Microbial Cell Factories

Tailored culture strategies to promote antimicrobial secondary metabolite production in *Diaporthe caliensis*: a metabolomic approach

Laura V. Hoyos[1], Luis E. Vasquez-Muñoz[1], Yuliana Osorio[1], Daniela Valencia-Revelo[1,2], Daiana Devia-Cometa[1], Miriam Große[2], Esteban Charria-Girón[2,3*] and Nelson H. Caicedo-Ortega[1,4*]

Abstract

Background In the search for new antimicrobial secondary metabolites of fungi, optimizing culture conditions remains a critical challenge, as standard laboratory approaches often result in low yields. While non-selective methods, such as modifying culture media, have been effective in expanding the chemical diversity of fungal metabolites, they have not yet established a direct link to key process parameters crucial for further optimization. This study investigates the capacity of *Diaporthe caliensis* as a biofactory for biologically active secondary metabolites, employing tailored culture media to explore the relationship between chemical diversity and critical process variables.

Results The metabolomic profiles, antibacterial activities, and production yields of the extracts were analyzed to progressively adjust the culture conditions. This study was conducted in five steps, evaluating carbon and nitrogen source concentration, nitrogen source type, salt supplementation, and pH adjustment. Altering the rice starch concentration affected biomass yield per unit of oxygen consumed, while modifications to the nitrogen source concentration influenced both the bioactivity and chemical space by *Diaporthe caliensis*. Despite changes at the metabolome level, the extracts consistently exhibited potent antibacterial activities, influenced by the nitrogen source, added salts and pH adjustments. For instance, when using corn steep liquor and rice starch, supplemented with micronutrients, different metabolites were produced depending on whether buffer or water was used, though both conditions showed similar antibacterial activities ($IC_{50} \approx 0.10$ mg mL^{-1} against *Staphylococcus aureus* and ≈ 0.14 mg mL^{-1} against *Escherichia coli*). In the treatment where buffer was used to stabilize pH change, there was an increase in the production of phomol-like compounds which are associated with known antibiotic properties. In contrast, in the treatments using water, the drop in pH stimulated the production of previously unidentified metabolites with potential antimicrobial activity.

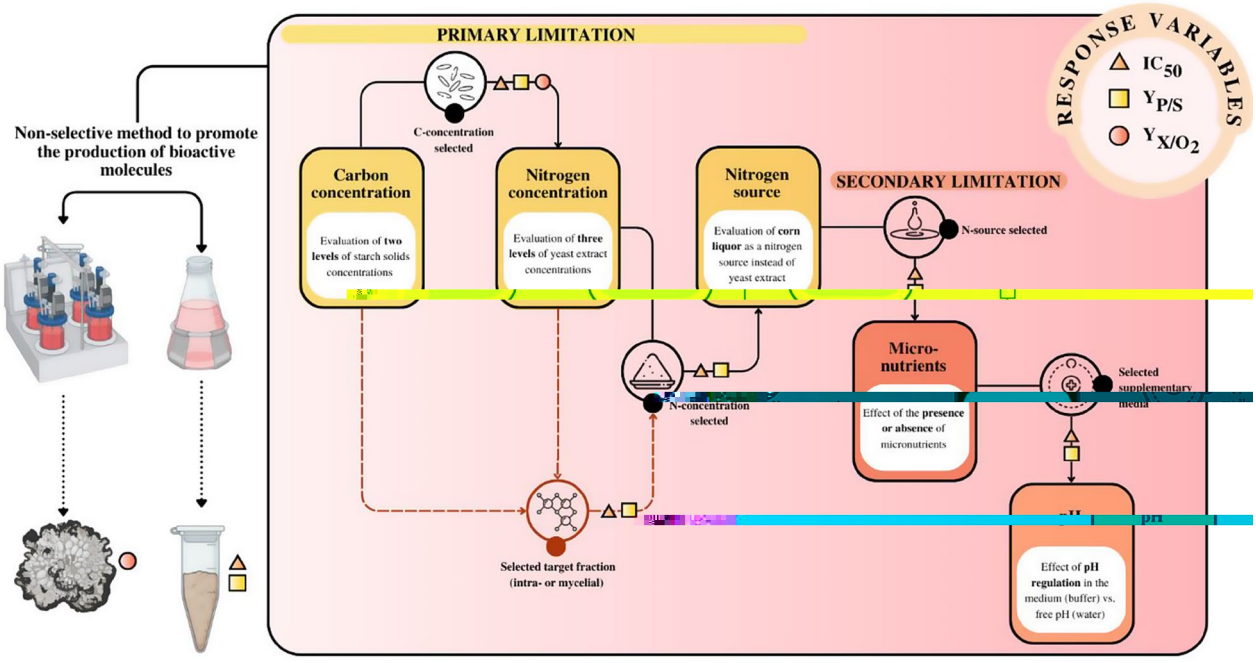
*Correspondence:
Esteban Charria-Girón
esteban.charriagiron@helmholtz-hzi.de
Nelson H. Caicedo-Ortega
nhcaicedo@icesi.edu.co

Full list of author information is available at the end of the article

Conclusions This study proposes a strategic methodology for the tailored formulation of culture media aiming to promote the biosynthesis of diverse secondary metabolites. This approach revealed the critical role of nutrient limitation and pH regulation in stimulating the production of polyketide-lactone derivatives, including the antibiotic phomol. Ultimately, the systematic, custom-designed culture conditions developed in this work o□er a promising strategy for expanding the chemical diversity of *Diaporthe caliensis*, while providing valuable insights into the key parameters needed for optimizing this fungal biofactory.

Keywords Nutrient limitation, Natural products, Antibacterial activity, Metabolomics, Submerged fermentation, Biofactory, Polyketides

Graphical Abstract



Background

Secondary metabolites (SMs) are low-molecular-weight compounds produced by bacteria [1, 2], fungi [3], or plants [4, 5]. Unlike primary metabolites, which are directly involved in the growth, reproduction, or development of microorganisms, SMs often facilitate and mediate microbial interactions within their environment [6]. ey are involved in cell–cell signaling, pathogenesis, and increased nutrient bioavailability [7]. In addition to conferring advantages to the producer organisms, SMs exhibit biological activities of great industrial value. SMs produced by endophytic fungi are characterized by diverse and attractive bioactivities, such as immunomodulatory [8], anticancer [9], antioxidant [10], and antimicrobial [11, 12] activities. However, traditional methodologies such as bioactivity-guided isolation face limitations, as certain molecules relevant to specific ecological niches are not produced under laboratory conditions [13]. Two main approaches are commonly used to induce their production in controlled settings: Selective and non-selective methods. e first one involves manipulating specific biosynthetic pathways using molecular biology tools, while the second one includes strategies such as the modification of culture media and fermentation conditions, o ering two alternative approaches [14].

Solid-state fermentations are usually employed within non-selective methods [15, 16]. Although they o er the advantage of increased productivity [17, 18], these fermentations require large quantities of solid substrate, which negatively a ects mass transfer at the substrate-fungus interface, especially oxygen di usion [19]. In contrast, liquid cultures allow for greater mass transfer by controlling agitation and/or aeration [20]. In addition to improving the bioavailability of nutrients, it is possible to test the e ect of nutritional limitations in liquid fermentation by modifying the concentrations of substrates in the medium and other culture parameters [21].

In non-selective methods, the one-strain-many compounds (OSMAC) strategy has proven e ective in promoting the production of novel chemistry by modifying

easily accessible cultivation parameters [22, 23]. Recent studies have explored the role of exogenous elicitors, such as chitooligosaccharides and lipids, in triggering the production of SMs in *Aspergillus fumigatus* [24]. Under nutrient-limited conditions, metabolic fluxes can shift in various organisms, favoring specific biosynthetic pathways at the expense of biomass formation [25, 26]. For example, Kottmeier et al. found that intentionally restricting phosphate increased the yield of green fluorescent protein (GFP) yield 1.87-fold in glucose-based media used to cultivate the yeast *Hansenula polymorpha* [25]. In fungi, oxygen-limiting conditions (static fermentation) can influence the production of fungal extracts with anticancer properties [9]. Another study used a coculture strategy combined with nutrient deprivation to induce cryptic biosynthetic pathways and novel antimicrobial metabolites in *Eutypa lata* and *Botryosphaeria obtusa* [27]. Furthermore, the e ects of nitrogen and phosphorus limitations on the fatty acid profile produced by *Aspergillus oryzae* have been examined through transcriptome analysis [28].

