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Ambient pH Modulates Aflatoxin Biosynthesis in *Aspergillus parasiticus* NRRL2999 through Transcriptomic Reprogramming

Rukaiya Almin Akhtar[1], Mohd. Ikram Ansari[1]✉, Akshay Kumar[2], Malik M. Ahmad[2]✉
[1]Department of Biosciences, Integral University, Lucknow, [2]Department of Agriculture, Integral Institute of Agricultural Science and Technology (IIAST), Integral University, Lucknow
Email: mohd.ikram.ansari@gmail.com; Email: malik4948@gmail.com
(Corresponding Authors)

Abstract
Ambient pH is a critical environmental factor influencing fungal development and secondary metabolism, yet its molecular role in regulating aflatoxin biosynthesis in *Aspergillus parasiticus* remains incompletely understood. This study investigated the effects of four pH regimes (3.5, 4.5, 5.5 and 6.5) on aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) production and associated transcriptomic responses in *A. parasiticus* NRRL2999 using RNA-sequencing. Acidic conditions (pH 3.5–4.5) significantly enhanced aflatoxin accumulation, whereas toxin production was markedly reduced at pH 5.5 and nearly suppressed at pH 6.5. Transcriptome analysis revealed pronounced pH-dependent transcriptional reprogramming, with principal component analysis showing clear separation of acidic treatments from the control. Differential expression analysis identified significant pH-responsive genes associated with oxidative stress adaptation, carbon metabolism, transport functions and aflatoxin biosynthetic processes. Upregulation of genes related to redox homeostasis, glycolytic flux, fatty acid synthesis and FAD-dependent oxidation suggested that acidic pH promotes a metabolic and regulatory environment favourable for aflatoxin biosynthesis. Conversely, repression of selected cytochrome P450s, methyltransferases and transporter-associated genes indicated selective pathway-level modulation rather than uniform cluster activation. Integrated analyses support a model in which acidic pH modulates aflatoxin biosynthesis through *PacC*-linked pH signalling, oxidative stress responses, metabolic rewiring and coordinated transcriptional regulation. The findings highlight ambient pH as a central ecological signal shaping toxin biosynthesis and fungal adaptive responses. This study provides new mechanistic insights into pH-mediated regulation of aflatoxin production and identifies candidate regulatory genes that may serve as targets for future mycotoxin mitigation strategies.
Keywords: *Aspergillus parasiticus*, Aflatoxin biosynthesis, secondary metabolism, ambient pH regulation, RNA-seq, oxidative stress signalling.
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1. Introduction

Aflatoxins are highly toxic, carcinogenic secondary metabolites produced predominantly by *Aspergillus flavus* and *A. parasiticus*, contaminating staple crops worldwide and threatening food safety, public health, and economies[1,2,3]. Chronic dietary exposure to aflatoxin B_1 (AFB_1) – the most potent member of this group – is strongly associated with hepatocellular carcinoma, immune suppression, and impaired child growth[4,2]. Indeed, AFB_1 has been classified as a Group I carcinogen by IARC. Outbreaks of acute aflatoxicosis have caused human fatalities, and aflatoxin contamination incurs annual economic losses on the order of USD 6–18 billion through lost crops, rejected exports, and health burdens[2]. Aflatoxin B_2 (AFB_2) is less abundant and slightly less toxic than B_1, but often co-occurs in the same grain commodities. Tight regulatory limits (parts-per-billion levels) underscore the global significance of controlling aflatoxins to ensure safe food and feed[3].

A. parasiticus is a major producer of aflatoxins B_1 and B_2 (and often G_1/G_2), infecting a wide range of plants[5]. The biosynthesis of aflatoxins is encoded by a ~75 kb gene cluster containing roughly 25–30 genes. Within this cluster, the Zn_2Cys_6 transcription factor *AflR* directly activates pathway genes by binding promoters, while its co-activator *AflS* (*AflJ*) enhances *AflR* function. Deletion of *aflR* abolishes expression of

all structural genes and aflatoxin production, whereas disruption of *aflS* causes a severe reduction. The cluster includes key enzymes such as a polyketide synthase (*AflC*) that catalyzes the first committed step and various oxidoreductases (e.g. *AflM*/REDOX, *AflQ*/*ORD*) and methyltransferases that convert intermediates to AFB_1 and B_2[4] (Li et al., 2020). Beyond cluster-specific factors, conserved global regulators link aflatoxin biosynthesis to fungal physiology. The Velvet complex (*VeA–VelB–LaeA*) is a light-responsive regulator of development and secondary metabolism; nuclear *VeA* binds *LaeA* to epigenetically activate numerous secondary metabolism gene clusters, including aflatoxin[6]. Other master regulators such as CreA (carbon catabolite repression) and AreA (nitrogen signaling) also modulate the aflatoxin cluster[4]. Collectively, these factors position the aflatoxin pathway under the control of the fungus's developmental and metabolic state[6].

Environmental cues profoundly influence aflatoxin production, and among these, ambient pH is a key regulatory signal. In many Aspergilli, the *PacC/Rim101* pathway senses external pH and reprograms gene expression accordingly. At neutral to alkaline pH, *PacC* is proteolytically activated and promotes alkaline-adaptive genes while repressing acid-expressed genes; conversely, at acidic pH a non-processed form of *PacC* permits expression of acid-induced genes. In *A. parasiticus* and *A. flavus*, aflatoxin biosynthesis typically occurs only under acidic conditions[7,3]. Accordingly, acidic pH conditions generally favor higher aflatoxin B_1/B_2 output, whereas alkaline pH or buffering strongly suppresses the cluster[7]. The *PacC* ortholog in *A. parasiticus* likely acts as a repressor of aflatoxin genes when activated under high pH[3]. Experimental evidence suggests that artificially raising culture pH (e.g. with buffers or alkaline ions) inhibits aflatoxin synthesis, partly because AFB_1's coumarin-lactone ring opens at high pH and because PacC-mediated signalling downregulates secondary metabolism. Conversely, culturing *A. parasiticus* at lower initial pH (around 3.5–5.5) typically accelerates aflatoxin gene expression and toxin accumulation[8]. These pH-dependent effects may also interact with nutrient status and oxidative stress pathways to coordinate the cluster's regulation. In sum, pH is an important environmental variable that modulates aflatoxin biosynthesis through well-known fungal pH signalling systems and transcription factors[3,8].

To dissect the molecular mechanisms linking ambient pH to aflatoxin output, it is valuable to integrate phenotypic assays with global transcriptomics. Quantifying AFB_1 and B_2 levels under defined pH conditions provides the observable outcome, while RNA-seq reveals the genome-wide transcriptional response driving it. Such combined "phenotype-omics" approaches have proven powerful in mycotoxin research. Complementary transcriptomic and proteomic profiling of *A. flavus* growing on different crop substrates identified differential expression of aflatoxin cluster genes and regulators[4]. In our context, measuring both aflatoxin titers and RNA-seq under multiple pH treatments will enable linkage of specific gene expression changes (e.g. in *aflR/S*, regulatory factors, or pH-responsive genes) to increases or decreases in toxin biosynthesis. This integrative approach can uncover the networks by which external pH cues are transduced into metabolic outcomes, going beyond single-gene studies to reveal pathway-level regulation[4,8].

