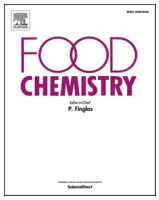
Food Chemistry 473 (2025) 143051

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Insights into PUFA and oxylipin production from incubation of *Aspergillus novoparasiticus* in sugarcane juice

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ARTICLE INFO

Keywords:
PUFA
Oxylipin
Kojic acid
Dereplication
Aspergillus section *Flavi*

ABSTRACT

Understanding the metabolism of toxigenic fungi in food is crucial for public health. Fungi of the *Aspergillus* section *Flavi* are widespread in tropical and subtropical regions, infecting and producing mycotoxins in peanuts, nuts, cereals, and fruits. Classical Molecular Networking on the Global Natural Products Social Molecular Networking (GNPS) platform was applied to organize and guide the analysis of polyunsaturated fatty acids (PUFAs) and oxylipins, which are strongly associated with chemical communication between fungi and host plants during infection. Dereplication and molecular networking enabled visualization of the metabolome of *A. novoparasiticus* (ITAL-Y174 strain) incubated in sugarcane juice to mimic the fungal chemical response to plant lipids. The ITAL-Y174 strain produced PUFAs that differed from those of sugarcane. Oxylipins were detected early in the incubation (PGE_1, 9,10-DiHOME, 13*S*-HODE). No known mycotoxins for *A. novoparasiticus* were detected, and kojic acid was identified, indicating a novel pathway to produce this important industrial natural product.

1. Introduction

Communication between plants and fungi occurs through lipids, particularly oxylipins, biosynthesized from polyunsaturated fatty acids (PUFA). These oxygenated and unsaturated lipids, the oxylipins, alter various internal biological responses and neighboring individuals. PUFAs regulate essential processes for the survival of *Aspergillus spp.* (Gessler et al., 2017) and there are reports that fungi can use oxylipins not only for their development but also to promote changes in their host's responses, supporting infection success, mycotoxin production, and host defense impairment, among others (Fischer & Keller, 2016; Gessler et al., 2017). The functions of oxylipins in plants and mammals are well established, while the concept of host-pathogen cross-communication has only recently emerged. The discovery of several microorganisms that utilize oxylipins to contribute to their infection is important to know some patterns that may occur, leading to the understanding of oxylipin and mycotoxin production of the biogenesis pathway and the maintenance of fungal development in the plant host (Noverr et al., 2003).

Brazil has incredible biodiversity of fungi, and the genus *Aspergillus* has been widely studied extensively due to its significant impact and importance in various research areas, *e.g.*, as pathogens in animals and humans, as spoilage agents in food, as producers of toxic metabolites or, on the other hand, as beneficial microorganisms in food fermentation

Abbreviations: 1D-NMR, one-dimensional nuclear magnetic resonance; CID, collision-induced dissociation; CMN, classical molecular networking; ESI, electrospray ionization; GC-EI-Q, gas chromatography with electron impact ionization coupled to quadrupole mass analyzer; GC–MS, gas chromatography coupled to mass spectrometry; GNPS, global natural products social molecular networking; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; LDS, linoleate dioxygenase; LOX, lipoxygenase; NMR, nuclear magnetic resonance; PG, prostaglandin; Ppo, psi factor-producing oxygenase; PUFA, polyunsaturated fatty acid; QTOF, quadrupole tandem time-of-flight mass analyzer; UPLC-HRMS-MS^2, ultra-performance liquid chromatography coupled to high-resolution mass spectrometry tandem.

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https://doi.org/10.1016/j.foodchem.2025.143051
Received 16 September 2024; Received in revised form 8 January 2025; Accepted 22 January 2025
Available online 26 January 2025

and biotechnological applications (Amaike & Keller, 2011; Katsurayama et al., 2018; Martins et al., 2017). The interaction between a fungus and a plant host shows a certain predominance of specific fungi and specific plants, even from the same group (*Aspergillus* section *Flavi*). It is hypothesized that the interactions must occur due to some components of the substrate (plant host) and the ability of the fungus to produce a series of metabolites that may be specific to the species. PUFA and oxylipin are the main compounds involved in the interaction (Fischer & Keller, 2016; Tsitsigiannis & Keller, 2007).

In addition, many species of *Aspergillus* section *Flavi* are known to produce kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone) (El-Aziz, 2013; Frisvad et al., 2019; Sharma et al., 2023), including *A. novoparasiticus* (Abdallah et al., 2020). Kojic acid has unique properties and is important in the cosmetic, food, and pharmaceutical industries (Chib et al., 2023). Futamura et al. (2001) reported that the production cost of kojic acid is about $10/kg. It must be reduced to less than $2/kg before being used in many applications (Yamada et al., 2014). Therefore, an efficient and cost-effective kojic acid production process would be desirable.

In this work, we monitored the PUFA and oxylipin profile of *A. novoparasiticus* strain ITAL-Y174 in the incubation using sugarcane juice, by Ultra-Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry tandem analysis (UPLC-HRMS-MS^2). We have discussed the production and occurrence of PUFAs and oxylipins detected in the sugarcane and *A. novoparasiticus* metabolome using HRMS-MS^2 dereplication in the GNPS online platform, including the organization of all incubation experiments analyzed by HRMS in the form of Classical Molecular Networking (CMN). We did not find any mycotoxin that has been reported in the literature for *A. novoparasiticus,* and that was not a target compound for evaluating the chemical response of strain ITAL-Y174, unlike the lipid profile of sugarcane, which is more valuable for understanding the chemical communication between a fungus and plant host.

2. Experimental section

2.1. General analytical equipment

The polar samples were analyzed at the Analytical Centre of the Chemistry Institute of the University of São Paulo using a Shimadzu liquid chromatograph coupled to the Bruker QTOF Maxis 3G spectrometer from Bruker Daltonics, operating in positive mode with a mass resolution of 60,000 FWHM. All chromatographic separations to acquire mass spectra were performed using a reverse phase column Kinetex EVO C18 (100 mm × 2.1 mm, 2.6 μm, 100 Å). Additional mass spectrometry analyses were performed in the UPLC-HRMS-MS^2 system Shimadzu Nexera X2 chromatography system coupled with a Bruker micrOTOF-QII-ESI mass spectrometer in positive mode at 18,000 FWHM mass resolution at NIPE, UNIFESP, Diadema *campus* (SP).

The spectral data from Gas Chromatography with Electron Impact Ionization coupled to Quadrupole (GC-EI-Q, also GC–MS) were recorded in Agilent 6850 gas chromatograph coupled to Agilent 5975C MSD mass spectrometry, equipped with an HP5-MS capillary column (Agilent, length 30 m, ID 250 μm, 0.25 μm film thickness). The spectra were recorded at the Institute of Biosciences (IB) of the University of São Paulo.

Semi-preparative High-Performance Liquid Chromatography (HPLC) system: Shimadzu liquid chromatograph equipped with LC-20 AT pumps, DGU-20 A3 degasser, SIL-20A automatic injector, and SPD-M20A diode array detector (60 mm flow cell). The chromatograph was equipped with an automatic collector to perform the purification. A reverse phase column Kinetex EVO C18 (10.0 × 250 mm, 5 μm, 100 Å) was used for the separation. The purification of kojic acid was conducted at CESM-ICAQF, UNIFESP, Diadema *campus* (SP).

