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The white-rot fungus, *Phanerochaete chrysosporium*, under combinatorial stress produces variable oil profiles following analysis of secondary metabolites

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Abstract

Aims: The effects of combinatorial stress on lipid production in *Phanerochaete chrysosporium* remain understudied. This species of white-rot fungi was cultivated on solid-state media while under variable levels of known abiotic and biotic stressors to establish the effect upon fungal oil profiles.

Methods and Results: Environmental stressors induced upon the fungus included the following: temperature, nutrient limitation and interspecies competition to assess impact upon oil profiles. Fatty acid type and its concentration were determined using analytical methods of gas chromatography and mass spectrometry. Growth rate under stress was established using high-performance liquid chromatography with ergosterol as the biomarker. Fungi grown on solid-state agar were able to simultaneously produce short- and long-chain fatty acids which appeared to be influenced by nutritional composition as well as temperature. Addition of nitrogen supplements increased the growth rate, but lipid dynamics remained unchanged. Introducing competition-induced stress had significantly altered the production of certain fatty acids beyond that of the monoculture while under nutrient-limiting conditions. Linoleic acid concentrations, for example, increased from an average of 885 ng μl^{-1} at monoculture towards 13 820 ng μl^{-1} at co-culture, following 7 days of incubation.

Conclusions: Interspecies competition produced the most notable impact on lipid production for solid-state media cultivated fungi while the addition of nitrogen supplementation presented growth and lipid accumulation to be uncorrelated. Combinatorial stress therefore influences the yield of overall lipid production as well as the number of intermediate fatty acids produced, deriving similar oil profiles to the composition of vegetable and fish oils.

Significance and Impact of the Study: Fungal secondary metabolism remains highly sensitive following combinatorial stress. The outcome impacts the research towards optimizing fungal oil profiles for biomass and nutrition. Future investigations on fungal stress tolerance mechanisms need to address these environmental factors throughout the experimental design.

Introduction

The white-rot fungus, *Phanerochaete chrysosporium*, is a species belonging to the Basidiomycota phylum of filamentous fungi (Burdsall and Eslyn 1974; Burdsall 1985) which possesses both ecological and genomic interest within the mycological research community (Kersten and Cullen 2006; Singh and Chen 2008; Liu and Qu

2019). White-rot fungi generally function as important members within both the carbon and nitrogen cycles by converting carbon-rich lignocellulose into glucose and other sugars from non-living organic substrates via decomposition. This complex process is achieved by utilizing a specialized array of hydrolytic enzymes which allow these organisms to colonize a variety of niches while continuing the recycling of nutrients. However, the adopted consensus by which fungal lignocellulosic degradation achieves the production of glucose remains incomplete as notable by-products are also derived. These include single cell oils (SCOs). This species, among other fungi, have been characterized as oleaginous organisms, capable of producing lipids of variable chain lengths throughout growth as microbial oils (Cohen and Ratledge 2010; Athenaki et al. 2018). These secondary metabolites are derived from de novo lipid accumulation, a mechanism comprising various enzymes specialized in altering the chain length and double bond number of saturated fatty acids (SFAs) and converting them to monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) (Chemler et al. 2006; Tang et al. 2015; Ochsenreither et al. 2016; Athenaki et al. 2018). These specialized metabolites are commonly produced by oleaginous bacteria, fungi and microalgae with some species accumulating SCOs higher than 20–70% w/w of their dry cell weight (Ratledge 2004; J.P and C 2005). Although this adaptation has been fully characterized among many species of fungi, the overarching purpose behind long-chain fatty acid production remains partially obscure. Multiple studies have attempted to ascribe various functions behind de novo lipid accumulation (Sumner 1973; Da Rocha Campos et al. 2008), particularly in filamentous fungi, with many speculations falling under the assumption of storage material production, cell membrane constituents as well as extracellular products for cell signalling, energy supply and cell death (Klug and Daum 2014). Genomic sequencing of P. chrysosporium from 2004 (Martinez et al. 2004; Kersten and Cullen 2006) also supports the potential for flexible yet specialized secondary metabolite production and therefore presents an avenue for mycological research. The complexity of this remarkable process highlights the possible basis of how certain species of filamentous fungi can adapt within constantly changing environmental conditions by altering their metabolism and enzymatic activity. Currently accepted reports of lipid accumulation have specified the process to occur under stressful, nutrient-limiting conditions throughout the stationary phase of growth, specifically, along the lines of simultaneous carbon excess and nitrogen/phosphorous limitation (Ratledge 2004; J.P. and C 2005; Kuttiraja et al. 2016; Ochsenreither et al. 2016; Wang et al. 2020). Therefore, suggesting growth and lipid

accumulation have the potential to be independent of one another. Notable PUFA production is thus proposed to change as a result of inducing different types of abiotic/biotic stress regardless of fungal mass. The ability to significantly alter the yields of multiple types of short-/-long-chain fatty acids presents oleaginous fungi as a potential resource for SCOs of commercial value. This commercial value extends towards using fungal oil for human nutrition in the form of omega-3/-6 PUFAs which are important for human health as intermediates for eicosanoids (Shinmen et al. 1989; Stressler et al. 2013; Bellou et al. 2016). The aim of this investigation, therefore, is to understand the environmental factors which trigger the shift of lipid production towards creating PUFAs from SFAs. Phanerochaete chrysosporium itself has been identified as a fungal model organism with notable environmental adaptations towards temperature fluctuations and nutritional alteration (Burdsall and Eslyn 1974; Lamar et al. 1987). This flexibility in adaptation presents this species as a suitable organism to analyse the oil profiles while grown under varying conditions beyond the optimal range. In this work, we investigated the ability of the fungus to convert SFAs to PUFAs by inducing combinatorial stresses towards P. chrysosporium to mediate stress tolerance secondary metabolism. We then assessed the extent environmental factors have towards influencing the abundance of specific PUFAs for this species. The results gathered allude to the possible functions lipid accumulation possesses towards tolerating environmental stressors. Multiple stressors were considered such as introducing the fungi to nutrient-rich and nutrient-limiting agar media. These included the mycological peptone-rich malt extract agar (MEA) and the sucrose-based soil bacterium media Czapek Dox agar (CDA), as well as others, all possessing variable carbon and nitrogen sources. Organic waste biomass such as wheat straw was also considered as a feedstock. Wheat straw not only contains the appropriate lignocellulose needed for growth but is also a common by-product of the agricultural industry. Assessing the effect nutritional composition has on specific secondary metabolite production may allow the optimization of acquiring a suitable substrate required to yield commercially desirable PUFAs for nutritional needs. Wheat-straw-cultivated fungi were also treated with variable nitrogen-based supplements: the organic amino acids glycine and L-methionine. Comparing the growth rate with the oil profiles will further deduce whether the link between fungal biomass and sequential lipid accumulation should be considered for future investigations.

