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Endophytic fungal production rates of volatile organic compounds are highest under microaerophilic conditions

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Abstract

Volatile organic compound (VOC) production from an endophytic fungus was quantified at four oxygen concentrations (0, 1, 13 and 21 %) throughout culture growth phases. The filamentous fungus, a *Nodulisporium* sp. (designated TI-13), was grown in a solid-state reactor with an agricultural byproduct, beet pulp, as the solid substrate. The VOCs, with potential applications as biofuels, natural flavour compounds and bioactive mixtures, were measured with a recently introduced platinum catalyst and proton transfer reaction mass spectrometry quantification system. The highest-specific production rates of carbon number four and higher VOCs were observed under microaerophilic conditions, which is the expected environment within the plant host. Specific production rates of two ester compounds increased by at least 19 times under microaerophilic conditions compared with those under any other oxygen concentration studied. Total VOC production, including small molecules such as ethanol and acetaldehyde, increased by 23 times when compared between aerobic and anoxic conditions, predominately due to increased production of ethanol. Additionally, total specific production for all 21 compounds quantified was highest under reduced oxygen conditions.

INTRODUCTION

Fungal endophytes have been the focus of many recent studies due to their ecological role and their production of numerous compounds of potential economic importance [1, 2]. Endophytes are micro-organisms that live within plants without causing apparent harm, but fungal interactions with the plant host may range from mutualistic to parasitic depending on the species and environmental conditions [2–4]. Endophytes may provide several benefits to the plant host, including resistance to microbial pathogens, insects, nematodes, animal herbivores, drought and heat [2, 3]. One means of interaction between fungal endophytes and their plant host is the production of volatile organic compounds (VOCs); bioactive fungal VOCs may signal the plant host to raise its own defences or may work directly against detrimental organisms [2, 4].

Endophytic fungal VOCs have potential applications as biofuels, flavour compounds and biofumigants [1]. The VOCs may deter insects, and inhibit bacteria and other fungi, which makes them potential biofumigants [1]. Many fungal endophytic VOCs also have the same carbon number as compounds found in gasoline and diesel fuel [5]. Additionally, fungal endophytes produce ethanol, which is a major source of bioenergy, with more than 23 billion gallons produced in 2013 [6]. However, the higher carbon number fungal biofuel compounds explored here are more energy dense and, therefore, could replace a higher percentage of gasoline than ethanol [5, 7]. Finally, fungal endophytes produce valuable flavour compounds, which are considered all natural and are typically worth substantially more than their synthetic counterparts [1, 8].

Many of the carbon number four (C4) and higher endophytic fungal VOCs are secondary metabolites [9]. The types and quantities of secondary metabolites produced depends on the fungal strain, medium, temperature, oxygen and growth phase of the culture [10–12]. Triggers for the production of many secondary metabolites are not yet well understood, especially in less-studied fungi such as endophytes [13, 14]. Varying oxygen concentrations have been shown to promote secondary metabolite production in fungi [15, 16], and it has been suggested that lowering oxygen concentration increases yields of C4 and higher VOCs in endophytes [5, 17].

Few studies have been performed that use strong quantification methods, such as proton transfer reaction mass spectrometry (PTR-MS), to study fungal VOC production throughout growth phases or between culture conditions [18, 19]. However, many studies have utilized solid-phase micro-extraction gas chromatography mass spectrometry (SPME GC-MS) to identify and quantify known VOCs [20–22]. While SPME GC-MS readily determines the identity of known compounds, it is prone to inaccurate quantifications in complex mixtures of VOCs, such as those typically produced by microbes [18, 23]. Measurements with SPME GC-MS can be improved by using standard additions [20] of each class and size of compound being studied in different mixtures. This tedious process can be avoided by using PTR-MS, which is an inherently quantitative method [24]. Strong quantification can be informative, as it has been suggested that some compounds may be beneficial to plants at low concentrations, but inhibitory at high concentrations [25].

In the current study, a fungal endophyte, a *Nodulisporium* sp. (designated TI-13), was grown on an agricultural byproduct, beet pulp, with minimal pretreatment and was shown to directly produce potential biofuel, flavour and biofumigant compounds. Recently, endophytes have been shown to produce VOCs with potential as biofuels, flavour compounds and biofumigants [19]; however, quantification data under different culture conditions are limited. In this study, production of VOCs was accurately measured at different oxygen conditions using a recently introduced quantification system composed of a platinum catalyst and CO_2 detector combined with PTR-MS. These methods were used to measure total VOCs as well as individual metabolites produced throughout the growth phases of a solid-state fungal culture under different oxygen conditions.

METHODS

Micro-organism

Using established collection and isolation methods as described previously [26], a *Nodulisporium* sp. (heretofore referred to as TI-13) was discovered as an endophyte of *Cassia fistula* in the highlands of Thailand [19]. TI-13 was characterized as having the perfect stage of *Hypoxylon* sp. and as *Nodulisporium* sp. based on its ITS1-5.8S-ITS2 ribosomal gene sequence, which is available in GenBank as KJ558391 [19]. The filamentous fungus is stored as sample NRRL 50502 in the Agriculture Research Service Culture Collection at the US Department of Agriculture [19].

Growth conditions

Inoculum cultures were generated by growing TI-13 on potato dextrose agar until hyphae covered $\geq$60 % of the plate surface. A 20 % glycerol solution (10 ml) was added to each plate, scraped with a sterile glass rod, and the suspended fungal biomass solution was collected. The solution was mixed thoroughly, added to Microbank microbead vials (Pro-Lab Diagnostic) and stored at −80 °C.

The composition of the inoculum medium was 60 g glucose l^{-1} and 0.5 g yeast extract l^{-1}. Sterile filtered (0.22 µm) medium (250 ml) was added to 500 ml sterile baffled flasks. Microbeads (three) were added to each flask and grown for 9 days at 160 r.p.m. and 30 °C. All inoculum flasks were covered with sterile Kimguard (Kimberly-Clark) to allow gas exchange, but prevent microbial contamination.

