

## ARTICLE



# Drosophila attack inhibits hyphal regeneration and defense mechanisms activation for the fungus *Trichoderma atroviride*

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The capability to respond to wounding is a process shared by organisms of different kingdoms that can result in the regeneration of whole-body parts or lost structures or organs. Filamentous fungi constitute a rich food source that ensures survival and reproduction of their predators and are therefore continuously exposed to mechanical damage. Nevertheless, our understanding of how fungi respond to wounding and predators is scarce. Fungi like plants and animals respond to injury recognizing Damage- and Microbe-Associated Molecular Patterns (DAMPs/MAMPs) that activate  $\text{Ca}^{2+}$  and Mitogen-Activated Protein Kinase dependent signaling for the activation of defense mechanisms. During herbivory, plants, in addition to activating pathways related to injury, activate specific responses to combat their predators. Using a transcriptional approach, we studied the capacity of the filamentous fungus *Trichoderma atroviride* to activate specific responses to injury and attack by different arthropods. Attack by *Drosophila melanogaster* inhibited the transcriptional activation of genes required for hyphal regeneration, and the fungal innate immune and chemical defense responses. We also provide mechanistic insight of this inhibition involving components of the *D. melanogaster* salivary glands that repress the expression of a set of genes and block hyphal regeneration.

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## INTRODUCTION

As cosmopolitan organisms, fungi share a niche with multiple organisms where they are exposed to different competitors and predators [1]. Fungi are absorptive heterotrophs, playing a major role in recycling dead and decayed matter and represent a nutrient source rich in amino acids and sugars for their predators [2].

Multicellular organisms have developed specific strategies to respond to mechanical injury and pathogen attack. A primary defense mechanism is the activation of innate immunity, which involves proteins that recognize molecules produced by foreign organisms or by themselves [3, 4]. These molecules are classified in MAMPs, derived from microbes, and DAMPs that are self-derived [5]. Pattern Recognition Receptors (PRRs) are in charge of recognizing MAMPs [6]. During the animal and plant innate immune responses, PRR-dependent signaling pathways trigger cell death. In plants, this occurs via the hypersensitive response and in animals via inflammasome assembly [6–8].

The existence of fungal PRRs, with a similar structure to plant and animal nucleotide-binding leucine-rich repeat (NLR) immune receptors, has been documented [8, 9]. Fungal NLRs have been associated with heterokaryon incompatibility during cell fusion [10] and with a fungal innate immune system [11]. Fungi can recognize MAMPs [12, 13] and DAMPs [14, 15] that trigger defense responses. The main defense of fungi is chemical, i.e., the production of toxins impairing growth, development, or viability of their antagonists. In addition, fungi possess an innate immune system that allows them to distinguish self from non-self and results in cell death or autophagy. The immune system restricts horizontal transmission of deleterious cytoplasmic elements, such

as viruses, and prevents resource plundering by parasitic genotypes and a pro-survival function in response to bacteria [12, 13, 16]. Recognition of these cues involves activation of ATP, calcium ( $\text{Ca}^{2+}$ ), and Reactive Oxygen Species (ROS) dependent signaling pathways. As observed in plants and animals, activation of programmed cell death and reactivation of the cell cycle is part of the fungal damage response, which is linked to the innate immune response [14, 15, 17].

*D. melanogaster*, the springtail *Folsomia candida*, and the nematode *Aphelenchus avenae* have been used as fungivorous models in interactions with *Aspergillus* spp. and *Coprinopsis cinerea*, demonstrating the importance of the fungal chemical defense against predators [18–24]. In addition, *C. cinerea* initiates a transcriptional response to different fungivorous and non-fungivorous organisms such as bacteria, nematodes, and to wounding, including the activation of defense-related genes [23]. The capacity of fungi to defend themselves against competitors and predators is also linked to the innate immune system. In *Fusarium graminearum* bacterial MAMPs induce processes associated with innate immunity in animals, such as mitochondrial activity, activation of ROS production-related genes, and the transcriptional upregulation of PRRs [12].

The filamentous fungus *Trichoderma atroviride* is a model to study regeneration due to the similarities of its response to mechanical injury to that of higher eukaryotes. *T. atroviride* regenerates its hyphae upon mechanical injury, which triggers asexual reproduction in the damaged area [17]. Mitogen-Activated Protein Kinases (MAPKs) and  $\text{Ca}^{2+}$  mediated signaling, as well as the transcriptional activation of NLR (HET) encoding genes are

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considered key for hyphal regeneration in *T. atroviride* [14, 15]. Furthermore, the attack on *T. atroviride* by *D. melanogaster* larvae represses the production of putative defense compounds and the expression of chemical-defense related genes [25].

Here we use *T. atroviride* in a model system to study antagonistic interactions of fungi with arthropods. We describe how *T. atroviride* establishes a different transcriptional response to mechanical injury and to chewing arthropods. We show that salivary gland components of *D. melanogaster*, during larval grazing on *Trichoderma*, inhibit hyphal regeneration and defense, by blocking the expression of genes critical to signal perception and the fungal innate immune response.

## METHODS

### Trichoderma spp. strain

*Trichoderma atroviride* IMI 206040 used throughout was preserved as described [25].

### Arthropod models

To compare the fungal response to arthropod grazing, we used *D. melanogaster* SD-5 and the Collembolan *Orthonychiurus folsomi* (obtained from the Faculty of Sciences, UNAM, Mexico). *D. melanogaster* SD-5 reared as previously described [25], and the collembolans maintained in sterile soil at 23–25 °C, 70% humidity in darkness and fed mushrooms every seven days.

### Response to arthropod grazing

To compare the morphological response of *T. atroviride* to arthropods and mechanical injury, we inoculated  $1 \times 10^8$  fresh conidia and incubated them in Petri dishes containing PDA for 36 h in darkness at 27 °C. To examine the grazing response, 20 larvae or collembola were placed on the mycelia for 10 min in darkness and immediately withdrawn. Mechanical injury was inflicted with a sterile star-shaped metal cookie cutter. After grazing or mechanical damage, plates were incubated 48 h in darkness at 27 °C and photographed. Five independent biological replicates were performed, each with three technical replicates.

### RNA preparation

For differential gene expression analyses, RNA was extracted from *T. atroviride* grown on PDA plates covered with sterile cellophane sheets and incubated for 36 h in the dark at 27 °C. Interactions between *T. atroviride* and the attackers were performed as mentioned above, but using 100 arthropods. For mechanical injury, the complete fungal colony was cut rapidly using a sterile scalpel. After arthropod attack or mechanical injury, the plates were incubated for an additional 30 min, 90 min, four and eight hours, collected, and immediately frozen in liquid nitrogen. Control mycelia plates were opened for 2 min in the dark and incubated for an additional 30 min and 8 h. Each treatment had three biological and three technical replicates. RNA was extracted using the TRIZOL method.