The one-dimensional Nuclear Magnetic Resonance (1D-NMR) spectra of ^{1}H and ^{13}C were recorded with a Bruker Advance III Ultrashield 300 spectrometer at 300 MHz for the proton and 75 MHz for the carbon nuclei. The experiments were recorded at 25 °C with BCU 0.5 and a 5.0 mm TXI probe. The experiments were performed at CESM-ICAQF, UNIFESP, Diadema *campus* (SP).

2.2. Incubation of A. Novoparasiticus strain ITAL-Y174 in sugar cane juice

A sample of sugarcane juice was obtained from a local market near ITAL (Campinas, SP). The fresh juice was filtered through simple filter paper and autoclaved at 121 °C for 15 min. The sterilized juice (100 mL) was transferred to 500 mL Erlenmeyer flasks and covered with hydrophobic cotton and gauze plugs. The strain ITAL-Y174 of *A. novoparasiticus* (= 86IT-SGC strain reported in Silva et al. (2022), GenBank accession numbers CaM = ON643029; BenA = ON642981 and RPB2 = ON642933) was inoculated into the liquid medium of sugarcane juice and the incubation experiment was carried out at 25 °C in a shaker (140 rpm). The metabolism of the ITAL-Y174 strain was studied through daily extraction over a period of 7 days. The incubation experiment was duplicated, with samples labeled as *Ycdc* and *Yga* available in data set MSV000090498 (see section 2.6). A similar experiment was conducted with a 28-day incubation to purify kojic acid.

2.3. Preparation of extracts for chromatographic and spectrometric analysis

Daily extraction of incubation periods and sugarcane extract was performed thrice with ethyl acetate, using ultrasonication for 10 min. The organic extracts were concentrated in a rotary evaporation concentrator. Each extract was redissolved in methanol and ultrapure water (9:1, *v/v*) three times for liquid/liquid partition with hexane (1:1, *v/v*). Finally, the hydromethanolic and hexanic phases were concentrated and analyzed by UPLC-HRMS-MS^2 (Bruker QTOF Maxis 3G Bruker Daltonics) and GC–MS (Agilent 6850 coupled with Agilent 5975C MSD), respectively.

2.4. UPLC-HRMS-MS^2 analysis

The hydromethanolic phases were prepared at an initial concentration of 10 mg/mL (MeOH:H_2O, 1:1 *v/v*) and centrifuged at 5 × 10^6*g*-force for 10 min. The final concentration for injection into the chromatograph was 500 μg/mL. The analyses were performed in gradient mode with a C18 column (100 mm × 2.1 mm, 2.6 μm, 100 Å), mobile phase A (H_2O + 0.2 % formic acid), and B (ACN), a flow rate of 300 μL/min and a constant temperature of 50 °C in the column chamber. UV–Vis detection was performed at 4.6 Hz in a window from 200 to 700 nm (PDA). The polarity gradient of the mobile phase was adjusted according to time and percentage of organic phase B as follows: 0 to 1 min at 5 % B, 1 to 6 min from 5 % to 25 % B, 6 to 8 min from 25 % to 40 % B, 8 to 10 min from 40 % to 60 %, 10 to 12 min from 60 % to 100 % B, 12 to 14 min at 100 % B, 14 to 15 min from 100 % to 5 % B, constant at 5 % for 3 min.

All spectral data were obtained using HRMS with Electrospray Ionization (ESI) and Quadrupole tandem Time-of-Flight (QTOF) mass analyzer (Bruker QTOF Maxis 3G Bruker Daltonics). Ionization was performed for *m/z* 80 Da to 1200 Da in positive mode at 4500 V at a capillary voltage and an end plate offset of 500 V, drying gas (N_2) at 8.0 mL/min, a pressure of 4.0 bar and a temperature of 200 °C. Two precursor ions were selected for fragmentation by auto-MS/MS. Collision-induced dissociation (CID) was performed with an energy ramp of 20 eV to 45 eV, varying from 80 Da to 1200 Da, depending on the ions with an *m/z* ratio. The line and profile spectra of the Base Peak Chromatogram in HRMS were detected at a 2 × 4 Hz ratio with smart exclusion of 3 spectra. The ionization methodology followed the protocol of Aron et al. (2020) with adaptations. The HRMS calibration was performed with sodium formate clusters in positive mode with an internal error below 2 ppm in Enhanced Quadratic mode.

2.5. Apolar compounds analysis by GC–MS

The apolar phase (hexane fraction) was analyzed by gas chromatography on Agilent GC 6850 coupled to an Agilent 5975C MSD mass spectrometer containing a HP-5MS column with a length of 30 m × 0.25 mm and a 0.25 μm film. The samples (0.1 mg) were derivatized with *N*, *O*-Bis-(trimethylsilyl)-trifluoroacetamide (50 μL) in pyridine (50 μL), both from Sigma-Aldrich®, and the reaction was performed at 70 °C in a dry bath for 1 h. After this procedure, the sample injection volume was 1 μL in splitless mode with helium as the carrier gas at a 1 mL/min rate. The method used had an initial oven temperature of 100 °C, which was maintained for 5 min, and a heating rate of 5 °C/min until a temperature of 320 °C was reached, which was maintained for a further 8 min at the end of the heating process. The injector, quadrupole, and ion source temperatures were 300 °C, 180 °C, and 280 °C, respectively (Carvalho et al., 2021). Mass spectra were obtained by electron impact ionization at 70 eV in full scan acquisition mode in the *m/z* range of 50–800 Da and a detection rate of 2.66 scans/s according to the above reference. GC–MS analyses were performed at the USP Biosciences Institute in collaboration with Prof. Dr. Marcelo J. P. Ferreira.

2.6. Spectral organization and dereplication

The Global Natural Products Social Molecular Networking (GNPS) online platform (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) (Wang et al., 2016) organized all UPLC-HRMS-MS^2 data in the form of Classical Molecular Networking (CMN) and performed spectral data alignment for dereplication using its spectral library. The parameters for clustering the high-resolution mass spectra and dereplication are listed below.

The first step was the conversion of manufacturer/raw data into 'mzXML' using MSConvert® (Chambers et al., 2012) and uploading through WinSCP® (Wang et al., 2016). The experimental "Precursor Ion Mass Tolerance" and "Fragment Ion Mass Tolerance" were compared within a range of ±0.02 Da for ".mzXML" data. The experimental ions detected in HRMS were clustered using the MSCluster algorithm (Frank et al., 2008) with a "Minimum Cluster Size" of 2 spectra and each cluster was compared with a "Minimum Pair of cosines" of 0.7, "Network TopK" of 10, "Minimum Matched Fragment" of 3 ions with a Maximum shift" of 1999 Da and for the "Maximum Connected Component Size" of 100. For dereplication in GNPS, the workflow was set with "Library Seach Min Matched Peaks" of 4 and a "Score Threshold" of 0.5. The molecular network created in the GNPS platform was processed with Cytoscape (Shannon et al., 2003).

Annotations of metabolites detected by GC–MS were performed by comparing the retention time of standards and the NIST spectral library (v2.0, 2008) using Match and R-Match comparison values above 900 (Carvalho et al., 2021).

All data were deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) database (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). The incubation was performed in duplicate (samples coded as *Ycdc* and *Yga*), and the two sets of analysis of ITAL-Y174 incubation extracts from the first to the seventh day (d1 to d7) in HRMS can be found under data set MSV000090498 (doi:10.25345/C5XD0R28W). All GC–MS can be found under data set MSV000088004 (doi:10.25345/C5954J).

