COMPARATIVE TRANSCRIPTOMIC ASSAY OF *PHELLINUS TREMULAE* ISOLATES TO IDENTIFY THE GENETIC RESPONSES OF ENVIRONMENTAL STRESS

by

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A Senior Honors Thesis Submitted to the Faculty of
The University of Utah
In Partial Fulfillment of the Requirements for the

Honors Degree in Bachelor of Science

In

The School of Biological Science

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May 2023

ABSTRACT

Saprotrophic fungi are a diverse ecological group that can breakdown organic matter to obtain carbon. Due to their metabolic processes, they play an essential role in nutrient cycling within the microbial soil community. Despite the importance of saprotrophic fungi, little is known about their response to climate change. Mimicking real world situations and using transcriptomic analysis can help us characterize what genetic mechanisms saprotrophic basidiomycetes use to respond to environmental changes. This research utilizes cultures of a common saprotrophic fungus, *Phellinus tremulae*, which is known to cause white trunk rot and parasitize aspen trees. We have conducted a culture growth assay where isolates are grown under varying conditions that mimic ecological stressors, to induce a stress response in the saprotrophic fungus. We then extracted and sequenced mRNA from the cultures to identify the genes that are differentially expressed in the presence or absence of an ecological stressor. Specifically, we analyzed the impacts of heat stress, high and low pH, and recalcitrant carbon compounds as the sole carbon source. Differential gene expression analysis of these conditions revealed that environmental stress, particularly habitat stress, had a greater impact on isolates compared to nutritional stress. Future work includes identifying if these fungi utilize these genes to adapt to environmental stress in their natural habitats and what cellular functions are involved with adapting to these stressors.

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INTRODUCTION

Saprotrophic fungi are a diverse ecological group of fungi that can decompose organic matter to obtain carbon (Várnai et al., 2014). These organisms can be found living in diverse habitats, such as soil, dung, and wood as well as marine environments. Saprotrophic fungi utilize secreted extracellular enzymes that break down complex organic molecules such as cellulose, lignin, or chitin into simple sugars (Clocchiatti et al., 2020). Due to this ability, saprotrophic fungi play an essential role in the ecosystem by decomposing organic biomass and recycling nutrients (Dighton, 2007). Increased human activity may lead to environmental impacts that can alter functions of fungi. For example, the growth of saprotrophic fungi can be impacted by climate change, as it can alter its surrounding environment such as soil composition, meteorological patterns, and the carbon-nitrogen cycle (Damialis et al., 2015; Dighton, 2007). Yet, many of these impacts are poorly characterized in most species, and the genetic pathways used to overcome stress are unknown. As climate change continues, it is important to study how environmental stress will impact saprotrophic fungi, and identify the molecular mechanisms used to tolerate stress. Identifying these mechanisms will also give insights into the tools used for evolutionary adaptation.

Decomposition contributes to the release of nutrients that can be later utilized by other organisms such as plants and animals. These nutrients predominantly include nitrogen and phosphorus. Saprotrophic fungi are the primary decomposers of forest ecosystems and are abundant in the litter layer where there is often strong competition among them, as well as other ecological guilds, for resources (Bödeker et al., 2016). Without

saprotrophic fungi, dead organic matter would accumulate and nutrients would become inaccessible for future generations, leading to ecosystem collapse. Yet, while extensively researched, the process of decomposition is still poorly understood. Many of the genes likely involved in decomposition are uncharacterized, despite evidence of differential regulation during wood decay (Floudas et al., 2020). Decomposition is a complex process and understanding how these organisms thrive under stress can contribute to valuable insights into the mechanisms that are used by saprotrophic fungi to grow under variable environmental conditions.

There are two primary types of wood decay fungi: white- and brown-rot. White-rot fungi are known to break down lignin and cellulose, leaving a white (mainly cellulose) residue. Brown-rot fungi only utilize cellulose and leave a brown (lignin) residue (Krah et al., 2018). White-rot species have on average more genes associated with decomposition than the brown rot species (Kohler et al., 2015). A pan-genomic comparison of differential gene analysis of four white-rot species found that enzymes responsible for lignin decay were more diverse than those associated with polysaccharide decomposition (Miyauchi et al., 2020). These enzymes play key roles in degrading organic matter, such as polysaccharides found on plant cell walls, which is not only important for ecosystem health but also have important industrial uses. In our research, we seek to provide insights into the transcriptional responses of a common fungal pathogen to stress. These insights will elucidate the diverse mechanisms employed in decomposition and shed light on how climate change may disrupt these mechanisms.

