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Functioning of a synthetic lignocellulolytic microbial consortium

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Chapter 3

Defining the eco-enzymological role of the fungal strain *Coniochaeta* sp. 2T2.1 in a tripartite lignocellulolytic microbial consortium

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Abstract

Coniochaeta species are versatile ascomycetes that have great capacity to deconstruct lignocellulose. Here, we explore the transcriptome of *Coniochaeta* sp. strain 2T2.1 from wheat straw-driven cultures with the fungus growing alone or as a member of a synthetic microbial consortium with *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4. The differential expression profiles of carbohydrate-active enzymes indicated an onset of (hemi)cellulose degradation by 2T2.1 during the initial 24 hours of incubation. Within the tripartite consortium, 63 transcripts of strain 2T2.1 were differentially expressed at this time point. The presence of the two bacteria significantly upregulated the expression of one galactose oxidase, one GH79-like enzyme, one multidrug transporter, one laccase-like protein (AA1 family) and two bilirubin oxidases, suggesting that inter-kingdom interactions (e.g. amensalism) take place within this microbial consortium. Overexpression of multicopper oxidases indicated that strain 2T2.1 may be involved in lignin depolymerization (a trait of enzymatic synergism), while *S. multivorum* and *C. freundii* have the metabolic potential to deconstruct arabinoxylan. Under the conditions applied, 2T2.1 appears to be a better degrader of wheat straw when the two bacteria are absent. This conclusion is supported by the observed suppression of its (hemi)cellulolytic arsenal and lower degradation percentages within the microbial consortium.

Keywords

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1 Introduction

A major challenge for biorefineries is the low saccharification efficiency of agricultural residues. Improving this microbial process will facilitate the production of commodity chemicals (e.g. biofuels) (Robak and Balcerek 2018). Due to the complexity of the polysaccharides found in plant biomass, a multitude of enzymes secreted by lignocellulolytic microbial consortia may be required to enhance the saccharification process (Zhu et al. 2016; Alessi et al. 2017). Recently, several studies have unveiled the lignocellulolytic capacity of complex microbiomes (e.g. forest soils) (Cardenas et al. 2015; Žifčáková et al. 2017; Wilhelm et al. 2019), but an in-depth eco-enzymological understanding of these communities is still lacking. Here, eco-enzymology is defined as the study of enzymes and their roles in microbial interactions and modification of the surrounding environment. Thus, the design and characterization of microbial consortia (i.e. low-complexity communities) can improve our understanding of plant biomass degradation processes (Jiménez et al. 2016; Lazuka et al. 2018; Serrano-Gamboa et al. 2019). There are two strategies used to build a microbial consortium: (i) selection and diversity reduction from nature (top-down enrichment) and (ii) design of a synthetic community with bacterial and/or fungal isolates (bottom-up) (Jiménez et al. 2017; Puentes-Téllez and Falcao Salles 2018; Gilmore et al. 2019). Synthetic approaches are excellent ways to study the functional traits, enzymatic dynamics/expression and interactions among the members of lignocellulolytic microbial consortia (Evans et al. 2017; Jiménez, Chaib De Mares and Salles 2018). For instance, Cortes-Tolalpa, Salles and van Elsas (2017) examined the wheat straw degradation potential of synthetic microbial consortia (i.e. bicultures and tricultures of different microbial species). They found high levels of synergism between two bacterial degraders, *Sphingobacterium multivorum* w15 and *Citrobacter freundii* so4, revealing an 18.2-fold increase in cell biomass and significantly enhanced (hemi)cellulolytic activities when grown in biculture compared with monoculture. On the other hand, Daly et al. (2017) evaluated the transcriptional responses of three ascomycete fungi on cultures that contained wheat straw. They showed that the expression of carbohydrate-active enzymes (CAZymes) was lower in fungal bicultures compared to the respective monocultures.

Some *Coniochaeta* species (Ascomycetes; Sordariomycetes) are recognized as versatile microbes due to their capacity to remove toxic compounds from plant biomass hydrolysates (Trifonova et al. 2008; Nichols, Dien and Cotta 2010) and secrete lignocellulolytic enzymes when grown on plant residues (López et al. 2007; Ravindran, Adav and Sze 2012). These organisms have been isolated from different environments (e.g. decaying Acacia trees, Picea abies trees, *Euphorbia polycaulis* plants and the interior of the living moss *Pleurozium*

schreberi) (Weber 2002; van Heerden et al. 2011; U'Ren et al. 2012; Nasr et al. 2018). Although the phylogenetic relationships between different *Coniochaeta* species might be blurred at present, it appears that they all thrive in lignocellulose-rich habitats. Thus, strains affiliated with *Coniochaeta* are frequently found in soil, sediment and decaying wood-derived microbial consortia, along with bacteria belonging to the orders Sphingobacteriales and Enterobacteriales (Cortes-Tolalpa et al. 2016; de Lima Brossi et al. 2016), suggesting that *Coniochaeta* spp. grow in synergy with these bacterial types. In previous work, *Coniochaeta* sp. strain 2T2.1 was isolated from a heat-treated wheat straw-degrading microbial consortium (Jiménez, Korenblum and van Elsas 2014). Based on bacterial 16S rRNA gene and fungal ITS sequencing, we observed that in this consortium (denoted TWS) *Coniochaeta* spp. were highly abundant, along with *Sphingobacterium*, *Klebsiella*, *Pseudomonas* and *Trichosporon* species (Jiménez, Dini-Andreote and van Elsas 2014). Strain 2T2.1 is a dimorphic fungus that forms black colonies on potato dextrose agar and shows yeast-like and mycelial growth in liquid medium containing wheat straw (Figure 1). Genome sequencing and transcriptome analysis showed that strain 2T2.1 has an exceptional lignocellulolytic machinery (Mondo et al. 2019), with significant upregulation of several families of endoxylanases (GH10 and GH11), feruloyl and acetyl xylan esterases (CE1), alpha-L-arabinofuranosidases (GH51 and GH62), endoglucanases (GH12), cellobiohydrolases (GH7) and lytic polysaccharide monooxygenases (AA9) when the fungus was grown on wheat straw compared to glucose. Additionally, strain 2T2.1 was found to have undergone a genome expansion due to a hybridization event (i.e. allopolyploidization) that is thought to endow it with a fitness advantage in plant biomass degradation processes (Mondo et al. 2019).
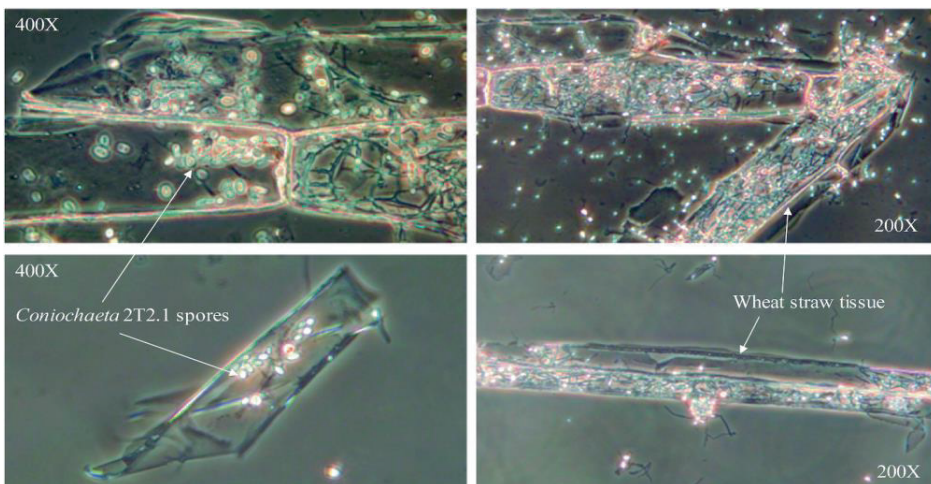
Figure 1. Growth of *Coniochaeta* sp. strain 2T2.1 (yeast appearance) after 24 hours of cultivation in liquid medium containing wheat straw as the sole of carbon and energy source. Microphotograph was taken using a BX60 microscope (Olympus Life Science, Waltham, MA, USA) with Nomarski interference contrast. Pictures at left and right are at 400X and 200X magnification, respectively.