When developing customized culture media to enhance the production of targeted molecules or trigger the biosynthesis of novel ones, a key challenge is integrating non-selective methods with easily measurable in situ variables, which are essential for obtaining early insights into culture performance [25]. One variable that can be monitored to identify culture patterns, such as nutrient limitations (including oxygen) or diauxic growth, is the oxygen transfer rate (OTR) in shaking flasks [29, 30]. In addition to real-time monitoring of biological variables, it is crucial to assess changes in secondary metabolite production to evaluate the e ectiveness of these non-selective methods. Modifying the culture media or operating conditions can disrupt the intricate biochemical networks within fungi [31, 32]. erefore, incorporating metabolomics analyses into bioprocess design is crucial for identifying conditions that maximize metabolite diversity and enhance the observed biological properties.

As part of our investigation into the biotechnological potential of endophytic fungi isolated from *Otoba gracilipes*, a medicinal tree native of Valle del Cauca, Colombia, we have identified promising candidates for producing laccase enzymes [33], flavors [34], antioxidant compounds [35], and secondary metabolites with antibacterial activity [36], such as phomol [37]. Inspired by these finding, and in response to the global issue of antimicrobial resistance, which was linked to 1.27 million deaths in 2019 [38–40], the aim of the present study was to design a methodology for the stepwise formulation of tailored culture media to stimulate the biosynthesis of antimicrobial metabolites in *Diaporthe caliensis*. To achieve this, we evaluated the e ect of two di erent nitrogen sources (i.e., corn steep liquor and yeast extract) during the submerged fermentation of this fungus, using a rice starch solution as the carbon source. e e ects of varying the carbon-to-nitrogen ratio were assessed in terms of (*i*) biomass yield produced per oxygen consumed ($Y_{X/O2}$), (*ii*) OTR, (*iii*) half-maximal inhibitory concentration (IC_{50}), (*iv*) yield of crude organic extracts per substrate consumed ($Y_{P/S}$), and metabolomics data.

Materials and methods

Microorganisms and reagents

e endophytic fungus *Diaporthe caliensis* CM-UDEA-H27, which belongs to the fungal collection of the Universidad de Antioquia (Medellín, Colombia), was used in this study. Agar was obtained from ermo Scientific. Malt extract, monobasic potassium phosphate, sodium tartrate dihydrate, magnesium sulfate, and iron (II) sulfate heptahydrate were obtained from Sigma–Aldrich. Manganese (II) sulfate monohydrate from Honeywell. *D*-glucose from Scharlab. Tween 80 was obtained from Loba Chemie. Corn steep liquor (SUST PROT, lot 1866013) was obtained from Ingredion®. e following Colombian brand reagents were used: yeast extract (EXLV-LS®-3111, lot 218671) from Levapan S.A. and Caribe® rice from e Diana group.

Assessment of nutrient limitation in submerged cultivation of *Diaporthe caliensis*

A five-step experimental procedure was used to systematically evaluate the e ect of primary and secondary nutrient limitations on the production of bioactive metabolites by *Diaporthe caliensis*. e strategy involved modifying one factor at a time and carefully selecting the most e ective treatment before advancing to the next phase. e response variables were yield of biomass formed on a dry basis per oxygen consumed ($Y_{X/O2}$), oxygen transfer rate (OTR), half-maximal inhibitory concentration (IC_{50} mg mL^{-1}), and yield of crude organic extracts obtained per substrate consumed ($Y_{P/S}$). Table 1 specifies the variables used in each research step. Each experiment was performed in duplicate.

Diaporthe caliensis was grown in RAMOS® shaking flasks to obtain the $Y_{X/O2}$ ratio and monitor the OTR over time. ese data were used to ensure shaking conditions that prevented oxygen limitation during the cultivation. After confirming this during the RAMOS® experiments, the relative centrifugal force (RCF) was maintained by adjusting the speed according to the shaking diameter [41] in the Erlenmeyer flask experiments. ese experiments were used to evaluate the $Y_{P/S}$ and IC_{50} parameters using a setup strategy similar to that of Philip et al. [42]. In addition, residual sugars were quantified in both cases using HPLC to determine glucose, fructose, maltodextrin, and sucrose levels (see section 'Chromatography').

us, it was confirmed that both setups had similar consumption profiles.

As a first step of this experimental approach, two different concentrations of the carbon source, formulated as the limiting nutrient, were evaluated using a rice starch solution as a substrate. is stock was prepared by heat treatment of long-grain white rice from the Colombian brand Caribe®. Initially, the rice grains were milled and sieved (425 µm). Subsequently, 16 g of the resulting flour was added to 400 mL of deionized water. e resulting suspension was heated in a water bath at 90 °C for 15 min.

Once the concentration of starch was chosen, the e ect of two concentrations of yeast extract were tested in the second step, considering the contributions of nitrogen and carbon to the medium formulation. In the third step, the yeast extract was replaced with corn steep liquor, while maintaining the same nitrogen contribution (i.e., 90 mg L^{-1}). is concentration was based on the total nitrogen content measured using the Kjeldahl method [43]. Prior to use, the corn steep liquor was centrifuged (4 °C and 4500 rpm) to remove suspended solids, accounting for 40%, which may have included insoluble carbohydrates. e total carbon contribution was estimated following the NREL protocol [44].

Until this point (from the first to the third step), nutrient solution A (0.5 g L^{-1} of KH_2PO_4 and $C_4H_4Na_2O_6 \cdot 2H_2O$) had been used to supplement the culture medium [45–48]. In the fourth step, the replacement of solution A for micronutrient solution B (0.5 g L^{-1} of $MgSO_4 \cdot H_2O$, 1.0 g L^{-1} of $FeSO_4 \cdot 7H_2O$ and 1.0 g L^{-1} $MnSO_4 \cdot H_2O$) was tested according to de Souza et al. [49] as well as a combination of both solutions. For the fifth step, the influence of pH regulation using water instead of 0.05 M phosphate bu er (pH 6.3) was evaluated. Eight di erent treatments with varying nutritional formulations were evaluated (Table 1).

Cultivation of *Diaporthe caliensis* in a shaking ask for online OTR assessment

e fungus *Diaporthe caliensis* CM-UDEA-H27 was cultivated on yeast malt agar (YMG) at 29 °C for seven days. Subsequently, a mycelium suspension was prepared by scraping the surface of three Petri dishes and mixing with 12 mL of 0.01% Tween 80. is suspension was used as the inoculum for the respiration activity monitoring system experiments, RAMOS® (HiTec Zang GmbH, Herzogenrath, Germany). e experiments were conducted in shaking flasks with online OTR parameter measurements. After adding 1 mL of the inoculum to 49 mL of the indicated medium, the mixture was incubated

OTR was monitored throughout the cultivation period. After 7 days, the biomass was separated from the supernatant by vacuum filtration through a cellulose filter paper (20–30 µm; FAST 101, Ø 12.5 cm; Chicago, Illinois, United States). e washed biomass (with deionized water) was dried using a convection oven (Binder, Germany) at 110 °C for two hours to determine the final biomass concentration (g DW L^{-1}). e presence or absence of residual sugars in the broth was confirmed through HPLC analyses (see section 'Chromatography'). Finally, the parameter $Y_{X/O2}$ was determined using the following equation:

$$Y_{X/O_2} = \frac{X_f}{TOT} \quad (1)$$

In the equation, X_f represents the concentration of bio-

Antibacterial activity assays

e antibacterial activity of the crude extracts was determined following the methodology described by Charria-Girón et al. [36]. Initially, the extracts were evaluated against *Staphylococcus aureus* ATCC 25,923, and those with the best IC_{50} values were subsequently tested against *Escherichia coli* ATCC 25,922. Bacterial cultures were prepared by taking 100 µL of each strain preserved in a cryovial and adding it to a flask with 50 mL of Luria–Bertani (LB) broth at pH 7.0. After 20 h of incubation (37 °C and 150 rpm), the culture was sampled and seeded on an LB agar plate. After another 20 h at 37 °C, four colonies were taken to prepare a bacterial suspension using 0.9% (v/v) saline solution (OD_{620} nm between 0.08 and 0.1). According to the McFarland scale, the estimated cell concentration was 1×10^8 CFU mL^{-1}, so dilutions were performed to reach a concentration of 1×10^4 CFU mL^{-1}.

e obtained crude extracts were evaluated at concentrations ranging from 2 to 10 mg mL^{-1} and were dissolved in a mixture of 20% methanol and 80% DMSO (1% v/v). Measurements were performed on six serial dilutions in triplicate, with LB broth and methanol-DMSO (1% v/v) serving as negative controls. e assay plates were incubated for 20 to 24 h at 37 °C and 140 rpm before the OD_{620} was measured using a spectrophotometric reader (ermo Fisher Scientific™ Varioskan™ LUX, United States).

