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Comparative metabolomics charts distinct chemical repertoires across phytopathogenic fungi

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Abstract

Introduction: Numerous fungal species have caused harm to non-cereal crops resulting in reduced crop yield and economic losses. Although different methods are applied to limit damage caused by fungal diseases in non-cereal plants, there are still knowledge gaps which hamper the formulation of sustainable strategies to eradicate these fungal infections and their downstream consequences. These limitations include lack of understanding of molecular mechanisms that govern fungal developmental stages and lifestyles, and the chemical or molecular lexicon that drives fungal pathogen-host interactions.

Objectives: Hence, the study examines the metabolic signatures of five non-cereal infecting fungal species: *Sclerotinia sclerotiorum, Cladosporium cucumerinum, Colletotrichum higginsianum*, *Phytophthora cinnamomi*, and *Phytophthora capsici* by employing computational metabolomics approaches involving the use of AI-driven methods.

Methods: Non-cereal pathogenic fungal isolates were cultured and sampled at three growth phases (4, 8, and 12 days post-inoculation, d.p.i.) to elucidate temporal chamges in the metabolic activities of non-cereal fungi species. Intracellular metabolites were extracted and analyzed on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system and the resulting metabolomics data was used for data analysis and biological interpretation.

Results: Principal component analysis (PCA) revealed species-specific and time-dependent sample clustering patterns, showing dynamic metabolic profiles. Spectral data annotation, supported by tools like microbeMASST and feature-based molecular networking (FBMN), allowed the detection of over 130 metabolites of various chemical classes, such as polyketides, alkaloids, nucleotides, and fatty acids. Key metabolites annotated were several anthraquinones which included sulochrin, fallacinol, and endocrocin in *P. cinnamomi*, indicating specialized virulence and adaptation mechanisms of the pathogen. Additionally, differential pathways were regulated across species and time points such as purine metabolism, riboflavin metabolism, and nucleotide sugar metabolism among other processes demonstrating the role of metabolic flexibility in lifestyle transitions.

Conclusion: These findings shed light on novel biochemical approaches of non-cereal infecting fungi isolates and revealed fungal metabolic complexity. As such, this study proposes a metabolomic blueprint for creating novel strategies to protect non-cereal crops against phytopathogenic fungi. These actionable insights contribute towards the realization of various sustainable development goals such as SDG 2 (zero hunger) and SDG 3 (good health).

1. Introduction

Plant pathogenic fungi constitute among the most important microorganisms that cause diseases in plants which comprise diverse fungal species with distinct infection mechanisms and lifestyles which have resulted in substantial economic losses and a threat to global food security. Farmers are therefore

challenged to create favorable growing environments along with protecting plants from fungal pathogens to produce high-quality crops with optimal yields. Hence, the extensive application of herbicides, insecticides and fungicides that has led to increased resistance among target microbial species (Hahn, 2014). Furthermore, abuse and misuse of chemical treatments have led to degradation of the environment and has raised concerns about their sustainability in agriculture. The emergence of fungicide-resistant fungal isolates has made the control of plant diseases even more challenging, resulting in large economic losses for farmers worldwide (Samal et al., 2024; Corkley et al., 2022). Therefore, understanding the biochemical reactions and molecular mechanisms governing the growth and lifestyle of plant-infecting fungi is crucial for developing effective methods for crop disease management.

Non-cereal crops' global consumption has significantly increased over the past 30 years, largely due to their vital function in supplying health-promoting dietary components such as vitamins, minerals, antioxidants and fiber (Kłapeć et al., 2021). However, non-ceral crops are highly susceptible to spoilage by a variety of microorganisms, including bacterial and fungal species (Tournas, 2005; Al-Abdalall, 2010; El-Mougy et al., 2011). Various fungal pathogens have been noted as major contributors to diseases affecting fruits, vegetables, and legumes. These microorganisms include species from *Phytophthora, Sclerotinia, Colletotrichum, Cladosporium, Alternaria*, and *Glomerella* genera (Kroon et al., 2012; Saharan and Mehta, 2008; Yan et al., 2018; Birker et al., 2009; Jiang et al., 2021; Pinto and Patriarca, 2016; Wang et al., 2015). Moreover, these fungi not only contribute to large agricultural losses but also pose risk to safety and quality of food products. Despite the agricultural disruption effects of many non-cereal infecting fungal species, their growth dynamics and survival strategies remain poorly characterized. Elucidating the metabolic profiles of these pathogens is crucial for understanding their biochemical processes, survival mechanisms, and interactions with host plants. Such actionable insight is essential for expanding the development of crop protection strategies and practical methods of fungal disease management.

Metabolomics, a quantitative and qualitative assessment of all detectable metabolites within a biological system, provides crucial insights into the biological system under investigation (Tugizimana et al., 2013). Metabolomics is, however, significantly hindered by several challenges that include confident metabolite identification and characterization of plant pathogenic fungi. These bottlenecks hinder the comprehensive exploration of microbial-derived metabolites, many of which hold potential for innovative medical and agricultural applications (Chaleckis et al., 2019; Novoa-del-Toro et al., 2024). The emergence and the use of advanced computational tools such as mass spectral mining and molecular networking strategies that include feature-based molecular networking (FBMN) and MicrobeMASST tools within the Global Natural Products Social Molecular Networking (GNPS) platform, show great potential to decode the fungal metabolic strategies explored in this study (Zuffa et al., 2024; Zuffa et al., 2023; Phelan, 2020). The increasing popularity of these computational tools in untargeted metabolomics is based on their ability to enhance metabolite annotations, simplify data visualization, and support collaborative annotations across the scientific community (Perez De Souza et al., 2020; Aron et al., 2020). For instance, molecular networking provides the visualization of non-targeted tandem mass

spectrometry (MS^2) data by constructing a molecular network which involves the clustering of experimentally identical spectra into a single node (*i.e*., a single metabolite) and grouping structurally similar spectra (*i.e*., nodes or metabolites) into molecular families that collectively form a molecular network for visualization and organization of large-scale mass spectral datasets (Watrous et al., 2012; Aron et al., 2020). This network approach not only facilitates the visualization and interpretation of complex metabolomics data but also can support the putative identification of unknown metabolites by their similarities with known annotations.

Furthermore, molecular networking (MN) offers a powerful method for deciphering the "dark metabolome"- which is a vast array of uncharacterized spectral features. Emerging tools such as MicrobeMASST have improved the characterization of metabolomic features from microbial monocultures by searching against libraries with microbial mass spectra datasets and facilitating in identifying the metabolite origin (Zuffa et al., 2024; Zuffa et al., 2023). Thus, this study presents a computational metabolomics approach for exploring and applying MN strategies to examine the metabolome and chemical diversity of non-cereal-infecting fungal species, particularly *Cladosporium cucumerinum, Colletotrichum higginsianum, Phytophthora capsici, Phytophthora cinnamomi, Sclerotinia sclerotiorum*. Additionally, this study seeks to discover key metabolic signatures that differentiate the different growth phases of non-cereal-infecting fungi, offering beneficial insights into time-specific adaptations and possible targets for disease control strategies. Overall, the study reports a metabolomics work to profile the metabolomic landscape of fungal isolates that are pathogenic to non-cereal crops.

2. Materials and methods

All chemicals for sample analyses (from the pre-analytical step to the data acquisition stage) were of pure-grade quality and obtained from various international suppliers. Briefly, the organic solvents used, methanol and acetonitrile, were LC-MS grade quality (Romil, SPS, Cambridge, UK). Water was purified by Milli-Q Gradient A10 system (Siemens, Fahrenburg, Germany).

2.1. Fungal culture, metabolite extraction, sample analysis and multivariate data exploration

Five fungal isolates *C. cucumerinum, S. sclerotiorum, C. higginsianum, P. cinnamomi, P. capsici* were collected from Omnia Group Ltd, South Africa and from the National Fungi culture collection of Agricultural Research Council (ARC), South Africa. The isolates were maintained and cultured on half-strength Potato Dextrose Agar (PDA) solid media on petri dishes which were sealed with parafilm and incubated in a dark room at 25°C (most fungi thrive in a temperature range of approximately 20°C to 30°C) for 12 days and a growth of the fungal lawn was observed over time. Agar plates were prepared under sterile conditions, and the PDA is an effective media for cultivating fungal isolates serving as a readily available source of carbohydrate required for the growth of fungi. The colonies of the isolates were re-isolated on pure sterile liquid medium potato dextrose broth (PDB). One hundred mL (100 mL)

Erlenmeyer flasks containing 50 mL of the PDB were inoculated with 3 mycelial plugs of the pathogenic isolates *C. cucumerinum, S. sclerotiorum, C. higginsianum, P. cinnamomi, P. capsici* from the pre-cultures grown on solid half-strength PDA media. Each mycelial plug was about 2 mm in diameter and taken from a 12-day old culture plates. The flasks were incubated at 25°C on a rotary shaker at 120 rpm cycle. The flasks of the different pathogen isolates were harvested at 4, 8, 12 days post inoculation (d.p.i), *i.e.*, time points derived from the growth curve (reported in section 3). Furthermore, to account for biological variability, the experiment was conducted three times. Non-inoculated liquid media were also incubated and harvested under the same conditions at the same time points to account for any changes that may occur in the media over time.

