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Modulation of volatile organic compound formation in the Mycodiesel-producing endophyte-*Hypoxylon* sp. CI-4

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An endophytic *Hypoxylon* sp. (strain CI-4) producing a wide spectrum of volatile organic compounds (VOCs), including 1,8-cineole, 1-methyl-1,4-cyclohexadiene and cyclohexane, 1,2,4-tris(methylene), was selected as a candidate for the modulation of VOC production. This was done in order to learn if the production of these and other VOCs can be affected by using agents that may modulate the epigenetics of the fungus. Many of the VOCs made by this organism are of interest because of their high energy densities and thus the potential they might have as Mycodiesel fuels. Strain CI-4 was exposed to the epigenetic modulators suberoylanilide hydroxamic acid (SAHA, a histone deacetylase) and 5-azacytidine (AZA, a DNA methyltransferase inhibitor). After these treatments the organism displayed striking cultural changes, including variations in pigmentation, growth rates and odour, in addition to significant differences in the bioactivities of the VOCs. The resulting variants were designated CI-4B, CI4-AZA and CI4-SAHA. GC/MS analyses of the VOCs produced by the variants showed considerable variation, with the emergence of several compounds not previously observed in the wild-type, particularly an array of tentatively identified terpenes such as α-thujene, sabinene, γ-terpinene, α-terpinolene and β-selinene, in addition to several primary and secondary alkanes, alkenes, organic acids and derivatives of benzene. Proton transfer reaction mass spectroscopic analyses showed a marked increase in the ratio of ethanol (mass 47) to the total mass of all other ionizable VOCs, from ~0.6 in the untreated strain CI-4 to ~0.8 in CI-4 grown in the presence of AZA. Strain CI4-B was created by exposure of the fungus to 100 μm SAHA; upon removal of the epigenetic modulator from the culture medium, it did not revert to the wild-type phenotype. Results of this study have implications for understanding why there may be a wide range of VOCs found in various isolates of this fungus in nature.

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INTRODUCTION

Fungi are one of the least explored taxonomic groups with regard to the extent of their biodiversity, biochemical properties and biotechnical promise. This is in spite of the fact that more than 1000 new species are characterized every year and thousands more await isolation and characterization (Heywood, 1995; Hawksworth, 2001). Thus, despite the incredible record of new taxonomic discoveries, the chemistry and utility of fungal products are largely unexplored. The potential of fungi is further limited by the presence of 'cryptic biosynthetic pathways' as indicated by the whole-genome sequencing and subsequent genome mining of several organisms (Bok *et al.*, 2006; Challis, 2008; Williams *et al.*, 2008).

One of the most effective ways to search for metabolically active fungi, producing a wide diversity of secondary metabolites, is to look for these organisms in unique ecological niches. One niche that is relatively unexplored is the living tissues of higher plants. The fungi living in these niches are known as endophytes. Endophytes produce a wide variety of secondary metabolites that may assist their adaptation and survival within higher plants (Strobel, 2006). The plant–microbe interaction may influence some of the otherwise cryptic biosynthetic pathways of endo-

Abbreviations: AZA, 5-azacytidine; PTR-MS, proton transfer mass spectrometry; SAHA, suberoylanilide hydroxamic acid; SPME, solid-phase micro-extraction; VOC, volatile organic compound.

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phytic fungi, resulting in the production of new molecules and overproduction of other secondary metabolites. Laboratory-based methods have been applied to express these latent pathways to produce new chemical entities; these include media engineering and optimization of growth conditions (Knappe *et al.*, 2008), co-culture (Schroeckh *et al.*, 2009), genetic manipulation (Bergmann *et al.*, 2007) and epigenetic modulation (Williams *et al.*, 2008; Henrikson *et al.*, 2009).

The complete analyses of fungal genomes in recent times indicate that many putative biosynthetic gene clusters are located in the distal regions of the chromosomes and exist in a heterochromatin state, with the constitutive genes often transcriptionally controlled by epigenetic regulation such as histone deacetylation and DNA methylation (Shwab *et al.*, 2007). Several studies indicate that the inhibition of histone deacetylase activity, through gene disruption or use of epigenetic modulators, leads to the transcriptional activation of gene clusters, resulting in enhanced production of secondary metabolites (Shwab *et al.*, 2007; Williams *et al.*, 2008).

In fungi, both class I and class II histone deacetylases, and lysine- as well as arginine-specific methyltransferases have been identified (Brosch *et al.*, 2008). The modification of histones via acetylation and methylation reactions can have important effects on the production of fungal secondary metabolites (Shwab *et al.*, 2007; Bok *et al.*, 2009). These modifications can induce heritable epigenetic changes (Mooibroek *et al.*, 1990; Birch *et al.*, 1998; Cheng *et al.*, 2003). In addition, fungi treated with DNA methyltransferase and histone deacetylase inhibitors exhibited natural product profiles with enhanced chemical diversity, demonstrating that these small-molecule epigenetic modifiers are effective tools for rationally controlling the native expression of fungal biosynthetic pathways and generating biomolecules not previously known from the organism (Williams *et al.*, 2008; Cichewicz, 2010).

