

Secondary metabolism and host responses during the amoebicidal interaction between *Beauveria bassiana* and *Acanthamoeba castellanii* revealed by dual RNA-seq

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Abstract

Beauveria bassiana is a well-known entomopathogenic fungus, yet its interactions with free-living amoebae remain poorly understood. Here, we combined phenotypic assays with dual RNA-seq to investigate the amoebicidal potential of *B. bassiana* against *Acanthamoeba castellanii* and to evaluate the transcriptional responses of both organisms during their interaction. Phagocytosis of conidia by *A. castellanii* occurred under all conditions evaluated, whereas conidial germination and amoebicidal activity varied markedly among culture media, indicating strong environmental modulation. Cell-free supernatants from fungal–amoeba co-cultures exhibited greater amoebicidal activity than fungal monocultures, supporting the production of secreted factors in response to the interaction. Dual RNA-seq identified 894 differentially expressed genes in *B. bassiana*, with enrichment of pathways related to detoxification, stress response, and protein synthesis, whereas genes involved in metal-ion homeostasis and siderophore-mediated transport were downregulated. Analysis of biosynthetic gene clusters revealed the coordinated activation of multiple secondary-metabolite pathways, including the oosporein and beauveriolide clusters, and purified oosporein reduced *A. castellanii* viability in a dose- and time-dependent manner, functionally validating its contribution to the interaction. Moreover, *A. castellanii* displayed extensive transcriptional remodeling, with 3,785 differentially expressed genes: cytoskeleton organization and vesicular-trafficking pathways were upregulated, while stress-response, DNA-damage-response, and DNA-repair pathways were suppressed. Together, these findings demonstrate that the amoebicidal activity of *B. bassiana* is a context-dependent, multifactorial process in which environmentally regulated secondary metabolism and virulence-associated programs in the fungus are met by a coordinated reorganization of stress and cytoskeletal machinery in the amoeba.

INTRODUCTION

Free-living amoebae (FLA) are ubiquitous protozoa distributed across a wide range of ecological niches (Soler et al., 2023). Among them, species of the genus *Acanthamoeba* are the most prevalent and can infect humans through contaminated water sources, contact lenses, and medical devices (Bellini et al., 2022). The life cycle of *Acanthamoeba* comprises two stages: a resistant cyst and a metabolically active trophozoite. This biphasic life cycle facilitates infection, as cysts withstand adverse environmental conditions, while trophozoites can invade host tissues and disseminate systemically (Kot et al., 2021; Wang et al., 2023).

Certain *Acanthamoeba* species are responsible for severe human infections, including granulomatous amoebic encephalitis (GAE) and amoebic keratitis (AK) (Reyes-Batlle et al., 2022). Although GAE remains relatively rare, it is associated with mortality rates exceeding 90% (Keane et al., 2020). In contrast, AK is an emerging global ocular infection that poses a significant threat to vision, with a high risk of blindness when not promptly treated. Epidemiological studies indicate substantial geographic variation in incidence, with high disease burdens reported in regions such as India, New Zealand, Egypt, Portugal, and the United Kingdom (Zhang et al., 2023). Given the limited availability of effective treatments, the development of new therapeutic strategies remains a critical challenge (Morán et al., 2023).

The interaction between fungi and FLAs has emerged as a relevant model for understanding microbial survival and resistance mechanisms. Several soil-dwelling fungi, including species of *Aspergillus*, *Beauveria*, and *Cryptococcus*, effectively withstand amoebic predation and persist following interaction with these protists (Hillmann et al., 2015; João et al., 2024; Udompaisarn et al., 2020). Although the molecular basis of these interactions is not fully understood, FLAs are recognized as important regulators of fungal populations in natural environments (Casadevall et al., 2019). In response to this predatory pressure, fungi have evolved adaptive strategies that enable not only evasion of phagocytic uptake but also survival following internalization, including the ability to modulate the phagosomal environment (Radosa and Hillmann, 2021). These adaptations involve the activation of diverse virulence-associated mechanisms, including the secretion of hydrolytic enzymes such as proteases, chitinases, and lipases, which contribute to host interaction, nutrient acquisition, and cellular damage (Corrêa-Junior et al., 2025). Collectively, these features reflect a complex evolutionary landscape in which selective pressures imposed by amoebae have shaped fungal defense strategies that overlap with classical virulence traits observed in host–pathogen systems (Da Silva Ferreira et al., 2021).

Entomopathogenic fungi, such as *Beauveria bassiana*, represent a particularly relevant model within this framework due to their ecological versatility and biotechnological potential in the control of insect and arthropod pests (Dannon et al., 2020). The ecological versatility of *B. bassiana* extends beyond insect parasitism, as the fungus also establishes endophytic associations with a broad range of plant hosts, colonizing internal tissues without causing disease and, consequently contributing to protection against herbivores and phytopathogens (Liu et al., 2025). This capacity to move among insect, plant, and soil-microbial niches places *B. bassiana* at the center of multitrophic interactions, in which the same fungal traits may be repeatedly recruited across hosts and environmental antagonists, including free-living amoebae. Beyond their entomopathogenic role, species of the genus *Beauveria* produce a wide range of bioactive compounds, many of which remain unexplored (Ávila-Hernández et al., 2020). Furthermore, these fungi are also capable of resisting amoebic predation, further supporting their adaptation to complex environmental interactions (Emaru et al., 2024; Wang et al., 2021).

Among the mechanisms underlying fungal survival, the production of secondary metabolites (SMs) with antipredatory activity plays a central role (Conrado et al., 2022). These compounds can impair or inhibit amoebic grazing, thereby promoting fungal persistence across diverse environmental conditions (Wadhwa et al., 2024). In fungi, genes involved in SM biosynthesis are typically organized into biosynthetic gene clusters (BGCs), which enable coordinated regulation and efficient production of structurally diverse compounds (Zhgun, 2023). These clusters generally include backbone enzymes responsible for the core structure of the metabolite, along with tailoring enzymes that expand chemical diversity (Wenski et al., 2022).

Despite this progress, the molecular events that occur during interactions of *B. bassiana* and free-living amoeba remain largely uncharacterized. We hypothesized that *B. bassiana* employs an environmentally modulated, multifactorial antipredatory strategy against *A. castellanii*. To test this, we combined phenotypic assays, dual RNA-seq, genome annotation refinement, and analyses of virulence-associated

genes and biosynthetic gene clusters to characterize the fungal response during interaction. We further evaluated the contribution of secreted metabolites using co-culture supernatants and directly assessed the amoebicidal activity of selected secondary metabolites, including oosporein and beauveriolide.

MATERIALS AND METHODS

Strains and growth conditions

B. bassiana AS272 strain, isolated from soil in Rio Grande do Sul and provided by Prof. Dr Augusto Schrank (Centro de Biotecnologia, UFRGS), was grown at 28 °C in Yeast Peptone Dextrose (YPD) medium. Conidia were collected from cultures on agar plates and centrifuged (5,000 × g, 10 min). *A. castellanii* Neff strain (ATCC 30010) was grown axenically at 25 °C in peptone-yeast extract-glucose (PYG) medium supplemented with 20 U/mL chloramphenicol in 12-well cell culture plates. The detailed composition and physicochemical characteristics of all culture media used in this study are provided in Supplementary File 1.

Phagocytosis and germination assay

To determine phagocytosis and conidial germination rates, interactions between *B. bassiana* and *A. castellanii* trophozoites were monitored using an EVOS™ FLoid Imaging System. For each condition, one hundred amoebae per replicate were examined, and the number of trophozoites containing internalized *B. bassiana* conidia was recorded. For phagocytosis analysis, amoebae were classified into three categories according to the number of internalized conidia: one conidium, two conidia, or three or more conidia. Phagocytosis was evaluated after 1, 2, 4, and 6 h of co-culture at 28 °C. Conidial germination was assessed by quantifying amoebae associated with germinated conidia after 24 and 48 h of incubation in the different culture media (PDB, potato dextrose broth; CM, Cove medium; CCM, Complete Cove medium; PYG, Peptone Yeast Glucose medium; and YPD, Yeast Peptone Dextrose medium). The extent of intracellular germination over the assay period is documented in Fig. S1. All assays were performed in three independent biological replicates.

Amoebicidal activity assay

The mortality of *A. castellanii* trophozoites was assessed after 24 and 48 h using the trypan blue exclusion method, in which stained cells were considered non-viable and unstained (dye-excluding) cells were considered viable. *B. bassiana* conidia were co-cultured with *A. castellanii* trophozoites at a 1:1 ratio (1×10^6 cells mL⁻¹ each) in 24-well plates at 28 °C in the different culture media (PDB, CCM, PYG, and YPD). Axenic amoeba cultures maintained under the same conditions were used as controls. All assays were performed in three independent biological replicates.