### Sequencing and RNA-seq data analysis

RNA sequencing libraries were prepared following the Illumina TruSeq Stranded Total RNA Sample Preparation kit instructions. A total of 42 TruSeq libraries were sequenced using the NextSeq500 platform (Illumina) in the  $1 \times 75$  single-end mode, obtaining an average of 12 million raw reads per library (Supplementary Table 1).

Raw RNA-seq data were processed with FastQC Version 0.11.6 [26], obtaining about 10 million high-quality reads per library. Raw RNA-seq data are available at Gene Expression Omnibus (GEO) accession number: GSE152652. High-quality reads were aligned to the *T. atroviride* genome using HISAT2 version 2.1.0 [27] and counted using HTseq version 0.14.1 [28]. The genome sequence is available on NCBI genomes, accession number: JAEAGS000000000. Differential expression analyses were carried out using *Edge R* version 3.11 [29]. We detected no significant differences between control (T0 and T8) libraries, except for CT0-2, which was discarded due to its poor quality. Contrasts between libraries were performed using an FDR < 0.005. Venn Diagrams were drawn using: <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

We performed hierarchical clustering followed by the K-means analysis, supported by a Pearson correlation  $P > 0.85$  and 50 iterations per core.

Codes used for RNA-seq data analysis are available at <https://github.com/Karina-atriztan/RNA-seq-data-analysis>.

### Motif prediction and orthologues searching

To determine if gene cores contained specific DNA binding motifs in their promoters, we performed a motif prediction on 11 selected cores. We extracted 700 bp upstream of the ATG using the script: <https://github.com/AgustinPardo/upDownStreamSeqsFromGbk>. For prediction of enriched ungapped motifs we used STREME of the MEME suite (<http://meme-suite.org>) with maximum and minimum lengths of 15 and eight nucleotides, respectively ( $E$  value threshold >0.05). As a control, we used the same number of random upstream sequences per core. De novo prediction was performed using MEME with a maximum of 20 motifs ( $E$  value >0.05), and maximum and minimum length of 15 and six nucleotides, respectively. For motif comparison, we used the Tomtom tool from MEME suite, using *S. cerevisiae* and Yeast database with default options. An overlap <70% between motifs and a  $p$  value >0.001 were used to select homologous motifs.

To find TF orthologues, we extracted the sequences for *S. cerevisiae* and *A. nidulans* from the Yeast database (<http://www.yeastextract.com/index.php>) and AspGD (<http://www.aspergillusgenome.org/>) databases. Candidates were obtained by Bidirectional Blast between the *T. atroviride*:*Saccharomyces cerevisiae*:*Aspergillus nidulans* proteomes. Candidates were supported by Hidden Markov Model analysis using HMMER V.3.2.1 ([hmmer.org](http://hmmer.org)).

Phylogenetic relationships between protein candidates were analyzed using Neighbor-Joining trees with 1000 Bootstrap resampling using MAFFT V.7 (<https://mafft.cbrc.jp/alignment/server/>). Additional fungal sequences were obtained from UNIPROT (uniprot.org) and FungiDB (fungidb.org).

### GO enrichment analysis

To determine which processes were enriched in selected gene sets, we performed GO enrichment analysis using *topGO* version 3.11 [30] ( $P > 0.05$ ) for enriched Biological process or Molecular Function. Redundancy was eliminated using REVIGO [31]. Heatmaps were generated using the *Heatmap2* function of the *gplots* package from Bioconductor R.

### Regeneration inhibition assays

To test larval salivary gland components we dissected 300 salivary glands of 3rd instar *D. melanogaster* larvae under a stereoscope Zeiss Stemi 2000. Ten glands were homogenized in 30  $\mu$ l PBS and centrifuged at maximum velocity for 10 seconds at 4 °C. We used approximately 0.024  $\mu$ g/ $\mu$ l of total protein for regeneration inhibition assays [32]. For fungal microcultures, 50 conidia were inoculated on sterile slides containing 2 ml PDA and incubated for 16 h at 27 °C in darkness.

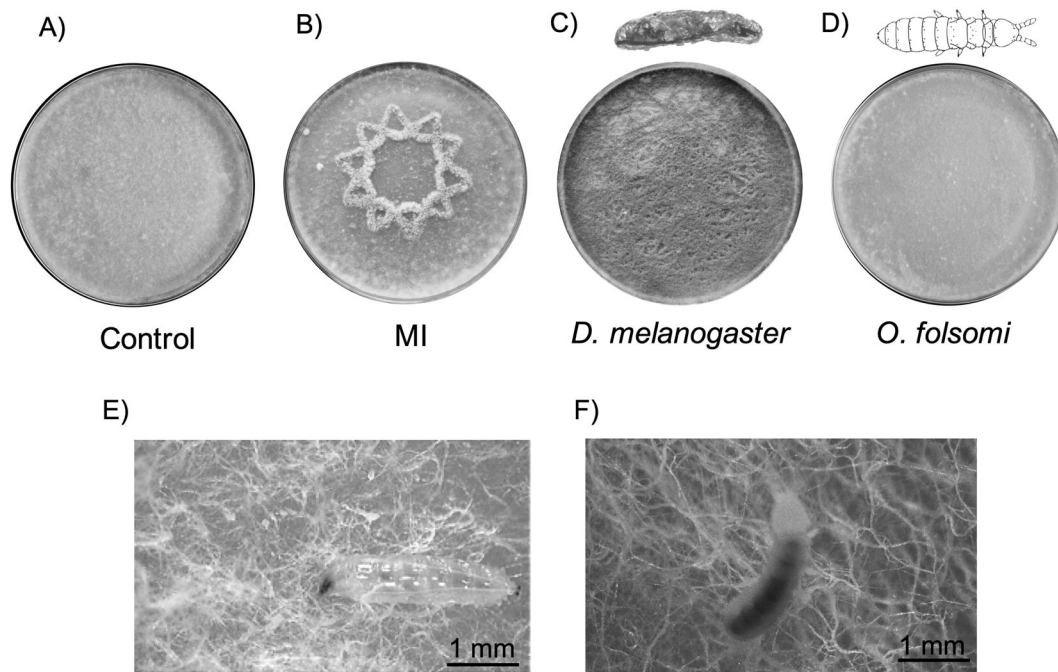
A sterile scalpel with 1  $\mu$ l of homogenized tissue was used per cut on the mycelia (MI + SGE), after cutting microcultures were incubated at 27 °C in darkness for one and two hours. We observed no significant differences when counting 1 or 2 h after the treatment.

Hyphae were stained with lactophenol blue and observed under a Leica DM6000-B microscope fitted with a 100x objective HCX PL Fluotar (0.75 N. A) and photographed with a Leica DFC 429 C camera. As a control, we used a clean scalpel to cut the mycelia (MI). Again, all experiments used three independent biological and three technical replicates. We determined the proportion of damaged hyphae that regenerated out of two hundred (100%) using a one-way ANOVA and Tukey test ( $P < 0.05$ ).