Chromatography

Detection of residual sugars

Samples of cell-free medium (supernatant) were filtered using a 0.2 µm cellulose filter, and the pH was adjusted to between 1 and 6 using 5 mM sulfuric acid. e presence of residual sugars was then detected by HPLC analysis (ermo Fisher Scientific, Waltham, MA, USA) using an Aminex HPX-87 H column (hydrogen-form, 300×7.8 mm, from Bio-Rad) and an RI detector. is analysis used a sulfuric acid solution (5 mM) as the mobile phase with a flow rate of 0.6 mL min^{-1} and an operating temperature of 45 °C. Five solutions with different concentrations of glucose, fructose, maltodextrin, and sucrose were injected as standards. ese results are not shown in the article since they correspond to a comparison of the type of nutritional limitation achieved in both configurations (RAMOS and Erlenmeyer flasks).

Metabolomics

e crude extracts were dissolved in acetone: methanol (1:1 v/v) to a concentration of 4.5 mg mL^{-1} for subsequent analysis via LC/MS. e measurements were recorded using an UltiMate 3000 Series UHPLC (ermo Fischer Scientific, Waltham, MA, USA) with a C18 Acquity UPLC BEH column (2.1×50 mm, 1.7 µm; Waters, Milford, MO, USA) connected to an AmaZon speed ESI-Iontrap-MS (Bruker Daltonics, Bremen, Germany). e analyses were performed using a 0.6 mL min^{-1} flow rate, an injection volume of 2 µL, and 40 °C. For the mobile phase, H_2O+0.1% FA (Solvent A) and ACN+0.1% FA (Solvent B) were used with the following gradient: 5% B for 0.5 min, 5–100% B for 20 min and 100% B for 4.5 min. e UV/Vis data were recorded using a Diode Array Detector (DAD) between 190 and 600 nm, and the results were analyzed using the software Data Analysis 4.4 (Bruker Daltonics, Bremen, Germany).

e concentration of each sample was measured using the instrumental strings and conditions reported by [51] for metabolomic analyses. e raw data were preprocessed with a MetaboScape 2022 (Bruker Daltonics, Bremen, Germany) in the retention time range of 1.0–20 min, and the obtained features were dereplicated based on their accurate molecular weight and MS/MS spectra against the compounds as previously reported for the genus *Diaporthe* or *Phomopsis* in the Natural Product Atlas (NP Atlas) database [52]. For this purpose, MetaboScape automatically performed in silico MS/MS matching based on the InChI-encoded structures using the MetFrag algorithm without MS/MS reference data [53]. Subsequent analyses were performed using R and RStudio software (version 4.2.1).

Data processing and statistical analysis

Determination of IC_{50} values was conducted using R and RStudio software (version 4.2.1) with the ggplot2 and drc packages fitted to a four-parameter model. Hierarchical cluster analysis was performed using the Ggdendro package. Statistically significant di erences in production yields and IC_{50} values among the various treatments were analyzed using MINITAB® 19 statistical software. An ANOVA was performed to assess the variation in production yields and IC_{50} values.

Results

e e ect of nutrient limitations (i.e., carbon, nitrogen, and micronutrients) on the production of secondary metabolites during the fermentation of *Diaporthe caliensis* was assessed through the evaluation of three response variables: $Y_{P/S}$, IC_{50}, and $Y_{X/O2}$. e results are presented in two stages, where primary and secondary substrates are studied by the systematic formulation of culture media.

Assessment of nutrient limitation in submerged cultivation of *Diaporthe caliensis*: primary limitation

e impact of carbon limitation on the production of secondary metabolites by *Diaporthe caliensis* was investigated by evaluating two di erent concentrations of a rice starch solution as a substrate (15 and 25 g L^{-1} starch). Figure 1A shows the oxygen transfer rate (OTR) curves

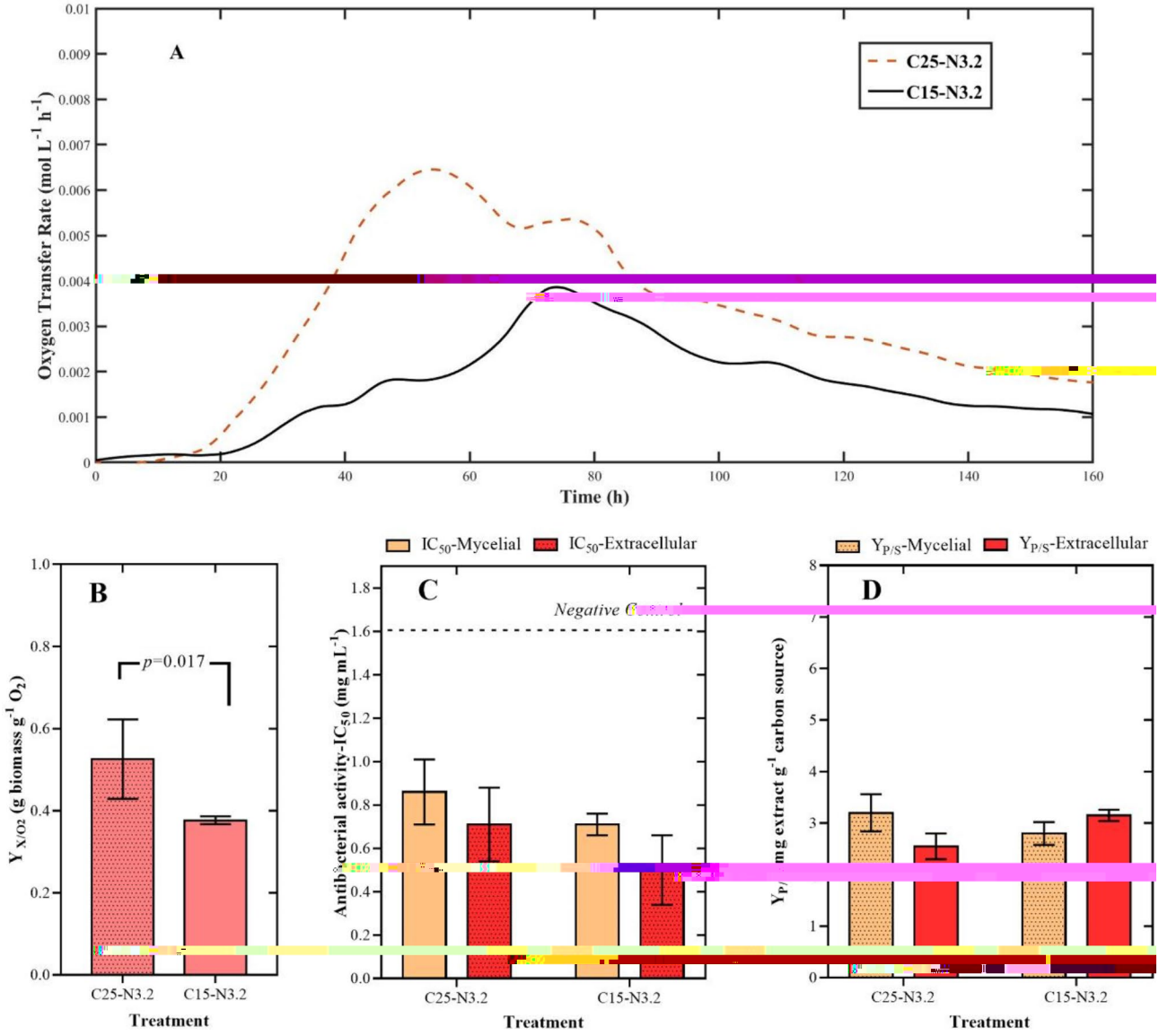


Fig. 1 E□ect of carbon source concentration (rice starch) on the performance of a batch culture of *Diaporthe caliensis* and its respective crude extracts: C15-N3.2 and C25-N3.2 treatments. **(A)** Changes in OTR (mol L^{-1} h^{-1}) over time for each treatment. **(B)** Calculated $Y_{X/O2}$ (g biomass g-1 O_2) for each treatment. **(C)** Antibacterial activity (IC_{50} in mg mL^{-1}) evaluated against *Staphylococcus aureus* (ATCC 25923). Statistically insigni□cant variable (p value > 0.05). **(D)** Calculated $Y_{P/S}$ (mg extract g-1 carbon source) for mycelial and extracellular extracts. *Statistically insigni□cant variable (p value > 0.05).* The antibacterial activities of the strains were contrasted with that of the negative control (black dotted line). Each value represents the mean of two biological replicates (n = 2). Cultivation conditions: **(A)** RAMOS® device (250 mL unba□ed shake □ask with 50 mL of medium, shaking diameter of 50 mm, and shaking frequency of 140 rpm); **(B)** and **D)** orbital shaker (250 mL unba□ed shake □ask with 80 mL of medium, shaking diameter of 20 mm and shaking frequency of 221 rpm)

for the two treatments, indicating no oxygen limitation throughout the cultivation period and consumption of more than one substrate, according to [54]. Online OTR measurements also revealed a delayed growth phase between 15 and 20 h for each treatment. While the C15-N3.2 treatment lacked the typical exponential increase (74 h for OTR max), the C25-N3.2 treatment reached the maximum peak at 53 h. e lower slope for the C15-N3.5 treatment may be associated with a metabolic difference in starch hydrolysis dynamics. In both cases, an OTR value of zero was not reached, probably due to the cell maintenance condition. Overall, lower total oxygen consumption was observed for the experiment with a low carbon source concentration.