Supernatant assay

To evaluate the contribution of secreted factors to amoebicidal activity, culture supernatants were obtained from *B. bassiana* monocultures and from *B. bassiana*–*A. castellanii* co-cultures. For this, *B.*

bassiana conidia were incubated alone (1×10^6 cells mL⁻¹) or co-cultured with *A. castellanii* trophozoites at a 1:1 ratio (1×10^6 cells mL⁻¹ each) for 48 h under the conditions described above. After incubation, the supernatants were collected and filtered through a 0.45 µm membrane filter to remove cells and debris. Newly harvested *A. castellanii* trophozoites (1×10^6 cells mL⁻¹) were then exposed to the cell-free supernatants and incubated at 28 °C for 24 and 48 h. Amoebic viability was subsequently assessed using the trypan blue exclusion method, under the conditions described in above. All assays were performed in three independent biological replicates.

RNA isolation and dual RNA-Seq analysis

For transcriptomic analysis, *B. bassiana* monocultures, *A. castellanii* monocultures, and *B. bassiana*–*A. castellanii* co-cultures were incubated in PDB medium for 48 h. Three independent biological replicates were performed for each condition. Following incubation, samples were harvested and ground to a fine powder in pre-chilled mortars with the gradual addition of liquid nitrogen. The homogenized material was transferred to bead-beating tubes containing 1 mL TRIzol reagent (Thermo Fisher Scientific) and 200 µL of glass beads. Cell disruption was performed using a FastPrep®-24 5G system, followed by RNA extraction according to the manufacturer's instructions for TRIzol. RNA samples were further purified using the RNeasy kit (Qiagen) and treated with TURBO™ DNase (Thermo Fisher Scientific) to remove residual genomic DNA. RNA integrity was verified by 1% agarose gel electrophoresis in Tris-Borate-EDTA buffer. RNA concentration and purity were assessed using a NanoDrop spectrophotometer and the Qubit RNA HS Assay (Thermo Fisher Scientific). Only samples with A_{260}/A_{280} ratios between 1.9 and 2.1 were selected for downstream analysis. Finally, samples were normalized to 2 µg of total RNA in a final volume of 20 µL and submitted to the Instituto para Pesquisa do Câncer (IPEC, Guarapuava, Brazil) for library construction and sequencing on an Illumina NovaSeq 6000 platform, generating paired-end reads for three biological replicates per condition.

Read processing and alignment

Raw sequencing reads were quality-filtered and adapter-trimmed with fastp (v0.24) using default parameters (Chen et al., 2018). The filtered reads were then aligned with STAR v2.7 (Dobin et al., 2013) against the concatenated genomes of *B. bassiana* AS272 (GenBank assembly GCA_052426205.1) and *A. castellanii* Neff (GCA_000313135.1). A concatenated reference was used so that reads originating from each organism were assigned to their genome of origin during a single alignment, and only uniquely mapped reads were retained for downstream quantification to avoid cross-assignment between the two genomes. Gene-level read counts were obtained using the GeneCounts option implemented in STAR. The resulting fungal and amoebal count matrices are provided in Supplementary files 2 and 3, respectively.

Gene prediction, functional annotation, and differential expression analysis of *B. bassiana*

To generate high-quality gene models for *B. bassiana* AS272, RNA-seq reads uniquely aligned to the fungal genome from the monoculture condition were used as extrinsic evidence for gene prediction with BRAKER3 v3.0.3 (Gabriel et al., 2024). The resulting gene models were subsequently processed using

the Funannotate pipeline v1.8.15 (Palmer and Stajich, 2020) to obtain comprehensive structural and functional annotations. To assess the improvement in gene discovery and structural accuracy, the newly generated models were compared against a previous *B. bassiana* annotation (Soares et al., 2026) using gffcompare v0.12.10 (Pertea and Pertea, 2020). Sensitivity and precision were calculated at multiple levels, including base, exon, intron, transcript, and locus levels; the comparative annotation metrics are summarized in Table 1. The resulting validated and refined gene models, whose gene codes received the index ACM6JR_ as described in the BioProject PRJNA1222367 that reported the genome sequence, served as the reference for all subsequent transcriptomic analyses. Differentially expressed genes (DEGs) were identified using the edgeR package (Robinson et al., 2010) in R (R Core Team, 2025) from the fungal count matrix (Supplementary file 2).

Raw count data were filtered to retain only *B. bassiana* genes and to remove lowly expressed genes (filterByExpr) prior to analysis. Library-size normalization was carried out using the trimmed mean of M-values (TMM) method. Dispersion estimates were calculated, and differential expression between the interaction and control conditions, each represented by three biological replicates, was assessed using an exact test. P-values were adjusted for multiple testing using the Benjamini–Hochberg procedure. Genes with a false discovery rate (FDR)-adjusted P-value < 0.05 and an absolute $\log_2$ fold change ≥ 1 were considered differentially expressed (Supplementary file 4). Functional enrichment analysis was then performed to identify enriched Gene Ontology (GO) terms associated with the differentially expressed genes. GO annotations obtained from eggNOG-mapper (Cantalapiedra et al., 2021) were used to generate the gene-to-GO mapping (Supplementary file 5). Enrichment analysis was conducted using the topGO package (Alexa and Rahnenführer, 2024) considering the Biological Process ontology, and overrepresentation of GO terms was evaluated using Fisher's exact test with the classic algorithm.

2.8 Differential expression and functional enrichment analysis of *A. castellanii*

Differential expression analysis of *A. castellanii* was performed in R (R Core Team, 2025) using the edgeR package (Robinson et al., 2010). From the gene-level counts obtained against the concatenated genome, only transcripts corresponding to *A. castellanii* (rna-XM_* identifiers) were retained. Transcript identifiers were converted to ACA locus tags using a custom mapping table generated from the reference GFF annotation of the *A. castellanii* Neff genome. Filtering, TMM normalization, dispersion estimation, and exact-test differential expression were performed as described for *B. bassiana* above, applying the same FDR < 0.05 and $|\log_2$ fold change| ≥ 1 thresholds. The resulting amoebal DEGs are provided in Supplementary file 6. Functional enrichment analysis of *A. castellanii* DEGs was performed using the topGO package (Alexa and Rahnenführer, 2024) considering the Biological Process ontology. GO annotations for *A. castellanii* Neff were obtained from AmoebaDB release 68 using the corresponding Gene Ontology annotation (.gaf) file available through the EuPathDB/VEuPathDB platform (Amos et al., 2022; Supplementary file 7).

Prediction of fungal biosynthetic gene clusters

Biosynthetic gene clusters (BGCs) in the *B. bassiana* genome were predicted using the fungal version of antiSMASH (fungiSMASH, v8.0.0) (Blin et al., 2025). The analysis was performed using 'relaxed' detection parameters to identify genomic regions associated with secondary metabolite biosynthesis (Supplementary file 8). Predicted clusters were classified according to their biosynthetic type, including non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpene, NRPS-like, NRP-metallophore, isocyanide, cyclodipeptide synthase (CDPS), and hybrid NRPS–PKS clusters. Genes located within predicted BGC regions were subsequently cross-referenced with the RNA-seq differential expression dataset to identify clusters transcriptionally regulated during interaction with *A. castellanii*.

Identification of virulence-related genes

The predicted proteome of *B. bassiana* was queried against the Pathogen–Host Interactions Database (PHI-base) (Urban et al., 2022) using BLASTp (Camacho et al., 2009). Hits were filtered to retain only alignments with at least 50% sequence identity and 50% query coverage, and for each *B. bassiana* protein only the best hit was retained based on the highest bit score (Supplementary file 9). PHI-base entries were parsed to extract associated metadata, including UniProt accession numbers, orthologous gene names, host species, and mutant phenotypes. Functional annotation of poorly characterized proteins was refined through queries in UniProtKB (The UniProt Consortium, 2023). To identify virulence-associated genes relevant during interaction, PHI-base hits were integrated with the differentially expressed genes (DEGs) obtained from the RNA-seq analysis using edgeR; gene identifiers were standardized prior to integration to ensure accurate matching across datasets. In addition, PHI-base hits were further intersected with genes predicted to belong to biosynthetic gene clusters (BGCs) identified using antiSMASH.

Oosporein toxicity analysis

Toxicity of oosporein was evaluated using the AlamarBlue® (Thermo Fisher Scientific) reagent. *A. castellanii* trophozoites were collected by centrifugation at $5,000 \times g$ for 5 min, resuspended in PYG medium, and adjusted to 1×10^6 cells mL^{-1} in a Neubauer chamber. Aliquots of 50 μL were distributed in 96-well plates and incubated at 28 °C for 1 h for adhesion. Oosporein (MedChemExpress HY-N7719, CAS No. 475-54-7) solutions were prepared from a stock (100 mM in DMSO) and diluted in PYG to final concentrations of 20, 50, 100, 150, 200, and 500 μM . The following controls were used: negative (cells + medium), vehicle (0.3% DMSO), blank (medium + AlamarBlue®), and interference (medium + compound + AlamarBlue®, without cells). The amoebae were treated with 50 μL of each solution, for a final volume of 100 μL per well, in technical and biological triplicate. The plates were incubated at 28 °C for 24 and 48 h. AlamarBlue® (10 μL) was then added to each well, with incubation protected from light for 8 h. Fluorescence was measured with excitation at 530 nm and emission at 615 nm in a Varioskan LUX reader (Thermo Fisher Scientific). Relative viability was calculated by normalizing the fluorescence of treated wells to the vehicle control (0.3% DMSO) after subtraction of the interference signal.