2.2. Metabolomics analysis: microbial metabolite extraction

A methanol-based extraction protocol was used to extract metabolites from the biological sample due to the efficiency of methanol, a versatile solvent that can extract a broad range of metabolites including polar, semi-polar and non-polar compounds (Sellick et al., 2011; Bi et al., 2013). The mycelia from each flask were harvested at 4, 8, and 12 d.p.i filtered through a double layer of Miracloth under a vacuum to separate the fungal cells from the liquid media. The wet mass of the mycelia was weighed and recorded using the ratio 1:10 (w/v), and 100% methanol (LC-MS grade) was used to extract metabolites from fungal cells.

The weighed cells, resuspened in the extraction solvent (100% methanol), were placed in Eppendorf tubes and immediately homogenized using Ultra Turrax homogenizer for 2 minutes and sonicated with a probe sonicator (Bandelin Sonopuls, Germany) which was set at 55% for a minute cycle. The whole extraction process was repeated twice to ensure efficient and thorough recovery of metabolites from the fungal cells. Methanol served also as a quenching agent by denaturing proteins and stopping the enzymatic activities (Dietmair et al., 2010; de Jonge et al., 2012; Tugizimana et al., 2013). The homogenized samples were centrifuged at 5000 ×g for 15 minutes at 4°C. The supernatants were evaporated to about 1 mL using a Rotavapor R-200 in a 50 mL round-bottom flask, and the 1 mL was evaporated to completeness on a dry block heater at 45°C. The dried fungal samples (metabolites) were reconstructed in 300 µL of 50% aqueous methanol and filtered, using 0.22 µm nylon filters, into pre-labeled HPLC glass vials fitted with 500 µL inserts (Shimadzu, South Africa). Un-inoculated PDB media was used as control samples.

2.3. UHPLC analysis: data acquisition

The fungal metabolite extracts were analyzed using a liquid chromatography–quadrupole time-of-flight tandem MS instrument (LCMS-9030 qTOF, Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was performed on a Shim-pack Velox C18 column (100 mm × 2.1 mm with particle size of 2.7 µm) (Shimadzu Corporation, Kyoto, Japan) at 55°C. For all fungal samples, an injection volume of 3 µL was used and run using a binary mobile phase gradient which consisted of solvent A: 0.1% formic acid in Milli-Q water and solvent B: Methanol with 0.1% formic acid. The flow rate was set to 0.3 mL/min

throughout the set 15 minutes gradient with the following separation conditions: 10% B maintained for 2 min, the gradient was increased from 10–60% B over 2–5 min, then the gradient was changed to 90% B between 5–8 min and maintained at 90% B for 3 min. The gradient was returned to initial conditions between 11–13 min which was followed by a 2-min column re-equilibration time.

The chromatographic eluents were further analyzed using the qTOF high-definition mass spectrometer which involves the identification and quantification of metabolites based on their mass-to-charge ratio (*m/z*) and was set to acquire negative electrospray ionization data. The subsequent parameters were set as: interface voltage of 4.0 kV, interface temperature of 300°C, nebulization and dry gas flow 3 L/min, heat block temperature of 400°C, DL temperature of 280°C, detector voltage of 1.8 kV and the flight tube temperature at 42°C. Sodium iodide (NaI) was used as a calibration solution to monitor high mass accuracy. MS^1 and MS^2 (through data dependent acquisition, DDA) were generated simultaneously for all ions with an *m/z* scan range between 100–1000 surpassing an intensity threshold of 1000 counts. Fragmentation was performed using argon as the collision gas at a collision energy of 30 eV with an energy spread of ± 5 eV and the data was acquired in centroid mode. To ensure analytical stability and accuracy, quality control (QC), *i.e.*, pooled samples, were used to condition the chromatography and mass spectrometry (LC-MS system) in monitoring the performance of the analytical instrument, detecting any changes or drifts in the instrument response. QC samples were injected at the start and end of each batch, as well as every 10 injections.

2.4. Data preprocessing, matrix creation and statistical analysis

The generated electrospray ionization (ESI)-negative mass spectral datasets (raw files) from Shimadzu LCMS-9030 qTOF were converted to mzML, an open-source file format that is machine-readable. The mzML files were uploaded onto the Mass Spectrometry-Data Independent Analysis (MS-DIAL) platform for data-processing and mass spectral deconvolution. The data processing parameters used were as follows: analysis retention time (rt) range of 0–15 min, mass (*m/z*) range of 100–1000 Da, mass accuracy (MS^1 and MS^2 tolerance) was set at 0.05 Da, the minimum peak height was set at 1000 amplitude, mass slice width set at 0.1 Da for peak detection, a sigma window value set at 0.5 and retention time tolerance set at 0.05 min. Following data processing, the resultant feature matrix files, *i.e.*, GnpsMgf and GnpsTable (feature quantification table), were then uploaded into the Global Natural Products Social (GNPS2; https://gnps2.org/homepage) environment for molecular networking. Furthermore, the feature matrices were also exported into MetaboAnalyst (MetaboAnalyst), a web-based platform for chemometrics analysis, visualization, and interpretation. An unsupervised method, Principle Component Analysis (PCA), was employed to reduce the dimensionality of the datasets, thereby enabling the visualization of any trends, similarities, and differences within the datasets (Cook and Rutan, 2014). Statistical differences between different fungal species and growth phases were assessed using one-way analysis of variance.

2.5. Metabolite annotation using molecular networking, pathway analysis and biological interpretation

Molecular networking analysis (feature-based molecular network, FBMN) was performed by uploading the spectral data (*i.e.*, mgf files) to the GNPS2 platform and applying consistent parameters across all computed networks for *C. cucumerinum, S. sclerotiorum, C. higginsianum, P. cinnamomi*, and *P. capsici* spectral data. The precursor and fragment ion mass tolerance for MS-Cluster and library searching were set to 0.05 Da. Edges (interconnections between metabolites/similarity) were formed only if the minimum cosine score of 0.65 was exceeded with more than 4 matched peaks, and the other molecular networking parameters were kept as the default values. Experimental spectra (in the network) were searched across spectral databases (libraries) such as MassBank, PubChem, GNPS library, ReSpect, METLIN, and NIST for metabolite annotation. The resulting molecular networks were visualized using Cytoscape software (version 3.8.2), as described in previous studies (Wang et al., 2017; Ramabulana et al., 2021; Aron et al., 2020; Shannon et al., 2003; Smoot et al., 2011).

In addition to GNPS ecosystem, various emerging platforms and tools were employed to validate and enhance the metabolite annotations. These include (i) SIRIUS (Sum formula Identification by Ranking Isotope patterns Using mass Spectrometry), a software tool that utilizes mass spectrometry data to identify metabolites (Dührkop et al., 2019) and (ii) microbeMASST, an AI-driven platform that primarily focuses on analyzing and annotating microbial-derived metabolites using mass spectrometry (Gaudêncio et al., 2023; Gomes et al., 2023; Zuffa et al., 2024). All validated metabolites and GNPS job links are listed in in the supplementary materials.

Metabolites were putatively annotated at level 2 of the Metabolomics Standards Initiative (MSI) (Sumner et al., 2007; Godzien et al., 2018). Level 2 is applied to compounds that are putatively annotated based on their physicochemical characteristics and their similarity to known compounds in commercial or public spectral libraries in terms of MS/MS or MS^n spectral similarities. The metabolites annotated from non-cereal fungi were used for metabolic pathway analysis, as shown in **section 3.4**. This functional analysis was performed using the Metabolomics Pathway Analysis (MetPA) tool. MetPA can discover the affected biological pathways and provides further insights on the biochemistry underlying growth, pathogenicity, or stress tolerance. The high-quality KEGG metabolic pathways serve as the foundation for MetPA's knowledge and with this approach allowing a more detailed mapping of the molecular landscape of the metabolism under study (Tugizimana et al., 2019).