Based on these findings, we hypothesized that a synthetic consortium comprising *Coniochaeta* sp. 2T2.1, *S. multivorum* w15 and *C. freundii* so4 may constitute an excellent microbial system for the development of novel enzyme cocktails useful in plant biomass saccharification, taking into account that the three consortium constituents might exert enzymatic synergism. In this study, we analyzed the differential gene expression profiles of strain 2T2.1 at 24 and 72 hours of cultivation, either alone or as a member of the mixed consortium using wheat straw as the sole source of carbon and energy. We explored the CAZyme profiles in order to assess the expression of fungal lignocellulolytic enzymes in the presence or absence of the two bacteria. We found upregulation of specific multicopper oxidases (MCOs) and glycosyl hydrolases (GHs) during growth with the bacteria, which allowed us to build hypotheses about the eco-enzymological role of strain 2T2.1 within the tripartite consortium. Apparently, *Coniochaeta* sp. 2T2.1 plays a key role in lignin depolymerization, as evidenced by overexpression of a gene for a laccase-like enzyme. This result can be considered as a token of enzymatic synergism. However, the presence of the two bacteria was found to suppress the 2T2.1 (hemi)cellulolytic arsenal, probably due to inter-kingdom competition and/or amensalism events.

2 Materials and methods

2.1 Synthetic tripartite consortium experiment and RNA extraction

Sphingobacterium multivorum w15 and *Citrobacter freundii* so4 were isolated previously from decaying wood and a soil-derived lignocellulolytic microbial consortium, respectively (Cortes-Tolalpa et al. 2016). The three microorganisms used in this study (*Coniochaeta* sp. 2T2.1, and the bacterial strains w15 and so4) were individually precultured at 30°C with shaking at 250 rpm in mineral medium (25 mM KH_2PO_4, 25 mM Na_2HPO_4, 0.1% $(NH_4)_2SO_4$ and 0.1% Hutner mineral base; final pH 6.8) containing 1% (w/v) glucose. The cell density (OD600; 1.0 cm pathlength) of each preculture was measured after overnight growth. Cell pellets, sufficient to achieve a starting OD600 of 0.1 in each flask, were collected by centrifugation and washed with mineral medium. The experimental flasks, containing 100 ml

of mineral medium and 1% (w/v) wheat straw, were autoclaved, cooled to room temperature and inoculated with the cell pellets (OD600 of 0.1), collected from each preculture (starting inoculum in relation 1:1:1). Cultures containing the 2T2.1 strain (alone) and the tripartite consortium were each carried out in triplicate (30°C, with shaking at 250 rpm) and harvested at 24 and 72 hours to extract total RNA (Figure 2). To harvest 2T2.1 propagules, flasks were gently shaken and solids were allowed to settle, then the liquid fractions were removed by pipetting. Cell pellets were then collected from the liquid fractions by centrifugation, suspended in 1.0 ml RNALater solution (Qiagen, Venlo, Netherlands) and stored at -80°C. Total RNA was isolated using the Qiagen RNAEasy plant mini kit followed by DNase digestion, and quantified using the Qubit RNA HS assay (ThermoFisher Scientific, Waltham, MA, USA). RNA quality was also assessed using RNA bleach gels (Aranda, LaJoie and Jorcyk 2012).
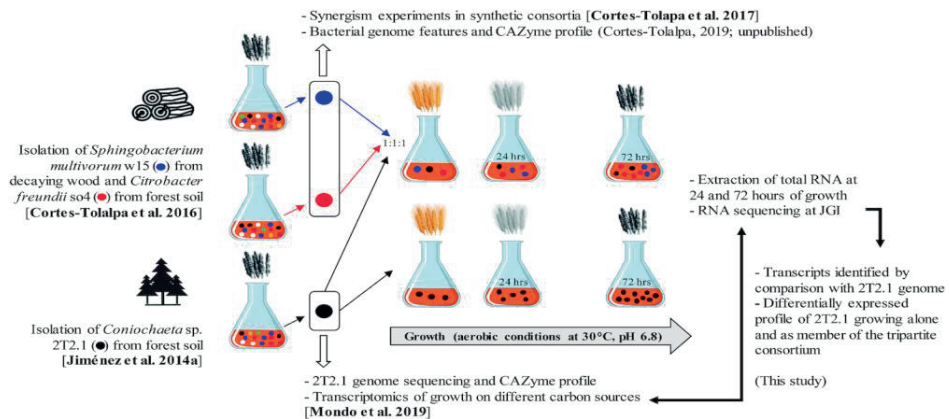
Figure 2. Schematic representation of methodology used in this study. Isolation of the three microbial species (*Coniochaeta* sp. 2T2.1, *S. multivorum* w15 and *C. freundii* so4) and use of the synthetic microbial consortium to compare the transcriptome of fungal strain 2T2.1 alone and in tripartite consortium with the two bacteria when degrading 1% raw wheat straw at 24 and 72 hours of cultivation. Previous studies are shown between brackets and in bold letters.