2.7. Purification of kojic acid

The polar main component of the *A. novoparasiticus* ITAL-Y174 strain was purified using a Shimadzu semi-preparative HPLC system. The mobile phase was polarized with water acidified with 0.2 % acetic acid (A) and a mixture of acetonitrile:methanol (1:1, *v/v*) (B) according to the time and percentage of organic phase (B) in a gradient: 0–2 min, 10 % B; 2–8 min, 10–25 % B; 8–16 min, 25 %; 16–18 min, 25–50 % B; 18–19 min, 50–100 % B, 19–21 min, 100 % B; 21–22 min, 100–10 % B and constant to 25 min.

Kojic acid: ESI-(+)-HRMS-MS^2 $[M + H]^+$ 143.0345 Da (calcd. For $C_6H_6O_4 + H^+$ 143.0339). Acetone-d_6 in ^{1}H-RMN 300 MHz, δ_H: 6.44 (s, 1H, H-3), 7.96 (s, 1H, H-6), 4.44 (s, 2H, H-7). ^{13}C-RMN 75 MHz, δ_C: 169.8 (C-2), 109.8 (C-3), 174.7 (C-4), 146.8 (C-5), 138.8 (C-6), 61.0 (C-7). ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) acquired in acetone-d_6. The chemical shifts based on Zeringue Jr. et al. (1999): DMSO-d_6, ^{1}H NMR (500 MHz), and ^{13}C NMR (150 MHz).

3. Results and discussion

The *A. novoparasiticus* ITAL-Y174 strain was chemically monitored from the first day to the seventh day of incubation using sugarcane juice as a medium culture. This experiment aimed to mimic *in vitro* the initial chemical responses of *A. novoparasiticus* in the presence of sugarcane lipids without mimicking an *in vivo* growth process in the host plant.

GC–MS and dereplication with the NIST14 database investigated the variation in the lipid fraction. UPLC-HRMS-MS^2 was used to analyze the polar fraction of sugarcane and incubation periods. Compared to GC–MS, the medium culture and incubation period polar fractions were particularly informative in UPLC-HRMS. They provided more information on the PUFA and oxylipin profile of sugarcane and *A. novoparasiticus*. This helped in the evaluation of fungal oxylipin and PUFA production. In addition, the dereplication process effectively discovered the secondary metabolite profile of *A. novoparasiticus*.

Monitoring of *A. novoparasiticus* in HPLC-DAD also revealed the presence of a major polar compound produced after 24 h of incubation and accumulated until the seventh day. Unfortunately, the GNPS workflow could not annotate this compound, then isolated by HPLC-DAD and subsequently identified as kojic acid by NMR and HRMS-MS^2. The results show that strain ITAL-Y174 can efficiently produce kojic acid by consuming glucoside from sugarcane juice (Sharma et al., 2023). The UPLC-HRMS and UV chromatograms showed that the strain does not consume phenolic compounds or phytosteroids as observed in GC–MS, as shown and discussed below.

3.1. Compounds detected by ultra performance liquid chromatography coupled to high-resolution mass spectrometry tandem (UPLC-HRMS-MS^2)

The incubation extracts were analyzed by UPLC-HRMS and organized using the GNPS workflow to create the Classical Molecular Networking (CMN) and perform dereplication to identify the chemical profile of *A. novoparasiticus* and the sugarcane metabolites. The CMN and HRMS spectra annotations were organized by removing blank, artifact, and impurity spectra. In this work, a CMN was applied to obtain a comprehensive overview of the changes in fungal metabolism in the presence of the plant host metabolites, especially the lipid changes. The GNPS workflow organization of all data includes each incubation period and sugarcane juice extract. This led to the visualization of compounds that disappeared and the discovery of some compounds that appeared during each day of the incubation experiment, which are presented and discussed below.

Glycosides (mono-, di- and trisaccharides), amino acids, aromatic amino acids (from the shikimate pathway), benzoic acids, flavonoids, phenylpropanoids, and coumarins were detected in sugarcane juice. Only a few compounds from the mevalonate pathway (terpenoids) were found. All annotations found in the spectral data of sugarcane juice can be found in Supplementary Material A. In addition, PUFA and oxylipin precursors were expected to be detected as secondary metabolites in the sugarcane extract sample. Once the PUFAs of the host plant are present, there are some possibilities for conversion to novel PUFAs and/or production of oxylipins by *A. novoparasiticus*.

In ITAL-Y174 *A. novoparasiticus* incubation extracts aimed exclusively at the detection of fungal metabolites, the spectral annotations indicated nitrogen compounds such as amino acids, diketopiperazines, nucleosides and peptides: arginine, Fru-Leu-Ile, cyclo(L-Val-L-Pro), 5-

hydroxyindole-3-acetic acid, *N*-acetyl-*l*-phenylalanine, riboprine (isopentenyladenosine), Val-Leu, *N*-(1-hydroxy-3-phenylpropan-2-yl)benzamide, fungisporin; shikimate derivatives: vanillylmandelic acid, hydroxyphenyllactic acid, bergenin); polyketides (7-hydroxy-3-(2-hydroxypropyl)-5-methylisochromen-1-one, scopoletin, chrysophanol; lipid-like polyketides: phytoceramide C2, 9,12,15-octadecatrienoic acid, avocadene 2-acetate, 5α,8α-peroxyergosterol, phytonadione, protoporphyrin IX; a mevalonate derivative: 1,4-dimethyl-9-oxo-7-propan-2-yl-3,4,10,10-tetrahydro-2*H*-phenanthrene-1-carboxylic acid. All spectra relating to *A. novoparasiticus* can be found in Supplementary Material B.

Previous work has shown that *A. novoparasiticus* produces mycotoxins in conventional medium cultures (Abdallah et al., 2020; Frisvad et al., 2019; Katsurayama et al., 2018). Strain ITAL-Y174 did not produce mycotoxin derivatives in this work, as revealed by dereplication using HRMS-MS^2 and UV–Vis spectra, and may be associated with one oxylipin found in the dereplication. Still, it produced kojic acid as the major polar compound in sugarcane juice-based incubation. The secondary metabolism of the ITAL-Y174 strain is described below to better understand the observed oxylipin formation.

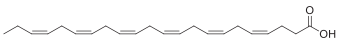
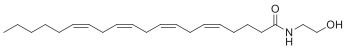
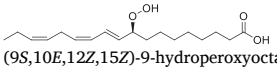
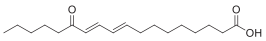
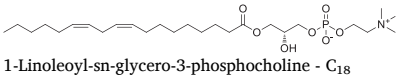
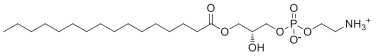
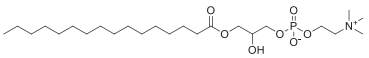
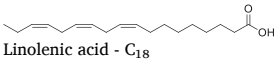
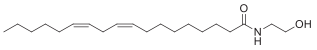
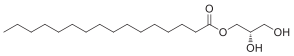
3.2. Investigation of PUFA and oxylipin precursors

The dereplication and the spectral organization in the CMN provided a comprehensive overview of the variation in PUFA of sugarcane compared to the metabolism of *A. novoparasiticus* (Tables 1 and 2). The Mini Graphic in Fig. 1 presents the global variation of PUFA and oxylipins, as shown in Tables 1 and 2. In Table 1, 'M' is coded as the spectrum detected in the sugarcane juice sample, and the codes 'd1' to 'd7' refer to the days of incubation and 24 h (day 1) to 168 h (day 7) of incubation, respectively. Table 2 contains only the fungal spectra with the codes d1 to d7, indicating the incubation times in the days the individual metabolites were detected.