3. Results and Discussion

3.1. Fungal growth on different growth media

Non-cereal infecting fungal isolates were cultured on both solid potato dextrose agar (PDA) and liquid potato dextrose broth (PDB), which functioned as nutrient–rich media offering an optimal *in vitro* environment for fungal proliferation, to examine the growth of the non-cereal infecting fungi (Westphal et

al., 2021; Griffith et al., 2007). Fungal growth was assessed by tracking glucose consumption and measuring biomass accumulation overtime. The first six days post-inoculation in the non-cereal infecting fungal species, showed a substantial increase in fungal biomass and a simultaneous decrease in glucose concentration in the liquid media (**Figure S1** and **S2** (Barboráková et al., 2012; Tugizimana et al., 2019). This suggests both active fungal growth and active carbon metabolism. However, the biomass decreased significantly by day 12 due to nutrient depletion, metabolic shifts, or secondary metabolite production that affects fungal growth (Calvo et al., 2002; Vrabl et al., 2019). The observed fungal proliferation in potato dextrose media not only validates rapid fungal growth but also confirms that plant pathogenic fungi can thrive on carbon-rich environment. The pattern of glucose consumption across all pathogens further reflected the role of carbohydrate metabolism in fungal development and emphasized the importance of nutrient availability in pathogen establishment (Fleck et al., 2011; Ene et al., 2014).

3.2. Mass spectral molecular networking for unraveling the chemical landscape of non-cereal Infecting Fungi

Principle component analysis (PCA) was employed to reduce the dimensionality of the metabolomic fungal datasets, thereby enabling the visualization of any trends, similarities, and differences within the metabolomics datasets. The unsupervised PCA score plot of fungal samples collected at 4 days post-inoculation (d.p.i) showed distinct clustering in metabolite profiles of the five non-cereal infecting fungal species (Fig. 1A). This indicates species-specific metabolic differences of *Sclerotinia sclerotiorum* (Ss), *Phytophthora capsici* (Pca), *Colletotrichum higginsianum* (Ch), *Phytophthora cinnamomi* (Pci), and *Cladosporium cucumerinum* (Cc). The model was robust with statistically significant quality ($R^2 > 0.90$; p-value of 0.001) highlighting reliable grouping between the different developmental stages. To further investigate metabolic changes during fungal growth, the PCA of samples collected at 4, 8, and 12 d.p.i. The results showed clear temporal grouping of fungal samples corresponding to the lag, exponential and stationary growth phases, which demonstrates progressive metabolic reprogramming during fungal growth (**Figure S4**). Species such as *Sclerotinia sclerotiorum* and *Colletotrichum higginsianum* showed clear growth stage specific clustering highlighting substantial shifts in metabolite composition between the different growth stages. These results therefore, demonstrate how fungal developmental stages are a major source of metabolic variation in the metabolomics datasets and how each fungal species have distinct temporal pathway overtime. The fungal metabolic profiles of the pathogens were then annotated to identify the metabolites contributing to the observed temporal changes overtime and species-specific metabolic patterns.

Feature-based molecular networking (FBMN) was used to visualize and annotate the spectral data of the 5 fungal isolates (**Figure S3**). The molecular networks for of *S. sclerotiorum, C. cucumerinum, C. higginsianum, P. capsici, and P. cinnamomi*, consisted of 4 353, 6 464, 6 474, 5 402, and 5 202 molecular features/metabolites (*i.e.*, nodes or MS/MS spectra), respectively. Additionally, FBMN enabled the annotation of 65, 67, 80, 70, and 72 compounds in the networks of *S. sclerotiorum, P. capsici, P. cinnamomi, C. cucumerinum, and C. higginsianum*, respectively. These annotations were achieved through spectral library matching against the GNPS spectral databases and they revealed a diverse array of

secondary metabolites belonging to the chemical superclass's of benzenoids and substituted derivatives, lipids and lipid-like molecules, organic oxygen compounds, nucleosides, nucleotides and analogues, organoheterocyclic compounds, organic acids and derivatives, phenylpropanoids and polyketides (Fig. 1B; **Table S1** and **Figure S3**). These annotations provide valuable insights into the biochemical processes of these fungal species. For instance, notable differences in metabolite class composition were observed between the fungal species, with *C. cucumerinum* rich in lipids and lipid-like molecules as well as organics; *C. higginsianum* rich in nucleosides and their derivatives; *P. capsici* rich in phenylpropanoids; *P. cinnamomi* rich in benzenoids; and *S. sclerotiorum* rich in organoheterocyclic compounds (**Figure S3**).

Recent studies have described several key roles of these chemical classes in fungal pathogens. Lipids for example, not only function in fungal cell membrane structure, signal transduction, differentiation, migration, and maintenance of the cellular integrity, but also play significant roles in stress responses, regulating developmental processes such as cell proliferation, spore differentiation and pathogenicity (Li et al., 2025; Zhu et al., 2023; Christensen and Kolomiets, 2011). Polyketides, terpenoids, alkaloids, phenylpropanoids and their derivatives metabolite classes have been reported in pathogenic fungi, and are closely associated with their infection strategies (Bills and Goer, 2016). Polyketides, for example, anthrones, anthraquinones, and naphthopyrones, are produced by diverse fungal genera including *Aspergillus*. These compounds possess virulence factors that promote oxidative stress, disrupt the integrity of host cells, or interfere with plant defense systems (Contigli et al., 2023). For example, anthraquinones as major biosynthetic intermediates produce aflatoxins and other toxic compounds, indicating how specialized metabolites can improve both fungal survival and pathogenicity. These findings demonstrate the importance of secondary metabolites in shaping fungal growth and infection outcomes (Bansal et al., 2023; Contigli et al., 2023). Thus, venn diagram analysis was done to highlight the differential distribution of the annotated metabolites between the fungal isolates.

Venn diagram analysis revealed clear chemotaxonomic differences between the species, with each showing unique metabolic fingerprints that reflect both conserved and species-specific biochemical characteristics (Fig. 1C and **Table S1**). A total of 28 metabolites were annotated across all fungal isolates. Some of the common metabolites annotated were from the glycerophospholipids, fatty acids, and carboxylic acids metabolite classes, which were also annotated in cereal-infecting fungal isolates, suggesting a shared core metabolome between cereal-infecting and non-cereal infecting pathogenic fungi. However, significant species-specific metabolites were also observed. For example, *P. cinnamomi* produced distinctive benzenoids compounds such as methyl asterrate (*m/z* 361.064) and emodin (*m/z* 269.046), which added to its profile of 22 species-specific metabolites (Fig. 1C). Other fungi, including *S. sclerotiorum*, *P. capsici*, *C. cucumerinum*, and *C. higginsianum* showed 11, 6, 14, 9 distinct metabolites respectively (Fig. 1C). This distinct metabolite fingerprints among species highlights the metabolic heterogeneity and possible pathogenic mechanisms employed by each organism

Additionally, molecular networking provided a relative quantitative assessment of the measured metabolome. Distinct differences between the distribution of annotated and unknown metabolites were

outlined for fungal species *S. sclerotiorum*, *C. cucumerinum, P. capsici, P. cinnamomi*, and *C. higginsianum*, which illustrates this differential distribution with pie charts using different colors, showing the relative abundance of the different metabolites (Fig. 2). For example, hydroxycinnamic acid derivative, 3_Phenyllactic_acid (*m/z* 353.119), was observed to be predominantly produced by *C. higginsianum*, while it appeared in much lower levels in other species. Additionally, compounds such as equisetin and sulochrin, with *m/z* values of 372.219 and 331.094 respectively, were found in high abundance in *S. sclerotiorum* and *P. cinnamomi*, as illustrated in Fig. 2. This heterogeneity in metabolite profiles highlights species-specific differences in metabolite profiles and suggests a strong connection of these compounds to the ecological strategies or pathogenic lifestyles of the distinct fungi. Moreover, the unique metabolic profiles signify important biological, ecological, or adaptive functions in different species. These differential expressions therefore further emphasize the uniqueness of the fungus in producing specific metabolites linked to functions such as defense, competition, or interaction with organisms (Keller et al., 2005; Macheleidt et al., 2016; Keller, 2019).

The biological significance of these differences is further supported by the functional roles played by these annotated metabolites. Several metabolite classes such as glycerophospholipids, fatty acids and oxylipins, nucleotide sugars, play a role in fungal adaptation by promoting processes such as spore germination, hyphal development, and colonization of host tissues, while also mediating interactions with the environment, including competition with other microorganisms (Boddy and Hiscox, 2016; Beccaccioli et al., 2019; Beccaccioli et al.,2022; Gow et al., 2011). These results therefore, suggest that the unique chemical diversity of each fungal species contribute adaptive strategies and pathogenic potential of each fungal pathogen. Furthermore, some of these secondary metabolites produced by these pathogens have broad applications as natural products in pharmaceuticals, agriculture, food preservation, textiles, and biotechnology research. It has been reported that approximately 78% of antimicrobial and 75% of anticancer currently in use are natural bioactive compounds (Singh, 2021; Khalid et al., 2024; Noor et al., 2020; Goyal et al., 2017), highlighting their importance in the fight against human and plant diseases (Vizcaino et al., 2005).