2.2 Fungal transcriptome sequencing and analysis

Before sequencing, we removed tRNA and rRNA from the total RNA using the polyA selection protocol. Stranded RNAseq libraries were created and quantified by qPCR. mRNA

sequencing was performed using an Illumina HiSeq HiSeq-2500 1 TB 1 × 101 instrument at the Joint Genome Institute (JGI). Using BBDuk (https://sourceforge.net/projects/bbmap/), raw reads were evaluated for artifact sequences by k-mer matching (k-mer = 25), allowing one mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the Phred trimming method set at Q6. Finally, reads under the length threshold were removed (minimum length 25 bases or 1/3 of the original read length—whichever is longer). Filtered reads from each library were aligned to the 2T2.1 genome (https://genome.jgi.doe.gov/Conioc1/) (Mondo et al. 2019) using HISAT2 version 2.1.0 (Kim, Langmead and Salzberg 2015). FeatureCounts (Liao, Smyth and Shi 2014) was used to generate the raw gene counts file using gff3 gene models. Only primary hits assigned to the reverse strand were included in the gene counts. Raw gene counts were used to evaluate the levels of similarity between biological replicates using Pearson's correlation. DESeq2 (version 1.18.1) (Love, Huber and Anders 2014) was subsequently used to determine which genes were differentially expressed between pairs of conditions. A table with the Log2 FC (fold change), adjusted Pval (padj-value) and whether the gene is significantly and differentially expressed (TRUE/FALSE/NA) for each pair of conditions was generated. In addition, FPKM (Fragments Per Kilobase Million) and TPM (Transcripts Per Kilobase Million) normalized gene counts were obtained using the RNA-Seq gene expression analysis pipeline at JGI. TPM data was only used to build the heatmaps because these are considered more comparable values between samples of different origins (Conesa et al. 2016). Differentially expressed genes were annotated using Pfam and KEGG databases. In addition, annotation of CAZymes was performed using combined BLAST (Altschul et al. 1997) and HMMER (Eddy 1998) searches against the CAZy database (Lombard et al. 2014; Mondo et al. 2019).

2.3 Bacterial genome sequencing and CAZyme annotation

Genomic DNA from *C. freundii* so4 and *S. multivorum* w15 was extracted using the UltraClean DNA Isolation Kit (MoBio® Laboratories Inc., Carslab, USA). Whole-genome sequencing was performed using the Illumina NextSeq 500 V2 platform by 150 bp paired-end reads (LGC Genomics., Berlin, Germany). Genome assembly and scaffolding was performed using SPAdes v3.5.0 (Bankevich et al. 2012), according to the workflow described by Nurk et al. (2013). The assembly resulted in 49 and 92 contigs for C. freundii so4 and S. multivorum w15, respectively. Gene detection/annotation was performed by using the RAST pipeline (Aziz et al. 2008) and predicted proteins were also annotated with the dbCAN

webserver (http://bcb.unl.edu/dbCAN2/) (Yin et al. 2012) for identification of CAZymes using a coverage value above 0.5 with an e–value < 1e-18. We here use data pertinent to the current study, whereas a full description of the sequencing data will be given in a separate manuscript (Cortes-Tolalpa et al. 2021.).

2.4 Evaluation of 2T2.1 growth and wheat straw degradation within the tripartite consortium

As a parallel experiment, triplicate 100 ml Erlenmeyer flasks containing 25 ml of mineral medium, vitamins, trace element solution (Jiménez, Korenblum and van Elsas 2014; Cortes-Tolalpa et al. 2016) and 1% (w/v) of autoclaved raw wheat straw (cut into pieces of about ≤1 mm, washed twice with distilled water, filtered with 210 μm filter and dried at 50°C for 48 hours) were inoculated with (i) 2T2.1 alone; and (ii) 2T2.1 with *C. freundii* so4 and *S. multivorum* w15 in relation 1:1:1. To prepare the inoculum, bacterial strains were recovered from -80°C on TSA plates at 28°C for 48 hours, and the fungal strain was pre-grown on PDA plates at 28°C for 72 hours. Then, a fresh colony of each strain was transferred into LB (bacteria) and potato dextrose (fungus) liquid medium at 28°C for 18 hours (bacteria) or 48 hours (fungus). The cell densities of the bacteria and fungus were then checked by microscopy using a Bürke-Turk chamber (Blaubrand®, Wertheim, Germany) according to a standard protocol, after which they were adjusted to cell density of about 5 Log cells per ml. The cultures were incubated at 28°C with shaking at 180 rpm. Bacterial and fungal growth was measured at regular time intervals (e.g. 24, 48, 96 and 144 hours). At each time point, flasks were gently shaken and solids were allowed to settle, then 1 ml of culture was harvested by pipetting and serial dilutions were plated on trypticase soy agar (TSA) containing 0.05 mg/ml of streptomycin, chloramphenicol and tetracycline to prevent bacterial growth. The inoculated plates were incubated at 28°C for 24–48 hours, after which colonies were counted. Thus, growth was evaluated by CFU/ml following incubation. Flasks with substrate, but without inoculum, were used as negative controls. After 10 days of microbial growth, the residual wheat straw matter in the cultures was retrieved and washed twice with distilled water and then dried at 50°C for 48 hours. Then, its weight was measured and compared to the negative control. The percentage of weight loss was defined as the degradation percentage, as calculated by the following formula: $[(Wt−Wi)/Wt] \times 100$; where Wt is total substrate weight before microbial growth (negative control); and Wi is the residual substrate weight after microbial growth (de Lima Brossi et al. 2016; Serrano-Gamboa et al. 2019). Statistical comparisons of weight losses were performed using 'Independent-Sample T Test' of the

means per treatment.

2.5 Analysis of multicopper oxidases (MCOs)

Differentially expressed MCOs were classified according to the laccase and multicopper oxidase engineering database v6.4 (LED) (Sirim et al. 2011). Additionally, we used the server SignalP v.4.1 (Nielsen 2017) to detect signal peptide cleavage sites. All AA1_2/3 proteins and two significantly upregulated bilirubin oxidases (JGI-ID 977649 and 496210) retrieved from the 2T2.1 genome were aligned with sixteen MCOs from different origins (bacteria, fungi and plants) using MAFFT (Katoh and Toh 2008) and MUSCLE (Edgar et al. 2004). Maximum-likelihood phylogeny was reconstructed using RAxML (Stamatakis 2014) under the WAGF substitution model and gamma rate distribution, with 1000 bootstrap replicates. In order to predict the putative 3D structural conformation of AA1_3-like protein (JGI-ID 1292083), we used the Phyre2 web portal with the intensive modeling mode (Kelley et al. 2015). With this structural prediction, the PDB files were uploaded to the EZmol web portal (Reynolds, Islam and Sternberg 2018) in order to compare these against a laccase-like protein from *Thielavia arenaria* (PDB-ID 3PPS), and conserved histidine residues involved in active site and copper ligands were identified.

3 Results

3.1 Expression of genes for fungal CAZymes in the absence or presence of two bacteria

Coniochaeta species have recently become recognized for their great potential in the degradation of lignocellulose. However, interactions with other microbes in these processes have not been extensively explored. In this study, we examined the expression profile of *Coniochaeta* sp. strain 2T2.1 as influenced by the presence of *S. multivorum* w15 and *C. freundii* so4 on wheat straw-driven cultures. On average, 18 ($\pm$2.8) million filtered raw reads were obtained per sample through RNA sequencing (Table S1, Supporting Information). Pearson correlation between the biological replicates was higher than 0.97, indicating that the expression profiles were very consistent across the replicates. Based on pairwise comparisons (24 vs 72 hours of cultivation, with 2T2.1 growing alone), we identified 90 transcripts in 2T2.1 that were significantly and differentially expressed (padj-value $\leq$ 0.05, Wald test; Log2 FC $\geq$ 6) (Figure S1, Supporting Information). Among these, only 32 had functional assignments using the KEGG database. The high percentage of transcripts lacking functional annotation highlights the substantial genetic novelty found in this genus.