Accordingly, the present study investigated the impact of different ambient pH conditions– pH 3.5, 4.5, 5.5, and 6.5 (near-neutral) for aflatoxin AFB_1 and B_2 production on cultures of *Aspergillus parasiticus* NRRL2999 performed RNA-seq to elucidate molecular mechanisms underlying pH-mediated regulation of aflatoxin biosynthesis. We hypothesize that the lowest pH treatments (f1, f2) will markedly upregulate aflatoxin cluster genes and associated regulators (for example via relief of PacC repression), resulting in higher toxin levels, whereas the near-neutral control pH (6.5) will show attenuated cluster expression. By linking toxin quantitation with genome-wide expression patterns, this work aims to illuminate how ambient pH controls aflatoxin biosynthesis in *A. parasiticus*, testing the idea that pH shifts modulate global regulatory networks (including PacC and Velvet signaling) that in turn govern the aflatoxin pathway.

2. Materials and Methods

2.1 Fungal Strain and Culture Conditions

The aflatoxigenic fungal strain *Aspergillus parasiticus* NRRL2999 was used in the present study to investigate the influence of ambient pH on aflatoxin production and global transcriptomic responses. The culture was maintained on Yeast Extract Sucrose (YES) slants at 4°C and periodically subcultured to preserve viability. Fresh conidial suspensions were prepared from actively growing 5–7 day-old cultures by flooding plates with sterile 0.01% Tween-80 solution and gently scraping the colony surface.

Conidial concentration was determined using a hemocytometer and adjusted to approximately 1 × 10^6 spores mL^{-1} for inoculation.

For pH treatment experiments, fungal cultures were grown in aflatoxin-inducing liquid medium (YES-media) adjusted to four pH levels designated as f1 (3.5), f2 (4.5), f3 (5.5), and f4 (6.5, untreated control). The pH of media was adjusted prior to sterilization using sterile 1 N HCl or 1 N NaOH and verified after autoclaving. Each treatment was established in biological triplicates in 250 mL Erlenmeyer flasks containing 100 mL medium inoculated with equal volumes of conidial suspension.

Cultures were incubated under stationary conditions at 25±2°C for the designated experimental period (insert incubation duration, e.g., 7 days), after which fungal biomass and culture filtrates were harvested separately for aflatoxin quantification and RNA extraction.

2.2 Quantification of Aflatoxins

Following incubation, culture broths were filtered through sterile Whatman No. 1 filter paper to separate mycelial biomass from extracellular culture filtrates. Extraction was performed using 40 mL of chloroform (twice with 20 mL each), and then the chloroform phase was filtered through filter paper and concentrated to dryness under 50 °C in an incubator. The residue was redissolved in 20 µL of methanol, and 10 µL of this solution was spotted and developed on a Si250 silica gel plate (Haiyang, Qingdao, China) with a solvent system of chloroform/acetone (90:10, v/v)[9]. Aflatoxin concentrations were expressed as µg mL^{-1} (or appropriate unit) and analysed statistically using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test at $p < 0.05$.

2.3. cDNA Preparation and Illumina Sequencing

Seven day-old mycelium was removed from the cellophane surface for isolation of RNA, and cDNA was prepared according to a protocol with some modifications[10]. Genomic DNA was digested using DNase (New England Biolabs, Beijing, China), and total RNA was isolated using TRIzol reagent (Invitrogen, Shanghai, China). A Nano Drop 2000 and Agilent 2100 were used to evaluate the quality of RNA.

2.4 Pre-processing and quality control of RNA-Seq data

The raw RNA-seq data in FASTQ format underwent stringent quality control to ensure reliability and accuracy of downstream transcriptomic analyses. Initial quality assessment was conducted using FastQC (v0.11.9)[11], which generated comprehensive reports on sequence quality metrics including per-base quality scores, per-sequence GC content, sequence length distribution, and potential adapter contamination.

To improve read quality, Trimmomatic (v0.39)[12] was employed for adapter trimming and filtering of low-quality bases using a sliding window approach. Bases with a Phred quality score below 20 were trimmed, and reads shorter than 36 bp following trimming were discarded to eliminate unreliable sequences that may compromise the accuracy of alignment. The resulting high-quality clean reads were subsequently aligned to the *Aspergillus parasiticus* reference genome (retrieved from the NCBI Genome database or Ensembl Fungi) using the STAR RNA-seq aligner (v2.7.9a)[13] under default parameters. This spliced aligner ensures efficient and accurate mapping of reads to the reference, accommodating the complex transcript structures of eukaryotic genomes. Post-alignment, mapping statistics and alignment quality were evaluated using SAMtools (v1.15)[14], which provided insights into the percentage of uniquely mapped reads, read distribution across genomic features (e.g., exonic, intronic, and intergenic regions), and potential duplication artifacts. These comprehensive pre-processing steps ensured high-confidence input data for subsequent quantification and differential gene expression analyses.

2.5 Differential gene expression analysis

Gene-level expression quantification was carried out by counting the number of reads aligned to annotated genes using HTSeq-count (v0.12.4)[15]. To correct for differences in sequencing depth and expression variability, raw read counts were normalized using the variance-stabilizing transformation (VST) implemented in DESeq2 (v1.32.0, Bioconductor)[16]. This normalization approach enabled robust downstream comparisons across samples and conditions. Differentially expressed genes (DEGs) were defined based on the following thresholds: an absolute $\log_2$ fold change ($|\log_2 FC|$) greater than 1.0, corresponding to at least a two-fold change in expression, and a false discovery rate (FDR) < 0.05, adjusted using the Benjamini-Hochberg correction to control for multiple hypothesis testing[17]. To explore global transcriptomic variance and assess sample clustering by treatment groups, Principal Component Analysis (PCA) was performed. Furthermore, hierarchical

clustering analysis and heatmap visualizations of DEGs were generated using the Complex Heatmap R package[18], providing insight into condition-specific expression signatures and clustering patterns across biological replicates.

2.6 Functional annotation and pathway enrichment analysis

Functional annotation of differentially expressed genes (DEGs) was carried out using the Blast2GO suite (v5.2.5)[19], which enabled assignment of Gene Ontology (GO) terms spanning biological processes, molecular functions, and cellular components. To further elucidate the biological significance of DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the KAAS (KEGG Automatic Annotation Server)[20], allowing for the mapping of DEGs to known metabolic and signalling pathways. Integrated visualization of these enriched pathways, in conjunction with transcriptomic fold-change data, was achieved through the Pathview package (v1.38.0, Bioconductor)[21], which provided an intuitive graphical representation of DEG perturbations within biochemical networks.Special emphasis was placed on genes associated with the aflatoxin biosynthetic gene cluster, given its relevance to toxin production and environmental regulation. These included polyketide synthase genes such as *aflC* and *aflD*, which catalyze the synthesis of the polyketide backbone; methyltransferase genes (*aflA, aflB*) involved in methylation steps; and cytochrome P450 monooxygenases (*aflM, aflO*), which facilitate oxidative modifications in the aflatoxin biosynthetic pathway[22]. In addition, regulatory genes *aflR* and *aflS* were assessed for their role in activating cluster expression, and the efflux transporter gene *aflT* was evaluated for its potential involvement in the cellular export of aflatoxins[23]. Together, these analyses provided a comprehensive framework for interpreting the transcriptional regulation of aflatoxin biosynthesis under varying environmental stress conditions.

2.7 Statistical analysis

All experiments were conducted in biological triplicates. Statistical analyses were performed using GraphPad Prism (v9.0)[24]. For pairwise comparisons, Student's t-test was used; for comparisons across multiple groups, one-way ANOVA followed by Tukey's post hoc test was applied. A significance threshold of $p < 0.05$ was adopted. All p-values were adjusted using the Benjamini-Hochberg method to control the false discovery rate (FDR), ensuring statistical rigor and minimizing type I errors.