## RESULTS

### *T. atroviride* distinguishes between mechanical damage and arthropod attack

We hypothesized that, like plants, *T. atroviride* discriminates between chewing arthropods and mechanical injury. We, therefore, analyzed the response of *T. atroviride* to two chewing arthropods (Fig. 1), namely *D. melanogaster* (Dm) larvae and the springtail *Orthonychiurus folsomi* (Of), and compared it to that displayed upon mechanical injury (MI). As expected, attack by *Drosophila* larvae and mechanical injury triggered conidiation in the damaged area (Fig. 1B, C) [25]. Interestingly, the collembolans caused no evident response (Fig. 1D); the fungal colony looked just like the undamaged control (Fig. 1A). When we observed the arthropods' behavior under a magnifying lens, it became evident



**Fig. 1** *T. atroviride* displays an attacker specific response. The upper panel of the figure shows photographs of *T. atroviride* growing on PDA plates for 48 h after being subjected to (A) No challenge (Control); (B) Mechanical injury (C) Grazing by *D. melanogaster* or (D) *O. folsomi*, as indicated. The lower panel shows photographs of (E) A *D. melanogaster* larva eating *T. atroviride* mycelia. (F) A springtail on *T. atroviride* mycelia.

**Table 1.** Differentially Expressed Genes (DEG) obtained after differential expression (DE) analysis for mechanical injury and attack (by *D. melanogaster* larvae, and the collembola *O. folsomi*) when comparing the response against the control condition.

Mechanical injury	Total of DEG	Upregulated genes	Downregulated genes
30 min AI	3398	1695	1703
90 min AI	2698	1222	1476
4 h AI	798	464	334
8 h AI	858	653	205
<i>D. melanogaster</i>	Total of DEG	Upregulated genes	Downregulated genes
30 min	2804	1136	1668
90 min	1971	650	1321
4 h	542	281	261
8 h	1111	650	461
<i>O. folsomi</i>	Total of DEG	Upregulated genes	Downregulated genes
30 min	136	9	127
90 min	0	0	0
4 h	0	0	0
8 h	0	0	0

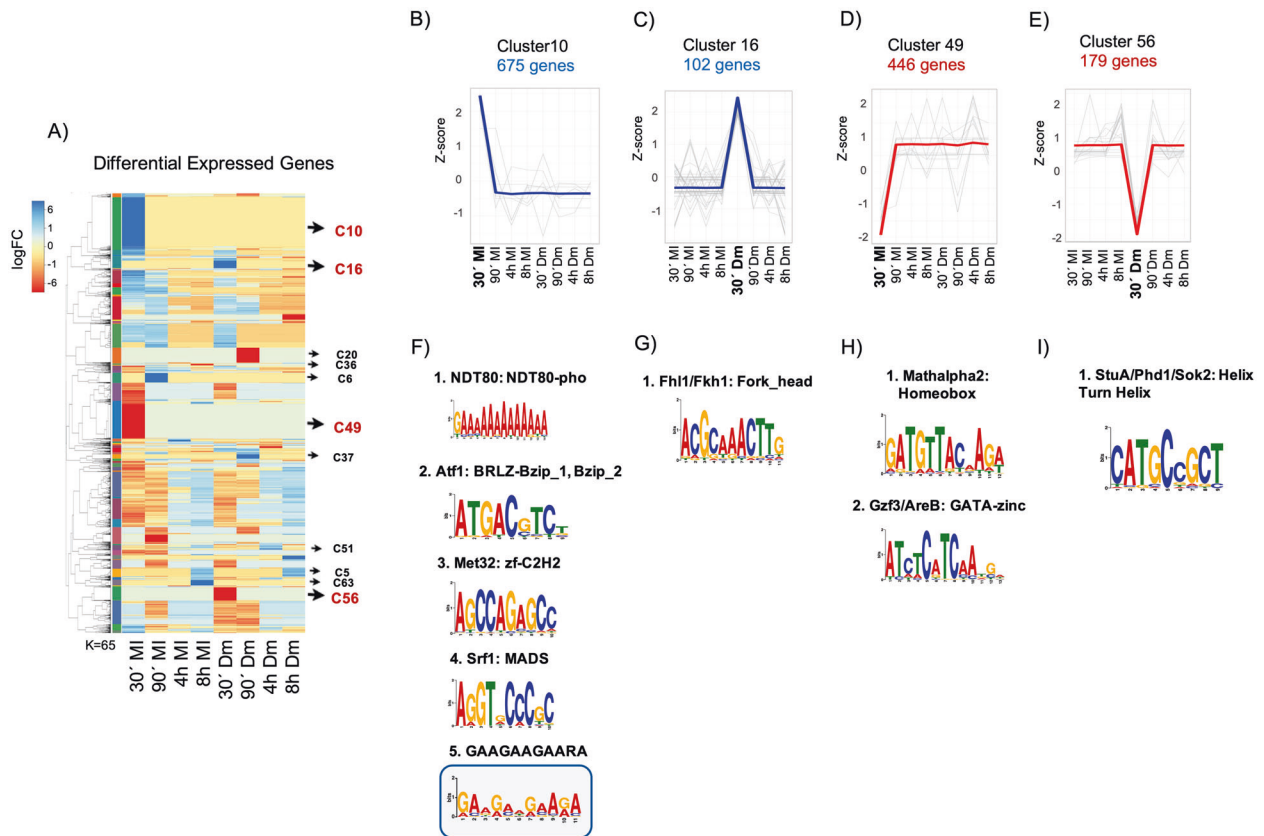
that while *Drosophila* larvae chewed and pulled the fungal mycelium (Fig. 1F, Supplementary Video 1) *O. folsomi* walked on *T. atroviride* without causing any apparent damage (Fig. 1E, Supplementary Video 2), and made no attempt to ingest mycelium. Furthermore, the fungus emerged from dead *Drosophila* bodies but we found no evidence of its presence in collembolans' bodies, which would indicate ingestion of *Trichoderma* mycelium (Supplementary Fig. 1).

We analyzed the fungus' transcriptomic response when exposed to the two arthropods and upon mechanical injury in a time course. The collembolans induced a weak transcriptional response at 30 min (nine upregulated and 127 downregulated genes), and none later (90 min to 8 h; Table 1; Supplementary

Data 1). In contrast, mechanical injury and grazing by *D. melanogaster* provoked significant changes in the expression level of thousands of genes at 30 and 90 min, and hundreds after 4–8 hours, sharing a substantial number of genes and displaying specific modifications of the transcriptional landscape (Table 1; Supplementary Fig. 2A). We, therefore, analyzed in detail only the fungal response to *Drosophila* attack and to mechanical injury (Supplementary Table 2).

