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Note

Carbon chain length of biofuel- and flavor-relevant volatile organic compounds produced by lignocellulolytic fungal endophytes changes with culture temperature

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ABSTRACT

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.

Endophytes live within plant tissues without causing apparent symptoms and have been found in all plant species 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). Production of biofuels from waste lignocellulosic biomass is a major global research goal due to finite petroleum reserves and atmospheric greenhouse gas increases (Sánchez 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

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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.

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