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Three fungal endophytes from the genus Nodulisporium were studied for volatile organic compound (VOC) production. All three fungi grew on a wide range of carbon substrates ranging from simple sugars to waste biomass sources. The fungi synthesized a number of long and short-chain VOCs, including eucalyptol; 1-butanol, 3-methyl; 1-octen-3-ol; and benzaldehyde, all with potential applications as biofuel or flavor compounds. As culture temperature decreased, average VOC carbon chain length increased, especially for VOCs associated with fatty acid metabolism. The results provide a template for controlling synthesis of desired VOCs through selection of species and culturing conditions.

Keywords: Fatty acid metabolism Filamentous fungi Lignocellulosic processing Secondary metabolites

Endophytes live within plant tissues without causing apparent symptoms and have been found in all plant spe-cies examined to date (Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011). Fungal endophytes produce a large range of compounds with antibacterial, antifungal or anti-tumor activity like the anticancer agent Taxol as well as industrially relevant volatile organic compounds (VOCs)(Strobel et al. 1996; Keller et al. 2005). VOC synthesis by fungal endophytes has been known for years (Strobel et al. 1996; Stinson et al. 2003), but the examination of biofuel and flavor compounds produced by endophytes is relatively new (Ahamed and Ahring 2011; Mallette et al. 2012). Pro-duction of biofuels from waste lignocellulosic biomass is a major global research goal due to finite petroleum reserves and atmospheric greenhouse gas increases (Sanchez and Cardona 2008). Endophytes also produce flavor molecules, which can be marketed as 'all natural' making them as much as three orders of magnitude more valuable than

compounds produced via synthetic chemistry (Krings and Berger 1998).

Filamentous fungi utilize at least four major pathways to produce VOCs. The fatty acid and mevalonate pathways, referred to here collectively as fatty acid synthesis, can produce alkanes, fatty alcohols, terpenes and terpenoids (Strobel et al. 2008; Grigoriev et al. 2011; Mallette et al. 2014). The Ehrlich pathway is another VOC production pathway which is used to catabolize amino acids as a source of nitrogen and can be associated with either primary or secondary metabolism (Hazelwood et al. 2008). Oxidation of linoleic acid by lipoxygenases is a third VOC producing pathway resulting in the formation of C_8 alcohols and ketones, such as 1-octen-3-ol and 3-octanone (Gianoulis et al. 2012). Finally, fermentative metabolism can produce VOCs, such as glycerol and 2,3-butanediol (Huang et al. 2007).

The current study analyzes three new endophytic *Nodulisporium* isolates in detail. The *Nodulisporium* isolates are from distinct, tropical locations (Ecuador, Thailand, Colombia) and distinct plant hosts (Supplementary Material). The isolates were selected to study similarities and differences in growth properties and VOC production profiles across geographical location and to expand the number of physiologically-characterized fungal endophytes in the literature (Ahamed and Ahring 2011; Mallette et al. 2014).

Substrate utilization and optimal growth conditions

The three *Nodulisporium* fungi isolates (EC, CO, TI, deposited as NRRL 50503, NRRL 50500 and NRRL 50502, respectively, in the Agriculture Research Service Culture Collection of the U.S. Department of Agriculture, Peoria, Illinois) grew on a range of simple and complex carbon sources including xylose, glucose, sucrose and cellobiose as well as the polymers cellulose and xylan (see Supplementary Material for culturing details). The three fungi also demonstrated robust growth on complex agricultural wastes including sugar beet pulp and corn stover (Table 1; Supplementary Fig. S1). The three fungi did not grow under anoxic conditions on potato dextrose agar (Sigma–Aldrich, St. Louis, MO, USA).

Table 1 – Growth of three *Nodulisporium* fungal endophyte isolates (EC, CO, TI) on different carbon sources at room temperature.