Fig. 1B summarizes the biomass-oxygen yields for each treatment, indicating that a higher starch concentration led to increased biomass formation at the expense of transferred oxygen (0.53 g biomass g^{-1} O_2±0.1). Figure 1C and D present the antimicrobial activity (IC_{50}) and $Y_{P/S}$ results for the mycelial and extracellular extracts,

respectively, even though neither variable was significant (p value > 0.05). Notably, a lower biomass-oxygen yield ($Y_{X/O2}$) might result in greater secondary metabolite production. erefore, 15 g L^{-1} of the starch solution was chosen for further experiments (corresponding to treatment C15-N3.2)

During the second step, yeast extract was tested as the sole nitrogen source at three di erent concentrations, while the starch concentration was kept at 15 g L^{-1}. Figure 2A illustrates that, compared to those of the carbon-limiting experiments, the OTR curves of these experiments were very similar. After 16 h of fermentation, both the C15-N0.75 and C15-N1.5 treatments started their exponential growth phase and reached the maximum OTR values after 80 h. Similarly, changes in $Y_{X/O2}$ were not statistically significant (Fig. 2B). In contrast, significant di erences were found in some treatments for the $Y_{P/S}$ and IC_{50} variables. In general, the crude extract yield increased with decreasing concentrations of yeast extract (Fig. 2D). In all the treatments, the extracellular extracts had higher yields. e highest $Y_{P/S}$ value (6.9 mg of crude extract per g of starch ± 0.43) was obtained for the C15-N0.75 treatment.

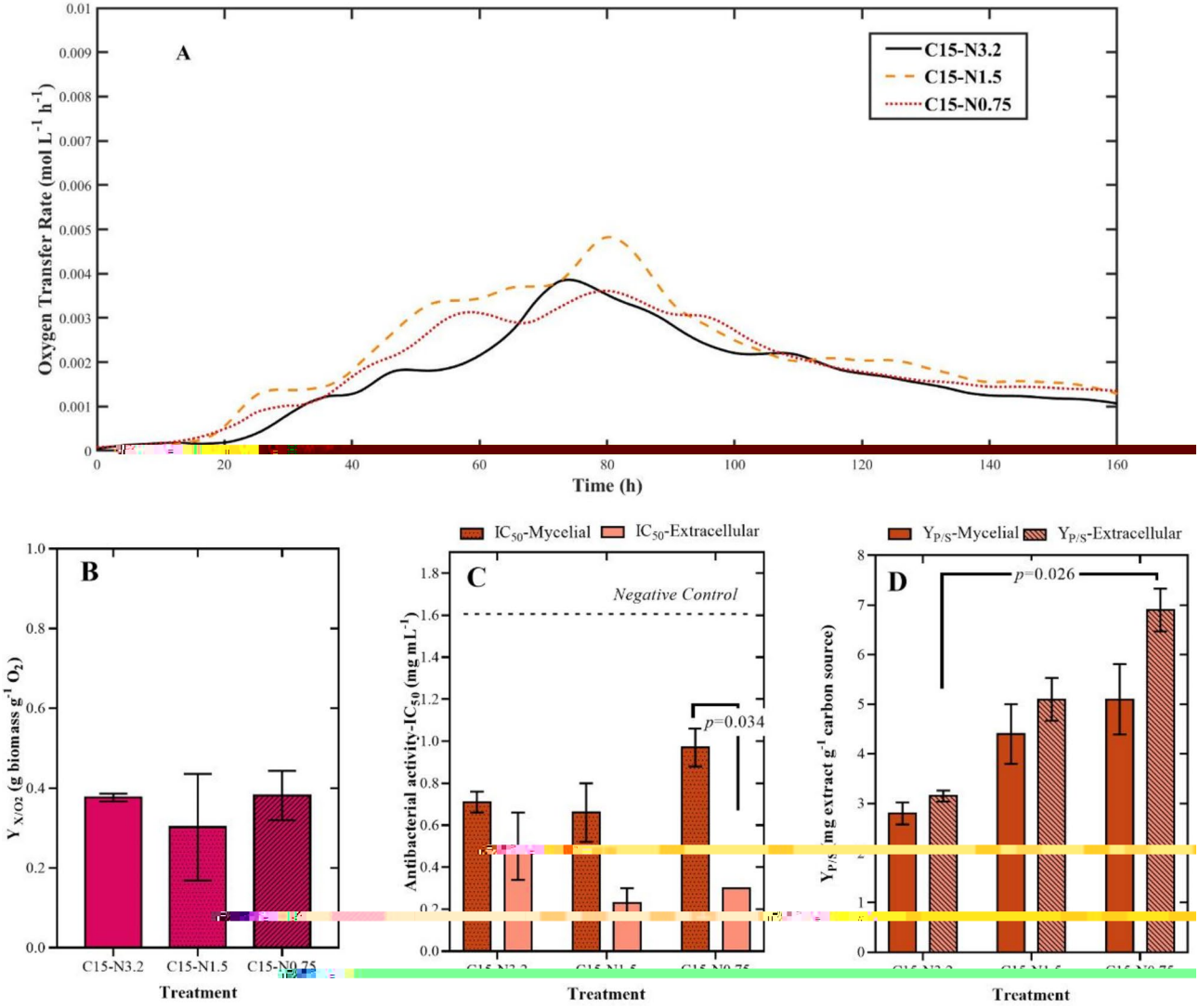
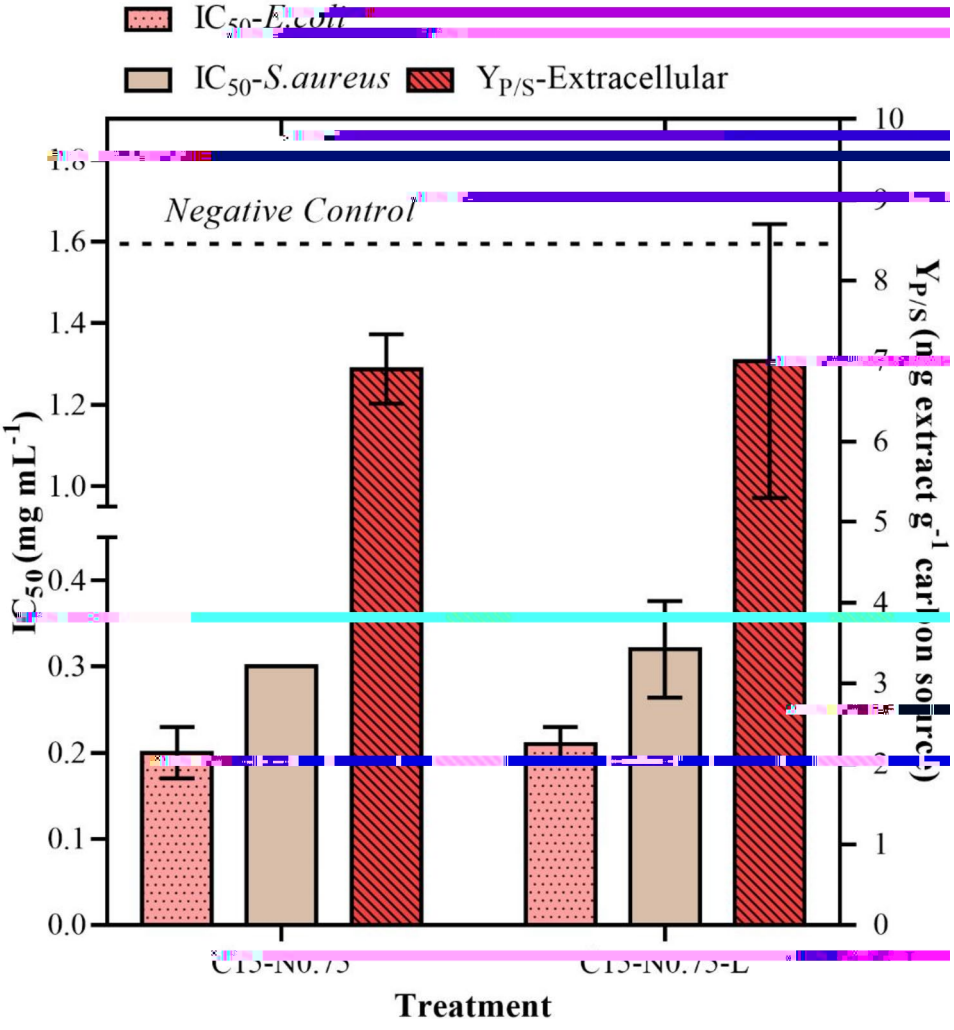


