Comprehensive analysis of the *Alternaria* mycobolome using mass spectrometry based metabolomics

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Abbreviations

alternariol (AOH), alternariol monomethyl ether (AME), altertoxin I (ATX I), altertoxin II (ATX II), alterperylenepoxide A-9-mercaptolactate (APML), alterperylenol (ALTP), bicycloalternarene (BCA), correlated spectroscopy (COSY), Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), Heteronuclear Multiple Bond Correlation (HMBC), Heteronuclear Single Quantum Coherence (HSQC), Kyoto Encyclopedia of Genes and Genomes (KEGG), Principal component analysis (PCA), stemphyltoxin III (STTX III), tentoxin (TEN), tenuazonic acid (TA), threshold of toxicological concern (TTC), tricycloalternarene (TCA)

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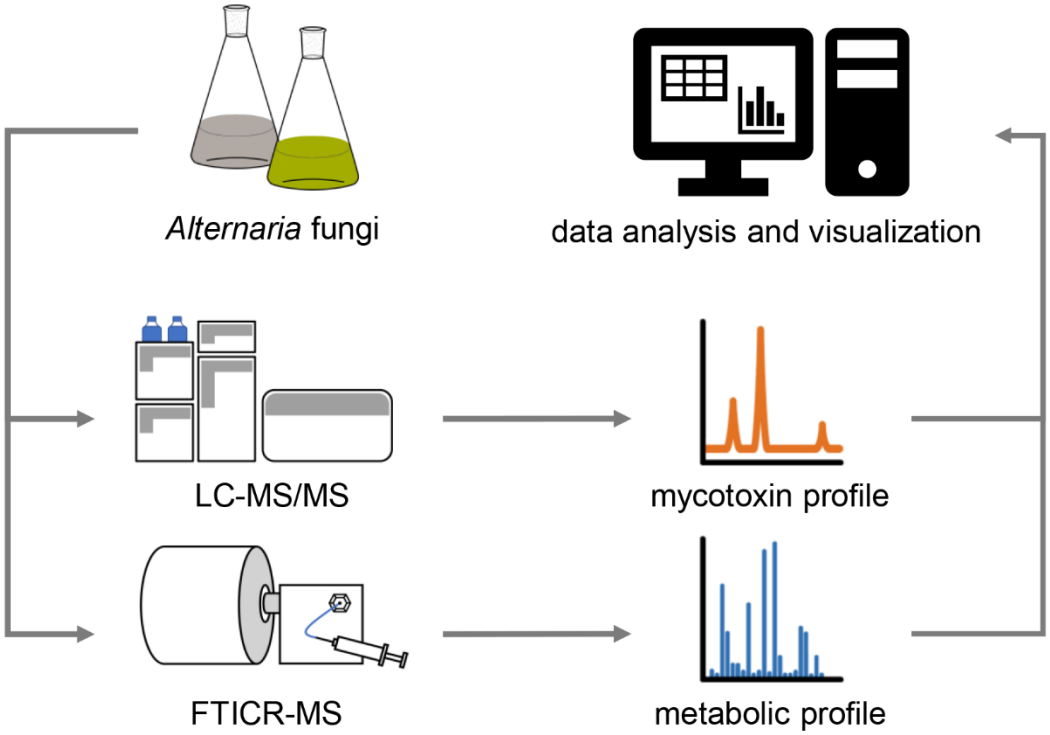
Abstract

Scope: *Alternaria* fungi are widely distributed plant pathogens infecting grains and vegetables and causing major harvest losses in the field and during postharvest storage. Besides, consumers are endangered by the formation of toxic secondary metabolites. Some of these secondary metabolites are chemically characterized as mycotoxins, but the majority of the *Alternaria* mycobolome remains still unknown.

Methods and Results: Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) and LC-MS/MS were combined for the non-targeted and targeted analysis of the metabolome of three *A. alternata* isolates and one *A. solani* isolate. Due to the ultra-high resolution of FTICR-MS, unique molecular formulae were assigned to measured *m/z* signals. The molecular formulae were matched to entries of the databases Antibase and KEGG. The non-targeted analysis of the fungal extracts revealed variations in the secondary metabolite profile of *A. alternata* and *A. solani*. Differences in the biosynthesis of dibenzo-α-pyrones, perylene quinones, tentoxin, and tenuazonic acid of the *A. alternata* and *A. solani* isolates were determined applying targeted LC-MS/MS.

Conclusion: FTICR-MS analyses revealed clear differences in the metabolic profile of the *A. solani* and the *A. alternata* isolates and gained deeper insights into the metabolome of the food contaminating pathogen *Alternaria*.



Fungi of the genus *Alternaria* are plant pathogens infecting agricultural foods. *Alternaria* isolates were cultivated in a chemically defined liquid medium and the extracts were analyzed by ultra-high resolution mass spectrometry (FTICR-MS) and LC-MS/MS. The targeted and non-targeted analysis of the fungal extracts uncovered differences in the mycotoxin and metabolic profile of the *A. alternata* and the *A. solani* isolates.

1 Introduction

Fungi of the genus *Alternaria* are widely distributed on seeds, plants, animals, in the soil, and in the atmosphere.[1] Growing in various regions and during different seasons, *Alternaria* species exhibit endophytic, saprophytic, or pathogenic growth.[1,2] The fungi decompose natural as well as artificial substrates [3] and cause plant diseases such as black rot of tomato, olive, and carrots and black and grey rot of citrus fruits.[2,1] Besides, cereals such as wheat, barley, oats, and sorghum are frequently infected by *Alternaria* fungi [4] resulting in losses of agricultural products in the field and during postharvest storage.[5] Common *Alternaria* species are *A. alternata*, *A. tenuissima*, *A. arborescence*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria*.[6]

In addition to losses of agricultural goods, *Alternaria* fungi endanger consumers by the production of mycotoxins and secondary metabolites with partly unknown toxicological potential.[7] The mycotoxins accumulate in agricultural products leading to a decline of the food quality.[8] Over 70 different secondary metabolites are described in the literature and more than 30 exhibit toxicological potential. [9]

Common *Alternaria* mycotoxins belong to five different structural classes, namely (1) tetramic acid derivatives (tenuazonic acid (TA)), (2) dibenzo-α-pyrones (alternariol (AOH), alternariol monomethyl ether (AME), and altenuene), (3) perylene derivatives (altertoxin I (ATX I), altertoxin II (ATX II), alterperylenol (ALTP), and stemphyltoxin III (STTX III)),[2] (4) miscellaneous structures (tentoxin (TEN)), and (5) *A. alternata* f. sp. *lycopersici* toxins (AAL-toxins).[9] In previous studies, the benzopyrones exhibited genotoxic, cytotoxic, and mutagenic effects *in vitro* [5,2] and caused DNA-damages in human colon carcinoma cells.[10] Additionally, AOH and AME were described to act as topoisomerase poison and to inhibit the catalytic activity of topoisomerase IIα.[5] While the acute toxicity of AOH and AME is rather low,[4] TA is acutely toxic to mice, chicken, and dogs.[2] While no mutagenicity was observed for the tetramic acid derivative in bacterial systems,[11,5] the altertoxins were mutagenic in the *Salmonella* Ames Test [11,12] and strongly genotoxic in mammalian and human cells.[13,14] Contrarily to the other mycotoxins, TEN is characterized as phytotoxin and inhibits the cyclic photophosphorylation in chloroplasts.[15]
As *Alternaria* mycotoxins are neither legislatively controlled nor routinely analyzed,[16] they are considered as “emerging mycotoxins”.[17] Besides, the modifications of AOH and AME with sulfates and glucosides attract increasing attention.[18] These so called “modified mycotoxins” are either produced by the fungi themselves or the mycotoxins are metabolized by plants for detoxification purposes.[19–21] After oral consumption, the modification is potentially hydrolyzed during digestion, which releases the aglycon.[22–24] In a recently developed LC-MS/MS method, AOH-9-glucoside and AME-3-sulfate were detected in naturally contaminated foods demonstrating the necessity to routinely analyze also the chemically modified mycotoxins in agricultural products.[17] Furthermore, the modified forms should be included in future risk evaluations [19] and also added to mass spectrometric databases.

In 2011, the Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority assessed the risk to human health originating from *Alternaria* mycotoxins in agricultural products. The estimated chronic dietary exposure of AOH and AME exceeded the threshold of toxicological concern (TTC) of 2.5 ng/kg body weight per day and, therefore, additional toxicity data are indispensable for further risk evaluations. TA and TEN are non-genotoxic and the estimated

chronic dietary exposure did not reach the TTC value of 1500 ng/kg body weight per day.[9] However, recent studies particularly on infant foods highlighted that these TTC values were exceeded[25] and required the establishment of a legal limit for TA in food products for infants.[26]

To perform reliable risk assessments, the metabolic capabilities of *Alternaria* fungi need to be investigated and various species need to be identified correctly. Traditionally, the systematics of *Alternaria* fungi was based on morphological characteristics.[3,8] But, morphology alone was insufficient due to overlapping traits of closely related species.[2] In addition to three-dimensional sporulation patterns on agar plates,[27,28,8] *Alternaria* species were also classified based on the sequence variation in the translation elongation factor 1-α.[8] The analysis of further genes resulted in distinct *Alternaria* species clusters.[3] As DNA-based studies did not always match the species-groups defined in morphological surveys,[1] excreted metabolites produced by the fungi were analyzed by HPLC-UV and LC-MS/MS and used for their differentiation.[29–31] The species group of *A. infectoria* was separated from *A. arborescence*, *A. alternata*, and *A. tenuissima,* but the latter were indistinguishable based on the mycotoxin profile obtained by LC-MS/MS.[30,31] As high-resolution mass spectrometers offer the simultaneous detection of hundreds to thousands of metabolites, these instruments are increasingly in use for the differentiation of fungal species.[32,33]

In addition to the species segregation, high-resolution mass spectrometers improve the holistic characterization of the fungal mycobolome. The comprehensive detection of low molecular weight metabolites of an organism is called metabolomics.[34] As the metabolome of organisms varies depending on genotype, cell cycle stage, or environment,[35] metabolomics approaches focus on the detection of a wide range of possibly produced compounds.[34] It has to be kept in mind, that sample preparation and the selection of the ionization mode in the electrospray ionization source will significantly influence the detected metabolite profile.[34,36] Metabolomics approaches commonly use time-of-flight (TOF), Orbitrap™, and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers.[34] As FTICR-MS combines ultra-high mass resolution and superior mass accuracy,[37,38,34] unique elemental compositions can be assigned clearly to measured *m/z* signals. Although the allocation of molecular formulae to metabolites is possible,[39] the structural identification of molecular formulae remains the bottleneck in mass spectrometric metabolomics studies.[36] FTICR-MS analysis was utilized to investigate alterations between *Clamydia pneumoniae*-infected and non-infected Hep-2 cells [38] and to identify differences in the growth stages of bacteria.[39] Applying FTICR-MS to foods is called “foodomics” aiming to analyzing the functionality, the nutritional value, and the safety of agricultural products.[24] Regarding mycotoxins and related food contaminants, foodomics investigations can support the identification of new or fungal metabolites on foods and can provide further data for proper risk assessments.[24]

In the present study, various fungal isolates of *A. alternata* and *A. solani* were cultivated in a chemically defined liquid medium and the extracts were analyzed by direct infusion FTICR-MS. Due to the determination of hundreds of metabolites, we aim at obtaining a more holistic insight into the fungal mycobolome. Assigned molecular formulae are intended to be allocated to metabolites using the Antibase and KEGG databases. Complementing this non-targeted FTICR-MS approach with targeted LC-MS/MS analysis [25] will help to clearly identify *Alternaria* mycotoxins as LC-MS/MS offers advantages in selectivity [40] and the possibility to chromatographically separate isomers such as ATX II and ALTP. Additionally, the LC-MS/MS measurements are intended to provide quantitative results on intra- and extra-cellular mycotoxin contents.