Statistical analysis

All statistical analyses were conducted in R 4.5.1 (R Core Team, 2025), and all experiments were performed with three independent biological replicates (and technical replicates where indicated). The full code for all analysis is provided as Supplementary file 10.

RESULTS

Interaction time and culture media modulate phagocytosis of *B. bassiana* and subsequent conidial germination

To investigate the influence of nutritional conditions on amoebic phagocytosis, we monitored the phagocytic uptake of *B. bassiana* conidia by *A. castellanii* trophozoites over a 6 h period. Incubation was limited to 6 h to avoid biasing the results due to high internal germination rates (Fig. S1). Phagocytic load was categorized by the number of internalized conidia per trophozoite (1, 2, or ≥ 3) for each time point and culture medium. Phagocytosis dynamics were strongly influenced by the culture conditions. In PYG and YPD, the proportion of amoebae containing three or more internalized conidia increased progressively over time, peaking at 80–90% by 6 h (Fig. 1a). In contrast, PDB, CM, and CCM displayed similar phagocytic kinetics (Fig. 1a). Across these conditions, the proportion of amoebae containing a single internalized conidium peaked within the first 1–2 h of interaction, followed by a gradual decline. Meanwhile, the proportion of amoebae containing two internalized conidia exhibited medium-dependent variability. These results demonstrate that both interaction time and nutrient availability modulate the phagocytic interaction between *A. castellanii* and *B. bassiana*.

To determine the intracellular fate of *B. bassiana*, we evaluated the germination of phagocytosed conidia under the same culture conditions. After 24 h, the highest germination rates were observed in PDB. By 48 h, germination reached near-saturation in PDB, YPD, and PYG (Fig. 1b). These findings indicate that both nutrient availability and host interaction shape fungal developmental outcomes, highlighting the importance of environmental context in modulating fungal responses during amoeba–fungus interactions.

Evaluation of the amoebicidal activity of *B. bassiana*

Given the varying phagocytic kinetics and germination rates observed, we next assessed the amoebicidal potential of *B. bassiana* under the same nutritional conditions. Amoeba viability was assessed using trypan blue exclusion after 24 and 48 h of interaction. After 24 h, co-culture with *B. bassiana* resulted in a significant increase in amoeba mortality in PDB and YPD relative to axenic amoeba controls. After 48 h, mortality was higher in all individual media tested when comparing treatment and controls (Fig. 2a), suggesting that the amoebicidal effect is time-dependent. At 48 h, culture in PDB and YPD induced the most pronounced amoeba mortality rates. Overall, these results demonstrate that the outcome of the *B. bassiana*–*A. castellanii* interaction is strongly influenced by environmental conditions, with culture medium composition shaping the extent of amoeba mortality.

To determine whether the observed toxicity was mediated by secreted fungal factors, we evaluated the effect of cell-free supernatants obtained from *B. bassiana* monocultures and *B. bassiana*–*A. castellanii* co-cultures. Among tested conditions, PDB showed numerically higher mortality, reinforcing the influence of environmental conditions on the production of extracellular factors associated with amoeba killing (Fig. 2b and Fig. S2). Consequently, PDB was selected for subsequent omics analyses, as it optimized both amoebicidal potency and fungal development among the media tested.

Transcriptional reprogramming of *B. bassiana* during interaction with *A. castellanii*

To investigate the molecular basis underlying the interaction between *B. bassiana* and *A. castellanii*, we performed transcriptome analysis of fungal cells co-cultured with amoebae. Prior to differential gene expression analysis, RNA-seq data were used to improve the genome annotation of *B. bassiana* AS272 using BRAKER3. Compared to a previous annotation based primarily on protein homology from *B. bassiana* ARSEF 2680 (Soares et al, 2026,), which lacked condition-specific transcriptional evidence, the BRAKER3-based annotation incorporates RNA-seq data to support gene prediction and exon–intron structure definition. The updated annotation resulted in 9,654 predicted gene models, compared to 10,783 mRNAs (9,702 loci) in the previous annotation (Supplementary file 11). Comparative analysis using gffcompare showed that 68.1% of transcripts and 75.6% of loci from the reference annotation were recovered. At the nucleotide level, the updated annotation achieved 91.5% sensitivity and 95.2% precision, while exon and intron predictions reached approximately 75% sensitivity and > 83% precision, indicating strong agreement in gene structure with improved boundary definition. Importantly, 7.3% of previously annotated loci were not supported by the RNA-seq–guided annotation, suggesting potential overprediction in the original protein-based models. Conversely, 5.9% of loci were newly identified, representing putative novel genes supported by transcriptional evidence under the experimental conditions tested. Structural refinement was further supported by the identification of 7,342 matching transcripts and 5,191 exact intron chain matches, indicating improved reconstruction of exon–intron architecture for a substantial fraction of genes. Together, these results demonstrate that the incorporation of RNA-seq data substantially improves the structural accuracy and biological support of gene models in *B. bassiana* AS272, providing a more reliable reference annotation for downstream transcriptomic analyses (Table 1).

Using the refined genome annotation, differential gene expression analysis of the raw count matrix revealed 894 differentially expressed genes (DEGs) in fungal cells co-cultured with amoebae compared to control conditions (absolute $\log_2$ fold change ≥ 1 and FDR-adjusted P-value < 0.05) (Fig. S3). Among the DEGs, the most highly upregulated genes included *ACM6JR_003426*, encoding a putative fungal transcription factor (PF11410), and *ACM6JR_005842*, encoding an acetylornithine aminotransferase (PF00202), potentially involved in arginine biosynthesis. In contrast, the most downregulated genes included *ACM6JR_009040* and *ACM6JR_006181*, both encoding uncharacterized proteins lacking identifiable conserved domains. To further characterize the transcriptional response, we analyzed the top 50 DEGs by absolute $\log_2$ FC, which revealed distinct and reproducible expression profiles between control and interaction conditions (Fig. 3a). Functional enrichment based on Gene Ontology (GO)

annotation of the upregulated genes highlighted a significant overrepresentation of pathways related to cellular detoxification and stress response, alongside a marked induction of ribosome biogenesis, rRNA processing, and translation-related machinery (Fig. 3b). Conversely, downregulated genes were predominantly associated with metal ion homeostasis, specifically iron coordination, and siderophore-mediated transport (Fig. 3c). These results indicate that *B. bassiana* activates a coordinated response to the predatory activity of amoeba, characterized by enhanced protein synthesis and detoxification capacity, while simultaneously suppressing several ion acquisition systems. This transcriptional shift reflects a functional reprogramming aimed at survival and persistence within a hostile habitat.

Activation of virulence-associated genes during fungal–amoeba interaction

To investigate the involvement of virulence-associated mechanisms during the interaction between *B. bassiana* and *A. castellanii*, the predicted fungal proteome was queried against the Pathogen–Host Interactions Database (PHI-base). A total of 1,449 *B. bassiana* proteins showed significant similarity to entries in PHI-base based on identity and coverage thresholds, revealing an extensive repertoire of putative virulence-associated genes. Cross-referencing the PHI-base annotation results with differential expression data revealed that 77 of these genes were differentially expressed during interaction with amoebae, suggesting the activation of virulence-related pathways under these conditions (Supplementary file 12). To further explore the functional properties of these gene products, DEGs were grouped into broad categories potentially associated with detoxification/stress response, nutrient acquisition and metabolism, or regulatory processes. Genes assigned to regulatory processes showed differential expression (Fig. S4a), including kinases, transcription factors, and other proteins potentially involved in cellular adaptation. In addition, *ACM6JR_007482*, annotated as an O-acetylhomoserine aminocarboxypropyltransferase, was strongly upregulated, suggesting that metabolic remodeling may occur alongside regulatory responses during interaction. Genes related to detoxification/stress response represented a prominent group, with most transcripts being upregulated during interaction (Fig. S4b). Genes associated with nutrient acquisition and metabolic functions included proteases, hydrolases, transporters, and other proteins that may contribute to resource utilization during co-culture (Fig. S4c). The most highly induced transcript in this group was *ACM6JR_000908*, annotated as a nitrate/nitrite transporter, suggesting modulation of nitrogen uptake pathways. Together, these findings suggest a multifaceted transcriptional adjustment of *B. bassiana* during interaction with *A. castellanii*.

