Received: 28 June 2023 | Revised: 9 December 2023 | Accepted: 13 December 2023

DOI: 10.1111/pce.14794

ORIGINAL ARTICLE

Plant, Cell & Environment WILEY

Untargeted metabolomics reveals PTI-associated metabolites

Lina Muñoz Hoyos[1] | Wan Petra Anisha[1] | Chen Meng[2] | Karin Kleigrewe[2] | Corinna Dawid[2,3,4] | Ralph Hückelhoven[1] | Remco Stam[1,5]

[1]Chair of Phytopathology, TUM School of Life Sciences, Technical University of Munich, Freising, Germany

[2]TUM School of Life Sciences, Bavarian Center for Biomolecular Mass Spectrometry, Technical University of Munich, Freising, Germany

[3]Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Freising, Germany

[4]Functional Phytometabolomics, TUM School of Life Sciences, Technical University of Munich, Freising, Germany

[5]Department of Phytopathology and Crop protection, Institute of Phytopathology, Kiel University, Kiel, Germany

Correspondence
Remco Stam, Department of Phytopathology and Crop Protection, Institute of Phytopathology, Kiel University, Kiel, Germany.
Email: remco.stam@phytomed.uni-kiel.de

Funding information
Deutsche Forschungsgemeinschaft

Abstract

Plants employ a multilayered immune system to combat pathogens. In one layer, recognition of Pathogen- or Microbe-Associated Molecular Patterns or elicitors, triggers a cascade that leads to defence against the pathogen and Pattern Triggered Immunity. Secondary or specialised metabolites (SMs) are expected to play a role, because they are potentially anti-fungal compounds. Tomato (*Solanum lycopersicum*) plants inoculated with *Alternaria solani* s.l. show symptoms of infection after inoculation. Plants inoculated with *Alternaria alternata* remain symptomless. We hypothesised that pattern-triggered induction of resistance related metabolites in tomato contributes to the resistance against *A. alternata*. We compared the metabolomic profile (metabolome) of tomato after treatments with *A. alternata*, *A. solani* and the fungal elicitor chitin, and identified SMs involved in early defence of tomato plants. We revealed differential metabolome fingerprints. The composition of *A. alternata* and chitin induced metabolomes show larger overlap with each other than with the *A. solani* induced metabolome. We identify 65 metabolites possibly associated with PTI in tomato plants, including NAD and trigonelline. We confirm that trigonelline inhibits fungal growth *in vitro* at physiological concentrations. Thus, a true pattern-triggered, chemical defence is mounted against *A. alternata*, which contains anti-fungal compounds that could be interesting for crop protection strategies.

KEYWORDS
Alternaria, early blight, plant defence, *Solanum lycopersicum*, specialised metabolites, trigonelline

1 | INTRODUCTION

Tomato is one of the most important vegetable crops worldwide, with a total fresh production that exceeded 187 million tons in 2020 (FAOSTAT, 2020). It can be grown in a wide range of climates from tropical to temperate and it can also be cultivated under cover conditions when outdoor temperatures are not favourable. Because of its wide use and nutritional values, there is a high demand for both fresh market and processed tomato varieties. However, a diversity of bacterial, viral, nematode and fungal diseases have made it difficult to grow tomatoes for commercial purposes (Bozbuga et al., 2022).

Due to intensive selection and inbreeding through domestication, the cultivated tomato has a limited genetic variation; as a result, they are more vulnerable to disease epidemics (Schauer et al., 2008).

