HHS Public Access

Author manuscript

Biodegradation. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Biodegradation. 2018 October ; 29(5): 463–471. doi:10.1007/s10532-018-9843-z.

Evaluating the Mycostimulation Potential of Select Carbon Amendments for the Degradation of a Model PAH by an Ascomycete Strain Enriched from a Superfund Site

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Abstract

Although ecological flexibility has been well documented in fungi, it remains unclear how this flexibility can be exploited for pollutant degradation, especially in the Ascomycota phylum. In this work, we assess three mycostimulation amendments for their ability to induce degradation in *Trichoderma harzanium,* a model fungus previously isolated from a Superfund site contaminated with polycyclic aromatic hydrocarbons. The amendments used in the present study were selected based on the documented ecological roles of ascomycetes. Chitin was selected to simulate the parasitic ecological role while cellulose and wood were selected to mimic bulk soil and wood saprobic conditions, respectively. Each amendment was tested in liquid basal medium in 0.1% and 1% (w/v) suspensions. Both chitin and cellulose amendments were shown to promote anthracene degradation in *Trichoderma harzanium* with the 0.1% chitin amendment resulting in over 90% removal of anthracene. None of the targets monitored for gene expression were found to be upregulated suggesting alternate pathways may be used in *T. harzanium.* Overall, our data suggest that mycostimulation amendments can be improved by understanding the ecological roles of indigenous fungi. However, further research is needed to better estimate specific amendment requirements for a broader group of target fungi and follow up studies are needed to determine whether the trends observed herein translate to more realistic soil systems.

Keywords

Mycoremediation; polycyclic aromatic hydrocarbons; *Trichoderma harzianum*; bioreactors; bioremediation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of legacy pollutants found at 155 currently active Superfund sites [1]. PAHs have been shown to be particularly recalcitrant to degradation, especially after weathering for an extended length of time [2]. In general, due to their recalcitrant properties and overall slow biodegradation, bacteria-centered

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bioremediation tends to be difficult to implement for high molecular weight PAHs [3]. As a result, site managers often resort to physico-chemical treatment such as excavation, *ex situ* treatment and landfilling in order to remediate PAHs [4]. Most bioremediation studies targeted at PAHs have focused on bacteria which often require contaminant transport into the cell for degradation and tend to have narrow degradative abilities [5]. By contrast, fungi have evolved numerous promiscuous enzymatic systems many of which can act on PAHs extracellularly [6]. The reliance of fungi on these extracellular enzymes are particularly attractive as many PAHs, especially heavier PAHs, are extremely hydrophobic and their diffusive transport across cell membranes is slow thereby severely limiting their biodegradation potential. Extracellular fungal enzymatic systems have evolved primarily to increase the breadth of plant materials fungi can use as food sources, however these enzymatic systems are known to be promiscuous enough to also act on PAHs [7, 8]. Several fungal phyla have been shown to take advantage of enzymatic substrate promiscuity, leading fungi to either transform these pollutants cometabolically or to metabolize them by incorporating intermediates in their growth cycles [9]. Several research groups have demonstrated the involvement of multiple promiscuous enzyme systems even when fungi are exposed to stressful conditions such as temperature, salinity and pH extremes [7, 10–14]. Lade et al. [15] also showed that synergistic relationships can develop between fungi and bacterial degraders where fungi initiate degradation using their non-specific enzymatic systems and then bacteria carry the degradation forward. Fungi with hydrophilic filaments have been shown to conduct bacterial degraders throughout the network, aiding in dispersal and resulting in better biodegradation than in the absence of their filaments [16, 17]. Finally, some fungi have been observed to overcome hydrophobic limitations on PAH transport by actively transporting PAHs over the range of centimeters to bacteria [18]. From all these previous studies, it is clear that fungi have advantageous properties which could be exploited in the context of soil bioremediation.

In previous work, we found that fungi belonging to the phylum *Ascomycota* were especially numerous at a heavily contaminated PAH Superfund site [19]. Thus, herein, we sought to identify an ascomycete resistant to high PAH concentrations and test various lineage-targeted mycostimulation treatment approaches. In particular, we applied an amplicon based metagenomic approach to scan soil obtained from the Atlantic Woods Industries (AWI) Superfund Site [20] to: 1) isolate a model fungus from the site; and 2) test a range of amendments to identify the most promising mycostimulation strategy on a model PAH. In addition, gene expression assays were developed to monitor the expression of several enzyme families with putative activities towards PAHs.

