperature. Then, the samples were filtered in vacuo, and the eluents were transferred to separatory funnels. The aqueous phases (~100 mL) were partitioned against water-saturated *n*-butanol (2 × 100 mL). The separated layers were dried.

The resultant aqueous and *n*-butanol fractions were dissolved in H_2O and H_2O/MeOH (2:1 v/v), respectively, and purified via reverse phase flash chromatography (C18) using a gradient solvent system of H_2O–MeOH at a 64 mL/min flow rate and 12.5 column volumes over 25.3 min to afford several fractions. These fractions were dried, and the presence of methylidene mevalonolactone (**2**) was detected via NMR. $CHCl_3$ was added to the identified fractions; compound **2** is soluble in this organic solvent, facilitating its isolation (>98 % pure; Scheme 1). Of the two, the *n*-butanol fraction had a higher relative concentration of the target compounds, and this facilitated the chromatographic purification step, particularly for gram-scale quantities needed for this stage of development.

Conversion of compound **2** to compound **1**

To a vial containing **2** (11.44 mg, 0.08 mmol), 1 N NaOH (80.4 µL, 0.08 mmol) and deionized H_2O (0.7 mL) were added and stirred with a magnetic stir bar for 2 h at 25 °C [13]. The product was dried to afford **1** (Scheme 1).

Quantification

Calibration curves (Table 1) were prepared using standards isolated from fungal strain MSX92917. Identification of both **1** and **2** was performed by NMR (Fig. S4) (JEOL ECS 400 MHz) and HRMS (Fig. S5) (Thermo LTQ Orbitrap XL) analyses. Both compounds were isolated and purified (ISCO flash chromatography), and UPLC-HRMS was used to verify the purity (>98 %). These standards of **1** and **2** were dissolved in deionized H_2O or MeOH, respectively, and analyzed 0.5–75 ppm for **1** and from 0.5 to 50 ppm for **2** (Table 1). Aqueous and *n*-butanol dried fractions were dissolved in deionized H_2O or MeOH, respectively, to a concentration of 1 mg/mL.

Table 1. The calibration curve data used for the quantitative analysis of mevalocidin (**1**) and methylidene mevalonolactone (**2**)

	(1)	**(2)**
Retention time (min)	3.20	3.68
Observed mass	161.08057	143.06972
Calculated mass	161.08084	143.07027
Linear equation	$y = 3078692x - 1500577$	$y = 61765810x + 138182908$
R^2	0.999	0.995
Range (ppm)	0.5–75	0.5–50

A UPLC Acquity system equipped with a BEH C18 (1.7 mm; 50 × 2.1 mm) column heated to 40 °C was run at a flow rate of 0.2 mL/min with a solvent system of CH_3CN–H_2O (0.1 % formic acid) (0–1.0 min, 0:100; 1.0–6.0 min, from 0:100 to 25:75; 6.0–6.1 min, from 25–75 to 100 % CH_3CN and hold for 1 min; 7.0–7.1 min, return to initial conditions and hold for 1 min). PDA (190–400 nm) and HRMS (Thermo LTQ Orbitrap XL system) were used to analyze the samples. Compound **1** eluted at 3.20 min and compound **2** eluted at 3.68 min. The standards of **1** and **2** were run in triplicate and the areas under the curves were calculated to create the calibration curve. Similarly, extracts were run in triplicate, the areas were averaged, and the concentrations of **1** and **2** were extrapolated from the calibration curve.

Surface sampling

A droplet-liquid microjunction-surface sampling probe (droplet–LMJ–SSP) was converted from a CTC/LEAP HTC PAL auto-sampler (LEAP Technologies Inc.) using in-house developed software dropletProbe Premium [20, 21, 22, 23, 24]. Microextractions were performed using Fisher Optima LC/MS grade H_2O. An initial 5 µL of H_2O was drawn into the syringe and 4 µL droplets were dispensed onto the surface of the sample at a rate of 2 µL/s, held on the surface for 2 s, and withdrawn back into the syringe at the same rate. This extraction process was repeated a total of three times using the same droplet, then injected in a UPLC-MS system using the same chromatographic conditions as above except into a Thermo Q Exactive Plus mass spectrometer. Heat mapping experiments showed the relative concentration of mevalocidin (**1**) at a particular location directly on the fungal culture. The area was calculated for the chromatographic peak of **1** (*m/z* 161.0808 ± 5 ppm) at 3.53 min. Retention times are shift compared to those for the quantitative study due to the incorporation of the droplet–LMJ–SSP.