Carbon source	EC	CO	TI
Glucose	+++	+++	+++
Xylose	+++	+++	+++
Glycerol	+++	+++	+++
Sucrose	+++	+++	+++
Cellobiose	+++	+++	+++
Xylan	+++	+++	+++
Cellulose	+++	+++	+++
Lignin	–	–	–
Sugar beet pulp	+++	+++	+++
Corn stover	+++	+++	+++
Grass	+++	+++	+
Paper	+	+	–
Woodchips	+	+	+

+++: Significant growth. More than 2 cm radius of fungal growth in 10 d.
+: Growth. Visible growth in 10 d.
–: No significant growth. No growth in 10 d.

Cultures grew as hyphal suspensions in shake flasks and demonstrated exponential biomass accumulation rates followed by a brief linear biomass accumulation phase (Supplementary Fig. S2). Reported specific biomass accumulation rates were taken from the initial growth phase, which formed a straight line on a cell dry weight vs. time semi-log plot. The effect of pH on specific biomass accumulation rate and final biomass titer was measured over a range of initial pH values (4, 5, 6, or 7) at 30 °C (Supplementary Fig. S3). The fastest specific biomass accumulation rates were observed at pH 6 with specific biomass accumulation rates of 1.1–1.2 d^{-1}; all three fungi had their highest final biomass titer at pH 6. The effect of temperature (room temperature, 27, 30, 33, 37 °C) on fungal growth was tested using glucose medium with an initial pH value of 6 (Supplementary Fig. S4). The optimal temperature range, based on specific biomass accumulation rate, was 30–33 °C. The liquid cultures did not show consistent growth at room temperature (data not shown). Maximum specific biomass accumulation rates and final biomass titers ranged from 1.1–1.6 d^{-1} and 12.9–19.0 g cell dry weight L^{-1}, respectively, for the three isolates. Biomass yields on glucose were calculated for each isolate after exponential biomass accumulation phase (Supplementary Table S1). Typical biomass yields on glucose ranged from 0.15 to 0.3 g cell dry weight per g glucose consumed.

VOC production

VOC production was measured as a function of culture pH and temperature because fungal secondary metabolites are well known to vary with culturing conditions (Keller et al. 2005; Mallette et al. 2014). Thirty-six biofuel- and flavor-relevant compounds were identified using SPME GC–MS. Table 2 lists the identified VOCs with quality match, relative concentration, as well as the associated fungal isolate and culturing condition. It should be noted that SPME GC–MS-based quantification of metabolite concentration can be complicated by competitive adsorption of compounds (Mallette et al. 2012). VOCs were classified as biofuel-relevant if the carbon backbone fell within the range of molecules used in gasoline (C_4 – C_{12}) or diesel fuel (C_8 – C_{25}) (U.S. Department of Energy 2013). Classification of a chemical as a flavor compound was based on the compound being described in a published report as a flavor compound (Jager et al. 1996; Rodriguez-Bustamante and Sanchez 2007; Berger 2009). All three fungal isolates produced flavor compounds including benzaldehyde; 1-octen-3-ol; and 1-butanol, 3 methyl. Some additional VOCs of interest include 2,3-butanediol secreted by isolate CO, eucalyptol, a cyclic ether terpenoid, secreted by isolate TI (Nigg et al. 2014), and limonene, also secreted by isolate TI. The total concentration of secreted VOCs increased substantially with increasing pH for all three isolates, while the highest total VOC concentration measured for all strains was at 27 °C (Supplementary Fig. S5). Isolate CO secreted the highest amount of total VOC, up to 277 mg L^{-1}, of the studied isolates with the majority of the VOC being 1-octen-3-ol.

Table 2 – Biofuel- and flavor-relevant VOCs produced by fungal endophyte isolates (EC, CO, TI) growing on glucose medium. Temperature and pH indicate growth conditions where the compound was detected. Compounds were sorted by production associated with the following metabolic pathway types: Fatty acid synthesis, Ehrlich pathway, Linoleic acid oxidation, Fermentative metabolism, or Undetermined.