Early blight (EB) is one of the most common diseases of tomato caused by several species of *Alternaria* fungi, predominantly *Alternaria solani* s.l. (Adhikari et al., 2017). The typical disease symptoms are dark brown to black lesions with concentric rings on the leaves, which result in leaf browning and leaf drop in severe cases, these lesions can occur over a wide range of environmental conditions causing severe reduction (35%–78%) in yield (Özer, 2011). During the infection process, *Alternaria spp*. produce germ tubes to penetrate host tissues or directly invade from stomata or wounds adjacent to epidermal cells. It secretes metabolites to break down host cell inclusions, thereby causing infection (Adhikari et al., 2017). Complete resistance of tomato against *A. solani* s.l. has not been observed (Chaerani et al., 2007; Ray et al., 2015). Notably, some tomato genotypes like 'Turkish cherry' from France and 'Rio Grande', 'H.a.s 2274' from the United States are resistant to *Alternaria alternata*, another causal agent of EB diseases (Alizadeh-Moghaddam et al., 2020). However, whereas weak correlations can be found between several morphological properties and *A. alternata* resistance, it remains unclear which specific biochemical properties are responsible for the resistance of the genotypes. Another study evaluating tomato genotypes for EB disease caused by different *Alternaria* species, did not find any genotype that was resistant to *A. alternata* isolates tested (Akhtar et al., 2019). Overall, while some tomato genotypes may show resistance to some *Alternaria* species or isolates, the mechanism underlying this resistance is not well understood yet.

Pathogens use different strategies to inhibit constitutive and induced plant defences, including the degradation of preformed antimicrobial compounds and the production of molecules that suppress induced plant defences. Necrotrophic pathogens like *A. solani* colonise the cell through the production of cell wall-degrading enzymes and phytotoxins killing them and acquiring nutrients from the dead plant cells (Chaerani & Voorrips, 2006; Sadeghi et al., 2022). Plants are able to perceive these pathogens or their Pathogen or Microbe-Associated Molecular Patterns (PAMPs or MAMPs, or patterns in short), also referred to as elicitors, such as the cell-wall component chitin, with specific receptors that trigger the mitogen-activated protein kinase cascades and activating hormone (jasmonates [JA]) and ethylene [ET])-dependent and hormone-independent signalling, which facilitates the mounting of defence response against these necrotrophs and results in Pattern-Triggered Immunity (PTI). This response involves the activation of specific transcription factors that result in the production of antifungal proteins or accumulation of specialised metabolites (SM) (also known as phytoalexins) (Muñoz-Hoyos & Stam, 2023; Pandey et al., 2016). The three main groups of secondary metabolite products in plants are terpenoids, phenolics and nitrogen-containing compounds (Taiz & Zeiger, 1991). Cultivated tomato, *Solanum lycopersicum* and its wild relative in this genus contain multiple representatives of these three major groups (Duffey & Stout, 1996). In addition, tomatoes and wild tomatoes produce methyl ketones and acylsugars, which are present in trichomes and play an important role in resistance to some microorganisms (Glas et al., 2012) and the presence of flavonoids, alkaloids, saponins, tannins, terpenoids, glycosides and steroids in plant extracts reduced the growth of *A. solani* in vitro (Ahmad et al., 2017). Like most of the members of the family Solanaceae, tomatoes also contain alkaloids. A well-known steroidal glycoalkaloid and saponin constitutively produced in tomato plants is *α*-tomatine. In healthy tomato leaf tissue, *α*-tomatine exists at sufficient concentrations to inhibit the growth of many fungi in vitro (Roddick, 1974) and it is therefore considered the major preformed compound that protects plants against attack by a wide range of potential fungal pathogens (Osbourn, 1996a). Tomato fungal pathogens are resistant to *α*-tomatine in vitro (Sandrock & VanEtten, 1998) because of their capability to detoxify this compound (Oka et al., 2006). Pathogens, such as *A. solani*, *Botrytis cinerea* and *Fusarium oxysporum* s. sp. *lycopersici* are known to produce extracellular enzymes that hydrolyse sugars from *α*-tomatine in various ways to be able to infect the plant (Roldán-Arjona et al., 1999). Seeing the important roles that SM play in defence against different pathogens, we hypothesise that in addition to *α*-tomatine, induced compounds must be synthesised by tomato plants to confer resistance to *Alternaria* species, and that elicitors such as chitin can trigger the production of these defence compounds.