3.3. Differential metabolite expressions and time-resolved distribution of specialized metabolites cross non-cereal pathogenic fungi

Thus, to further functionally assess these specialized metabolites from metabolomic charts of the pathogenic fungi under consideration (section 3.2), quantitative analyses, such as heatmaps and PLS Variable importance in projection (VIP), were performed on the specialized metabolite profiles. The temporal distribution and production pattern of secondary metabolites by microbial populations provides critical information on the functional activities of specific secondary compounds (Maithani et al., 2022). The heatmap on various specialized metabolites revealed variation in the quantitative profiles across the fungi (Fig. 3A). For instance, a pyrrolindole derivative, roquefortine C with *m/z* of 388.18, had significantly increased levels in *C. cucumerinum* compared to other fungal isolates. Furthermore, *P. cinnamomi* had higher concentrations of several specialized compounds, such as pannaric acid,

sulochrin, fallocinol, and aloe-emodin, with *m/z* values of 315.045, 331.083, 299.057, and 269.046, respectively (Fig. 3A). Similarly, studies from Shaaban et al., 2023 and Tan et al., 2024 also annotated sulochrin, methyl asterrate, and roquefortine C in fungal species such as *Penicillium* and *Aspergillus*, further supporting their widespread role in fungal metabolism.

Moreover, these metabolites appeared to be associated with fungal growth and infection strategies. Sulochrin, for example, possess antioxidant properties, which enable the fungi to inhibit oxidative stress arising during the colonization of the host and environmental challenges (Hussein et al., 2022; Masi et al., 2020; Shaaban et al., 2023; Diaz-Munoz et al., 2018). Interestingly, the production levels of these metabolites varied among species as some metabolites were absent or present in trace amounts in species such as *P. capsici, S. sclerotiorum*, and *C. higginsianum* (**Table S1**). Such observation represents the species-specific characteristics of diverse metabolic profiles of fungi and suggests varying dependence on metabolite-mediated processes of infection.

Additionally, PCA revealed distinct fungal sample separation at different time points (days 4, 8, and 12), suggesting growth stage-specific metabolic reprogramming (Figs. 3B and S4). Furthermore, the VIP scores plot showed different specialized metabolites annotated from *P. cinnamomi* as significant discriminant features underlying the metabolic changes observed at different growth phases (Fig. 3C). Such compounds were endocrocin (*m/z* 313.036), methyl asterrate (*m/z* 361.094), and isorhodoptilometrin (*m/z* 313.072), which were primarily detected in the stationary phase. This highlights the functional significance of specialized metabolism in the late-stages of fungal development, particularly for survival, adaptation to nutrient limitation, and progress of infection. Polyketides and polyketides derivatives, with indole alkaloids and indole-related compounds produced by non-cereal infecting fungal isolates are reported to possess different biological and ecological functions. Polyketides play critical functions in microbial communication, defense, virulence, and environmental adaptation. These metabolites are widely distributed among microorganisms like fungi and bacteria, as well as in higher organisms such as plants (Fischbach and Walsh, 2006; Hertweck, 2009). In this study, several polyketides such as endocrocin (*m/z* 313.036), equisetin (*m/z* 372.219), and andrastin A (*m/z* 485.255) were annotated from *P. cinnamomi*, *S. scerotiorum*, and *P. capsici*, respectively (Fig. 4). Interestingly, endocrocin and equisetin were predominantly expressed during the stationary phase (12 d.p.i), suggesting their possible roles in oxidative stress, and defense mechanisms that facilitate long-term survival of the fungi and its host colonization processes.

Furthermore, some of these polyketides have been reported to have other functional applications outside the fungi, *i.e*., not related to the fungal metabolism and lifestyles. For example, equisetin is a well-characterized polyketide with known antibacterial activity against Gram-negative pathogens, and when combined with colistin can increase antibacterial activities against Gram-negative and colistin-resistant pathogens (Radka and Rock, 2022; Zhang et.al., 2021). It also possesses anti-inflammatory properties and has been reported to exhibit activity against HIV-1 integrase (Zhang et al., 2021; Pandey, 2019). These observations suggest that these phytopathogenic fungi can be explored as source of natural products with pharmaceutical benefits. The results also showed high levels of andrastin A in the

early growth phases (4–8 d.p.i), (Fig. 4), which suggests its involvement in core metabolic functions during fungal establishment in new environments. This compound is a polyketide-terpenoid hybrid which acts as a farnesyltransferase inhibitor, an enzyme critical for isoprenoid biosynthesis thereby conferring competitive advantages during early colonization (Rojas-Aedo et al., 2017; Overy et al., 2005; Metin, 2023). Additionally, indole and indole-derivative metabolites, including roquefortine C and DL-3-indolelactic acid, were annotated from *C. cucumerinum* and *C. higginsianum*, respectively (Fig. 4). These metabolites were most prevalent during the lag and exponential growth phases, which suggest their role in early fungal defense, biomass accumulation, and the metabolic transitions required for host penetration. For instance, DL-3-indolelactic acid is involved in the tryptophan metabolism and plays a crucial role in fungal growth, hyphal formation, and the conversion from primary to secondary metabolism through the indole pyruvate pathway (Fu et al., 2015; Meng et al., 2020; Costantini et al., 2020).

Moreover, indole derivatives are also involved in the plant-pathogen interactions by regulating host immune responses and change in plant growth dynamics (Feng et al., 2023). Therefore, the identification of such specialized metabolites using different AI-driven tools such as MicrobeMASST (**Figures S5-S7** and **Table S1**), is essential to understanding their functional importance in fungal lifecycle, regulation, and pathogenic strategies. In addition to these specialized metabolic profiles, other primary metabolites such as nucleotides, were also annotated (**Table S1**), which are known to play equally important roles in growth and survival of fungi, under consideration.

3.4. Role of nucleotides, nucleosides, and their analogues in regulating the metabolism of non-cereal pathogenic fungal isolates

Multiple classes of metabolites from the nucleotide, nucleosides and analogues superclass were annotated in non-cereal infecting fungal isolates (Fig. 1B). These include nucleotides, nucleotide sugars, flavin nucleotides, nucleosides, and deoxynucleosides (**Figure S3**). Some of these metabolites were flavin adenine dinucleotide (FAD), UPD-alpha-D-galactose, and uridine 5’-diphospho-acetylglucosamine, with *m/z* of 565.05, 565.05, and 606.076, respectively. These compounds displayed differential profiles across the studied fungal pathogens, *i.e*., *C. cucumerinum, S. sclerotiorum, C. higginsianum, P. capsici*, and *P. cinnamomi* (Fig. 5A). Among these, UPD-alpha-D-galactose, a known nucleotide sugar involved in glycosylation pathways, was found at significantly higher levels in *C. higginsianum* compared to other fungal isolates, suggesting a potential species-specific role in cell wall biosynthesis or pathogenicity (Schwerdt et al., 2021). Contrary, other metabolites such as UDP-glucose, FAD, and uridine 5’-disphospho-N-acetylglucosamine were detected across all isolates, albeit at varying concentrations (Fig. 5A).

These compounds not only serve as building blocks for nucleic acids, but are also chemical energy carriers (*e.g*., ATP, GTP), intracellular messengers (*e.g*., cAMP, cGMP), and are essential co-factors in different biochemical reactions (*e.g*., CoA, FAD, FMN, NAD^+). Nucleosides (the phosphate-free versions of nucleotides) are integral to the functions of fungal biochemistries. In addition to acting as precursors

to nucleic acid biosynthesis, they participate in sugar and lipid metabolism, polyamine biosynthesis, and methylation reactions, contribute to energy transfer, among other biochemical functions (Rosemeyer, 2005; Zhang et al., 2020; Phan et al., 2018). Among these metabolites, purine nucleotides and nucleosides such as adenosine and guanosine are particularly prevalent and useful. Purine metabolism, including *de novo* synthesis, salvage, and catabolism, is well characterized in model fungi *Saccharomyces cerevisae* and is central to cell proliferation, differentiation, and adaptation to environmental shifts (Ljungdahl and Daignan-Fornier, 2012; Moffatt and Ashihara, 2002).

Metabolically, nucleotides and their derivatives were found to be involved in key biological pathways, including riboflavin metabolism, amino sugar and nucleotide sugar metabolism, and purine metabolism (Fig. 5B). The distribution and existence of these metabolites demonstrate their role in essential biological processes. In riboflavin metabolism, FAD, a coenzyme produced from riboflavin functions as a key compound in a number of redox reactions. It serves as a cofactor for many flavoenzymes that are important in oxidative metabolism, mitochondrial energy production, protein folding, reactive oxygen species (ROS) regulation, and defense against oxidative stress (Giancaspero et al., 2017). Moreover, FAD is also involved in cellular differentiation and stress responses (Kavitha and Chandra, 2014; Malik et al., 2025; Sheridan et al., 2015; Bahn et al., 2007). The annotation of riboflavin in the fungal isolates highlights the metabolic dependence of the riboflavin-FAD pathway in the metabolism of these organisms (Fig. 5D). The purine metabolism pathway was another well characterized pathway comprising of metabolites such as guanosine (*m/z* 282.077), guanosine monophosphate (*m/z* 362.054), adenosine (*m/z* 312.105), and adenosine 5’-diphosphate (*m/z* 426.023) (Figs. 5B and S8). These metabolites’ distribution differed among the different fungal species (**Figure S8**), suggesting species-specific metabolic adaptations. Additionally, the biosynthesis and accumulation of nucleotide related metabolites were analyzed in different fungal growth phases to determine their functional roles during fungal development and pathogenesis. For example, FAD was significantly high throughout the exponential and stationary phase across all fungal isolates (**Figure S9**). This highlights continuous relevance in their metabolic activity, stress response, and cellular maintenance during pathogenicity.