Materials and Methods

Isolation of a Model Fungus from the Ascomycota Phylum

Approximately 0.5–1 g of soil from the AWI site was plated on potato-dextrose agar (VWR, Radnor, PA) and incubated at ~20°C until growth was visible (~2–5 days). This short incubation time was chosen in order to avoid plate overgrowth. Soils were also diluted 10-800 fold and plated on Rose Bengal agar (BD, Franklin Lakes, NJ). Phenotypically distinct colonies were transferred to fresh potato-dextrose agar plates until pure cultures

were isolated. Pure cultures were confirmed by DNA sequencing and using NCBI's nucleotide BLAST tool [21]. Spore suspensions were obtained by rinsing mature cultures with basal medium (containing 5 g $(NH_4)_2SO_4$/L, 2.5 g $MgSO_4 \cdot 7H_2O$/L, 20 mg $CaCl_2 \cdot 2H_2O$/L, 1.0g KH_2PO_4/L, 1.01 mg $MnSO_4 \cdot H_2O$/L, 0.24 mg $Na_2MoO_4 \cdot 2H_2O$/L, 0.10 mg $NiCl_2 \cdot 6H_2O$/L, 0.17 mg $CuCl_2 \cdot 2H_2O$/L, 1.36 mg $FeSO_4 \cdot 7H_2O$/L, 0.24 mg $CoCl_2 \cdot 6H_2O$/L and 0.58 mg $ZnSO_4 \cdot 7H_2O$/L) and centrifuging at speeds greater than 10,000xg in microfuge tubes (Eppendorf, Hamburg, Germany) [22, 23].

Amendment Materials and Degradation Experimental Design

Degradation experiments were carried out in 20 mL borosilicate glass vials with solid top fluoropolymer resin-lined screw caps (I-Chem 200 Series, Thermo Scientific, Waltham, MA). Reactors were set up to test the influence of three amendments (cellulose, chitin, and wood substrates) supplied at two different concentrations (0.1% and 1% w/v) on the degradation of anthracene introduced at three concentrations (0.4 mg/L, 4mg/L, and 40 mg/L). Anthracene was selected as the model PAH as it tends to be ubiquitous at PAH contaminated sites and has been well studied previously [19, 24, 25]. Carbon amendments were selected to mimic the substrates used by fungi in natural ecosystems. Chitin powder (Alfa Aesar, USA) was selected to mimic cell walls of fungi, simulating the conditions of a parasitic ecological role. Cellulose (microcystalline cellulose powder 97–102%, MP Biomedicals, USA) was chosen as a proxy for natural organic material under mostly bulk soil saprobic conditions. Wood flour (Brown wood flour, System Three, USA) was selected for baseline comparison purposes as wood has been commonly used historically for fungal biostimulation. Powdered amendment substrates were used to eliminate any biases based on surface area differences as well as to ensure adequate anthracene recovery in analytical methods.

To prepare the 4 mg/mL stock anthracene solution, 80 mg of anthracene (Sigma-Aldrich, St. Louis, MO) was dissolved in 20 mL of acetone, which was serially diluted ten-fold to prepare two more stock solutions (0.4 mg/mL, and 0.04 mg/mL anthracene). A Hamilton syringe was sterilized over a flame, heated to red hot, allowed to cool and then rinsed thrice in acetone before adding 50 μL of the stocks to the bottom of the glass vials so each contained either 200, 20, or 2 ng. Reactors containing a range of anthracene were set up to ensure inhibitory levels were not reached. Each amendment was added in either a 0.1 or 1% w/v ratio in basal medium and autoclaved prior to inoculum addition. Five mL of basal medium and 10^7 fungal spores (as determined by serial dilution and hemacytometer counts) were added to the reactors in a laminar hood. Vials were sealed using sterile cotton plugs to ensure sterile conditions were maintained throughout reactor set up and incubation. Nine reactors were prepared for most treatment conditions for each of four timepoints: four replicates for all biological conditions and five for chemical analysis except for controls, which had three replicates.