All nutrient types were combined with three different temperatures (22, 30 and 37°C) to analyse the enzymatic limitation of the lipid accumulation pathway and thus

assess the effect combinatorial stress has on metabolism. Growth was observed across multiple time intervals (7, 14 and 21 days) and oil profiles of the fatty acids produced were analysed and quantified using gas chromatography (GC). Fungal growth itself is challenging to quantify visually and ergosterol assays were used to determine growth on the molecular scale with straw-cultivated fungi and high-performance liquid chromatography (HPLC). Limitations of this assay do persist however, as ergosterol from dead fungi is just as stable as when collected from living fungal biomass. Nevertheless, the assay was still used, following repeats, as several studies still incorporate the assay as a tool with recommended caution throughout data interpretation (Mille-Lindblom *et al.* 2004; Zhao *et al.* 2005; Young *et al.* 2006). Besides temperature and nutrient limitation, interspecies competition was also introduced. Competition among resources is a known biotic stressor which drives secondary metabolite production among fungi as well as other micro-organisms while representing a true, dynamic environment (Magdouli *et al.* 2016). *Phanerochaete chrysosporium* fungi were grown alongside another species of white-rot fungus, *Trametes versicolor*, which occupies a similar niche alongside the same temperature gradient and nutritional needs. Combining competition with both nutrient-limiting substrates and steep temperature gradients presents a tripartite combination of stressors which may induce the production of unusual secondary metabolite. Fungal cultivation across combinatorial stressors could reveal a significant trend in the relationship between *de novo* lipid accumulation and stress tolerance mechanisms. The results gathered had demonstrated that *P. chrysosporium* itself can tolerate a wide range of temperatures and nutrient limitations, but the oil profiles reveal an underlying, complex reaction towards combinatorial stresses which appears to influence the production of unusual fatty acids. The results obtained have implications for future studies considering the usage of fungal oil for nutritional benefit and delivery.

Materials and Methods

Fungal cultivation (media culture)

Freeze-dried cultures of *P. chrysosporium* were delivered from the Leibniz Institute DSMZ—German Collection of Micro-organisms and Cell Cultures GmbH and stored at 4°C. Stock fungi were cultivated using rye grain and plated under sterile conditions (100 mm diameter plates). Fungi on media culture were grown over three weekly intervals of 7, 14 and 21 days on individual growth media (30 ml per plate) including PDA (potato dextrose agar), SDA (sauboraud dextrose agar), CDA and MEA at

varying temperatures (22, 30 and 37°C) away from sunlight. See Table 1 for nutrient composition and Table S1 for experimental design.

Stocks of *T. versicolor* were received as plug spawn from Gourmet Woodland Mushrooms. PDA was inoculated with plug spawn under sterile conditions before cultivation on autoclaved rye grain.

Fungal cultivation (wheat straw)

Stock fungi were also grown on dry, autoclaved wheat straw (10 g) added with 13 ml of sterile water in sealed jars. Straw-cultivated fungi was grown along the same weekly intervals (7, 14 and 21 days) and the same temperatures (22, 30 and 37°C).

Individual jars of straw were also inoculated with nitrogen supplements. The following supplements L-Methionine (Sigma-Aldrich, Dorset, UK, catalogue no. M-9625) and Glycine (Sigma-Aldrich, catalogue no. 33226) were made in solution with a concentration of 12 g l^{-1} in sterilized water. 1 ml of each solution was added to straw-cultivated fungi and left to grow along the same time intervals.

Table 1 The nutritional composition of all five agar substrates used throughout the study. Ingredients with (C) are the primary carbon source of the media while ingredients with (N) are the primary nitrogen source.

Media type	Ingredients	g l^{-1}
PDA	Dextrose (C)	20·0
	Potato peptone (N)	4·0
	Agar	15·0
SDA(PD)—Pancreatic Digest	Dextrose (C)	20·0
	Pancreatic digest of casein (N)	5·0
	Pancreatic digest of animal tissue (N)	5·0
	Agar	15·0
SDA(MP)—Mycological Peptone	Dextrose (C)	40·0
	Mycological peptone (N)	10·0
	Agar	15·0
CDA	Sucrose (C)	30·0
	Sodium nitrate (N)	3·0
	Potassium chloride	0·5
	Magnesium sulphate heptahydrate	0·5
	Iron (II) sulphate heptahydrate	0·01
	Di-potassium hydrogen phosphate	1·0
	Agar	15·0
MEA	Malt extract (C)	30·0
	Mycological peptone (N)	5·0
	Agar	15·0

Transesterification of fungal lipids

Under sterile conditions, <100 mg of fungal mycelia from each agar plate was taken and placed into a 2 ml screw-cap vial (Supelco® Sigma-Aldrich—27079) to be transesterified directly, once the growth interval was complete. 10 ml of 25 mg ml^{-1} 15:0 FFA (free fatty acid) internal standard was added to the sample and 0·5 g of Tripentadecanoin (Sigma-Aldrich Catalogue no. T4257) was added to 20 ml of HPLC-grade Chloroform (Rathburn RH1009). This was followed by addition of 500 ml of 1 mol l^{-1} MeOH/HCl, 600 ml 1 mol l^{-1} MeOH/HCl made from 200 ml 3 mol l^{-1} MeOH/HCl (Sigma-Aldrich 33050-U) and 400 ml HPLC-grade Methanol (Rathburn RH1019)). Once combined, the vials were capped (Supelco® Sigma-Aldrich, 27261-U) and vortexed to allow mixing and release of lipids, and then transferred to a rack and incubated at 80°C for 24 h to ensure complete derivatization.