Regarding the transcripts that could be assigned to CAZymes, one was predicted to encode a cellulose 1,4-beta-cellobiosidase (CAZy family GH7) (JGI-ID 385552). This transcript was highly expressed at 24 hours of growth (96.1 ± 9.5 FPKM) compared to 72 hours (0.73 ± 0.19 FPKM). In contrast, three transcripts (JGI-IDs 982287, 588474 and 519774), all annotated as L-ascorbate oxidases/ferroxidases (AA1_2), were highly expressed at 72 hours of growth (Figure S1, Supporting Information). A complete list of transcripts and their read counts, FPKM and TPM values can be found in Table S1 (Supporting Information).

Previous studies reported that *Coniochaeta* species secrete an array of different hemicellulases when grown on agricultural residues (Ravindran, Adav and Sze 2012; Mondo et al. 2019). Based on our data, it appears that 2T2.1 shifts its gene expression from exocellulases to AA1-like enzymes (MCOs, probably involved in lignin transformation) during its growth as a monoculture. In nature, leaf litter-degrading fungal communities are predicted to have a similar dynamic behavior (i.e. from cellulose to lignin decomposers) (Voříšková and Baldrian 2013). In addition, it has been reported that lignin-modifying enzymes are highly expressed by a sugarcane bagasse-degrading microbial consortium after 192 hours of cultivation (Jiménez, Chaib De Mares and Salles 2018). Our results show that, after 72 hours of cultivation, *Coniochaeta* sp. 2T2.1 upregulated the expression of genes involved in the metabolism of carbohydrates, lipids, amino acids, cofactors and vitamins. Interestingly, eight significantly upregulated transcripts were predicted to be alcohol dehydrogenases (Table S2, Supporting Information), suggesting a shift from plant polymer degradation (at 24 hours) to the metabolism of released monosaccharides and lignin transformation (at 72 hours).

We then compared the expression profile of strain 2T2.1 after 24 hours of cultivation on wheat straw in the monoculture with that in the tripartite consortium (with strains w15 and so4). This analysis revealed that 63 genes of 2T2.1 were differentially expressed (padj-value ≤ 0.05; Log2 FC ≥ 6). Of these, 38 could be annotated with the KEGG database (Table S2, Supporting Information). In the presence of bacterial strains w15 and so4, strain 2T2.1 significantly downregulated the transcription of 21 genes predicted to encode CAZymes involved in cellulose and arabinoxylan deconstruction. Specifically, these genes encode proteins annotated as alpha-L-arabinofuranosidases (GH51 and GH62), beta-L-arabinofuranosidases (GH127 and GH146), endo-1,4-beta-xylanases (GH11), xylan 1,4-beta-xylosidases (GH3), acetyl xylan esterases (CE1), endoglucanases (GH12) and cellobiohydrolases (GH7) (Figure 3). Thus, it appears that after 24 hours of cultivation on wheat straw and without associated bacteria, *Coniochaeta* sp. strain 2T2.1 can upregulate its (hemi)cellulolytic machinery. We suggest that, under the applied conditions, *S. multivorum*

and *C. freundii* occupy specific niches that influence the physiology of strain 2T2.1. As a consequence, strain 2T2.1 decreased expression of its enzymatic arsenal, similar to what was reported by Daly et al. (2017) in bifungal cultures containing *Aspergillus niger*/*Trichoderma reesei* and *A. niger*/*Penicillium chrysogenum*. The subsequent pairwise comparison of the expression profiles of 2T2.1 after 72 hours of growth with and without the two bacteria showed that 62 genes were differentially expressed. Nine of these corresponded to transcripts predicted to encode CAZymes (families GH12, GH62, GH71, GH3, GH88, GH146 and PL3) (Fig. S2, Supporting Information).
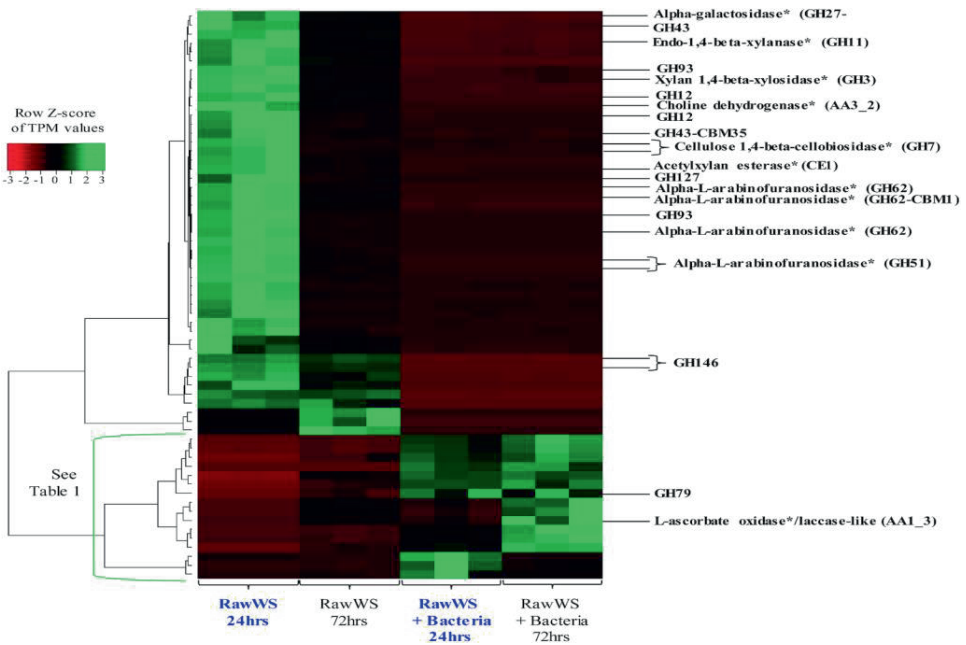

Figure 3. Heat map using row Z-score of TPM (Transcripts Per Kilobase Million) values of 63 differentially expressed (padj-value ≤ 0.05, Wald test; and Log2 FC ≥ 6) transcripts (rows) from *Coniochaeta* sp. strain 2T2.1 growing alone versus in a tripartite microbial consortium (+ Bacteria: *S. multivorum* w15 and *C. freundii* so4) at 24 hours of incubation using wheat straw as the sole of carbon and energy source. Pairwise comparison: RawWS 24 hours versus RawWS + Bacteria 24 hours, in blue. Included are the TPM values of the transcripts in RawWS 72 hours (monoculture) and RawWS + Bacteria 72 hours (tripartite consortium). Differentially expressed genes were annotated using Pfam, KEGG and CAZy databases. Lines at right side: transcripts annotated with KEGG (*) and/or CAZy. Double lines: duplicated protein-encoding genes.