Results and discussion

Molecular identification of the fungal strains

Results from uncorrected *p* distance using ITS data suggested that the two MSX strains were 99 % identical. Based on a BLAST search of NCBIs GenBank nucleotide database, the closest hits using the ITS sequence were *Coniolariella limonispora* (basionym: *Rosellinia limonispora*) (strain CBS 283.64) (GenBank KF71998; identities = 502/518 (97 %), gaps = 8/518 (1 %), followed by *Coniolariella hispanica* (strain ATCC MYA-4453)] [GenBank FJ172294; identities = 503/524 (96 %), gaps = 10/524 (1 %)].

Maximum likelihood analysis of the ITS and LSU regions indicated that the MSX strains are phylogenetically related to the genus *Coniolariella* D. García, Stchigel & Guarro [11]. The two strains were clustered within the clade containing most species of *Coniolariella* [4, 39] including the type species, *C. gamsii* (Asgari & Zare) Garcia, Stchigel & Guarro (Fig. S1, S2). Based on both ITS BLAST search as well as ML analysis using portions of ITS and LSU regions, the MSX strains were identified as belonging to *Coniolariella* (Xylariales, Ascomycota). In an earlier study, Gerwick et al. [13] identified MSX56446 (which they termed as DA056446) as *Fusarium* and MSX92917 (which they termed as DA092917) as *Rosellinia* sp, respectively. We compared the D2 region of GenBank accession no: KF698738 for DA092917, and it matched the D2 region of MSX56446 with 100 % identity. Thus, it is likely that both the strains MSX92917 and MSX56446 are congeneric (Fig. S2), but Gerwick et al. used a different name due to erroneous interpretation of BLAST search data, especially since Coniolariella shows a close phylogenetic relationship with *Rosellinia*-like genera within the Xylariales [4].

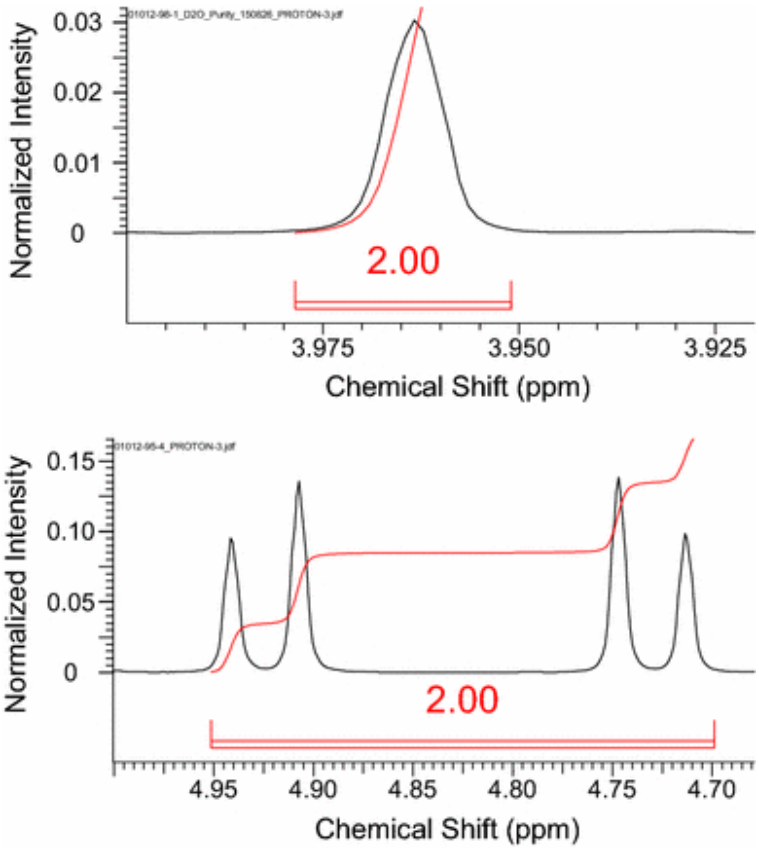


Fig. 1 The zoomed in NMR spectra for the H-5 signals of mevalocidin (*top* D_2O) and methylidene mevalonolactone (*bottom* $CDCl_3$) display the identifying signals for each compound

Purification protocol

Due to its H_2O solubility, mevalocidin (**1**) was difficult to isolate directly using the traditional protocols common for natural products discovery [7, 9]. To overcome this, the isohypsic properties of the open and closed forms were exploited. After drying the reverse phase flash chromatography fractions, they were screened for methylidene mevalonolactone (**2**) using NMR. Compounds **1** and **2** can be clearly distinguished from each other through ^{1}H NMR (Fig. S4). Due to the rigidity of the lactone, the proton signals are coupled at the C-5 position resulting in a very distinct differentiation between the two compounds (Fig. 1). Furthermore, isolation of compound **2** was readily achieved by dissolving the chromatography fractions in $CDCl_3$. Once isolated, compound **2** was opened to **1** by introducing a base. This methodology was utilized for the isolation of the standards for the quantitation study.