The above procedure was suitable for straw-cultivated fungi. 10 ml of ethanol (Sigma-Aldrich) and 30 ml of dichloromethane (Sigma-Aldrich) were added to the jars after each time period and shaken at 250 rev min^{-1} over 24 h at room temperature to separate the fatty acids from biomass. The biomass was then pressed using a FRENCH® Pressure Cell Press to remove the fatty acids contained in the liquid. The liquid contents were then stored at −20°C for transesterification. 50 ml of the liquid (containing fungal lipids) was used for derivatization and placed into the screw-cap vials, the rest of the procedure was commenced exactly as follows for fungal mycelia-based transesterification.

Once derivatization was complete for both straw-/agar-cultivated fungal samples, the vials were left to reach room temperature. 250 ml of 0·9% KCl (5·4 g KCl (Fisher Scientific—7447407) added to 600 ml of H$_2$O) was added followed by 250 ml of HPLC-grade Hexane (Rathburn RH1002). The vials were vortexed briefly and left to stand for 10–30 min. Around 500 ml of the fatty acid methyl ester (FAME) layer (upper hexane layer) was removed and placed into a fresh vial and stored at −20°C.

Gas Chromatography

Samples were run on an Agilent Technologies 6850 Series Gas Chromatograph with GC/MSD ChemStation software (Agilent Technologies, Stockport, UK). Nitrogen (carrier gas), Hydrogen and Air (detector gases) provided air flow within a BPX70 column (length 10 m, diameter 0·1 mm, 0·2 mm film). Nitrogen gas had a constant flow of 30 ml min^{-1}. The Detector Flame Ionisation Detector was set at 240°C and the oven temperature programme at 150°C (0·1 min), 15°C min^{-1} to 240°C (6 min). Hexane (Sigma-Aldrich, catalogue no. 34859-M) was used to wash the needle and provided as a control sample. 1 ml of 10 mg ml^{-1} Supelco 37 component FAME standard (CRM47885) was used to calibrate the sequence. Samples were loaded as 1ml per run. GC reports were collated as .txt files after running through a Perl script (Raku) as individual fatty acid methyl esters (FAMEs) concentrations (ng ml^{-1}).

GC-Mass Spectrometry

Samples prepared for GC were also prepared for GC-MS to identify fatty acids which were not specified on the FAME standard (>24 carbons long). After GC, 800 ml of HPLC-grade Hexane (Rathburn RH1002) was added to the samples to increase volume for drying. 200 mg of anhydrous sodium sulphate (Sigma-Aldrich—7757826) was added to remove water. After drying, the sample was filtered (0·2 mm filter—Fisher Scientific—10509821) into new vials and sent off for analysis with pure hexane (control) and 1 mg ml^{-1} of Supelco 37 component FAME standard (CRM47885) for calibration. The samples were analysed by the Gas Chromatography Agilent Technologies 7890B and the Mass Spectrometer Agilent Technologies 5977B MSD.

Statistical Analysis of FAME concentrations

Fatty acid concentrations collated from GC analysis were grouped as SFAs, MUFAs and PUFAs. The data were processed using IBM® SPSS® Statistics 24 with an overall 95% confidence level ($P < 0.05$) to be taken as significant. Data were tested for normality with a Shapiro–Wilk test and transformed when necessary. Datasets proven to have normal distribution were tested for equal variance with a Levene's Test. The results suitable for parametric testing were tested with a one-way analysis of variance (ANOVA) or a Welch's ANOVA if data lacked equal variance but carried a normal distribution. Datasets which gave significance for the Shapiro–Wilk test were put through nonparametric testing with a Kruskal–Wallis H test and a Dunn's post hoc tests if $P < 0.05$. The Mann–Whitney U Test was another nonparametric test to assess significance between two groups.

Ergosterol Assay

After time intervals, 1400 mg of straw-cultivated mycelia was placed in a 15-ml falcon tube under sterile conditions and freeze-dried (Christ Alpha 1-2 LDplus—SciQuip) for 24 h at −50°C between 0·1 and 0·25 mBar. The sample was removed and placed in a 20-ml

scintillation vial with 2 ml of ethanol and 3-mm Tungsten Carbide Beads (QIAGEN No. 69997). Once vortexed, the vials were placed in an orbital shaker for 1 h at 25°C (350 rev min^{-1}) in complete darkness. Vials were then allowed to precipitate at room temperature and aliquoted (1·5 ml) into a 2-ml microcentrifuge tube. Once spun for 10 min at 4°C (11 000 rev min^{-1} approx. 13 627 g), the supernatant is filtered into a new 1 ml HPLC dark vial (Chromacol^{TM}) and kept at −20°C prior to HPLC.

High-performance liquid chromatography

Samples of ergosterol were analysed using HPLC Agilent Technologies 1260 Infinity machine with a Luna® 5 mm C18(2) 100A LC column (250 mm × 4·6 mm). Conditions for the analysis were optimized at UV detection rate of 282 nm with only HPLC-grade methanol (Fisher Chemical M/4056/17) as the mobile phase. The methanol flow rate was maintained at 1·5 ml min^{-1} with a column pressure of $1·15 \times 10^7$ Pa and column temperature of 25°C. Different concentrations of Ergosterol standard (DrEhrenstorfer^{TM}—57874) were used for calibration with pure ethanol as the control sample.