In separate experiments for anoxic growth testing, fungal cultures were grown on potato dextrose agar plates until they covered $\geq$60 % of the surface. Fungi and agar plugs, approximately 1×1 cm, were placed on beet pulp plates in an anoxic jar (Gas Pak System; Becton Dickinson) at room temperature. Control beet pulp plates inoculated with the same procedure were allowed to grow under aerobic conditions.

Solid-state fungal reactor system

A solid-state reactor constructed from a 2-litre, air-tight, borosilicate glass container with three stainless steel mesh shelves was inoculated with 250 mg biomass as determined by a correlation with protein concentration using a modified Bradford method [27]. As a substrate, beet pulp (50 g) was autoclaved for 20 min with 250 ml water and allowed to cool. The reactor, hydration flask and all tubing (see Fig. 1) were autoclaved at 121 °C for 20 min before use. Inoculum culture was spun down and the supernatant discarded. The remaining fungal biomass was added to the beet pulp, mixed by inversion and evenly distributed to the three shelves of the reactor. The reactor was placed in an incubator at 27 °C during experiments. For each of the four oxygen conditions, biological duplicate experiments were run until total VOC production rates approached zero.

The solid-state reactor was continuously sparged with 100 ml hydrated gas min^{-1} (industrial grade nitrogen and medical grade air) mixed with two mass-flow controllers to concentrations of 21, 13, 1 or 0 % oxygen. Oxygen concentrations were switched from 21 % to 0, 1 or 13 % in mid-exponential phase to allow for the initial accumulation of fungal biomass. The air was delivered via a stainless steel tube (0.5 cm internal diameter) running to the bottom centre of the reactor. Reactor off-gas was then diluted with 900 ml medical grade air min^{-1} and pulled through a CO_2 detector and PTR-MS with the PTR-MS diaphragm pump, as described previously [28]. The reactor off-gas flow path was switched every 3 h using an automated three-way valve to direct the off-gas either straight to the CO_2 detector to measure respiratory CO_2 only or through the VOC-oxidizing platinum catalyst and then to the CO_2 detector, measuring total CO_2 from both VOCs and fungal respiration. The PTR-MS sampled the gas streams that entered and bypassed the platinum catalyst once per day. Background CO_2 and VOC values were measured for each tank of air and nitrogen and were subtracted from these values.

Total VOC and respiratory CO_2 measurement

An LI-840 CO_2/H_2O non-dispersive infrared gas analyzer (Li-Cor Biosciences) was used to measure respiratory CO_2.

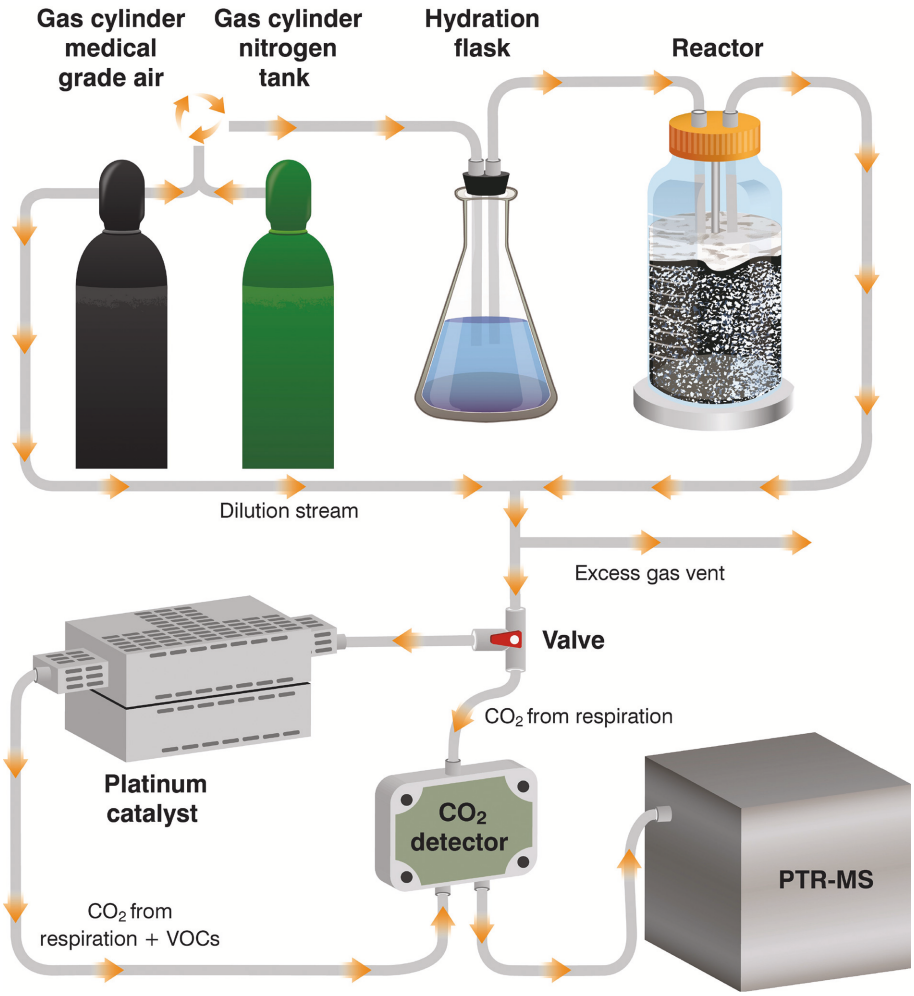

Fig. 1. Experimental setup for monitoring VOC production from a fungal solid-state reactor. Air and nitrogen were mixed, hydrated and sparged through the bottom of the reactor. Reactor off-gas was sampled from the top of the reactor, diluted and sent to a CO_2 detector to measure respiratory CO_2 and then to the PTR-MS for quantification of major VOC species. Alternatively, the off-gas was sent to a platinum catalyst where VOCs were oxidized to CO_2 and measured by the CO_2 detector as the sum of the VOCs produced and respiratory CO_2.