It was observed that certain compounds disappeared before the completion of the first 24 h of incubation (detected only in sugarcane) (Table 1, Fig. 1), including 1-palmitoyl-sn-glycero-3-phosphoethanolamine, himbacine, tryptophan, and adenosine. The UPLC-HRMS-MS^2 analysis revealed an increase in the detection of the linolenic acid spectrum from the medium to the second day of incubation, suggesting that the ITAL-Y174 strain was producing this PUFA. However, the spectra decreased on the third day and disappeared on the fourth day, suggesting consumption. The saturated C_{18} fatty acid derivative 1-palmitoyl-sn-glycerol also increased during the first 24 h of incubation. This was followed by a decrease from the second day onward, with a continuous decline until the seventh day. These observations suggest that the ITAL-Y174 strain may be involved in the metabolism of the palmitoyl derivative during the incubation period from the second to the seventh day. The 1-palmitoyl-sn-glycerol is a precursor for PUFA and subsequent oxylipin production (Qiu et al., 2021; Tsitsigiannis & Keller, 2007). In any case, it can be assumed that the number of spectra of each detected compound ion decreases when there are fluctuations in concentration and/or consumption by the fungus during incubation. The flow chart in Fig. 2 shows the pathway of PUFA and oxylipin production discovered in this work, linked to the Mini Graphic (Fig. 1), which shows the global variation of PUFAs and oxylipins detected in the incubation experiments also associated with the spectral organization from the CMN (Fig. 3) using GNPS CMN manipulated and treated in Cytoscape (see data set, section 2.6).

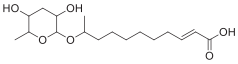
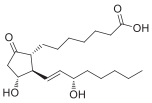
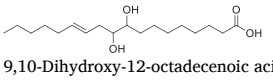
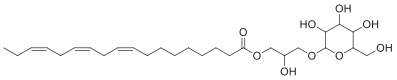
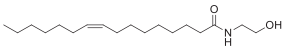
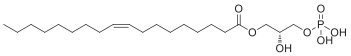
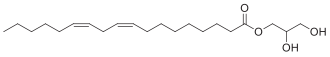
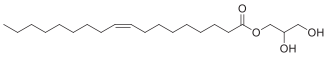
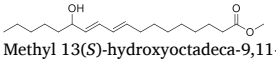
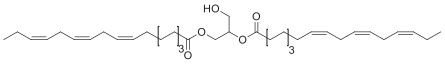
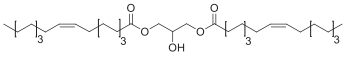
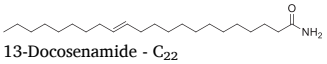
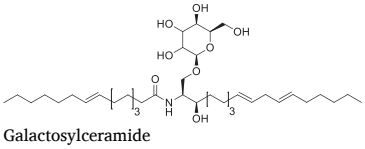
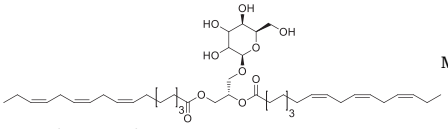
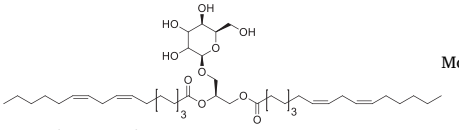
The CMN (Fig. 3) revealed the presence of additional unannotated nodes linked to the annotated PUFAs and oxylipins detected in the metabolome of sugarcane and ITAL-Y174. The connections between these nodes indicate a similarity between the HRMS-MS^2 spectra of different ions, suggesting the presence of analogous structures. The color

Table 1
Annotations of Sugarcane PUFA and oxylipin derivatives obtained from the GNPS workflow. Spectra were acquired from the first to the seventh day of incubation of A. novoparasiticus ITAL-Y174 (d1, d2, d3, d4, and d7) in sugarcane juice (M).

Code	Compound	RT (min)	Precursor *m/z*	Adduct	Error (ppm)	Cosine	Sample
S1	 Docosahexaenoic acid - C_{22}	647.1	329.3	$[M + H]^+$	4	0.57	M d1-d3
S2	 Arachidonoyl ethanolamide - C_{20}	666.4	348.3	$[M + H]^+$	34	0.67	M d1-d2
S3	 (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxyoctadeca-10,12,15-trienoic acid 9*S*-HpOTrE (or 9*S*-HPOTE) - C_{18}	734.1	293.2	$[M + H\text{-}H_2O]^+$	14	0.55	M d1-d3
S4	 13-oxooctadeca-9,11-dienoic acid 13-KetoODE - C_{18}	745.6	295.2	$[M + H]^+$	13	0.57	M d1-d3
S5	 1-Linoleoyl-sn-glycero-3-phosphocholine - C_{18}	753.6	520.3	$[M + H]^+$	6	0.77	M d1
S6	 1-Palmitoyl-sn-glycero-3-phosphoethanolamine - C_{16}	762.7	454.3	$[M + H]^+$	4	0.59	M
S7	 1-Palmitoylphosphatidylcholine - C_{16}	765.1	496.3	$[M + H]^+$	8	0.84	M d1
S8	 Linolenic acid - C_{18}	771.1	279.2	$[M + H]^+$	14	0.87	M d1-d3
S9	 Linoleyl ethanolamide - C_{18}	804.9	324.3	$[M + H]^+$	12	0.57	M d1-d3, d7
S10	 1-Palmitoyl-sn-glycerol- C_{16}	825.9	331.3	$[M + H]^+$	24	0.50	M d1-d7

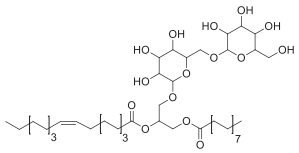
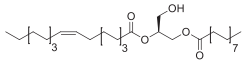
Medium culture sample (sugarcane juice, M). Extracts of incubation periods from the first day (d1) to the seventh day (d7). Codes ***S1*** to ***S10*** are present in the molecular networking in Fig. 3 and represent annotations of lipids detected in sugarcane juice.

Table 2
Annotations of *A. novoparasiticus* ITAL-Y174 PUFA and oxylipin derivatives obtained from the GNPS workflow. Spectra were acquired from the first to the seventh day of incubation of *A. novoparasiticus* ITAL-Y174 (d1, d2, d3, d4, and d7) in sugarcane juice.