Volatile organic compounds (VOCs) produced by microorganisms are regarded as important infochemicals in the biosphere which influence the dynamics of the ecosystem and vice versa (Wheatley, 2002). Microbial species produce consistent and reproducible VOC profiles under standard culture conditions. With the discovery of the Mycodiesel-producing organism, previously identified as *Gliocladium roseum* and subsequently reclassified as *Ascocoryne* sp. (Strobel *et al.*, 2008; Griffin *et al.*, 2010), and the knowledge acquired through the investigation of VOCs of *Muscodor* species (Strobel, 2006), it has become clear that fungi produce a wide array of VOCs with great potential for industrial and agricultural applications. These applications include alternative fuels, perfumery, biodegradation and decontamination of human and animal wastes, as well as post-harvest food processing (Daisy *et al.*, 2002; Ezra *et al.*, 2004a; Strobel, 2006). Fungi producing VOCs with biological activities and potential as fuels and fuel additives have been reported from several other fungal genera such as *Hypoxylon* (Tomsheck *et al.*, 2010), *Phomopsis* (Singh *et al.*, 2011) and *Phoma* (Strobel *et al.*, 2011).

Recently, a *Hypoxylon* sp. has been described as an endophyte of *Persea indica*, producing bioactive VOCs (Tomsheck *et al.* 2010). The possible use of these products as fuel additives increased our interest in this strain. Thus, initial work was undertaken to affect the gene expression of this organism via compounds known to act as epigenetic modifiers and to explore the possibility of the appearance of new products and/or the increased or decreased production of other VOCs already known from this organism. This study shows that treatment of this fungus with known epigenetic modulators can result in phenotypic changes as well as modifications in bioactivity and VOC profiles.

METHODS

Development of epigenetic variants. The wild-type *Hypoxylon* sp. used in this study had previously been found as an endophyte in *Persea indica* growing in the Laurisilva, Tenerife, Spain (Tomsheck *et al.*, 2010). The fungus, growing on potato dextrose agar (PDA), was hyphal tipped in order to obtain a genetically pure strain, CI-4. This strain was used for epigenetic modulation using a histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA) and a DNA methyltransferase inhibitor, 5-azacytidine (AZA) as the modulating compounds (Cichewicz, 2010). Stock solutions of these compounds were prepared in DMSO and sterile deionized water, respectively. The fungus was grown on PDA supplemented with different concentrations of the epigenetic modulators (1, 5, 10, 20, 30, 50 and 100 μM) for 15 days. The numerous variants resulting from concentrations of modulators producing the most notable cultural, biological and VOC changes were selected for future study. The strains studied here were CI-4 (wild-type), CI4-B (strain selected after a 15 day exposure of CI-4 to 50 μM SAHA, hyphal tipped, transferred to PDA and studied without subsequent exposure to SAHA), CI4-SAHA (strain CI-4 exposed to 50 μM SAHA in all studies), and CI4-AZA (strain CI-4 exposed to 100 μM AZA in all studies).

Qualitative/quantitative analyses of fungal VOCs

Weight measurements of VOCs (Carbotrap experiments). In order to obtain quantitative weight measurements of the VOC production by the fungi, they were grown for 14 days in 7 l potato dextrose (PD) broth, supplemented with 50 μM SAHA or 100 μM AZA as required, in 10 l flasks with shaking at 200 r.p.m. at 22 °C, with an inflow of 800 ml sterile compressed air min^{-1} through a 20 μm filter. The outlet was connected to a custom-designed stainless steel column containing Carbotrap materials (Supelco; Carbotraps A and B specifically for hydrocarbons) for adsorption of the hydrocarbon-like VOCs from each culture for quantitative and qualitative measurements.