3.2 Fungal-bacterial interaction: synergy or antagonism?

After 24 hours of cultivation with associated bacteria, 16 transcripts were significantly upregulated in strain 2T2.1 (padj-value ≤ 0.05; Log2 FC ≥ 6) (Table 1), whereas another set of 47 were downregulated (some of them discussed above). The former 16 genes were mainly affiliated to hypothetical proteins (10 transcripts), one galactose oxidase (FPKM_RWS+Bacteria24hrs / FPKM_RWS24hrs = ~155) and three MCOs (two bilirubin oxidases and one laccase-like protein of CAZy family AA1_3). The gene encoding a laccase-like protein (JGI-ID 1292083) was most highly upregulated at 72 hours of growth (FPKM_RWS+Bacteria24hrs / FPKM_RWS24hrs = ~437; FPKM_RWS+Bacteria 72 hrs / FPKM_RWS 72 hrs = ~5.19; FPKM_RWS+Bacteria 72 hrs / FPKM_RWS 24 hrs = ~5417) (Table 1 and Table S1, Supporting Information). One transcript predicted to encode a protein belonging to CAZy family GH79 was also upregulated in strain 2T2.1 when grown in the presence of both bacteria (Table 1; Figure 3). Using SignalP v.4.1 (Nielsen 2017), we found that the three MCOs, the galactose oxidase (JGI-ID 1286908) and the GH79-like enzyme (JGI-ID 1046446) contain N-terminal signal peptide sequences, indicating that they are likely secreted by 2T2.1.

Table 1.Genes of *Coniochaeta* sp. strain 2T2.1 that were significantly upregulated (padj-value ≤ 0.05, Log2 FC ≥ 6) at 24 hours when the fungus was grown as a member of synthetic tripartite lignocellulolytic microbial consortium (see Figure 3).

JGI protein ID	FPKM values (standard deviation)			Pfam/Interpro domain	Best Blastp hit [taxon] (sequence identity)
	RWS 24hrs	RWS+Bacteria 24hrs	RWS+Bacteria 72hrs		
1210207	0	1.75 (0.95)	0.28 (0.05)	NI	Hypothetical protein CONLIGDRAFT_716048 [*Coniochaeta ligniaria* NRRL 30616] (79%)
1278719	0	3.62 (1.25)	0.67 (0.33)	NI	Hypothetical protein CONLIGDRAFT_440819 [*Coniochaeta ligniaria* NRRL 30616] (44%)
1286908	0.22 (0.06)	34.10 (8.26)	1.15 (0.37)	NI	Galactose oxidase protein [*Rutstroemia* sp. NJR-2017a WRK4] (43%)
302642	0.77 (0.18)	53.63 (7.40)	117 (4.44)	Domain of unknown function (DUF3328)	Tat pathway signal sequence [*Coniochaeta ligniaria* NRRL 30616] (68%)
501728	2.65 (1.17)	540.24 (65.85)	2065.39 (180.59)	NI	Hypothetical protein CH63R_10743 [*Colletotrichum higginsianum* IMI 349063] (57%)
977649 [a]	4.05 (1.74)	715.78 (107.07)	2366.56 (190.62)	Multicopper oxidase	Bilirubin oxidase [*Colletotrichum higginsianum* IMI 349063] (73%)
1292083*	0.17 (0.05)	74.30 (32.32)	920.94 (210.37)	Multicopper oxidase	Multicopper oxidase [*Meliniomyces bicolor* E] (75%)
1084612	3.82 (0.95)	316.75 (145.34)	4236.81 (1263.20)	NI	Hypothetical protein CONLIGDRAFT_68186

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					[Coniochaeta ligniaria NRRL 30616] (64%)
390783	2.93 (0.95)	285.23 (122.35)	2551.26 (795.94)	NI	NM
1046446	0	1.39 (0.31)	1.24 (0.35)	Glycoside hydrolase superfamily	Glycoside hydrolase family 79 protein [Hyaloscypha variabilis F] (74%)
1079026	3 (0.36)	446.63 (8.36)	507.85 (91.67)	NI	Hypothetical protein DL546_000384 [Coniochaeta pulveracea] (50%)
1232275	5.14 (1.25)	415.10 (24.74)	444.79 (33.37)	Conidiation protein 6	Putative protein of unknown function [Podospora comata] (83%)
1211584	0.80 (0.12)	261.08 (34.52)	306.38 (49.42)	NI	Hypothetical protein FIE12Z_3876 [Fusarium sp. FIESC_12] (44%)
1071462	4.77 (0.06)	686.83 (77.11)	880.60 (81.03)	NI	Hypothetical protein DL546_000384 [Coniochaeta pulveracea] (52%)
496210 [a]	4.88 (2.00)	532.48 (69.90)	777.69 (74)	Multicopper oxidase	Bilirubin oxidase [Colletotrichum incanum] (70%)
496226	3.07 (2.04)	552.45 (56.75)	880.08 (80.04)	NI	Hypothetical protein VD0004_g3696 [Verticillium dahliae] (61%)

RWS: Raw wheat straw; NI: Not identified; NM: Not match within NCBI; *AA1_3 CAZy family (superfamily B—ascomycete laccases).

[a] superfamily I—bacterial bilirubin oxidases.

Galactose oxidases (EC 1.1.3.9) are enzymes secreted by numerous fungi that catalyze the oxidation of D-galactose with concomitant reduction of oxygen to hydrogen peroxide (H_2O_2) (Paukner et al. 2015). This enzymatic reaction suggests that 2T2.1 might fight against bacteria by producing the bacteriostatic/bactericidal agent H_2O_2 (Linley et al. 2012). Moreover, GH79-like enzymes can degrade arabinogalactan-proteins (i.e. proteoglycans) found in plant and bacterial cell walls (Knoch, Dilokpimol and Geshi 2014). Konishi et al. (2008) reported that fungal GH79-like enzymes release both glucuronic acid (GlcA) and 4-O-methyl-GlcA from arabinogalactan-proteins. We surmised that the overexpression of this enzyme by 2T2.1 might be a strategy to attack the proteoglycans on the surface of bacterial cells. However, GH79-like enzymes were also significantly upregulated in a brown-rot fungus (*Postia placenta*) after 20 days of growth in poplar residues (Skyba et al. 2016), suggesting that they might still have a role in lignocellulose deconstruction. Given the absence of such genes in the bacterial genomes (Table 2), it is plausible to assume that the fungal strain 2T2.1 uniquely supplies this enzymatic function to the tripartite consortium. Conversely, the two bacteria revealed considerable genomic potential to deconstruct arabinoxylan, which is an abundant component of the plant cell wall. In particular, the genome of *S. multivorum* w15 was found to contain many genes encoding enzymes of families GH43 and CE1 compared with *C. freundii* so4 (Table 2). CE1-like enzymes (i.e. acetyl xylan esterases; EC 3.1.1.72) hydrolyze ester bonds in polysaccharides, releasing acetic acid from acetylated arabinoxylan. It has been reported that these enzymes enhance the hydrolysis of pretreated wheat straw and giant reed (*Arundo donax*) (Zhang et al. 2011). In addition, GH43-like enzymes (i.e. alpha-L-arabinofuranosidases) have been considered key proteins that release arabinose moieties from external linkages of arabinoxylan. However, they may have dual activity, also acting as beta-xylosidases (Maruthamuthu, Jiménez and van Elsas 2017).