Results

Stress tolerance upon alteration of nutritional medium

All five media types were composed of variable nutrient types which each appeared to impact the production of unusual fatty acids, particularly PUFAs. Fungal growth was consistent across all media types and fungal biomass became more evident over time. It was clear that *P. chrysosporium* had an optimal temperature range above 30°C as the fungus became more established within a shorter span of time when compared to room temperature. With the GC-FID data, only one media, CDA, could not provide any oil profiles at 30°C with limited production at the other temperature variants (22 and 37°C) (see Fig. 1). It became evident that CDA was not as nutritionally accessible as the other media as CDA applies for soil micro-organisms, rather than Basidiomycota. Across all media, distinct fatty acids were repeatedly observed across the given time frame which all fell into the categories of saturated and unsaturated fatty acids. SFAs typically produced were palmitic acid (**16:0**) and lignoceric acid (**24:0**) which were both observed either simultaneously or at distinct time points across all media types and temperature constraints. Commonly observed unsaturated fatty acids included the MUFAs, nervonic acid (**24:1(n-9)**), vaccenic acid (**18:1(n-9)**) and PUFAs, linoleic (**18:2(n-6)**) omega-6 fatty acid, docosahexaenoic (**22:6(n-3)**) omega-3 fatty acid. Nutritional composition, specifically carbon

and nitrogen source, appeared to influence the production of these fatty acids (see Fig. 1) with unsaturated fatty acids overtaking SFA production with exception to MEA and CDA at 22°C. Moreover, the shift from SFA to PUFA production correlates with increased temperature constraint as *P. chrysosporium* was shifted to optimal temperature range. However, the production of certain individual fatty acids was not consistent over time, with some fatty acid types diminishing while others became more common. Nevertheless, the GC-FID data depict that the cultivation of *Phanerochaete* white-rot fungi on different substrates confers the production of unusual fatty acids associated with fish and vegetable oils.

The GC-MS approach was able to detect a much wider range of unusual fatty acids which the GC-FID was unable to give due to FAME standard limitations (see Table S2). All samples had been analysed from 37°C which was the closest temperature for optimal growth, allowing nutritional composition to become a standalone factor towards initiating the stress response. Commonly observed fatty acids, **C16:0** and **C18:2(n-6)**, were present along with a variety of very-long-chain fatty acids (VLCFAs) which fitted the category of saturated/unsaturated. These VLCFAs included melissic acid (**30:0**) as well as octacosapentaenoic (**28:5**) and hexacosatrienoic acid (**26:3**). However, the GC-MS failed to detect certain fatty acids confirmed by GC-FID, such as nervonic acid, with low detections of vaccenic and docosahexaenoic acid. Fungi cultivated on CDA gave the lowest count of fatty acids with rare occurrences of LCFAs while production shifted towards medium-chain fatty acids (Table S2). *Phanerochaete chrysosporium* cultivated on MEA for 7 days at 37°C gave the largest variety of lipids ranging from enanthic acid (**7:0**) to **C29:6** fatty acid. SDA-PD-cultivated fungi, grown after 14 days, gave both the longest and shortest fatty acid detected (**30:0** and caproic (**6:0**) acid), both produced simultaneously. To conclude, the nutritional composition, particularly the nitrogen source, impacts the degree of production of unusual PUFAs, going further with the simultaneous production of VLCFAs and short-chain fatty acids (SCFAs).

Stress tolerance upon supplementation of organic substrate

With wheat straw as the substrate, lipid accumulation appeared to remain similar when compared to agar-based substrate with production of previously recognized fatty acids (**16:0, 24:0, 22:6(n-3), 24:1(n-9)**), albeit a few others were observed including the SFA, undecylic acid (**11:0**) and the PUFA, arachidonic (**20:4(n-6)**) omega-6 fatty acid (see Fig. 2). With control straw, total PUFA production was marginally higher than SFA, although this
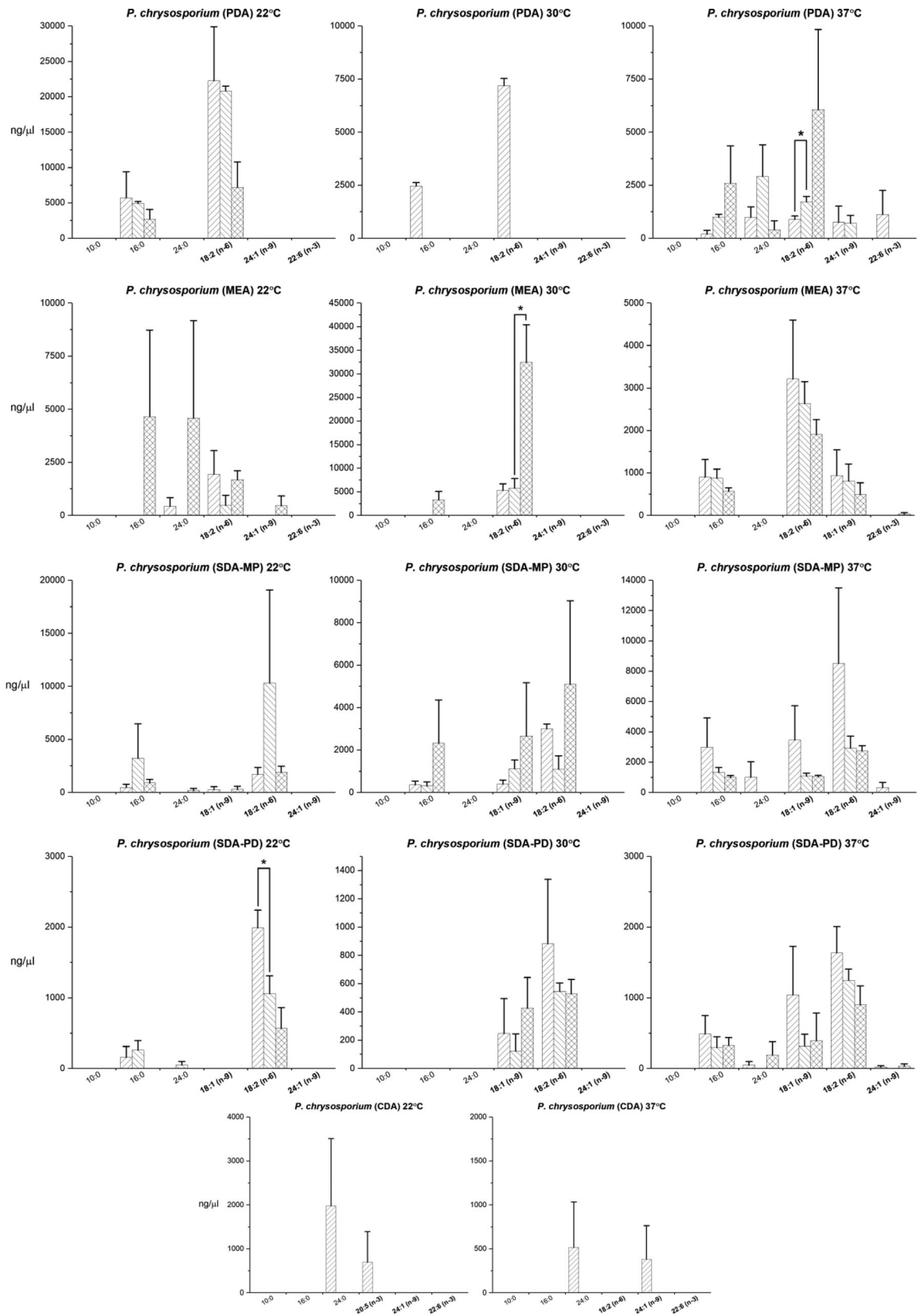
Figure 1 Alteration of substrate nutritional composition produces variable concentrations of long-chain PUFAs over a 21-day time period. Combined with temperature, the composition of lipids is impacted even further. Six of the most common fatty acids (SFAs/PUFAs) found during the investigation are displayed as patterned bar charts with each plot representing a unique substrate and temperature combination to induce varying stress responses. Time series: (▨) 7 days, (▧) 14 days, (▩) 21 days. PUFAs are labelled in bold on the *x*-axis. *$P \leq 0.05$.