Phellinus tremulae is both a saprotrophic white-rot fungus and an important plant parasite. Due to this dual lifestyle, *P. tremulae* plays an essential role in both the ecosystem as a nutrient cycler, and in plant communities as a parasite. It specifically parasitizes quaking aspens (*Populus tremuloides*) which can be found in many areas in the western United States. In Utah, quaking aspens are a keystone species and are an essential part of the ecosystem. For example, one of the largest organisms on earth, Pando, is a quaking aspen clone found at Fishlake National Forest. Climate change may impact the function of *P. tremulae* which can have cascading impacts across the ecosystem. Previous work has shown that temperature alters the rate at which *P. tremulae* parasitizes aspen trees (Koide et al., 2017). As temperatures rise due to climate change, the abundance, distribution, and impact of *P. tremulae* may change, which will change its impacts on aspen trees. Through comprehensive research, we can improve insights of genes involved in decomposition and how *P. tremulae* thrives under changing environmental factors. Such environmental stressors include access to low pH conditions, rapid daily temperature spikes to 40°C, and recalcitrant forms of sugar such as D-xylose.

METHODS

Sample Collection

Tissue samples of *Phellinus tremulae* were collected from Minnesota by Bryn Dentinger on August 2004. Cultures of *Phellinus tremulae* were originally cultured on MMN (Modified Melin-Norkrans adapted from the Talbot Lab Protocol from Boston University) slant cultures. After isolation, cultures grown on slants were transferred and maintained on MMN pH 6 petri dishes. To mimic environmental stressors, we subcultured *P. tremulae* into six growth conditions and grew for 21 days: 1) base MMN pH 4.7 as our control condition, 2) base MMN but pH adjusted to 7, 3) base MMN pH 4.7 but glucose and malt extract replaced with 2g/l of D-xylose, 4) base MMN pH 4.7 but glucose and malt extract replaced with 2g/l of D-mannose, 5) base MMN pH 4.7 but glucose and malt extract replaced with yeast extract, and finally 6) base MMN pH 4.7 with a slow heat ramp stress applied starting at 24°C to 40°C, increasing 1°C every 10 minutes, after 21 days (Lamb, 1974).

After three weeks of growth, culture plates were flash frozen in liquid nitrogen. Fungal mycelium was scraped from the frozen agar into a 15 mL conical tube in a laminar flow hood and immediately transferred to a -80°C freezer to preserve RNA from degradation.

RNA Extraction and Sequencing

RNA tissue samples were homogenized using a mortar and pestle in the presence of a small amount of liquid nitrogen. RNA from frozen tissue samples was extracted using

Sigma GeneElute Total RNA Extraction kit as per manufacturer's instructions and stored at -70˚C. Eluted samples were kept on ice to prevent further degradation. Gel electrophoresis was used to assess RNA integrity. Tris-acetate-EDTA-based 1% agarose gels along with a low concentration of bleach is used to get rid of any remaining RNase and analyze RNA quality. Gels are analyzed after running samples at 120V for ~20 minutes and samples are stored at -70˚C after loading into the gel. For detection and quantification of RNA concentrations in the eluted samples, Qubit RNA HS (High Sensitivity) Assay Kit was used as per manufacturer's instructions. Nanodrop spectrophotometer was used as another means to quantify sample purity. Paired-end sequencing (150bp paired end) and library preparation were performed by Novogene (USA) on the NovoSeq platform.

Bioinformatics and Differential Expression

To assess transcript abundance, we first produced a de-novo reference transcriptome. All sequences were combined and a meta-transcriptome was assembled using Trinity-v2.9.1. After assembly we removed redundant transcripts with CD-HIT-est using default settings. After reference construction, sequences were first adapter-trimmed and quality filtered with fastp. Filtered sequences were aligned to the reference transcriptome with hisat2, and transcript abundance was quantified with Salmon. Highly differentially expressed transcripts were identified after alignment and quantification using the R package DESeq2 (Love et al., 2014)

We compared each environmental condition to our control (base MMN pH 4.7) to identify gene with more than 2 log-fold change in expression levels. The Wald test is a default test in DESeq2 which is used to compare two groups. Using the Wald test with an adjusted significance level of 0.05, we were able to determine which genes were upregulated or downregulated (Figure 1). After assemble, transcripts were annotated using eggNOG-mapper (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019). After identifying genes differentially expressed against the control for each condition, we then identified genes unique to each type of stress. We categorized our stressors into two groups as nutritional stress (sugar types and yeast-derived adjuncts) and "habitat" stress (pH and temperature shock) and compared them. The top three hits that have predicted genes functions for each condition were analyzed (Table 1).