The Carbotrap columns were eluted by heating in a programmable oven and purged with a flow of nitrogen gas as previously described (Booth *et al.*, 2011) and weighed. Gravitimetric analysis provided information on VOC yields. The efficiency of the column trapping method ranges from 65 to 70 % according to Booth *et al.* (2011). In addition, the gas-trapping vial, having a septum, was gently warmed and thus directly prepared for qualitative GC/MS gas analysis. 4

GC/MS analyses. Qualitative gas analysis of the compounds in the gas-trapping vials and regular Petri plate cultures was done on a

preconditioned solid-phase micro-extraction (SPME) syringe (Supelco) consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a Stable Flex fibre for 5 min (Booth *et al.*, 2011). For Petri plate analysis (8-day-old cultures) it was placed through a small hole drilled in the side of the plate and exposed to the vapour phase for 5 min or 30 min (Strobel *et al.*, 2001). The syringe was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph containing a 30 m × 0.25 mm i.d. ZB Wax capillary column with a film thickness of 0.50 μm. The column was temperature programmed as follows: 30 °C for 2 min, increasing to 220 °C at 5 °C min^{-1}. The carrier gas was ultrahigh-purity helium, and the initial column head pressure was 50 kPa. A 30 s injection time was used to introduce the sample fibre into the chromatograph. The gas chromatograph was interfaced to a Hewlett Packard 5973 mass selective detector (mass spectrometer) operating at unit resolution. The spectra were acquired at a rate of 2.5 scans per second over a mass range of 35–360 amu. Data acquisition and data processing were performed on the Hewlett Packard ChemStation software system. Tentative identification of the fungal compounds was made via library comparison using the NIST database, and all chemical compounds described in this report use the NIST database chemical terminology. Only compounds with a quality match score better than 72 % are listed and described in this report. All other unidentified compounds are lumped and summed for each strain tested. For some compounds, confirmatory identification was made with available authentic standards obtained from Sigma/Aldrich. This was done by comparing the GC/MS data of the standards, including 1,8-cineole, sabinene and others, with the GC/MS data of fungal products. The GC/MS tests were conducted several times with different exposure times of the fibre to fungal gases. Compounds appearing in the control flasks or plates were removed from the analysis and the data presented.

Proton-transfer mass spectrometry (PTR-MS). PTR-MS was used to quantify the production of fungal volatiles on a one-time monitoring basis on 10-day-old cultures growing in 10 l flask containing 7 l liquid medium as described above for the Carbotrap experiments. During the PTR-MS measurement, which lasted ~1 h, the sterile air flow through the culture flask was reduced from 800 to 50–100 ml min^{-1}. The outflow from the culture flask was disconnected from the Carbotraps and diluted with dry air at 500–1000 ml min^{-1} before being directed to the inlet of the PTR-MS. This 1/20–1/10 dilution kept the measurements within the linear dynamic range of the instrument and prevented water from condensing in the sample lines. The PTR-MS instrument was operated in the bar scan mode and mass spectral scans were obtained from 20 to 200 Da. These ion intensity measurements were converted to concentrations as previously described (Ezra, *et al.*, 2004b; Bunge, *et al.*, 2008; Strobel, *et al.*, 2008). The same analysis procedure was performed for both inoculated and uninoculated samples. At the end of the PTR-MS analysis the original flows were re-established and the outflow from the culture flasks was reconnected to the Carbotraps.

It should be recognized that the PTR-MS instrument ionizes organic molecules in the gas phase through their reaction with H_3O^+, forming mostly protonated molecules (MH^+, where M is the neutral organic molecule) which can then be detected by a standard quadrupole mass spectrometer (Lindinger *et al.*, 1998). This process can be run on real air samples with or without dilution, since the primary constituents of air (nitrogen, oxygen, argon and carbon dioxide) have a proton affinity less than water and thus are not ionized. Most organic molecules (except alkanes) have a proton affinity greater than water and are therefore ionized and detected. A further advantage of PTR-MS is that from the known or calculated quantities, the reaction time, the amount of H_3O^+ present and the theoretical reaction rate constant for the proton transfer reaction, the absolute concentration of constituents in a sample can be quantified (Lindinger *et al.*, 1998). Finally, an enormous advantage of PTR-MS is that it can be run in real time and continuously produce data on the concentrations of specific ions of interest.

Concentrations of molecules derived from the PTR-MS measurements were calculated using equations derived from reaction kinetics and assume that a reaction rate coefficient to 2×10^{-9} ml s^{-1} is appropriate for all compounds (Lindinger *et al.*, 1998; Ezra *et al.*, 2004b). This method provides a simple means by which the measured ion intensity at any mass can be expressed as an equivalent concentration. In particular, the ion whose mass is 47 (ethanol) was monitored along with the total concentration of all other ions appearing in the spectrum and the ratios were calculated. Computation of the ethanol concentration is complicated by the fragmentation of MH^+ through loss of H_2O. Since we only used the ethanol concentration to compare the samples on a relative basis, no attempt has been made to correct the reported concentrations for this loss of signal.