To measure total gas-phase organic carbon, a heated platinum catalyst (High Sensitivity Catalyst 630-00996 maintained at 400 °C; Shimadzu) completely oxidized VOCs to CO_2 before the CO_2 detector, so that total volatile organic carbon and respiratory CO_2 were measured. Data from the CO_2 detector were analysed using IGOR 6 (Wavemetrics) as described previously [28] to determine respiratory CO_2 and total VOC production.

The platinum catalyst and CO_2 detector measured carbon as CO_2 (in p.p.m.) that was created by oxidizing VOCs, by measuring the difference between respiratory CO_2 and respiratory CO_2 plus oxidized VOC CO_2 as described by Schoen *et al.* [28]. VOC units were then converted to moles of carbon produced per hour based on the temperature, pressure and gas flow rate through the system [production rate (mol carbon h^{-1})].

Selectivity

Selectivity was determined by dividing the total amount of VOC product in p.p.m. C by respiratory CO_2 in p.p.m. C (numerator and denominator were calculated as described by Schoen *et al.* [28]). Selectivity is an instantaneous ratio of the carbon channelled into VOCs instead of respiratory CO_2. This measurement does not examine carbon utilized to produce biomass or non-VOC metabolites, but selectivity is a relative measure of desired VOC products to carbon converted to respiratory CO_2 measured in the gas phase.

Fungal biomass estimates

Measuring fungal biomass on solid substrates can be extremely difficult, so a correlation was determined based on TI-13 CO_2 evolution when grown on inert filters on beet pulp plates where biomass can be readily measured. Fungal

biomass was then estimated using respiratory CO_2 measurements of reactor off-gas in the primary experiments as described by Couriol *et al.* [29]. Estimates were made by determining growth-associated CO_2 production, as well as maintenance CO_2 evolution, using the correlation shown as equation 1:

$$\frac{FC}{V} \approx A\frac{dx}{dt} + BX \quad (1)$$

where C is gas-phase respiratory CO_2 (concentration), F is the flow rate of gas leaving the reactor and V is volume of the reactor, such that the term on the left-hand side represents the total respiratory CO_2 leaving the reactor. X is the total biomass in the system (mass). A is a constant (unitless) associated with CO_2 evolved due to growth, and B is a constant (time^{-1}) associated with maintenance CO_2 evolution [29]. The constants A and B were estimated from two biological replicate experiments relating respiratory CO_2 evolution to growth of TI-13 grown on agar beet pulp plates made with 15 g noble agar l^{-1} (Becton Dickinson) and 20 g l^{-1} ground beet pulp. Nitrate cellulose filters (0.2 µm; GE Healthcare) were dried at 80 °C overnight, weighed and placed on the beet pulp plates. Abiotic beet pulp plates with filters were placed in the reactor to measure CO_2 evolution as a control, and this very small concentration of CO_2 was subtracted from respiratory CO_2 in the experiments. To determine the constants A and B, inoculated plates were placed in the reactor, sparged with medical grade air, and duplicate plates were removed twice per day to measure biomass. Fungus and membrane filters were dried again at 80 °C overnight and weighed to determine dry weights. Constants were identified using linear regression in Excel (Microsoft Corporation), which gave an R^2 of 0.82 (data not shown). The constants A and B were then applied to respiratory CO_2 measurements to estimate biomass dry weights throughout the solid-state reactor experiments.

Proton transfer reaction mass spectrometry (PTR-MS)

PTR-MS was used to provide compositional information about the VOCs in the reactor off-gas. PTR-MS uses H_3O^+ ions to protonate molecules (e.g. VOCs) with proton affinities greater than water [24]. The singly charged ions are then typically detected as protonated molecules (ions with a mass-to-charge ratio, m/z, equal to the molecular weight plus 1 for the proton) by a quadrupole mass spectrometer [24]. Carbon number used for each mass-to-charge ratio was based on HS-SPME GC-MS data, known fungal metabolites and identification of endophytic fungal VOCs in previous studies [30] as further discussed in the supplementary text (available in the online version of this article).

A multicomponent standard containing methanol, acetaldehyde and alpha-pinene (Apel-Riemer Environmental) was diluted dynamically to determine PTR-MS sensitivity factors for those compounds. Ethanol calibration was performed separately using a permeation tube at a variety of humidities as described previously [28]. Sensitivity factors of 4.1, 17.4, 18.2 and 2.2 ncps p.p.b.v^{-1} (normalized counts per second per parts-per-billion by volume) for ethanol (m47, m65, m93), methanol (m33, m51), acetaldehyde (m45), and terpenes and terpenoids (m137) were used, respectively. The off-gas of the abiotic control and fungal cultures were both analysed by PTR-MS, and VOCs measured in the abiotic control were subtracted from the fungal VOC production measurements. Concentrations of all other ions reported were estimated using equation 2:

$$[R] = \frac{I_{RH^+}}{ktz(I_{m_{m/z21}} * 500 + I_{m/z39} * 250)} \quad (2)$$

where R is the concentration of the compound of interest, k is the reaction rate constant (here assumed to be k=2×10^{-9} cm^3 molecule^{-1} s^{-1}), t is the reaction time [24] and z is a mass-dependent correction factor for transmission bias [31, 32] determined for this PTR-MS. Ions representing 2 % or greater of the total ion signal were quantified, with the exception of isotopes.

PTR-MS measured counts of ions, which were converted to moles of carbon produced per hour [production rate (mol carbon h^{-1})] using the system conditions as described above. To examine the specific biomass VOC productivity, this number was divided by the grams of biomass {specific production rate [mol carbon h^{-1}(g biomass)$^{-1}$]}. To find the production of VOCs throughout an experiment, the daily PTR-MS measurements were first converted to production rates and then integrated over time using the trapezoid rule and divided by the biomass estimate {total specific production [mol carbon (g biomass)$^{-1}$]}. To determine the experiment which produced the most VOCs per time on average, the total specific production of VOCs per experiment was divided by the length of the experiment in hours {average specific production rate [mol carbon h^{-1}(g biomass)$^{-1}$]}.