Compound	Carbon number	Isolate and conditions								
		EC			CO			TI		
		pH	T (°C)	Concentration (mg L^{-1})/ Quality match	pH	T (°C)	Concentration (mg L^{-1})/ Quality match	pH	T (°C)	Concentration (mg L^{-1})/ Quality match
Fatty acid synthesis										
1-Propanol[a]	3				7	30	3.89/90	6	37	1.91/90
Pentane	5				6	30	20.8/91	6	30	7.77/78
Hexanal[a]	6				6	30	2.29/87			
Hexanoic acid[a]	6				6	37	2.31/83			
2-Heptanone[a]	7	7	30	1.15/80						
Nonanoic acid	9				4	30	0.80/93			
Nonanal[a]	9	6	27	1.76/91						
Eucalyptol[a]	10							6	27	32.5/90
Limoene[a]	10							6	30	1.45/70
3-Cyclohexene-1-methanol, α,α 4-trimethyl-	10				7	30	6.83/87	6	27	23.3/91
Octane, 2,6-dimethyl-	10				6	27	3.39/72			
Octadecanoic acid	18	6	27	41.6/99						
Ehrlich pathway										
1-Propanol, 2-methyl-	4							6	37	2.47/72
Butanal, 3-methyl	5							7	30	4.29/90
1-Butanol, 3-methyl-[a]	5	7	30	2.04/90	6	27	5.07/78	6	30	2.38/78
3-Buten-1-ol, 3-methyl-	5	6	27	2.96/90	6	27	3.57/90			
		6	30	1.44/94	6	30	1.48/70			
Benzaldehyde[a]	7	6	30	1.24/97	6	30	1.31/97	6	27	21.6/97
		7	30	1.79/97	7	30	0.49/97	7	30	7.93/97
								6	37	5.65/97
Methylbenzaldehyde[a]	8	4	30	3.00/97	6	30	1.44/94	6	30	1.53/97
		6	30	1.79/96						
Phenylethyl alcohol[a]	8				7	30	6.16/94	6	27	17.1/97
					6	37	2.29/93		30	7.29/95
									37	19.6/94
Dimethylbenzaldehyde[a]	9	6	30	1.01/90	6	27	2.29/87			
					6	30	0.97/70			
Linoleic acid oxidation										
1-Octen-3-ol[a]	8	6	27	233/78	6	27	18.0/86	7	30	27.7/83
		7	30	5.95/90	6	30	2.44/90			
3-Octanone[a]	8	6	27	5.71/94						
Fermentative metabolism										
2-Butanone, 3-hydroxy-	4				7	30	3.29/78	6	37	14.4/78
					6	37	18.1/83			
2,3-Butanediol	4				6	37	3.83/90			
Butyrolacetone[a]	4	6	37	1.00/72						
2,4-Pentanedione	5	6	37	1.29/87						
Undetermined										
2-Furancarboxaldehyde, 5-methyl-[a]	6	6	37	1.16/94						
2-Furancarboxaldehyde, 5-hydroxymethyl	6	6	30	1.15/70						
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	12	6	27	11.7/80						
1,3-Propanedione, 1,3-diphenyl-	15	6	27	15.3/93						
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	16	6	27	30.4/87						

Table 2 – (*continued*)

Compound	Carbon number	Isolate and conditions								
		EC			CO			TI		
		pH	T (°C)	Concentration (mg L^{-1})/ Quality match	pH	T (°C)	Concentration (mg L^{-1})/ Quality match	pH	T (°C)	Concentration (mg L^{-1})/ Quality match
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	16				6	30	1.99/87			

[a] VOCs that have documented roles as flavor compounds. Classification of a chemical as a flavor compound was based on the compound being described in a published report as a flavor compound (Jager et al. 1996; Rodriguez-Bustamante and Sanchez 2007; Berger 2009).