was only observed at the later stages of growth. With ʟ-methionine as the supplement, PUFAs were also produced however, at a much smaller scale with lipid accumulation peaking at 14 days. With glycine, lipid accumulation was diminished further but fatty acids were still observed following 91 days of growth although this production was shifted towards long-chain SFAs. Glycine-supplemented fungi were left to grow for much longer to observe whether stress can still be observed beyond initial establishment of the substrate. In contrast to the oil profiles, the ergosterol assays gave a much different perspective. Typically, with the control, fungal biomass increased marginally overtime at 22°C but as predicted, fungal biomass increased initially with addition of nitrogen-based supplements. Glycine supplements greatly increased fungal biomass in a shorter span of time with fungal biomass more than doubling in scale continually increasing even

at 91 days post-supplementation. However, with ʟ-methionine, despite fungal biomass beginning with a large increase, ergosterol content begins to decrease over time with ergosterol levels below that of glycine but still marginally higher than the control.

As can be seen in the GC-MS output (see Table S3), palmitic acid was the most commonly detected lipid despite the GC-FID data conferring low levels of C16:0. However, low detection rates of C24:0, C18:2(n-6), C22:6 (n-3), C24:1(n-9) and C20:4(n-9) were observed opposite to the GC-FID data. Instead, very unusual lipids, the GC-FID cannot detect, were seen, again similar to the results gathered of the agar-cultivated fungi. Although lipid variety was notably reduced with the shortest fatty acid at 12 carbons (Table S3), all straw-cultivated fungi were still able to give readings of VLCFAs. Addition of glycine gave the lowest average count of fatty acid types across the
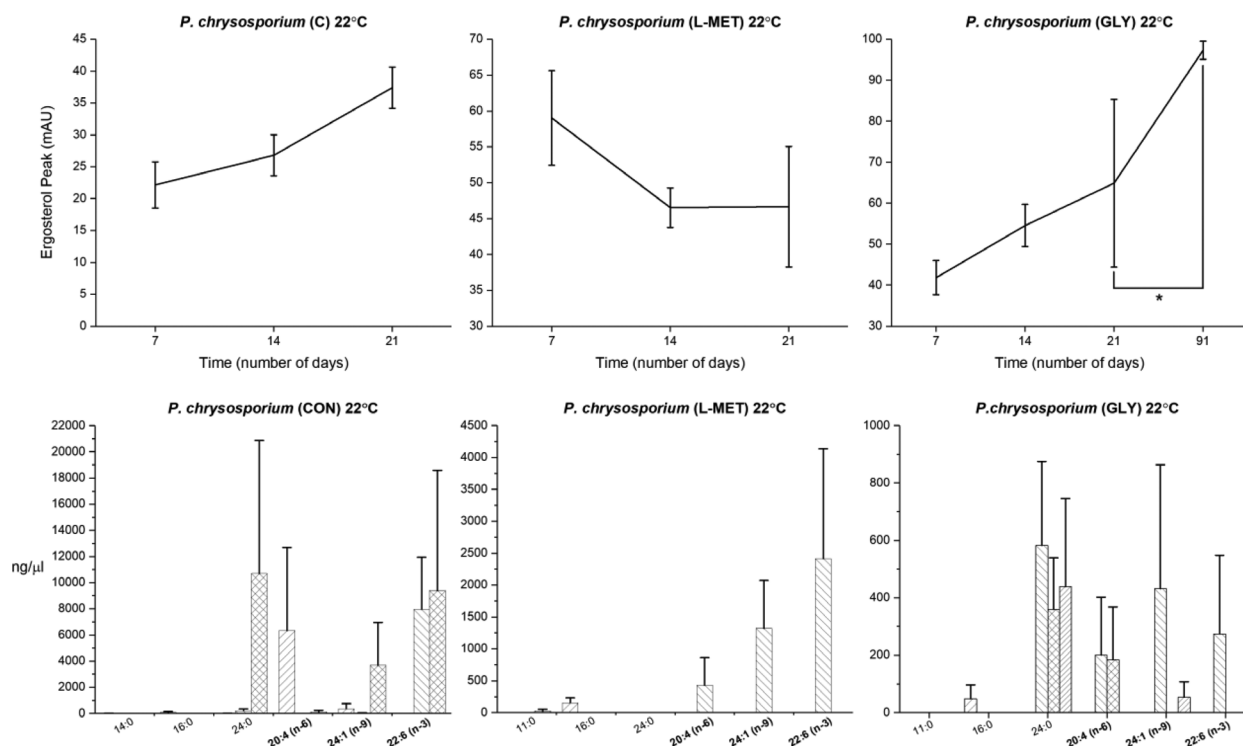