Code	Compound	RT (min)	Precursor *m/z*	Adduct	Error (ppm)	Cosine	Sample
A1	 10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid - C_{11}	689.8	331.2	$[M + H]^+$	6	0.60	d2-d3, d7
A2	 Prostaglandin E1 PGE_1 - C_{20}	715.3	337.2	$[M + H\text{-}H_2O]^+$	15	0.61	d1-d2
A3	 9,10-Dihydroxy-12-octadecenoic acid 9,10-DiHOME - C_{18}	735.1	315.3	$[M + H]^+$	16	0.63	d1
A4	 3-(hexopyranosyloxy)-2-hydroxypropyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate - C_{18}	758.2	532.4	$[M + NH_4]^+$	9	0.59	d1
A5	 Palmitoleoyl Ethanolamide - C_{16}	758.5	298.3	$[M + H]^+$	13	0.51	d2-d4
A6	 1-Oleoyl lysophosphatidic acid - C_{18}	774.0	437.3	$[M + H]^+$	11	0.50	d4
A7	 Monolinolein - C_{18}	783.7	355.3	$[M + H]^+$	17	0.65	d1
A8	 Monoolein - C_{18}	803.8	357.3	$[M + H]^+$	14	0.50	d1
A9	 Methyl 13(*S*)-hydroxyoctadeca-9,11-dienoate 13(*S*)-HODE methyl ester - C_{18}	842.9	293.3	$[M + H\text{-}H_2O]^+$	17	0.64	d1-d2
A10	 1,2-di-*O*-Linolenoylglycerol - C_{18}	892.76	613.5	$[M + H]^+$	10	0.56	d1
A11	 1,3-Diolein 1,3-Dioleoylglycerol - C_{18}	892.77	638.6	$[M + NH_4]^+$	9	0.60	d1
A12	 13-Docosenamide - C_{22}	895.9	675.7	$[2\ M + H]^+$	12	0.62	d1-d7
A13	 Galactosylceramide GalCer(d18:2/16:1)	901.3	696.5	$[M + H]^+$	5	0.57	d2-d3
A14	 Monogalactosyldiacylglycerol MGDG(18:3/18:3)	908.5	792.6	$[M + NH_4]^+$	8	0.52	d1
A15	 Monogalactosyldiacylglycerol MGDG(18:2/18:2)	955.6	796.6	$[M + NH_4]^+$	6	0.50	d1

(*continued on next page*)

Table 2 (*continued*)

Code	Compound	RT (min)	Precursor *m/z*	Adduct	Error (ppm)	Cosine	Sample
A16	 Digalactosyldiacylglycerol DGDG(16:0/18:1)	970.3	936.7	$[M + NH_4]^+$	7	0.64	d1-d3
A17	 1-Palmitoyl-2-oleoyl-sn-glycerol DG(16:0/18:1/0:0)	972.0	595.5	$[M + H]^+$	10	0.66	d1

Extracts of incubation periods from the first day (d1) to the seventh day (d7). Codes ***A1*** to ***A17*** are present in the molecular networking in Fig. 3 and represent annotations of lipids detected in *A. novoparasiticus* ITAL-Y174 metabolism.

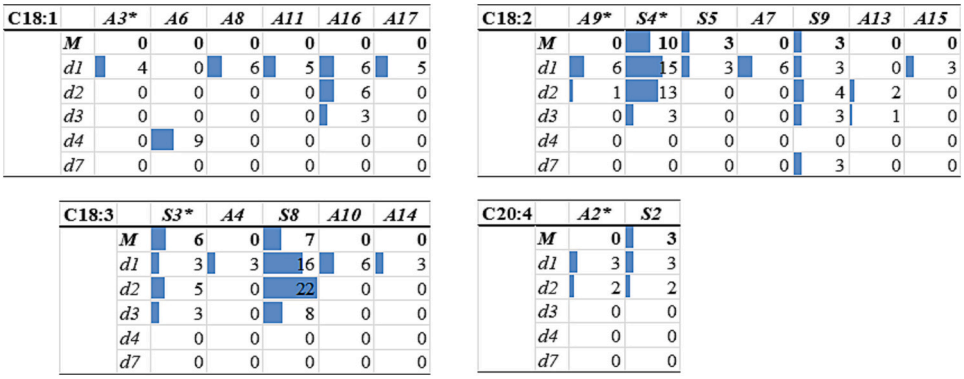


Fig. 1. Vertical mini graphics (blue bars) showing the global variation of detected spectra of PUFAs C18:1, C18:2, C18:3, and C20:4 produced by *A. novoparasiticus* ITAL-Y174 (***A4***, ***A6***, ***A7***, ***A8***, ***A10***, ***A11***, ***A13***, ***A14***, ***A15***, ***A16***, and ***A17***) and detected in sugarcane juice (***S2***, ***S5***, ***S8***, and ***S9***), including the oxylipins marked with an * (***A2***, ***A3***, ***A9***, ***S3***, and ***S4***). Each number represents the spectra detected during incubation (from d1 to d7) or medium culture (***M***). Codes '***A***' and '***S***' summarize the annotations in *A. novoparasiticus* metabolome and sugarcane, respectively, organized in Tables 1 and 2. The graphics are based on the manipulation, data curation, and processing of the CMN in cytoscape. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

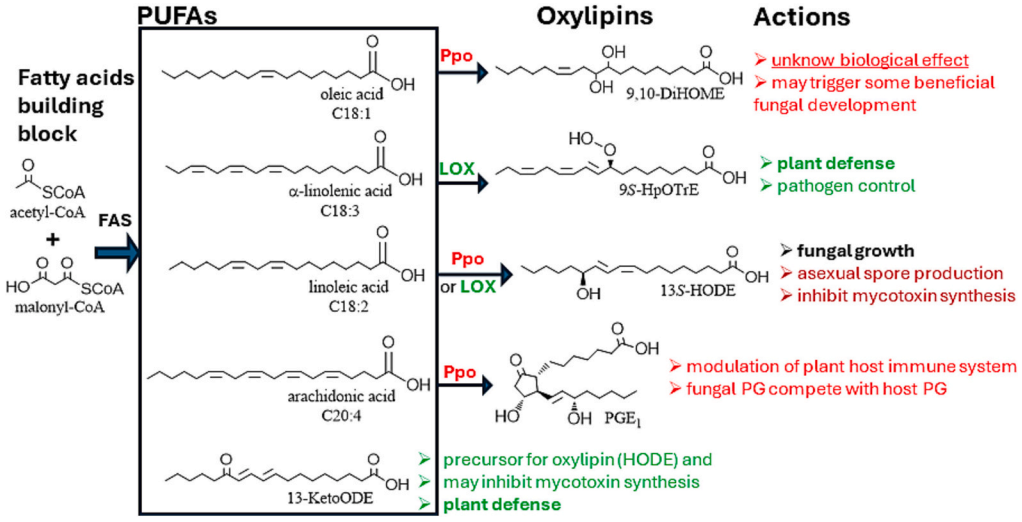


Fig. 2. A flow chart of oxylipins and their PUFA precursors discussed in this work. Acetyl-CoA and malonyl-CoA are the building blocks for fatty acid synthesis with the enzyme "fatty acid synthase" (FAS) to produce PUFA. Specific fungal enzymes (Ppo) and plant enzymes (LOX) reduce the PUFA into diverse oxylipins with specific biological responses.

coding of the nodes further facilitates the interpretation of the data, as it indicates the presence of spectra obtained during different incubation periods. This includes spectra from sugarcane metabolites and from incubations d1 to d7, a seven-day incubation of *A. novoparasiticus* Y174-ITAL.