Headspace solid-phase micro-extraction gas chromatography mass spectrometry (HS-SPME GC-MS)

HS-SPME GC-MS was performed (Montana State University Mass Spectrometry Facility, MT, USA) as described previously [18, 33] to assist in assignment of identities to PTR-MS ions. Briefly, a SPME fibre (divinylbenzene/carboxen on polydimethylsiloxane; Supelco) was conditioned at 240 °C for 15 min before being exposed to the headspace of the sample for 35 min. The exposed fibre was inserted into a modified gas chromatography (GC) injection port operated at 240 °C. The Zebron ZB-WAX (30 m×0.25 mm, internal diameter 0.50 µm) GC column (Phenomenex) temperature was held at 30 °C for 2 min and then ramped to 230 °C at 7 °C min^{-1}. Compounds were identified via library comparison using the National Institute of Standards and Technology search program (version 2.0 f). All compounds reported had quality matches of at least 70; direct manual

comparisons were made to spectra of known compounds when there were multiple high-quality matches.

RESULTS AND DISCUSSION

Respiratory and total volatile organic carbon

The endophytic fungus, a *Nodulisporium* sp. (designated TI-13), was grown in a solid-state reactor on the agricultural byproduct beet pulp at varying oxygen conditions. Respiratory CO_2 and total VOC production were measured by a recently introduced monitoring system [28], providing the raw data (Fig. 2a). A CO_2 detector measured respiratory CO_2, as well as VOCs, which had been completely oxidized to CO_2 by a heated platinum catalyst. By subtracting the respiratory CO_2 measurement from the respiratory CO_2 plus VOCs measurement, an accurate quantification of total VOC carbon was obtained (Fig. 2b). The sparge gas in the solid-state reactor was switched from air to nitrogen at day 2, to allow time for biomass accumulation. Total VOC production increased quickly and substantially after the introduction of nitrogen gas at the beginning of day 2 (Fig. 2b). In addition to the previously described parameters monitored [28], biomass was also estimated (Fig. 2c) using the respiratory CO_2 measurement and a modified correlation method based on that of Couriol *et al.* [29].

Total VOC production by TI-13 was measured using a heated platinum catalyst and CO_2 detector under four oxygen conditions. The VOC production rates (Fig. 3) were calculated directly from results such as those shown in Fig. 2(b) using the system conditions as described in the Methods. Total VOC production increased significantly with decreasing oxygen (Fig. 3), with data shown starting at the time the gas conditions were changed, at approximately mid-exponential phase. The initial slope of total VOC production for 21 % oxygen (Fig. 3) was low, and total VOC production peaked after approximately 12 h and then decreased towards zero. The slopes of total VOC production were initially very similar for 13, 1 and 0 % oxygen, and the plots had a growth-curve-like shape. Slopes started to decrease at 20 and 24 h, respectively, for 13 and 1 % oxygen. Increasing total VOC production continued longer for 0 % oxygen (45 h) compared with 13 and 1 % oxygen (32 h), with a slow decline that lasted approximately 9 days, compared with 1 to 2 days for the other oxygen conditions. Total VOCs included small molecules such as ethanol and acetaldehyde, as well as larger, tentatively identified compounds such as 2-methylpropan-1-ol and benzaldehyde. The total VOC production of the reactor increased from 0.052 ±0.017 g VOC carbon (mean and standard error) under fully aerobic conditions (21 % O_2) to 1.2±0.060 g VOC carbon under anoxic conditions (0 % O_2). This represents a 23-fold increase in total VOC production between oxic and anoxic conditions. Total VOC production rates were also highest anoxically at 4.0±0.15 mg carbon h^{-1}. This increase was predominately the result of increased ethanol production and was in part due to the fungus entering an extended VOC production state without growing under anoxic conditions. All

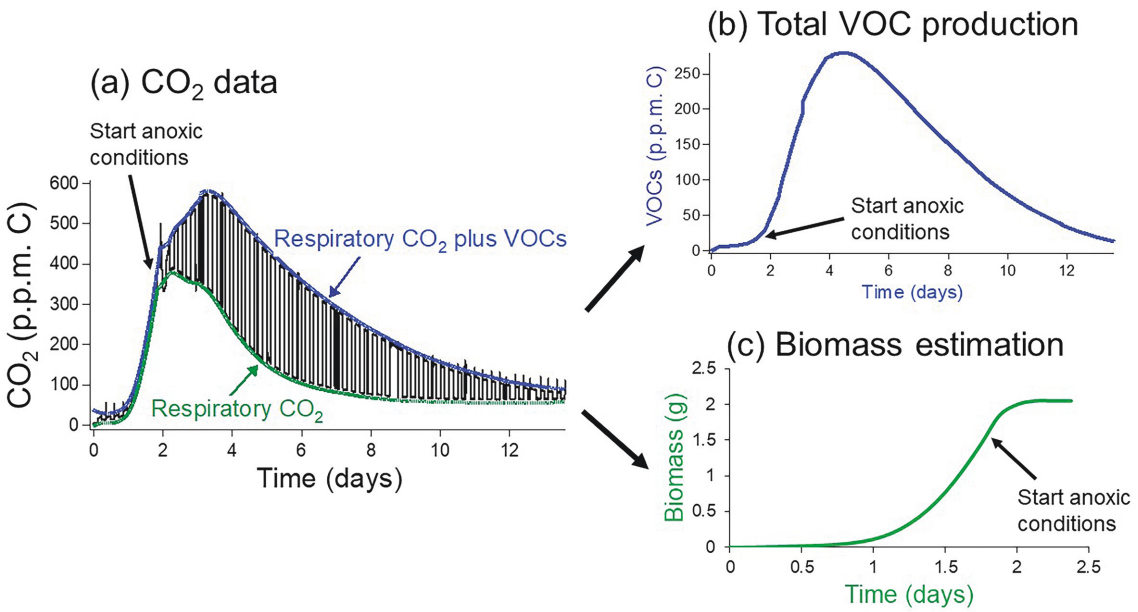

Fig. 2. Raw CO_2 data of the endophytic fungus, *Nodulisporium* sp. (designated TI-13), grown in a solid-state reactor are shown in black in (a). The sparge gas was switched from air to nitrogen at the beginning of day 2. Here, the lower green line represents the interpolation of respiratory CO_2 (off-gas measured with the CO_2 detector) and the upper blue line is the interpolation of respiratory CO_2 plus VOCs (includes the addition of VOCs oxidized to CO_2 with a heated platinum catalyst). The difference between these two lines results in the accurate quantification of total VOC production as shown in (b). Equation 1 was used to estimate biomass, shown in (c), based on the green interpolated respiratory CO_2 data.