Genomic inference and transcriptional regulation of secondary metabolism during interaction

To investigate the secondary metabolic potential of *B. bassiana* AS272, biosynthetic gene clusters (BGCs) were predicted using fungiSMASH. A total of 43 BGCs were identified in the genome, encompassing diverse classes of secondary metabolites, including nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpene clusters, and hybrid NRPS–PKS systems (Supplementary

file 13). These clusters collectively comprised 706 genes, representing a substantial portion of the fungal genome potentially associated with secondary metabolism. To identify transcriptionally active BGCs during interaction with amoebae, the predicted BGC genes were cross-referenced with the transcriptomic dataset. Of the 706 genes identified within BGCs, 608 were detected in the transcriptomic dataset, indicating widespread expression of secondary metabolism-related genes under the evaluated conditions. Among these, 96 genes were differentially expressed, suggesting that specific secondary metabolic pathways are actively modulated during fungal–amoeba interaction. To infer the potential role of these clusters in pathogenesis, we investigated their overlap with virulence-associated genes identified through PHI-base analysis. Among the 608 BGC-associated genes detected in the transcriptome, 84 showed similarity to PHI-base entries (Supplementary file 13), indicating a substantial convergence between secondary metabolism and virulence-related functions. We subsequently focused on the core biosynthetic (backbone) genes of each predicted cluster, as these enzymes are critical for the synthesis of secondary metabolites. The expression profiles of the backbone genes detected in the transcriptome, resolved by cluster identity and biosynthetic class – NRPS, PKS, terpene, NRPS–PKS hybrids, NRPS-like, NRP-metallophore, isocyanide, and CDPS clusters – are summarized in Fig. 4. Integration with the transcriptomic data revealed that 33 backbone genes were differentially expressed during interaction with amoebae (Supplementary file 13), spanning multiple biosynthetic classes and indicating the targeted transcriptional activation of key biosynthetic pathways in response to the amoebae.

Five backbone genes were both differentially expressed and annotated in PHI-base, suggesting an overlap between specialized metabolism and virulence-associated functions. Following the global BGC analysis, we prioritized clusters with the most pronounced transcriptional changes during the fungal–amoeba interaction. Clusters were selected based on backbone gene DEGs, the proportion of upregulated genes per cluster, and their overall magnitude of induction relative to the control condition. Based on these criteria, the beauveriolide and oosporein BGCs were selected for further characterization, as they correspond to well-characterized metabolites and displayed highly coordinated transcriptional induction. Within the oosporein BGC (Fig. S5a), seven genes were identified as DEGs (Fig. S6). These included the core biosynthetic gene (*ACM6JR_005733*), the MFS transporter *OpS2* (*ACM6JR_005729*), and the cluster-specific transcription factor *OpS3* (*ACM6JR_005730*). The concurrent upregulation of biosynthetic, regulatory, and transport-related components indicates coordinated activation of the oosporein pathway. Similarly, the beauveriolide BGC (Fig. S5b) showed significant activation, with six of its 14 genes identified as DEGs (Fig. S6). This included two core backbone genes (*ACM6JR_008267* and *ACM6JR_008264*) and genes associated with precursor uptake and auxiliary functions (e.g., *ACM6JR_008260*). This pattern supports activation of the beauveriolide biosynthetic machinery during interaction. In both clusters, the upregulation of backbone, regulatory, and accessory genes demonstrates that these pathways are induced in response to the amoebae. Together, these results highlight selective activation of specific secondary metabolic pathways, suggesting a targeted metabolic response of *B. bassiana* under these conditions.

Oosporein exposure reduces *A. castellanii* viability

While both the oosporein and beauveriolide clusters were significantly upregulated, the oosporein pathway exhibited a more comprehensive transcriptional profile, involving the concurrent induction of biosynthetic, regulatory, and efflux machinery. This robust coordination indicated full biosynthetic activation and provided the rationale for selecting oosporein for subsequent functional validation. To functionally evaluate the role of the secondary metabolites identified by transcriptomic profiling, we assessed the effects of oosporein on *A. castellanii* viability. Trophozoites were treated with a range of oosporein concentrations (20, 50, 100, 150, 200, and 500 μ M), and cell viability was normalized to the vehicle control (0.3% DMSO). At 24 h, oosporein concentrations ≥ 200 μ M significantly reduced viability, while lower concentrations (20–150 μ M) showed no statistically significant difference from the control. After 48 h, a more pronounced effect was detected, with significant reductions in viability at all concentrations tested (Fig. 5). Comparison of overall viability between 24 and 48 h revealed increased sensitivity to oosporein at the later time point, as indicated by area under the curve (AUC) analysis ($AUC_{24} = 361.99$; $AUC_{48} = 280.49$; $t = 6.15$; $P = 0.0043$). Collectively, these data demonstrate that oosporein exerts a concentration- and time-dependent toxic effect on *A. castellanii*, supporting a functional role for this metabolite during the fungal–amoeba interaction.

Global transcriptional response of *A. castellanii* during interaction with *B. bassiana*

To characterize the host response, differential expression analysis was performed on the *A. castellanii* read counts obtained from the same co-culture experiment. Applying the same thresholds used for the fungal analysis (absolute $\log_2$ fold change ≥ 1 and FDR-adjusted P-value < 0.05), 3,785 amoebal genes were differentially expressed during interaction with *B. bassiana*, comprising 1,857 upregulated and 1,928 downregulated genes (Supplementary file 12 and Fig. S7). Among the most upregulated genes, *ACA1_051000* encoded a protein containing an SMC ATP-binding domain, *ACA1_378200* a putative RecQ-family helicase (both associated with chromosome maintenance and DNA repair), and *ACA1_397960* a putative Rab9-related protein, implicating vesicular trafficking and endosomal transport. Conversely, the most downregulated genes included two Hsp20/ α -crystallin superfamily proteins (*ACA1_390590* and *ACA1_391470*), a putative glutathione S-transferase (*ACA1_060440*), a universal stress domain-containing protein (*ACA1_244800*), a putative $\alpha(1,3)$ -fucosyltransferase (*ACA1_364520*), and a CBS domain-containing protein (*ACA1_246090*). These profiles indicate that interaction with *B. bassiana* induces extensive transcriptional reprogramming in *A. castellanii*, affecting pathways related to intracellular organization and cellular stress regulation.

Functional enrichment analysis of the amoebal DEGs revealed extensive modulation of biological processes associated with cellular organization and stress response. Upregulated genes were significantly enriched for terms related to regulation of cellular processes, cytoskeleton organization, and cellular component organization and biogenesis, consistent with structural and intracellular rearrangements associated with motility, vesicular trafficking, and phagocytic activity during fungal interaction (Fig. 6a). In contrast, downregulated genes were enriched for cellular response to stress, DNA damage response, and DNA repair-related pathways (Fig. 6b and Supplementary file 13), in agreement with the reduced expression of genes encoding heat shock proteins, glutathione S-transferases, and

universal stress proteins. Together, these results indicate that interaction with *B. bassiana* induces a coordinated reorganization of both cellular architecture and stress-response programs in *A. castellanii*.

DISCUSSION

Entomopathogenic fungi such as *B. bassiana* have evolved diverse strategies to resist predation and host immune defenses, frequently involving the production of secondary metabolites (SMs) with cytotoxic or immunomodulatory properties, as documented in insect–fungus interactions (Liu et al., 2025). Because *B. bassiana* occupies a broad ecological range that spans insect hosts, the soil environment, and endophytic association with plants (Liu et al., 2025), the same metabolic and virulence-associated traits may be recruited across multiple biotic interactions, including those with free-living amoebae (FLAs). Supporting this view, *Metarhizium anisopliae* survives and replicates within amoebae (Bidochka et al., 2010), pointing to functional parallels between resistance to amoebic predation and evasion of insect immune responses, and resistance to amoebae has been reported across phylogenetically diverse fungi, including human-associated pathogens such as *Aspergillus* spp. (Hillmann et al., 2015), *Cryptococcus* spp. (João et al., 2024), and *Paracoccidioides* spp. (Albuquerque et al., 2019). Together, these observations support the hypothesis that FLAs act as environmental selective pressures that shape fungal defense strategies, which may subsequently be co-opted for pathogenicity in animal hosts (Mungroo et al., 2021).

Secondary metabolites play a central role in the ecological success of many fungal species (Eagan and Keller, 2025), and their biosynthesis is highly plastic, responding to environmental cues such as nutrient availability, light, temperature, pH, and specific elicitors (Reshi et al., 2023; Zhou et al., 2012). Consistent with this plasticity, our phenotypic data show that the culture medium markedly influences the amoebicidal activity of *B. bassiana*. The enhanced amoebicidal potential observed in nutrient-rich media, such as YPD and PDB, suggests that these conditions favour the activation of biosynthetic pathways associated with antipredatory responses, potentially increasing the production of SMs with cytotoxic or defensive functions (Breitling et al., 2013; Tudzynski, 2014). These findings highlight the extracellular environment as a key regulator of fungal metabolic output and defense strategies (Eagan and Keller, 2025; Keller, 2019).