All three isolates demonstrated a strong relationship between the number of carbon atoms in the secreted VOCs and the culturing temperature (Table 2; Fig. 1). VOCs from cultures grown at 27 °C had an average length of 9 carbon atoms, while VOCs from cultures grown at 37 °C had an average length of 5 carbon atoms. The data was further analyzed as a function of four VOC synthesis pathways (production associated with i. fatty acid synthesis, ii. Ehrlich pathway, iii. oxidation of linoleic acid and iv. fermentative metabolism) and fungal isolate (Table 2; Fig. 2). The fatty acid synthesis-associated VOCs showed an increase in carbon length with decreasing culture temperature; the relationship was observed for all three isolates. Ehrlich pathway-associated VOCs demonstrated a similar increase in carbon chain length with decreasing temperature in TI cultures, while CO and EC had an opposite trend. VOCs associated with oxidation of linoleic acid (8 carbon atoms) were detected more often at lower culturing temperatures. Fermentative metabolism VOCs were shorter in carbon chain length and detected more often at higher temperatures. The VOC carbon chain length analysis was also performed as a function of starting culture pH. Generally, a smaller number of VOC compounds were detected at lower pH values (Table 2).

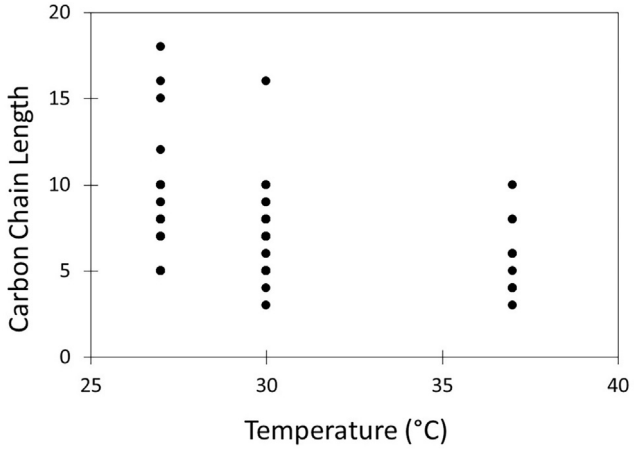

Fig. 1 – Carbon chain length for VOCs produced by three fungal endophyte isolates (EC, CO, TI) as a function of temperature at pH 6 during batch growth on glucose media.

Additional metabolic byproducts

Culture supernatants were analyzed for soluble organic byproducts using HPLC. All three *Nodulisporium* isolates produced substantial amounts of glycerol, up to 4 g L^{-1} (Supplementary Fig. S6). Glycerol production was highest near optimal temperatures and did not demonstrate a statistically significant trend with pH. Ethanol production was detected for isolates CO and TI under a variety of conditions. Ethanol concentrations increased as the specific biomass accumulation rate decreased suggesting a correlation to culturing stress like oxygen depletion (Supplementary Fig. S7).

Implications

Filamentous fungi produce a wide variety of bioactive and industrially relevant metabolites, including numerous VOCs (Strobel et al. 1996; Keller et al. 2005). Longer carbon chain lengths are desirable for biofuels, since they are more energy dense (Strobel et al. 2008). The presented data showed longer chain VOCs were favored at lower culturing temperatures; this is the first report of such behavior. This is opposite of the trend observed in fungal membrane fatty acid carbon chain length; typically membrane fatty acids decrease in length as temperatures decrease to maintain membrane properties (Suutari et al. 1997). The increase in secreted VOC chain length may be due to temperature dependent kinetics of fatty acid synthesis reactions (Kates and Baxter 1962) or temperature dependent differences in cell membrane properties that increase excretion of longer carbon chain length VOCs (Liu et al. 2011; Kawahara et al. 2016). Alternatively, many of the VOCs detected are bioactive and their production may have evolved to support endophyte ecological functions. Plant transpiration, the transport and evaporation of water, can maintain plant tissues at cooler than ambient temperatures which could be a key ecological parameter for these tropical isolates (Edwards and Hanson 1996; Stockfors 2000). Several large bioactive compounds, including terpenes, were detected at lower culturing temperatures and may play a role protecting the living plant host against pathogens and herbivores (Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011). Endophytes spend part of their lifecycle within live plants but may switch to saprophytic phenotypes upon host death or otherwise live outside the host (Porras-Alfaro and Bayman 2011). Warmer culturing temperatures may replicate conditions associated with