Fig. 2 E□ects of di□erent concentrations of nitrogen source (yeast extract) on the performance of a batch culture of *Diaporthe caliensis* and its respective crude extracts: C15-N3.2, C15-N1.5, and C25-N0.75 treatments. **(A)** Changes in the oxygen transfer rate (OTR, mol/L/h) over time for each treatment. **(B)** Biomass-oxygen yield ($Y_{X/O2}$, g biomass g^{-1} O_2) for each treatment. *Statistically insigni cant variable (p value > 0.05).* **(C)** Antibacterial activity (IC_{50} in mg mL-1) evaluated. *Staphylococcus aureus* (ATCC 25923). **(D)** Crude extract-substrate yield ($Y_{P/S}$, mg extract g-1 carbon source) for mycelial and extracellular extracts. The antibacterial activities of the strains were contrasted with that of the negative control (black dotted line). Each value represents the mean of two replicates ($n = 2$). Cultivation conditions: **(A)** RAMOS® device (250 mL unba□ed shake □ask with 50 mL of medium, shaking diameter of 50 mm, and shaking frequency of 140 rpm); **(B)** and **(D)** orbital shaker (250 mL unba□ed shake □ask with 80 mL of medium, shaking diameter of 20 mm and shaking frequency of 221 rpm)

For antibacterial activity (Fig. 2C), the mycelial extracts demonstrated less desirable IC_{50} values. e lowest IC_{50} value was 0.66 mg mL^{-1} ± 0.14, which was greater than the 0.3 mg mL^{-1} ± 0.0 obtained from the same treatment (C15-N1.5). Notably, a more pronounced di erence between the fractions was observed for the C15-N0.75 treatment, with IC_{50} values of 0.97 mg mL^{-1} ± 0.09 and 0.3 mg mL^{-1} ± 0.0 for the mycelial and extracellular extracts, respectively. Based on these findings, it was determined that *(i)* the extracellular extract was of greater interest, as the antibacterial activity of the mycelial extract was generally lower across all treatments. *(ii)* Treatment C15-N0.75 was chosen as the starting point for subsequent experiments due to its highest $Y_{P/S}$. In addition, *(iii)* for the subsequent steps, the variable $Y_{X/O2}$ was not considered, as it was insignificant in the context of the nitrogen limitation experiments.

e e ect of the type of nitrogen source was assessed by replacing yeast extract with corn steep liquor, using the C15-N0.75 treatment as a reference. e performance of each treatment was evaluated by comparing the $Y_{P/S}$ and IC_{50} values against two reference bacteria (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) as the response variables. When using corn steep liquor, $Y_{P/S}$ was 5.7 mg of extract per g of carbon source, which was lower than that when using yeast extract (6.9 mg of crude extract per g of starch±0.43) (Fig. 3). No clear di erences in the IC_{50} were detected between the treatments for either bacterial strain (0.21 mg mL^{-1} ± 0.02 for *Escherichia coli* and 0.32 mg mL^{-1} ± 0.056 for *Staphylococcus aureus*). Although the findings were not statistically significant, we decided to continue experimenting with corn steep liquor (C15-N0.75-L) because



it is a low-cost source of bioactive secondary metabolites that has been relatively unexplored.

Assessment of nutrient limitation in submerged cultivation of *Diaporthe caliensis*: secondary limitation

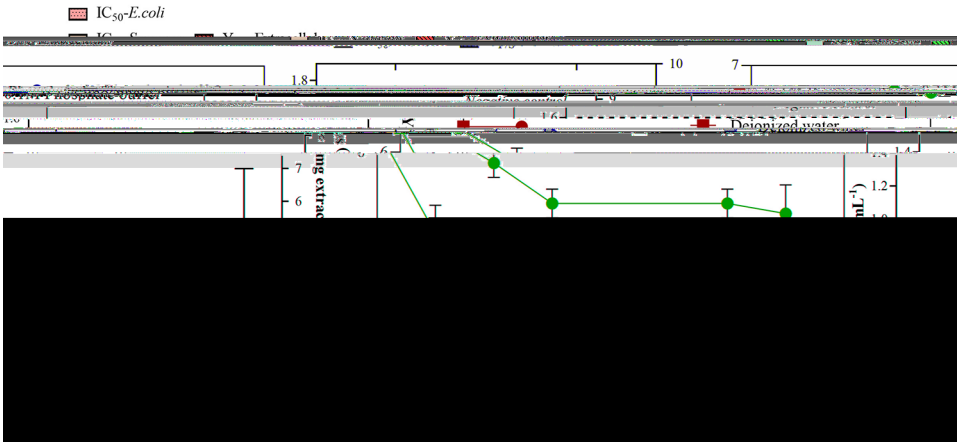


Fig. 5 E□ect of pH on the performance of a submerged batch culture of *Diaporthe caliensis* and their respective crude extracts: C15-N0.75-L-M (bu□ered) and C15-N0.75-L-M-W (not bu□ered) treatments. (A) Antibacterial activity (IC_{50} in mg mL^{-1}) of the extracellular extracts evaluated against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) and product-substrate yield ($Y_{P/S}$, mg extract g^{-1} carbon source). *Statistically insigni*ﬁ*cant variables (p value > 0.05)* were also included. (B) pH culture value over time (mean value of two replicates)

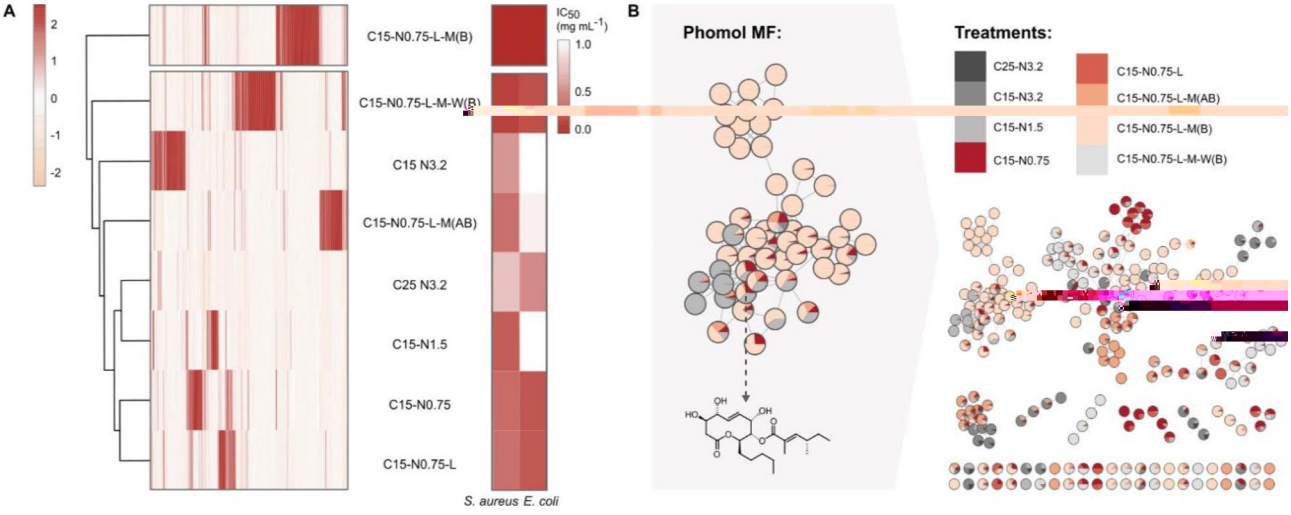


Fig. 6 (A) Heatmap following a hierarchical clustering of MS features detected in the extracellular crude extracts of all treatments derived from the cultivation of *Diaporthe caliensis* (left) using the complete linkage method and the Euclidean distance metric. The heatmap displays feature abundance values with hierarchical clustering of features and crude extracts obtained from di□erent media. The scaled and centered abundances are color coded from red (high abundance) to light orange (low abundance). Heatmap of the antibacterial activities (IC_{50} in mg mL^{-1}) of the extracellular crude extracts of all the treatments evaluated against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) (right). A heatmap with dendrograms was generated with the R package pheatmap. **(B)** Feature-based molecular networking (FBMN) plot of the compounds detected in all the treatment groups (right), with pie charts representing the treatments in which the spectra of the compounds were detected. The Molecular Family (MF) encompassing phomol-like features (left)

biological activity, resulting in a pH of 4.0 starting from the second day of cultivation.