Figure 2 Addition of nitrogen supplements in tandem with straw cultivation appears to reduce the concentration of fatty acids produced during growth despite fungal biomass increasing beyond the control (C/CON). The top three scatter plots depict ergosterol quantity changes over time measured with HPLC while the bottom three bar charts represent the related fatty acid profiles detected using GC-FID within the same time points. Time series: (▨) 7 days, (▧) 14 days, (▩) 21 days, (▨) 91 days. All supplement experiments were grown at 22°C. PUFAs labelled in bold on *x*-axis. *$P \leq 0.05$.

three time points and gave the longest VLCFAs at 7 days of growth. But the trend in low lipid count was also observed among the other variable including L-methionine addition and the control itself which gave the most counts at 7 days following inoculation. The GC-MS results therefore depict straw-cultivated fungi conferring reduced output of very unusual fatty acids when compared to agar-cultivated fungi.

Stress tolerance following competition between species within closed system

Combining two species of white-rot fungi, both competing for the same substrate, within differing temperature gradients, has led to different responses in secondary metabolite production when compared to the monoculture variants of both *P. chrysosporium* (Fig. 1) and *T.*

versicolor (see Fig. S1). The induction of tripartite stress has produced an assortment of unusual PUFAs simultaneously with SFAs including the medium-chain fatty acid, capric acid (**10:0**) and the PUFA, eicosapentaenoic (**20:5 (n-3)**) omega-3 fatty acid. At 22°C, it was clear that this temperature gradient was more optimal for *T. versicolor* than *P. chrysosporium* by visually observing growth (see Fig. 3). Over time, *Trametes* growth excluded *Phanerochaete* growth on the substrate but at higher temperatures, the opposite was observed. At 30°C, *Trametes* growth was reduced to small outcrops among *Phanerochaete* mycelia while at 37°C, *Phanerochaete* remained dominant throughout initial growth. The corresponding oil profiles at 30°C were most interesting, however, with the simultaneous production of medium-chain SFAs (**10:0**) and long-chain PUFAs (**22:6(n-3)**) after 7 days. This outcome became less apparent over time with fatty
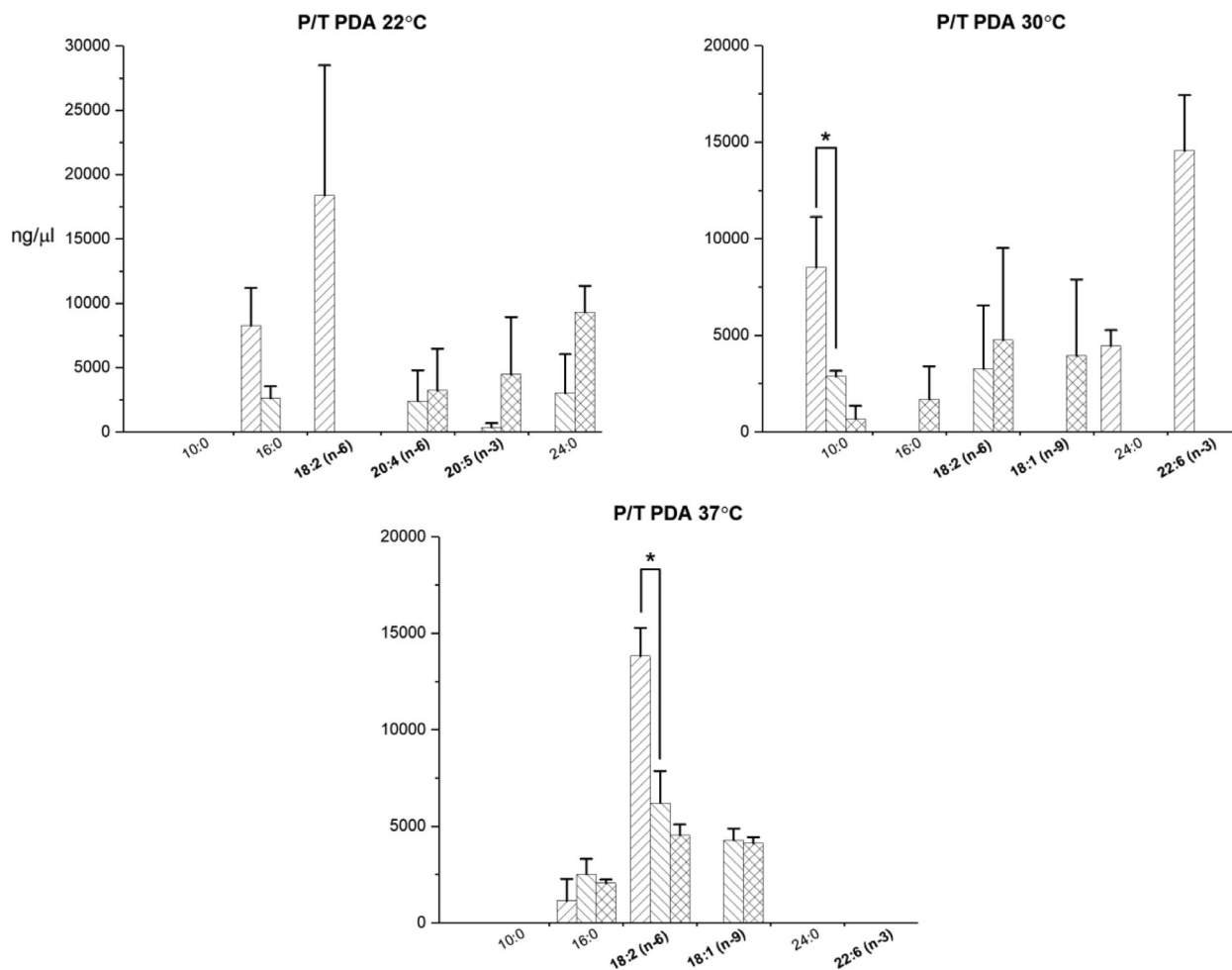