Biological and Chemical Anthracene Degradation Analysis

Four reactors were sacrificed at each sampling time point for biological analysis while five reactors were used for most chemical analyses. The sampling time points consisted of: 1) day zero; 2) after growth onset was first sufficient to be observed by visual inspection (6

days); 3) when most of the reactors had grown spores into mats (15 days); and 4) after the mats had matured (30 days). At each sampling time, 2 mL of cells were split between two 2 mL microfuge tubes (Eppendorf, Hamburg Germany). Cell pellets were obtained by centrifuging the tubes for ten minutes at 10,000xg. The supernatant was removed from the pellets before they were submerged in 500 μL RNAlater ® (Sigma-Aldrich, St. Louis, MO). The pellets were stored at −80°C until RNA was extracted. Only the mid-level anthracene reactors (nominally 20 ng) from the start and the end of the experiment were analyzed. Briefly, samples were acidified to pH<2 with 25 μL 6 M HCl, and extracted three times by sonicating in 1:1 hexane:dichloromethane. The organic layers were combined and diluted to 25 mL. A 0.25 mL aliquot was transferred to an autosampler vial, spiked with 100 μL D_{10}-anthracene (AccuStandard, New Haven, CT) as an internal standard, and diluted to 1 mL with dichloromethane. Analysis was performed via gas chromatography with mass spectrometry in electron ionization mode with selected ion monitoring on an Agilent 7890A GC and 5975C mass spectrometer. Separation was conducted on an Agilent HP-5MS column (30 m x 0.25 μm film thickness) over a thermal gradient (45°C for 2 min, to 300°C at 9°C/min, hold 300°C for 15 min) at constant flow (1.2 mL/min).Anthracene and D_{10}-anthracene were detected at 178 and 188 *m/z*, respectively. Method detection limit was 0.30±0.08 ng/mL .

The fungal pellet was washed twice by adding 1 mL of molecular biology grade water, centrifuging at 10,000xg and decanting the supernatant to remove the RNA Later solution. RNA was extracted from the resulting cell pellets using the FastRNA SPIN Kit for Yeast (MP Biomedicals, USA) and the MiniBeadbeater 16 (Biospec Products, USA) per the manufacturer's instructions. Following extraction, RNA concentrations were measured via fluorescence using the Qubit BR RNA kit (Invitrogen Thermo FisherScientific, Waltham, MA). RNA concentrations above 200 μg/mL were further diluted as to not exceed the maximum recommended RNA concentrations for cDNA synthesis. The TurboDNAse free kit (Ambion , Thermo Fisher Scientific, Waltham, MA) was used to remove DNA from the RNA samples per the manufacturer's routine treatment protocol. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was then used to synthesize cDNA from the RNA according to the manufacturer's instructions. cDNA was stored at −20 C until analysis. Briefly, 2 μL 10X RT Buffer, 2 μL 10X RT Random Primers, 0.8 μL 25X dNTP Mix (100mM), 1 μL MultiScribe® Reverse Transcriptase (50U/μL), and 4.2 μL nuclease free water were mixed and added to 10 μL cleaned RNA on ice to prepare each cDNA reverse transcription reaction. Reverse transcription conditions of 25°C for 10 minutes, then 37°C for 120 min, then 85°C for 5 min were carried out on a T100 Thermocycler (Bio-Rad, Hercules, CA). The resulting cDNA was stored at −20°C until qPCR analysis.

Gene expression assays were developed for genes classified as homologous according to the Carbohydrate Active Enzymes database (http://www.cazy.org/) [26]. In particular, we focused on genes within the glycoside hydrolase family 7 (cellulase and hemicellulase activity), glycoside hydrolase family 18 (chitinase activity), and glycoside hydrolase family 33 (chitinase and cellulase activity lacking a substrate-binding site), as well as adherence to hydrophobic surfaces (*qid74,* a proxy for hydrophobins). The glycoside hydrolase 7 (GH 7) assay (Table 1) was derived from a previously published primer set originally used to amplify genes coding for cellobiohydrolases expressed in soils [27]. The glycoside

hydrolase 18 (GH 18) primer set was developed by aligning sequences from closely related species to the chosen isolate (i.e. *T. virens, T. atroviridi* with *T. harzianum* CBS 226.95 v1.0) using the Mycocosm portal to find sequence homology within the GH 18 locus [28, 29]. Primers were designed with redundancy to complement the identified conserved sequence homology that spanned between 100 and 150 bp in order to account for strain-strain differences. Primer sets for *chitinase 33*, were chosen because it is a chitinase that lacks a substrate-binding domain and has been shown to be involved in mycoparasitism [30, 31]. The primer set for *qid74* was selected because it was shown to mediate hydrophobic surface attachment during mycoparasitism [32]. For quantification purposes, each target was normalized to the 18S gene using previously published methods [33]. All qPCR assays were performed on a LightCycler 96 thermocycler (Roche Diagnostics, Indianapolis, IN). The qPCR reactions were performed in a total reaction volume of 20 μL using a QuantiTect SYBR Green assay. Relative quantification of target gene expression levels were subjected to one-way ANOVA in qBase Plus to ascertain statistical significance [34]. Significance analyses for degradation experiments were carried out using basic stats in R and the BSDA package [35].