#### Mechanical injury and attack by *Drosophila* differentially regulate the expression of specific gene sets

We visualized all (5561) Differentially Expressed Genes (DEG) in the comparison of the responses to *Drosophila* attack and mechanical



**Fig. 2 Mechanical injury and attack by *Drosophila* differentially regulate the expression of specific gene sets.** **A** Heatmap showing all DEG at all time points. A Blue-Yellow-Red color scale displays the Gene expression level (logFC). Heatmap columns indicate the time of sample collection and the condition: Mechanical injury (MI) or attack by *D. melanogaster* (Dm). At the left of the heatmap, color bars indicate the 65 clusters formed. Lettering at the right indicates the name assigned to the selected clusters. Graphs from (**B–E**) show the behavior of the gene set based on their Pearson correlation value, letters indicate the number of cluster and the number of genes in each of them. Blue lines correspond to upregulated genes and red lines to downregulated genes. (**F–I**) show the enriched motifs found for each cluster. Blue box is the de novo predicted motif. MI: mechanical injury, Dm: *Drosophila* attack.

injury in a heatmap (Fig. 2A). Hierarchical clustering followed by K-means analysis resulted in 65 gene cores, of which 11 were unique for a time or condition (Fig. 2A; Supplementary Data 2).

We decided to focus on four of the 11 clusters, which contain the earliest responsive genes and are more likely part of the primary response to the challenge. Clusters (C10 and C49) contain the most extensive sets of genes (675 and 446, respectively) that modify their expression only at 30 min after mechanical injury but are not altered in any other condition analyzed (Fig. 2B & D). Similarly, the expression of clusters C16 (102 genes) and C56 (175 genes) was modified only 30 min after attack by the arthropod (Fig. 2C & E). We observed other, smaller, and/or less specific gene clusters (Supplementary Data 2).

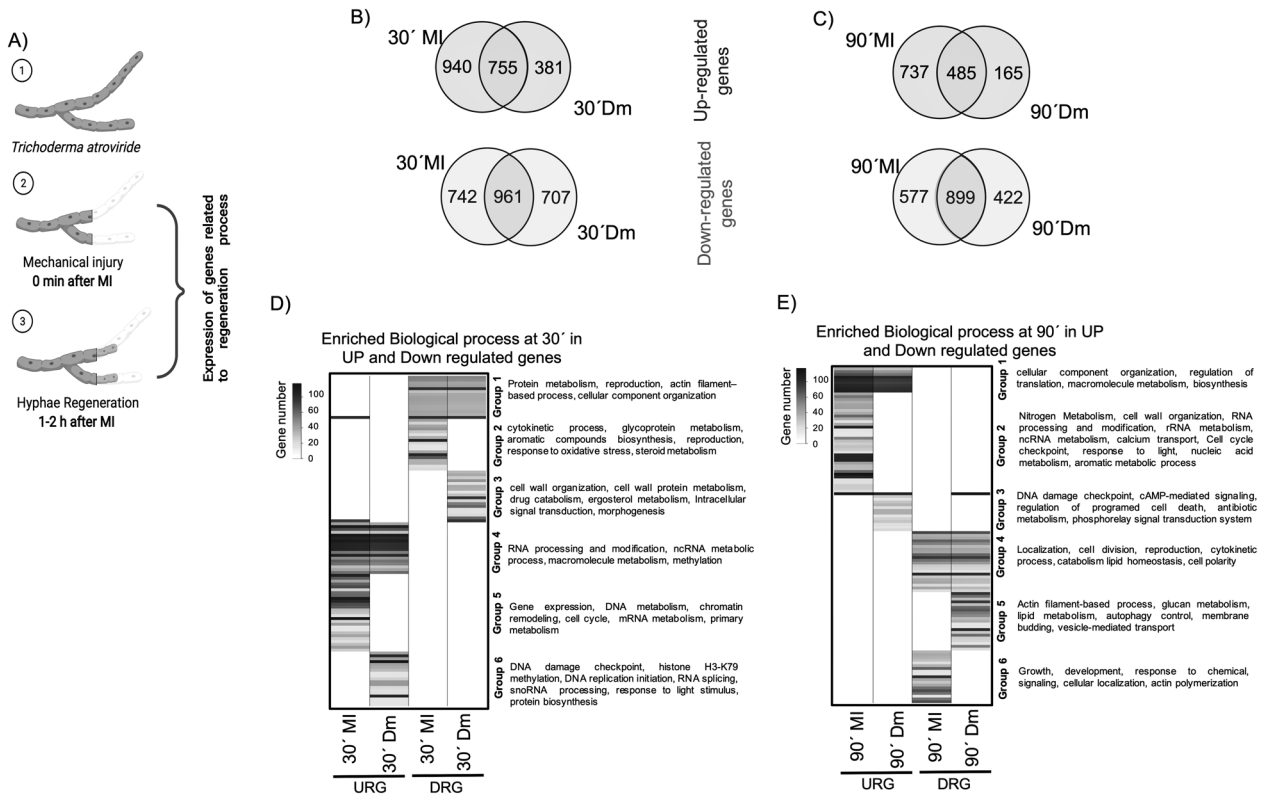
Transcription Factor (TF) binding motif predictions for the promoters of the genes belonging to the 11 clusters (Supplementary Data 3) revealed the enriched motifs for the contrasting cores C10, C16, C56, and C49 (Fig. 2F–I). All of them contain a recognition sequence for an *Aspergillus nidulans* TF for which we found an orthologue in *T. atroviride*. Genes in C10 contain four enriched motifs recognized by NDT80-pho (Tatro\_010668-T1, Tatro\_003887-T1 and Tatro\_001315), Sfr1 (Tatro\_003671-T1), Aft1 (Tatro\_009572-T1), and Met32 (Tatro\_001433-T1) (Fig. 2F; Supplementary Figs. 3A–C). C16 genes contain motifs recognized by Fkh1 (Tatro\_011463-T1) (Fig. 2G; Supplementary Fig. 4A). C49 contains two regulatory elements recognized by Mat alpha2 (Tatro\_004149-T1) and AreB (Tatro\_003849-T1) (Fig. 2H; Supplementary Fig. 4B, C). The core of downregulated genes at 30 min in response to

*Drosophila* attack contains a motif recognized by the orthologue of StuA (Tatro\_009561-T1) (Fig. 2I; Supplementary Fig. 4D). Using MEME for a de novo approach we found as significant ( $E$  value  $<0.05$ ) the novel “GAAGAAGAARA” motif, present in 2264 sites in the 645 sequences for C10 (Fig. 2F) that could be specifically related to the injury response.

We performed Gene ontology and enrichment analyses for the 11 selected clusters (Supplementary Data 2 and Supplementary Fig. 5). For the four clusters shown in Fig. 2, mechanical injury-induced, within minutes, the expression of genes related to DNA metabolic process, DNA repair, and response to stress, among other processes (C10). In contrast, the expression of genes related to cell redox homeostasis was downregulated (C49), as previously reported [15, 16], (Supplementary Fig. 5A). C16 contains genes upregulated only at 30 min after exposure to *Drosophila*, related to DNA packaging, protein DNA assembly, oxidation-reduction, DNA conformation change, and response to oxidative stress (Supplementary Fig. 5B). In contrast, C56 contains 179 genes with decreased expression only at 30 min of exposure to *Drosophila*, which is related to transcription regulation, cellular metabolic process, and macromolecule metabolic process, among others (Supplementary Fig. 5B).