2.8 Data visualization

To comprehensively visualize transcriptomic alterations and extract biologically meaningful patterns, a suite of graphical approaches was employed. Heatmaps and hierarchical clustering were generated using the Complex Heatmap package in R[18] to depict the expression profiles of differentially expressed genes (DEGs) across all treatment groups. These visualizations enabled identification of condition-specific gene clusters and co-expression modules. Volcano plots, constructed using the ggplot2 package[25], were used to illustrate the distribution of DEGs based on $\log_2$ fold change and adjusted p-values, effectively highlighting genes with both statistical and biological significance. Principal Component Analysis (PCA) plots were created to evaluate global sample variance and to assess clustering patterns among replicates and treatment conditions, providing an overview of the consistency and separability of transcriptomic profiles. Additionally, KEGG pathway diagrams were generated via Pathview[21] to map differentially expressed genes onto known metabolic and signalling pathways, allowing for integrative interpretation of stress-responsive transcriptional shifts within biochemical networks. Collectively, these visualization strategies facilitated a holistic understanding of the transcriptional landscape and its modulation under environmental stress in *Aspergillus parasiticus*.

3. Results

3.1 Aflatoxin Production at Different pH

Aflatoxin levels were strongly pH-dependent. Under the most acidic conditions (f1 pH 3.5 and f2 4.5) *A. parasiticus* produced maximal toxin, whereas at near-neutral pH (f4 6.5 control) production was nearly abolished. Mean AFB_1 was 0.0033±0.05 µg/mL at pH 3.5 versus 0.00857± 0.04 µg/mL at pH 6.5 (N=3 each, ANOVA $p<0.01$, Tukey's post-hoc $p<0.05$). AFB_2 mirrored this trend (Table 1). Differences between f1/f2 and f3 were significant (post-hoc $p<0.05$). The AFB_1 and AFB_2 titers were highly correlated (Pearson $r=0.98$, $p<0.01$), confirming a coordinated biosynthetic output. These data indicate that growth at pH ≤4.5 triggers strong aflatoxin biosynthesis, whereas neutral pH represses it (Fig.1).

Table 1. Effect of different pH levels on mycelial biomass and aflatoxin production on *A. parasicitus* NRRL2999. The table presents aflatoxin content (ppb) including B1, B2, and G fractions for samples

grown at varying pH conditions (f1 = 3.5, f2 = 4.5, f3 = 5.5).

Sample no.	pH	Aflatoxin content (µg/mL)		
		B1	B2	G
f1	3.5	0.0033±0.05	0.0026±0.03	ND[#]
f2	4.5	0.00269±0.02	0.00242±0.02	ND
f3	5.5	0.00398±0.06	0.00406±0.04	ND
f4 (control)	6.5	0.00857±0.04	0.00745±0.03	0.00252±0.04

[#]*ND = Not detected*

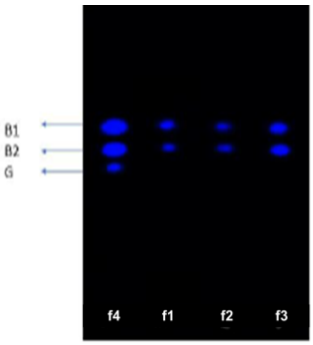

Fig. 1: Thin Layer Chromatography (TLC) analysis of aflatoxin production under different pH conditions. Samples correspond to pH treatments: f1 (3.5), f2 (4.5), f3 (5.5), and f4 (6.5; control). Fluorescent bands represent aflatoxin fractions, with B1, B2, and G indicated. Variation in band intensity across treatments reflects differences in aflatoxin production at different pH levels.

3.2 RNA-seq Overview and Data Quality

To elucidate the molecular basis of pH-mediated modulation of aflatoxin biosynthesis in *Aspergillus parasiticus* NRRL2999, transcriptome profiling was conducted under four pH treatments, namely f1 (pH 3.5), f2 (pH 4.5), f3 (pH 5.5), and f4 (pH 6.5; control). Sequencing generated high-quality datasets for all samples, with total reads ranging from 32.59 to 39.81 million and total bases between 5.1 and 6.3 Gbp (Table 2). The uniform sequence length (159 bp) and narrow GC content range (51–53%) indicated consistency across libraries and ruled out major compositional biases in sequencing output. The highest sequencing depth was observed in f3 (39,805,584 reads), followed closely by f2 (38,795,287 reads), whereas the control generated 32,598,280 reads, which still provided sufficient depth for robust differential expression analysis.

Overall, these metrics confirmed that all libraries met accepted standards for fungal transcriptome analysis and provided adequate coverage for reliable gene expression quantification.

Principal component analysis (PCA) further demonstrated distinct transcriptomic responses associated with ambient pH (Fig. 2). Principal component 1 accounted for 61% of the total variance, while principal component 2 explained an additional 22%, collectively capturing 83% of global expression variability. Samples corresponding to acidic treatments (particularly f1 and f2) formed clusters distinct from the control (f4), indicating substantial transcriptional reprogramming in response to lowered pH. The clear separation of treatments along PC1 suggested that ambient pH was a dominant driver of transcriptomic variation. The greatest divergence from the control was observed for pH 3.5, implying that severe acidic stress imposed the strongest regulatory effects.

The clustering pattern further suggested treatment-specific responses rather than stochastic variation, supporting the reproducibility of the dataset and validating downstream analyses of differentially expressed genes.

Table 2. Sequencing metrics (total reads, total bases, sequence length, and GC content) for each experimental sample.

Sample no.	Total reads	Total bases (Gbp)	Sequence length	GC (%)
f1	34,288,450	5.4	159	52
f2	38,795,287	6.1	159	53
f3	39,805,584	6.3	159	51
f4 (Control)	32,598,280	5.1	159	52

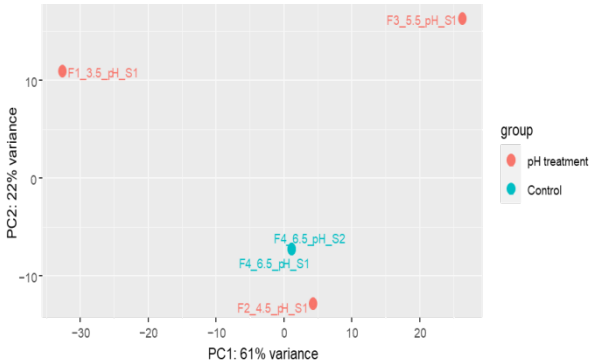

Fig. 2: Principal Component Analysis (PCA) score plot showing the clustering of *Aspergillus parasiticus* transcriptomic samples based on their global gene expression profiles under different pH conditions. Each point represents an individual sample, and samples are color-coded according to the treatment group: control (blue), and pH stress (red). Distinct separation of samples across the plot indicates variation in gene expression profiles in response to pH stress, with replicates from similar conditions clustering closer together.

3.3 Differential Gene Expression in Response to Acidic pH Stress

Comparative differential expression analysis between acidic treatments and the control identified a defined but biologically meaningful set of pH-responsive genes. Volcano plot analysis (Fig. 3a) revealed 22 significantly differentially expressed genes (DEGs) across treatments using thresholds of $|\log_2$ fold change$| > 1$ and adjusted p-value ≤ 0.05, comprising 14 upregulated and 8 downregulated genes.

A pronounced asymmetry toward gene induction under acidic conditions was observed, indicating activation of selective transcriptional programs rather than generalized suppression. Several genes exhibited strong induction, with log2 fold

changes exceeding 6–8, reflecting substantial transcriptional responsiveness to altered extracellular pH.