2 Materials and Methods

2.1 Chemicals and reagents

Reference compounds of AOH, AME, TA, and TEN were purchased from Sigma-Aldrich (Steinheim, Germany). TA was released from its commercial copper salt according to the literature.[41,42] ATX I, ATX II, ALTP, and STTX III were biosynthesized as described previously.[43] After biosynthesis, the analytical standards were purified by preparative HPLC and characterized by nuclear resonance spectroscopy (NMR) as reported earlier.[43]

Agar, ammonium formate (≥ 99.0 %, for mass spectrometry), ammonium sulfate, arginine, potassium dihydrogen phosphate, sodium acetate trihydrate, sodium nitrate, and sucrose were obtained from Sigma-Aldrich (Steinheim, Germany). Ammonia solution (25 %, for LC-MS), formic acid (> 98 %), glucose, iron sulfate heptahydrate, potassium chloride, potassium nitrate, and sodium hydroxide were received from Merck KGaA (Darmstadt, Germany). Tween 20 was purchased from AppliChem GmbH (Darmstadt, Germany). Calcium nitrate tetrahydrate, dichloromethane (technical grade), ethyl acetate (technical grade), formic acid (> 99 %, for mass spectrometry), magnesium sulfate heptahydrate, acetonitrile and water (HPLC grade, LC-MS grade), isopropanol (technical grade, HPLC grade, LC-MS grade), and methanol (HPLC grade) were obtained from VWR (Ismaning, Germany). Methanol (LC-MS grade) was purchased from Honeywell International Inc. (Seelze, Germany). Water was purified using a Milli-Q system (Millipore, Darmstadt, Germany).

2.2 Preparation of stock solutions

Stock solutions of *Alternaria* mycotoxins were prepared in acetonitrile (AOH, AME, TEN) or methanol (ATX I, ALTP, TA) in concentrations ranging from 10 to 100 µg/mL. For quantitative measurements, the stock solutions were further diluted. All solutions were stored in the dark at -20 °C. The absorptions of the solutions were measured by a Genesys, 10S, UV-Vis spectrophotometer (Thermo Fisher Scientific, Madison, Wisconsin, USA) and the concentrations were confirmed by applying the published extinction coefficients.[44] However, ATX II and STTX III were only qualitatively included in the method, as the available amounts of these reference compounds were not detectable by UV-Vis.

2.3 Preparation of SNA (synthetic nutrient-poor agar)

Glucose (0.2 g), magnesium sulfate heptahydrate (0.5 g), potassium chloride (0.5 g), potassium dihydrogen phosphate (1 g), potassium nitrate (1 g), and sucrose (0.2 g) were dissolved in 100 mL of water. To adjust the pH to 5.5, 600 µL of sodium hydroxide (1 mol/L) were added. 22 g of agar were solved in 900 mL of water, and after the unification of both solutions, the medium was autoclaved for 20 min at 121 °C.[45]

2.4 Isolation of *Alternaria* fungi

A. alternata was isolated from potato leaves (Uelzen, Germany, isolate 1), tomato leaves (Aitrang, Germany, isolate 2), and tomatoes (Aitrang, Germany, isolate 3). The *A. solani* isolate originated from potato leaves (Kirchheim, Germany). After harvesting, the plant leaves were dried. The surface of the dried leaves and fresh tomato was sterilized using 3 % of sodium hypochlorite. Subsequently, small pieces of plant tissue showing typical symptoms of infection were placed on SN agar and cultivated at 22 °C and 65 % relative humidity for one week. Alternately, the isolates were exposed to black light for 12 hours and subsequently cultivated in the dark for 12 hours. Single spores were isolated from the overgrown agar plates and cultivated on SNA at 22 °C and 65 % relative humidity

for two weeks. Again, 12 hours of black light exposure were followed by the cultivation in the dark. The overgrown agar plates with pure isolates were used for further experiments. All fungi were obtained in 2015.

2.5 Cultivation of fungal isolates and metabolite extraction for analysis

To obtain samples for FTICR-MS measurements, the *Alternaria* isolates 1–4 were cultivated in a synthetic liquid medium. The liquid medium contained ammonium sulfate (0.2 g/L), calcium nitrate tetrahydrate (0.3 g/L), glucose (4.0 g/L), iron sulfate heptahydrate (0.02 g/L), magnesium sulfate heptahydrate (0.25 g/L), potassium chloride (0.25 g/L), potassium dihydrogen phosphate (0.5 g/L), sodium acetate trihydrate (0.66 g/L), and sodium nitrate (2.0 g/L). After adjusting the pH to 5.5 using formic acid, 35 mL of the liquid medium were transferred into polycarbonate Erlenmeyer flasks and autoclaved at 121 °C for 20 min.

The sterile liquid medium was inoculated with defined spore suspensions. For the preparation of the spore suspensions, 3 mL of detergent solution (0.5 % tween 20) were pipetted on the overgrown agar plates and the mycelium and spores were scratched. The spores were counted using a Thoma chamber and the spore suspensions were diluted to $8.75 \cdot 10^5$ spores/mL for *A. alternata* and to $2 \cdot 10^5$ spores/mL for *A. solani*.[45] 25 µL of the spore suspensions of the *A. alternata* isolates and 100 µL of the spore suspension of the *A. solani* isolate were added to the liquid medium to receive equal amounts of total spores. During FTICR-MS measurements, contaminations originating from chemicals, solvents, plastic and glass surfaces were detected and these contaminations were compensated for by the analysis of control samples. The control samples were obtained by adding 25 µL of pure detergent solution to the liquid medium. All samples were prepared in replicates of five. The Erlenmeyer flasks were sealed with septa allowing sterile sampling during the cultivation process after four, seven, nine, and eleven days using cannulas and syringes. The fungi were cultivated in the dark (26 °C, 110 rpm) [46] and the isolates were exposed to artificial daylight for half an hour a day.

The liquid medium was analyzed by FTICR-MS after four, seven, nine, and eleven days of cultivation. 3 mL of the medium were sterilely taken from the Erlenmeyer flasks and centrifuged (15.000 x g, 10 min) to separate the mycelium. The pH of the supernatant was adjusted to pH 2 using formic acid. To protect the ESI source of the instrument from contamination, salts of the liquid medium were removed by solid phase extraction (Discovery® DSC-8, Supelco, Bellefonte, PA, USA). The C8 material was washed with 1 mL of methanol and conditioned with 1 mL of water (pH 2). After the sample loading (2 mL), the column was washed with 5 mL of water (adjusted to pH 2 using formic acid). The elution of the analytes was performed with 1 mL of methanol.

Besides, the mycelia of the fungi after eleven days of cultivation were also analyzed by FTICR-MS and LC-MS/MS. After separating the liquid medium from the mycelium by centrifugation, the latter was thoroughly washed with water to remove remaining liquid medium. 200 mg of the mycelium were weighed into a nucleo spin bead tube (type A, 0.6–0.8 mm, Machery Nagel, Düren, Germany), previously cleaned with 5 mL of water and 5 mL of methanol. After adding 1 mL of ice-cold methanol/water (90/10, v/v), the disruption of the cells was performed at 6800 rpm ($4 \cdot 30$ s) utilizing a Precellys homogenizer (bertin instruments, Montigny-le-Bretonneux, France). The homogenizer operated at -10 °C using liquid nitrogen. After the disruption, the cell suspension was centrifuged at 21.000 x g for 10 min, the supernatant was dried under nitrogen and the residue was

resolved in 2 mL of water (adjusted to pH 2 using formic acid). To ensure comparability of medium and mycelium samples, the aqueous solutions were desalted by solid phase extraction (Discovery® DSC-8, Supelco, Bellefonte, PA, USA). The C8 material was washed with 1 mL of methanol and conditioned with 1 mL of water (adjusted to pH 2 using formic acid). After loading the sample onto the column, the latter was washed with 5 mL of water (adjusted to pH 2 using formic acid). The elution of the analytes was performed with 1 mL of methanol.

2.6 FTICR-MS analysis

The acquisition of ultrahigh-resolution mass spectra was performed on a Bruker Solarix Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS) (Bruker Daltonics GmbH, Bremen, Germany) coupled to a 12 Tesla superconducting magnet (Magnex Scientific Inc., Yarnton, GB). The direct infusion of samples was performed with an APOLO II electrospray ionization source (Bruker Daltonics GmbH, Bremen, Germany) which operated in the negative ionization mode. The samples were diluted with methanol (1/10; v/v) prior to injection and were introduced into the ESI source at a syringe flow rate of 120 µL/h by a Gilson autosampler (Gilson, Inc., Meddleton, WI, USA). The spectra were externally calibrated by using ion clusters of arginine (10 mg/L in methanol) and were acquired with a time domain transient of four mega words in size. Measured masses ranged from *m/z* 150 to *m/z* 1000. For each sample, 300 scans were accumulated. This was equivalent to 13 min of analysis time. The capillary voltage was set to 3600 V and the spray shield voltage was -500 V. The drying gas flow rate and the drying gas temperature were adjusted to 4.0 L/min and 200 °C. The ion accumulation time was 0.3 s. A resolving power of 600.000 at *m/z* 300 was achieved. Subsequently, internal calibration was carried out on each mass spectrum by using a calibration list of *Alternaria* metabolites described in the literature. The calibration list covered a *m/z* range of 160 to 730 Da. The Data Analysis Version 4.2 (Bruker Daltonics GmbH, Bremen, Germany) was used to process raw spectra. The *m/z* values with a signal to noise ratio of 7 and a relative intensity threshold of 0.01 % were exported as mass lists. The mass lists were de-noised from the well-known Gibbs sidelobes (wiggles) by the use of a special program of denoising [47] and the clean mass lists were subsequently aligned using an in house written program (peak alignment window width: ± 1 ppm).[39,48] In total 120 spectra were measured.