In summary, the first minutes after mechanical injury appear critical to transcriptionally activate genes, like those involved with cell homeostasis and DNA damage, to protect against damaging effects of mechanical injury. In contrast, attack by *Drosophila* induced genes related to DNA protection. At (4–8 h), processes





**Fig. 3** Attack by *D. melanogaster* affects the transcriptional response of genes related to hyphal regeneration and immune response. **A** Cartoon representing the first two hours of the hyphal regeneration process after mechanical injury. (1) *T. atroviride* undamaged hyphae (Time 0). (2) Hyphal regeneration taking place in the damaged area during the first two hours after MI (16,18). **B, C** Venn diagrams illustrate the specific and shared DEG at 30 min (**B**) and 90 min (**C**) after MI and Dm. Upper circles correspond to upregulated genes and lower circles to downregulated genes. **D, E** Heatmap showing the Biological Processes (BP) enriched in up and downregulated genes 30 min (**D**) and 90 min (**E**) after MI and Dm. Groups of BP for each time and condition are indicated by the bold letters at the right of the heatmap. Black letters at the right of the heatmap highlight the most relevant BP for each group. MI: after mechanical injury. Dm: After *D. melanogaster* larvae attack, URG: upregulated genes, DRG: downregulated genes.

related to protein degradation and secondary metabolism were activated (Supplementary Data 2).

### Attack by *D. melanogaster* affects the transcriptional response of genes related to hyphal regeneration and immune response

Mechanical injury induces hyphal regeneration during the first two hours after damage, triggering the expression of genes related to regeneration and the putative innate immune system (Fig. 3A; [15, 16]). Therefore, we analyzed the impact of attack by *Drosophila* on the expression of genes activated early after the challenges. Figure 3B shows the sets of up- and downregulated genes 30 min after mechanical injury and insect attack. The expression of 940 genes was upregulated exclusively 30 min after mechanical injury, while *Drosophila* attack induced the expression of a different set of genes (381), and 755 genes were upregulated by both stimuli (Fig. 3B). A similar pattern existed for the downregulated genes at 30 min. Mechanical injury resulted in the specific downregulation of 742 genes, attack by the arthropod resulted in repression of 707, and 961 genes were downregulated in response to both treatments (Fig. 3B). At 90 min, mechanical injury resulted in the upregulation of 737 genes: 165 for *Drosophila* attack and 485 genes upregulated in common (Fig. 3C). Similarly, we found 577 specifically downregulated at 90 min for mechanical injury and 422 genes for *Drosophila* attack, with 899 genes in common (Fig. 3C).

GO term enrichment analysis of the DEG at 30 min showed upregulation of genes involved in RNA processing and modification, ncRNA metabolic processes, macromolecule metabolism, and

methylation in response to the two challenges (Fig. 3D, Group 4). At 30 min, mechanical injury resulted in upregulation of processes related to gene expression, DNA metabolism, cell cycle, mRNA metabolism, primary metabolism, and response to ion transport (Fig. 3D, Group 5). Interestingly, within DNA metabolism, genes related to DNA damage checkpoint, histone H3-K79 methylation, DNA replication initiation, RNA splicing, snoRNA processing, response to light, and protein biosynthesis were enriched (Fig. 3D, Group 6). Processes such as protein metabolism, reproduction, actin filament-based process, and cellular component organization were downregulated in response to both challenges (Fig. 3D, Group 1). Cytokinesis, glycoprotein metabolism, aromatic compounds biosynthesis, reproduction, and response to oxidative stress and steroid metabolism were downregulated only at 30 min after mechanical injury (Fig. 3D, Group 2). While only after arthropod grazing, the Biological Processes related to cell wall organization, cell wall protein metabolism, drug catabolism, ergosterol metabolism, intracellular signal transduction, and morphogenesis were downregulated (Fig. 3D, Group 3). GO enriched terms for groups shown in Fig. 3D are presented in Supplementary Data 4.

Ninety minutes after the challenges, the number of Biological Processes common to the two conditions, represented by cellular component organization, regulation of translation, macromolecule metabolism, and biosynthesis, diminished (Fig. 3E, Group 1). Mechanical injury provoked the upregulation of genes related to nitrogen metabolism, cell wall organization, RNA processing and modification, rRNA metabolism, ncRNA metabolism, calcium transport, cell cycle checkpoint, response to light, nucleic acid

metabolism, aromatic metabolic process, which were not induced upon attack by *Drosophila* (Fig. 3E, Group 2). After arthropod attack, genes involved in DNA damage checkpoint, histone H3-K79 methylation, and DNA replication were still upregulated. Processes such as cAMP-mediated signaling, regulation of programmed cell death, antibiotic metabolism, and phosphorelay signal transduction system were upregulated only 90 min after attack (Fig. 3E, Group 3), among other less represented processes.

Ninety minutes after the challenges, almost 900 genes were downregulated by both treatments, genes involved in establishing and maintaining localization, cell division, reproduction, cytokinetic process, catabolism lipid homeostasis, and cell polarity (Fig. 3E, Group 4). At this stage of the response, mechanical injury resulted in downregulation of genes related to growth, development, response to chemicals, signaling, cellular localization, and actin polymerization (Fig. 3E, Group 6). Whereas larval grazing negatively impacted the expression of genes involved in actin filament-based process, glucan metabolism, lipid metabolism, autophagy control, membrane budding, and vesicle-mediated transport (Fig. 3E, Group 5). GO enriched terms for groups from Fig. 3E are presented in Supplementary Data 4.

Overall, early (30–90 min) after the challenges, we observed that critical processes for hyphal regeneration and chemical response, such as heterokaryon incompatibility, calcium signaling, cell death, DNA metabolism, secondary metabolism, and cell cycle do not respond to *Drosophila*'s attack (Supplementary Data 5). Thus, we wondered if attack by *Drosophila* could affect hyphal regeneration.

#### Larvae salivary gland extracts block hyphal regeneration in *T. atroviride*

When looking at the upregulated genes considered necessary for hyphal regeneration [14] that did not respond to *Drosophila*'s attack, we found genes related to heterokaryon incompatibility, calcium signaling, cell death, and cell cycle (Supplementary Data 5). We selected two genes belonging to each of these processes for RT-qPCR analysis. As expected, all selected genes were induced by mechanical injury, five of them showing maximum expression level 15 min after mechanical injury and the remaining three at 90 min after the treatment (Fig. 4A). In contrast, only one encoding a cyclin-dependent kinase was slightly induced in response to *Drosophila*, confirming the RNAseq results.