The MA plot (Fig. 3b) supported these observations by showing that the significant DEGs were distributed across a broad range of expression levels, indicating that pH-responsive regulation was not restricted to highly expressed transcripts but affected genes spanning multiple abundance classes. While most genes remained centered near zero fold change, the subset of DEGs deviated markedly, underscoring biologically significant shifts rather than technical noise.

Heatmap-based hierarchical clustering (Fig. 3c) further resolved distinct expression patterns among the DEGs. Genes formed two major clusters. Cluster I: Acid-induced genes: This cluster comprised transcripts strongly upregulated under low pH conditions (particularly f1 and f2), including genes associated with redox metabolism, transport functions, transcriptional regulation, and aflatoxin-related biosynthetic processes. Cluster II: Acid-repressed genes: This cluster included genes progressively downregulated as pH decreased, several of which were linked to hydrolase activity, cytochrome P450-dependent functions, methyltransferases, and membrane-associated proteins.

The strongest transcriptional contrast was consistently observed between pH 3.5 and the pH 6.5 control, while pH 5.5 showed a more moderate profile, suggesting a graded rather than binary response to ambient pH. Collectively, these analyses indicate that acidic pH triggers selective and coordinated transcriptional remodelling in *A. parasiticus*.

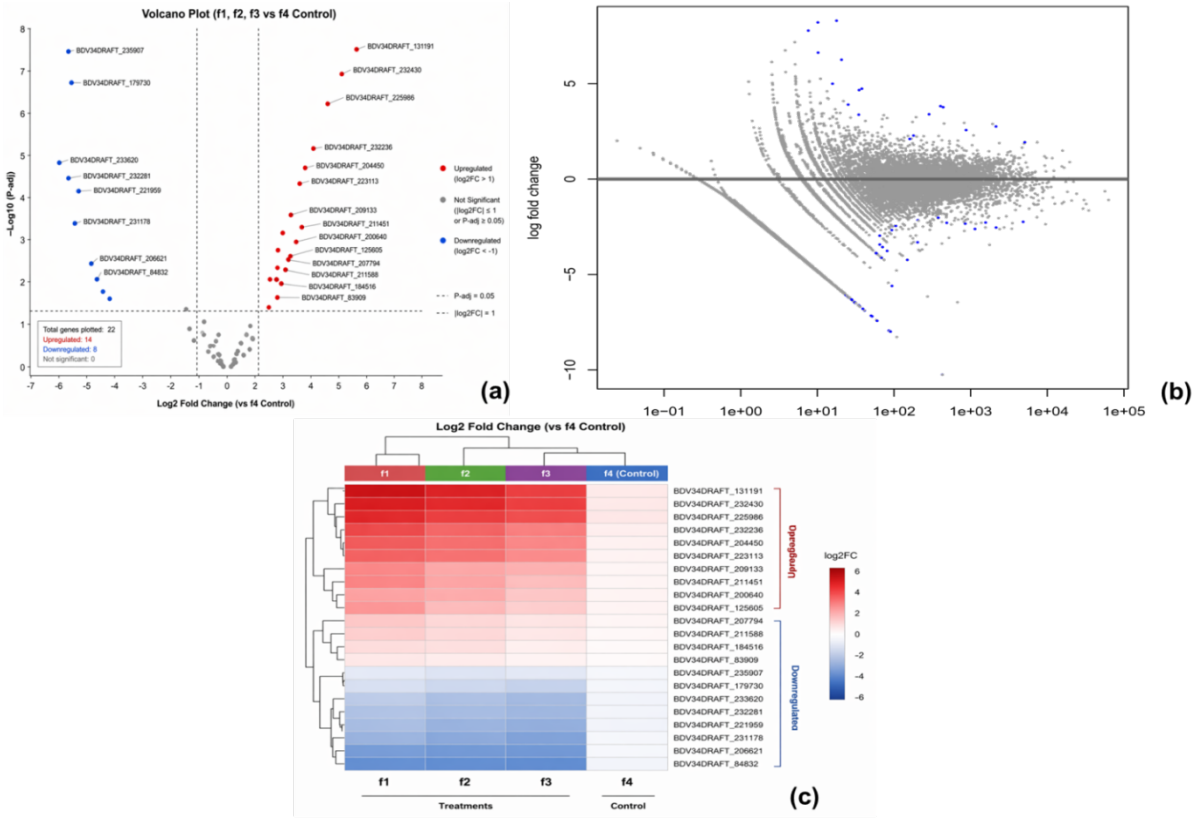

Fig. 3: Comprehensive analysis of differential gene expression in *Aspergillus parasiticus* NRRL 2999 under varying pH conditions. (a) Volcano plot showing significantly upregulated and downregulated genes in samples f1, f2, and f3 compared to the f4 control. (b) MA-plot depicting the distribution of gene expression changes between control and pH-stressed samples, highlighting differentially expressed genes (DEGs). (c) Heat map illustrating expression patterns of significant genes across treatments f1 (pH 3.5), f2 (pH 4.5), f3 (pH 5.5), and control f4 (pH 6.5), with hierarchical clustering indicating distinct groups of upregulated and downregulated genes.

3.4 Functional Characterization of pH-Responsive Genes

Functional annotation of the significant DEGs revealed that pH-mediated transcriptional changes involved genes associated with diverse biological processes and catalytic functions (Fig. 4). Gene Ontology (GO) categorization and KEGG analysis of the DEGs (combined from all acidic vs. control contrasts) revealed enrichment of specific functions representation of biological process, cellular component and molecular function categories, with catalytic activity and binding functions dominating the molecular function assignments.

A considerable proportion of responsive genes were associated with hydrolase activity, oxidoreductase-related functions, and transport processes, reflecting activation of secondary

metabolic and stress pathways under acidic stress. The prevalence of catalytic and binding-related functions implied that low pH does not merely alter structural or housekeeping components but modulates active biochemical pathways. Particularly, functional categories associated with oxidative metabolism and secondary metabolic processes were enriched among induced genes, consistent with the established linkage between redox homeostasis and aflatoxin biosynthesis. Fig. 4 shows that many upregulated DEGs are annotated as catalytic enzymes (hydrolases, oxidases) and membrane transporters, whereas downregulated genes include hydrolases and regulatory proteins. KEGG pathways such as "oxidative phosphorylation", "amino acid metabolism", and "ABC transporters" were overrepresented. An appreciable fraction of genes remained annotated as hypothetical or unknown proteins, highlighting the possibility that previously uncharacterized pH-responsive genes contribute to toxin regulation. These unidentified genes may represent novel regulatory components warranting future investigation. Taken together, functional annotation suggests that ambient pH affects interconnected pathways involving metabolism, stress adaptation, transport and secondary metabolite biosynthesis. These enrichments suggest that acid stress upregulates pathways for toxin biosynthesis, redox balancing, and compound export[26,4].