Bioactivities of CI-4 and its epigenetic variants. The VOCs produced by 7-day-old cultures of *Hypoxylon* sp. CI-4 and its variants were tested for inhibitory antimicrobial activity against selected pathogenic fungi according to a bioassay test system previously described for analysis of VOCs produced by *Muscodor albus* (Strobel *et al.*, 2001). The time frame of 7–9-day-old cultures is optimal for VOC production. The assays were conducted by growing the test organism in one well (PDA or PDA plus an epigenetic modulator) of a quadrant plate and placing 3 mm plugs of test fungi in each of the remaining wells (always PDA). The plate was then wrapped with a single piece of Parafilm and incubated at 23 °C. Growth of the filamentous test fungi was quantitatively assessed by making multiple measurements of growth extending from the edge of the inoculum plugs after 2–5 days and comparing them to those of corresponding controls as described previously (Strobel *et al.*, 2001). The tests were performed multiple times and the standard deviation calculated for each test organism (Strobel *et al.*, 2001).

RESULTS AND DISCUSSION

Epigenetic variants

Successful attempts were made to acquire a response of *Hypoxylon* sp. CI-4 to varying concentrations (1–100 μM) of epigenetic modifying agents. Growth of strain CI-4 on PD broth with 50 μM SAHA or 100 μM AZA produced the most striking changes in the morphology and bioactivities of the cultures; lower concentrations were seemingly ineffective. Independently, after culture in the presence of these agents two variants, CI4-SAHA and CI4-AZA, were produced that had altered cultural characteristics (Table 1). In order for them consistently to behave in the same manner, they were always grown on medium supplemented with the particular concentration of the specific modulator. On transferring these variants to normal PDA plates, one isolate, modified with SAHA, displayed morphological characteristics and bioactivity different from both the wild-type and the SAHA-treated strain growing on the supplemented medium. This strain was designated CI4-B. However, when this strain growing with AZA was cultured in normal PD medium, it regained the characteristic odour, cultural characteristics and bioactivity of the wild-type (data not shown). Thus, for this study, CI-

4 (wild-type) and epigenetic variants CI4-SAHA and CI4-AZA were used. CI4-B (selected from the 50 μM SAHA culture) was also included since it maintained some characteristics of the CI4-SAHA culture (Table 1). Interestingly, the growth rate of CI4-B exceeded that of the other strains, including the wild-type CI-4 (Table 1). This was also true for the total production of weighable VOCs as acquired in the Carbotrap experiments (Table 1). The coloration of each of the organisms was different especially on the reverse side of the plate, with CI4-SAHA having the greatest pigmentation and CI4-AZA having the least (Table 1).

Bioactivity of the fungi

Bioactivity tests done on the VOCs of each organism indicated several important features among these variants (Table 2). The bioactivity of the VOCs differed in specificity as well as the extent of the inhibition of the some of the test organisms (Table 2). The VOCs of all four strains inhibited *Ceratocystis ulmi*, *Cercospora beticola*, *Phytophthora palmivora*, *Phytophthora cinnamomi*, *Pythium ultimum*, *Rhizoctonia solani* and *Verticillum dahliae* completely. However, significant differences were found in the inhibition patterns of other test organisms. For instance, CI4-SAHA displayed 100 % inhibition against *Colletotrichum lagenarium* and *Muscodor albus* as compared to 46.2 % and 16.7 % inhibition exhibited by CI-4 to these same organisms, respectively (Table 2). However, the effect on *Sclerotinia sclerotiorum* was reversed, as CI4-SAHA produced an inhibition of 44.9 % compared to the complete inhibition by the wild-type CI-4 (Table 2). The epigenetic variant CI4-AZA inhibited *Botrytis cinerea* and *M. albus* completely while the wild-type showed only 54 % and 16.4 % inhibition, respectively. CI4-B showed an overall lower bioactivity than CI4-SAHA (Table 2). Overall, the results show that the epigenetic modulators are having an influence upon the VOC production by this fungus and further analytical chemical studies seemed warranted.

VOC profiles of *Hypoxylon* sp. CI-4 and its epigenetic variants on PDA

GC/MS analysis of 8-day-old cultures of the fungi grown on PDA revealed significant variations in the VOCs, with several new compounds appearing in the epigenetic variants and several others not produced by comparison to the CI-4 wild-type (Table 3). Most notably, the terpenes (−)-aristolene and cedrene were detected in CI4-AZA along with 1,5-cyclooctadiene, 3-bromo plus most of the other compounds associated with CI-4 (Table 3). In the VOCs of CI4-SAHA and CI4-B, benzene,1-ethyl-3-methyl, and naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1s-(1α,4aβ,8aα)]- were absent (Table 3). These compounds were present in the VOCs of CI-4. In addition to these changes, uniquely, CI4-B also produced bicyclo [4.2.0.] octa-1,3,5-triene along with spiro [4.4] nona-1,6-diene, (*S*)- and 5-methylenecycloocta-1,3-diene along with 1,4- cyclooctadiene, 6-bromo (Table 3). Some of these compounds are of major interest because of their potential as fuels. Other compounds with fuel potential include ethyl alcohol, 1,4-cyclohexadiene-1- methyl and cyclohexane, 1,2,4-tris(methylene)-, plus 1,8-cineole,

Table 1. Characteristics of the epigenetic variants of *Hypoxylon* sp. as compared to the CI-4 wild-type strain

The results presented are a compilation of data from at least three cultures of each organism. Numerical values are means ± SD.