Omics-based tools, such as untargeted metabolomics are potent techniques to investigate molecular changes operating during plant–pathogen interaction. Untargeted metabolomics involves global metabolic profiling with high throughput technology such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), followed by annotation, quantification and visualisation of generated metabolites (Allwood et al., 2021; Castro-Moretti et al., 2020; Muñoz-Hoyos & Stam, 2023). By analysing the metabolites present in plants that are resistant or susceptible to a particular stressor, biomarkers or patterns can be identified to develop strategies for improving plant resistance to different pathogens. In this study, we aim to identify pattern-triggered bioactive compounds that play a role in the resistance of cultivated tomato plants to *A. alternata* and validate their biological effects on the pathogen in vitro. For this, we perform untargeted metabolomics analysis with tomato leaves at 3 and 24 h after infection with *A. alternata*, *A. solani* and elicitation with chitin, representing the times in which the fungal spores germinate and try to penetrate the plant to establish disease. The identification of compounds as biochemical markers may be useful as screening tools for plant material in breeding programmes.

2 | MATERIALS AND METHODS

2.1 | Plant material

Seeds of *S. lycopersicum* (HEINZ 1706) were obtained from the Centre of Genetic Resources, the Netherlands (CGN). To ensure homogenic material, cuttings were made of a single mother plant after 6 weeks of sowing. Cuttings of 3 weeks old were used for the metabolomics comparison. Plants were grown in controlled chamber

conditions (long days condition 16 h light [µmol/m^2/s] 8 h dark 23°C and 60% humidity).

2.2 | Fungal isolates

A. alternata was isolated from the leaves of the wild tomato plant (*Solanum chilense*) showing signs of leaf spot (Schmey et al., 2022). *A. solani* (1117-1) was isolated from tomato in Freising. The identity of the fungal isolates was confirmed based on morphological (Simmons, 2007) and molecular features (Schmey et al., 2022).

2.3 | Pathogen inoculation, plant elicitation and plant infection assays

A. alternata and *A. solani* strains were cultured on synthetic, nutrient-poor agar (SNA) plates at 25°C, 12UV-A light, 12 h darkness and 85% humidity for 10 days. The spores were harvested by scraping them with an inoculation loop from the plates and placing them in water. The spore concentration was determined under the microscope with a Thoma counting chamber, and the suspension in water was diluted to a concentration of 3×10^4 spores/mL. Chitin derived from shrimp shell (C9752; Sigma-Aldrich) was ground and resuspended in water, a final concentration of 50 µg/mL was used for experiments. Spray inoculation was performed for elicitation and infection of the plants (5 mL per plant). A total of 24 plants of *S. lycopersicum* were sprayed with *A. alternata*, *A. solani*, chitin and water (mock) and the leaves were collected after 3 and 24 h postinoculation (hpi) (3 plants per treatment and time point). Similar samples were used for both chlorophyll measurements and for untargeted MS. Five leaves of similar developmental stages per plant (three replicates) were collected and immediately frozen in liquid nitrogen. Samples were ground to a fine homogenous powder using liquid nitrogen and porcelain mortars and pestles. For extraction, approximately 100 mg of the powder was weighed. Each of the samples was extracted using 1 mL of extraction solution (70% methanol, 30% water) shaking in a vortex for 30 min. Samples were then centrifuged for 15 min at 12 000 rpm and 200 µL of the supernatant was collected into glass tubes and stored at −20°C.

2.4 | Red light chlorophyll fluorescence measurement for cell death quantification

Red light emission of chlorophyll fluorescence can be measured for quantification of cell death (Landeo Villanueva et al., 2021). Leaf discs (Ø 4 mm) of *S. lycopersicum* treated with *A. solani*, *A. alternata* or water were floated on water in black 96-well plates and chlorophyll fluorescence was measured with a plate reader (Tecan Infinite F200 PRO, excitation 535 nm, emission 590 nm, 25 flashes, integration time 20 µs, 4 × 4 reads per well, gain set to 80) as relative fluorescence units. Values of all reads per well were summed up.