This medium-dependence is consistent with the OSMAC (One Strain–Many Compounds) framework, in which systematic variation of culture conditions uncovers otherwise cryptic metabolic capacities within a single fungal strain (Lutfia et al., 2024). Changes in nutritional parameters, such as nitrogen availability, can activate silent biosynthetic gene clusters, as exemplified by the discovery of sanghaspirodins A and B in *Aspergillus nidulans* under nitrogen-limited conditions (Tudzynski, 2014). These considerations reinforce that the choice of culture medium is a critical determinant for detecting biologically active fungal metabolites, since many biosynthetic pathways remain silent under standard conditions and are activated only by environmental variation (Brakhage, 2013; Keller, 2019). This rationale guided our selection of PDB, which combined robust amoebicidal activity with efficient fungal development, for the subsequent transcriptomic analyses.

Based on these phenotypic observations, dual RNA-seq provided insight into the molecular basis of the fungal–amoeba interaction. Beyond its role in the present study, the RNA-seq–guided re-annotation of the *B. bassiana* AS272 genome yields an improved, condition-informed functional reference that will support future genomic and comparative analyses of this strain. The global fungal transcriptional response revealed the upregulation of genes associated with detoxification, enzymatic activity, and cellular signalling, indicating that *B. bassiana* actively responds to a potentially hostile intracellular environment following amoebic uptake and deploys adaptive mechanisms to cope with oxidative stress and other amoebal defenses, thereby promoting survival during interaction.

The enrichment of genes associated with detoxification and oxidative-stress responses is consistent with the capacity of phagocytic predators, including amoebae, to generate reactive oxygen species (ROS) as part of their defense mechanisms (Bidochka et al., 2010; Hillmann et al., 2015; Mungroo et al., 2021). In this context, the upregulation of genes encoding oxidoreductases, cytochrome P450 enzymes, and superoxide dismutases supports the activation of protective responses that mitigate oxidative damage. Because these same mechanisms are frequently associated with fungal pathogenicity in animal hosts (Brakhage, 2013; Wang et al., 2021), their induction reinforces the hypothesis that environmental interactions with amoebae may select for traits that later contribute to virulence in other systems (Bidochka et al., 2010; Ramos et al., 2025).

Consistent with this interpretation, integration of the transcriptomic data with PHI-base analysis revealed a substantial overlap between differentially expressed genes and known virulence-associated factors. This overlap suggests that genes traditionally linked to pathogenicity may also operate in environmental interactions, particularly in predator–prey dynamics involving amoebae (Albuquerque et al., 2019; João et al., 2024), in line with the ecological view that virulence traits can evolve through selection imposed by environmental predators (Casadevall et al., 2019).

A prominent feature of the fungal response was the activation of secondary metabolism. The identification of multiple biosynthetic gene clusters (BGCs) and the differential expression of their backbone genes underscore the importance of SMs during the fungal–amoeba interaction, consistent with previous work linking BGCs to virulence-associated traits in entomopathogenic fungi (Ramos et al., 2025). Secondary metabolites mediate a broad range of ecological functions, including defense, competition, and communication (Ávila-Hernández et al., 2020; Conrado et al., 2022), and their coordinated induction here suggests a role in modulating amoebic viability or behaviour.

Among the activated clusters, the oosporein BGC showed strong transcriptional induction during interaction with *A. castellanii*. Oosporein is a dibenzoquinone SM with well-documented cytotoxic effects on insect hemocytes, where it reduces hemocyte numbers and disrupts immune homeostasis (Fan et al., 2017; Mascarín et al., 2024). Given the functional similarities between insect hemocytes and phagocytic amoebae, particularly in microbial recognition and phagocytosis, oosporein may exert analogous cytotoxic effects on *A. castellanii*, and the coordinated induction of core genes within the cluster supports the involvement of this pathway in the fungal response to amoebae (Feng et al., 2015).

Notably, transcriptional activation of the oosporein cluster was not restricted to biosynthetic genes but extended to its regulatory and transport components. The concurrent upregulation of the cluster-specific transcription factor OpS3 and the MFS transporter OpS2, alongside the core biosynthetic gene, indicates coordinated activation of the biosynthetic, regulatory, and export machinery of the pathway rather than the isolated induction of a single enzyme. This pattern is characteristic of a fully engaged biosynthetic response and provided the rationale for prioritizing oosporein for functional validation.

The beauveriolide BGC likewise exhibited coordinated transcriptional activation, including the core biosynthetic genes ACM6JR_008267 and ACM6JR_008264 and additional biosynthetic components (e.g., ACM6JR_008260). Although this metabolite was not functionally validated, the expression pattern suggests that beauveriolide biosynthesis contributes to the broader metabolic response of *B. bassiana* during amoebal interaction. Beauveriolides are cyclodepsipeptides with well-characterized activity, including inhibition of acyl-CoA:cholesterol acyltransferase (ACAT), which disrupts lipid metabolism and reduces lipid-droplet formation in eukaryotic cells (Kim et al., 2023; Namatame et al., 1999; Tomoda and Doi, 2008). Such effects on cellular lipid homeostasis suggest that beauveriolides may interfere with membrane dynamics, energy storage, or intracellular trafficking in amoebae. The concurrent activation of multiple BGCs indicates a multifaceted metabolic strategy in which distinct SMs may act in combination (Keller, 2019; Pedrini, 2022; Zhgun, 2023).

Functional assays using purified oosporein confirmed a dose- and time-dependent reduction in *A. castellanii* viability. However, the magnitude of this effect was smaller than that observed during direct interaction with *B. bassiana*, indicating that oosporein alone does not fully account for the amoebicidal activity of the fungus. Oosporein can participate in redox reactions that modify protein sulfhydryl groups, leading to enzyme inactivation, and can inhibit membrane-associated ATPases involved in essential processes such as phagocytosis and osmoregulation (Aleo, 1991; Ramesha et al., 2015). These mechanisms offer a plausible basis for the cytotoxic effects observed in amoebae, and the dose- and time-dependent profile parallels reports in mammalian cells (Li et al., 2023; Ramesha et al., 2015), suggesting that similar stress-related pathways may be affected in *A. castellanii*.

An important question is whether the amoebicidal activity reflects the action of SMs specifically or a broader set of fungal factors, such as hydrolytic enzymes and oxidative-stress-related and virulence-associated responses. Our data support a multifactorial model. Alongside the induction of genes associated with detoxification, enzymatic activity, and cellular signalling, we observed the coordinated activation of specific BGCs, particularly those for oosporein and beauveriolide, indicating that secondary metabolism is not an isolated feature but part of a broader adaptive response during the interaction (Reshi et al., 2023; Wadhwa et al., 2024). The measurable effect of purified oosporein supports a direct contribution of SMs, while the stronger activity of the intact fungus indicates that additional factors act in combination (Ávila-Hernández et al., 2020; Pedrini, 2022).

A. castellanii represents an initial experimental model for studying fungus–amoeba interactions. Extending these analyses to other free-living amoebae, including clinically relevant taxa such as

Naegleria fowleri (Siddiqui et al., 2025) and *Balamuthia mandrillaris* (Siddiqui et al., 2026), as well as diverse clinical isolates of *Acanthamoeba*, will be necessary to establish the broader relevance of these findings, including the effects of oosporein on cystic forms. In such settings, other fungal factors, including secreted proteases and chitinolytic enzymes previously associated with antipredatory and virulence-related functions in entomopathogenic fungi (Fan et al., 2017; Semenova et al., 2020), may act together with SMs to shape the overall amoebicidal response.

The dual RNA-seq design also showed that *A. castellanii* is far from a passive predator in this interaction. The 3,785 amoebal DEGs demonstrate that contact with *B. bassiana* triggers broad transcriptional remodeling, spanning cytoskeletal and vesicular-trafficking pathways, stress regulation, DNA repair, signalling, and metabolic adjustment. The enrichment of upregulated cytoskeleton- and organization-related genes, together with the induction of a Rab9-related GTPase, is consistent with the remodeling of motility, phagocytosis, and endosomal trafficking expected in an actively feeding trophozoite (Vaughn and Abu Kwaik, 2021).