Results and Discussion

Choice of Model Fungus for Mycostimulation Tests

Approximately 13 distinct ascomycete and zygomycete strains were isolated. Notable fungal isolates cultured here belonged to the *Mortierella* (previously *Zygomycota,* now *Mucoromycota* [36]), *Penicillium* (*Eurotiales* order), *Bionectria* and *Trichoderma* (*Hypocreales* order) genera (Figure 1). Because ascomycete fungi have been relatively mechanistically unstudied and found to contain numerous potential biostimulation candidates, we chose to focus on the Ascomycota phylum. Based on the availability of a full genome, sporulation capacity, and zone of clearing observed after plating on Rose Bengal agar, we selected the isolate *Trichoderma harzianum* for further studies. *T. harzanium* has been shown to sporulate under stressful conditions, is ubiquitous in soils and has ecological flexibility [37]. *T. harzianum* can assume various ecological roles including parasitic in the presence of certain nematodes and fungi as well as saprotrophic in the presence of organic material in bulk soil and wood [38, 39]. In addition, the genome of *T. harzianum* CBS 226.95 v1.0 contains a range of genes which may be useful for degrading PAHs including 73 genes that have chitinase activity, 15 genes that have cellulase activity, and 8 genes that encode hydrophobins [28, 29].

White-rot basidiomycete fungi have been found to degrade a variety of pollutants exhibiting a range of chemical structures including multiple aromatic rings (e.g., PAHs such as benzo(a)pyrene and anthracene), chlorinated substituted aromatic rings (e.g. dioxins and polychlorinated biphenyls), and long chain petroleum hydrocarbons such as hexadexanes [41, 42]. Exudates from white rot fungi have been thoroughly studied and the literature includes various surveys of lignolytic and non-lignolytic oxidoreductase enzymes and their respective degradative potential towards various contaminants. Basidiomycete fungi use their wood-degrading mechanisms to degrade pollutants in a way that might be analogous to mechanisms employed by ascomycete fungi in pollutant degradation. For instance,

filamentous fungi have evolved mechanisms of attachment to hydrophobic surfaces (via hydrophobins) in order to parasitize plants or other fungi [43–45]. These same mechanisms have also been shown to be active when challenged with non-living hydrophobic surfaces such as Teflon and to be upregulated during heavy-metal bioremediation [45–47].

Ascomycete fungi have also been shown to degrade a considerable range of pollutants [3] and, while they dominate fungal microbial communities in PAH polluted soils, they have not been well characterized for mycoremediation application. Some of our isolates were closely related to species with known degradation capacity although relatively little is known about the degradative pathways in ascomycete fungi. *Penicillium janthinellum* has been shown to degrade pyrene to 1-pyrenol and pyrenequinones [48]. *Bionectria* sp., *Trichoderma* sp. and *Penicillium* sp. have been seen to solubilize benzo[a]pyrene [49], *Bionectria* sp. in particular, have been shown to degrade it. Cerniglia and coauthors present a review where they indicate that many ascomycete fungi, including our isolates, degrade PAHs [5]. Harms and coauthors suggest that internal processes involving nitroreductases, and transferases may also play roles in degrading organic contaminants such as PAHs [3]. Ecological roles of fungi have also been proposed to determine which mechanisms are used in bioremediation, finding saprotrophic fungi to have hydrolases and miscellaneous enzymes targeting carbohydrates that may play also play a role in hydrophobic pollutant degradation [8].

Evaluation of Mycostimulation Treatment Strategies

Chitin and cellulose amendments positively stimulated the removal of anthracene by *T. harzanium* while the wood flour had no stimulatory effect (Figure 2). Approximately 29–33% of the anthracene was lost in the abiotic controls suggesting volatilization may have occurred over the course of the experiment. The wood treatments did not stimulate anthracene removal significantly over the control ($p>0.05$). This result is consistent with the limitations observed in previous wood-based mycostimulation tests in polluted soils [50] and also explains the persistence of woodchips in contaminated media also piled on the site where samples were collected [19].