RESULTS

Sequencing Results and Transcriptome Assembly

In our study, we sought to assess the impacts of heat stress, high and low pH, and recalcitrant carbon compounds as the sole carbon source, on gene expression of *P. tremulae*. A total of 18 plates were extracted and sequenced. Illumina sequencing of the *P. tremulae* mRNA resulted in an average of 13,128,196 reads per sample. Transcriptome assembly resulted in 59,950 non-redundant transcripts.

Differential gene expression analysis was used to compare the various environmental conditions (Figure 1). For the carbon sources, we found that D-xylose had an average of 102 upregulated genes and 168 downregulated genes. Similarly, D-mannose had an average of 255 upregulated genes and 188 downregulated genes. Out of the three carbon conditions tested, yeast extract exhibited the greatest number of differentially expressed genes with an average of 495 upregulated and 863 downregulated genes. The pH7 condition had an average of 378 upregulated and 514 downregulated genes. We found that heat ramp had the most differentially expressed genes overall with an average of 4016 upregulated genes and 2912 downregulated genes.

Different categories were compared against each other to see unique or overlapping genes that characterized each condition (Figure 2). D-mannose, D-xylose, and yeast extract shared a total of 84 downregulated genes and 8 upregulated genes. Heat ramp and pH7 shared a total of 279 downregulated genes and 89 upregulated genes. Habitat stress had

more differential genes in both upregulated and downregulated expression than nutritional stress conditions.

Differentially expressed genes unique to each condition were analyzed by matching them to their predicted gene functions (Table 1). The presence of genes involved in the cell cycle and cell cycle checkpoint loci was observed under conditions of habitat stress (GO:0030896). HUS1, a gene related to DNA damage checkpoint was found in the heat ramp (Kostrub et al., 1997). Transporter proteins have been found to be involved in responding to nutritional stressors (Sugar_tr). Genes utilized in decomposition were also found in D-mannose and yeast extract (Abhydrolase_3). Although nutritional stress resulted in the upregulation of genes, those specific functions remain unknown.