Interestingly, nucleotide sugar species such as UDP-glucose and UDP-alpha-D-galactose were largely synthesized from the early stages of growth of the fungal isolates, with low concentration recorded in the stationary phase (**Figure S9**). These dynamics in the time-resolved profiles of these metabolites demonstrates the vital roles of these metabolites in promoting cellular and structural activities during active cell division and hyphal elongation. For instance, UPD-alpha-D-galactose plays a critical role as a sugar donor for the galactomannan biosynthetic pathway, which plays an important role in fungal cell wall formation and correlates directly with virulence factors and host-pathogen interactions (Groß et al., 2024). It is also an intermediate in the synthesis of polysaccharides, which aids in the extracellular matrix formation and biofilm development (Park et al., 2014). Additionally, this metabolite may participate in proteins and lipids glycosylation by donating galactose residues in the biosynthesis of glycoproteins and glycolipids, both which are essential to the structure and function of fungal membranes and cell walls (Lee et al., 2014). Contrary, other nucleotide sugars such as uridine 5’-diphospho-N-acetylglucosamine (UDP-GlcNAc) presented a different profile, exhibiting low abundance

during the early growth phases in *S. sclerotiorum, C. higginsianum*, and *P. capsici* and higher accumulation during the stationary phase in several fungal species (**Figure S9**). This suggests a strategic metabolic change that occurs, where the fungi prioritize the synthesis of structural and protective components in response to environmental stress or nutrient limitation. UDP-GlcNAc is a key precursor in the synthesis of chitin which is a major structural polysaccharide of the fungal cell wall and plays an essential role in preserving cell wall integrity, particularly during non-proliferative stages (Ryczko et al., 2016; Song et al., 2024; Gaderer et al., 2017). Moreover, this metabolite functions as a sugar donor in the glycosylation of proteins and lipids which contributes to cellular plasticity including the ability to respond to stress and supporting membrane stability (Paneque et al., 2023; Fisi et al., 2017).

This phase specific production pattern of such a metabolite provides insight into the physiological status, pathogenic potential, and responses to both host and environmental cues of these fungi (**Figure S9**). It indicates on how and when these key metabolites are activated in a pathogenic life cycle. These findings reveal a complex regulation of nucleotide and nucleotide sugar metabolism across the different fungal growth stages. The annotation of different nucleosides and nucleotides analogues emphasizes their beneficial roles in promoting growth, pathogenicity, and survival of the fungi. Such molecules are not only metabolic indicators of the developmental conditions of these fungal isolates, but can also be potential agents in antifungal intervention, especially considering their role in key biological activities such as cell wall biosynthesis, redox reactions, and macromolecular modifications. Thus, this study provides insights into the chemical space of non-cereal phytopathogenic fungi, a metabolomic landscape characterized by a wide range of metabolite classes, of both primary and secondary metabolism. Several core metabolite categories were found to be consistently associated with virulence, adaptation, and host interaction (Fig. 6).

Thus, the metabolomic atlas of the five non-cereal infecting fungi species includes functional profiles of fatty acids and their derivatives, polyketides and quinones, glycerophospholipids, and carbohydrates and sugar derivatives, each represented by distinct examples of annotated metabolites within their respective classes (Fig. 6). Fatty acids and terpenoids, such as eicosanoic acid, 12, 13-DIHOME, 9-HOTrE, and andrastin A, exhibit significant structural diversity and are functionally involved in critical roles such as membrane integrity, intracellular signaling, and pathogenicity (De Carvalho and Caramujo, 2018; Bhat et al., 2023; Das, 2018). These compounds may also influence spore germination, and host penetration. Polyketides and quinones, including emodin and isorhodoptilometrin are typical secondary metabolites with redox activity and cytotoxic propertied that can enhance fungal survival and host colonization (Fouillaud et al., 2016; Singh et al., 2021; O'Brien and Wright, 2011; Dar et al., 2017; Christiansen et al., 2021). Furthermore, glycerophospholipids contribute to the biosynthesis and remodeling of cell membranes which are essential for maintaining cell integrity during host invasion and stress responses (Li et al., 2025; Hanafusa et al., 2020). Among primary metabolites, nucleotides (*e.g.*, riboflavin and adenosine monophosphate) and amino acids act as metabolic precursors for numerous biosynthetic pathways, including those leading to secondary metabolites (Sanchez and Demain, 2008). These compounds are essential for signaling, energy transmission, and fungal growth.

Carbohydrates and their derivatives, such as raffinose, melibiose, UDP-glucose, and, UDP-alpha-D-galactose, play significant roles in energy storage, osmoprotection, and mostly, in cell wall biosynthesis and remodeling (Jamal et al., 2026; Nehls, 2004; Latgé, 2007; Baltar et al., 2021). These metabolites are crucial to fungal pathogenicity, as they form structural elements such as chitin and 1, 3-beta-glucans, which are part of primary constituents of the fungal cell wall (Niaz et al., 2020; Schwerdt et al., 2021). Lastly, flavonoids and phenylpropanoids including hesperetin-7-O-rutinoside, 5-O-methylgenistein, DL-3-phenyllactic acid and p-hydroxyphenyllactic acid are aromatic secondary metabolites known to improve oxidative stress tolerance, regulate plant defense mechanism and promoting signaling or regulatory in host-pathogenic interactions and biochemical mimicry facilitating in infection and persistence in hosts (Lavermicocca et al., 2003; Mierziak et al., 2014; Pusztahelyi et al., 2015; Debonne et al., 2020 Ponzio et al., 2024). Thus, these profiles (Fig. 6) point to the metabolic flexibility and biochemical strategies employed by non-cereal fungi to support growth, infection, colonization and survival in diverse host environments.

4. Conclusion

This study provides the first comprehensive metabolomic atlas of non-cereal infecting pathogenic fungi, using untargeted computational metabolomics. Through systematic profiling of five agriculturally relevant species, *S. sclerotiorum*, *C. cucumerinum*, *C. higginsianum*, *P. cinnamomi*, and *P. capsici*, across multiple growth stages, the study reveals a dynamic and species-specific chemical landscape characterized by differential profiles of primary and specialized metabolites. The characterization of virulence-associated compounds such as sulochrin, equisetin, and endocrocin illustrates how closely linked fungal pathogenicity ensures that metabolic reprogramming is properly tuned. The temporal dynamics of nucleotide metabolism, particularly of purine and riboflavin pathways, indicate how these fungal pathogens modify their metabolism in response to distinct phases of growth and nutrient environments. Leveraging AI-powered tools such as molecular networking and cheminformatics analysis, this work supported the annotation of the "dark metabolome" and allowed functional dissection of metabolic pathways such as purine and riboflavin metabolism. Through the combination of temporal sampling and FBMN, the study shows a clear reprogramming of metabolite expression at different growth phases and reveals exciting biosynthetic strategies for host invasion and stress resilience. These actionable findings present critical opportunities for targeted biocontrol agents, antifungal therapeutics, and diagnostic biomarkers. In this way, this study constructs a hypothetical framework that describes chemical strategies of non-cereal pathogens. The ability to identify this metabolomic blueprint is essential for devising novel strategies to protect non-cereal crops against phytopathogenic fungi. As such, these actionable insights help advance a range of sustainable development goals such as SDG 2 (zero hunger) and SDG 3 (good health).

Declarations

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Author contributions M.B.D.: Methodology, data analysis, data curation, writing of original draft, writing-review and editing. M.M.T.: Review and editing. F.T: Conceptualization, methodology, data analysis, data curation, writing-review and editing, supervision and project administration. All authors read and approved the manuscript.

Data Availability Statement

The spectral data presented in this study is made available on the supplementary files as job links

Conflicts of Interest: Fidele Tugizimana (F.T.) is employee of Omnia Group, South Africa, a dual affiliation. All authors declare to have no conflicts of interest.