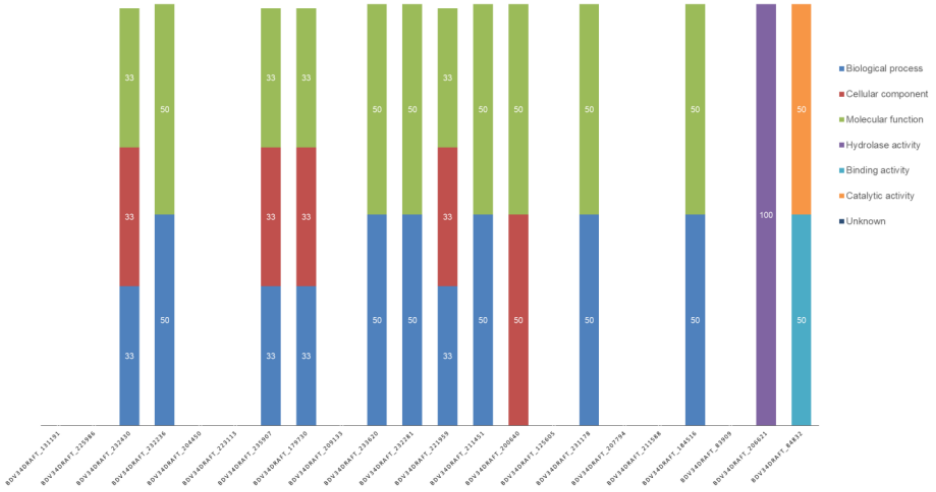

Fig. 4: Stacked bar chart showing the percentage distribution of functional annotations across different groups. Categories include biological process (blue), cellular component (orange), molecular function (gray), hydrolase activity (yellow), binding (light blue), catalytic activity (green), and unknown (dark blue). Each bar represents a group and sums to 100%, illustrating variation between general Gene Ontology classifications and more specific functional activities.

3.5 pH-Responsive Genes Associated with Aflatoxin Biosynthesis and Related Processes

Detailed examination of the 27 annotated candidate genes (Table 3) revealed substantial modulation of genes potentially linked directly or indirectly to aflatoxin biosynthesis. Among the strongly upregulated genes, BDV34DRAFT_232430, encoding a glyceraldehyde-3-phosphate dehydrogenase NAD(P)-binding domain-containing protein (*GapA*), exhibited the highest induction (log_2FC 8.30). Upregulation of this gene suggests increased carbon flux and reducing power generation under acidic conditions, potentially supporting enhanced precursor supply for secondary metabolism. Similarly, BDV34DRAFT_209133 (log_2FC 8.21) and BDV34DRAFT_211588 encoding a transcription factor domain-containing protein (log_2FC 7.78) were strongly induced, implying possible regulatory roles in coordinating stress-responsive and biosynthetic pathways.

Table 3. Functional annotation of selected genes associated with aflatoxin biosynthesis and related processes in *Aspergillus* species, including predicted proteins and functions.

GeneID	log2(FC)	p-adj	Putative Protein	ref_ID	Function	References
BDV34DRAFT_131191	3.822744556	3.76E-08	hypothetical protein	KAB8203513	-	-
BDV34DRAFT_225986	4.665989565	9.31E-05	hypothetical protein	KAB8204965	-	-
BDV34DRAFT_232430	8.302695827	9.31E-05	Glyceraldehyde 3-phosphate dehydrogenase NAD(P) binding domain-containing protein (GapA)	A0A5N6D4C8	In *Aspergillus flavus*, GapA is crucial for energy metabolism and is linked to the generation of NADPH for biosynthetic pathways, which can influence the production of aflatoxin B1.	[40]
BDV34DRAFT_232236	3.398702305	0.00020939	heme peroxidase	KAB8209450	Heme peroxidases, particularly those acting under oxidative stress conditions in *Aspergillus flavus*, increase aflatoxin production by modulating the biosynthesis, trafficking, and secretion of the toxin.	[41]
BDV34DRAFT_204450	6.256188282	0.00078631	DUF7703 domain-containing protein	A0A5N6D990	This protein is predicted to participate in regulatory or stress-associated processes.	[42]
BDV34DRAFT_223113	4.73767994	0.00097845	hypothetical protein	-	-	-
BDV34DRAFT_235907	-2.614786082	0.002065677	Major facilitator superfamily domain-containing protein	KAB8204076	The aflT gene encodes a Major Facilitator Superfamily (MFS) transporter located within the aflatoxin biosynthetic gene cluster of *Aspergillus parasiticus* and *Aspergillus flavus*. AFLT is a predicted 14-transmembrane domain transporter that resides between the polyketide synthase gene pksA and the P450-encoding gene cypA. It is part of the MFS, a large family of membrane transporters.	[43,44]
BDV34DRAFT_179730	-4.234778226	0.005358971	Major facilitator superfamily domain-containing protein	KAB8201067		
BDV34DRAFT_209133	8.211567266	0.00620392	hypothetical protein	KAB8210921	-	-
BDV34DRAFT_233620	-7.942200589	0.016087638	S-adenosyl-L-methionine-dependent methyltransferase	KAB8208046	SAM-dependent MTases (such as DmtA and OmtA) play a critical role in the late stages of the aflatoxin biosynthetic pathway. They catalyze the conversion of demethylsterigmatocystin (DMST) to sterigmatocystin (ST) and sterigmatocystin to O-methylsterigmatocystin (OMST), which are key intermediates in aflatoxin B1 and G1 formation.	[45]
BDV34DRAFT_232281	-7.194843685	0.020495471	Mycolic acid cyclopropane synthetase-domain-containing protein	KAB8209592	-	-
BDV34DRAFT_221959	-6.893615344	0.027467878	RNA cap guanine-N2 methyltransferase-domain-containing protein	KAB8209124	RNA cap guanine-N2 methyltransferase-domain-containing protein, identified as VepN (AFLA_006970), acts as a positive regulator of aflatoxin production, sclerotia formation, growth, and pathogenicity in *Aspergillus flavus*. This protein, which contains a Septin-type guanine nucleotide-binding (G) domain, is regulated by the global velvet regulator VeA.	[46]
BDV34DRAFT_211451	6.626863528	0.031558593	FAD-binding domain-containing protein	A0A5N6DRE9	FAD-binding domain-containing protein (A0A5N6DRE9) also known as AflX or OrdB that plays a critical role in the aflatoxin biosynthesis pathway, specifically in the conversion of versicolorin A (VA) to demethylsterigmatocystin.	[47]

BDV34DRAFT_200640	3.767843234	0.031558593	CFEM domain-containing protein	A0A5N6DC23	CFEM domain-containing protein is a member of the Common in Fungal Extracellular Membrane (CFEM) family in *Aspergillus flavus*. CFEM domain proteins are characterized by a unique eight-cysteine structural motif (PFAM ID: IPR008427) found in many fungi, often involved in pathogenicity, stress response, and cell wall stability. Oxidative stress plays a key role in regulating growth and aflatoxin production.	[48]
BDV34DRAFT_125605	3.903893355	0.03603234	hypothetical protein	KAB8202686	-	-
BDV34DRAFT_231178	-7.995633731	0.037240988	MAC/Perforin domain-containing protein	KAB8199481	The MAC/Perforin domain-containing protein is a pore-forming protein involved in fungal sporulation, morphogenesis, and environmental adaptation. Based on its functional domain, this protein acts as a membrane-attack complex/perforin (MACPF), a family of proteins that can disrupt host cell membranes.	[49]
BDV34DRAFT_207794	4.99855065	0.037240988	hypothetical protein	KAB8198951	-	-
BDV34DRAFT_211588	7.783107584	0.039203204	Transcription factor domain-containing protein	A0A5N6DR02	The protein with accession number A0A5N6DR02, likely from an *Aspergillus* species, is a transcription factor domain-containing protein. This class of transcription factors acts within the aflatoxin gene cluster and broader genome to control toxin production in response to environmental factors like pH, light, and nutrient availability.	[50]
BDV34DRAFT_184516	3.376466463	0.044846212	AhpD-like protein	KAB8211977	AhpD-like proteins and related peroxiredoxins in *Aspergillus* species, are crucial antioxidants that protect the fungus from oxidative stress, particularly during infection. Asp f3 is a 2-Cys peroxiredoxin that acts as a dimeric, thioredoxin-dependent peroxidase. Asp f3 functions as a scavenger of reactive oxygen species (ROS), decomposing hydroperoxides using cysteine residues (Cys31 and C61).	[51]
BDV34DRAFT_83909	2.281839416	0.044846212	C2 domain-containing protein	A0A5N6DM93	C2 domain-containing proteins serve as crucial regulatory links between environmental conditions and the metabolic pathways leading to aflatoxin contamination in crops	[52]
BDV34DRAFT_206621	-6.799342755	0.044846212	Carboxylic ester hydrolase	A0A5N6D300	Carboxylic ester hydrolase (corresponding to accession A0A5N6D300) catalyzes the conversion of versiconal hemiacetal acetate (often referenced as VHA or a related derivative) into versiconal (VAL).	[53]
BDV34DRAFT_84832	-6.606225582	0.045228455	Cytochrome P450	KAB8206309	The OrdA P450 is responsible for catalyzing multiple complex oxidative steps that modify the precursors O-methylsterigmatocystin (OMST) and 11-hydroxy-O-methylsterigmatocystin (HOMST) which further gets converted into aflatoxin B1 and G1.	[50,54]
BDV34DRAFT_184975	-7.428263139	0.046791678	Cytochrome P450	KAB8211630		
BDV34DRAFT_188594	-7.160750274	0.046791678	hypothetical protein	KAB8209563	-	-