Characteristic	CI-4	CI4-SAHA	CI4-B	CI4-AZA
Percentage lateral growth on PDA as compared to CI-4 over 7 days	–	94.4 ± 1.96	106.7 ± 1.44	49.0 ± 1.91
Dry cell weight in PD broth (total 6.5 l) after 2 weeks	27.9 ± 3.7	27.1 ± 2.2	28.7 ± 1.7	35.8 ± 1.4
Total quantity extracted via Carbotrap after 2 weeks (mg)	37.4 ± 2.0	33.7 ± 2.4	58.3 ± 3.5	45.3 ± 2.7
Growth on PDA	Covers whole plate in15 days	Similar to CI-4	Faster than CI-4, covers the plate in 13–14 days	Slower than CI-4, taking 20 days to cover the plate
Growth density	Dense, fuzzy	Dense, fuzzy	Dense, fuzzy	Thin and sparse
Pigmentation	Olive green to brownish mycelial pigmentation, varying to light brown towards the margins	Heavier mycelial pigmentation spreading from the centre towards the margin	Only one-third of the culture gets the light brown mycelial pigmentation; rest remains creamy	The characteristic pigmentation of the wild-type is absent, or very light in the centre
Reverse side characteristics	Discontinuous dark brown colour halfway from the centre and lighter colour towards the margin	Heavy olive green to brownish Pigmentation up to three-quarters of the diameter of the plate	Very thin pigmentation in the centre only	Creamy, lacking the olive to dark brown colour of the wild-type

Table 2. Bioactivity of the VOCs of 7-day-old cultures of *Hypoxylon* sp. CI-4 and its epigenetic variants against a panel of test organisms on PDA

Test organism	Inhibition (%)*			
	CI-4	CI4-SAHA	CI-4B	CI4-AZA
Aspergillus fumigatus	54.4±3.0	63.8±1.7	43.7±1.5	65.7±2.6
Botrytis cinerea	52.0±4.0	73.3±2.3	70.8±3.4	100±0.0
Ceratocystis ulmi	100±0.0	100±0.0	100±0.0	100±0.0
Cercospora beticola	100±0.0	100±0.0	100±0.0	100±0.0
Colletotrichum lagenarium	46.2±7.7	100±0.0	25.7±2.1	26.8±0.0
Fusarium solani	71.9±3.1	58.4±3.8	10.5±1.5	64.8±3.2
Geotrichum candidum	61.90±2.1	39.2±3.4	25.0±3.8	47.1±0.0
Mucodor albus	16.7±0.0	100±0.0	22.2±3.3	100±0.0
Phytophthora palmivora	100±0.0	100±0.0	100±0.0	100±0.0
Phytophthora cinnamomi	100±0.0	100±0.0	100±0.0	100±0.0
Pythium ultimum	100±0.0	100±0.0	100±0.0	100±0.0
Rhizoctonia solani	100±0.0	100±0.0	100±0.0	100±0.0
Sclerotinia sclerotiorum	100±0.0	44.9±1.5	82.3±1.6	100±0.0
Trichoderma viride	21.9±0.0	38.1±1.6	19.2±2.2	85.1±1.6
Verticillum dahliae	100±0.0	100±0.0	100±0.0	100±0.0

*Relative to a control culture grown on PDA. Data are means±SD.

11 which were detected in the VOCs of each variant (Table 3). The unknowns associated with the VOCs in these organisms are not shown. As surmised, the variations in VOC composition of these cultures offer some explanation as to the variation in bioactivity of the volatiles observed against different test organisms (Table 2), but the exact explanation for the variation is unknown.

VOC profiles of *Hypoxylon* sp. CI-4 and its epigenetic variants on PD broth

Commonly, fungi behave in a different biochemical manner when grown in liquid shaken culture versus solid stationary culture. For this reason each of the organisms was grown in 7 l of its respective PD medium, with shaking for 14 days. Compounds with hydrocarbon-like characteristics were trapped on Carbotraps and the eluents weighed and analysed by GC/MS. The weights of the samples varied over the range 33–55 mg (Table 1). It is also noteworthy that the technique of trapping larger amounts (tens of milligrams) of the fungal VOCs and subsequent analysis by SPME-GC/MS yielded much cleaner GC separation results and thus better mass spectral analyses of the fungal products (Booth *et al.*, 2011).