2.5 | Cultivation of fungal isolates for MS of growth media

The *Alternaria* isolates were cultivated in *S. lycopersicum* host broth medium to obtain the exometabolome (pool of exogenous metabolites). *S. lycopersicum* leaves (20 g) were boiled with 500 mL of distilled water for 15 min, the leaves were then filtered using a stainless-steel mesh strainer. One hundred and fifty millilitres of the liquid medium was transferred into three different polycarbonate Erlenmeyer flasks, the pH was adjusted to 7 using formic acid and was later autoclaved at 121°C for 20 min. The sterile liquid media was inoculated with 25 µL of the spores suspension of *A. alternata* (8.75×10^5 spores/mL) and 100 µL of the spores suspension of *A. solani* (2×10^5 spores/mL) to receive an equal amount of total spores. The fungi were cultivated in the dark (25°C, 100 rpm) and the isolates were exposed to day light for half an hour a day. After 11 days of cultivation, the liquid medium was filtered with filter paper Whatman No.1 to separate the mycelium. Five millilitres of the filtrate were pipetted and transferred to 15 mL Falcon tubes and stored at −20°C. Three different types of samples were measured: '*A. alternata* exo' (liquid broth media from *S. lycopersicum* after filtering *A. alternata* mycelium), '*A. solani* exo' (liquid broth media from *S. lyopersicum* after filtering *A. solani* mycelium) and 'control' (liquid broth media from *S. lycopersicum* without fungus inoculum). For extraction, 200 µL of the liquid media were used and each of the samples were extracted using 1 mL of extraction solution (70% methanol, 30% water) shaking in a vortex mixer for 30 min. Samples were then centrifuged for 15 min at 12 000 rpm and 200 µL of the supernatant was collected into glass tubes and stored at −20°C.

2.6 | Metabolomics analysis

The untargeted metabolite analysis was performed using a Nexera UHPLC system (Shimadzu) coupled to a Q-TOF mass spectrometer (TripleTOF 6600; AB Sciex). Separation of the samples was performed using a UPLC BEH Amide 2.1 × 100 mm, 1.7 µm analytic column (Waters) with a 400 µL/min flow rate. The mobile phase was 5 mM ammonium acetate in water (eluent A) and 5 mM ammonium acetate in acetonitrile/water (95/5, v/v) (eluent B). The gradient profile was 100% B from 0 to 1.5 min, 60% B at 8 min and 20% B at 10 min to 11.5 min and 100% B at 12 to 15 min. A volume of 5 µL per sample was injected. The autosampler was cooled to 10°C and the column oven heated to 40°C. Blank samples were measured and manually checked. Every tenth run a quality control (QC) sample, which was pooled from all samples, was injected. The samples were measured in a randomised order and in the Information Dependent Acquisition mode. MS settings in the positive mode were as follows: Gas 1 55 psi, Gas 2 65 psi, Curtain gas 35 psi, Temperature 500°C, Ion Spray Voltage 5500 V, declustering potential 80 V. The mass range of the TOF MS and MS/MS scans were 50–2000 *m/z* and the collision energy was ramped from 15 to 55 V. MS settings in the negative mode were as follows: Gas 1 55 psi, Gas 2 65 psi, Cur 35 psi,