BDV34DRAFT_46554	-3.431699264	0.046791678	Alpha/Beta hydrolase protein	KAB8208754	The protein KAB8208754 is a member of the alpha/beta-hydrolase superfamily, which in the context of *Aspergillus* aflatoxin biosynthesis, likely corresponds to the thioesterase (TE) or Claisen cyclase (CLC) domain associated with the polyketide synthase PksA. This alpha/beta-hydrolase domain is critical for the final stage of polyketide assembly, catalyzing the release of the polyketide chain and its subsequent cyclization to form the first stable precursor, norsolorinic acid anthrone (often associated with the formation of norsolorinic acid) in the aflatoxin pathway.	[55]
BDV34DRAFT_231555	-3.877115564	0.046791678	hypothetical protein	KAB8199085	-	-
BDV34DRAFT_207679	1.932101251	0.047967323	Fatty acid synthase subunit alpha	KAB8198991	Fatty acid synthase subunit alpha (encoded by aflA or fas-1A) is a specialized Type I FAS essential for aflatoxin biosynthesis in *Aspergillus* species. It partners with subunit beta (aflB) to provide a hexanoyl-CoA starter unit to polyketide synthase (PksA). This enables the synthesis of the initial precursor, norsolorinic acid (NOR).	[56]

Genes directly associated with aflatoxin biosynthesis also displayed marked differential regulation. The FAD-binding domain-containing protein BDV34DRAFT_211451 (putative *AflX/OrdB* homolog) was significantly upregulated (log_2FC 6.63), suggesting enhanced flux through oxidative conversion steps of the aflatoxin pathway. Likewise, BDV34DRAFT_207679, encoding a fatty acid synthase alpha subunit associated with starter unit formation for polyketide biosynthesis (*PksA/AflC*-related), was induced under acidic conditions, supporting activation of early biosynthetic events.

Oxidative stress-associated genes were prominently represented. Heme peroxidase (BDV34DRAFT_232236) a catalase-peroxidase, AhpD-like protein (BDV34DRAFT_184516) an alkyl hydroperoxide reductase AhpD-like protein, and CFEM-domain protein (BDV34DRAFT_200640) were upregulated, suggesting activation of antioxidant defense pathways. This observation is notable given the recognized interplay between oxidative stress signalling and aflatoxin biosynthesis. On the contrary, several genes implicated in later biosynthetic steps showed significant repression. These included: BDV34DRAFT_233620 as S-adenosyl-L-methionine-dependent methyltransferase (*OmtA*-like) (log_2FC −7.94), BDV34DRAFT_84832 (log_2FC −6.61) and BDV34DRAFT_184975 (log_2FC −7.43) as cytochrome P450 monooxygenase (*OrdA/AflQ*-like) and BDV34DRAFT_206621 as carboxylic ester hydrolase (*Nor-1* like) (log_2FC −6.79).

These patterns suggest that acidic pH does not uniformly activate the entire aflatoxin gene network but may selectively modulate different biosynthetic branches. Particularly notable was differential regulation of transporter-associated genes. Two major facilitator superfamily (MFS) domain proteins (one homologous to the cluster transporter *AflT*) (BDV34DRAFT_235907 and BDV34DRAFT_179730) were significantly downregulated, thus potentially facing difficulty in toxin export. Given their relationship to *aflT*-like transport functions, these responses may indicate altered metabolite trafficking or cluster-level feedback regulation under acid stress. Importantly, transcription factors VelB and SakA (HOG MAPK) did not change significantly, consistent with post-transcriptional control. However, expression of the pathway-specific activator *aflR* was elevated at low pH, suggesting its role in cluster induction.

Overall, these results reveal a complex transcriptional response involving simultaneous induction and repression of genes associated with precursor metabolism, oxidative processes, transport, and specific aflatoxin biosynthetic steps. In summary, acid pH induced a suite of genes that align with increased toxin biosynthesis, consistent with the observed high AFB_1/B_2.

3.6 Transcriptional Evidence for Metabolic Reprogramming Under Low pH

Beyond individual genes, the expression patterns collectively suggest substantial metabolic reorganization under acidic conditions. Upregulation of genes related to glycolytic flux (*GapA*), fatty acid synthesis, FAD-dependent oxidation, and redox homeostasis indicates that acidic pH may favor conditions supporting secondary metabolite precursor generation. This interpretation is reinforced by induction of regulatory proteins and stress-responsive factors, suggesting that pH acts not only as an environmental constraint but also as a signalling cue initiating broader transcriptional adaptation. The simultaneous repression of selected methyltransferases, cytochrome P450s and hydrolases further implies dynamic remodelling of pathway flux, possibly reflecting selective enhancement of specific aflatoxin intermediates or pathway balancing under stress. Importantly, several hypothetical proteins showed strong differential expression, including BDV34DRAFT_131191, BDV34DRAFT_225986, and BDV34DRAFT_125605, indicating the possible involvement of uncharacterized genes in pH-mediated regulation.

Given their magnitude of induction and statistical significance, these genes may represent novel candidate regulators linked to aflatoxin production.

3.7 Putative Regulatory Network Linking pH Signalling and Aflatoxin Biosynthesis

Integrated analysis of differential gene expression patterns, functional annotations, and clustering profiles suggested that ambient pH functions not merely as a growth parameter, but as a regulatory signal capable of modulating interconnected molecular networks associated with aflatoxin biosynthesis in *Aspergillus parasiticus* NRRL2999. The transcriptomic responses observed under acidic treatments, particularly at pH 3.5 and 4.5, support the existence of a coordinated pH-responsive regulatory network involving stress signalling, metabolic reprogramming, transport processes,

and modulation of aflatoxin-associated biosynthetic functions.

A principal feature emerging from the dataset was the strong induction of genes associated with redox homeostasis and oxidative stress adaptation under acidic conditions. Upregulation of the heme peroxidase-encoding gene (BDV34DRAFT_232236), the AhpD-like protein (BDV34DRAFT_184516), and the CFEM-domain-containing protein (BDV34DRAFT_200640) suggests activation of oxidative stress-responsive pathways in response to extracellular acidification. Given the recognized relationship between intracellular reactive oxygen species balance and aflatoxin biosynthesis, these expression patterns indicate that redox signalling may constitute a major upstream component of pH-mediated toxin regulation.