2.7 Molecular formula annotation and database assignments of FTICR-MS data

The *m/z* signals that occurred in at least two out of five biological replicates were assigned unequivocally to molecular formulae by an in-house written software tool named NetCalc. The annotation of the molecular formulae is based on a mass difference network consisting of nodes and edges. The nodes represent *m/z* values (metabolite candidates) and edges constitute biochemical reactions.[49] The biochemical reactions can be expressed as mass differences between substrates and products and are predefined in a mass difference list covering 191 reaction-equivalent mass differences such as oxidation, reduction, hydroxylation, methylation, and the loss of CO_2.[49,34] As starting points of the network, 41 reference masses (*Alternaria* metabolites) with exact deprotonated mass and molecular formula were specified. Originating from the references, measured *m/z* values were assigned to molecular formulae by comparing the mass differences of all signals of a mass spectrum to the mass difference list.[49,34] Signals of isotopes and masses with an unusual mass defect were not included in the network.[49,50] The assignments of the elemental compositions contained only C, H, N, O, S, and P. The molecular formula allocation was performed

on 8139 *m/z* values resulting in 4467 monoisotopic elemental compositions (55 %) with 3285 molecular formulae exhibiting an annotation error within ± 0.2 ppm (74 %).[51,50] During FTICR-MS measurements, adduct ions such as chloride adducts are formed for some metabolites. 402 adducts were removed and 2883 annotated elemental compositions were used for further investigations. The calculated mass difference network was constituted using Gephi 0.9.2 software.[52]

To assign the experimental *m/z* values to metabolite candidates, the annotated molecular formulae were matched to the entries of Antibase.[53] For *Alternaria* and other genus of fungi, 95–98 % of the metabolites described in the literature are included in the database.[54] Additionally, the molecular formulae were matched to the entries of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/compound/).[55]

The annotated elemental compositions were used to calculate the hydrogen to carbon ratio (H/C) and the oxygen to carbon ratio (O/C). Subsequently, the ratios were displayed in a two-dimensional *van Krevelen* diagram to visualize the variations within the metabolic profile of the fungal isolates. [36]

Statistical evaluations were only performed on *m/z* values that occurred in at least four out of five biological replicates. Principal component analysis (PCA), an unsupervised statistical method, was applied for reducing the complexity of the data. After z-score normalization, the variation in the data was displayed as a set of new independent variables called the principal components. It was used for providing an overview of the complex multivariate data and for detecting outliers and relations between samples.[56,57] The PCA was performed using Simca-P 9.0 software (Umetrics, Sweden).

To determine the most discriminative molecular formulae between the *A. alternata* and the *A. solani* isolates, volcano plots were created. Volcano plots display the $\log_2$ fold change (ratio of averaged intensities of measured *m/z* signals) on the x-axis. The y-axis shows the $-\log_{10}$ p-value of measured intensities to significantly determine discriminating molecular formulae between the *A. alternata* and the *A. solani* isolates.[58]

2.8 LC-MS/MS analysis of *Alternaria* mycotoxins

AOH, AME, TEN, ATX I, ATX II, STTX III, and ALTP were chromatographically separated on a Shimadzu Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan). As stationary phase, a HyperClone BDS-C18 column (150 · 3.2 mm, 3 µm, 130 Å, Phenomenex, Aschaffenburg, Germany) was utilized. Further details on the instrument conditions, the solvent mixtures, and the gradient were published recently.[25] TA had to be analyzed in an additional LC-MS/MS run, due to the more polar character of the molecule. A Gemini-NX C18 column (150 · 4.6 mm, 3 µm, 110 Å, Phenomenex, Aschaffenburg, Germany) served as stationary phase. The solvent mixtures, the gradient, and further details on the chromatographic separation of TA are listed in the literature.[25] The LC was connected to a triple quadrupole ion trap mass spectrometer (LCMS-8050, Shimadzu Corporation, Kyoto, Japan). All analytes were detected in the negative electrospray ionization (ESI) mode. Details on the mass spectrometric conditions were published recently [25] and are listed in the Supporting Information (Table S1). The LabSolutions software (Shimadzu, Kyoto, Japan) was used for data acquisition and data analysis.

3 Results and Discussion

3.1 Comprehensive insights into the fungal mycobolome

3.1.1 Selection of fungal isolates

A. alternata isolates were analyzed by FTICR-MS to obtain a comprehensive insight into the fungal mycobolome. To cover a "general" *A. alternata* metabolome irrespective from the origin of the fungi, *A. alternata* was isolated from different sources such as potato leaves, tomato leaves, and mouldy tomatoes. Apart from the three small-spored *A. alternata* isolates 1, 2, and 3, one *A. solani* isolate was analyzed to reveal inter-species variations. The fungi were cultivated in a chemical defined liquid medium consisting of salts and glucose facilitating the detection of metabolites solely biosynthesized by *Alternaria*. As most of the metabolites produced by the fungi are excreted into the media,[29] the cultivation in liquid medium circumvented the extensive extraction of metabolites after growing the isolates on solid medium. In the literature, metabolic profiling is mainly performed by growing the fungi on solid media due to a higher quantity and a higher number of produced metabolites,[29] In 2018, Zwickel et al. performed a study on the metabolic profiles of different *Alternaria* species grown on rice,[31] After the cultivation, the mycotoxins were laboriously extracted using a mixture of acetonitrile/water/acetic acid.[59]

3.1.2 Molecular formula annotations

The network established during the annotation process is shown in Figure 1. The black nodes in the network represent assigned molecular formulae which are linked via edges representing biochemical reactions.[49] The smaller sections of the network illustrate the biochemical connections between the molecular formulae of AOH and AME, and between the molecular formulae of ATX I, ATX II/ALTP, and STTX III. The elemental compositions of AOH and AME are linked via the accurate mass difference of CH_2, whereas the accurate mass difference of H_2 interconnects the molecular formulae of the perylene quinones ATX I, ATX II/ALTP, and STTX III. In addition to ATX I, ATX II/ALTP, and STTX III, molecular formulae of other perylene quinones described in the literature were detected in the data. The molecular formulae of ATX III ($C_{20}H_{12}O_6$),[60] STTX IV ($C_{20}H_{12}O_7$),[60] alterlosin I/STTX I ($C_{20}H_{14}O_7$),[61,60] stemphyperylenol ($C_{20}H_{16}O_6$),[60] and alterlosin II/7-*epi*-8-hydroxy-altertoxin I/stemphytriol/6-*epi*-stemphytriol ($C_{20}H_{16}O_7$) [61,60] were determined and Figure 2 displays their elemental compositions as well as their chemical structures. Applying LC-Orbitrap, Zwickel et al. determined ATX I, ATX II, STTX III, as well as hydrated and dehydrated forms of these mycotoxins corresponding to further perylene quinones.[31] Besides, the latter authors detected additional peaks in the ion chromatograms of ATX I, ATX II, and STTX III, sharing the same elemental compositions as the respective mycotoxins within the mass error range (± 5 ppm) of the applied LC-Orbitrap MS instrument.

Apart from the determination of mycotoxins, a comprehensive analysis of the fungal mycobolome also includes the detection of precursors of metabolites. Previous studies on the biosynthesis of perylene quinones of fungi postulated a dinaphthyl intermediate as a precursor.[62,63] The dinaphthyl intermediate is supposed to originate from two tetralone derivatives biosynthesized from one acetate and six malonyl units.[63,64] Molecular formulae of 1,3,6,8-tetrahydroxynapthalene ($C_{10}H_8O_4$), syctalone ($C_{10}H_{10}O_4$), 1,3,8-trihydroxynaphtalene ($C_{10}H_8O_3$), vermelone ($C_{10}H_{10}O_3$),[65] and perylene quinone ($C_{20}H_{10}O_4$) [60] were detected in our data and might represent precursors of the respective perylene quinones. Biosynthetic pathways of fungal secondary metabolites are often not clarified

yet and reference compounds for the precursors are often not available. Therefore, the identification of the precursors could not be performed in the present study.

Modified forms of mycotoxins attract increasing attention in fungal investigations and sulfo-conjugations were frequently detected by different research groups.[31,19,66] During FTICR-MS measurements, the molecular formulae of the sulfo-conjugated forms of the mycotoxins AOH, AME, altenuisol, and altenuene [31] were not detected, which is not surprising as the intensities of the *m/z* signals of AOH, AME, and altenuisol in the mass spectra were already low.

The number of possible structural suggestions and the information on the biological context were extended by matching the molecular formulae to entries of two different databases. For the annotation of secondary metabolites, the elemental compositions were checked against the subset *Alternaria* of the database Antibase.[53] In total, 86 of the 2883 molecular formulae were assigned to metabolites which equals 3 % annotation rate. 97 % of the molecular formulae could not be assigned to metabolites using Antibase. Applying only the subsets *A. alternata* and *A. solani* of Antibase to the data, mainly solanapyrones and altersolanols were annotated for *A. solani*, whereas bicyclo- (BCA) and tricycloalternarenes (TCA) were assigned for *A. alternata*. Detailed information on the detected experimental masses, the theoretical neutral masses, the molecular formulae as well as the annotated metabolites using the subsets *A. alternata* and *A. solani* are depicted in Table 1.

Besides the secondary metabolites, molecules of the primary metabolism can be detected by FTICR-MS analysis. The assigned molecular formulae were compared to the entries of the KEGG database and subsequently allocated to species-specific pathways.[55] Comparing the assigned molecular formulae to the entries of the KEGG database resulted in 527 annotations (18 %) and, due to molecules with the same molecular formula, in 1820 metabolite annotations. The database assignments of less than 20 % illustrate the extent of the still unknown metabolism of *Alternaria* fungi and demonstrate the low coverage of the total diversity of all existing metabolites.[67] Besides, great attention should be paid to multiple annotations to only one molecular formula also hampering the identification of the metabolites.[38] This problem was addressed by Nielsen et al., as the researchers determined multiple entries of Antibase 2008 exhibiting identical molecular formulae. For example, the elemental composition of $C_{15}H_{22}O_3$ resulted in 113 metabolite candidates.[54] Another example of multiple assignments was given by Zwickel et al..[31] ATX II and ALTP share the same molecular formula and, therefore, exhibit identical *m/z* values in the mass spectrum. In the survey of Zwickel et al., various *Alternaria* isolates were analyzed by high-resolution mass spectrometry and four chromatographically separated peaks in the ion chromatogram of ATX II were assigned to the same molecular formula. Only ATX II and ALTP were identified, whereas the other two peaks could not be allocated to metabolites.[31] The results of Nielsen et al. and Zwickel et al. demonstrate the difficulty in dealing with multiple assignments of molecular formulae to metabolites.[54,31] To support the identification of metabolites, MS/MS spectra, specific UV-Vis spectra, and authentic reference compounds are indispensable.[54,30,68] In our survey, the lack of reference compounds allowed solely the hypothetical identification of the database assignments. The unambiguous identification of the metabolites was only performed for AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA using targeted LC-MS/MS analysis (see section 3.2).