Temperature 500°C, Ion Spray Voltage −4500 V, declustering potential −80. The mass range of the TOF MS and MS/MS scans was 50–2000 *m/z* and the collision energy was ramped from −15 to −55 V. The 'msconvert' from ProteoWizard was used to convert raw files to mzXML (denoised by centroid peaks). The bioconductor/R package xcms was used for data processing and feature annotation (Smith et al., 2006). In more detail, the following steps were performed: to reduce noise peaks in the spectra, we applied intensity filters. Specifically, any peaks with an intensity lower than 30 in MS2 spectra and peaks with an intensity lower than 50 in MS1 spectra were removed. Chromatographic peaks in each sample were initially identified using the matched filter method. The settings included a full width at half maximum of the matched filtration Gaussian model peak set to 7.5 s, with other settings left at their default values. This was achieved using the 'findChromPeaks' and 'MatchedFilterParam' functions in the xcms package. To reduce chromatographic peaks driven by low-intensity peaks, we retained only those chromatographic peaks identified by at least five peaks with intensities greater than 3000. Features that were identified in at least half of the samples were further selected for correction of retention time differences between samples. This correction was performed using the lowest method, with the span parameter set to 0.5. This step was executed with the 'adjustRtimePeakGroups' and 'PeakGroupsParam' functions in the xcms package. The chromatographic peaks identified in each file were grouped into features to enable cross-sample comparisons, a process known as 'correspondence' or chromatographic peak grouping. During this step, the chromatographic peaks from each file were grouped based on their density distribution along the retention time axis within slices of overlapping *m/z* ranges. Default parameters of the 'PeakDensityParam' function were used for this grouping. Each sample was treated as a separate group, allowing the retention of features exclusively expressed in individual samples for later QC and potential outlier identification.

To annotate possible metabolites to identified features MS2 spectra were linked to each feature based on their retention time and precursor mass. For potential feature annotation using MS2 spectra, we downloaded public MS/MS spectra compiled by the MSDial programme (link: http://prime.psc.riken.jp/compms/msdial/main.html#MSP; download date: 3 September 2021). A detected MS2 peak was considered mapped with a public MS2 spectra peak if the mass difference was smaller than 20 ppm. The mapping between our experimental MS2 and database MS2 was manually checked using the visualisation tool xcmsViewer (GitHub link: https://github.com/mengchen18/xcmsViewer).

The resulting peak list was uploaded into MetaboAnalyst 5.0 (Xia et al., 2009), a web-based tool for metabolomics data processing, statistical analysis and functional interpretation where statistical analysis and modelling were performed. Missing values were replaced using a K-nearest neighbour missing value estimation. Data filtering was implemented by detecting and removing noninformative variables that are characterised by near-constant values throughout the experimental conditions by comparing their robust estimate interquartile ranges, subsequently sum normalised and autoscaled. The data were checked after normalisation (Supporting Information: Figure 1) and statistically analysed by analysis of variance (ANOVA) and continuous with Tukey's HSD test for determining the significant difference with $\alpha = 5\%$. Furthermore, the multivariate data analysis was performed using Partial Least Squares Discriminant (PLS-DA) (Lê Cao et al., 2011) by MetaboAnalyst Software version 5.0. Out of 4496 mass features originally detected, 2499 were used for PLS-DA after filtering. The PLS-DA model was validated based on cross-validation and permutation. For the annotation of candidate metabolites, the individual mass features that contributed to the separation between the different treatments were further characterised by applying a range of univariate and multivariate statistical tests to determine the importance including PLS-DA importance variables, *t*-test and random forest. The processed data are available at (MassIVE MSV000092161). Detailed description and implementation of the data processing steps can be found in our GitHub repository (https://github.com/mengchen18/xcmsViewer/).

2.7 | Bioassay on the antifungal effect of trigonelline on the growth of *Alternaria*

Physiological concentrations of trigonelline in tomato leaves were estimated based on literature (328 ppm) (Tyihák et al., 1988). *Alternaria* isolates were grown on SNA plates supplemented with different concentrations of trigonelline or nicotinic acid spanning both sides of the previously mentioned value (0.0328, 0.0656, 0.328, 1.64, 3.28 mg/mL). The fungi were grown at 25°C, 12UV-A light, 12 h darkness and 85% humidity. For growth rate assays, radial growth was measured at regular intervals (1, 2, 3, 4, 5, 7, 9 and 14 days postinoculation) after placing a 4 mm agar plug in the centre of the petri dishes. The spore germination was assessed microscopically. For each treatment and time point, 50 spores were observed.