Metabolomic analysis

To evaluate the e ect of media formulation on secondary metabolite production by *Diaporthe caliensis* during submerged cultivation in shaking flasks, all 7415 MS features detected in all the treatments were grouped by hierarchical clustering (HCA) after blank features removal.

e data were centered and scaled during preprocessing, resulting in normalized abundance values that indicate features with lower or higher intensities relative to the mean. ese standardized values are represented on the x-axis of the heatmap (Fig. 6A). Notably, treatment pairs such as C25-N3.2 and C15-N1.5, as well as C15-N0.75 and C15-N0.75-L, clustered together, indicating similarities in their metabolomes. Conversely, the remaining treatments formed distinct clades, highlighting substantial metabolome di erences. Notably, these metabolomic patterns did not directly correlate with the antibacterial properties against the evaluated bacterial strains (i.e., *Staphylococcus aureus* and *Escherichia coli*). In fact,

despite the metabolome being markedly altered in most cases, the antibacterial activities (IC_{50}) remained similar.

To further inspect the changes at the metabolome level depending on the media formulation, a feature-based molecular network (FBMN) was constructed. e analysis revealed that 237 features were grouped into 35 molecular families (MFs) with at least two clustered nodes (Fig. 6B) and 2670 singletons. e molecular families were di erentially distributed across the di erent treatments, even though a reduced chemical space was observed for the C25-N3.2 treatment compared to the other treatments. A decision was made to explore the chemical diversity within the obtained crude extracts by using CANOPUS to predict the respective natural product classes de novo based on the MS/MS spectra [55].

is analysis determined that the most abundant class of compounds corresponded to polyketides, although revealing a high chemical diversity within the major MFs. Accordingly, their respective features were classified as alkaloids, amino acids and peptides, carbohydrates, fatty acids, polyketides, shikimates, phenylpropanoids, terpenoids, and even some without annotation (Additional file 1). Out of the 2907 MS/MS features detected, only 39 were annotated, and 5 were traced back to di erent MFs (Additional file 2, Table 1), accounting for just 0.5% of total MS features and 1.3% at the MS/MS level.

After dereplication, phomol was identified and found to be absent in the C25-N3.2 treatment, but it appeared once the carbon and nitrogen contents were reduced, indicating a positive relationship between nitrogen limitation and phomol-like molecule production. However, phomol-like molecules were not detected in the C15-N0.75-L-M-W (B) treatment, which di ered only by the absence of bu er, leading to di erences in chemical space and pH by the end of fermentation. Accordingly, the highest production of phomol-like molecules was observed in the C15-N0.75-L-M (B) treatment, as shown in Fig. 6B, which also demonstrated higher production titers of phomol and its related derivatives compared to their production in solid oat medium, as previously reported (Additional file 2, Fig. S2) [37]. For phomodiol and chaetoaurin, which were grouped within MFs comprising 14 and 3 nodes respectively, a similar trend to phomol-like molecules was observed, showing higher abundance in the C15-N0.75-L-M (B) treatment. However, some features within the phomodiol MF appeared to be more prominent in treatments with higher carbon and nitrogen content. Meanwhile, phomophyllin D and E were produced widely across di erent treatments, though our FBMN analysis suggests that more complex terpenoid derivatives were almost exclusively produced in the C15-N0.75-L-M-W (B) treatment. Nevertheless, it is of utmost interest that the major MF in our FBMN analysis remains entirely unannotated. However, CANOPUS results suggest that the features within this MF are likely to be chemically diverse, indicating that the main SMs of *Diaporthe caliensis* are yet to be discovered.

Overall, both the HCA and FBMN analyses showed similar results, illustrating that the chemical space produced by *Diaporthe caliensis* was tailored by modifications in the culture medium. e highest number of MS/MS features was detected in cultures with a lower nitrogen content and supplemented with micronutrients. In addition, a greater chemical diversity was observed when the nitrogen content was reduced to its lowest level, resulting in a much richer metabolome beyond the previously identified polyketide-lactones produced by this fungus. Given the fact that the observed changes in the antibacterial e ects for the di erent treatments did not directly correlate with the production of phomol-related molecules, which were confined to a limited number of treatments, it is expected that other unidentified molecules are responsible for these biological e ects. e results of this study underscore that the chemical diversity produced by *Diaporthe caliensis* remains largely uncharted when compared to the known secondary metabolites of this genus, suggesting the potential for discovering other bioactive molecules beyond polyketide-lactones related to phomol.

Discussion

is work focused on the formulation of tailored media to improve the production of bioactive SMs during the cultivation of *Diaporthe caliensis*. Fungi from this genus are known for producing secondary metabolites with diverse biological activities, such as anti-inflammatory, antibacterial, cytotoxic, and neuroprotective e ects [31]. Despite the vast chemical diversity within the genus *Diaporthe*, to the best of our knowledge, no systematic study has been reported on the design of tailor-made media to improve the production of bioactive molecules. erefore, this study proposes a five-step approach for selecting an optimal culture medium to yield crude extracts with antibacterial activity.

e results presented herein suggested two trends: *(i)* primary metabolism was predominantly a ected when the carbon source concentration was modified, and *(ii)* altering the nitrogen source concentration (i.e., yeast extract) principally impacted secondary metabolite production. is observation is supported by monitoring changes on the $Y_{X/O2}$, IC_{50}, and $Y_{P/S}$ across the di erent treatments. Specifically, during the first step of this study, it was found that the higher concentrations of the starch solution led to an increased $Y_{X/O2}$ ratio and greater biomass formation (Additional file 3). In addition, the OTR curves (markedly Fig. 1A) indicated a tendency to consume multiple substrates, likely originating from both enzymatic transformation and thermal pretreatment,

during which starch is fractionated into dextrins and other simple sugars, thus leaving more than one substrate available. It is well understood that rapidly assimilable carbon sources are preferentially utilized during growth [56, 57]. Once depleted, more complex substrates are consumed, initiating the idiophase [57]. Consequently, a higher carbon source concentration delays the onset of the phase in which SMs are produced [58]. erefore, the antibacterial activity, $Y_{P/S}$ yield, and metabolomic profiles of the extracts from the C15-N3.2 and C25-N3.2 treatments did not di er significantly.

However, when the e ect of modifying the nitrogen source concentration was studied, it was found that lower levels of yeast extract resulted in greater yields of crude extract ($Y_{P/S}$). Although the antibacterial activity did not substantially improve when the concentration decreased from 3.2 g L^{-1} to 0.75 g L^{-1} nitrogen, a greater diversity of molecules was promoted, according to our metabolomics analyses. Generally, fungi respond to changes in nitrogen availability through very complex regulatory mechanisms [59]. For instance, *Aspergillus* spp. are known to produce SMs of interest (i.e., aflatoxin, ochratoxin, orsellinic acid, and spiroanthrones) when subjected to conditions of nitrogen repression or starvation [60–62]. It is possible that reduced nitrogen availability may accelerate the initiation of the idiophase, during which SMs are typically produced, as microorganisms can be sensitive to their own products during the trophophase [56].

e concentrations of carbon and nitrogen sources were first chosen to limit one nutrient over the other. For rice starch, the polymer content was measured by Lugol's iodine staining, while the sugar content was determined by HPLC to verify the experimental limitations. However, for nitrogen, no strategy was established to measure its content at the end of fermentation without interference from proteins or peptides secreted by the fungus. As a result, it could not be robustly confirmed whether nitrogen limitation was achieved in some of the treatments. As an indirect alternative, simple sugars were present in the C15-N0.75 treatment (see Additional file 4), suggesting that nitrogen could have been depleted by the end of the fermentation period. ese limitations highlight the need for future research employing more sensitive methods to confirm the nutritional conditions to which the fungus is exposed.

In addition to the nitrogen concentration, the e ect of changing the nitrogen source by substituting corn steep liquor with yeast extract was evaluated. e preliminary choice of corn steep liquor was because although it is a vastly available resource, it has not been frequently used in evaluating SM production [63]. However, any change in complex sources in the culture medium must be evaluated because di erent nutritional compositions can significantly influence metabolic pathways [64] and physiological and morphological responses [59, 65]. Although no significant di erences in the IC_{50}, $Y_{P/S,}$ or metabolome were found in this study, there was a change in the relative intensity of the phomol-like molecules (Additional file 5). is e ect of the nitrogen source on the production of a particular group of molecules has been reported for *Y. lipolytica*, where the substitution of yeast extract for corn steep liquor led to an increase in intracellular enzymes [63]. is phenomenon was explained by increased activity in the pyruvate carboxylation pathway, which suggested a redistribution of carbon flux [63].