Notably, the amoebal stress program appeared to be reorganized rather than uniformly activated: genes encoding small heat-shock proteins, glutathione S-transferase, and universal stress proteins were downregulated, and functional enrichment indicated a coordinated suppression of cellular stress-response, DNA-damage-response, and DNA-repair pathways. This signature is the counterpart of the fungal response on the opposite side of the interface, where *B. bassiana* upregulated detoxification and oxidative-stress genes and activated the oosporein biosynthetic pathway. The two DEG sets therefore represent complementary readouts of a shared oxidative-stress interface, in which the fungal secondary-metabolite and detoxification programs coincide with a reorganization of the amoebal stress and genome-maintenance machinery (Motavallihaghi et al., 2022). Because *A. castellanii* shares functional similarities with mammalian phagocytes, including a respiratory-burst-like generation of ROS during phagocytosis (Brooks and Schneider, 1985; Davies et al., 1991), this reciprocal molecular dialogue reinforces the value of fungal–amoebal systems as environmental models for the evolution of microbial persistence and virulence traits (Rayamajhee et al., 2022; Ženíšková et al., 2025).

CONCLUSION

In conclusion, this study demonstrates that interactions between *B. bassiana* and *A. castellanii* trigger coordinated transcriptional and metabolic responses in both organisms. By integrating phenotypic assays, dual RNA-seq, biosynthetic gene cluster analysis, and functional validation, we show that fungal secondary metabolism contributes directly to amoebicidal activity, while the amoebal transcriptome undergoes extensive reprogramming during predation. These findings support the concept that free-living amoebae act as environmental selective pressures shaping fungal defence strategies and provide new insights into the ecological and evolutionary links between environmental survival and fungal virulence.

Declarations

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Declaration of competing interest

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

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Data availability

All datasets generated and analyzed during this study are included in this published article. R scripts used in the analyses are available in ZENODO (<https://doi.org/10.5281/zenodo.21611127>). Raw fastq files are available under NCBI bioproject PRJNA1222367. Gene-level count tables for both *B. bassiana* and *A. castellanii*, together with the R scripts used for the fungal and amoebal transcriptome analyses, are included in the same repository.

Clinical trial number

Not applicable.

CRediT authorship contribution statement

MEDJ and CCS contributed to the study conception and design. Methodology was developed by MEDJ, HRMA, and CCS. Investigation was performed by MEDJ and CCS. Formal analysis and data curation were conducted by MEDJ, HRMA, and CCS. Visualization was performed by MEDJ and HRMA. The first draft of the manuscript was written by MEDJ and CCS. LK, CCS, and HRMA reviewed and edited previous versions of the manuscript. Resources were provided by LK and CCS. Supervision was carried out by CCS. Project administration was performed by LK and CCS. Funding acquisition was provided by LK and CCS. All authors read and approved the final manuscript.

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Table

Table 1. Comparative metrics of *B. bassiana* AS272 genome annotation versions

Feature / Level	Previous Annotation (Reference)	BRAKER3 Annotation (Query)	Sensitivity (%)	Precision (%)
Total Gene Models (Loci)	9,702	9,654	75.6	76.1
Total mRNAs (Transcripts)	10,783	9,654	68.1	76.1
Multi-exon Transcripts	8,028	7,006	—	—
Intron Chains	—	—	64.7	74.1
Exon Level	—	—	74.9	83.1
Intron Level	—	—	75.6	84.5
Base Level	—	—	91.5	95.2

Figures

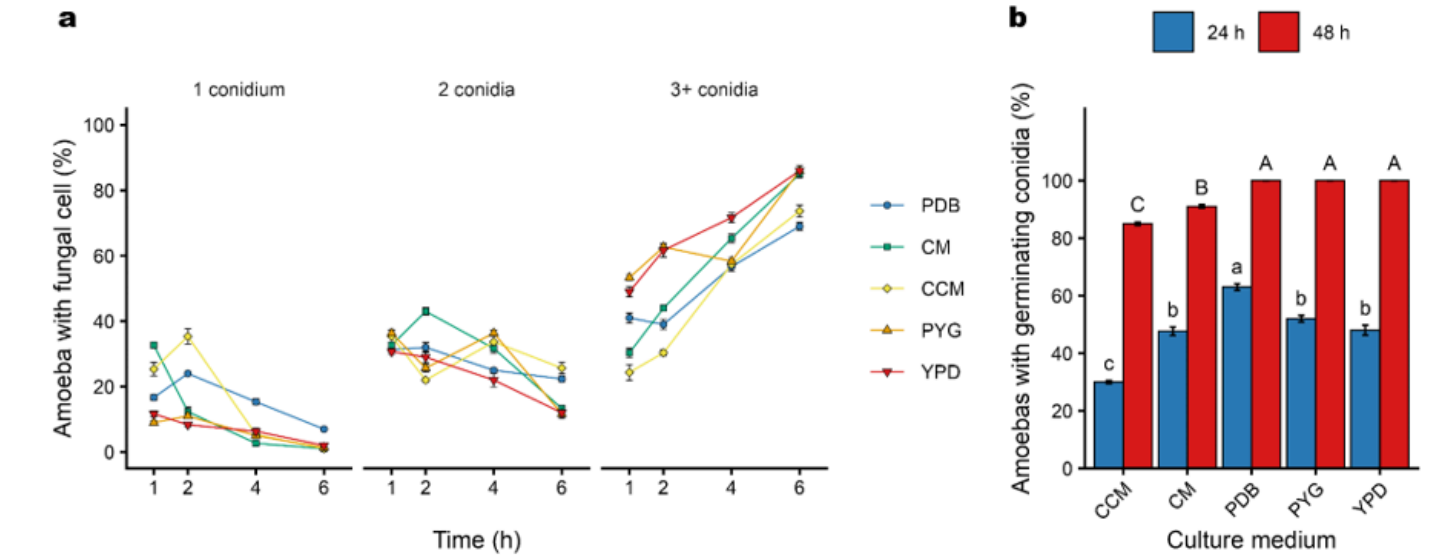


Figure 1

Phagocytosis and germination of *B. bassiana* conidia by *A. castellanii* across culture media. (q)

Percentage of amoebae containing internalized conidia (1, 2, or ≥ 3 per trophozoite) over 6 h in PDB, CM, CCM, PYG, and YPD (biological triplicates). (b) Germination of phagocytosed conidia after 24 h (blue) and 48 h (red); lowercase letters (a–d) denote differences among media at 24 h and uppercase (A–D) at 48 h (ANOVA, post-hoc, $P < 0.05$).