The dereplication for *A. novoparasiticus* data was matched to the

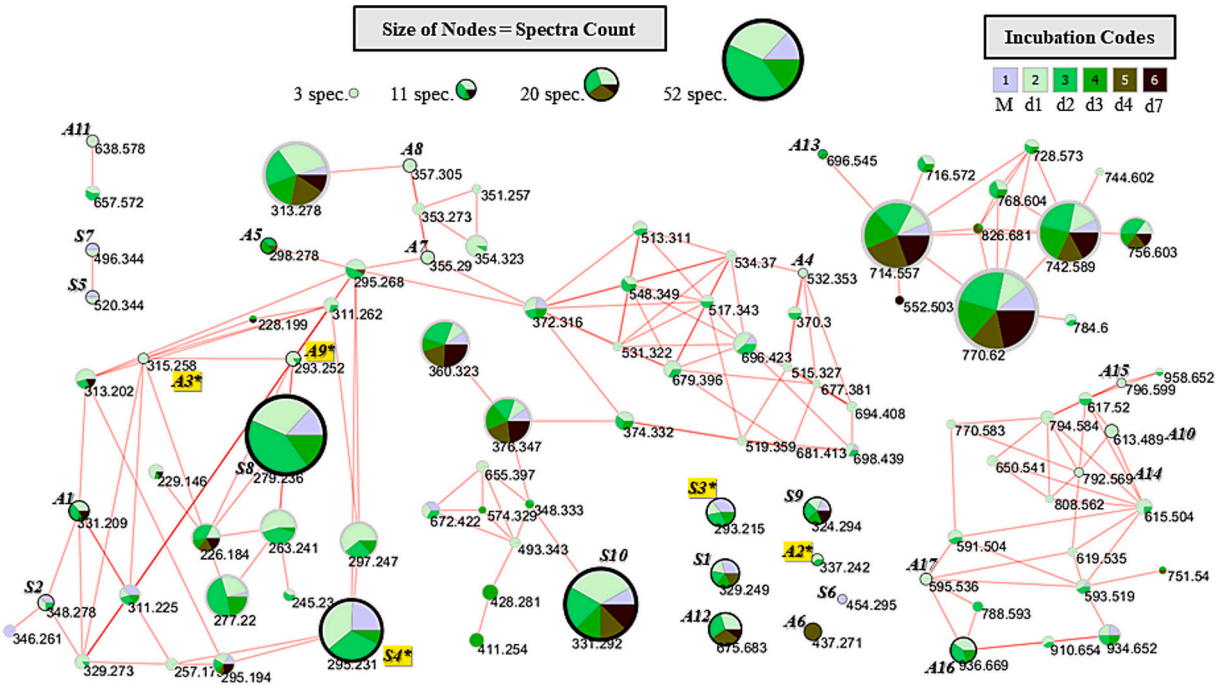


Fig. 3. Classical molecular networking created in the GNPS workflow targeting annotations (nodes with black border) of PUFA produced by sugarcane (***S***) and produced by ITAL-Y174 (***A***), and oxylipins produced by sugarcane (***S****) and produced by ITAL-Y174 (***A****) from seven-day incubation (d1 to d7). Each node represents the clustered spectra organized in the GNPS workflow. The size of a node is proportional to the total clustered spectra. The colors inside nodes, as well as the pizza graph, represent the number of spectra detected on each day of incubation. The connection of nodes shows that different clustered spectra present HRMS-MS^2 similarity as similar structures. Use data set MSV000090498 in the GNPS platform for reanalyzing.

PUFA derivatives 10-(3,5-dihydroxy-6-methyloxan-2-yl)oxyundec-2-enoic acid, prostaglandin E1 (PGE_1), 9,10-dihydroxy-12-octadecenoic acid (9,10-DiHOME), 3-(hexopyranosyloxy)-2-hydroxypropyl ester, palmitoyl ethanolamide, 1-oleoyl lysophosphatidic acid, monolinolein, monoolein, methyl 13-hydroxyoctadeca-9,11-dienoate (13*S*-HODE methyl ester), 1,2-di-*O*-linolenoylglycerol, 1,3-diolein, 13-docosenamide, and one galactosylceramide (18:2/16:1), two monogalactosyldiacylglycerol derivatives (MGDG, 18:3/18:3 and MGDG, 18:2/18:2) were annotated, along with digalactosyldiacylglycerol (DGDG, 16:0/18:1) and 1-palmitoyl-2-oleoyl-sn-glycerol (see Table 2).

Phospholipids and acylglycerides are the primary substrates for synthesizing saturated and unsaturated fatty acids. Oxylipins, in turn, are derived from C_{18} and C_{20} PUFA, including oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and arachidonic acid (C20:4). Lipases can hydrolyze PUFA, promoting the formation of oxylipins (Sakuradani et al., 2009). Fungal oxidation of arachidonic acid produces prostaglandin derivatives that regulate lipid synthesis and secretion. These derivatives have also been implicated in regulating cell growth, differentiation, and apoptosis and developing infectious processes caused by pathogenic microorganisms (La Camera et al., 2004; Noverr et al., 2003). While oxylipin receptors, genes, and their structure-function relationships have been the subject of extensive research in vertebrates, they remain to be thoroughly investigated in plants and fungi (Christensen & Kolomiets, 2011; Shah, 2005).

A cluster annotation of 9*S*-HpOTrE (or 9*S*-HPOTE), a hydroperoxy oxylipin precursor for 9*S*-HOTrE, and annotation of oxylipin 13-KetoODE (precursor for 13-HODE oxylipin) were found in sugarcane, suggesting that the plant was undergoing some defense action against fungal pathogeny. Oxylipins were detected in sugarcane juice but disappeared by the fourth day of incubation due to decreased concentrations. The C_{18} PUFA linolenic acid is metabolized by different plant lipoxygenase (LOX) enzymes to produce several oxylipins, including 9-HpOTrE. Potato tuber LOX has been shown to generate 9*S*-HpOTrE from α-linolenic acid (Grechkin et al., 1991), while soybean LOX produces the 13-oxygenated derivative, 13*S*-HpOTrE, and to a lesser extent 9*S*-HpOTrE (Liu et al., 2013; Sok & Kim, 1990). The hydroperoxides are readily converted to their hydroxylated derivatives, such as 9*S*-hydroxy-octadecatrienoic acid (9*S*-HpOTrE) (Cambiaggi et al., 2023), which have been observed to exhibit antimicrobial properties (Prost et al., 2005). There is significant evidence that sugarcane was developing an antimicrobial defense mechanism by synthesizing the 9*S*-HpOTrE oxylipin to control potential pathogens.

The investigation of fungal oxylipins resulted in three annotations. The first annotation was PGE_1 (cosine 0.61, 15 ppm error), detected within 48 h of incubation. The spectral comparison suggests that PGE_1 is a fungal derivative of C_{20} PUFA arachidonic acid (C_{20} PUFA was detected in sugarcane juice). The second detected oxylipin is a C_{18} PUFA derivative, 9,10-DiHOME (cosine 0.63, 16 ppm error), suggesting the presence of an oxylipin structure derived from a C_{18} PUFA linoleate derivative, which was only present on the first day of incubation. The last oxylipin was annotated as a C_{18} PUFA derivative 13*S*-HODE methyl ester, detected in the first 48 h of incubation, derived from C_{18} PUFA linoleate. The 13*S*-HODE methyl ester (cosine 0.64, 17 ppm error) corroborates and supports the oxylipin profile in this *in vitro* experiment involving the host metabolites and the "infectious fungus" (*A. novoparasiticus*). The low cosine index of spectra annotations may be due to the low concentration of ions during the HRMS acquisition, which could provide low-quality spectra. In addition, the CMN (Fig. 3) shows unknown nodes close to the PUFA and oxylipin annotations, suggesting derivatives present that may represent a novel target for exploring further studies about the chemical communication of fungi-plant host.