We visually observed growth on wood (data not shown), so we had expected to see upregulation of genes involved in cellulose degradation (i.e., GH7) as cellulose is one of the polymers in wood [51, 52]. However, the cellulose in wood is bound to a lignin matrix [52], which may explain why less removal was observed. Expression of the GH7 gene associated with the GH7 enzyme family was not upregulated (Figure 3). This either suggests that alternate pathways were used to support growth on wood, such as cytP450 monooxygenases [53], or that samples were collected at a time when GH7 was not active.

In the cellulose treatments, both the 0.1 and 1% cellulose amendments stimulated more removal than the control ($p \leq 0.05$). In fact, the cellulose treatment ($64 \pm 6\%$ and $79 \pm 9\%$, for the 0.1 and 1%, respectively) stimulated more than twice the removal of the abiotic control (~29%). The removal observed in the 1% cellulose treatment was not significantly higher ($p > 0.05$) than that observed in the 0.1% reactors. This finding is of note because a large portion of the organic materials typically entering soils consist of cellulose [54, 55]. This suggests that the organic material composition should be examined when considering adding more cellulose to PAH-polluted soils, as it may impact the amount of cellulose that

would need to be amended. Again, we had expected to see upregulation in the *GH7* gene, but none was observed. As there are up to 15 different genes which have been shown to be used by pathways degrading cellulose, it is possible that the wrong gene was targeted here [28, 29]. Also, some cellulase enzymes have been shown to exhibit broad substrate promiscuity because they lack binding sites to recognize parts of cellulose [56], while other cellulases have multiple binding sites [57]. To account for potential upregulation of such promiscuous genes, we targeted an additional gene known to bind cellulose: *chitinase33* [30], however no upreguation was observed for that gene as well.

The greatest substrate promiscuity was observed in the 0.1% chitin amendment where 93 ± 3% of the anthracene was removed. By contrast, only 53% anthracene removal was observed in the presence of 1% chitin. These data suggest a potential explanation for why PAHs persist in soils containing known chitin degraders: substrates such as chitin in the bulk soil may inhibit use of PAHs as substrates. One potential explanation for the high removal seen in the 0.1% chitin is that there was not enough carbon supplied to sustain the fungus alone, causing it to resort to anthracene as a supplementary carbon source to incorporate into its metabolism. This high level of removal is consistent with other published studies. In one study, over 90% removal was observed when ascomycetes relied on anthracene as the sole carbon source [25]. Another study found anthracene degradation linked to the up-regulation of chitin degradation genes, which suggests that the enzymes these genes encode may exhibit substrate promiscuity between chitin and anthracene [8]. We anticipated to detect upregulation with assays targeting *GH18* and *chitinase33* because these enzymes have been shown to bind to chitin [58, 59]. Furthermore, we expected upregulation in *qid74* because it aids attachment to hydrophobic surfaces [44], a first step in modifying the surface [45, 60]. Additionally, *qid74* and has been shown to be involved in pollutant coping mechanisms [47, 61], yet none of these genes were significantly upregulated at the times sampled and therefore we cannot draw any meaningful conclusion regarding mechanisms for this study. In *T. harzianum*, there are 73 genes that could potentially be stimulated by chitin [28, 29] and any of those genes could lead to anthracene removal. Interestingly, others have shown that *T. harzianum* can produce inhibitory substances in an attempt to protect its personal food source from other microbial invaders [8]. Fungal inhibitory substances have also been shown to enhance PAH removal [62, 63]. However, in the absence of any competitors, *T. harzianum* may have only produced inhibitory substances under extremely limited conditions, and not under the 1% chitin treatment scenario. This may explain why a lower anthracene removal was observed herein. Finally, the fact that we were unable to capture changes in gene expression, likely because alternate pathways were employed, suggests that an alternative method that captures differences across the whole transcriptome should be used to obtain mechanistic pathway information. In the future, perhaps methods such as subtractive library hybridization [32] performed on mRNA or RNAseq would be better suited to identify targets for subsequent gene expression studies.

Acknowledgments

Funding for this work was gratefully provided by the NIEHS-supported Duke University Superfund Research Center (NIEHS grant P42-ES010356).