Simultaneously, several genes involved in precursor generation and metabolic flux showed marked induction, indicating that acidic pH may favour metabolic states conducive to secondary metabolism. The strong upregulation of *GapA* **(**BDV34DRAFT_232430) implies enhanced glycolytic activity and NAD(P)H generation, potentially supporting reductive biosynthetic reactions. Likewise, induction of the fatty acid synthase alpha subunit (BDV34DRAFT_207679) suggests increased availability of acetate-derived starter units required for polyketide assembly, including aflatoxin biosynthetic intermediates. These responses collectively support the hypothesis that pH-mediated regulation involves coupling of environmental sensing with metabolic channelling toward secondary metabolite biosynthesis.

Further evidence for coordinated regulatory control emerged from genes associated with transcriptional regulation and signalling. The pronounced induction of a transcription factor domain-containing gene (BDV34DRAFT_211588) and C2-domain-associated proteins suggests activation of regulatory nodes potentially involved in pH-responsive transcriptional remodelling. Although, classical pH signalling components such as the PacC/Rim pathway were not explicitly represented among the significant DEGs, the observed downstream expression signatures are consistent with modulation of broader ambient pH regulatory circuitry.

Several genes linked to aflatoxin biosynthesis or pathway-associated oxidation steps exhibited significant differential expression, further supporting direct transcriptional impacts on toxin-related pathways. Upregulation of the FAD-binding domain-containing gene (BDV34DRAFT_211451, putative *aflX/ordB*-like homolog) implies stimulation of oxidative conversion steps within the aflatoxin pathway. In contrast, repression of cytochrome P450 genes (BDV34DRAFT_84832 and BDV34DRAFT_184975) and a methyltransferase gene (BDV34DRAFT_233620) suggests selective pathway-level modulation rather than uniform induction of the entire biosynthetic cluster.

This differential regulation is particularly noteworthy, as it indicates that acidic pH may influence aflatoxin biosynthesis through dynamic redistribution of pathway flux rather than simple cluster-wide activation. Such responses may reflect fine-tuning of intermediate conversion steps, precursor partitioning, or feedback regulation under stress conditions.

Transport-associated genes also appeared to be integrated into this regulatory network. Significant repression of major facilitator superfamily (MFS) transporter genes (BDV34DRAFT_235907 and BDV34DRAFT_179730) suggests that ambient pH may affect metabolite transport processes, potentially influencing intracellular trafficking of biosynthetic intermediates or toxin export mechanisms. Given the documented association of transporter systems with fungal secondary metabolite clusters, these findings imply that pH-responsive regulation extends beyond biosynthesis into transport-linked control mechanisms.

Taken together, these observations support a putative model in which ambient pH triggers a multilayered regulatory cascade involving: (1) pH perception and stress signalling activation: Acidic extracellular conditions initiate stress-responsive signalling pathways, likely involving conserved ambient pH sensing mechanisms and oxidative signalling components. (2) Redox and metabolic reprogramming: Activation of antioxidant enzymes, glycolytic flux genes, and precursor-generating pathways establishes a metabolic environment favourable for secondary metabolism. (3) Selective modulation of aflatoxin-associated genes: Key biosynthetic enzymes, oxidative tailoring genes, and pathway-related regulators undergo differential expression, reshaping aflatoxin biosynthetic flux. (4) Transport and pathway coordination: Changes in transporter-associated genes suggest coordinated regulation of metabolite trafficking alongside biosynthetic gene responses. (5)

Integration through broader transcriptional regulatory networks: Induced transcription factor-associated genes and several highly responsive hypothetical proteins may represent additional regulatory nodes linking environmental pH with toxin biosynthesis.

Based on these transcriptomic signatures, acidic pH appears to act as an ecological signal that integrates stress adaptation and secondary metabolic regulation through a coordinated regulatory network rather than through isolated gene-level responses. The strong responses observed at pH 3.5 and 4.5 particularly suggest that lower pH conditions intensify this regulatory circuitry, potentially shifting the balance of metabolism toward adaptive toxin-associated responses.

Moreover, the presence of several strongly induced hypothetical proteins lacking functional annotation raises the possibility that previously uncharacterized components contribute to pH-dependent aflatoxin regulation. These genes may represent novel regulators within this network and constitute promising targets for future functional validation.

Overall, the data support a putative regulatory framework in which ambient pH modulates aflatoxin biosynthesis through interconnected pH signalling, oxidative stress responses, metabolic rewiring, and selective pathway regulation, highlighting the complexity of environmental control over secondary metabolism in *A. parasiticus*.

4. Discussion

The present study demonstrates that ambient pH functions as a critical environmental regulator of aflatoxin biosynthesis in *Aspergillus parasiticus* NRRL2999 and that this regulation is mediated through coordinated transcriptional reprogramming involving stress signalling, metabolic adaptation, transport processes, and selective modulation of biosynthetic genes. The observed phenotypic pattern, characterized by elevated AFB_1 and AFB_2 accumulation under strongly acidic conditions (pH 3.5–4.5) and marked suppression of toxin production at pH 5.5–6.5, is consistent with classical and emerging reports indicating that acidic environments favour aflatoxin biosynthesis in aflatoxigenic aspergilli[27,3]. Importantly, the present results extend these observations by linking this pH-dependent phenotype with transcriptome-wide responses that suggest pH acts not merely as a permissive growth parameter, but as a signalling cue that restructures regulatory networks governing secondary metabolism.

The clear segregation of transcriptomic profiles observed in PCA, particularly under pH 3.5 and 4.5, indicates that extracellular acidification induced substantial global transcriptional remodelling. The strong treatment-dependent clustering supports the conclusion that ambient pH is a major determinant of gene expression variability in *A. parasiticus*. Comparable transcriptome-scale shifts have been reported in aflatoxigenic fungi under oxidative, temperature, and nutritional stress, where environmental signals trigger coordinated changes in signalling, metabolism, and toxin biosynthesis rather than isolated gene-specific responses[28,29]. The greater divergence observed at lower pH is also consistent with studies implicating the *PacC/Rim* ambient pH signalling system as a major regulator of fungal physiological adaptation and secondary metabolism[30-32].

Although known pH regulatory genes were not detected among the significantly expressed genes in the present dataset, the downstream transcriptional signatures strongly suggest involvement of *PacC*-mediated regulatory circuitry[33]. Under acidic conditions, *PacC* likely remains in its inactive unprocessed form, thereby relieving repression of acid-expressed genes, potentially including aflatoxin pathway regulators, whereas at near-neutral pH, processed *PacC* may contribute to repression of the aflatoxin gene cluster[34]. This interpretation is further supported by the observed induction of aflatoxin-associated genes under acidic conditions and their suppression under the control pH, suggesting that ambient pH may act through a regulatory switch governing secondary metabolic competence.

A notable feature of the present transcriptomic dataset is that only a relatively small but highly significant set of 22 DEGs was identified. Rather than indicating a limited response, this likely reflects selective regulatory rewiring, where a relatively small number of responsive genes exert disproportionate control over phenotype. Increasing evidence suggests that environmental signalling often acts through focused regulatory nodes capable of generating substantial downstream effects[29], and the present findings appear to support this model.

Volcano plot, MA plot, and hierarchical clustering analyses further revealed that acidic pH induced highly selective transcriptional responses involving both activation and repression of genes linked to metabolism and aflatoxin-related processes. The predominance of upregulated genes under acidic conditions

suggests induction of adaptive pathways rather than generalized stress suppression. Similar asymmetric transcriptional responses have been observed in *Aspergillus flavus* under oxidative and temperature stress, where pathway-specific activation accompanies repression of other processes, resulting in network-level reorganization[28,29].