Mevalocidin production optimization

Fungal strains MSX92917 and MSX56446 were tested for their viability for producing these compounds as bioherbicides for organic farming. Large-scale rice cultures were extracted using the traditional protocol incorporating $CHCl_3$, MeOH, and H_2O [1, 34]; however, partitioning the aqueous layer with *n*-butanol was performed to concentrate mevalocidin and remove it from the abundance of sugars present in the aqueous layer. While both layers contained significant proportions of **1** and **2** (Table 2), the more concentrated *n*-butanol fraction was more amenable for the gram quantity isolation of each compound needed for future testing. The productions of compounds **1** and **2** were compared for both cultures, ultimately determining the yields to be higher in MSX92917 by approximately a factor of two (Table 2). Therefore, subsequent analyses were performed only on MSX92917.

To optimize mevalocidin production, the fungus was subjected to a series of altering conditions, including media nutrient levels, media phase (liquid or solid), growth time, and incubation temperature. First, the nutrition levels of the media were examined to understand if production of mevalocidin would change as the sugar levels in the media changed. Incremental amounts of dextrose (0–12.5 %) were added to liquid media, since dextrose was shown to be readily utilized; it was also an inexpensive carbon and energy source. The yields of **1** and **2** were highest at 2.5 % dextrose (Table 2). This was evident when combining the calculated mass from both the *n*-butanol and aqueous partitions.

Solid and liquid media were compared by growing the fungal strain in two separate flasks of the same medium, but one with agar (solid) and the other without (liquid). The presence of agar displayed an increase in production for both compounds (Table 2).

Optimization of the growth time was performed by comparing the production over increments of 5 days before freezing. The combined calculated masses of **1** and **2** increased steadily until 20 days (Table 2). After 20 days, the rate of mevalocidin production began to slow; therefore, approximately 20 days was considered the preferred growth time for commercial production.

Additionally, the temperature in which the fungus was grown had an effect (Table 2). Cultures of MSX92917 were grown on rice, but the temperature for each culture varied (25, 30, or 35 °C). The cultures at 30 °C showed the highest production of **1** and **2** with a significant drop-off at 35 °C (Table 2).

Table 2. The concentrations of mevalocidin (**1**) and methylidene mevalonolactone (**2**) for each partition (*n*-butanol and aqueous) were determined by the LC–MS calibration curve

Fungal code	**1 (mg)[a]**			**2 (mg)[a]**			**Combined total (mg)[b]**	**Concentration for commercial production (mg/g)[c,d]**
	***n*-Butanol**	**Aqueous**	**Total 1**	***n*-Butanol**	**Aqueous**	**Total 2**		
MSX92917	28.48 ± 2.97	147.81 ± 8.51	**176.29**	48.40 ± 4.51	110.06 ± 4.52	**158.46**	**334.75**	**2.2**
MSX56446	29.11 ± 3.85	38.92 ± 6.83	68.03	58.36 ± 2.24	34.61 ± 6.18	92.97	161.00	1.1
MSX92917								
Dextrose (%)								
0.0	0.13 ± 0.01	0.84 ± 0.07	0.97	0.51 ± 0.02	0.42 ± 0.02	0.93	1.90	0.16
2.5	0.48 ± 0.03	3.43 ± 0.30	**3.90**	2.41 ± 0.08	2.42 ± 0.01	**4.83**	**8.74**	**0.73**
5.0	0.71 ± 0.03	1.54 ± 0.07	2.25	3.14 ± 0.07	1.43 ± 0.10	4.57	6.82	0.57
7.5	0.45 ± 0.05	2.37 ± 0.18	2.82	2.47 ± 0.12	1.72 ± 0.09	4.19	7.01	0.58
10.0	0.49 ± 0.05	2.65 ± 0.05	3.14	2.51 ± 0.03	0.65 ± 0.08[e]	3.16	6.30	0.53
12.5	0.59 ± 0.05	3.17 ± 0.06	3.76	1.71 ± 0.17	1.85 ± 0.18	3.56	7.32	0.61
Media phase								
YESD	0.06 ± 0.00	0.49 ± 0.11	0.55	0.01 ± 0.00[e]	0.04 ± 0.05[e]	0.05	0.60	0.050
YESD + 1.5 % agar	0.06 ± 0.00	0.64 ± 0.04	**0.70**	0.04 ± 0.00	0.20 ± 0.03[e]	**0.24**	**0.94**	**0.078**
Grow length (days)								
5	0.12 ± 0.01	1.71 ± 0.07	1.83	0.06 ± 0.00	0.11 ± 0.03[e]	0.17	2.00	0.17
10	0.34 ± 0.05	2.66 ± 0.16	3.00	0.98 ± 0.10	0.07 ± 0.07[e]	1.05	4.05	0.34
15	1.02 ± 0.11	1.66 ± 0.44	2.68	4.45 ± 0.13	0.51 ± 0.22[e]	4.95	7.64	0.64
20	1.56 ± 0.09	3.10 ± 0.11	4.66	4.37 ± 0.34	0.98 ± 0.27	**5.35**	10.01	0.83
25	1.19 ± 0.10	2.73 ± 0.36	3.92	3.66 ± 0.03	0.55 ± 0.14[e]	4.22	8.14	0.68
30	2.32 ± 0.28	6.14 ± 0.41	**8.46**	2.84 ± 0.51	1.36 ± 0.10	4.20	**12.67**	**1.1**
Temperature (°C)								
25	2.09 ± 0.14	4.83 ± 0.87	6.92	3.69 ± 0.02	3.52 ± 0.72	7.21	14.13	1.2
30	2.83 ± 0.17	5.69 ± 0.32	**8.52**	4.74 ± 0.31	4.07 ± 0.36	**8.81**	**17.33**	**1.4**
35	0.08 ± 0.00	1.49 ± 0.04	1.58	0.01 ± 0.00[e]	0.22 ± 0.04[e]	0.23	1.81	0.15