As expected, in the fungi treated with chemical modulators, new compounds appeared, and the production of other compounds was lost. For instance, as was the case with the solid culture, sesquiterpenoids also appeared in the CI4-AZA liquid culture (*m/z* 204) and they had not been previously observed in the wild-type culture (Tables 3 and 4). Likewise, numerous terpenoids also appeared in the epigenetically modulated fungi, with the greatest number of them in CI4-AZA (*m/z* 204) (Table 4). The monoterpenoid γ-terpinene appeared in each of the modulated fungi but not the wild-type (Table 4). On the other hand, 1,8-cineole and cyclohexane, 1,2,4-tris(methylene), well-established volatile products of CI-4, were also detected in each variant (Table 4). Finally, many VOCs found in the wild-type fungus did not appear in the variants, including cyclohexene, 1 (1-propynyl)-; undecane, 5-methyl-; 2,4,5-trimethyl-1,3-dioxolane; and *o*-cymene (Table 4).

Of interest was the appearance of several alkanes in the GC/MS analysis of the modulated fungi, including decane and dodecane, as well as several benzene derivatives including benzene, 1,2,3,4-tetramethyl along with benzene, 2-propenyl and *o*-cymene (Table 4). Other differences in VOC products were observed in the variants versus the wild-type fungus (Table 4).

Thus the epigenetic modification produced a range of outcomes: (1) the loss of the production of certain VOCs, (2) the appearance of new VOCs, and (3) no effect on the production of some VOCs (Tables 3 and 4).

Quantification of VOCs

Using PTR-MS analysis it was possible to do direct on-line 12 monitoring of the VOC production of each 7 l fungal culture. Monitoring was done at day 10 of the culture for 1 h with constant shaking. The total ion concentration of each of the epigenetic variants was reduced as compared to the control wild-type fungus (Fig. 1). However, the qualitative PTR-MS spectra obtained from each organism were all virtually identical (data not shown). This result seems at odds with conclusions drawn from the SPME-GC/MS analyses of Carbotrapped and concentrated VOCs in that, whereas striking differences in the VOCs were detected by that technique (Table 4), the amounts of most products were not great enough to be detectable by PTR-MS, which was conducted on the gas phase during the culture cycle. However, monitoring of the *m/z* at 47 (ethanol) along with the total ion concentration of VOC products showed that considerably less ethanol was produced by the variants, especially CI4-B and CI4-AZA. The most striking result from PTR-MS was the marked increase in the ratio of mass 47 (ethanol) to the total ion concentration in the variants: in the wild-type the ratio was about 0.65 but it increased to nearly 0.8 in the CI4-AZA culture (Fig. 1). It should be noted that ethanol was not recovered from the Carbotrap column since it was too polar for the column materials used and thus passed through the column.

Table 3. Comparison of VOCs in *Hypoxylon* sp. CI-4 and its epigenetic variants by the SPME fibre analytical technique on 8-day-old cultures on PDA plates

Wild-type CI-4	CI4-SAHA	CI4-B	CI4-AZA
Ethyl alcohol	Ethyl alcohol	Ethyl alcohol	Ethyl alcohol
1,4-Cyclohexadiene, 1-methyl-	1,4-Cyclohexadiene, 1-methyl-	1,4-Cyclohexadiene, 1-methyl-	1,4-Cyclohexadiene, 1-methyl-
	1,3,5-Cyclooctatriene		1,3,5-Cyclooctatriene
1,8-Cineole	1,8-Cineole	1,8-Cineole	1,8-Cineole
		Bicyclo [4.2.0.] octa-1,3,5-triene	
		Spiro [4.4] nona-1,6-diene, (*S*)-	
Benzene, 1-ethyl-3-methyl-			Benzene, 1-ethyl-3-methyl-
2,3-Heptadien-5-yne, 2,4-dimethyl-	2,3-Heptadien-5-yne, 2,4-dimethyl-	2,3-Heptadien-5-yne, 2,4-dimethyl-	2,3-Heptadien-5-yne, 2,4-dimethyl-
Cyclohexane, 1,2,4-tris(methylene)-	Cyclohexane, 1,2,4-tris(methylene)-	Cyclohexane, 1,2,4-tris(methylene)-	Cyclohexane, 1,2,4-tris(methylene)-
		1,4-Cyclooctadiene,6-bromo-	
Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1s-(1α,4aβ,8aα)]-			Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1s-(1α,4aβ,8aα)]-
Phenylethyl alcohol		Phenylethyl alcohol	Phenylethyl alcohol
			(−)-Aristolene
			1,5-Cyclooctadiene, 3-bromo-
6a-Methyl-hexahydropentalene-1,6			6a-Methyl-hexahydropentalene-1,6
	2,5-Methano-1H-inden-7 (4H)-one, hexahydro-3a,7a-dimethyl-		2,5-Methano-1H-inden-7 (4H)-one, hexahydro-3a,7a-dimethyl-
2-Naphtholenol, 3-methoxy-	2-Naphtholenol, 3-methoxy-	2-Naphtholenol, 3-methoxy-	2-Naphtholenol,3-methoxy-