Table 2.Number of genes encoding CAZymes within the genomes of the members of the tripartite microbial consortium.

CAZy family	Common activity	Putative Substrate	*Coniochaeta* sp. 2T2.1 [A]	*S. multivorum* w15 [B]	*C. freundii* so4 [B]
			Number of genes within each genome		
AA1_2	Ferroxidase/ Laccase-like	Lignin	5	0	0

AA1_3 [u]	Laccase-like multicopper oxidase		5	0	0
AA3_2 [d]	Glucose oxidase		19	4	0
GH3 [d]	Xylan-β-xylosidase	Xylan/ Arabinoxylan	18	6	4
GH11 [d]	Endo-β-xylanase		8	0	0
GH43 [d]	β-xylosidase/α-L-arabinofuranosidase		22	22	1
GH62 [d]	α-L-arabinofuranosidase		2	0	0
GH51 [d]	Endo-glucanase/α-L-arabinofuranosidase		2	3	0
CE1 [d]	Acetyl xylan esterase		13	19	6
GH93 [d]	Exo-α-L-arabinanase		2	0	0
GH127 [d]	β-L-arabinofuranosidase		3	2	2
GH146 [d]	β-L-arabinofuranosidase		1	0	0
GH7 [d]	Endo-glucanase/ cellobiohydrolase	Cellulose	6	0	0
GH12 [d]	Endo-glucanase		2	0	0
GH27 [d]	α-galactosidase	Glycolipids/ proteins	4	0	0
GH71	α-1,3-glucanase	Dextran	11	0	0
GH79 [u]	β-glucuronidase	Proteoglycans	4	0	0
GH88	β-glucuronyl hydrolase	NA	4	5	2
PL3_2	Pectate lyase	Pectin	1	0	0

Families shown correspond to genes for CAZymes that were down and/or upregulated in Coniochaeta sp. 2T2.1 (see Figure 3; Figures S1 and S2, Supporting Information).

[A] Data retrieved from Mondo *et al*. (2019) (number of genes after removal of duplicated content); [B] (Cortes-Tolalpa, 2020).

[u] Gene-encoding proteins significantly upregulated at 24 hours of cultivation when the fungus growth within the tripartite consortium.

[d] Gene-encoding proteins significantly downregulated at 24 hours of cultivation when the fungus growth within the tripartite consortium.

Moreover, at 72 hours and in the presence of w15 and so4, strain 2T2.1 showed significant overexpression of genes that encode hypothetical proteins, a PLAC8-domain-containing protein and a multidrug transporter of the MFS class (Table S3, Supporting Information). Fungal multidrug transporters are membrane proteins that provide resistance against a wide variety of toxic compounds. In particular, such transporters play an essential role in protection against plant defense compounds during pathogenesis. Thus, they can be considered as a 'first-line defense barrier' in fungal survival mechanisms (Del Sorbo, Schoonbeek and de Waard 2000; de Waard et al. 2006). In our study, the expression of the respective multidrug transporter could be a response of *Coniochaeta* sp. 2T2.1 to bacterial secondary metabolites that inhibit its growth (Dos Santos et al. 2014; Daghino et al. 2019).

Regarding the bacterial and *Coniochaeta* species co-growth, Cortes-Tolalpa, Salles and van Elsas (2017) set up synthetic microbial co-cultures, using different combinations of 22 bacterial strains and one *Coniochaeta* species (strain sedF1). In a triculture (containing w15, so4 and sedF1), *C. freundii* so4 showed a growth increase of 27.8 (± 0.8) and *S. multivorum* w15 of 28.2 (± 1.5) fold, compared to the respective monocultures. In contrast, strain sedF1 showed a decrease in growth (43.9 ± 2.7 fold) compared with its abundance in monoculture. In order to complement our transcriptomic analysis, we evaluated the growth and wheat straw degradation capacity of *Coniochaeta* sp. 2T2.1 growing alone or as a member of the tripartite consortium. Our results showed that, at 24 hours of cultivation, the fungus biomass was at ~ 6.6 Log10 CFU/ml, probably in the exponential growth phase. The presence of the two bacteria reduced (but did not abolish) fungal growth until 96 hours of incubation (Figure 4A). After 10 days of cultivation on wheat straw, 2T2.1 had degraded over 15% of the substrate (measured by dry weight), whereas this was around 10% in the tripartite consortium (Figure 4B). Recent parallel experiments with the tripartite consortium (containing 2T2.1, w15 and so4) confirmed that fungal growth is inhibited, but not stopped by the presence of the two bacteria, and both bacteria grew out, irrespective of the presence of 2T2.1, to population densities of about 10^8 to 10^9 CFU per ml, within 48 hours of growth (Wang et al. 2021.).
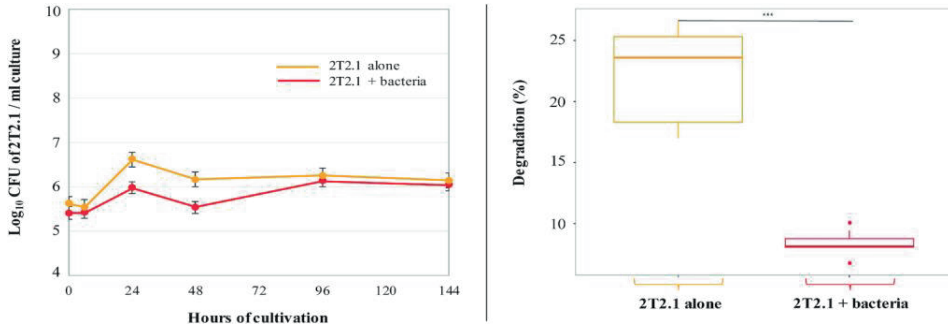
Figure 4. (A, left panel) Growth (Log_{10} CFU/ml) of *Coniochaeta* sp. 2T2.1 when grown alone or with bacteria (*S. multivorum* w15 and *C. freundii* so4) at 28°C in aerobic conditions (pH 7.2). Bars: standard deviations. (B, right panel) Wheat straw degradation percentages after 10 days of cultivation. Assays used three biological replicates. Asterisks (*) represent significant differences using Student's T test, ***P < 0.001.