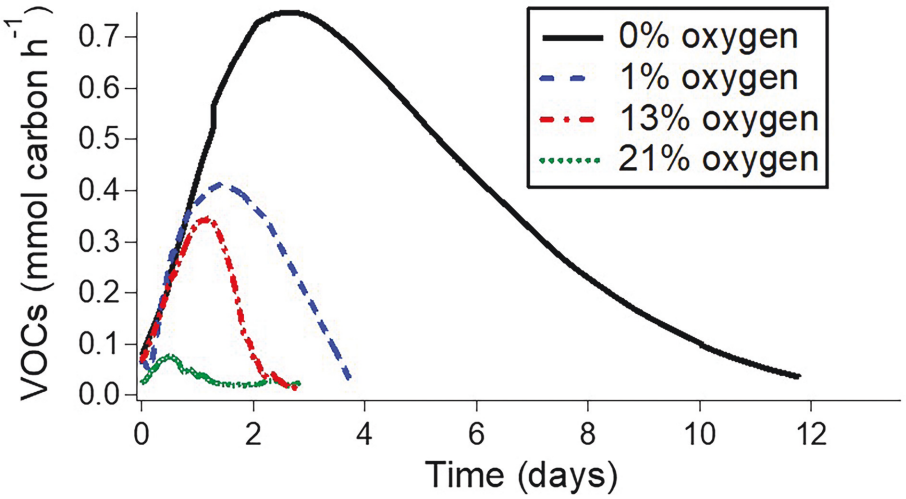

Fig. 3. Total VOC production rates at four different oxygen conditions as measured by a platinum catalyst and CO_2 detector. This figure shows a representative run for each of the four oxygen conditions starting at the point the oxygen conditions were changed at approximately mid-exponential phase.

experiments were sparged with 21 % oxygen through mid-exponential phase to allow for accumulation of biomass. TI-13 did not show growth on beet pulp after 1 month in an anoxic chamber. Most likely no growth occurred in the reactor once anoxic conditions were initiated, but the cells entered a metabolically active, VOC-producing state that lasted approximately 12 days.

Carbon number four and higher VOC production

While ethanol is a widely used biofuel, production rates observed here were low, and the higher-value products made by the fungus are of more novel interest as biofuels, flavour compounds and bioactive mixtures. These individual products were investigated using PTR-MS to quantify specific compounds. Putative identification of PTR-MS ions was assisted by using HS-SPME GC-MS (see Table S1 for SPME data and Table S2 for PTR-MS quantifications). No compounds with a carbon number greater than ten were identified. Table 1 shows putative identifications for potential biofuel and flavour compounds, such as 1,8-cineole, phenylethanol and ethyl butanoate. Table 1 also provides the total specific production of each ion under the four oxygen conditions, obtained by integrating daily PTR-MS measurements. Table S2 contains the raw data used in this analysis. All 21 products quantified had the highest total specific production at an oxygen condition below 21 % (Fig. 4a). Under anoxic conditions, 15 of the 21 products quantified had the highest total specific production (Fig. 4b). At 1 % oxygen, five of the 21 metabolites had the highest total specific production (Fig. 4c), and m/z 99 had the highest total specific production at 13 % oxygen (Fig. 4d).

The highest average specific VOC production rates were observed at reduced oxygen conditions for 19 of the 21 compounds quantified (Fig. 5a). 1,8-Cineole and m/z 59 had the highest specific production rates at 21 % oxygen. Anoxically, 8 of the 21 compounds had the highest specific production rates (Fig. 5b), including 3-methyl-1-butanol and 2-phenylethan-1-ol. At 1 % oxygen, 7 of the 21 compounds had the highest specific production rates at 1 % oxygen (Fig. 5c), including ethyl acetate and benzaldehyde. At 13 % oxygen, 4 of the 21 compounds had the highest specific production rates (Fig. 5d), including ethyl butanoate and acetaldehyde.

Overall for C4 and higher compounds, the highest specific production rates were observed under microaerophilic conditions [2.24±0.0080 μmol carbon h^{-1}(g biomass)$^{-1}$]. High specific production rates of VOCs under microaerophilic conditions may impart an ecological advantage to the endophytic fungus. As part of their relationship with the plant hosts, endophytic fungi may produce suites of bioactive VOCs to inhibit other organisms from invading the plant and to signal the plant to raise its own defences [2, 4]. Endophytes such as TI-13 most likely experience microaerophilic conditions inside the tree [5] and produce the highest yields of bioactive VOCs under similar conditions.

VOC production through culture growth phases

Total VOC production rates over time followed a typical growth curve (Fig. 3) where production increased with decreasing oxygen conditions, predominately due to the major product ethanol. Ethanol has a growth curve pattern (Fig. 6a), which was expected since ethanol is a primary metabolite [34]. Most of the tentatively identified compounds tracked in this study showed a similar production trend, such as phenylacetaldehyde (Fig. 6b). However, for two compounds, ethyl acetate (Fig. 6c) and another ester at m/z 103 (Fig. 6d) as described in the supplementary text, production was much higher at 1 % oxygen than under any other condition tested at the start of stationary phase, which is the expected trend for secondary metabolites [34]. The

Table 1. Quantification of ions by PTR-MS

Ions representing 2 % or greater of the total ion signal were quantified. The table provides tentative ion and compound identities based on HS-SPME GC-MS data, known fungal metabolites and identification of endophytic fungal VOCs in previous studies. Quantitative data represent the average of two biological replicates. Data for methanol, ethanol and 1,8-cineole were calibrated and quantified utilizing multiple ions and only one set of quantitative data is given. No compounds were clearly identified for m/z 59, 79, 89, 99, 103 or 135.