Additionally, the metabolites were allocated to metabolic pathways listed in the KEGG database.[55] Figure 3 displays various pathways of amino acids, carbohydrates, and lipids related to *A. alternata*.

The black bars represent the number of annotated metabolites belonging to one specific pathway. Contrarily, the shaded bars show the number of molecules, which belong to the respective pathway, but the molecular formulae of which were not detected in the data. The percentage of detected molecular formulae with regard to the number of all metabolites belonging to the pathways shown in Figure 3 was mainly below 50 %. This may be due to the restrictions of the applied methodology, e.g. a too small or large molecule size (beyond the detection limit of the MS analyzer), the presence of other elements than CHNOSP in the elemental composition, the SPE conditions during sample preparation, the ionization mode during MS measurements or simply due to low concentrations of the molecules.

After the annotation of *m/z* signals to molecular formulae, H/C and O/C ratios were calculated from the elemental compositions.[38] The H/C ratios of the molecular formulae were plotted against the O/C ratios [69] and each *Alternaria* isolate was displayed separately on such a *van Krevelen* diagram. In the *van Krevelen* plot, different metabolite classes have their specific position based on different elemental compositions (Fig. 4).[36] The positions of the metabolite classes of fatty acids, amino acids and peptides, carbohydrates, and polyphenols are displayed in Figure 4a. For all *Alternaria* isolates, 72 % of the molecular formulae corresponded to a CHO composition, followed by 17 % of a CHNO composition, and 11 % of CHOS and CHNOS compositions. Comparing the plots of Figure 4a–d with each other, the profiles of the mycobolome differ. In the *A. solani* samples, fatty acids and condensed terpenoids are displayed, whereas in the *A. alternata* samples, the polyphenols were dominant. All investigated *Alternaria* fungi share the presence of amino acids and peptides.

As the molecular formulae of the *Alternaria* mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, and TA, of further perylene quinones, possible precursors and other secondary metabolites were detected in the FTICR-MS data, we assumed a representative coverage of the *Alternaria* mycobolome under the given conditions. Besides, the suitability of the liquid medium for the cultivation of *Alternaria* isolates was confirmed and the sample preparation protocol as well as the conditions during the measurements allowed the detection of a wide range of fungal secondary metabolites.

3.2 LC-MS/MS detection of *Alternaria* mycotoxins

In addition to the non-targeted analysis of fungal extracts, the identification and quantification of the mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA were complemented by targeted LC-MS/MS analysis. The mycotoxins were compared to reference compounds and were identified based on retention times and mass transitions.[25] A chromatogram of AOH, AME, ATX I, ATX II, ALTP, STTX III, and TEN is displayed in the Supporting Information Figure S1a. An additional chromatographic run had to be performed for TA due to different polarity (Fig. S1b). As adequate amounts of stock solutions were not available for ATX II and STTX III, quantitative values could not be calculated for these mycotoxins. The sample preparation and LC-MS/MS analysis were not fully validated for the fungal cultures in this study and, therefore, no limits of detection and quantification were calculated. However, to precisely identify the mycotoxin signals in the LC-MS/MS run, a minimum peak area unit of 10^5 was stated. The peak areas of the mycotoxins are displayed as mean values of areas of the five biological replicates (Fig. $5a_1$–d_1). If one or two of the five replicates showed peak areas below the area cut off, a peak area of $1 \cdot 10^5$ was used for the calculation of mean values. Outliers were detected by applying Dixon's Q testing.

The *A. solani* isolate did not produce any of the targeted *Alternaria* mycotoxins above the peak area cut off, neither on the first day of sampling nor on the other days of cultivation. In the literature, *A. solani* is reported to produce AOH and AME.[70,71] AOH was consistently detected in the medium of the *A. alternata* isolate 2, whereas the *A. alternata* isolate 1 and the *A. solani* isolate did not produce AOH at all. AME was not detected in the media of any isolate. As the benzopyrones were neither detected in the extracts of *A. solani* nor often analyzed in the samples of *A. alternata*, the excretion of AOH and AME into the liquid medium might be low. The three *A. alternata* isolates produced various mycotoxins exceeding the minimum peak area (Fig. $5a_1$–d_1). The most frequently detected mycotoxins were the perylene quinones ATX I, ATX II, ALTP, and STTX III. These mycotoxins were produced by all *A. alternata* isolates and were detected at each day of sampling. During the cultivation, the peak area of ATX II and STTX III decreased to less than one tenth for all *A. alternata* isolates when comparing the peak area of the 4th to the 11th day of cultivation. Contrarily, a decrease of the peak area of ATX I and ALTP could not be observed. ATX II and STTX III structurally share an epoxy group,[60] probably sensitive to chemical degradation during the cultivation process. The *A. alternata* isolates 1, 2, and 3 produced TEN, which was detected from the 7th day of cultivation on. TA was produced by the isolates 2 and 3, while the isolate 1 did not produce TA at all. Due to different sensitivity of the analytes in the mass spectrometer, the peak areas of the mycotoxins were transferred into contents via one-point calibration (Fig. $5a_2$–d_2). Quantitative results could not be calculated for ATX II and STTX III due to the lack of adequate amounts of stock solutions. The determined contents of AOH were below 5 µg/kg for the isolates 2 and 3. ATX I and ALTP were detected in all *A. alternata* isolates in contents ranging from 28 to 233 µg/kg and from 40 to 182 µg/kg. TA was produced by the isolates 2 and 3 and the calculated amounts varied from 5400 to 20000 µg/kg. The highest content of TEN of 37 µg/kg was produced by the isolate 1. However, it has to be mentioned that the calculated contents were obtained using only one-point calibration and were not quantified by matrix matched calibration or by using isotopically labeled internal standards. Therefore, the given contents should only be classified as semi-quantitative.

The biosynthetic capabilities of diverse *Alternaria* species has been reported by Andersen et al. [30] and Zwickel et al.,[31] who both have been growing their isolates on solid media. Anderson et al. investigated 87 *Alternaria* isolates and allocated 22 isolates to the *A. arborescence*, *A. infectoria*, *A. tenuissima*, and *A. alternata* species groups.[30] Similarly, Zwickel et al. [31] performed studies on 93 isolates of *A. alternata*, *A. arborescence*, *A. tenuissima*, and *A. infectoria.* In the former study, the *A. alternata*, *A. arborescence*, and *A. tenuissima* isolates frequently produced AOH, AME, and altenuene, whereas TEN and TA were biosynthesized less frequently. In the study of Zwickel et al., 21 isolates belonged to the *A. alternata* species group and 90 % of the isolates produced ATX I, 81 % produced STTX III, and 76 % ATX II and ALTP. AOH and AME as a group as well as TA were biosynthesized by 81 % and 76 % of the *A. alternata* isolates, respectively. Interestingly, five out of the 93 *Alternaria* isolates did not biosynthesize any of the analyzed mycotoxins. These results are partly different to ours, as the benzopyrones were not detected in the liquid medium of our *A. alternata* isolate 1 and were rarely determined in the samples of the *A. alternata* isolate 3. One explanation for this discrepancy could be given by the study of Söderhäll et al.,[72] who investigated the mycotoxin production of *A. alternata* under the exposure of white light. Depending on the growth phase of the fungus, the biosynthesis of the benzopyrones was almost completely inhibited after the light exposure,[72] which may have been also the case for our cultures. The perylene quinones ATX I, ATX II, ALTP, and STTX III were produced by all *A. alternata* isolates, which is in good

agreement with the results of Zwickel et al..[31] Alike in the study of Zwickel et al.,[31] TEN was produced by all *A. alternata* isolates but was detected above the peak area limit only from the second day of sampling on. The results of Zwickel et al. that some of the *A. alternata* isolates did not produce any *Alternaria* mycotoxins clearly demonstrate the differences in the metabolic capabilities of isolates belonging to the same species group.[31,30] Due to the differences in the TA production of the three *A. alternata* isolates in our survey, large numbers of reliable isolates of the same taxon should be used for differentiation investigations based on mycotoxin profiles.[73]

The mycotoxins AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA were also determined in the extracts of fungal cells from the mycelium (Fig. 6a). Contrarily to the liquid medium, AOH was detected in the cells of all investigated *Alternaria* isolates and AME was determined in the cells of *A. solani* and of the *A. alternata* isolates 2 and 3. This confirmed the ability of the *A. solani* isolate to produce these two mycotoxins. The perylene quinones were verified in the cells of all *A. alternata* isolates. Interestingly, TEN was only determined in the cells of the isolate 2, whereas TA was not detected in any of the *Alternaria* cells. Figure 6b displays the contents of AOH, AME, ATX I, ALTP, and TEN calculated via one-point calibration. The highest contents were determined for ATX I and ALTP, ranging from 730 to 1900 µg/kg and from 400 to 1300 µg/kg, respectively. AOH was detected in contents from 11 to 120 µg/kg and AME from 0.5 to 2.7 µg/kg. Again, the contents have to be considered as semi-quantitative. Although the benzopyrones were detected frequently in the extracts of the disrupted cells, AOH and AME were only rarely analyzed in the liquid medium. As the benzopyrones were also detected in almost all *A. alternata* cultures in the study of Zwickel et al.,[31] this suggests that these compounds are generally formed, but only excreted under certain conditions to the medium. Contrarily, the perylene quinones ATX I, ATX II, ALTP, and STTX III were determined in the extracts of the cells and were excreted into the liquid medium. A different tendency was observed for TA as this mycotoxin was fully excreted into the liquid medium and was not detectable inside the fungal cells. The transport mechanisms to export these metabolites are still largely unknown. It can be hypothesized that extracellular vesicles reported to carry virulence factors [74] may be involved.