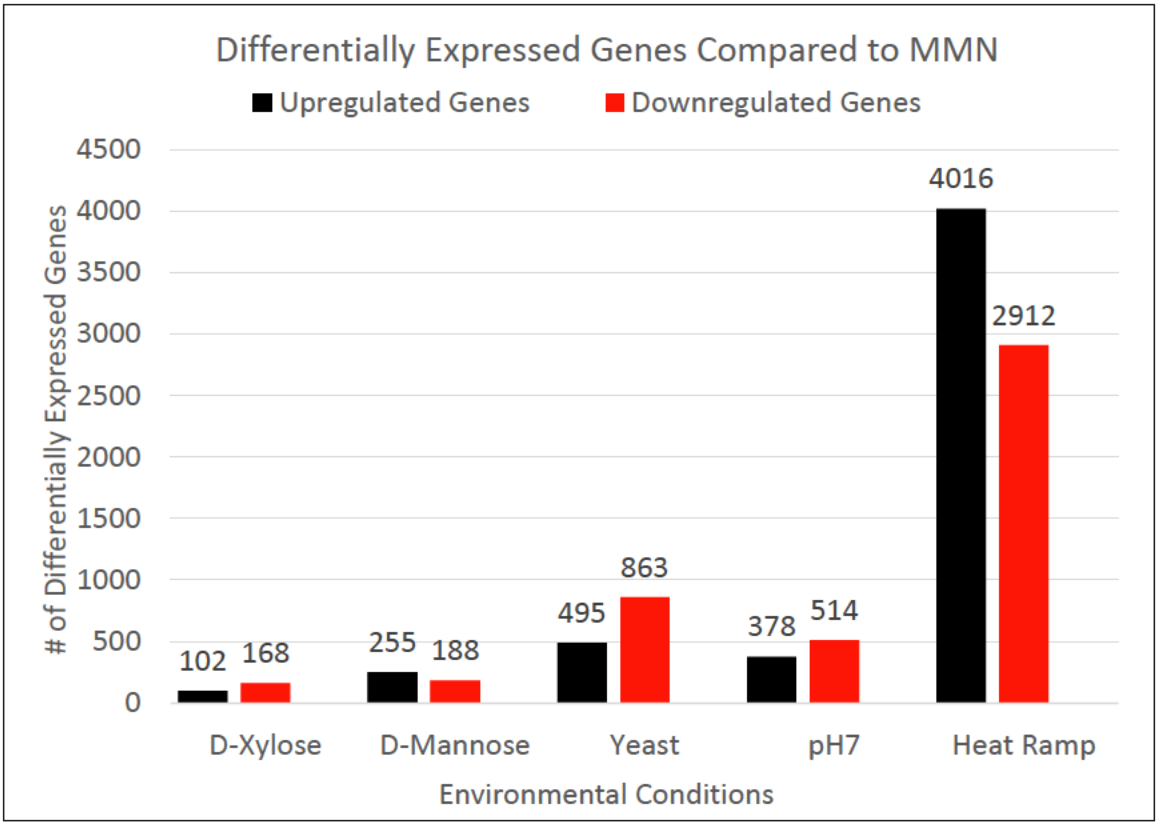

Figure 1: Differentially Expressed Genes Compared to MMN Bar Graph.
The graph depicts the number of differentially expressed genes from each of the five conditions found using a Wald test with a adj. p-value of 0.05. The bars on the left represents the upregulated genes with log fold change greater than 2 while the bars on the right represents the downregulated genes with log fold change smaller than 2.