Tentative compound identification	Ion	Tentative ion identification	Total specific production [mmol carbon (g biomass)$^{-1}$]			
			Oxygen percentage			
			0	1	13	21
Methanol	33	CH_4OH^+	0.368	0.250	0.0679	0.0171
	41	$C_3H_5^+$	0.00969	0.00391	0.00263	0.00111
	43	$C_3H_7^+$	0.101	0.0386	0.0505	0.00934
Acetaldehyde	45	$C_2H_4OH^+$	1.19	0.616	0.716	0.203
Ethanol*	47	$C_2H_6OH^+$	33.7	6.79	2.83	1.03
Methanol	51	$CH_4OH^+(H_2O)$	See ion 33			
2-Methylpropan-1-ol*†	57	$C_4H_9^+$	0.150	0.0906	0.0474	0.0273
C_3H_6O	59	$C_3H_6OH^+$	0.00938	0.00595	0.00432	0.00527
Ethanol*	65	$C_2H_6OH^+(H_2O)$	See ion 47			
Ethyl Acetate	61	$C_2H_5O_2^+$	0	0.0205	0	0
3-Methylbutan-1-ol*†	71	$C_5H_{11}^+$	0.0875	0.0313	0.011021	0.010505
Methyl acetate	75	$C_3H_6O_2H^+$	0.0370	0.00582	0.00126	0.00114
	79	$C_2H_5O_2^+(H_2O)$	0.0690	0.0237	0.00862	0.00867
1,8-Cineole*†	81	$C_6H_9^+$	See ion 137			
Hexan-1-ol*†	85	$C_6H_{13}^+$	0.00729	0.00198	0.00153	0.00122
	89	$C_4H_8O_2H^+$	0.0102	0.0425	4.03×10^{-4}	0.0115
Ethanol*	93	$H^+(C_2H_6O)_2$	See ion 47			
$C_6H_{10}O$	99	$C_6H_{10}OH^+$	2.12×10^{-4}	0.00144	0.00621	7.32×10^{-4}
$C_5H_{10}O_2$	103	$C_5H_{10}O_2H^+$	2.62×10^{-4}	0.00726	2.57×10^{-6}	0.000242
2-Phenylethan-1-ol*†	105	$C_8H_8^+$	0.0011	1.25×10^{-4}	1.90×10^{-4}	1.18×10^{-5}
Benzaldehyde*†	107	$C_7H_6OH^+$	0.00238	0.00268	3.78E-05	0.001743
Ethyl butanoate*†	117	$C_6H_{12}O_2H^+$	4.79×10^{-4}	6.03×10^{-4}	4.26×10^{-5}	1.32×10^{-4}
Phenylacetaldehyde*†	121	$C_8H_8OH^+$	0.00479	0.00241	3.76×10^{-4}	6.74×10^{-4}
$C_9H_{10}O$	135	$C_9H_{10}OH^+$	0.00296	7.47×10^{-5}	1.13×10^{-4}	1.69×10^{-4}
1,8-Cineole*†	137	$C_{10}H_{17}^+$	0.102	0.0808	0.0598	0.0535

*Denotes potential biofuel.

†Denotes flavour compound.

significantly modulated production rates, at least 19 times higher than the rates under other oxygen conditions tested, were also present in the biological replicates. Ethyl acetate is produced by acetyltransferases from ethanol and acetyl-CoA [35]. Ehrlich pathway alcohols can be esterified in a similar way, and some acetyltransferases act on multiple alcohols [36, 37]. The ion at 103 showed a similar pattern, but could not be clearly identified. However, this ion was identified in previous fungal work as propanoic acid, 2-methyl-methyl ester [38], and this m/z ratio in PTR-MS is most likely an ester in a fungal experiment [39] (see supplementary text for additional discussion).

High oxygen concentrations, anoxic conditions, or the presence of ethanol or fatty acid related compounds in the medium have been shown to repress expression of some acetyltransferases and lower ester production in *Saccharomyces cerevisiae* [35, 36, 40], which is similar to the ester responses seen here. Endophytic fungal VOCs can inhibit microbial growth by disruption of cell membrane integrity and damage to DNA [41]. Endophytic VOCs are synergistically toxic in combination, and esters were the most inhibitory individual class of fungal VOCs against other micro-organisms in a previous study [42]. Esters were the only carbon number four or higher metabolites with substantially varying production rates, at least 19 times higher under microaerophilic conditions (carbon grams) compared with any other oxygen concentration studied.

Selectivity

Selectivity, the total amount of gas phase VOC product in p.p.m. C divided by the amount of respiratory CO_2 in p.p.m. C, was determined (Fig. 7) for the four oxygen conditions (0, 1, 13 and 21 %), starting at the introduction of nitrogen at approximately mid-exponential phase. At 21 %