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Figures

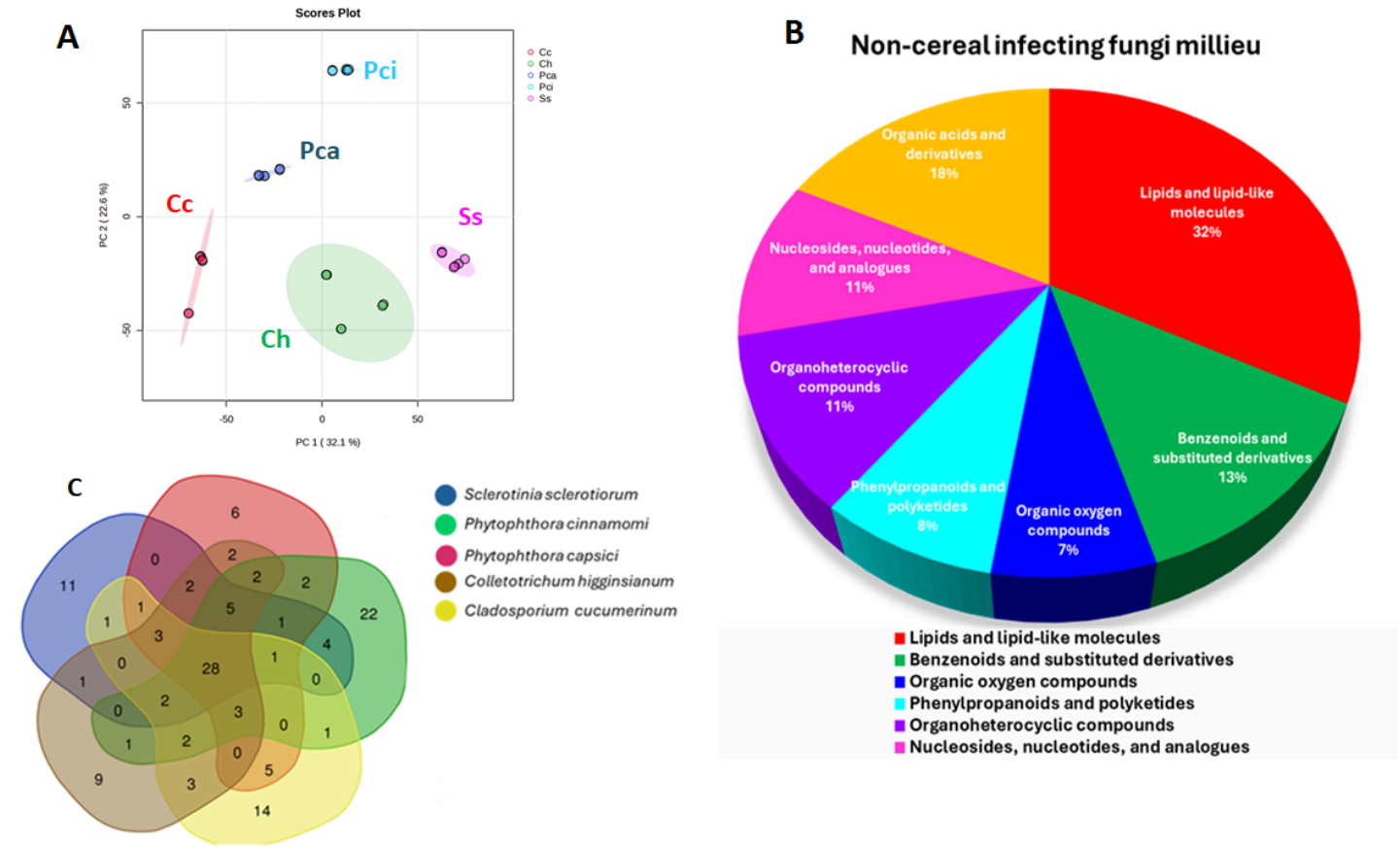


Figure 1

Overview of metabolome coverage of non-cereal infecting fungal species. (A)The PCA score plots of the UPLC-MS data of five non-cereal infecting fungal species' metabolite extracts collected at day 4 post inoculation (d.p.i). PCA scores plots (PC1 vs PC2) showed distinct clustering of the fungal sample reflecting species-specifc metabolic variation and the model showed good explanatory value with R^2 > 0.90 and statistically significant *p*-value of 0.001. (B**)** Pie chart displaying the percentage of the combined chemical class annotations from the 5 non-cereal infecting pathogens: *S. sclerotiorum, C. cucumerinum, P. capsici, P. cinnamomi, C. higginsianum.* **(**C**)** Venn diagram showing the distribution of the annotated metabolites (Table S1) across the fungal species Abbreviations: Cc = *C. cucumerinum*, Ch = *C. higginsianum*, Pca = *P. capsici*, Pci = *P. cinnamomi*, and Ss = *S. sclerotiorum*.

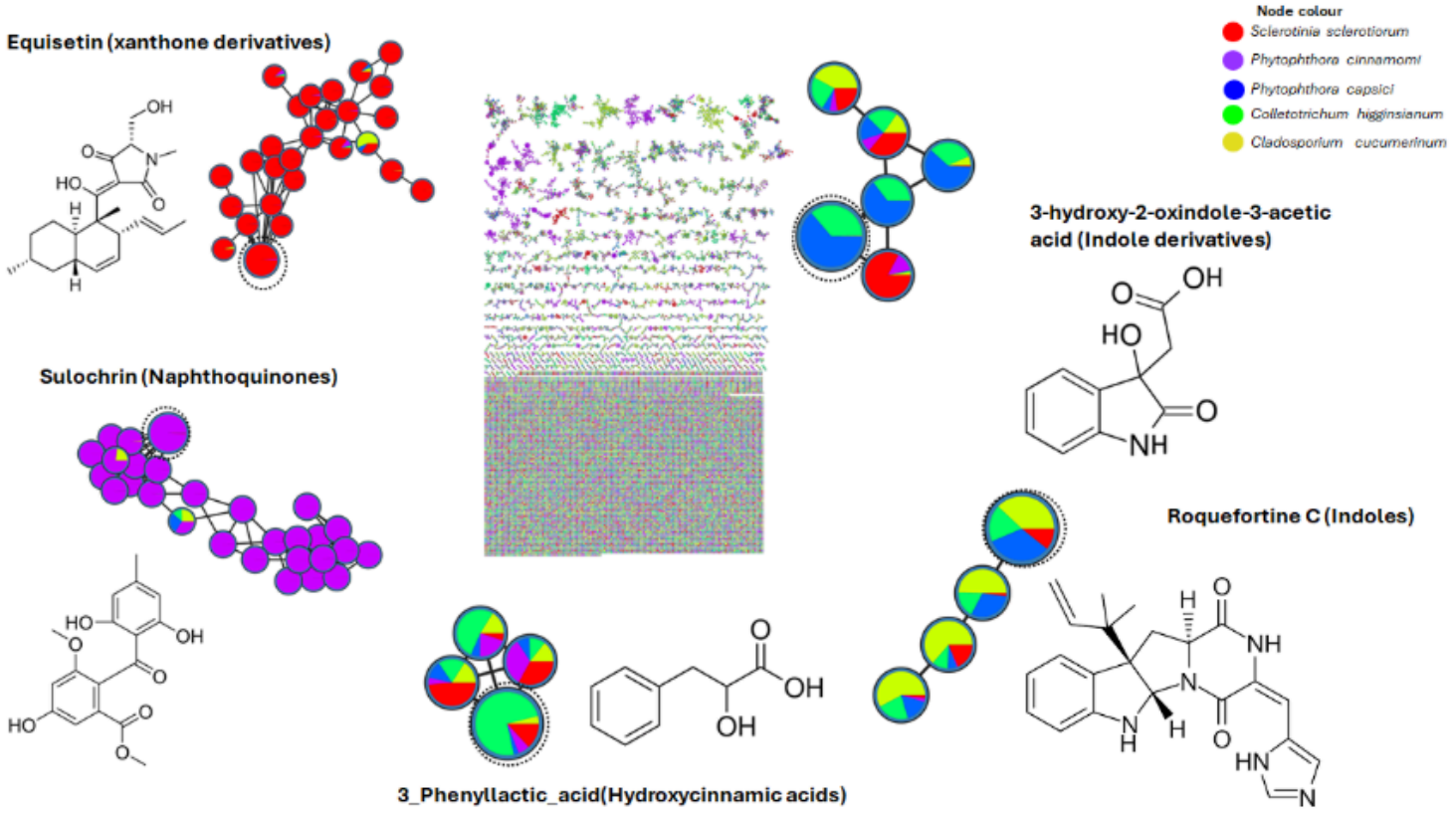


Figure 2

A feature-based molecular network (FBMN) of non-cereal infecting fungi *S. sclerotiorum*, *C. cucumerinum, P. capsici, P. cinnamomi,* and *C. higginsianum* MS/MS spectra. The molecular network visualizes the relative concentrations of metabolites produced by the respective fungal isolates, with the node colours indicating the abundance of the respective metabolite across the different fungal isolates. Annotated metabolites from molecular clusters/families belonging to chemical classes of phenylpropanoids and polyketides, benzenoids and substituted derivatives, and organoheterocyclic compounds are also highlighted in the molecular network.