3.3 Differentiation between *Alternaria* species

Apart from the comprehensive description of the *Alternaria* mycobolome, similarities and differences between samples can be detected by FTICR-MS and subsequent PCA. Before performing the PCA, the matrix was filtered by keeping only *m/z* signals that occurred in at least four out of five biological replicates ensuring the biological importance of the remaining signals. The PCA was performed on the whole data set and each day of sampling is displayed as an individual PCA plot (Fig. 7). In the scores plot of the 4th day of cultivation (Fig. 7a), the replicates of *A. solani* and the controls revealed distinct clusters displaying differences in the second component. For the *A. alternata* isolate 1, four of the five replicates clustered together while the fifth replicate was determined in the cluster of the *A. solani* isolate. The replicates of the *A. alternata* isolates 2 and 3 showed high variation and did not form distinct clusters. In the PCA model, 26.9 % of the total variance are explained in the PC1 and 8.6 % are explained in the PC2. On the 7th day of cultivation (Fig. 7b), clear clusters are formed by the replicates of the isolate 1, the *A. solani* isolate, and the controls. The clusters of the isolates 2 and 3 are partly overlapping and, therefore, the two isolates cannot be separated clearly. A similar cluster formation to the 7th day of cultivation is obtained on the 9th day of cultivation (Fig. 7c). However, the replicates of the *A. solani* isolate and the controls

are overlapping. At the 11th day of cultivation (Fig. 7d), the *A. solani* replicates and the controls form distinct clusters, which are clearly separated in the second component. The isolate 1 forms a distinct cluster and the replicates of the isolates 2 and 3 are overlapping. Again, the highest variation was determined between the five replicates of the isolate 3. Due to the formation of clusters in the PCA, differences in the mycobolome of the different *Alternaria* isolates are obvious. The distinction of the *A. alternata* and *A. solani* isolates in the PCA was based on more than 3000 *m/z* values, whereas the differentiation by LC-MS/MS was performed using eight mycotoxins. The FTICR-MS measurements confirmed the LC-MS/MS results according to which the *A. solani* isolate differs from the *A. alternata* isolates in the mycotoxin production. Besides, the *A. alternata* isolate 1 was proven to vary from the other two *A. alternata* isolates.

In the literature, different approaches were applied to differentiate between various *Alternaria* species. Zwickel et al. compared the metabolic capabilities of various *Alternaria* isolates belonging to *A. alternata*, *A. arborescence*, *A. tenuissima*, and *A. infectoria*.[31] Based on the metabolic profile and overall low mycotoxin production, the *A. infectoria* isolates were segregated from *A. alternata*, *A. arborescence*, and *A. tenuissima*. Contrarily, the analysis of various mycotoxins by HPLC-MS/MS could not separate the isolates of *A. alternata*, *A. arborescence*, and *A. tenuissima*.[31] Andersen et al. obtained identical results when analyzing the mycotoxin profiles of *A. infectoria*, *A. alternata*, *A. arborescence*, and *A. tenuissima*.[30] Besides the analysis of mycotoxin profiles, Andersen et al. performed a metabolic differentiation of *A. alternata*, *A. gaisen*, *A. limoniasperae*, *A. longipes*, *A. tangelonis*, and *A. turkisafria* based on direct infusion MS. In the mass spectra, 100 to 400 ions were detected, respectively, and the clustering of the isolates resulted in a separation of four of the six *Alternaria* species. *A. gaisen*, *A. turkisafria*, *A. tangelonis*, and *A. alternata* clustered in four separated clusters, whereas one isolate of *A. limoniasperae* and *A. longipes* clustered apart from the other isolates of the related species-group, respectively.[32] In our survey, we analyzed three different *A. alternata* isolates by FTICR-MS and the *A. alternata* isolate 1 clustered apart from the other two isolates in the PCA plots. Accordingly, Andersen et al. did not obtain distinct clusters for the *A. limoniasperae* and *A. longipes* isolates as one isolate clustered apart from the other isolates belonging to the same species-group.[32]

3.3.1 Determination of discriminating metabolites

To identify metabolites, which are responsible for the grouping in the PCA plots, volcano plots were created. Only *m/z* values that were assigned to molecular formulae were considered to be relevant metabolite candidates [50]. The volcano plots were created by plotting the $-\log_{10}$ p-value against the $\log_2$ fold change of the MS signal intensities of the *A. solani* and the *A. alternata* replicates.[58] A volcano plot was created for the *A. solani* and the *A. alternata* isolate 1 at the 11th day of cultivation (Fig. 8a). The horizontal line in the plot represents the significance value of 0.01. The higher the y value of one molecular formula is, the more significant is the difference. Interesting molecular formulae are located on the upper left part and upper right part of the plot.[58]

To allocate the discriminating molecular formulae to metabolites, all discriminating elemental compositions were checked against Antibase.[53] The assignments are displayed as blue triangles for discriminating molecular formulae of *A. solani* and as green hashes for *A. alternata*. If one allocated molecular formula was discriminating in the *A. solani–A. alternata* isolate 1 comparison, as well as in the *A. solani–A. alternata* isolate 2 and *A. solani–A. alternata* isolate 3 comparison (Supporting Information, Fig. S2) its *m/z* value, the theoretical neutral mass, the error of annotation, the

molecular formula as well as the metabolite assignment were listed in Table 2. For *A. solani*, four molecular formulae were significantly different in all comparisons and were allocated to the metabolites altechromone A,[75] quadrilineatinmethylether,[76] 2,4-dihydroxy-6-acetonyl-benzoic acid,[77] and (8R,9S)-9,10-epoxy-8-hydroxy-9-methyldeca-(2E,4Z,6E)-trienoic acid [78] (Table 2). For *A. alternata*, seven molecular formulae were assigned to metabolites (Table 2), e.g. ATX III [12]/STTX III,[79] xanalteric acid I/II,[80] alterlosin I, and alterlosin II.[61] One of the orange marked squares in the volcano plot a (Fig. 8) represents the molecular formula of the mycotoxin STTX III, identified by targeted LC-MS/MS analysis.[81] In the LC-MS/MS studies, the perylene quinones ATX I, ATX II, ALTP, and STTX III were only produced by the *A. alternata* isolates. In the literature, the production of the perylene quinones by *A. alternata* and, additionally, by *A. arborescence* and *A. tenuissima* is reported. Contrarily, the biosynthesis of these mycotoxins by *A. solani* is not mentioned.[31] Interestingly, the molecular formulae of ATX I ($C_{20}H_{16}O_6$) and ATX II/ALTP ($C_{20}H_{14}O_6$) were also detected in the samples of *A. solani* in our FTICR-MS measurements but were not identified as ATX I, ATX II, and ALTP by LC-MS/MS analysis using reference compounds. As these molecular formulae were also detected in the samples of *A. solani*, these elemental compositions are not identified as discriminant masses by the volcano plots. Obviously, our *A. solani* isolate produces different compounds with the same molecular formulae as the perylene quinones, which points to the need of using these complementary methods for differentiating the metabolomes. The second orange marked square in Figure 8a represents the molecular formula ($C_{23}H_{20}O_9S$) of a discriminating metabolite named APML. In this study, APML was extracted from overgrown rice, purified by various stationary and mobile phases, and characterized by ^{1}H and ^{13}C NMR analysis as well as ^{1}H–^{1}H COSY, HSQC, and HMBC 2D NMR analysis (Supporting Information, Table S2-S4, Fig. S3-S11).

Due to the lack of reference compounds, the other assigned elemental compositions were only tentatively allocated to metabolites. The remaining molecular formulae that were also significantly different among *A. solani* and *A. alternata* could not be assigned by Antibase.[53] This reveals the still unknown metabolites produced by *Alternaria* fungi and the necessity for additional studies on the comprehensive analysis of the *Alternaria* mycobolome.

Apart from the differences in the mycobolome of *A. solani* and *A. alternata*, variations between different *A. alternata* isolates can be determined. The volcano plot b in Figure 8 displays the differences in the mycobolome of the *A. alternata* isolates 1 and 2. The discriminating elemental compositions were checked against the entries of Antibase [53] and five (three) molecular formulae were assigned to metabolites of the isolate 1 (isolate 2) (Table 3). The orange marked square (Fig. 8b) represents the molecular formula of the mycotoxin TA, which was already proven to be discriminant by targeted LC-MS/MS analysis. Although the two isolates belong to the same species group and were cultivated under the same conditions, some metabolites differ in averaged signal intensities or are only produced by one of the two isolates. To confirm and improve the results, more isolates from the species groups of *A. alternata* and *A. solani* need to be analyzed by FTICR-MS and also fungal isolates from different origins should be included. As it is not possible to fully characterize the fungal mycobolome using only one type of instrument,[68] different approaches and various analytical techniques are required to receive a more holistic picture of the mycobolome of *Alternaria* fungi.[82] A more holistic knowledge on the secondary metabolism of the food contaminating fungus *Alternaria* enables proper risk evaluations on food and feed. After isolating and characterizing known *Alternaria* mycotoxins as well as new secondary metabolites, their

toxicological capabilities should be investigated. Besides, accurate quantitative analytical methods are needed to obtain more data on the occurrence and contents of *Alternaria* metabolites in agricultural commodities. A sound knowledge on the toxicology as well as on the occurrence of the fungal food contaminants is essential to allow the EFSA to perform proper risk evaluations on food and feed products.

4 Concluding remarks

The mycobolome of various *Alternaria* isolates was analyzed by FTICR-MS and complemented with LC-MS/MS analyses. From the total number of detected *m/z* signals of the FTICR-MS, 35 % could be assigned to unequivocal molecular formulae of potential metabolites. Of these formulae, only 3 % could be verified as specific fungal metabolites using the Antibase database, which particularly focuses on fungi. Performing an additional database search against the KEGG database resulted in only 18 % of assignments of the 2883 molecular formulae to general cellular metabolites. This result indicates that only about one fifth of the metabolome signals are potentially known. However, this estimation of the current database knowledge becomes even worse, when we consider that only a small number of the metabolites, whose molecular formulae were effectively detected by FTICR-MS, could be assigned by targeted LC-MS/MS using authentic reference compounds as revealed by the missing perylene quinones in the *A. solani* extracts in the LC-MS/MS measurements. On the other hand, complementing the FTICR-MS data with targeted LC-MS/MS analyses is necessary, as the molecular formulae of the direct infusion FTICR-MS measurements generally can be assigned to several metabolites, due to lack of retention time information in direct infusion FTICR-MS. Another striking result from the pathway analyses was the missing detection of over 50 % of expected metabolites in both LC-MS/MS and FTICR-MS measurements, which also indicates that our coverage of the metabolome is still very low. The low percentage of database allocations demonstrates the need for further comprehensive investigations of the *Alternaria* mycobolome. The clear identification of interesting metabolites remains the bottleneck of metabolomics and authentic reference compounds of fungal metabolites are necessarily needed for identification purposes. The non-targeted analysis of the fungal mycobolome by FTICR-MS unraveled variations in the metabolome of *A. solani* and *A. alternata* and enabled the detection of discriminating metabolites. One of these discriminating metabolites was identified as alterperylenepoxide A-9-mercaptolactate by ^{1}H, ^{13}C, and 2D NMR analysis after isolating the metabolite. In addition to the inter-species variations, differences within the *A. alternata* species were determined. Further isolates from the species group of *A. alternata* and *A. solani* need to be analyzed by FTICR-MS to confirm the intra- and inter-variation of the mycobolome. Additionally, isolates from different small spored *Alternaria* species such as *A. tenuissima* and *A. arborescence* should be included in future investigations. Combined to additional analytical tools, FTICR-MS is a promising tool for the chemotaxonomic differentiation of fungal isolates. In future mycobolome investigations, FTICR-MS should also be applied to *Alternaria* fungi-plant interactions or in the field of food contaminants related to *Alternaria* mycotoxins.