3 | RESULTS

3.1 | *S. lycopersicum* (Heinz 1706) is resistant to *A. alternata* (CS046)

Prior investigation into whether the secondary or SM differ between our treatments, for example, elicitors, and compatible or incompatible pathogens, we performed infection assays on *S. lycopersicum* plants inoculated with *A. solani*, *A. alternata* and water (mock). To confirm the infection phenotype, we studied a subset of leaves for up to 4 dpi. We visualised the necrotic lesions by measuring chlorophyll fluorescence (Figure 1a), which is associated with cell death (Landeo Villanueva et al., 2021). We observed necrotic lesions in plants treated with *A. solani*, whereas the plants treated with *A. alternata* did not show any symptoms (Figure 1b). In the same manner, leaves treated with *A. solani* showed increased chlorophyll fluorescence compared with *A. alternata* and mock treatments, validating the ability of our *A. solani* isolate to infect *S. lycopersicum* plants in contrast to our *A. alternata* isolate.

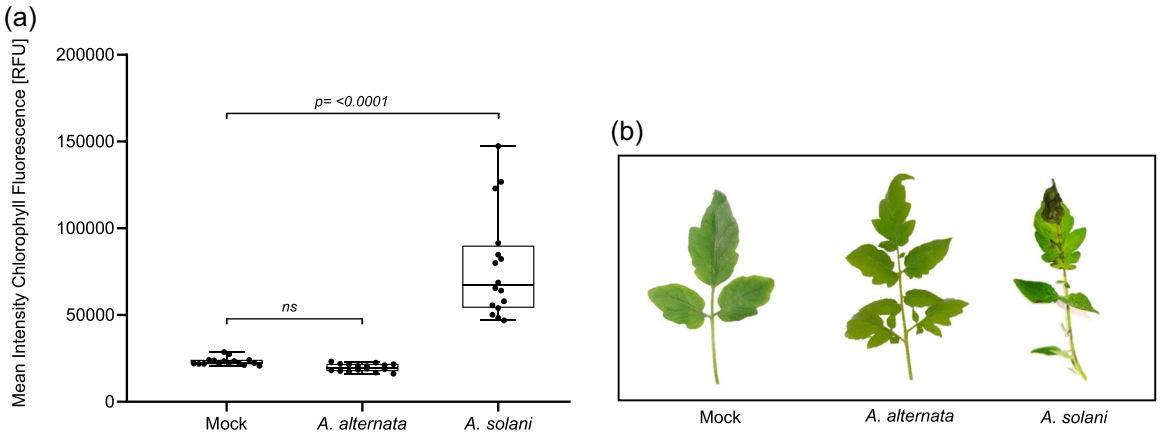

FIGURE 1 (a) Chlorophyll fluorescence measurement serves as proxy for cell death upon infection of tomato plants inoculated with *Alternaria alternata* or *Alternaria solani*. $n = 16$ leaf discs. *Y*-axis show fluorescence in relative fluoresence units. Differences between treatments were analysed with one-way ANOVA with Dunnett's multiple comparison test, $\alpha = 0.05$. (b) Tomato leaves after 4 days of spray inoculation with *A. alternata*, *A. solani* and water (mock).