Figure 3 *Phanerochaete chrysosporium* produces PUFAs (*x*-axis labelled in bold) while under tripartite stress from nutritional limitation, temperature change and competition with another white-rot species. The three charts show the change in concentration of particular fatty acids over the course of 21 days with each chart representing each temperature. Time series: (▨) 7 days, (▧) 14 days, (▩) 21 days. *$P \leq 0.05$.
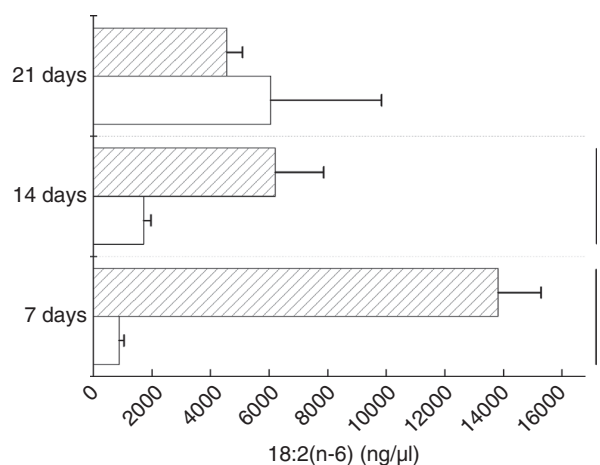
Figure 4 Bar graph depicting average concentration of PUFA, linoleic acid (18:2(n-6)), over time between PDA-cultivated *Phanerochaete chrysosporium* monoculture and PDA-cultivated co-culture, both at 37°C. Culture series: (▨) Co-culture, (☐) Monoculture. *$P \leq 0.05$.

acid production shifting towards PUFA production across all three temperatures. What is notable when comparing co-culture to *Phanerochaete* monoculture was the number of different fatty acids produced. At 22 and 30°C, the oil profiles appeared much more dynamic in composition when compared to monoculture variants with PDA as the substrate. Whereas, at 37°C, lipid composition in the co-cultures was reduced in range although the production of individual lipids such as linoleic acid was significantly higher at both 7 and 14 days than monoculture (see Fig. 4) which then dropped after 21 days. Total fatty acid production, however, diminished over time for co-culture. The same was true for co-culture at 22°C when compared to *Phanerochaete* monoculture but was much higher when compared to *Trametes* monoculture at 22°C (Fig. S1). Overall, the induction of multiple stressors results in the oil profiles remaining non-consistent with that of monoculture variants, thereby establishing competition as another valid parameter towards overall fatty acid production.

Lipid profiles were also run through GC-MS which again gave a wide variety of fatty acids ranging from C6:1 to C28:5 (see Table S4). All three temperature gradients were taken as both *Trametes* and *Phanerochaete* possessed opposing optimal temperature ranges which would confer the extent competition itself imposed towards lipid accumulation as one species excludes the other. Palmitic acid was detected the most along with linoleic acid. Unusual PUFAs including C17:2 were found to appear in almost all samples despite the adjacent SFA, margaric acid (**17:0**) only appearing once after 21 days at 37°C. Capric acid (**10:0**), which was found at high concentrations at 30°C with the GC-FID, was only detected at 22 and 37°C for

the GC-MS with only decenoic acid (**10:1**) detected for 30°C. Lignoceric acid gave a similar case conferring with both GC-MS and GC-FID at 30°C but was not observed at 22°C. Individual lipid count appeared to be highest at 22°C at the beginning but decreased over time while the opposite was true for 37°C with lipid counts comparable towards the monoculture version across the same temperature gradient. Altogether, similar lipids were observed when compared to monoculture despite the reduction across the range of fatty acid types. Nonetheless, the results conferred a significant change towards the concentration of particular fatty acids by introducing competition to initiate a tripartite stress response which again manifested towards the production of unusual VLCFAs simultaneously with SCFAs.

Discussion

Understanding the extent combinatorial stress has towards sequential production of fungal fatty acids remains challenging. It is therefore important to assess the impact tripartite stress has towards lipid accumulation overall, rather than just one stress parameter, as combinatorial stress remains a big effector of fungal metabolism; linking secondary metabolism with that of a true environment. Analysing the oil profiles of fungi under combinatorial stress indicates a level of sensitivity for lipid accumulation and thus secondary metabolism overall. By examining the GC-FID/GC-MS data accrued from lipids derived from agar-cultivated fungi, it is again demonstrated that the nitrogen source has a clear impact on the extent of lipid accumulation as certain solid-state media (PDA/SDA) possess the same carbon source (dextrose). However, the comparison between two versions of SDA with differing nitrogen sources (pancreatic digest and mycological peptone) failed to support significant differences in lipid production. This may have followed from SDA-MP having a higher concentration of dextrose thus affecting the initial growth rate of fungi and potential secondary metabolite production. General fungal growth across PDA, SDA-MP/PD and MEA appeared consistent, although ergosterol assays are needed to compare solid-state growth rate at the molecular scale. CDA, on the other hand, is a media type typically used for the cultivation of soil-based micro-organisms which was why *P. chrysosporium* grew so poorly, but to observe lipid production at such small levels of biomass may suggest the link between growth and lipid production at solid-state media is uncorrelated. Nevertheless, the included temperature shift beyond optimal range on solid-state media gave an unpredictable effect on the lipid profiles with the unexpected production of VLCFAs in tandem with SCFAs. The most common fatty acids observed across the