Furthermore, we also evaluated the addition of two di erent supplementary solutions. e media containing solution B exhibited improved IC_{50} values compared to those with solution A alone (see Materials and Methods section) or a combination of both solutions. e magnesium in solution B is crucial for protein synthesis [66], while iron is essential for transcription, replication, and energy production [67]. Although the exact function of manganese is poorly understood, its significance in fungal homeostasis is well known [68]. Sodium and potassium are vital for maintaining osmotic balance, generating membrane potential, and regulating ion or molecule transport [69]. While both solutions e ectively promoted the production of antibacterial compounds, their combinations might have induced osmotic stress, leading to observable changes in the metabolome (Fig. 6A) and alterations in antibacterial activity, as evidenced by lower IC_{50} values against *Escherichia coli* observed with treatments supplemented with solutions A and B.

In the last step of this study, treatments were carried out with 0.05 M phosphate bu er and water. In both cases, the pH tended to decrease due to fungal metabolic activity. However, this change was much more pronounced in the absence of bu er. Although neither the IC_{50} nor the $Y_{P/S}$ ratio reflected these di erences, the metabolomic profiles were markedly di erent. For instance, the production of phomol-like molecules was greater in the C15-N0.75-L-M (B) bu er treatment (Additional file 5). is could be attributed to *(i)* the availability and consumption of nutrients, which depend on the pH of the medium, particularly for the absorption of iron [67], a component of solution B. us, di erent biosynthetic pathways might be stimulated, as some substrates are more readily utilized in one treatment compared to others. On the other hand, *(ii)* it is possible that the production of molecules such as phomol is pH dependent, similar to the polyketides hibarimicins A-G [70]. Although di erent molecules were produced, the crude extracts exhibited similar bioactivities. us, rather than focusing on a specific class of molecules, this metabolomics-guided strategy highlights how variations

in the culture conditions of *Diaporthe caliensis* can alter its metabolome and enhance desirable biological activities. Our in-depth metabolomics analysis demonstrated that the proposed methodology e ectively expanded the chemical diversity of *Diaporthe caliensis*, prompting the production of SMs besides the previously associated polyketide-lactones known from this fungus. Moreover, despite our dereplication strategy relying on in-silico annotation based on mass accuracy, isotopic pattern, and MS/MS spectra, the combination of di erent approaches revealed a high chemical diversity across all treatments.

e putative annotation of chaetoaurin, phomodiol, and phomophyllins, whose presumed biosynthetic origins were in agreement with the CANOPUS predictions further support this hypothesis [71–74]. In fact, these SMs are reported to exhibit diverse biological activities, with phomophyllins, in particular, belonging to the protoilludane, illudalane, and botryane sesquiterpenoid classes.

ese compound classes represent indeed promising targets for further development, as in the case of the melleolides produced by *Armillaria* spp. (Agaricales, Basidiomycota) and the anticancer lead compounds illudins from the genus *Omphalotus* (Agaricales, Basidiomycota) [75, 76].

In summary, this study developed a stepwise methodology for formulating culture media tailored to *Diaporthe caliensis*. By integrating metabolomic profiling, we gained insights into the chemical diversity of this fungus and its relationship with factors like carbon and nitrogen source concentrations, type of nitrogen source, supplemental solutions, and pH regulation. Among our findings, it is noted that the addition of micronutrients and the presence of bu er significantly influenced the production of polyketide-lactones related to phomol, setting the path towards future optimization of its production. In fact, this methodology not only facilitates the targeted discovery of novel molecules but also highlights the potential of this fungus for producing metabolites from distinct compound classes while maintaining desirable biological properties, which targeted purification, will be the endeavor of future studies.

Conclusions

is study presents a practical approach for designing tailored media based on nutritional limitations to enhance the production of antimicrobial SMs during the submerged fermentation of *Diaporthe caliensis*. By systematically analyzing key bioprocess parameters, we evaluated both metabolomic changes and biological activities in response to nutritional adjustments. is demonstrated that the biosynthetic potential of the studied fungus could be further explored by inducing specific nutritional stress conditions. Notably, our findings revealed that the antibacterial e ects observed across di erent treatments are not solely dependent on the production of phomol-like molecules, which were only significantly promoted in a limited number of treatments. is indicates that *Diaporthe caliensis* has the ability to produce a diverse range of antimicrobial compounds under varying conditions. Consequently, our study highlights the need for future research aimed at enhancing our understanding of the chemical and biosynthetic diversity of this rather unexplored fungal biofactory, which could be employed for the sustainable production of novel antimicrobial agents.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02567-y.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements
The genetic resource access contract RGE244-44#190 was provided by the Autoridad Nacional de Licencias Ambientales (ANLA) from the Ministerio de Ambiente y Desarrollo Sostenible in Colombia.

Author contributions
NC conceived and designed the research. NC, LH, LV, YO, DV, DD, and MM designed the experiments. LH, LV, YO, DV, DD, and MM performed the experiments and acquired the data. NC, LH, LV, DV, and EC analyzed and interpreted the data. LH, LV, and DV drafted the manuscript, which was critically revised by NC, EC, and MG. All authors read and approved the nal manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL.
This work was supported by the Ministerio de Ciencias, Tecnología e Innovación (MinCiencias), through the Sistema General de Regalias (SGR) code BPIN 2022000100068, and by the project titled "Incorporación de jóvenes investigadores e innovadores en las regiones para atención de demandas de nidas por los CODECTI de los departamentos de Chocó, Valle del Cauca, Cauca y Nariño". E. Charria-Girón was funded by the HZI POF IV Cooperativity and Creativity Project Call.

Data availability
Data is provided within the manuscript or supplementary information les.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Departamento de Ciencias Biológicas, Bioprocesos y Biotecnología. Facultad de Ingeniería, Diseño y Ciencias Aplicadas, Universidad Icesi, Cali, Colombia

[2]Department Microbial Drugs, Helmholtz Centre for Infection Research (HZI), German Centre for Infection Research (DZIF), Partner Site Hannover-Braunschweig, Inho□enstrasse 7, 38124 Braunschweig, Germany
[3]Institute of Microbiology, Technische Universität Braunschweig, Spielmannstraße 7, 38106 Braunschweig, Germany
[4]Centro BioInc, Universidad Icesi, Cali, Colombia

Received: 6 August 2024 / Accepted: 16 October 2024
Published online: 05 December 2024