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MG, MR, and PSK designed the experiments. MG performed the cultivation of fungi and the salting of samples. MG and BK analyzed the samples by using FTICR-MS and MG performed the LC-MS/MS measurements. FS accomplished the extraction and purification of APML and FS, RH, OF, and TH confirmed the structure of APML by NMR analyses. MG wrote the manuscript. MR, BK, and PSK revised the manuscript. All authors contributed to the revision of the manuscript.

6 Conflict of Interest

The authors declare that they have no conflict of interest.

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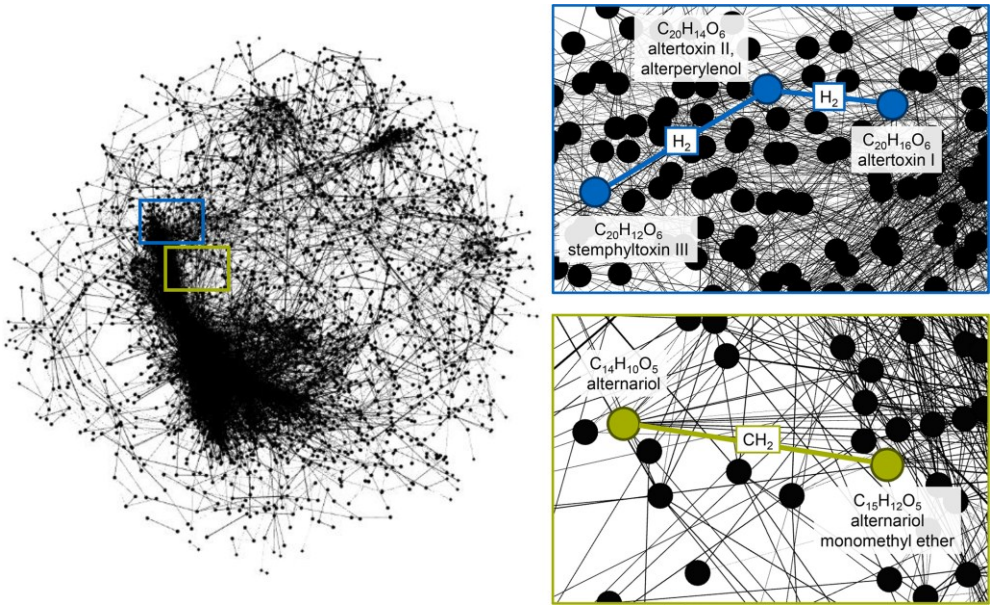

Fig. 1 Network generated for annotation purpose. The blue box shows the connections between STTX III, ATX II/ALTP, and ATX I and the green box displays the computational connection between AOH and AME

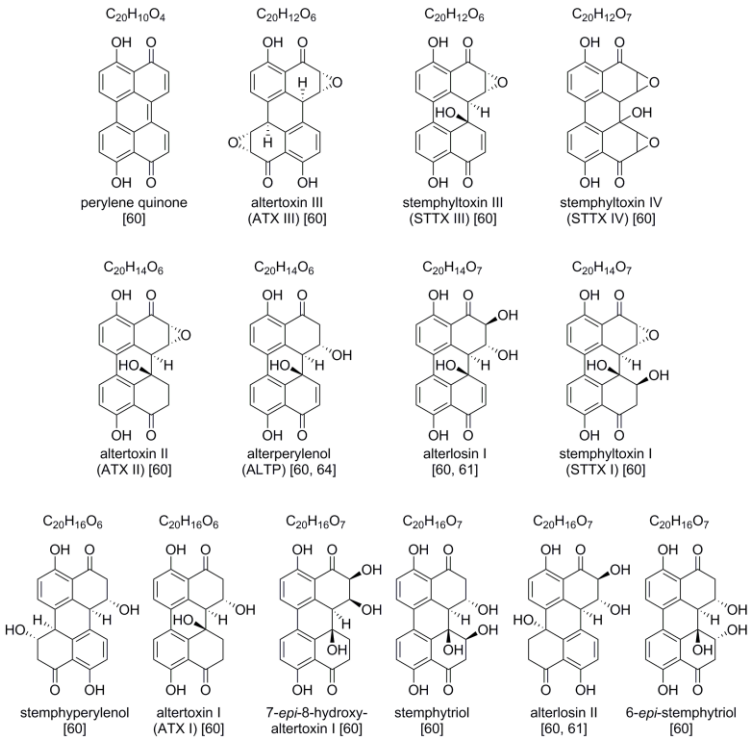

Fig. 2 Molecular formulae and structures of perylene quinone derivatives in the literature possibly detected in the FTICR-MS data

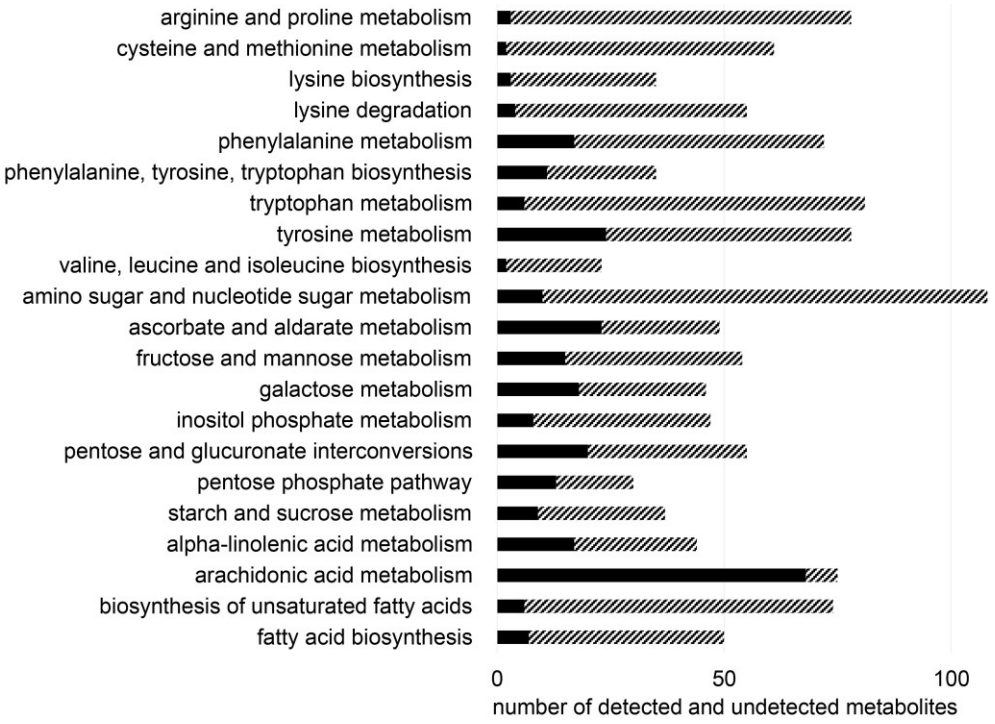

Fig. 3 The illustration displays the number of detected molecular formulae (black bars) of various metabolic and biosynthetic pathways in the KEGG database.[55] The shaded bars represent metabolites belonging to the respective pathways, but the molecular formulae were not detected in the FTICR-MS data

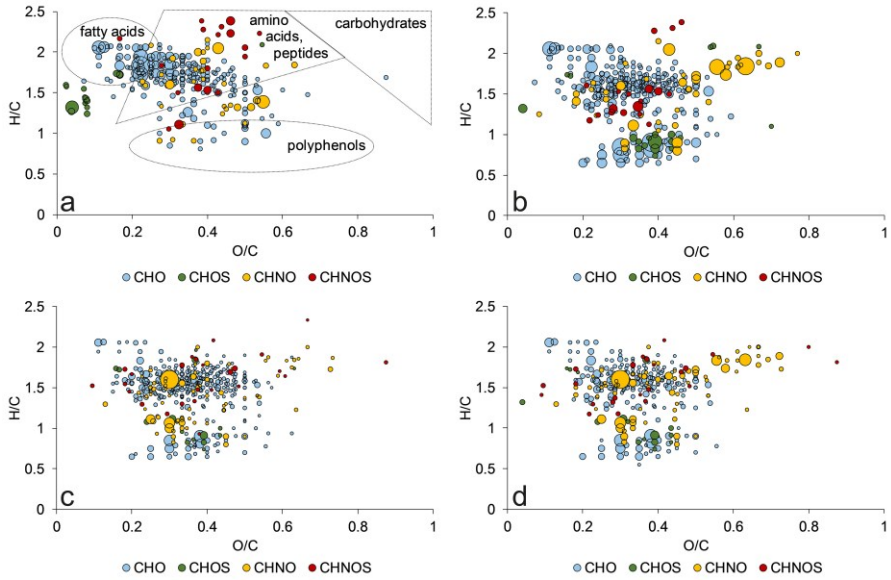

Fig. 4 Van Krevelen diagrams of *A. solani* (a), *A. alternata* isolate 1 (b), *A. alternata* isolate 2 (c), and *A. alternata* isolate 3 (d). The labelling of the chemical groups was performed according to Roullier-Gall et al.,[36] Liu et al.,[114] and Schmitt-Kopplin et al.[115]