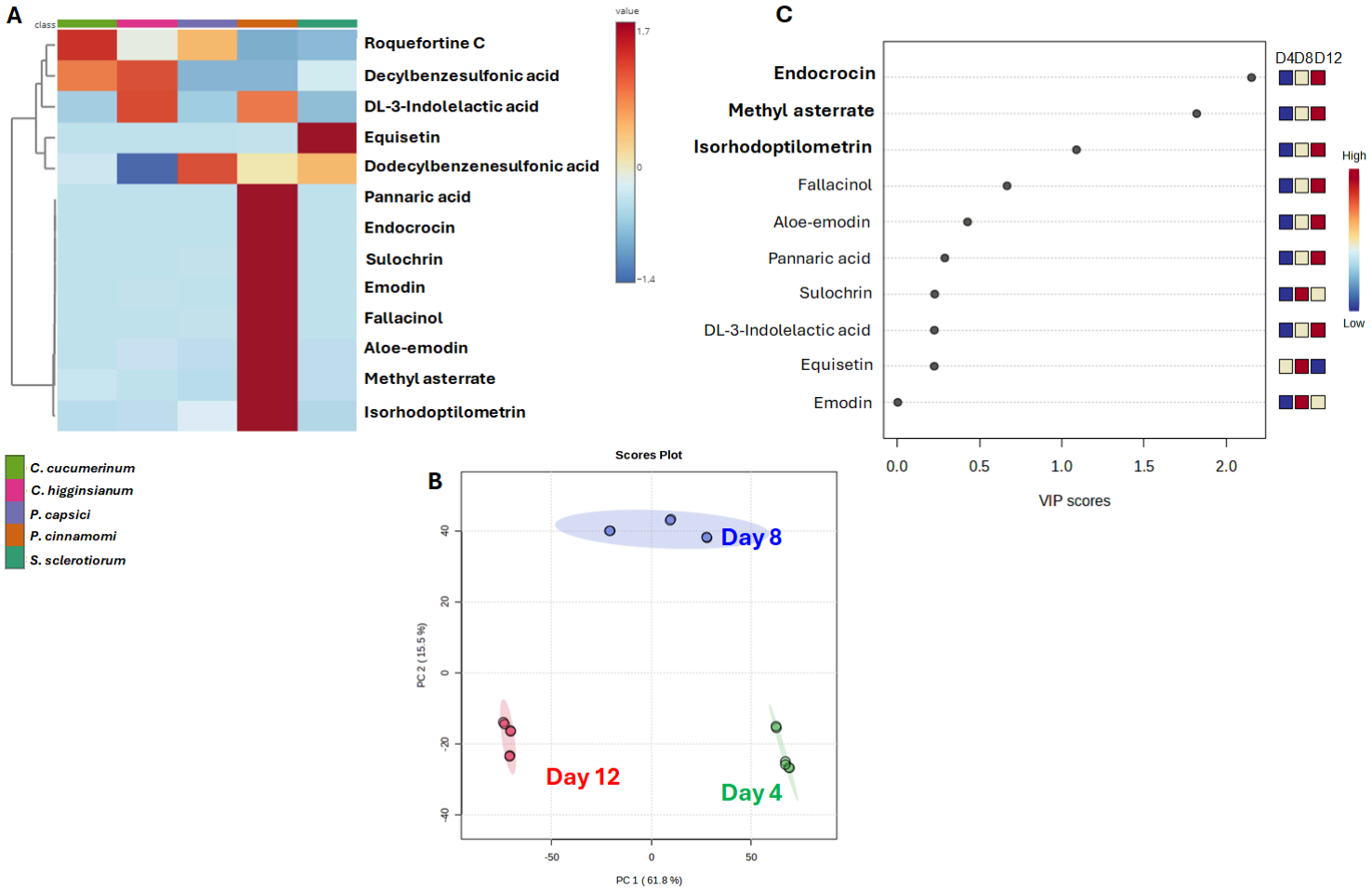


Figure 3

The relative quantification of specialized compounds of different chemical classes and chemometrics analysis. (a**)** Heatmap showing the relative quantification of different secondary metabolites produced by different non-cereal pathogenic fungal isolates. **(**b**)** Principal component analysis (PCA) shows the separation between samples of *P. cinnamomi* grown and harvested at different time points with a R^2 values greater than 0.60 and *p*-value of 0.001. **(**c**)** Variable importance in projection (VIP) revealing some of the key discriminant metabolites (secondary metabolites with VIP scores > 1.0) contributing to the groupings shown in PCA score plot of *P. cinnamomi*.

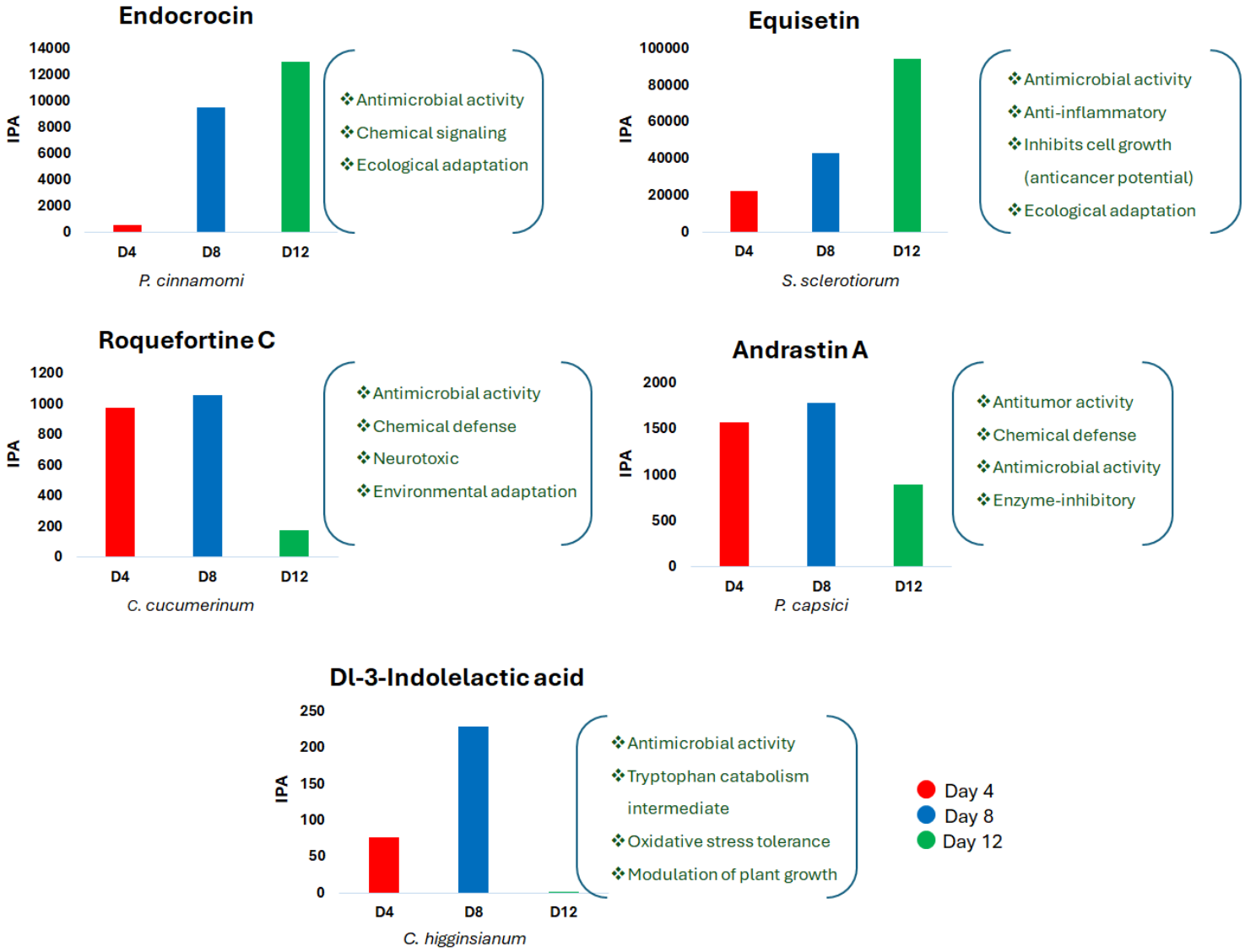


Figure 4

The relative quantification of polyketides and indole alkaloids compounds. The bar graphs show the distribution of metabolites at different growth phases for the selected metabolites produced by the respective non-cereal infecting fungi isolates *P. cinnamomi*, *S. sclerotiorum*, *C. cucumerinum*, *P. capsici*, and *C. higginsianum*. Each bar represents the average of integrated peak area (IPA) from six replicates (n=6). All differences between the fungal growth phases were statistically significant at a threshold of *p*-values ≤ 0.05. Abbreviations: D4 = day 4, D8 = day 8, and D12 = day 12, IPA = Integrated Peak Area.