While the chemical characterization of the subject has been investigated using linoleic acid as a substrate, psi factor-producing oxygenase (Ppo) proteins have been observed to oxidize oleic and linolenic acid to synthesize certain C_{18} oxylipins, including arachidonic acid to synthesize certain prostaglandins, as summarized in the review by Fischer and Keller (2016). Fungal prostaglandins (PGs) have been shown to compete with host PG, resulting in interference in the modulation of host immune

responses regulated by host PG. A growing body of research has underscored the pivotal role of the eicosanoid (C_{20}) pathway in regulating morphogenesis and biofilm formation in *C. albicans*. These findings highlight the potential of these lipids as regulators of *Candida* virulence. According to Kuroda and Yamashita (2003) and Singh and Del Poeta (2011), PGE_2 damages the Th1 cell's protective response to infection. (Erb-Downward & Noverr, 2007) suggest that developing prototypes that target fungal prostaglandin pathways could be a potential strategy for controlling fungal colonization and infection. Additionally, it was observed that the C_{20} arachidonoyl ethanolamide from the sugarcane was detected at the 48 h incubation stage but not at the 72 h stage, and the fungal PGE_1 was detected similarly. This supports that the arachidonoyl derivative from sugarcane is the precursor to producing fungal C_{20} oxylipin PGE_1.

Researchers have characterized Ppo homologs, including linoleate diol synthases, linoleate dioxygenases, and linoleate dioxygenases (LDS), in fungi other than aspergilli. LDS-encoding genes have also been identified in pathogenic and non-pathogenic fungi (Fischer & Keller, 2016). For instance, *Fusarium verticillioides* contains an LDS termed Fvlds1 that produces oxylipins, which regulate fungal development. The deletion of Fvlds1 led to a decrease in the levels of oxylipins, including 11-HPODE, 12,13-DiHOME, 12-EpoOME, 9,10-DiHOME, 9-EpoOME, 8,13-DiHODE, 8-HPODE, and 8-HODE (Scala et al., 2014). During the initial 24 h incubation period, the medium culture exhibited the presence of C_{18} PUFA 1-linoleoyl-sn-glycero-3-phosphocholine. Furthermore, linoleyl ethanolamide was detected from the medium to the seventh day, as well there were present three oleic acid derivatives (1-palmitoyl-sn-glycero-3-phosphoethanolamine, 1-palmitoylphosphatidylcholine, and 1-palmitoyl-sn-glycerol), and the *A. novoparasiticus* may metabolize them to produce oxylipin 9,10-DiHOME. This finding indicates that *A. novoparasiticus* ITAL-Y174 can synthesize 9,10-DiHOME from C_{18} PUFA linoleyl derivatives present in sugarcane. The action of DiHOME derivatives is unknown, but it has been suggested that 5,8-DiHOME may trigger hyphal branching, while its analog 12,13-DiHOME lacks this effect (Niu et al., 2020).

The oxylipin 13*S*-HODE is produced by LOX from C_{18} PUFA in plants or Ppo in fungi. The 13*S*-HODE has been identified in cucumber seeds, where it functions as a lipid reserve to facilitate initial plant growth and is utilized for β-oxidation during germination (Feussner et al., 1997, 2001). However, when fungi produce the oxylipin 13*S*-HODE, it may inhibit its production of mycotoxins (Burow et al., 1997; Gao et al., 2019) and induce asexual spore reproduction, promoting the production of the cAMP gene through GPCR signaling, inducing fungal growth and development (Calvo et al., 1999). Similar actions to HODE have been observed in 5,8-dihydroxyoctadecenoic acid (5,8-DiHOME), an oxylipin derived from oleic acid and produced by *Aspergillus spp.* (Garscha et al., 2007), and 7,8-dihydroxyoctadecadienoic acid (7,8-DiHODE), an oxylipin produced by the plant pathogen *Magnaporthe grisea*. Both induced hyperbranching behavior similar to that observed in 5,8-DiHODE (Cristea et al., 2003). In contrast, analogous HODE oxylipins did not influence branching, suggesting that structural specificity is imperative. This notion may be associated with the positioning of hydroxyl groups, the number of double bonds, and/or acyl chain length (Niu et al., 2020), corroborating the hypothesis that the ITAL-Y174 strain detected the presence of sugarcane PUFA linoleic acid derivatives and produced 13*S*-HODE as a fungal secondary metabolite to promote growth (Gessler et al., 2017) but altering its secondary metabolism, which action was reported once for *A. parasiticus* (Burow et al., 1997).

The actual application of oxylipins in food requires a detailed understanding of their different antifungal and antimycotoxin mechanisms, as each oxylipin moiety may have other sites of action. The presence of PGE_1, 9,10-DiHOME, and 13*S*-HODE methyl ester as fungal oxylipins (as well as more neighbor nodes present in the CMN, Fig. 3) provide some insight into the development of the strain ITAL-Y174 in the presence of sugarcane metabolites based on the *in vitro* model. The production of fungal oxylipins ceased during the early incubation period (Fig. 1), suggesting that in the absence of a response from an active plant host, oxylipin production does not affect any host but only its development and inhibits mycotoxin synthesis (by 13*S*-HODE) (Burow et al., 1997; Gao et al., 2019).

A host's oxylipins may influence the fungus, leading the fungus to conserve energy by not producing oxylipins or to head energy to produce specific lipids. ITAL-Y174 strain consumed the C_{18} PUFA plant host to synthesize ester derivatives MGDG(18:3/18:3), MGDG(18:2/18:2), 3-(hexopyranosyloxy)-2-hydroxypropyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate, 1,2-di-*O*-linolenoylglycerol and monolinolein. The synthesis of linoleate and linolenate PUFA derivatives by *A. novoparasiticus* ITAL-Y174 appears to be an attempt to conserve the fatty acid obtained from the medium culture or represent a different path to synthesize its PUFA and subsequentially synthesize oxylipins. It was also observed in CMN that the spectral rate detection was vanishing from the third and fourth day, and on the seventh day, almost no lipids were present in the MS detection. It may be associated with the lack of response of host plant PUFA and oxylipins, making the fungus stop its own PUFA and oxylipins synthesis to save energy. These observations could indicate consistent production of PUFA and oxylipins as chemical messengers to sugarcane (Figs. 1, 2, and 3). The *in vivo* experimental models using sugarcane cultures will better explore the various chemical responses and interactions.

3.3. Compounds detected by gas chromatography coupled to mass spectrometry

Apolar metabolites in the hexane phase were dereplicated using GC–MS based on the NIST database (v2.0, 2008). During the short incubation period of ITAL-Y174 from the first to the seventh day with daily extractions, there was only a slight increase in lipids compared to sugarcane metabolites (Fig. 4). The fungal apolar metabolites include annotations of adamantane derivatives (20.3 to 21.3 min), methyl 8-methylnonanoate ester (24.8 min), methyl hexadecanoate (26.1 min), *cis*-11-eicosenoic acid (29.8 min), oleic acid (31.5 min), and octadecenoate ester derivatives (33.5 and 34.2 min).

On the first day of incubation, oleic acid (31.5 min) was detected as ITAL-Y174 metabolite, while 2-hexyl-1-decanol (23.2 min) and carbonic acid derivative (35.5 min) decreased their intensity. Phytosteroids from sugarcane derived from stigmasterol and sitosterol predominated in the retention time above 42.5 min, and they were neither consumed nor produced by *A. novoparasiticus* ITAL-Y174. The sugarcane compound detected at 25.5 min was annotated as 9(*Z*)-octadecenoate methyl ester, and it was interesting to observe its detection increasing and then decreasing on the second day of incubation, while methyl hexadecanoate (26.1 min) and *cis*-11-eicosenoic acid (29.8 min) increased (Fig. 4). Saturated and monounsaturated lipids, including those identified by GC–MS and UPLC-HRMS-MS^2, are essential for fungal development, influencing and contributing to growth (Qiu et al., 2021).