References

1. Sharrar AM, Crits-Christoph A, Méheust R, Diamond S, Starr EP, Ban□eld JF. Bacterial secondary metabolite biosynthetic potential in soil varies with phylum, depth, and vegetation type. Appl Environ Microbiol. 2020;11.
2. Singh BP, Rateb ME, Rodriguez-Couto S, De Moraes Polizeli LT, Li M, Editorial WJ. Microbial secondary metabolites: recent developments and technological challenges. Front Microbiol. 2019;10.
3. Keller NP. Fungal secondary metabolism: regulation, function and drug discovery. Nat Rev Microbiol. 2019;17:167–80.
4. Guerriero G, Berni R, Muñoz-Sanchez JA, Apone F, Abdel-Salam EM, Qahtan AA et al. Production of plant secondary metabolites: examples, tips and suggestions for biotechnologists. Genes (Basel). 2018;6:9.
5. Teoh ES. Secondary Metabolites of Plants. Medicinal Orchids of Asia. 2016. pp. 59–73.
6. Seyedsayamdost MR. Toward a global picture of bacterial secondary metabolism. J Ind Microbiol Biotechnol. 2019;46:301–11.
7. Santamaria G, Liao C, Lindberg C, Chen Y, Wang Z, Rhee K et al. Evolution and regulation of microbial secondary metabolism. Elife. 2022;11.
8. Liu Y, Bastiaan-Net S, Wichers HJ. Current understanding of the structure and function of Fungal Immunomodulatory proteins. Front Nutr. 2020;7.
9. Ramírez-Villalobos JM, Gomez-Flores R, Velázquez-Flores PV, Morán-Santibáñez KS, Tamez-Guerra P, Pérez-González O et al. E□ect of Culture conditions of Lophocereus Marginatus Endophytic Fungi on yield and anticancer and antioxidant activities. Int J Environ Res Public Health. 2023;20.
10. Kousar R, Naeem M, Jamaludin MI, Arshad A, Shamsuri AN, Ansari N et al. Exploring the anticancer activities of novel bioactive compounds derived from endophytic fungi: mechanisms of action, current challenges and future perspectives. Am J Cancer Res. 2022;12.
11. Fasinu PS, Okoye FBC, Abiodun OO, Kamdem RST, Ogbole OO. Editorial: fungal bioactive metabolites of pharmacological relevance. Front Pharmacol. 2022;13.
12. Jakubczyk D, Dussart F. Selected fungal natural products with antimicrobial properties. Molecules. 2020;25.
13. Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov. 2021;20:200–16.
14. Craney A, Ahmed S, Nodwell J. Towards a new science of secondary metabolism. J Antibiot. 2013;66:387–400.
15. Yang X, Wu P, Xue J, Li H, Wei X. Cytochalasans from endophytic fungus *Diaporthe sp.* SC-J0138. Fitoterapia. 2020;145.
16. Yu JJ, Yang HX, Zhang FL, He J, Li ZH, Liu JK, et al. Secondary metabolites from cultures of the kiwi-associated fungus *Diaporthe phragmitis* and their antibacterial activity assessment. Phytochem Lett. 2021;46:143–8.
17. Patil RH, Patil MP, Maheshwari VL. Bioactive secondary metabolites from endophytic Fungi: a review of Biotechnological Production and their potential applications. Stud Nat Prod Chem. 2016;49:189–205.
18. Srivastava N, Srivastava M, Ramteke PW, Mishra PK. Solid-state fermentation strategy for microbial metabolites production: An overview. New and Future Developments in Microbial Biotechnology and Bioengineering: Microbial Secondary Metabolites Biochemistry and Applications. 2019. pp. 345–54.
19. Webb C. Design aspects of Solid State Fermentation as Applied to Microbial Bioprocessing. J Appl Biotechnol Bioeng. 2017;4.
20. Aidoo R, Kwo□e EM, Adewale P, Lam E, Ngadi M. Overview of single cell protein: production pathway, sustainability outlook, and digital twin potentials. Trends Food Sci Technol. 2023;138:577–98.
21. Zhao YS, Eweys AS, Zhang JY, Zhu Y, Bai J, Darwesh OM et al. Fermentation a□ects the antioxidant activity of plant-based food material through the release and production of bioactive components. Antioxidants. 2021;10.
22. Bode HB, Bethe B, Höfs R, Zeeck A. Big e□ects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem. 2002;3:619–27.
23. Rodríguez Martín-Aragón V, Trigal Martínez M, Cuadrado C, Daranas AH, Fernández Medarde A. Sánchez López JM. OSMAC Approach and Cocultivation for the induction of secondary metabolism of the Fungus *Pleotrichocladium opacum*. ACS Omega. 2023;8.
24. Meena MG, Lane MJ, Tannous J, Carrell AA, Abraham PE, Giannone RJ et al. A glimpse into the fungal metabolomic abyss: novel network analysis reveals relationships between exogenous compounds and their outputs. PNAS Nexus. 2023;2.
25. Kottmeier K, Müller C, Huber R, Büchs J. Increased product formation induced by a directed secondary substrate limitation in a batch *Hansenula polymorpha* culture. Appl Microbiol Biotechnol. 2010;86:93–101.
26. Youngquist JT, Rose JP, P□eger BF. Free fatty acid production in *Escherichia coli* under phosphate-limited conditions. Appl Microbiol Biotechnol. 2013;97.
27. Azzollini A, Boggia L, Boccard J, Sgorbini B, Lecoultre N, Allard PM et al. Dynamics of metabolite induction in fungal co-cultures by metabolomics at both volatile and non-volatile levels. Front Microbiol. 2018;9.
28. Lv G, Xu Y, Tu Y, Cheng X, Zeng B, Huang J et al. E□ects of Nitrogen and Phosphorus limitation on fatty acid contents in *aspergillus oryzae*. Front Microbiol. 2021;12.
29. Rodríguez-Torres M, Romo-Buchelly J, Orozco-Sánchez F. E□ects of oxygen transfer rate on the L(+) lactic acid production by *Rhizopus Oryzae* NRRL 395 in stirred tank bioreactor. Biochem Eng J. 2022;187.
30. Dinger R, Lattermann C, Flitsch D, Fischer JP, Kosfeld U, Büchs J. Device for respiration activity measurement enables the determination of oxygen transfer rates of microbial cultures in shaken 96-deepwell microtiter plates. Biotechnol Bioeng. 2022;119.
31. Jiang L, Ma Q, Li A, Sun R, Tang G, Huang X et al. Bioactive secondary metabolites produced by fungi of the genus *Diaporthe (Phomopsis)*: structures, biological activities, and biosynthesis. Arab J Chem. 2023;16.
32. Liu X, Locasale JW, Metabolomics. A primer. Trends Biochem Sci. 2017;42:274–84.
33. Hoyos LV, Chaves A, Grandezz D, Medina A, Correa J, Ramirez-Castrillon M, et al. Systematic screening strategy for fungal laccase activity of endophytes from *Otoba gracilipes* with bioremediation potential. Fungal Biol. 2023;127:1298–311.
34. Mendez MJ, Caicedo NH, Salamanca C. *Trametes Elegans*: a fungal endophytic isolate from *Otoba gracilipes* as biocatalyst for natural □avors production. N Biotechnol. 2018;44.
35. Caicedo NH, Davalos AF, Puente PA, Rodríguez AY, Caicedo PA. Antioxidant activity of exo-metabolites produced by *Fusarium oxysporum*: an endophytic fungus isolated from leaves of *Otoba gracilipes*. Microbiologyopen. 2019;8.
36. Charria-Girón E, Espinosa MC, Zapata-Montoya A, Méndez MJ, Caicedo JP, Dávalos AF et al. Evaluation of the antibacterial activity of crude extracts obtained from cultivation of native endophytic Fungi belonging to a Tropical Montane Rainforest in Colombia. Front Microbiol. 2021;12.
37. Charria-Girón E, Marin-Felix Y, Beutling U, Franke R, Brönstrup M, Vasco-Palacios AM et al. Metabolomics insights into the polyketide-lactones produced by *Diaporthe caliensis* sp. nov., an endophyte of the medicinal plant *Otoba gracilipes*. Pidot SJ, editor. Microbiol Spectr. 2023.
38. Dolecek C, Shakoor S, Basnyat B, Okwor T, Sartorius B. Drug-resistant bacterial infections: we need urgent action and investment that focus on the weakest link. PLoS Biol. 2022;20.
39. Salam MA, Al-Amin MY, Salam MT, Pawar JS, Akhter N, Rabaan AA, et al. Antimicrobial Resistance: a growing serious threat for Global Public Health. Healthcare (Switzerland; 2023.
40. Tang KWK, Millar BC, Moore JE. Antimicrobial Resistance (AMR). Br J Biomed Sci. 2023;80
41. Duetz WA, Witholt B. Oxygen transfer by orbital shaking of square vessels and deepwell microtiter plates of various dimensions. Biochem Eng J. 2004;17:181–5.
42. Philip P, Meier K, Kern D, Goldmanns J, Stockmeier F, Bähr C et al. Systematic evaluation of characteristics of the membrane-based fed-batch shake □ask. Microb Cell Fact. 2017;16.
43. Sáez-Plaza P, Navas MJ, Wybraniec S, Michałowski T, Asuero AG. An overview of the Kjeldahl Method of Nitrogen Determination. Part II. Sample Preparation, Working Scale, Instrumental Finish, and Quality Control. Crit Rev Anal Chem. 2013;43.
44. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of structural carbohydrates and lignin in biomass determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory (NREL); 2010.

45. Harms K, Surup F, Stadler M, Stchigel AM, Marin-felix Y. Morinagadepsin, a depsipeptide from the fungus morinagamyces vermicularis gen. Et comb nov Microorganisms. 2021;9.
46. Kemkuignou B, Lambert C, Stadler M, Kouam Fogue S, Marin-Felix Y. Unprecedented antimicrobial and cytotoxic polyketides from cultures of *Diaporthe Africana* sp. nov. J Fungi. 2023;9:781.
47. Kemkuignou B, Schweizer L, Lambert C, Anoumedem EGM, Kouam SF, Stadler M, et al. New polyketides from the liquid culture of *Diaporthebreyniae sp.* nov. (*Diaporthales, Diaporthaceae*). MycoKeys. 2022;90:85–118.
48. Pfütze S, Khamsim A, Surup F, Decock C, Matasyoh JC, Stadler M. Calamene-type Sesqui-, Mero-, and Bis-sesquiterpenoids from cultures of *Heimiomyces sp*