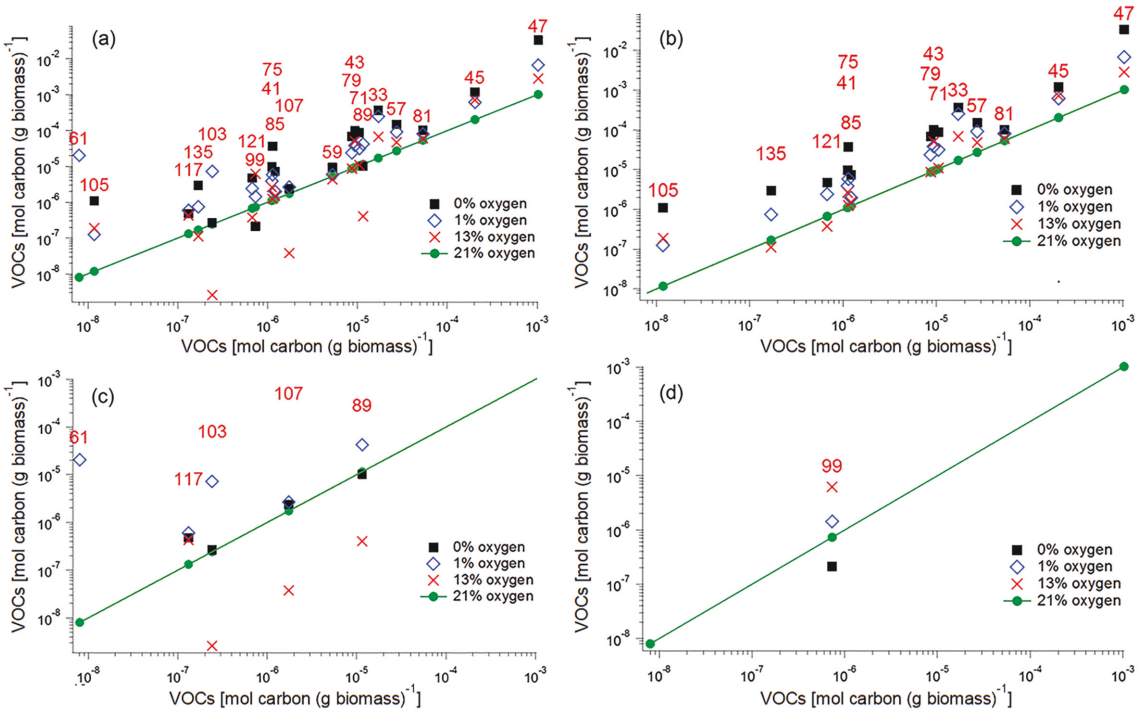

Fig. 4. (a) Total specific VOC production (normalized to fungal biomass), average of two biological replicates, for each compound or ion quantified. Total specific VOC production at 21 % oxygen is displayed as the green line, where both the y and x axes are the production rate. Total specific VOC production values at 13 % oxygen (×), 1 % oxygen (♦) and 0 % oxygen (■) are displayed on the y axis versus production at 21 % oxygen on the x axis, with red ion designations matching those in Table 1. (b) Subset of (a) showing only ions for which total specific production is highest at 0 % oxygen. (c) Subset of (a) showing only ions for which total specific production is highest at 1 % oxygen. (d) Subset of (a) showing only ions for which total specific production is highest at 13 % oxygen.

oxygen, selectivity increased slightly for 4 h to 0.037 and then decreased slowly. At 13 % oxygen, selectivity increased for 1 day to 0.14 and then decreased quickly towards zero. The shape of 1 % oxygen selectivity was similar to that for 13 % oxygen, except VOC production increased for 45 h to a maximum of 0.3. Anoxically, selectivity increased for significantly longer, 5 days, and increased 49-fold compared with aerobic conditions, to a maximum of 2.3±0.09, again predominately due to ethanol production. This high selectivity is above the maximum theoretical yield of two for ethanol or VOC metabolism, compared with the CO_2 evolved, as one molecule of CO_2 must be evolved for each molecule of ethanol produced [34], likely due to volatilization of ethanol from the liquid phase outpacing ethanol production. However, ethanol appears to be the only major product of isolate TI-13 fermentation, and no significant amounts of acetate were identified here or in a previous study [43]. Other well-studied cellulosic fungi, such as *Fusarium oxysporum*, *Trichoderma reesei* and *Ascocoryne sarcoides*, produce significant amounts of acetate during fermentation, reducing ethanol yields [17, 44, 45].

TI-13 is of interest for its production of numerous bioactive carbon number four and higher VOCs with potential as biofuels, flavour compounds and biofumigants, which is uncommon in yeasts and other fungi. Overall production here was low, but a potential production scheme is offered that could be used in conjunction with genetic engineering. A packed-bed reactor, which produced bioethanol, 55 m in length, was constructed in China with sweet sorghum as the substrate [46]. A similar solid-state reactor design could be applied to the fungal production of VOCs, which takes advantage of increased ethanol and C4 and higher compound production at low oxygen conditions. Fungal inoculum, air and a waste cellulosic feedstock would enter in one end, and the air would allow the accumulation of biomass. Further, in the reactor, oxygen concentrations would decrease due to utilization, and production of ethanol and higher value VOCs would increase significantly. Such a reactor may provide an environmentally friendly biofuel, flavour or biofumigant production scheme.

While lowering oxygen concentrations significantly increased secondary metabolite production, the specific production rates of high-value biofuel, flavour and biofumigant VOCs were low, at 2.24±0.0080 μmol carbon h^{-1}(g biomass)$^{-1}$, for C4 and higher products. Optimizing process conditions such as medium, temperature and gas sparge rate may increase yields, but genetic engineering is most likely required to achieve industrially viable production