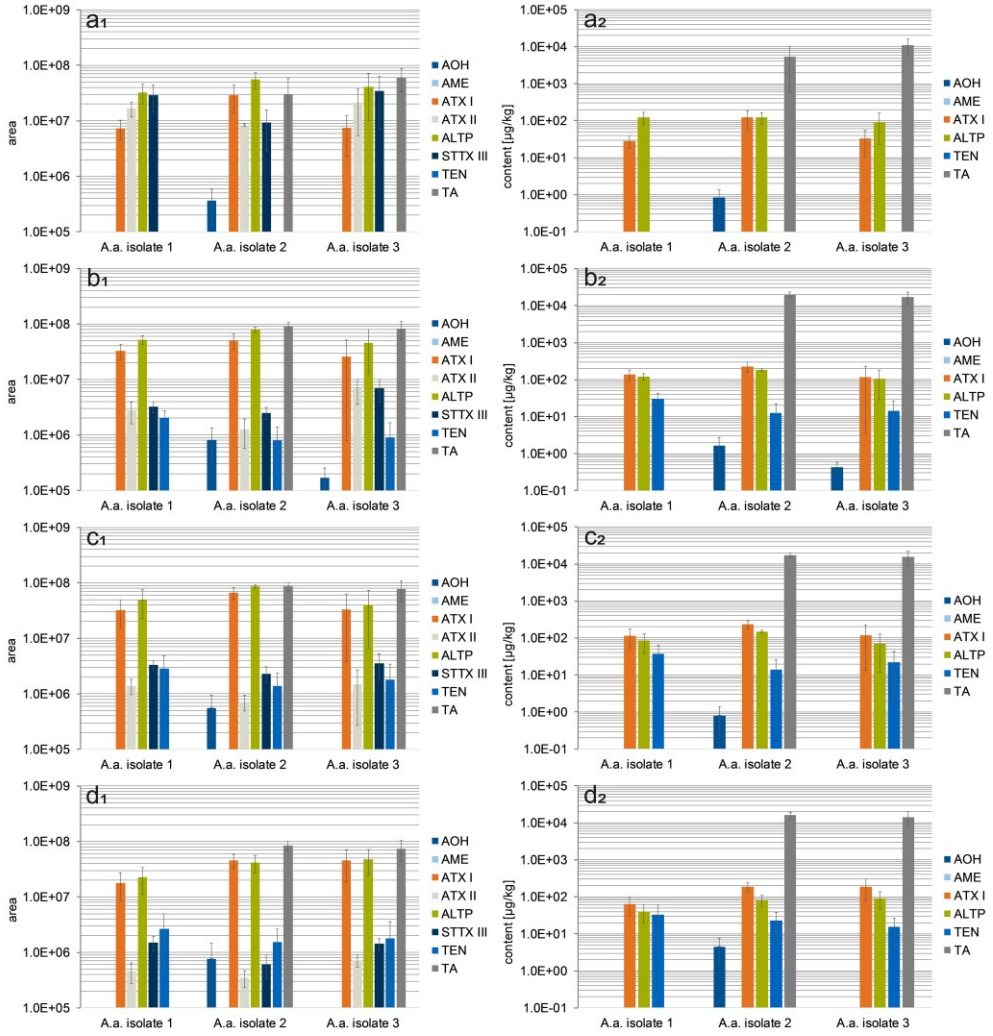

Fig. 5 The peak areas of AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA are displayed for the 4th day (a_1), 7th day (b_1), 9th day (c_1), and 11th day (d_1) of cultivation. The peak areas are shown as mean values and standard deviations of the five replicates. The *A. solani* isolate is not displayed, as this isolate did not produce any mycotoxin above the peak area limit. The diagrams a_2–d_2 show contents of the mycotoxins AOH, AME, ATX I, ALTP, TEN, and TA in the liquid medium of the 4th, 7th, 9th, and 11th day of cultivation calculated via one-point calibration. Please, note the logarithmic axis of the peak areas and of the mycotoxin contents

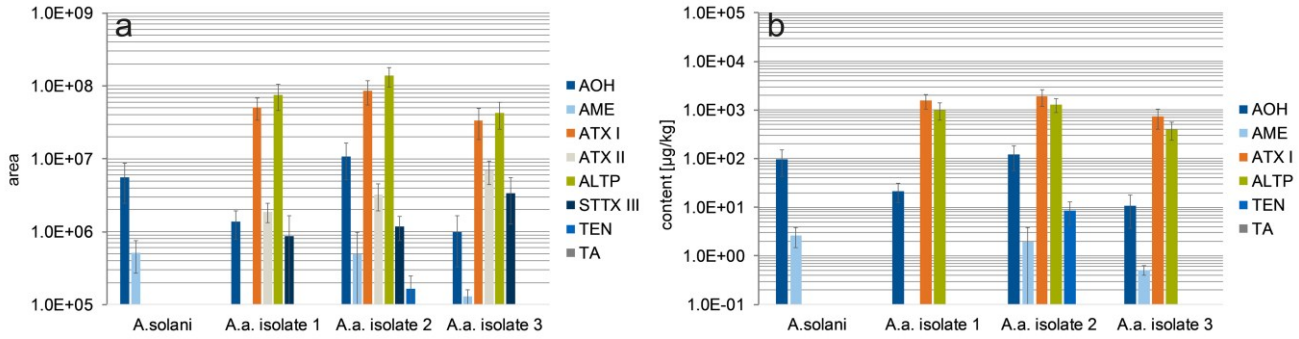

Fig. 6 The diagram a displays the peak areas of AOH, AME, ATX I, ATX II, ALTP, STTX III, TEN, and TA in the disrupted cells form the mycelium. The peak areas are shown as mean values and standard deviations of the five replicates. The diagram b shows the contents of the mycotoxins AOH, AME, ATX I, ALTP, and TEN in the fungal cells calculated via one-point calibration. Please, note the logarithmic axis of the peak areas and of the mycotoxin contents

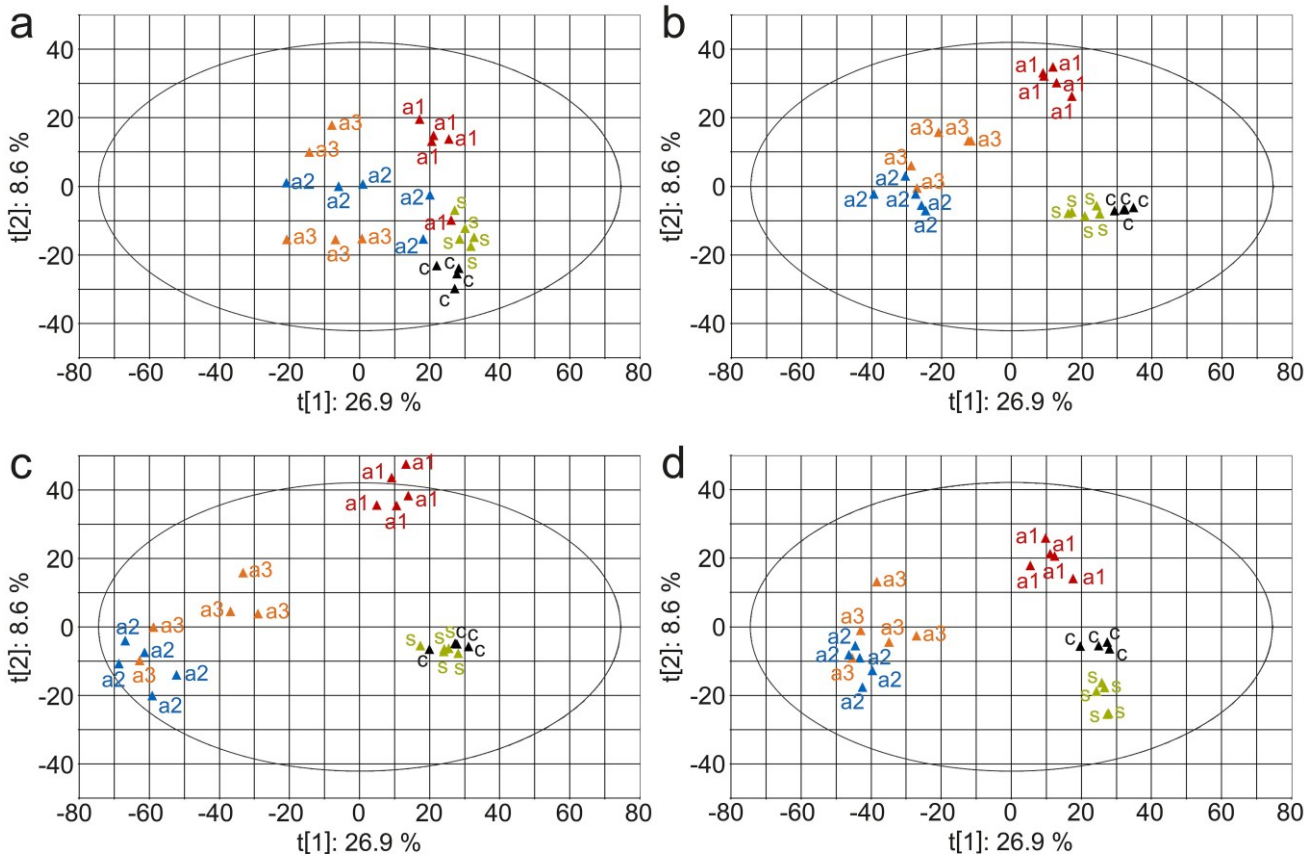

Fig. 7 Scatter plots of the Principal Component Analysis (PCA) of the FTICR-MS data. The plots display the *A. solani* (▲s), the three *A. alternata* isolates 1 (▲a1), 2 (▲a2), and 3 (▲a3) and the control samples (▲c) on the 4th day (a), the 7th day (b), the 9th day (c), and the 11th day (d) of cultivation