The concentrations of **1** and **2** obtained from the quantitation study were converted from ppm to mg and then combined as a total mass. This was then subsequently converted to (mg of total compound)/(g of rice) to effectively represent the optimal production conditions. Bold indicate the highest total for each individual study

[a][Conc of mevalocidin] × total volume of extract = mass of mevalocidin in extract $\frac{\text{ug}}{\text{mL}}\frac{\text{mL}}{1}\frac{\text{mg}}{1000\text{ug}} = \frac{\text{mg}}{\text{extract}}$

[b]Combined total is to account for any potential shift in equilibrium between compounds **1** and **2** during the processing steps

[c]Based on large-scale 2.8-L Fernbach cultures (150 g of rice)

[d]Based on small-scale screener cultures (12 g of rice)

[e]Indicates that concentrations were above limit of detection but below limit of quantitation

As mevalocidin progresses into commercial development, further optimization may be required. While all of these data are based on single growths, the goal of these experiments was to gauge relative trends, so that we could choose the best culture conditions. This may include a combination of the above conditions, as well as, probing additional conditions, such as the

FERMEX methods [2], particularly for kg or larger-scale production. Studies on the efficacy of mevalocidin as an organic herbicide are ongoing, as well.

Probing biosynthetic queries

It was originally unknown whether the fungi were producing both mevalocidin (**1**) and its lactone form or if the lactone was formed due to the use of acid through the extraction/isolation process. To address this, a droplet–LMJ–SSP coupled to a UPLC-HRMS system [21, 22, 23] was utilized to sample a solid-state rice culture of MSX92917. This system has recently been shown to be a viable tool for sampling the chemistry of fungal cultures in situ [31]. When sampling fungal cultures MSX56446 and MSX92917, compounds **1** and **2** were detected readily off the surface; the HRMS and retention times were confirmed by comparison with standards of **1** and **2** (Fig. S6). Furthermore, if any of the mevalocidin was being converted during the UPLC method, it would have been observed in the analysis of the standard as well. This supports the premise that the fungus produces both **1** and **2**, at least under these conditions.

Additionally, many questions regarding the biosynthesis of a fungal metabolite with herbicidal properties abound, particularly in fungi that are presumed to be saprobes. For instance, does the fungus interact with its environment, perhaps using the herbicidal compound to kill plant materials, giving the next generation a source of nutrients? If so, where does the fungus store the compound and how does it release it into its surroundings? To explore these questions, both mevalocidin-producing cultures were grown on agar. Interestingly, it was observed that guttates (Fig. 2) were produced by both fungal isolates when grown in this manner. Guttates are liquid droplets that are produced by the fungi and are exuded out via the mycelium to the surface [18, 32]. Previously, it has been observed that bioactive secondary metabolites concentrate in such structures [10, 31, 35]. We hypothesized that they are a means for the fungus to interact with its surrounding habitat, as these are typically found upon the surface of the culture.