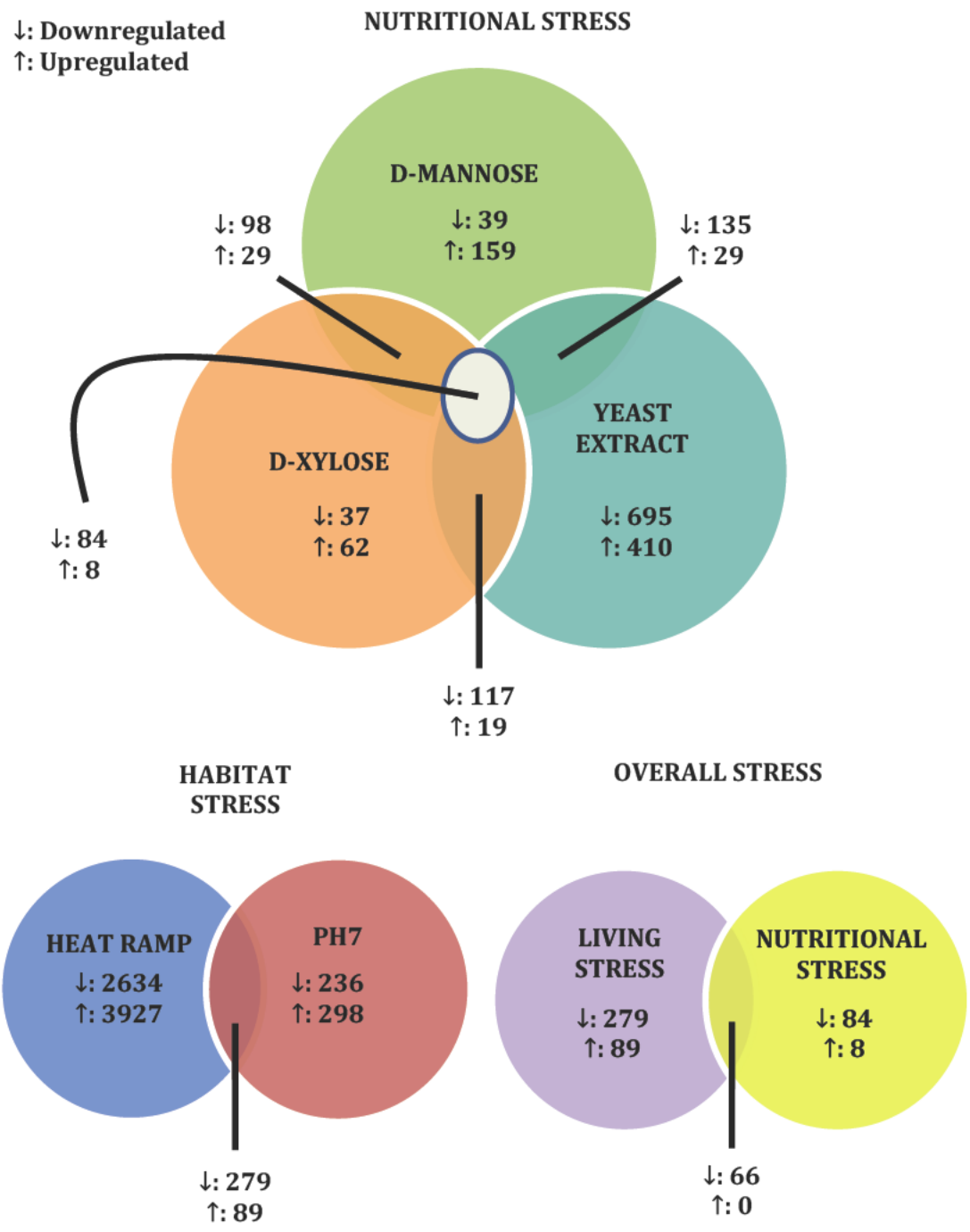

Figure 2: Venn Diagram of the Breakdown of Differentially Expressed Genes
Colors have no indication factor other than for differentiating the conditions. Up and downregulated genes are indicated by arrows. Overlap of circles are an indicator of overlapping genes between conditions compared.

CONDITIONS	PATHWAYS		
D-mannose Downregulated	rRNA (uridine-2'-O-)-methyltransferase activity (RNA Methylation)	Not1 N-terminal domain, CCR4-Not complex component (Global Regulator of Gene Expression)	N/A
D-mannose Upregulated	Inhibitor of growth proteins N-terminal histone-binding (Inhibitory Enzymes)	Alpha beta-hydrolase (Decomposition)	O-methyltransferase family 3 protein (DNA Methylation)
D-xylose Downregulated	WD40 repeat-like protein (Biological Functions)	NACHT domain (Conserved Protein Domain)	ribosomal protein (rRNA)
D-xylose Upregulated	The model conserved GMC regions and catalytic active site residues (Enzyme-Catalyzed Reactions)	chromatin remodeling protein (Cellular Processes)	2OG-Fe(II) oxygenase superfamily (Metabolic Pathways)
Yeast Extract Downregulated	Fungal specific transcription factor domain (Transcription Factor)	Belongs to the major facilitator superfamily. Sugar transporter (TC 2.A.1.1) family (Transporter)	Protein transport protein Sec61 subunit beta (Transport Protein)
Yeast Extract Upregulated	Dienelactone hydrolase family (Metabolite Degradation)	ATP-dependent DNA helicase (DNA Replication)	Plasma membrane osmosensor that activates the high osmolarity glycerol (HOG) MAPK signaling pathway in response to high osmolarity. (Stress-activated MAPK pathway)
PH7 Downregulated	P-loop containing nucleoside triphosphate hydrolase protein (Nucleotide-Binding Protein Folds)	C2H2-type zinc finger (Transcription Factors)	WD40 repeat-like protein (Biological Functions)
PH7 Upregulated	Mitochondrial carrier (Metabolite Transporters)	GPI-GlcNAc transferase complex, PIG-H component (Membrane Protein Transportation/Cell Wall Synthesis)	WD40 repeat-like protein (Biological Functions)
Heat Ramp Downregulated	CorA-like Mg2+ transporter protein (Transporter)	Calcium proton exchanger (Exchanger)	RNA-binding, Nab2-type zinc finger (Transcription Factors)
Heat Ramp Upregulated	Cell cycle checkpoint (Cell-Division Cycle)	Signal recognition particle receptor (Docking Protein)	alpha/beta hydrolase fold (Decomposition)
Nutritional Stress Downregulated	LCCL domain-containing protein	cytosine-purine permease (Active Transporter)	N/A
Nutritional Stress Upregulated	N/A	N/A	N/A
Habitat Stress Upregulated	L-lysine 6-monooxygenase (NADPH-requiring)	Inhibitor of growth proteins N-terminal histone-binding (Inhibitory Enzymes)	DASH complex subunit Dam1 (Biological/Cellular Functions)
Habitat Stress Downregulated	UDP-glucose:Glycoprotein Glucosyltransferase (Glucose Metabolism)	rRNA (uridine-2'-O-)-methyltransferase activity (RNA Methylation)	Ribosomal protein S6 (Translation)

Table 1: Biological Processes
A table of top three hits that have predicted functions from differentially expressed genes. These are from genes that are unique to each condition.