given temperature range were palmitic acid (16:0) and linoleic acid (18:2(n-6)). Palmitic and linoleic acid are both typically associated as the precursors of long-chain MUFAs and PUFAs in the lipid accumulation pathway (Ochsenreither et al. 2016). Enzymes, including desaturases and elongases, add extra double bonds or carbons once under stress, leading to the shift towards VLCFAs from medium-chain SFAs. Temperature changes may thus alter the enzymatic activity which directs the conversion rate of these two fatty acids into omega 3/6 PUFAs. Therefore, palmitic and linoleic acid could be used as an indirect indicator to assess the effects of combinatorial stress by linking elongase and desaturase activity. Assuming lipid accumulation has an influence on cell membrane constituents, it can be argued that the shift from SFA to PUFA production is related to membrane fluidity with the phospholipid bilayer switching from saturated hydrophobic tails to unsaturated (Garba et al. 2017). However, increasing PUFA content at rising temperatures can affect cellular activity due to steric hindrance and therefore would be detrimental towards fungal growth (Ferrante et al. 1983; Hatziantonioy and Demetzos 2008; Santomartino et al. 2017; Tezaki et al. 2017). Therefore, lipid accumulation cannot be fully associated with contributing towards membrane components which suggests other functions are at work, following temperature increase. Previous studies have described and demonstrated linoleic acid itself as a link or marker to fungal biomass cultivated on either solid (Yu et al. 2009; Liu et al. 2017) or liquid media (Eiland et al. 2001; Klamer and Bååth 2004). However, this study has failed to establish this using solid media/substrate as there was no correlation observed between increasing ergosterol content and overall lipid production with straw-cultivated fungi. The GC-MS/GC-FID failed to detect high levels of linoleic acid throughout the experiment no matter how far along the growth curve. Despite the increase in fungal biomass following the addition of organic nitrogen supplement, lipid production was observed to be much lower than the control. The outcome therefore further establishes the association of lipid production with nutrient-limited-induced stress rather than an increase in fungal biomass. The complex relationship between growth and secondary metabolite production remains underreported and presents a complication when shifting lipid production to the commercial scale. This investigation, as well as other research (Nurika et al. 2018), supports the consensus by which linoleic acid is unsuitable to determine fungal biomass itself. Ergosterol assays, however, are still a preferred method (Seitz et al. 1979; Gessner 2005) to accurately measure fungal growth, although some reports claim unreliability and therefore express caution when using this biomarker alone to describe fungal growth

(Mille-Lindblom et al. 2004; Zhao et al. 2005; Young et al. 2006). The effects from competition, meanwhile, present an alternative outlook in lipid production. Fungi–fungi interactions are of great interest to research as the results gained are a more realistic comparison to a dynamic ecological community (Hiscox et al. 2018). This study has managed to observe a significant difference in lipid production by introducing another stressor into the closed system: interspecies competition. The effects of two species of white-rot fungi have resulted in the production of linoleic acid significantly increasing beyond the monoculture control even while abiotic conditions were optimal for one species over the other. The results observed appear to mimic the effects of hyperbiotrophy, where a tripartite interaction results in competition which indirectly benefits the host depending on which species becomes displaced (Laur et al. 2018). Trametes fungus temperature range is below optimal for 37°C which is instead more suited for Phanerochaete. But despite Phanerochaete gaining growth majority, the lipid profiles suggest combinatorial stress had triggered a shift towards prioritizing the production of linoleic acid, again demonstrating linoleic acid as an indicator of stress-induced lipid accumulation. The reduction of linoleic acid after 21 days below the monoculture control may have been induced once competition became less apparent as Trametes began to 'die off' while incubating at temperatures beyond the normal temperature range at 37°C (Jo et al. 2010). This assumption hypothesizes that as one species becomes displaced, stress factors are reduced to monoculture levels and fatty acid production begins to diminish overtime. However, another assumption for changing oil profiles is the shift towards producing other secondary metabolites associated with competition. Resources and energy expenditure may go into the production of volatile organic compounds as a defensive mechanism (Hynes et al. 2006). Secondary metabolites may be produced to alter the pH of the environment (Hiscox et al. 2018) or contain antifungal properties with the release of aromatic compounds (Viiri et al. 2001). Nevertheless, the experiment confirms competition-induced stress as a viable parameter towards impacting lipid production. Future studies may consider assessing the impact of combining more species of white-rot fungi into closed systems to observe the effects on oil profiles and specific PUFAs as biomarkers for stress. The presence of certain unusual fatty acids was unexpected as complex, lesser-known intermediates of PUFAs were observed. The oil profiles of P. chrysosporium itself match very closely to the long-chain PUFAs derived from fish oils as well as vegetable and algal oils with the production of docosahexaenoic acid (22:6(n-3)) and oleic acid (18:1(n-9c)), both typically observed in cod liver oil and linoleic acid

found in vegetable oil. After following combinatorial stress, the observed oil profiles included the VLCFA melissic acid (30:0) (also known as triacontanoic acid), which in one study has been associated with anti-fungal activity *in vitro* (Bordoloi *et al.* 2017). Distinct omega-9 fatty acids such as nervonic acid were also found which have been linked with mammalian neural tissue (Martínez and Mougan 2002). It remains unclear whether unusual intermediate fatty acids or groups of similar fatty acids possess specialized roles within fungal metabolism, but what is demonstrated is that combinatorial stress notablydisrupts these processes along the lipid accumulation pathway. To conclude, studies assessing the effects of combinatorial stress upon lipid accumulation are limited, especially towards white-rot fungal species. But fungi naturally encounter combinatorial stress within their natural environment and therefore oil profiles under tripartite stress remain an accurate representation of secondary metabolism. This study has managed to derive significant changes in fatty acid production following combinatorial stress on solid-state media and highlights how lipid accumulation and fungal growth are uncorrelated. Competition-induced stress was also able to highlight a notable change in oil profiles which should be observed as a suitable parameter while designing future experiments. Overall, combinatorial stress should be considered as a valid parameter when either yielding notable PUFAs or understanding the underlying mechanisms and sensitivity of secondary metabolism. Further optimization of the growth conditions is necessary to derive PUFAs of commercial value with regard to human nutritional application.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Experimental design referred towards the whole investigation and which variables each experiment falls under.

Table S2. GC-MS data. Inducing stress via nutrient limitation and alteration results in the production of unusual very-long chain fatty acids both saturated and unsaturated.

Table S3. GC-MS data. Addition of nitrogen-based supplements leads to detection of very-long chain fatty acids as well as medium-chain.

Table S4. GC-MS data. Tripartite stress response features the simultaneous production of short-chain and very-long-chain fatty acids.

Figure S1. Fatty acid profile of *Trametes versicolor* over 21-day incubation at 22°C with PDA as the substrate. PUFAs labelled in bold.