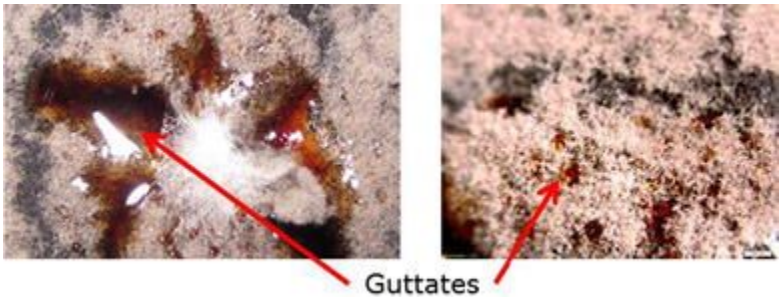


Fig. 2 Guttates (liquid droplets) residing on the surface of fungal culture MSX56446

To gain a better understanding of the biological and ecological purpose of these guttates, the cultures were analyzed by the droplet–LMJ–SSP coupled to an UPLC-HRMS. For both fungal cultures grown on agar, the mycelium, the guttates, and the surrounding agar were all explored in search for the molecular ion of mevalocidin (*m/z* 161.0808 ± 5 ppm). It was observed in both cultures that the guttates contained the highest concentrations of mevalocidin relative to the surrounding mycelium, as noted by the size of the blue circles in the heat map (Fig. 3). Likewise, more mevalocidin was detected in the surrounding agar than directly on the surface of the mycelium. This indicated that mevalocidin was actively exuded out of the fungi into its surroundings. This supports the postulate that guttates are concentrated droplets of secondary

metabolites and that the fungi are using the herbicidal compound to potentially kill plants in its environment. Importantly, the use of the droplet–LMJ–SSP allowed us to probe the chemistry of the guttates, a feat that would have been impossible with traditional natural product protocols [31].

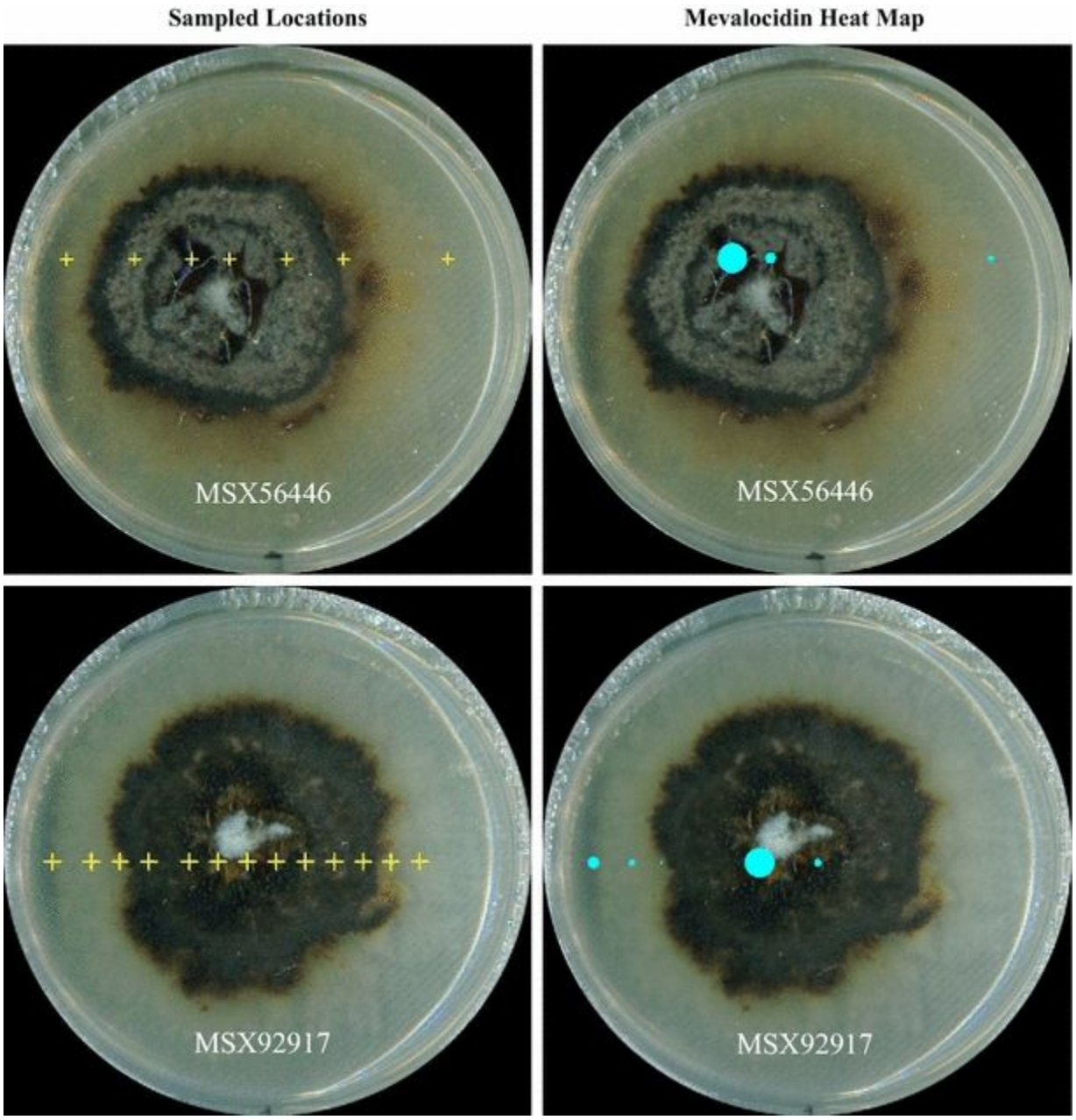


Fig. 3 Heat map displaying the presence of mevalocidin (**1**) at various locations. The *two images* on the *left* indicate the locations that were sampled with the droplet–LMJ–SSP. The *two images* on the *right* show the heat map. *Larger circles* indicate higher concentrations of mevalocidin at that location