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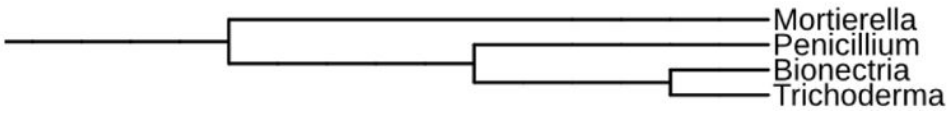

Figure 1.
Cladogram according to NCBI taxonomy of genera for 13 fungal isolates from AWI (http://phylot.biobyte.de/) [40].

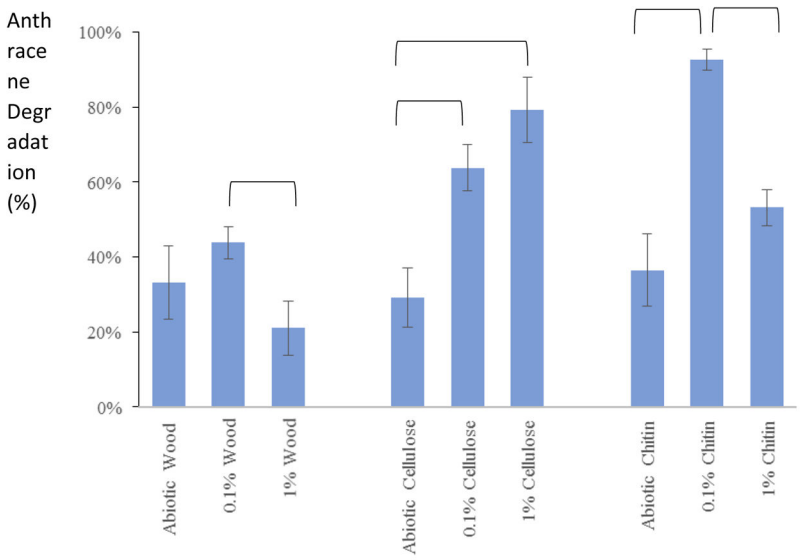

Figure 2.

Anthracene loss observed in treatments. Error bars represent standard error (N=3) while brackets represent significantly different degradation within amendment type ($p<0.05$)

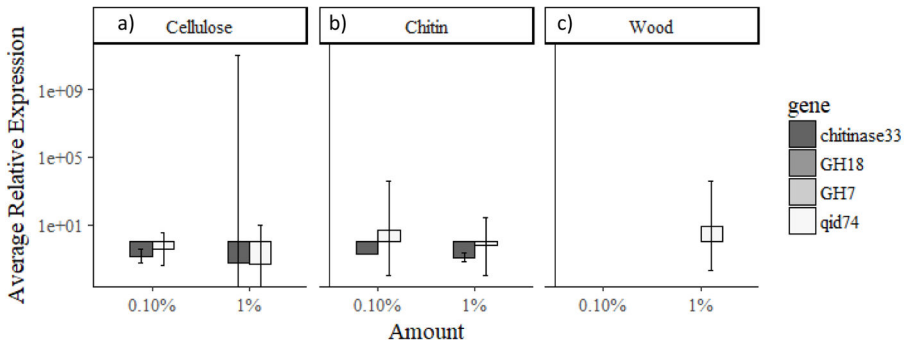

Figure 3.

Average relative expression data suggests that no significant differences can be inferred based on the 95% confidence intervals indicated by error bars.

Table 1

Primers used in gene expression assays.

Name	Primer (F/R)	Sequence
GH 7	Forward	5'-ACC AAY TGC TAY ACI RGY AA-3'
	Reverse	5’-GCY TCC CAI ATR TCC ATC-3'
GH 18	Forward	5’-GTC GAC GTA CTT CTT GAT AA-3'
	Reverse	5’-AAC TTC GGC TTG ACT ATG-3'
Chitinase 33	Forward	5’-TGG AGC TCA ACA GGC GCT GC-3'
	Reverse	5’-ACG-ACG-GCA-CTG-CCA-AAG-GG-3'
qid74	Forward	5’-CAG AAG AAG TGC GTG TGC AAC AAG-3'
	Reverse	5’-AGC TAG CAT CTT TGC CGC AGT TTG-3'
18S	Forward	5’-CGA TAA CGA ACG AGA CCT-3'
	Reverse	5’-AIC CAT TCA ATC GGT AIT-3’