DISCUSSION

To study the impacts of various stressors potentially due to climate change on *Phellinus tremulae,* we identified differentially expressed genes of isolates grown on different media compositions, as well as a heat shock treatment, to mimic changes in gene regulation under environmental stress. Through our analysis, we found that habitat stress had more differentially expressed genes than those of nutritional stress. Out of the carbon sources, there were more differentially expressed genes in isolates grown on yeast extract than D-xylose and D-mannose. These results could be due to the complexity of habitat stresses and the wide variety of compounds found in yeast extract. Habitat stresses are factors that affect the environment these fungi grow in while nutritional are factors that affect metabolism. Utilization of other genes for metabolism is a simpler process than dealing with temperature that can affect multiple aspects of an organism's metabolism. While many of the differentially expressed genes did not have known functions, some are well characterized and play important roles in cellular growth and essential biological processes (Table 1). Not surprisingly, we found that under nutritional stress, nutrient transporters were found to be differentially regulated.

Decomposition is a complex process not solely dependent on enzymes that break down carbohydrates but often involves many enzymes that do not actively facilitate decomposition. Transporters, such as amino-acid transporters, are an important class of enzymes used in decomposition (Rineau et al., 2013). They play an important role in

transporting these nutrients in and out of cells. Under the condition with yeast extract added, we found a downregulation of differentially expressed genes linked to sugar transporters. This could be due to the mix of different components in yeast extract, like amino acids and glucans, that these fungi have a harder time utilizing. These sugar transporters are known to be essential in utilizing the lignocellulose after degradation (Nogueira et al., 2018). Although the precise mechanism of sugar transporters in fungi is yet to be fully understood, they are associated with transporting sugars into cells. This finding indicates there is a change in the transport of sugar in the cell, which can lead to an effect on growth because sugar is the main source of carbon for most fungi.

The heat stress and pH conditions have some upregulation in growth inhibition and cell cycle checkpoints. These enzymes are associated with cell cycle regulations and have inhibitory properties that slow down or stop the growth of cells. This suggests that stressors may affect cell communication on when to initiate or halt growth to conserve energy in poor environmental conditions. Temperature and pH are environmental factors that are known to influence biological activities. This aligns with prior knowledge that high temperatures and fluctuating pH levels require greater energy and decreased yields in growth (Papagianni, 2004). The observation of increased gene expression in cell cycle checkpoints and growth-inhibiting enzymes during environmental pressures provides valuable information on how fungi conserve growth under stress.

Carbohydrate modification enzymes, such as hydrolases, were observed to be differentially regulated under nutritional stress. Hydrolases are known to be utilized in degradation, especially in carbon and nitrogen mineralization (Tan et al., 2020). When grown in the presence of D-mannose, *P. tremulae* isolates were found to have an upregulation of alpha/beta hydrolase expression, also seen in the treatment with yeast extract. Hydrolase enzymes play a significant role in breaking down nutrients for cells to uptake and use, so it is not surprising to see that there is a hydrolase differentially regulated to either increase or decrease these activities in response to nutritional stress.

The results of our study show habitat stress had more differentially expressed genes compared to those of nutritional stress in *P. tremulae*. Decomposition enzymes were found in nutritional stress while growth-inhibiting pathways were found in habitat stress. As *P. tremulae* growth is impacted by climate change due to stressors, such as nutritional and habitat stress, aspen tree parasitism rates can shift. This study can help us further understand the effects of climate change on saprotrophic fungi, including their decomposition abilities and their interactions with plants. Most fungal proteins remain functionally uncharacterized. Further research is needed to fully elucidate the role the proteins encoded by the differentially expressed genes play across a range of environmental conditions. In addition, future experimentation on other ecological guilds of fungi, such as ectomycorrhizal fungi, would provide a more comprehensive view of the impact of climate change on fungi and the consequences for ecosystem health.

ACKNOWLEDGEMENTS

A special thanks to the University of Utah School of Biological Science, Mountaineer Endowed Fund for Undergraduate Research, Natural History Museum of Utah, the National Science Foundation (NSF#2114785), and Honors College at the University of Utah for their support on this research. I would also like to thank Dr. Bryn Dentinger, Keaton Tremble, Alexander Bradshaw, Kendra Autumn, Sariah VanderVeur, and the rest of the lab for their guidance and support throughout this process.

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Date of Submission: April 30, 2023