3.4. Purification and structural identification of major compound of A. novoparasiticus

The ITAL-Y174 strain produced a major compound obtained after a 28-day incubation. Mass spectrometry detected the substance as an ion with m/z 143.0345 (calcd. For $C_6H_7O_4^+$ 143.0339). The UV spectrum showed absorption at 265 nm, suggesting the presence of a conjugated π system of gamma-pyrone backbone. The gamma-pyrone was purified using semi-preparative reverse-phase HPLC (Supplementary Material C).

The ^1H NMR spectrum of the fungal metabolite clearly showed the presence of aromatic or olefin hydrogens, as evidenced by chemical shifts of δ_H 7.96 (s, 1H), δ_H 6.44 (s, 1H), and δ_H 4.44 (d, 2H). The ^{13}C NMR signals exhibited the presence of a carboxyl group at δ_C 174.7, two oxygenated sp^2 carbons at δ_C 169.8 and δ_C 146.8, one oxygenated methine sp^2 carbon at δ_C 138.8, one sp^2 carbon at δ_C 109.8, and an aliphatic carbinolic methylene at δ_C 61.0. The compound obtained from *A. novoparasiticus* ITAL-Y174 was identified as kojic acid by NMR

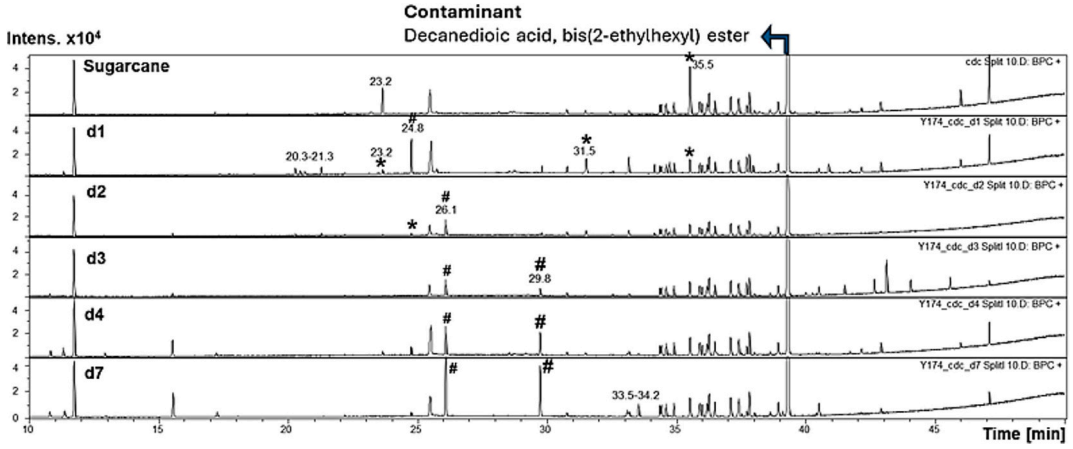


Fig. 4. Base peak chromatograms from GC–MS analysis of sugarcane and incubation extracts (d1 to d7). Decreasing compound concentration is marked with the *, and the # is related to increasing the compound concentration. The contamination of decanedioic acid, bis (2-ethylhexyl) ester is highlighted.

(Zeringue Jr. et al., 1999) (Supplementary Material D) and HR-ESI-(+)-MS-MS^2 spectrum with a fragmentation propose map (Supplementary Material E).

Kojic acid possesses potential biological properties as an anticancer prototype for melanoma due to NF-kB inhibition in human keratinocytes and its effects on melanin and tyrosinase (Ma et al., 2011; Moon et al., 2001; Saeedi et al., 2019). About 68 species of *Aspergillus*, mainly from *A.* section *Flavi*, can produce kojic acid (El-Aziz, 2013; Frisvad et al., 2019; Sharma et al., 2023). *A. novoparasiticus* has been reported to produce aflatoxin B_1, B_2, G_1, G_2, aspergillic acid, aspirochlorin, ditryptophenaline, flavacol, hydroxyaspergillic acid, kojic acid, leporin B, leporin C, miyakamides, neoaspergillic acid, neohydroxyaspergillic acid, *O*-methylsterigmatocystin and parasiticolides (Abdallah et al., 2020; Frisvad et al., 2019) but of these secondary metabolites we only found kojic acid as the major polar compound produced in sugarcane juice incubation, but no mycotoxin. The strain ITAL-Y174 examined in this work may be applied in industrial processes. Industrial production of kojic acid was optimized using glucose with yeast extract, but ongoing research aims to enhance these conditions (Sharma et al., 2023). Sugarcane juice is a simple, accessible, low-cost medium for producing kojic acid using *A. novoparasiticus*.

4. Conclusion

Aspergillus section *Flavi*, often associated with toxigenic fungi in food, exhibits potential for biotechnological applications. In conclusion, utilizing dereplication and automated mass spectra organization in the GNPS platform, we examined the metabolome of *A. novoparasiticus* (strain ITAL-Y174) in the presence of sugarcane metabolites. Our analysis revealed the presence of three fungal oxylipins (PGE_1, 9,10-DiHOME, 13*S*-HODE) produced during incubation of *A. novoparasiticus*, suggesting their role in fungal initial development in the presence of C_{18} and C_{20} PUFA from sugarcane. Evidence for 9,10-DiHOME oxylipin bioactivity is unknown, while 13*S*-HODE is linked to the fungal development with asexual spore production with inhibition of the mycotoxin production, which may explain why no mycotoxin of *A. novoparasiticus* was found in this work. Finally, we suggest that PG_1 was produced to modulate the plant host immune system.

Interestingly, strain ITAL-Y174 demonstrated the ability to produce kojic acid from sugarcane glucose as the unique major polar metabolite, offering a cost-effective method for industrial kojic acid production. Moreover, this strain holds promise for biotechnological advancement due to its diverse secondary metabolites accessed through GNPS workflow dereplication, including phenolic polyketides, diketopiperazine, peptides, and terpenoids, as well the absence of mycotoxin in the incubation by simply using sugarcane juice as medium culture.

Declaration of generative AI in scientific writing

While preparing this work, the main author used Grammarly® to help improve grammar check. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Acknowledgements and funding

This study was financed by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) according to the following processes: 2016/24985–4, 2017/50349–0, 2020/16554–9, 2021/00956–3, 2021/12590–3, 2022/09202–4; and Conselho Nacional de Desenvolvimento Científico e Tecnológico: CNPq Process 304,036/2022–6. This study was registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado [SisGen A8A72E0].

CRediT authorship contribution statement

Augusto Leonardo dos Santos: Writing – original draft, Formal analysis, Data curation. **Marcelo José Pena Ferreira:** Writing – review & editing, Methodology, Funding acquisition, Data curation. **Patricia Sartorelli:** Writing – review & editing, Funding acquisition, Data curation. **Josué José da Silva:** Writing – review & editing, Conceptualization. **Beatriz Thie Iamanaka:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Jens Christian Frisvad:** Writing – review & editing, Conceptualization. **Marta Hiromi Taniwaki:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.143051.

Data availability

I have shared the link to my data in the manuscript.

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