3.3 Expression of novel fungal MCOs in the tripartite consortium

A major finding in this study was the overexpression of transcripts predicted to encode MCOs by *Coniochaeta* sp. 2T2.1 in the presence of the two bacteria at 24 and 72 hours of cultivation (Table 1). MCOs catalyze the oxidation (at a mononuclear copper center T1) of a wide variety of aromatic compounds, with the concomitant reduction of molecular oxygen to water (Copete et al. 2015). Across all MCOs (i.e. laccase-like enzymes, ascorbate oxidases, ferroxidases, bilirubin oxidases and pigment oxidases), sequence homology is low. However, copper-binding motifs have been reported to be highly conserved, as follows: HXHG, HXH, HXXHXH and HCHXXXHXXXXM/L/F (Reiss et al. 2013). Using the LED database v6.4 (Sirim et al. 2011), two of the upregulated MCOs (JGI-ID 977649 and 496210) were classified within superfamily I (bacterial bilirubin oxidases), while the third one, JGI-ID 1292083, belongs to superfamily B ('ascomycete' laccases) (Sirim et al. 2011). The former two bilirubin oxidase encoding transcripts (663 amino acids each) correspond to a duplicated gene within the strain 2T2.1 genome, clustering together with similar genes from *Colletotrichum* (around 71% amino acid identity), *Verticillium* and *Myrothecium* species (Figure 5). It has been reported that bilirubin oxidases (EC 1.3.3.5) catalyze the oxidation of bilirubin, an open chain tetrapyrrole, to biliverdin (a green tetrapyrrolic pigment). These enzymes have been used in the detection of bilirubin in serum and therefore in the diagnosis of jaundice (Mano 2012). Although bilirubin has been typically thought of as an animal

compound, it has also been found in plants (e.g. in angiosperms) (Pirone et al. 2010), so a role for bilirubin oxidases in deconstruction of plant biomass might be possible. Unlike laccases (EC 1.10.3.2), bilirubin oxidases display high thermal stability, high activity at neutral pH and high tolerance towards chelators, which makes them excellent candidates for the pretreatment of agricultural residues. These enzymes can be useful in different biotechnological applications, e.g. pulp bleaching, decoloring of dyes, bilirubin biosensors operating in serum, and bioremediation (Durand et al. 2012).
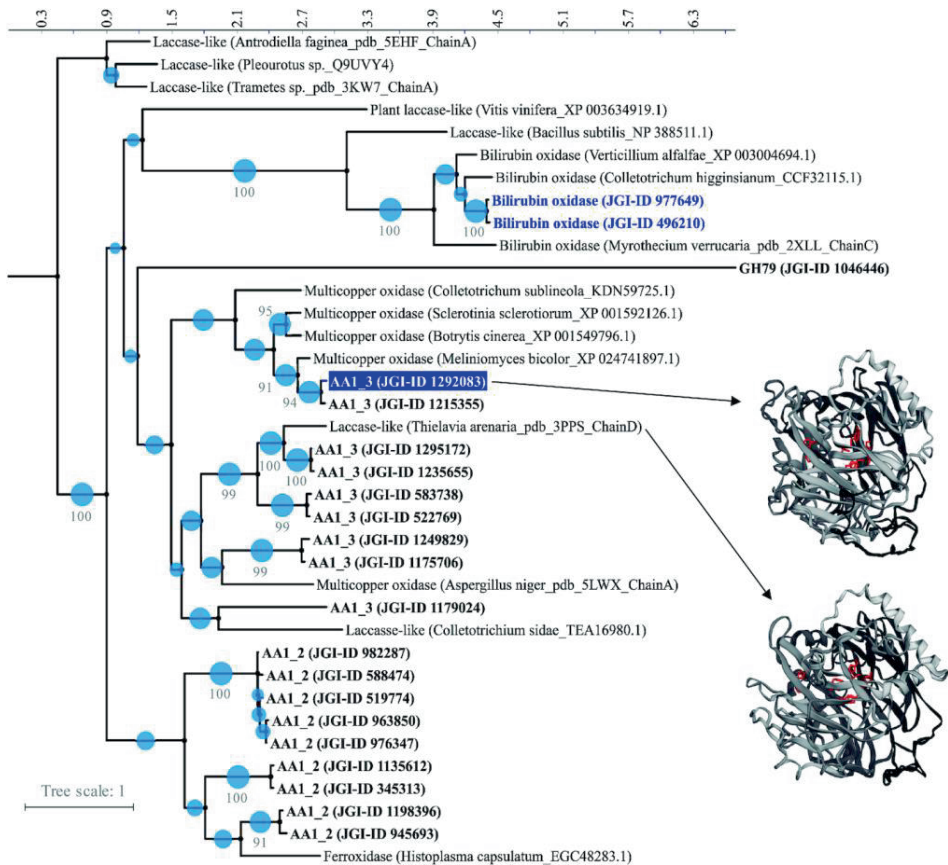
3

Figure 5. RAxML phylogenetic tree of MCOs that were significantly upregulated (padj-value ≤ 0.05 and Log2 FC ≥ 6) in strain 2T2.1 in the presence of *S. multivorum* w15 and *C. freundii* so4 (JGI-IDs 496210, 977649 and 1292083) (see Table 1). Proteins in bold: AA1_2 and AA1_3 enzymes from fungal strain 2T2.1. Bootstrap range (10–100) is shown in blue circles. Numbers: >90 values. As an outgroup sequence, we used GH79-like protein from strain 2T2.1. Right: 3D structural modelling of laccase-like proteins from *T. arenaria* (PDB-ID

3PPS, chain D) and (JGI-ID 1292083) from *Coniochaeta* sp. strain 2T2.1 (Model dimensions (Å): X: 64.919 Y: 66.405 Z: 79.800). Red: conserved histidine residues (H121, H123, H165, H167, H470, H473, H475, H528, H530 and H534) involved in copper-binding motifs and active sites.

The predicted laccase-like protein (JGI-ID 1292083) of strain 2T2.1 had a size of 583 amino acids (59.19 kDa), sharing 75% sequence identity and 99% coverage with a multicopper oxidase of *M. bicolor*. It clustered together with several asco-laccases from *Colletotrichum*, *Botrytis* and *Sclerotinia* species (Figure 5). The 3D structure of this protein was predicted using as a template the crystal structure of an ascomycete laccase of *T. arenaria* (PDB-ID 3PPS, chain D) (Kallio et al. 2011). In this analysis, 95% of the residues were modeled at >90% confidence. In addition, the 3D modeling and multiple sequence alignment revealed the presence of ten highly conserved histidine residues, which is indicative of copper-binding motifs and active sites: T1-T2-T3 sites (H121, H123, H165, H167, H470, H473, H475, H528, H530 and H534) (Figure 5). Fungal laccases are essential for diverse ecological functions in which attack on lignocellulose is important. Consequently, they have been implicated in plant pathogenicity, as described for *Colletotrichum orbiculare* (Lin et al. 2012), as well as in ligninolytic complexes of wood-decaying organisms. Laccase-like proteins can oxidize a range of lignin-derived aromatic compounds, phenolic moieties, amines, benzenethiols and hydroxyl indols (Janusz et al. 2017). These enzymes, mainly from *Pleurotus*, *Trametes*, *Aspergillus* and *Trichoderma*, have been widely studied with respect to their role in lignin depolymerization (Levasseur et al. 2010; Ramos et al. 2011; Yang et al. 2017). However, little is known about their role in *Coniochaeta* spp. and the ligninolytic capacity of this fungus has so far been poorly explored.