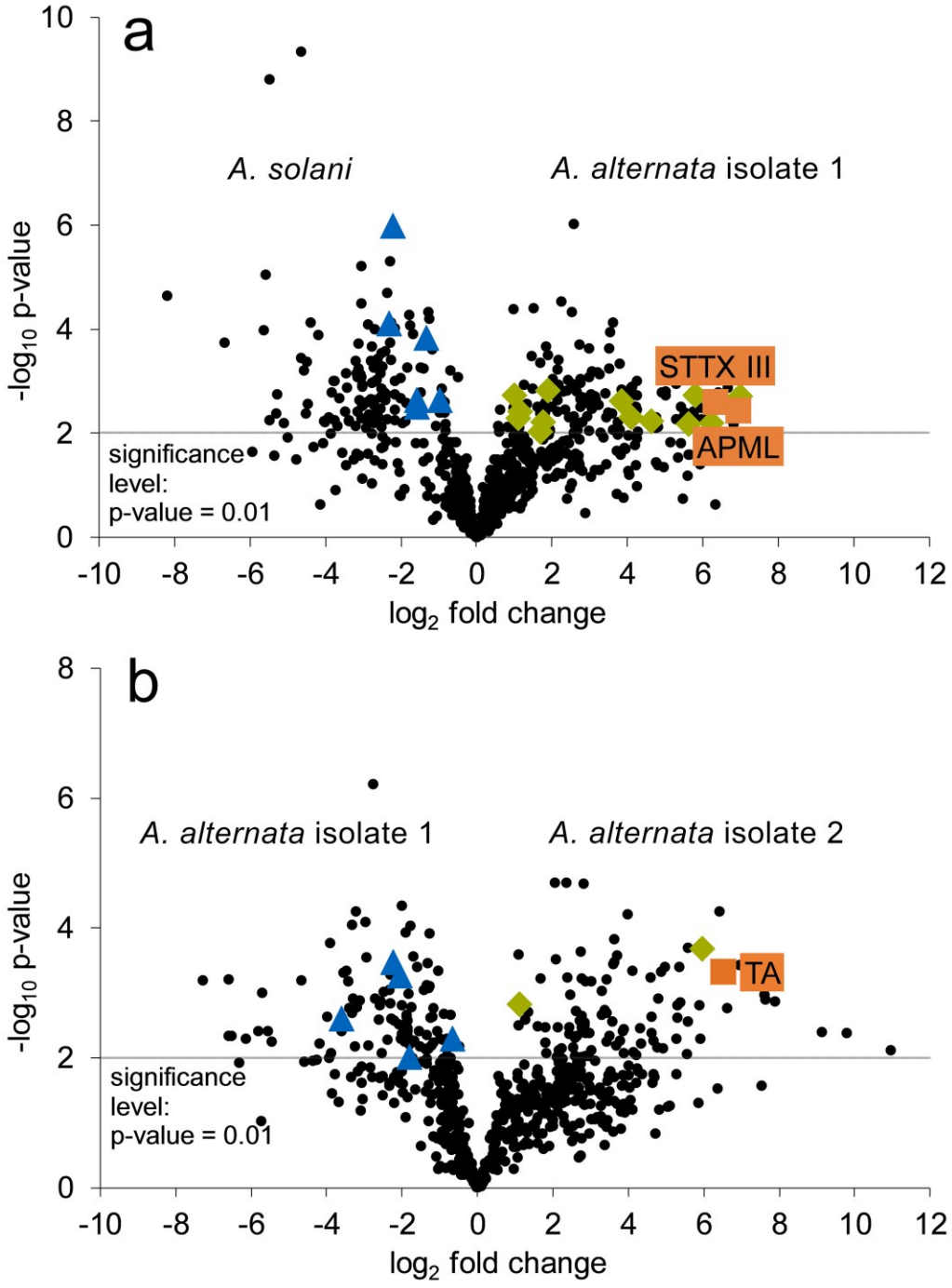

Fig. 8 The volcano plot a displays the discriminant masses between *A. solani* and the *A. alternata* isolate 1 at the 11th day of cultivation. The volcano plot b depicts the discriminant masses between the *A. alternata* isolate 1 and 2 at the 11th day of cultivation. The triangles and hashes represent molecular formulae that were assigned to metabolites by the Antibase database.[53] Due to the lack of reference compounds, the identification of these metabolites could only be performed for STTX III, APML, and TA (displayed as squares) using targeted LC-MS/MS analysis. Details on the extraction and purification, on the structure elucidation, on the mass spectrometric fragmentation pattern, and UV/Vis absorption spectrum of APML are displayed in the Supporting Information.

Table 1 Detected experimental masses, theoretical neutral masses, annotation errors, annotated molecular formulae, and metabolite candidates of the database match in Antibase for the subsets *A. alternata* and *A. solani* [53]

Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Subset in Antibase	Annotated molecular formula	Assignments during database search for *A. solani* and *A. alternata*
301.14452	302.15181	-0.044	*A. solani*	$C_{18}H_{22}O_4$	solanapyrone A,[83] solanapyrone E,[84] prosolanapyrone III [85]
330.17112	331.17836	0.103	*A. solani*	$C_{19}H_{25}NO_4$	solanapyrone C [86]
333.09796	334.10526	-0.059	*A. solani*	$C_{17}H_{18}O_7$	altersolanol G [87]
335.07725	336.08452	0.016	*A. solani*	$C_{16}H_{16}O_8$	altersolanol A,[88] altersolanol D-F [89]
271.06118	272.06848	-0.064	*A. alternata*	$C_{15}H_{12}O_5$	AME [90]
319.15511	320.16238	0.030	*A. alternata*	$C_{18}H_{24}O_5$	TCA A [91]
323.15002	324.15729	0.022	*A. alternata*	$C_{17}H_{24}O_6$	AF toxin II, AF toxin IIA, AF toxin IIC [92]
345.20716	346.21441	0.083	*A. alternata*	$C_{21}H_{30}O_4$	ACTG Toxin D,[93] TCA 2a/b, TCA 8a,[94] ACTG Toxin E [95]
347.22279	348.23006	0.028	*A. alternata*	$C_{21}H_{32}O_4$	TCA 1a/b,[96] BCA 3, BCA 9 [97]
361.20204	362.20933	-0.015	*A. alternata*	$C_{21}H_{30}O_5$	TCA C [91]
361.23843	362.24571	-0.006	*A. alternata*	$C_{22}H_{34}O_4$	TCA 11a/b,[94] BCA 4, BCA 5 [97]
363.21769	364.22498	-0.015	*A. alternata*	$C_{21}H_{32}O_5$	TCA 6a/b,[94] BCA 2, BCA 8,[97] TCA E [91]
365.23334	366.24063	-0.017	*A. alternata*	$C_{21}H_{34}O_5$	BCA 1 [97]
367.08232	368.08961	-0.032	*A. alternata*	$C_{20}H_{16}O_7$	alterlosin II [61]
377.23334	378.24063	-0.034	*A. alternata*	$C_{22}H_{34}O_5$	TCA 7a/b,[94] BCA 10 [97]
379.24899	380.25628	-0.027	*A. alternata*	$C_{22}H_{36}O_5$	BCA 11 [97]
381.22825	382.23554	-0.029	*A. alternata*	$C_{21}H_{34}O_6$	BCA 6 [97]
387.21768	388.22498	-0.047	*A. alternata*	$C_{23}H_{32}O_5$	TCA B [91]
389.23332	390.24063	-0.085	*A. alternata*	$C_{23}H_{34}O_5$	TCA D [91]
395.24390	396.25119	-0.045	*A. alternata*	$C_{22}H_{36}O_6$	BCA 7 [97]
413.21944	414.22671	0.030	*A. alternata*	$C_{22}H_{30}N_4O_4$	TEN [98]
415.23507	416.24236	-0.018	*A. alternata*	$C_{22}H_{32}N_4O_4$	dihydrotentoxin [99]
423.20244	424.20972	-0.013	*A. alternata*	$C_{22}H_{32}O_8$	AF Toxin III, AF Toxin 3A [100]
439.19734	440.20464	-0.033	*A. alternata*	$C_{22}H_{32}O_9$	AF Toxin 1, AF Toxin A1 [100]

Table 2 Discriminant molecular formulae between *A. alternata* and *A. solani* assigned to metabolites by Antibase.[53] For *A. solani*, four discriminant molecular formulae were annotated to metabolites, whereas for *A. alternata*, seven molecular formulae were assigned to metabolite candidates. The identification of STTX III was performed using targeted LC-MS/MS analysis, while the identification of the other assigned metabolites of Table 2 could not be performed due to the lack of reference compounds

Species	Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Molecular formula	Metabolite assignments by Antibase [53]
A. solani	189.05572	190.06300	0.007	$C_{11}H_{10}O_3$	altechromone A [75]
A. solani	207.06628	208.07356	-0.008	$C_{11}H_{12}O_4$	quadrilineatin methylether [76]
A. solani	209.04554	210.05283	-0.026	$C_{10}H_{10}O_5$	2,4-dihydroxy-6-acetonyl-benzoic acid [77]
A. solani	209.08194	210.08921	0.005	$C_{11}H_{14}O_4$	(8R,9S)-9,10-epoxy-8-hydroxy-9-methyldeca-(2E,4Z,6E)-trienoic acid [78]
A. alternata	207.02990	208.03718	-0.009	$C_{10}H_8O_5$	iso-ochracinic acid [101]
A. alternata	221.08193	222.08921	-0.014	$C_{12}H_{14}O_4$	3-epideoxyradicinol [102]
A. alternata	267.08741	268.09469	-0.002	$C_{13}H_{16}O_6$	9,10-epoxy-3-methoxy-3-epiradicinol [103]
A. alternata	347.05612	348.06339	0.009	$C_{20}H_{12}O_6$	ATX III [12], STTX III [79]
A. alternata	363.05102	364.05831	-0.020	$C_{20}H_{12}O_7$	xanalteric acid I/xanalteric acid II [80]
A. alternata	365.06667	366.07396	-0.026	$C_{20}H_{14}O_7$	alterlosin I [61]
A. alternata	367.08232	368.08961	-0.032	$C_{20}H_{16}O_7$	alterlosin II [61]

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Table 3 Discriminant elemental compositions of the *A. alternata* isolates 1 and 2 detected by FTICR-MS and assigned to metabolite candidates by Antibase.[53] The discriminant molecular formulae of Table 3 could not be identified by LC-MS/MS due to the lack of reference compounds. The only exception was TA, which was identified by targeted LC-MS/MS analysis and was already identified as discriminant mycotoxin between the *A. alternata* isolates 1 and 2

A. alternata isolate	Experimental mass	Theoretical neutral mass	Mass error of annotation [ppm]	Molecular formula	Metabolite assignments by Antibase [53]	Log_2 fold change	$-Log_{10}$ p-value
isolate 1	193.05063	194.05791	-0.002	$C_{10}H_{10}O_4$	silvaticol,[104] porriolide [105]	-2.23	3.48
isolate 1	211.09758	212.10486	-0.027	$C_{11}H_{16}O_4$	3-carboxy-2-methylene-4-pentenyl-4-butenolide,[106] methylenolactocin,[107] depudecin [108]	-0.67	2.29
isolate 1	221.04554	222.05283	-0.031	$C_{11}H_{10}O_5$	tenuissimasatin [109]	-3.59	2.61
isolate 1	251.16526	252.17255	-0.024	$C_{15}H_{24}O_3$	deoxyuvidin B [110]	-2.05	3.27
isolate 1	415.23507	416.24236	-0.018	$C_{22}H_{32}N_4O_4$	dihydrotentoxin/cyclo(L-leucyl-N-methyl-L-phenylalanylglycyl-N-methyl-L-alanyl) [99]	-1.80	2.01
isolate 2	182.08227	183.08954	-0.014	$C_9H_{13}NO_3$	isopropyl tetramic acid [111]	5.96	3.69
isolate 2	196.09792	197.10519	-0.001	$C_{10}H_{15}NO_3$	L-TA, [112,113] isobutyl tetramic acid [111]	6.52	3.32
isolate 2	363.05102	364.05831	-0.020	$C_{20}H_{12}O_7$	xanalteric acid I/xanalteric acid II [80]	1.12	2.82