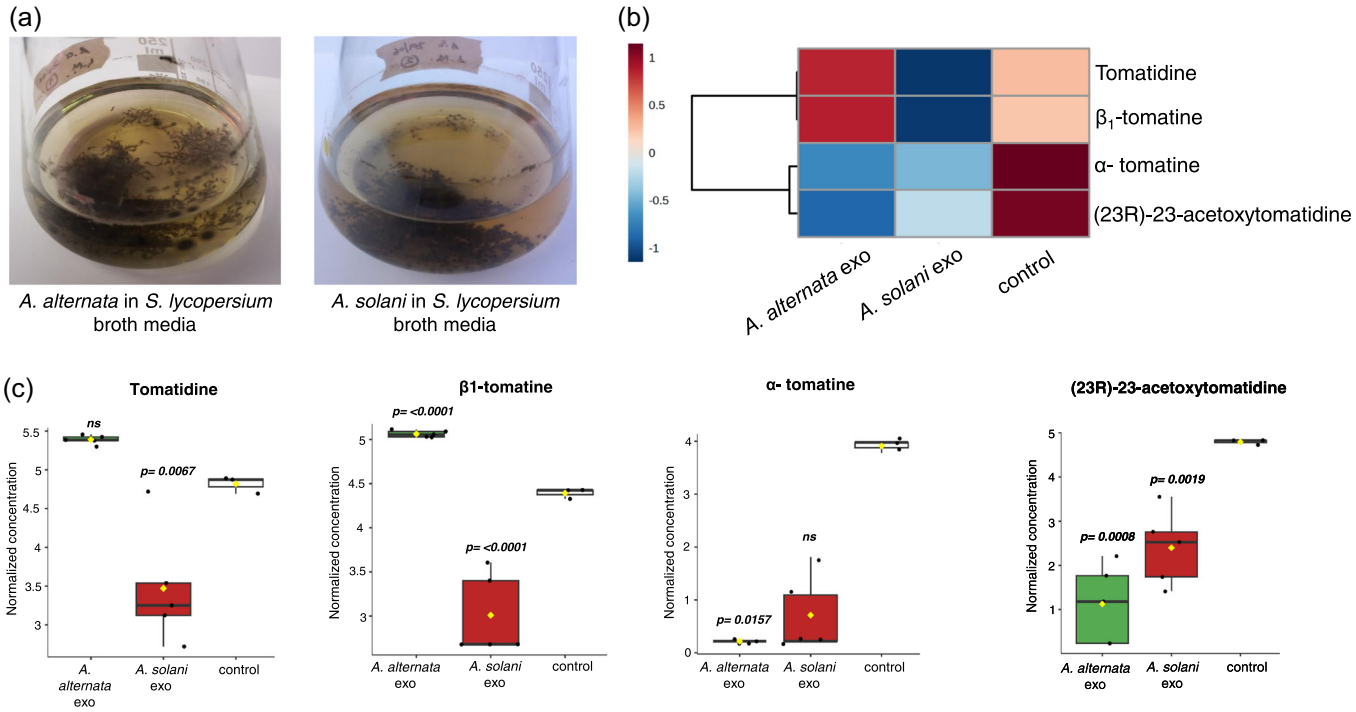

FIGURE 2 (a) *Solanum lycopersicum* broth liquid media after 11 days of inoculation with *Alternaria alternata* and *Alternaria solani*. (b) Heatmap representing the relative levels of α-tomatine and derivatives in the control, *A. alternata* exo and *A. solani* exo treatments. Values range from −2 (dark blue) to 2 (dark red). Distance measure using Euclidean, and clustering algorithm using ward.D. (c) Normalised concentration of α-tomatine and derivatives in *A. alternata* exo, *A. solani* exo and control treatments. Differences between treatments were analysed with one-way ANOVA with Dunnett's multiple comparison test, $\alpha = 0.05$.

3.2 | Both fungi can break down α-tomatine

It is known that α-tomatine is an important phytoanticipin against fungi that needs to be detoxified before successful infection (Oka et al., 2006; Ökmen et al., 2013). To assess the presence of phytoanticipins that could inhibit the growth of *A. alternata* on tomato, we performed inoculation in tomato leaf broth medium, hypothesising that this medium contains all phytoanticipins, but that it cannot show induced defence responses. Both fungi are able to grow in this medium, confirming that phytoanticipins do not play a major role in the resistance against *A. alternata* (Figure 2a). To corroborate these results and to show that both fungi are able to detoxify α-tomatine, we performed untargeted metabolomics on the Heinz exometabolome (exo) (*S. lycopersium* broth liquid medium after inoculation with the fungi