With respect to *Coniochaeta* species, Ravindran, Adav and Sze (2012) reported that strain LF2 can degrade around 32% of lignin after 72 hours of cultivation on sawdust. In addition, López et al. (2007) found that *C. ligniaria* NRRL301616 can degrade up to 40% of the lignin fraction from pepper plant residues in semi-solid culture after 20 days. In this latter study, lignin and manganese peroxidase activities were quantified, but laccase activity was not detected. In a recent examination of the genomes of *Coniochaeta* sp. 2T2.1, *C. ligniaria* CBS111746, *C. ligniaria* NRRL30616 and *Coniochaeta* sp. PMI546, it was found that each of these strains contains 16 to 18 genes that encode laccase-like proteins belonging to CAZy family AA1 (Mondo et al. 2019). Notably, genes from families AA1_2 and AA1_3 were absent in the genomes of both bacteria used in this study, while the genome of strain 2T2.1

contains 10 such genes (Table 2). Based on all this information, we posit that a major role for *Coniochaeta* sp. strain 2T2.1 in the tripartite microbial consortium studied here is the transformation of lignin by secretion of the AA1-like proteins and bilirubin oxidases. This would allow release of lignin-derived aromatic compounds and increased access of enzymes to plant polysaccharides as a result of the broken bonds. The process is predicted to enhance bacterial growth and synergism within the tripartite consortium, as observed by Cortes-Tolalpa, Salles and van Elsas (2017). Certain extracellular laccases-like enzymes can be induced due to competitive interactions in mixed consortia without an abiotic inducer (Flores et al. 2009). Thus, by exploiting inter-kingdom competitive interactions, these types of MCOs can be expressed/produced and are potentially of use in biotechnological applications (Zhang et al. 2018). In this respect and as a practical spinout, inclusion of MCOs in enzyme cocktails could improve the saccharification of wheat straw. For instance, Deng et al. (2019) reported an increase of ~26% in wheat straw saccharification after a pretreatment with laccases.

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Conclusion

We conclude that, in the tripartite microbial consortium growing on wheat straw, gene expression in *Coniochaeta* sp. 2T2.1 is modified to exert a major role in the transformation of lignin. Meanwhile, *S. multivorum* w15 and *C. freundii* so4 have the genomic potential to deconstruct the arabinoxylan fraction. This division of catabolic labor may be an indication of synergy within the tripartite consortium. In this regard, the secreted fungal MCOs could potentially facilitate the growth of the bacterial consortium members. This conclusion is partially supported by recent growth data produced by Wang et al. 2021, but needs to be confirmed by in-system evaluations of bacterial growth (e.g. using 16S rRNA gene qPCR). From the bacterial genome analyses, we concluded that the two bacterial strains (so4 and w15) lack the capacity to transform lignin by laccases-like enzymes of AA1 family.

Clearly, the presence of the bacteria (and/or their secondary metabolites) suppresses fungal growth to some extent, and inhibits the expression of enzymes related to arabinoxylan and cellulose deconstruction (e.g. alpha-L-arabinofuranosidases, acetyl xylan esterases, endoxylanases, endoglucanases and cellobiohydrolases). Overall, *Coniochaeta* 2T2.1, as a single organism, might be the better deconstructor of wheat straw, as seen in our degradation data (Figure 4B). The reduction of growth and enzyme expression in strain 2T2.1 could be explained by carbon source competition, nutritional stress, secondary bacterial metabolites and/or fast metabolic activity of the bacteria. However, the upregulation of the GH79-like

enzyme and galactose oxidase hinted at a fungal anti-bacterial defense mechanism becoming operational.

Finally, several key up- and downregulated genes encoding plant biomass-degrading enzymes were identified, which typify the eco-enzymological role of strain 2T2.1 within the tripartite microbial consortium. In addition, genes encoding novel *Coniochaeta*-derived MCOs could be the starting point for future biotechnological applications. Further studies of specific proteins (e.g. for CAZy families GH43, CE1 and lytic polysaccharide monoxygenases) in both the bacteria and the fungus are required to unravel the in-depth enzymatic mechanism of interaction between these organisms. This three-member synthetic consortium could serve as a model for understanding the eco-enzymological roles of fungi and bacteria in naturally lignocellulose-degrading microbial processes.

Data availability

The transcriptome data were deposited at DDBJ/EMBL/GenBank databases under the following SRA accessions numbers (SRP170794, SRP170796, SRP170779, SRP170797, SRP170780, SRP170786, SRP170788, SRP170787, SRP170776, SRP170781, SRP170790 and SRP170778).

Author Contributions

DJJ, LCT, NNN and JDvE conceived this project. REH, JAM and NNN performed most of the wet lab experiments and RNA extraction. JL, JJ, AL, KB SM and IG generated the transcriptome data. DJJ, MCDM and SM analyzed transcriptomic and CAZy data. LCT provided the bacterial genome data. YW performed the fungal growth and degradation experiments. DJJ wrote the manuscript, with comments and revisions by MCDM, YW, JAM, REH, SM, IG, NNN and JDvE. All authors read and approved the final manuscript.

Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY INFORMATION CHAPTER 3

Defining the eco-enzymological role of the fungal strain *Coniochaeta* sp. 2T2.1 in a tripartite lignocellulolytic microbial consortium

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Supplementary materials chapter 3: Table S1. Raw data; **Table S2.** Differentially expressed (padj-value ≤ 0.05; Log2 FC ≥ 6) transcripts under each pair comparison annotated with the KEGG database; **Table S3.** Significant overexpression of strain 2T2.1 at 72 hours in the presence of w15 and so4 by pfam; **Figure S1.** Heat map using row Z-score of TPM (Transcripts Per Kilobase Million) values of 63 differentially expressed (padj-value ≤ 0.05, Wald test; and Log2 FC ≥ 6) transcripts (rows) from *Coniochaeta* sp. strain 2T2.1 growing alone at 24 and 72 hours of incubation using wheat straw as the sole of carbon and energy source. Pairwise comparison: RawWS 24 hours versus RawWS 72 hours, in blue; **Figure S2.** Heat map using row Z-score of TPM (Transcripts Per Kilobase Million) values of 62 differentially expressed (padj-value ≤ 0.05, Wald test; and Log2 FC ≥ 6) transcripts (rows) from *Coniochaeta* sp. strain 2T2.1 growing alone versus in a tripartite microbial consortium (+ Bacteria: *S. multivorum* w15 and *C. freundii* so4) at 72 hours of incubation using wheat straw as the sole of carbon and energy source. Pairwise comparison: RawWS 72 hours versus RawWS + Bacteria 72 hours, in blue.

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