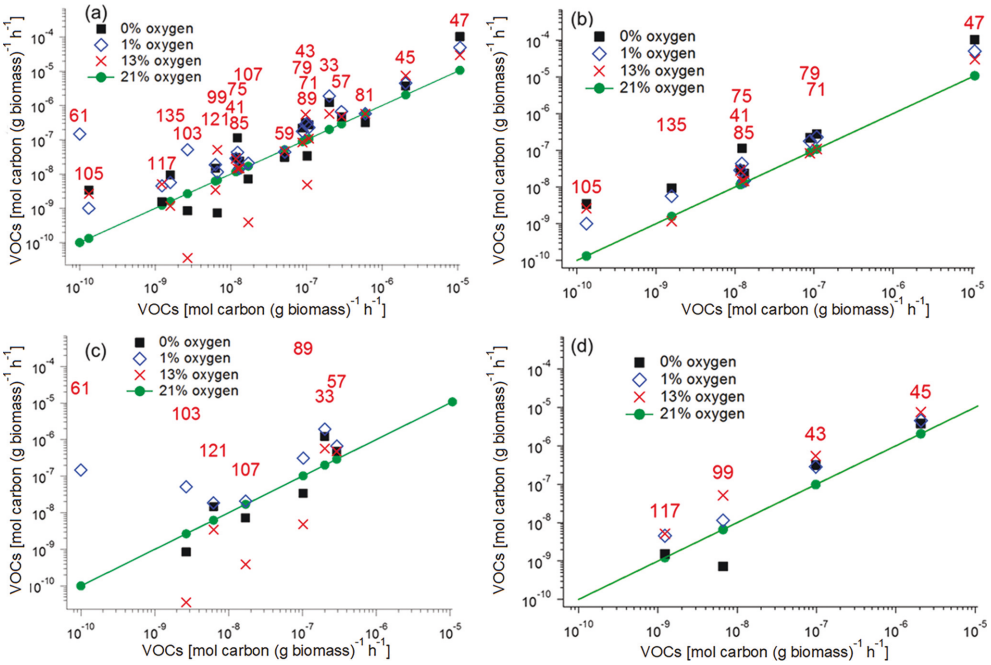

Fig. 5. (a) Total specific rates (normalized to fungal biomass), average of two biological replicates, of VOC production for each ion quantified. The 21 % oxygen VOC production rate is displayed as the green line, where both the y and x axes are the production rate. Total specific VOC production rates at 13 % oxygen (×), 1 % oxygen (♦) and 0 % oxygen (■) are displayed on the y axis versus 21 % oxygen production on the x axis, with red ion designations matching those in Table 1. (b) Subset of (a) showing only ions for which total specific production rates are highest at 0 % oxygen. (c) Subset of (a) showing only ions for which total specific production rates are highest at 1 % oxygen. (d) Subset of (a) showing only ions for which total specific production rates are highest at 13 % oxygen.

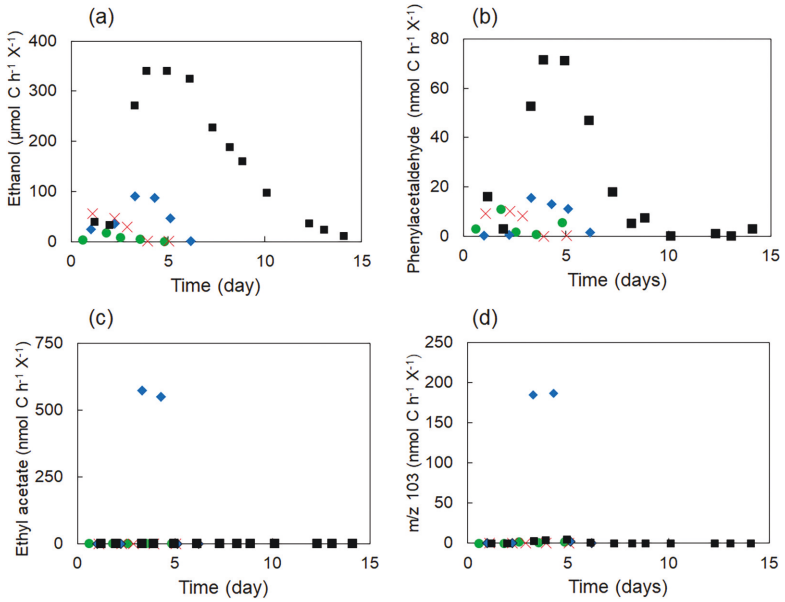

Fig. 6. Specific production rates (depicting instantaneous production as normalized to fungal biomass) of ethanol (a), phenylacetaldehyde (b), ethyl acetate (m/z 61) (c) and an ester at m/z 103 (d) at four different oxygen conditions as measured by PTR-MS and reporter in moles carbon h^{-1}(g biomass)$^{-1}$. On each graph, data symbols are 21 % oxygen (•), 13 % oxygen (×), 1 % oxygen (♦) and 0 % oxygen (■) for a representative run under each condition.

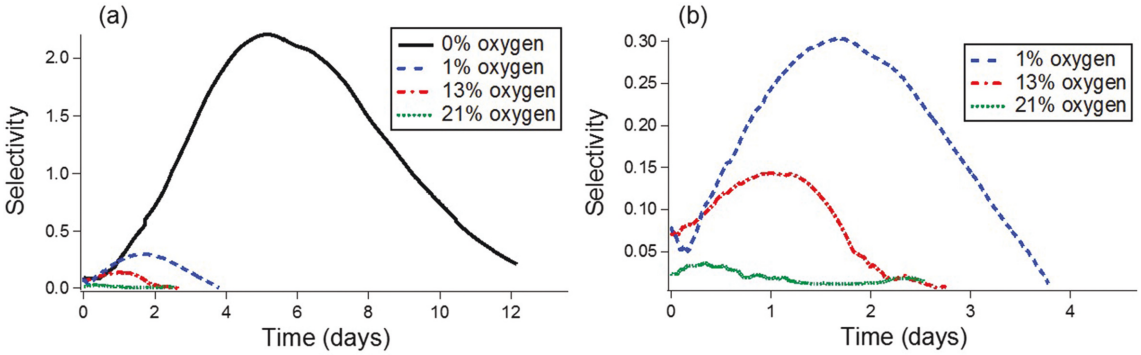

Fig. 7. Selectivity, the amount of volatile organic carbon divided by respiratory CO_2 carbon, at all four oxygen concentrations. (a) A representative run for each of the four oxygen conditions starting at the point the oxygen conditions were changed at approximately mid-exponential phase, and (b) just the three conditions containing oxygen.

rates, even of higher-value flavour and biofumigant compounds. The techniques used to identify and quantify VOCs used here could be combined with transcriptomics and metabolic modelling to identify target genes for genetic engineering [47, 48]. Alternatively, TI-13 has several advantageous properties including cellulose utilization, VOC production, resistance to high concentrations of VOCs and ethanol-specific fermentation [43] that could be further studied and engineered into other organisms.

Conclusions

An endophytic fungus produced numerous potential biofuel, flavour and biofumigant compounds from the cellulosic agricultural byproduct beet pulp. Lower oxygen conditions increased the total specific production of ethanol and all other metabolites quantified by *Nodulisporium* sp. (designated TI-13), with 23 times more carbon grams of VOCs produced under anoxic conditions than aerobically, predominately due to ethanol. Total production of C4 and higher VOCs was also highest anoxically. However, specific production rates of C4 VOCs were highest at 1 % oxygen [2.24 ±0.0080 μmol carbon h^{-1}(g biomass)$^{-1}$]. Two ester-specific production rates increased by 19 times or more under microaerophilic conditions. The methods used here could be combined with other techniques to learn more about VOC-producing pathways. This information could then be combined with genetic engineering to increase production.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study does not include experiments on humans or animals.

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