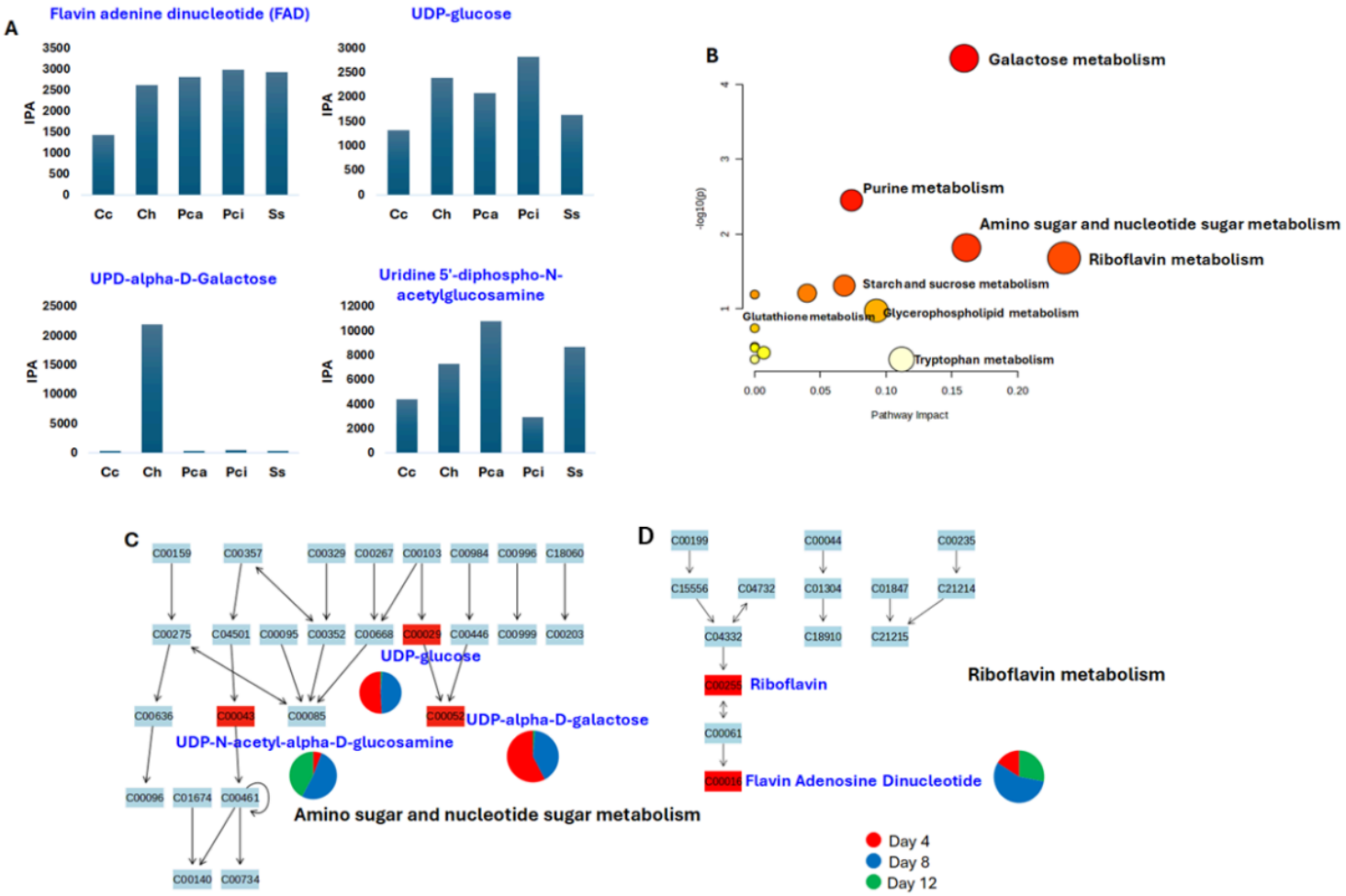


Figure 5

Nucleotides and nucleotide sugars annotated from non-cereal infecting fungal isolates. (a) Bar graphs showing relative quantification of different nucleotides and nucleotide sugars annotated from the non-cereal infecting fungal isolates. All differences between the fungal isolates between the bar graphs were statistically significant with *p*-values ≤ 0.05. (b) Metabolomics Pathway Analysis visualization of all metabolic pathways identified from the resultant annotations, organized by their significance (pathway impact) and p-values (-log10(p)). Amino sugar and nucleotide sugar (c) and Riboflavin (d) metabolism pathways, mapping the matched nucleotide metabolites (highlighted in red) in the pathway.
Abbreviations: Cc = *C. cucumerinum*, Ch = *C. higginsianum*, Pca = *P. capsici*, Pci = *P. cinnamomi*, and Ss = *S. sclerotiorum*, IPA = Integrated Peak Area.

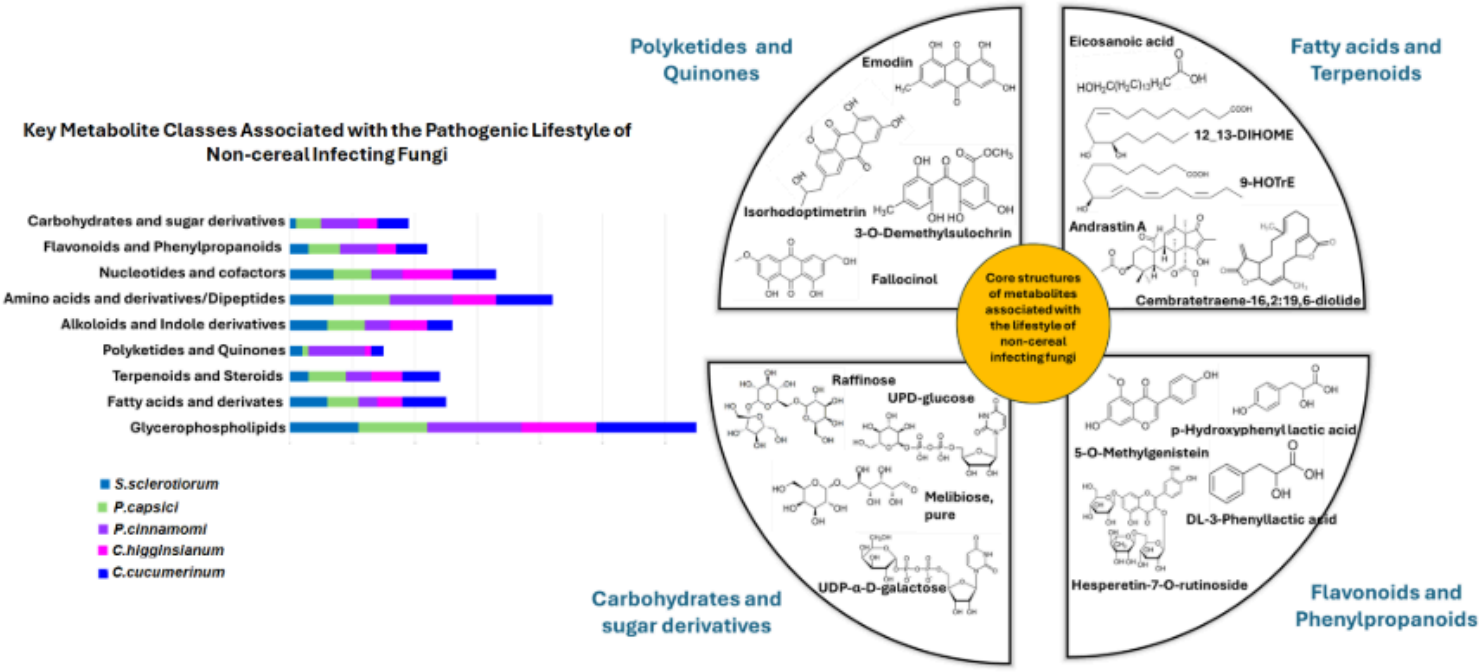


Figure 6

Metabolite classes and examples of metabolites associated with the pathogenic lifestyle of non-cereal infecting fungi. An illustration of diversity and distribution of key metabolite classes that are linked to the pathogenicity of five non-cereal infecting fungi species: *S.sclerotiorum, P.cinnamomi, P.capsici, C.higginsianum*, and *C.cucumerinum*. The horizontal stacked bar chart displays the relative representation of metabolite classes across the five species including carbohydrates and sugar derivatives, nucleotides and cofactors, amino acids and derivatives/dipeptides/dipeptides, flavonoids and phenylpropanoids, alkaloids and indoles derivatives, polyketides and quinones, terpenoids and steroids, fatty acids and derivatives, and glycerophospholipids. Each color segment represents a different fungal species. The four quadrant panels depict representative of some of the chemical metabolite structures from key metabolite categories.

Supplementary Files

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