Importantly, the graded responses observed across pH treatments indicate that pH-dependent regulation operates along a continuum rather than through binary switching. This is biologically plausible, as ambient pH serves as a dynamic ecological signal capable of influencing developmental programs, nutrient sensing, and resource allocation. The coexistence of both induced and repressed aflatoxin-associated genes in this study further suggests that pH modulates pathway flux through selective control of biosynthetic nodes rather than through uniform induction of the entire aflatoxin cluster. Similar pathway-level modulation has been proposed as a common regulatory strategy in fungal secondary metabolism[32].

One of the most significant findings was the induction of multiple oxidative stress-associated genes under acidic conditions, including heme peroxidase, AhpD-like proteins, and CFEM-domain proteins, suggesting that redox signalling is a central component of the pH response. The relationship between oxidative stress and aflatoxin biosynthesis has been extensively documented. Reactive oxygen species are now recognized not only as stress by-products but also as signalling molecules that influence aflatoxin pathway activation[35,28]. Moreover, aflatoxin biosynthesis itself has been proposed to function in oxidative stress alleviation, indicating a bidirectional relationship between toxin production and redox homeostasis[36,37].

The present data strongly support this framework. Upregulation of oxidative stress-responsive genes under acidic pH may reflect activation of a signalling environment favourable for aflatoxin pathway induction rather than merely damage mitigation. Acidic conditions may promote ROS accumulation or activate stress-responsive kinase pathways such as *Hog1*/*SakA*, which could further stimulate redox-sensitive transcriptional regulators linked to aflatoxin biosynthesis, as proposed previously[35]. This convergence of pH signalling and oxidative stress responses likely represents a major mechanism underlying the enhanced toxin production observed under acidic conditions.

Another notable feature of the transcriptome was the strong induction of genes associated with central metabolism, particularly *GapA* and fatty acid synthase-related genes. These responses suggest that acidic pH may promote metabolic states favourable for secondary metabolism by enhancing precursor availability and reducing power generation. Such an interpretation is consistent with previous evidence linking glycolytic flux, acetyl-CoA supply, and NADPH-dependent metabolism to aflatoxin biosynthesis[28,38]. Given the high energetic demand of aflatoxin production, induction of carbon metabolism and lipid-associated pathways may reflect metabolic channelling toward secondary metabolite biosynthesis under acid stress.

The enrichment of genes linked to carbon and amino acid metabolism also suggests broader metabolic reprogramming under low pH, potentially shifting flux toward malonyl-CoA-derived polyketide biosynthesis. Simultaneous repression of selected hydrolases and putative transcriptional repressors may further indicate relief from catabolite repression, thereby favouring secondary metabolism, as proposed previously[39].

The differential regulation of FAD-dependent oxidoreductases, cytochrome P450s, methyltransferases, and transporter-associated genes further indicates that ambient pH influences aflatoxin-associated pathways at multiple regulatory levels. Particularly noteworthy was induction of a putative *aflX/ordB*-like gene alongside repression of specific cytochrome P450 and methyltransferase genes. This mixed expression pattern suggests dynamic redistribution of pathway flux rather than simple uniform activation of the entire biosynthetic pathway. Similar selective modulation of biosynthetic branches and tailoring reactions has been reported under other environmental stresses affecting aflatoxin production[33,29].

The observed induction of transporter-associated genes, including MFS-domain proteins with putative *AflT*-like functions, further suggests that metabolite export or intracellular trafficking forms part of the acid stress response. Increasing evidence indicates that transport systems are integral components of fungal secondary metabolite clusters and can influence toxin secretion, compartmentalization, and pathway coordination[26]. Their induction here implies that pH-mediated regulation extends beyond

biosynthetic enzyme expression into transport-linked coordination.

The present findings also suggest involvement of broader regulatory systems beyond pH signalling levels and through integration of environmental cues such as light and nutrient status[6]alone. Although, major changes in the velvet complex genes (*VeA, VelB, LaeA*) were not evident, this does not exclude their participation, as velvet-mediated regulation is often governed at post-translational[6]. Nonetheless, the present data suggest that pH signalling through *PacC*-like pathways may represent a dominant regulatory input under these experimental conditions.

Taken together, the results support a model in which acidic pH acts upstream of interconnected regulatory modules involving ambient pH sensing, oxidative stress signalling, metabolic reprogramming, transport coordination, and selective modulation of aflatoxin-associated genes. In this framework, low pH relieves *PacC*-mediated repression, activates oxidative and metabolic adaptation pathways, promotes precursor generation, modulates biosynthetic and transporter genes, and ultimately reshapes aflatoxin pathway activity. Such a model is consistent with emerging views that fungal secondary metabolism is embedded within broader adaptive regulatory networks rather than controlled as an isolated biosynthetic module[37,29].

An additional notable feature of this study is the induction of several strongly responsive hypothetical proteins lacking functional annotation. These genes may represent previously uncharacterized components of pH-responsive toxin regulation and offer promising targets for future functional characterization. Likewise, experimental validation through *PacC* disruption, *aflR* expression studies, and gene-specific functional assays will be necessary to further resolve the mechanistic basis of the proposed model.

From an ecological and food safety perspective, these findings have significant implications (). Environmental pH can fluctuate substantially during crop colonization, fermentation, storage, and such changes may directly influence transcriptional networks associated with aflatoxin risk. Understanding how acidic conditions activate signalling pathways linked to toxin biosynthesis may therefore aid in developing intervention strategies aimed at disrupting environmental cues that favour contamination.

Overall, this study supports the emerging concept that ambient pH functions as an ecological signal integrating stress responses, metabolic adaptation, and secondary metabolism in *A. parasiticus*. Rather than acting solely as a growth condition, pH appears to operate as a central regulator of transcriptional networks that shape aflatoxin biosynthesis and fungal adaptive responses.

5. Conclusion

This study demonstrates that ambient pH is a critical environmental determinant governing aflatoxin biosynthesis and associated transcriptional regulation in Aspergillus parasiticus NRRL2999. Acidic conditions, particularly pH 3.5 and 4.5, significantly promoted AFB_1 and AFB_2 production, whereas toxin biosynthesis was markedly suppressed at higher pH levels, confirming a strong pH dependence of aflatoxin production. RNA-seq analysis revealed that these phenotypic responses were accompanied by distinct transcriptomic reprogramming, including differential expression of genes associated with oxidative stress adaptation, precursor metabolism, transport processes, and aflatoxin-related biosynthetic functions. The results support a model in which acidic pH acts not merely as a growth condition but as a signalling cue that reshapes regulatory networks linked to secondary metabolism. Induction of oxidative stress-responsive genes, metabolic pathway components, and candidate aflatoxin-associated genes suggests that pH-mediated toxin regulation involves coordinated integration of redox signalling, carbon flux modulation, and selective biosynthetic pathway control. The likely involvement of PacC-mediated ambient pH signalling, together with contributions from broader regulatory networks, provides mechanistic insight into how environmental acidity influences toxin production. Importantly, the identification of several strongly responsive hypothetical genes further suggests the existence of previously uncharacterized regulators involved in pH-responsive aflatoxin biosynthesis. Collectively, this study provides transcriptome-wide evidence that ambient pH functions as a major ecological regulator of aflatoxin production and offers new insights into molecular mechanisms underlying environmental control of fungal secondary metabolism. These findings may contribute to the development of targeted strategies for mitigating aflatoxin contamination through manipulation of environmental conditions or disruption of pH-responsive regulatory pathways.

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Conflict of interest

The authors declare no conflict of interest.

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