Conclusions

The concept of using epigenetic modulation to enhance or change the production of volatile secondary metabolites in the VO- producing fungus *Hypoxylon* sp. CI-4 yielded some results that have been observed with non-volatile products in other fungi (Cichewicz, 2010). For instance, VOCs not previously observed as products of this organism appeared after exposure to the epigenetic modulators SAHA or AZA (Tables 3 and 4). The majority of newly appearing products were mono- and sesquiterpenoids (Table 4). As previously proposed, work on the epigenome represents a valuable opportunity to obtain new secondary metabolites from fungi (Cichewicz, 2010). Furthermore, the new products arising in the epigenetic variants, especially the monoterpenoids, have potential as fuels, as exemplified by γ-terpinene, various benzene derivatives and 1,3,7-octratriene, 3,7-dimethyl-.

It is interesting that the epigenetic modulating compound SAHA generally caused a reversed effect on the overall biological properties of the wild-type fungus. That is, once the fungus was removed from a culture medium containing SAHA it generally reverted to an organism having the same cultural characteristics as the wild-type. However, in one case, a hyphal tip from a SAHA culture developed into an organism, CI4-B, having a set of phenotypic characteristics including coloration, colony type, bioactivity and VOC production that were unique to it, and these characteristics were maintained even without the presence of SAHA (Tables 1–4). Thus, it seems that simple chemical epigenetic methods can result in stable mutants having unique phenotypic characteristics. As expected, the variant CI4-B did have some characteristics of CI4-SAHA, including its ability to make benzene, 1-methyl-2-(1-methylethyl)-, and its PTR mass spectrum was nearly identical to that of CI4-SAHA with respect to ethanol production and the ratio of ethanol to the total ion concentration (Table 4, Fig. 1). This suggests that the CI4-B was not a random mutant and that the influence of SAHA on the fungus resulted in one or more permanent epigenetic modifications.

This work also demonstrates that *Hypoxylon* sp. CI-4 has the genetic potential to produce a wide spectrum of hydrocarbon-related molecules (Mycodiesel), and collectively these should be examined as potential fuels or other resource materials. Regular diesel fuel, from many locations, has a number of major families of molecules represented in it, including straight-chained and branched hydrocarbons, cyclohexanes, benzenes and naphthene-like molecules. The VOCs made by *Hypoxylon* sp. CI-4 and its epigenetic variants include representative molecules within these major families. This is exemplified by straight-chained and branched hydrocarbons (decane, dodecane, undecane, 5-methyl- and decane, 3,6-dimethyl-), cyclohexanes [cyclohexane, 1,2,4-tris(methylene); cyclohexene, 1(1-propynyl)-] benzyl derivatives [benzene, 1-ethenyl-2-methyl-, benzene, 1,2,3,4-tetramethyl-, benzene, 1-methyl-4-(1-methylethyl)-, benzene, 1-methyl-2-(1-methy-

Table 4. Comparison of VOC production of *Hypoxylon* sp. CI-4 and its epigenetic variants, collected and eluted from Carbotraps and subsequently analysed by SPME-GC/MS

The fungi were grown with shaking in 7 l PD broth (10 l flasks) for 2 weeks at 23 °C. RT, retention time.