The fact that a set of genes considered necessary for hyphal regeneration did not respond to attack by *Drosophila* suggested that products of their salivary glands could block the activation of gene expression. Therefore, we decided to determine the effect of salivary glands extracts on hyphal regeneration and gene expression. Using a scalpel soaked in Salivary Gland Extract (SGE), we injured hyphae, quantified hyphal regeneration, and compared these numbers with those obtained upon damage with a clean scalpel. As previously reported [17], upon hyphal injury, cytoplasm leakage stops immediately, regardless of the presence of salivary gland extract (Fig. 4B). However, hyphal regeneration dropped from 70% to 30% when the scalpel was soaked in the extract (Fig. 4B & C). To test if SGE blocked the activation of gene expression, we selected four genes induced by mechanical injury and repressed by *Drosophila* attack (Het-1, Het-2, cyclin, and calmodulin) for RT-qPCR analysis (Supplementary Table 3). As hypothesized, the presence of the salivary gland extract blocked the transcriptional activation of regeneration-related genes (Fig. 4D). Thus, a component of the larval salivary glands blocks the transcriptional response and consequently hyphal regeneration.

To determine the nature of the product of the salivary glands that could exert the blocking effect, we quantified regeneration frequency upon treating the extract with heat, proteases, and protease inhibitors. Heat treatment of the extract allowed almost twice as many hyphae to regenerate compared to the untreated

SGE (Supplementary Fig. 6), indicating that the inhibitory molecules are heat sensitive. Protease treatment of the extract and the addition of protease inhibitors produced similar results (Supplementary Fig. 6), suggesting that the inhibitory component present in the extracts is proteinaceous, very likely proteases.

#### DISCUSSION

Here we describe how *T. atroviride* responds specifically to different types of damage. Attack by *D. melanogaster* larvae resulted in a strong transcriptional response. In contrast, exposure to the springtail *O. folsomi* resulted in a feeble transcriptional response, which could be explained by nearly complete inhibition of any response from the fungus. Nevertheless, no fungi emerged from collembolan bodies. As fungi emerged from dead *D. melanogaster* bodies, it suggests that the collembolans did not ingest *T. atroviride* mycelia. In this regard, collembolans are considered generalist feeders but in most laboratory studies they are selective when given fungi as a food source, feeding on specific fungal taxa or structure [33]. Thus, the mere presence of a potential predator is insufficient to trigger a response. Possibly the transient, weak transcriptional response to the collembolan being triggered by the mechanical perturbation from walking on the mycelial mat.

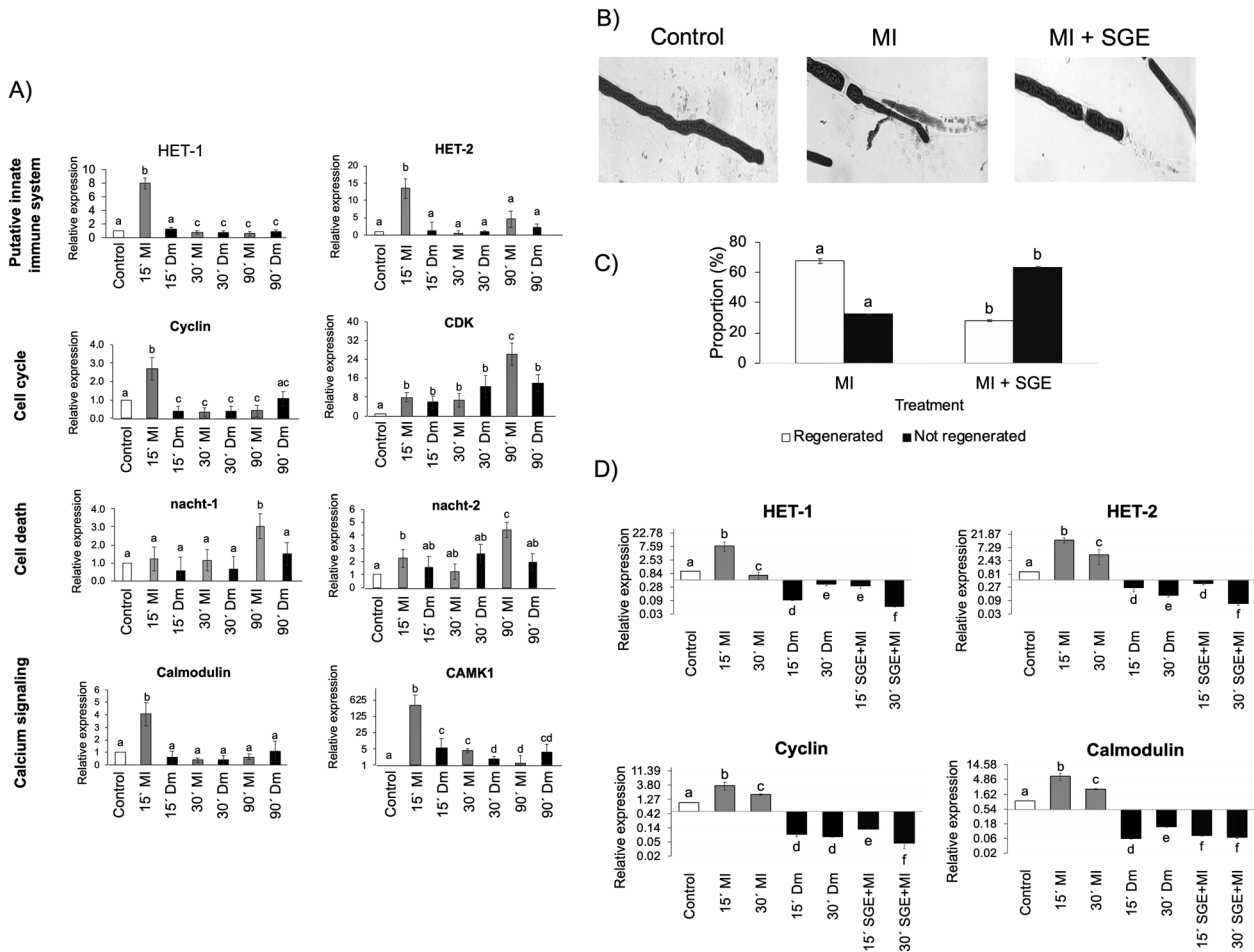
*Drosophila* attack inhibited the transcriptional activation of genes involved in ROS production, Ca<sup>2+</sup> and MAPK mediated signaling pathways, programmed cell death, and the innate immune system, previously reported as key processes for the injury response ([14, 15, 17]; Fig. 5).

We showed that mechanical injury and *Drosophila* attack provoke specific transcriptional responses, allowing us to propose regulatory elements present in genes co-expressed in response to damage, which resembles the differential response of plants to mechanical wounding and herbivory [34–37].

As in plant herbivory, larval oral secretions could be an important factor for the adverse effects observed on fungal fitness when challenged with *Drosophila* larvae [25, 38]. Larval saliva at the instar used here (late third instar) is composed mainly of proteins, sugars, and RNA, including glycoproteins, proteases, and protease inhibitors, among other components [39, 40]. Fungi recognize MAMPs and DAMPs to activate their innate immune system in response to injury or predation [1, 8, 12]. We proposed that salivary gland contents could inhibit the fungal response. Consequently, DAMPs released after the insect attack would not activate the signaling pathways necessary to trigger the transcriptional response required to initiate hyphal regeneration. In this regard, our transcriptional analysis showed that genes of the putative immune response acting as NLRs, like those encoding HET, WD40, and NACHT domain proteins, were not induced upon insect attack [8, 15]. In addition, specific *het* genes were induced within minutes upon mechanical injury, and only those activated late (8 h) responded to both mechanical injury and arthropod attack. In this sense, *het* genes, induced late could prepare the cell for future damage or participate in developmental processes, and those induced early after mechanical injury could be related to the innate immune system [15]. Consistently, salivary gland extracts repressed the expression of *het* genes and inhibited hyphal regeneration.