In conclusion, mevalocidin had previously displayed promise as an organic herbicide [13]. These experiments were pursued to optimize conditions for mevalocidin production in fungal strains

MSX92917 and MSX56446. Initial optimization studies argue that the best results could be realized on YESD agar medium containing 2.5 % dextrose, incubated at 30 °C for at least 20 days; ongoing studies are probing this further, with an aim towards commercial scale production. The conversions between **1** and **2** are prime examples of applying basic chemistry to solve isolation challenges. These results are currently being utilized to generate **1** for field trials as an organic bioherbicide. Furthermore, the data obtained from the droplet–LMJ–SSP demonstrate that the fungi are biosynthesizing both compounds **1** and **2**. The spatial distribution mapping experiments displayed valuable information about the production of mevalocidin, where the concentration of **1** in the guttates indicates an ecological use. Extruding this compound from the body of the fungus into the environment supports the hypothesis that the fungus is using mevalocidin to kill plants, which it can then decompose in its role as a saprobe.

Acknowledgments

This research was supported in part by a Grant from the US Department of Agriculture (NIFA 2012-33610-19523). The authors thank T. N. Graf of UNCG for assistance in isolation method development and Drs. Vilmos Kertesz and Gary J. Van Berkel (Mass Spectrometry and Laser Spectroscopy Group, Chemical Sciences Division, Oak Ridge National Laboratory) for inspiration and guidance with the droplet-LMJ-SSP.

References

1. Ayers S, Graf TN, Adcock AF, Kroll DJ, Matthew S, Carcache de Blanco EJ, Shen Q, Swanson SM, Wani MC, Pearce CJ, Oberlies NH (2011) Resorcylic acid lactones with cytotoxic and NF-kappaB inhibitory activities and their structure–activity relationships. J Nat Prod 74:1126–1131

2. Bills GF, Dombrowski AW, Goetz MA (2012) The "FERMEX" method for metabolite-enriched fungal extracts. Methods Mol Biol 944:79–96

3. Cantrell CL, Dayan FE, Duke SO (2012) Natural products as sources for new pesticides. J Nat Prod 75:1231–1242

4. Checa J, Arenal F, Blanco N, Rogers JD (2008) *Coniolariella hispanica* sp. nov. and other additions to *Coniolariella*. Mycol Res 112:795–801

5. Copping LG, Duke SO (2007) Natural products that have been used commercially as crop protection agents. Pest Manag Sci 63:524–554

6. Dayan FE, Cantrell CL, Duke SO (2009) Natural products in crop protection. Bioorg Med Chem 17:4022–4034

7. El-Elimat T, Raja HA, Day CS, Chen WL, Swanson SM, Oberlies NH (2014) Greensporones: resorcylic acid lactones from an aquatic *halenospora* sp. J Nat Prod 77:2088–2098

8. El-Elimat T, Raja HA, Graf TN, Faeth SH, Cech NB, Oberlies NH (2014) Flavonolignans from *Aspergillus iizukae*, a fungal endophyte of milk thistle (*Silybum marianum*). J Nat Prod 77:193–199

9. Figueroa M, Graf TN, Ayers S, Adcock AF, Kroll DJ, Yang J, Swanson SM, Munoz-Acuna U, de Blanco EJC, Agrawal R, Wani MC, Darveaux BA, Pearce CJ, Oberlies NH (2012) Cytotoxic epipolythiodioxopiperazine alkaloids from filamentous fungi of the Bionectriaceae. J Antibiot 65:559–564

10. Figueroa M, Jarmusch AK, Raja HA, El-Elimat T, Kavanaugh JS, Horswill AR, Cooks RG, Cech NB, Oberlies NH (2014) Polyhydroxyanthraquinones as quorum sensing inhibitors from the guttates of *Penicillium restrictum* and their analysis by desorption electrospray ionization mass spectrometry. J Nat Prod 77:1351–1358

11. Garcia D, Stchigel AM, Cano J, Calduch M, Hawksworth DL, Guarro J (2006) Molecular phylogeny of Coniochaetales. Mycol Res 110:1271–1289

12. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

13. Gerwick BC, Brewster WK, deBoer GJ, Fields SC, Graupner PR, Hahn DR, Pearce CJ, Schmitzer PR, Webster JD (2013) Mevalocidin: a novel, phloem mobile phytotoxin from *Fusarium* DA056446 and *Rosellinia* DA092917. J Chem Ecol 39:253–261

14. Gerwick BC, Graupner PR, Fields SC, Schmitzer PR, Brewster WK (2008) Methylidene mevalonates and their use as herbicides. US 7,393,812 B2

15. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27:221–224

16. Guindon S, Delsuc F, Dufayard JF, Gascuel O (2009) Estimating maximum likelihood phylogenies with PhyML. Methods Mol Biol 537:113–137

17. Hoagland RE (2001) Microbial allelochemicals and pathogens as bioherbicidal agents. Weed Technol 15:835–857

18. Hutwimmer S, Wang H, Strasser H, Burgstaller W (2010) Formation of exudate droplets by *Metarhizium anisopliae* and the presence of destruxins. Mycologia 102:1–10

19. Johnson WC, Boudreau MA, Davis JW (2013) Combinations of corn gluten meal, clove oil, and sweep cultivation are ineffective for weed control in organic peanut production. Weed Technol 27:417–421

20. Kertesz V, Paranthaman N, Moench P, Catoire A, Flarakos J, Van Berkel GJ (2014) Liquid microjunction surface sampling of acetaminophen, terfenadine and their metabolites in thin tissue sections. Bioanalysis 6:2599–2606

21. Kertesz V, Van Berkel GJ (2010) Liquid microjunction surface sampling coupled with high-pressure liquid chromatography-electrospray ionization-mass spectrometry for analysis of drugs and metabolites in whole-body thin tissue sections. Anal Chem 82:5917–5921

22. Kertesz V, Van Berkel GJ (2013) Automated liquid microjunction surface sampling-HPLC-MS/MS analysis of drugs and metabolites in whole-body thin tissue sections. Bioanalysis 5:819–826

23. Kertesz V, Van Berkel GJ (2014) Sampling reliability, spatial resolution, spatial precision, and extraction efficiency in droplet-based liquid microjunction surface sampling. Rapid Commun Mass Spectrom 28:1553–1560

24. Kertesz V, Weiskittel TM, Van Berkel GJ (2015) An enhanced droplet-based liquid microjunction surface sampling system coupled with HPLC-ESI-MS/MS for spatially resolved analysis. Anal Bioanal Chem 407:2117–2125

25. Oberholtzer L, Dunutru C (2009) Marketing US organic foods: recent trends from farms to consumer. Economic information bulletin, 58. USDA, Washington

26. Raja HA, Oberlies NH, Figueroa M, Tanaka K, Hirayama K, Hashimoto A, Miller AN, Zelski SE, Shearer CA (2013) Freshwater ascomycetes: *Minutisphaera* (Dothideomycetes) revisited, including one new species from Japan. Mycologia 105:959–976

27. Rehner SA, Samuels GJ (1995) Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Can J Bot 73:816–823

28. Schoch CL, Robbertse B, Robert V, Vu D, Cardinali G, Irinyi L, Meyer W, Nilsson RH, Hughes K, Miller AN, Kirk PM, Abarenkov K, Aime MC, Ariyawansa HA, Bidartondo M, Boekhout T, Buyck B, Cai Q, Chen J, Crespo A, Crous PW, Damm U, De Beer ZW, Dentinger BT, Divakar PK, Duenas M, Feau N, Fliegerova K, Garcia MA, Ge ZW, Griffith GW, Groenewald JZ, Groenewald M, Grube M, Gryzenhout M, Gueidan C, Guo L, Hambleton S, Hamelin R, Hansen K, Hofstetter V, Hong SB, Houbraken J, Hyde KD, Inderbitzin P, Johnston PR, Karunarathna SC, Koljalg U, Kovacs GM, Kraichak E, Krizsan K, Kurtzman CP, Larsson KH, Leavitt S, Letcher PM, Liimatainen K, Liu JK, Lodge DJ, Luangsa-ard JJ, Lumbsch HT, Maharachchikumbura SS, Manamgoda D, Martin MP, Minnis AM, Moncalvo JM, Mule G, Nakasone KK, Niskanen T, Olariaga I, Papp T, Petkovits T, Pino-Bodas R, Powell MJ, Raja HA, Redecker D, Sarmiento-Ramirez JM, Seifert KA, Shrestha B, Stenroos S, Stielow B, Suh SO, Tanaka K, Tedersoo L, Telleria MT, Udayanga D, Untereiner WA, Dieguez Uribeondo J, Subbarao KV, Vagvolgyi C, Visagie C, Voigt K, Walker DM, Weir BS, Weiss M, Wijayawardene NN, Wingfield MJ, Xu JP, Yang ZL, Zhang N, Zhuang WY, Federhen S (2014) Finding needles in haystacks: linking scientific names, reference specimens and molecular data for fungi. Database (Oxford) 2014

29. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, Miller AN, Wingfield MJ, Aime MC, An KD, Bai FY,

Barreto RW, Begerow D, Bergeron MJ, Blackwell M, Boekhout T, Bogale M, Boonyuen N, Burgaz AR, Buyck B, Cai L, Cai Q, Cardinali G, Chaverri P, Coppins BJ, Crespo A, Cubas P, Cummings C, Damm U, de Beer ZW, de Hoog GS, Del-Prado R, B D, Dieguez-Uribeondo J, Divakar PK, Douglas B, Duenas M, Duong TA, Eberhardt U, Edwards JE, Elshahed MS, Fliegerova K, Furtado M, Garcia MA, Ge ZW, Griffith GW, Griffiths K, Groenewald JZ, Groenewald M, Grube M, Gryzenhout M, Guo LD, Hagen F, Hambleton S, Hamelin RC, Hansen K, Harrold P, Heller G, Herrera G, Hirayama K, Hirooka Y, Ho HM, Hoffmann K, Hofstetter V, Hognabba F, Hollingsworth PM, Hong SB, Hosaka K, Houbraken J, Hughes K, Huhtinen S, Hyde KD, James T, Johnson EM, Johnson JE, Johnston PR, Jones EB, Kelly LJ, Kirk PM, Knapp DG, Koljalg UGMK, Kurtzman CP, Landvik S, Leavitt SD, Liggenstoffer AS, Liimatainen K, Lombard L, Luangsa-Ard JJ, Lumbsch HT, Maganti H, Maharachchikumbura SS, Martin MP, May TW, McTaggart AR, Methven AS, Meyer W, Moncalvo JM, Mongkolsamrit S, Nagy LG, Nilsson RH, Niskanen T, Nyilasi I, Okada G, Okane I, Olariaga I, Otte J, Papp T, Park D, Petkovits T, Pino-Bodas R, Quaedvlieg W, Raja HA, Redecker D, T R, Ruibal C, Sarmiento-Ramirez JM, Schmitt I, Schussler A, Shearer C, Sotome K, Stefani FO, Stenroos S, Stielow B, Stockinger H, Suetrong S, Suh SO, Sung GH, Suzuki M, Tanaka K, Tedersoo L, Telleria MT, Tretter E, Untereiner WA, Urbina H, Vagvolgyi C, Vialle A, Vu TD, Walther G, Wang QM, Wang Y, Weir BS, Weiss M, White MM, Xu J, Yahr R, Yang ZL, Yurkov A, Zamora JC, Zhang N, Zhuang WY, Schindel D, Fungal Barcode Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci USA 109:6241–6246

30. Shrestha A, Moretti M, Mourad N (2012) Evaluation of thermal implements and crganic herbicides for weed control in a nonbearing almond (*Prunus dulcis*) orchard. Weed Technol 26:110–116

31. Sica VP, Raja HA, El-Elimat T, Kertesz V, Van Berkel GJ, Pearce CJ, Oberlies NH (2015) Dereplicating and spatial mapping of secondary metabolites from fungal cultures in situ. J Nat Prod 78:1926–1936

32. Singh S (2014) Guttation: quantification, microbiology and implications for phytopathology. In: Lüttge U, Beyschlag W, Cushman J (eds) Progress in botany, vol 75. Springer, Berlin, pp 187–214. doi: 10.1007/978-3-642-38797-5_7

33. Swofford DL (2002) PAUP*: phylogenetic analysis using Parsimony (* and other methods). Version 4. Sinauer Associates, Sunderland

34. Sy-Cordero AA, Graf TN, Adcock AF, Kroll DJ, Shen Q, Swanson SM, Wani MC, Pearce CJ, Oberlies NH (2011) Cyclodepsipeptides, sesquiterpenoids, and other cytotoxic metabolites from the filamentous fungus *Trichothecium* sp. (MSX 51320). J Nat Prod 74:2137–2142

35. Wang X, Sena Filho JG, Hoover AR, King JB, Ellis TK, Powell DR, Cichewicz RH (2010) Chemical epigenetics alters the secondary metabolite composition of guttate excreted by an atlantic-forest-soil-derived *Penicillium citreonigrum*. J Nat Prod 73:942–948

36. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc Guide Methods Appl 18:315–322

37. Willer H, Kilcher L (2011) The world of organic agriculture. Statistics and emerging trends 2011. IFOAM, Bonn

38. Young SL (2004) Natural product herbicides for control of annual vegetation along roadsides. Weed Technol 18:580–587

39. Zare R, Asgari B, Gams W (2010) The species of *Coniolariella*. Mycologia 102:1383–1388