RT (min)	Compound	*m/z*	Relative area (%)				Quality match (%)
			CI-4	CI4 -SAHA	CI-4B	CI4-AZA	
1.85	Acetaldehyde*	44	2.40	1.31	2.01	0.93	74, 83, 74, 74
5.22	2,4,5-Trimethyl-1,3-dioxolane	116	0.74				87
5.24	Decane*	142				1.22	95
6.15	Undecane, 5-methyl-*	170	0.21				81
6.16	α-Thujene	136		9.61	13.18	2.63	94, 94, 94
6.70	2-Butenal*	70	0.47	1.37	0.92	1.40	91, 91, 91, 91
7.70	Decane, 3,6-dimethyl-	170	0.20				83
7.96	1,3,5-Trioxane, 2,4,6-trimethyl-	132	0.58				87
8.03	Dodecane*	170				1.86	80
8.55	Sabinene*	136			0.36		81
8.72	1-Butanol, 3-methyl-, acetate*	130	0.40	0.31			90, 72
8.83	1,3-Cyclopentadiene, 5-(1-methylpropylidene)-	120	0.21				72
9.15	Cyclohexene, 1(1-propynyl)-	120	0.23				87
9.82	β-Myrcene	136			1.86	4.75	94, 94
9.93	2-Butenoic acid, ethyl ester, (E)	114		0.37			86
11.67	4-Octanone*	128	0.55	0.59			90, 90
11.85	1, 8-Cineole*	154	33.39	36.77	25.03	3.39	93, 97, 93, 96
12.17	1,3,6-Octratriene, 3,7-dimethyl- (*trans*-ocimene)	136			0.75	0.82	95, 95
12.26	2-Butenoic acid, 2-methyl-, ethyl ester	128		0.53			93
12.30	Benzene, 1-ethenyl-2-methyl	118	0.19			0.83	76, 94
12.41	γ-Terpinene*	136		0.31	3.43	1.84	97, 96, 96
12.55	1,3,7-Octratriene, 3,7-dimethyl-	136			0.57		95
12.99	Benzene, 1,2,3,4-tetramethyl	134				16.39	76
13.1	Benzene, 1-methyl-4-(1-methylethyl) (*o*-cymene)	134	32.61				94
13.18	Benzene, 1-methyl-2-(1-methylethyl)-*	134		30.46	20.81		95, 95
14.22	Cyclohexane, 1,2,4-tris(methylene)	120	6.73	1.19	2.43	10.35	95, 95, 95, 97
13.39	α-Terpinolene*	136			1.11	0.52	98, 98
16.69	Benzene, 2-propenyl-	118			0.47		95
17.14	1-β-Pinene	136	0.88				91
19.26	1-Phellandrene	136	0.75				72
20.03	Linalool	154				0.36	95
20.18	2-β-Pinene	136		2.38			70
20.62	*cis-p*-2-Menthen-1-ol	154		0.48			93
21.05	α-Guaiene	204				0.50	99
21.60	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	154	0.27	0.48	0.46	3.18	97, 97, 97, 97
21.89	2-(2-Propenyl)-furan	108				0.96	70
22.02	4-(Cyclopentylidene)-2-butanone	138				1.27	72
23.78	1α-Terpineol	154				15.15	91
23.83	Linalyl propionate	210	0.90	1.57	2.12		91, 91, 91
24.02	β-Selinene	204				5.92	83
24.11	δ-Guaiene	204				0.65	99
24.47	Camphene	136				0.45	74
24.77	Cyclopentane, 1-methylene-3-(1-methylethylidene)	122				2.02	72
	Unknowns		18.09	12.27	24.47	22	

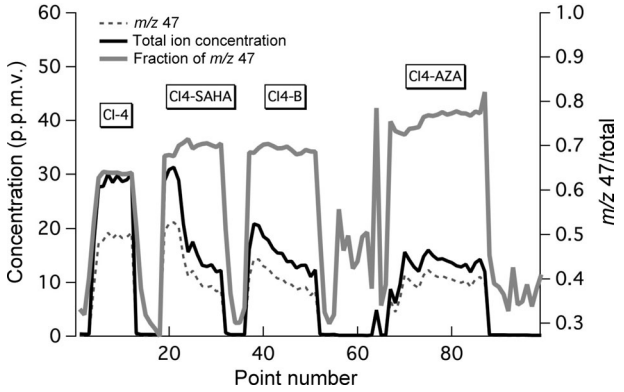

Fig. 1. Gas production by *Hypoxylon* sp. cultures that were grown in 7 l PD broth and were 10 days old at the time of monitoring of the VOCs of the culture by PTR/MS. The left axis represents the total ion concentration and the right axis represents the ratio of mass 47 (ethanol) to the total ion concentration. The PTR/MS traces of each fungal culture are labelled with the respective fungal culture designation.

lethyl)- and benzene, 2-propenyl-] and naphthene derivatives [naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1s-(1α,4aβ,8aα)]-] (Tables 3 and 4). Other useful fuel molecules are also produced by these organisms, including ethanol and 1,8-cineole (an octane derivative) (Table 4, Fig. 1). It appears that epigenetic modification experiments, such as these, provide a basis for a wider understanding of the genetic potential of an organism.

An extrapolation of the observations in this study to other fungi and biological situations implies that the genetic potential of any fungus is much greater than one may guess on first inspection. Furthermore, the phenomenon of epigenetics may help explain the observations that the VOC compositions of no two endophytic *Hypoxylon* isolates are ever the same (G. A. Strobel unpublished). While many make the standard VOCs such as 1,8-cineole and cyclohexane, 1,2,4-tris(methylene), other products also appear that no two organisms hold in common. It may be that epigenetic modulation of a fungus can occur via mechanisms associated with its respective host plant. This idea may have broad implications in pathology and industrial microbiology.

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