Transcriptomic response of the basidiomycete fungus *C. cinerea* to biotic and abiotic stress revealed the induction of lectin encoding genes during its interaction with the fungivorous nematode *A. avenae* [23]. This toxicity extended to the nematode *Caenorhabditis elegans* [41], the mosquito *Aedes aegypti*, and the amoeba *Acanthamoeba castellanii* [24]. Thus, fungal lectins are part of the first barrier of fungal defense against predators.

We found the early upregulation of six genes encoding Concanavalin A-like lectin/glucanases (galectins) after challenge with *Drosophila* (30 and 90 min). It is exciting that a gene encoding a Ricin-B lectin, a member of the lectin family related to



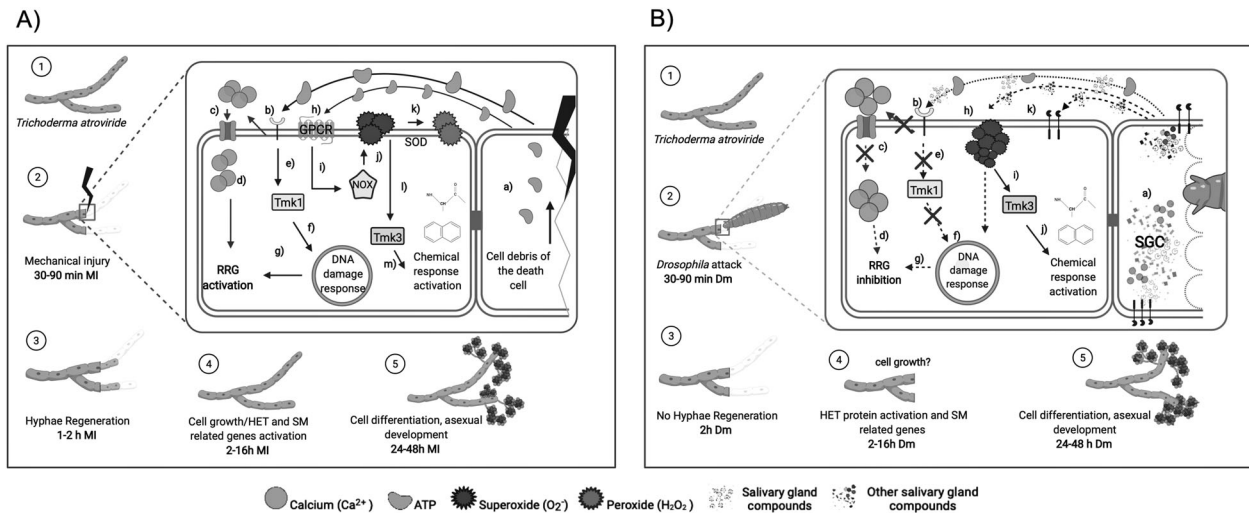
**Fig. 4 Larvae salivary gland extracts block hyphal regeneration in *T. atroviride*.** **A** RT-qPCR gene expression analyzed of selected genes belonging to different processes related to hyphal regeneration. Bars indicate the relative expression level of the indicated gene in the control condition (white), after MI (gray), and after attack by *D. melanogaster* larvae (black). **A–D** Gene expression relative to the not challenged control (arbitrarily set to one), was calculated by the  $2^{-\Delta\Delta CT}$  method. Different letters in the error bars indicate significant statistical differences with the control condition and constitutive control gene after ANOVA and Tukey tests ( $P > 0.05$ ). Control: not challenged, undamaged *T. atroviride*. MI: After a mechanical injury. Dm: After *D. melanogaster* grazing. **B** Micro-photographs of a *T. atroviride* hypha (control), and injured with a clean scalpel MI, or a scalpel dipped in Salivary Gland Extract (MI + SGE). Black arrows in micro-photographs indicate the damaged hypha—scale bars (20  $\mu$ m). **C** Hyphal regeneration after the indicated treatments represented as a percentage. Grey bars indicate the proportion of “Regenerated hyphae” and black bars the proportion of “Not regenerated hyphae”. Different letters above the bars indicate significant statistical differences between treatments ( $P < 0.05$ ). Control: Control condition. MI: After a mechanical injury. **D** RT-qPCR of the genes *het1*, *het2*, *cyclin*, and *calmodulin* after MI + SGE treatment of *T. atroviride* hyphae or grazing by *D. melanogaster* larvae (black bars), mechanical injury (MI, gray bars), and control condition (white bars). Control: not challenged, undamaged *T. atroviride*. Dm: After *D. melanogaster* grazing. SGE + MI: After mechanical injury with a scalpel soaked in salivary gland extract. MI: Mechanical injury.

the fungal defense against fungivorous nematodes [23, 24], was induced early by arthropod attack. Galectins, like Ricin-B lectins, have been linked to immune responses in fungi, plants, and animals recognizing carbohydrates present in pathogens or antagonists [23, 42–44]. Thus, Trichoderma lectins could serve as a natural defense against the larvae, as entomotoxic proteins as previously proposed [23, 24], causing larval death and developmental delays [25].

Calcium signaling is critical for initiating the regeneration processes [15, 45]. The lack of activation or repression of  $Ca^{2+}$  signaling related genes upon attack by *Drosophila* and injury by larval salivary gland extract is consistent with the severe reduction in hyphal regeneration capacity in *T. atroviride* (Fig. 5).

ROS participate as signal molecules at low levels to induce an immune response or to eliminate invading pathogens [46, 47] or to promote regenerative events in wounded Zebrafish and *Xenopus* [48, 49]. In *Trichoderma*, NADPH oxidase-dependent ROS production is essential to trigger conidiation in the damaged

area after mechanical injury [17]. However, at high concentration ROS induce severe DNA damage, leading to cell death, affecting tissue repair [50]. Here we show that mechanical injury induced the expression of 11 genes related to the DNA repair pathway, encoding four proteins involved in DNA mismatch repair (MutS-like), five genes encoding the DNA repair proteins (Rad18, Rad50, Rad52, Rad21/Rec8-like protein and RecA), and two DNA replication/checkpoint proteins MRC1 (homologs of the human Mediator protein MDC1). In contrast, *Drosophila* attack resulted in the downregulation of five DNA damage response genes, two encoding Rad, and three MutS-like proteins. The genes related to the DNA damage response pathways could respond to the previously described production of ROS after mechanical injury [17] and the subsequent activation of processes related to hyphal regeneration (Fig. 5, [51]). In contrast, damage caused by the larvae could activate the production of high concentrations of ROS, increasing DNA damage and leading to the activation of a protective mechanism mediated by the superoxide dismutase and