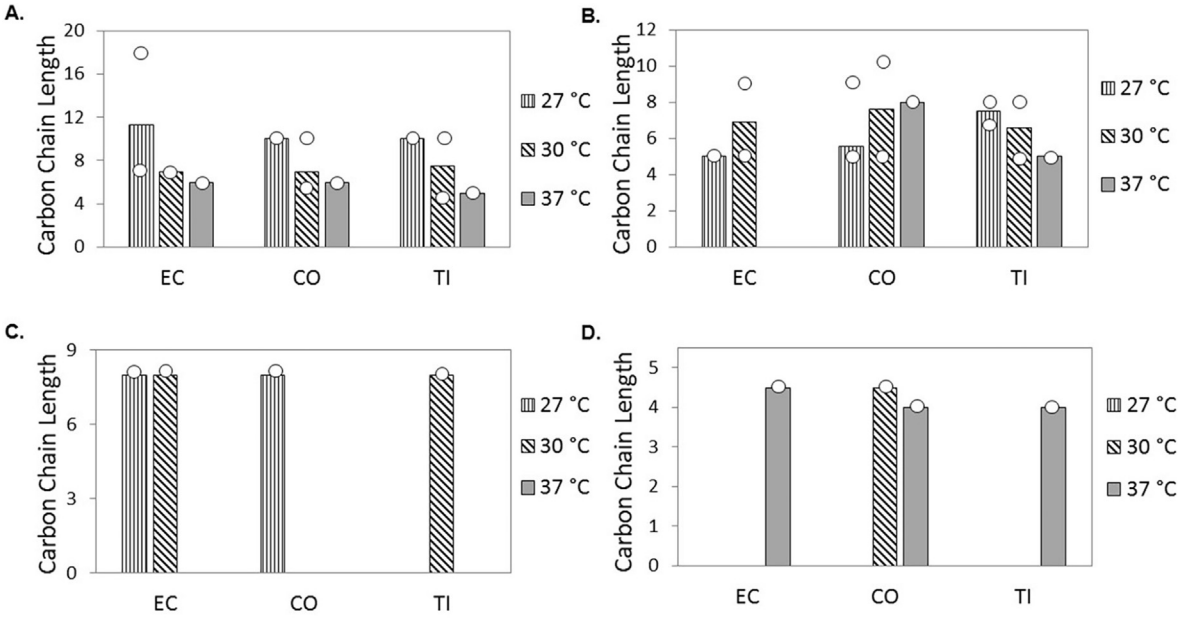

Fig. 2 – Fungal endophyte isolate (EC, CO, TI) VOC carbon chain length as a function of temperature at pH 6 during batch growth on glucose media and as a function of metabolism type (A: Fatty acid synthesis, B: Ehrlich, C: Oxidation of linoleic acid, D: Fermentative metabolism). The height of the bars represents the average carbon chain length of the VOCs and the dots the minimum and maximum carbon chain length identified at each condition.

fungal growth within a dead plant or in the tropical environment, where a switch toward fermentative metabolism would be ecologically competitive.

In summary, three *Nodulisporium* isolates produced biofuel- and flavor-relevant VOCs. The carbon chain length of the VOCs increased as culturing temperatures decreased. The fungi grew on a wide variety of low cost feedstocks, including sugar beet pulp, corn stover, and grass clippings. The *Nodulisporium* isolates grew quickly with specific biomass accumulation rates up to 1.6 d^{-1} and produced relatively high biomass titers with maximum concentrations ranging from 13 to 19 g cell dry weight L^{-1}. The presented work also increases the number of physiologically-characterized fungal endophyte isolates from four to seven.

Disclosure

The authors declare that they have no conflicts of interest.

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