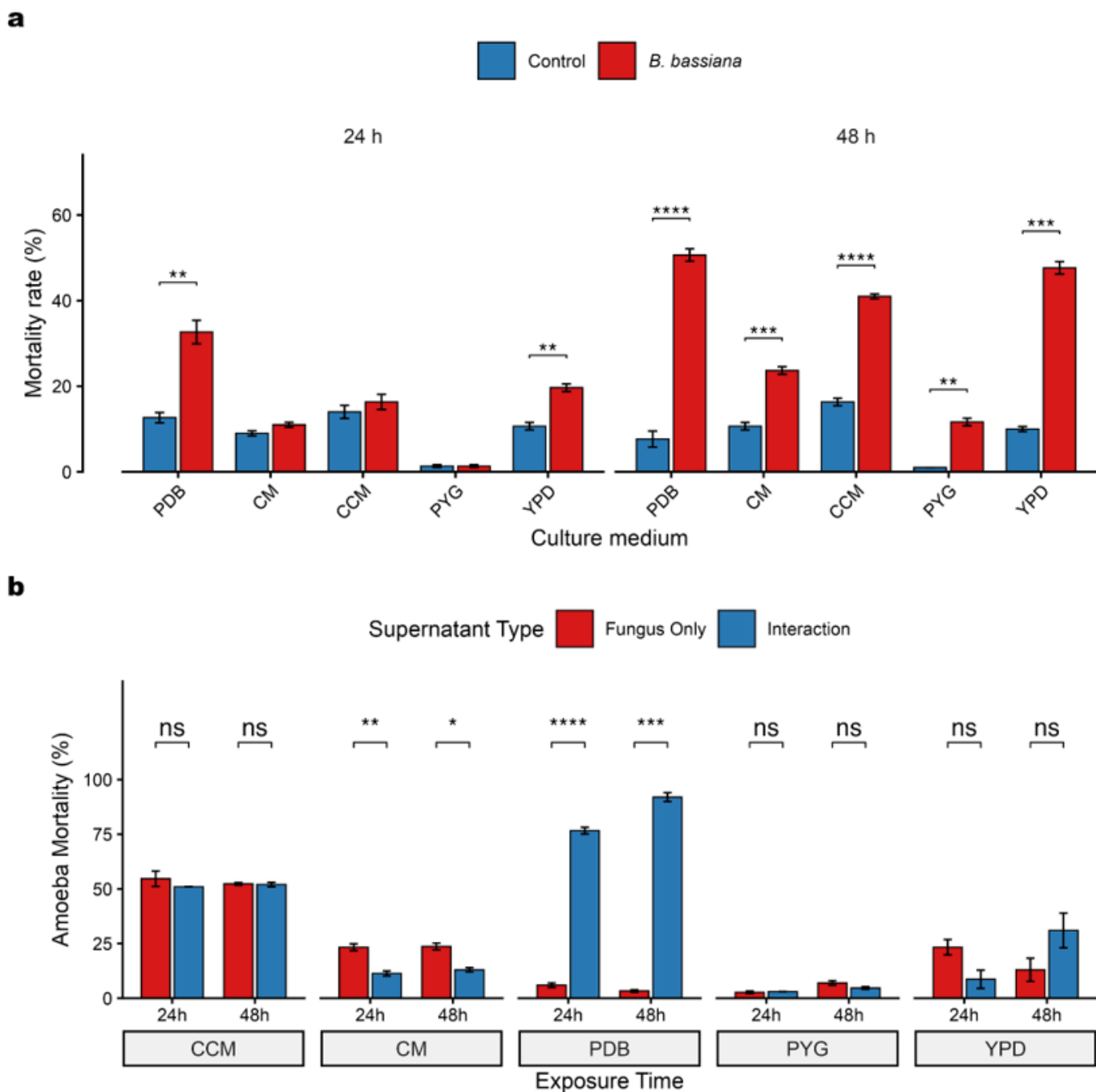


Figure 2

Amoebicidal activity of *B. bassiana* and its cell-free supernatants. (a) *A. castellanii* mortality (trypan blue) after 24 and 48 h of co-incubation; blue, amoeba-only control; red, co-culture (t-tests per medium/time; *P < 0.05 to ****P < 0.0001). (b) Mortality induced by cell-free supernatants from fungal monocultures (red) and co-cultures (blue); unpaired t-tests with Bonferroni correction (ns, not significant; *P < 0.05 to ****P < 0.0001).

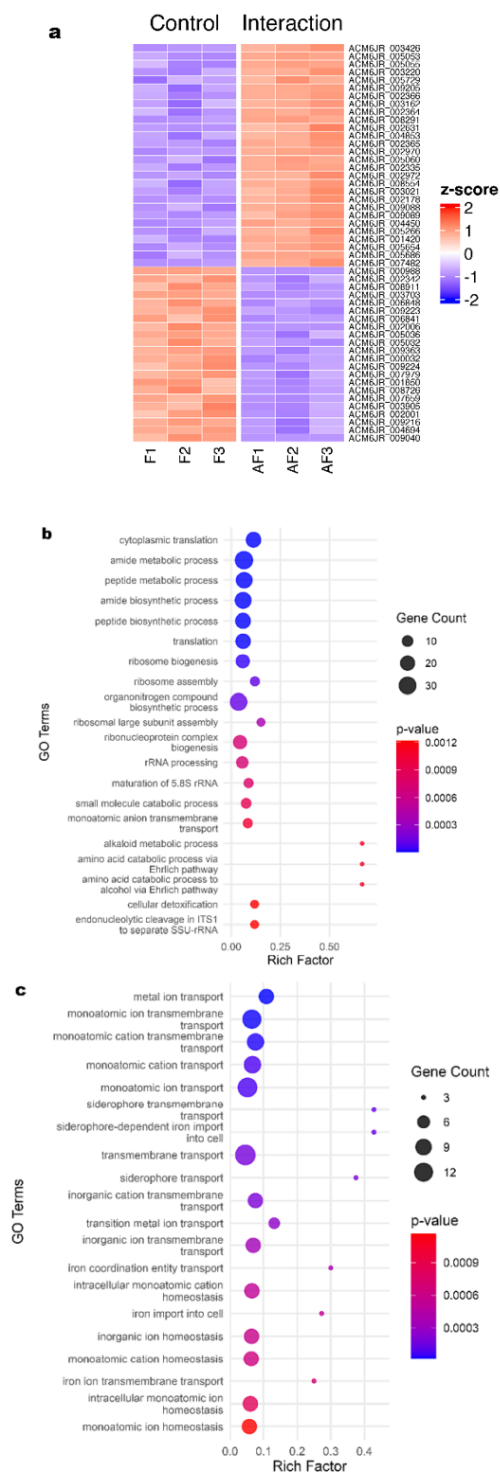


Figure 3

Global transcriptional response of *B. bassiana* during co-culture with *A. castellanii*. (a) Heatmap showing the top 50 differentially expressed genes (DEGs) ranked by absolute $\log_2$ fold change. Gene expression is displayed as row-wise z-scores of TMM-normalized CPM values. F1–F3, fungal monocultures; AF1–AF3, fungal–amoeba co-cultures. (b) Gene Ontology (GO) enrichment analysis of upregulated DEGs. (c) GO enrichment analysis of downregulated DEGs. Bubble size represents the

number of genes associated with each GO term, and bubble color indicates Fisher's exact test *P*value. Only significantly enriched terms (adjusted *P* < 0.05) are shown.

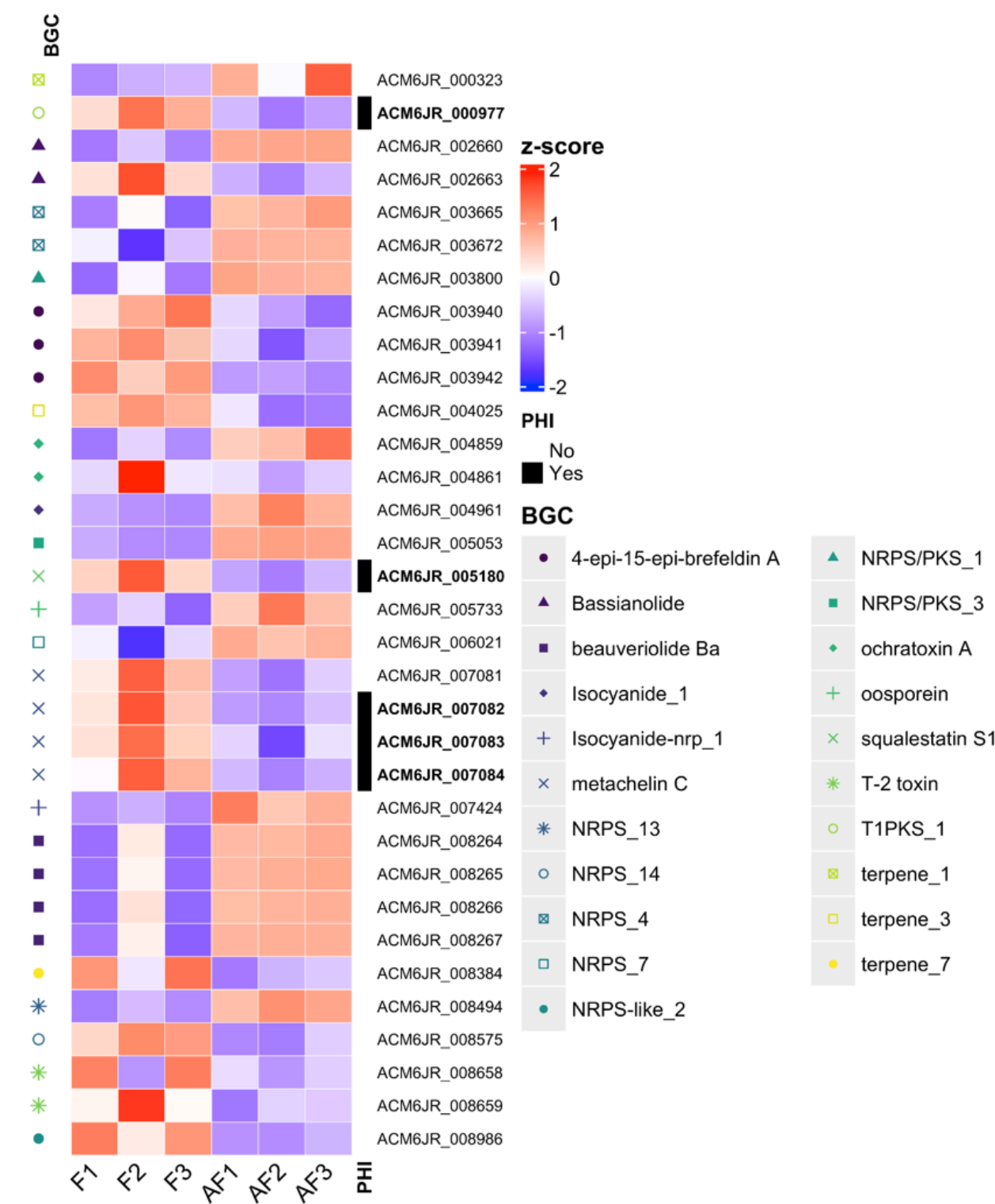


Figure 4

Expression of biosynthetic gene cluster (BGC) backbone genes in *B. bassiana* during co-culture with *A. castellanii*. Heatmap of core biosynthetic (backbone) genes from predicted BGCs. Expression values are

shown as row-wise z-scores of TMM-normalized CPM across fungal monocultures (F1–F3) and fungal–amoeba co-cultures (AF1–AF3). Rows are labeled by BGC (antiSMASH identifier or predicted product) and annotated by biosynthetic class (right color bar).