**Fig. 5 Model of the mechanisms involved in mechanical injury response and its alteration by arthropod attack. A** Model of the cellular response to Mechanical Injury (MI). 1. Cartoon of a *T. atroviride* hypha. 2. Early cellular response to MI (0–90 min) after damage. (a) After mechanical injury, damaged cells release ATP molecules, which are recognized by putative receptors localized in the cell membrane; (b) recognition of extracellular ATP allows influx of extracellular calcium (c), which promotes increases in cytosolic calcium concentration (d; [15]). ATP recognition activates the MAPK Tmk1 (e) that in turn activates the DNA damage response (f; [15]) (g). Extracellular ATP can also be recognized by a GPCR (h). The GPCR, activates the NADPH oxidase Nox1 (i; [17]). O<sub>2</sub><sup>-</sup> produced by Nox1 (j) is converted to H<sub>2</sub>O<sub>2</sub> by a superoxide dismutase (k). ROS activates Tmk3 (l) that triggers the chemical response to injury (m; [25]). 3. Hyphal regeneration is evident 1–2 h after MI. 4. Activation of the het and secondary metabolism-related genes and cell growth. 5. Cell differentiation and asexual development is observed 24–48 h after mechanical injury (MI). **B** Impact of *Drosophila* attack on the response. 1. Cartoon of a *T. atroviride* hypha. 2. Early cellular response to larval grazing (0 to 90 min). (a) During larval grazing, larval salivary gland components such as proteases are released, inhibiting genes related to calcium signaling (b, c, d) and the MAPK *tmk1* (e), consequently repressing the expression of DNA damaged (f) and regeneration (g) related genes. Grazing damage and the release of SGC increase ROS production and accumulation (h), which increases DNA damage (f), and contributes to inhibition of hyphal regeneration (g). Activation of the MAPK Tmk3 (i) is essential to trigger the chemical response to arthropod attack (j; [26]). SGC are recognized by lectins that could serve as a defense mechanism (k) 3. Inhibition of hyphal regeneration after Dm. 4. Activation of HET and SM related genes (red letters indicate that it is unclear when growth reinitiates). 5. Cell differentiation and asexual development processes observed 24–48 h after larval grazing. MI: mechanical injury, Dm: Grazing by *D. melanogaster* larvae. SGC: salivary gland components, Tmk1: MAPK Tmk1, Tmk3: MAPK Tmk3, RRP: Regeneration related processes, ROS: Reactive oxygen species, SM: Secondary metabolism, GPCR: G-Protein Coupled Receptors, Nox: NADPH oxidases, RRG: Regeneration-Related Genes. Model created in Biorender.com.

chloroperoxidase induced by *Drosophila* attack (Fig. 5). In conclusion, it appears that *Drosophila* attack does not activate the necessary mechanisms to repair possible DNA damage caused by an increase of ROS production and consequently, affects the hyphal regeneration process.

The first barrier of fungal defense against predators is the production of secondary metabolites [18, 19, 52]. Previously we reported that attack by *Drosophila* affected the production of secondary metabolites presumably related to *T. atroviride* chemical defense [25]. We found induced 12 secondary metabolism-related genes after mechanical injury. Among these genes, putative regulators of secondary metabolism, such as the methyltransferase *Talae1* and the bZIP transcription factors *TanapA* and *TametR* were induced by mechanical injury and repressed by arthropod attack [25]. Attack by *Drosophila* resulted in the upregulation of 15 secondary metabolism genes, mainly related to the production of non-ribosomal peptides and the orthologue of a gene coding for the *Clostridium difficile* insecticidal toxin TcdB. These genes may constitute a defense mechanism against an attacker, possibly even killing the predator. At late stages of the responses, mechanical injury and *Drosophila* attack activated many non-ribosomal peptide synthase and polyketide synthase encoding genes, that could play a role in conidiophore development and conidia pigmentation [53–55].

In regenerative organisms, the expression of genes related to cell cycle control and activation is vital to injury response and regeneration [56, 57]. Here, the transcript of the *T. atroviride* gene (Tatro\_009561-T1) orthologue of StuA decreased within minutes during the response to *Drosophila*. Consistently, genes required for cell cycle activation were downregulated, and, consequently,

hyphal regeneration inhibited. Accordingly, the StuA DNA binding motif was enriched in the promoters of genes downregulated early after insect attack.

Other TFs important for fungal development are the members of the NDT80 family. In *A. nidulans* NdtA, a member of this family, is required for sexual reproduction [58, 59]. In *N. crassa* Vib-1, one of the three members of the Ndt80 family, is required for expression of genes involved in heterokaryon-incompatibility and programmed cell death [59–61]. Here we reported three NDT80 homologues in *T. atroviride*, Tatro\_010668-T1 being the more closely related to Vib-1 of *N. crassa*, and Tatro\_003887-T1 to NdtA of *A. nidulans*. Although these TFs are not differentially expressed, we found an enrichment in the motif recognized by these TFs in the promoter of a large set of genes induced early after mechanical injury, containing genes related to heterokaryon-incompatibility.

The *T. atroviride* Srf1 and Fkh1 orthologues are induced by mechanical injury. In *A. nidulans* Srf1 is involved in sexual/asexual development [62], while the *S. pombe* orthologue of Fkh1 participates in cell cycle and sexual differentiation [63]. Thus, these TFs are likely involved in the regulation of hyphal regeneration in *T. atroviride*. Furthermore the *T. atroviride* orthologue of Atf1 is repressed by *Drosophila* attack and a set of genes enriched in its recognition motif are induced by mechanical injury. In *A. nidulans* Atf1 and Saka (the orthologue of the *T. atroviride* Tmk3) regulate oxidative and osmotic stress responses [64]. Earlier we described the participation of Tmk3 in the chemical and developmental responses to injury [14, 15, 25].

In conclusion, *T. atroviride* responds differently to damage caused by an attacker or mechanically and activates a



developmental program leading to conidiation to permit its survival. Thus, fungi display an inducible defense that allows “economically friendly” allocation of resources, likely to influence multitrophic interactions. We also provide transcriptomic evidence of the specific response of *T. atroviride* to injury and attack by *Drosophila* and how the latter affects processes related to chemical defense, the putative innate immune system, DNA damage repair, and hyphal regeneration (Fig. 5). In general fungi have a negative impact on *Drosophila* larval development [21]. In this regard, even though a *Trichoderma*-*Drosophila* interaction is unlikely to occur in nature, our model system indicates that *Drosophila* may block the fungal chemical defense and re-growth to avoid infections and possible deleterious effects.

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## AUTHOR CONTRIBUTIONS

KA-H carried out the experiments, analyzed data, and contributed to the experimental design. AH-E supervised K A-H, designed experiments, and obtained financial support. AH-E and KA-H wrote the manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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