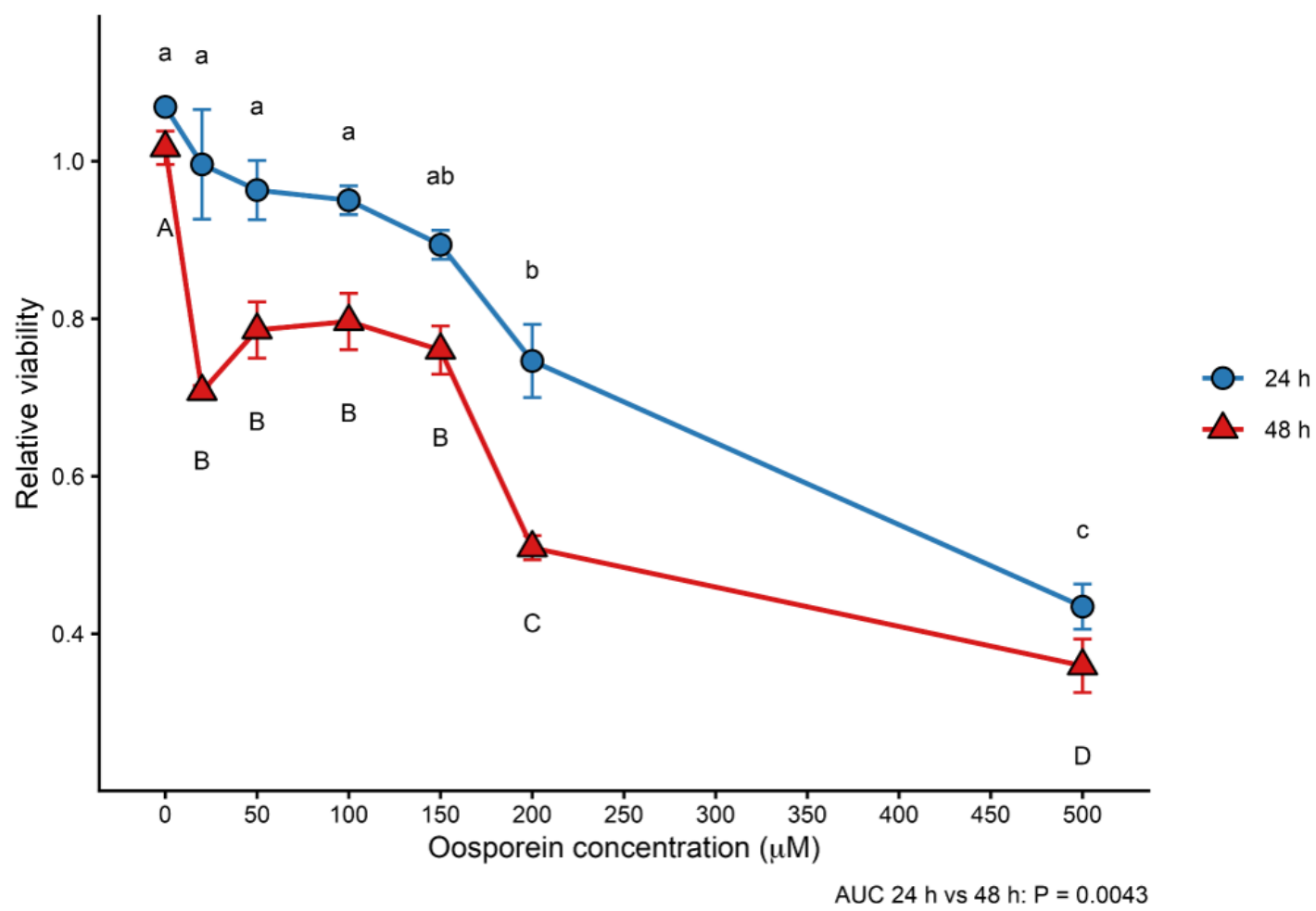


Figure 5

Dose- and time-dependent effect of oosporein on *A. castellanii* viability. Cell viability, measured by the AlamarBlue® assay, after 24 h (blue) and 48 h (red) exposure to 0–500 µM oosporein, expressed relative to the vehicle control. Points represent the mean and error bars indicate the standard error (three biological replicates). Different lowercase (24 h) and uppercase (48 h) letters indicate significant differences among concentrations within each time point (one-way ANOVA with post hoc test, $P < 0.05$). Overall dose–response curves were compared by area under the curve (AUC; $P = 0.0043$).

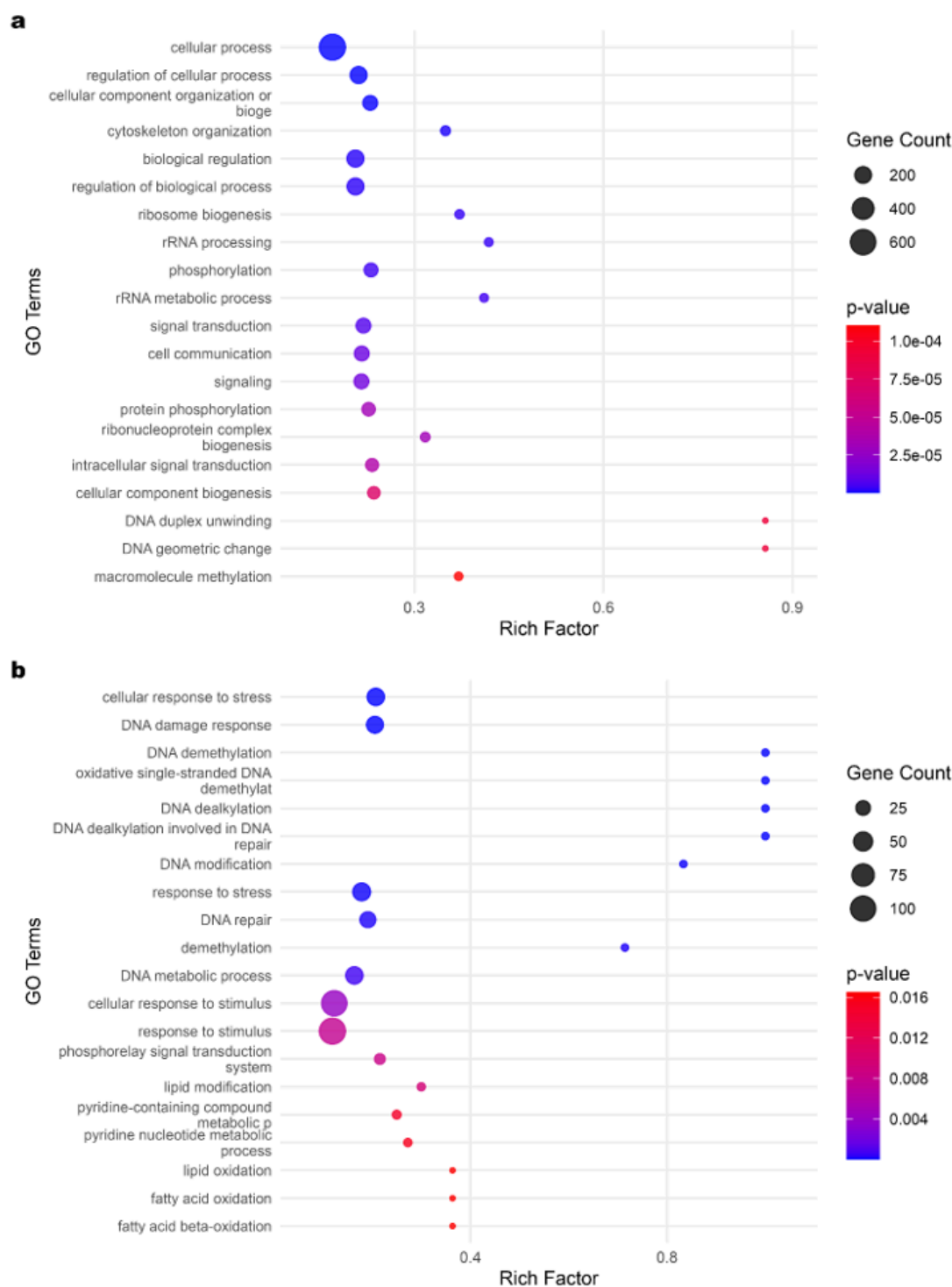


Figure 6

Functional enrichment of differentially expressed genes in *A. castellanii* during interaction with *B. bassiana*. Bubble plots showing enriched Gene Ontology (GO) Biological Process terms for (a) upregulated and (b) downregulated differentially expressed genes, based on topGO analysis using the AmoebaDB release 68 annotation. Bubble size represents the number of genes associated with each GO

term, and bubble color indicates enrichment significance. Only significantly enriched terms (adjusted $P < 0.05$) are shown.

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