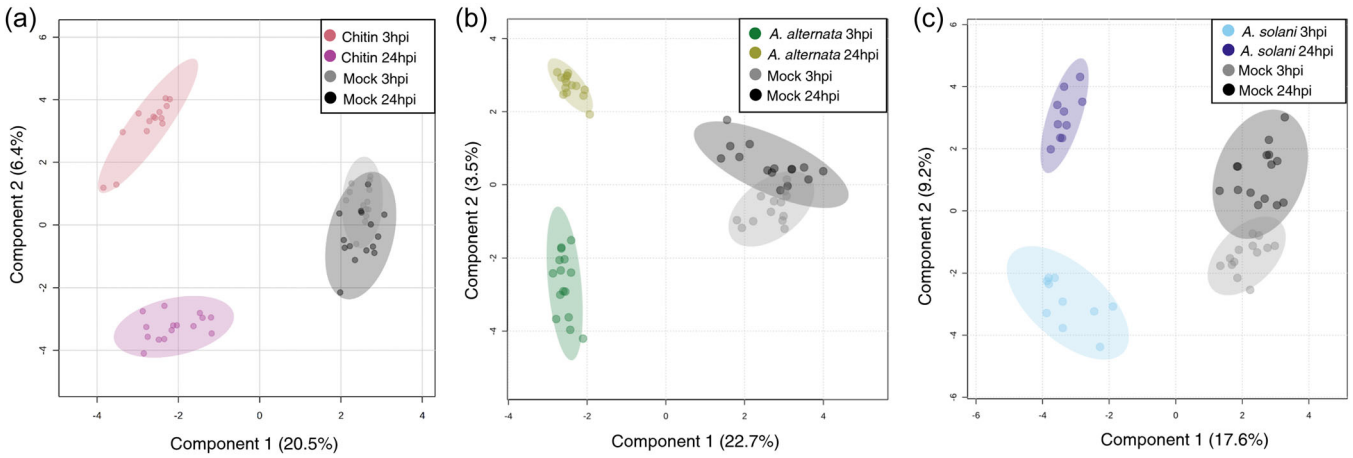

FIGURE 3 (a) Partial Least Squares (PLS) representation of statistical analysis for leaves treated with chitin, samples marked in pink and dark magenta. (b) PLS representation of the statistical analysis for leaves treated with *Alternaria alternata*, samples marked in dark yellow and green. (c) PLS representation for *Alternaria solani*, samples marked in soft blue and dark blue. All the different treatments were compared with mock, samples marked in grey and black. Sample size for every treatment ($n = 3$). Confidence regions of 95% are marked in circles for each group. Axes show the first (x-axis) and second (y-axis) components. The percentage variance explained is indicated on the axes. [Color figure can be viewed at wileyonlinelibrary.com]

and the mycelium being filtered out) and Heinz control medium (*S. lycopersicum* broth liquid medium without fungal inoculum). We then looked specifically for features that can be associated with typical known phytoanticipins, such as α-tomatine. We found that *A. solani* exo and *A. alternata* exo showed a significant decrease in the amount of α-tomatine compared with the control (Figure 2b) confirming a possible degradation or detoxification of this compound into other molecules. To confirm the degradation or detoxification of α-tomatine, we looked at the presence of known related compounds. Tomatidine, a glycoalkaloid and precursor and possible degradation product of α-tomatine (Oka et al., 2006), significantly accumulated in *A. alternata* exo compared with the control, but not in *A. solani exo* samples. We found a similar pattern for β_1-tomatine, a compound known to be the result of α-tomatine detoxification by fungi such as *B. cinerea* (Quidde et al., 1998), where an increase in accumulation levels was observed in *A. alternata* exo compared with *A. solani* exo samples. Similar to α-tomatine, (23*R*)-23-acetoxytomatidine, a steroidal alkaloid with known antifungal properties against *Fusarium* spp. (Nagaoka et al., 1995), strongly accumulated in the control compared with *A. alternata* exo and *A. solani* exo. This suggests that both fungi are able to degrade (different) phytoanticipins.

3.3 | Metabolic profiles differ after treatment with *A. alternata*, *A. solani* and chitin

To identify PAMP and pathogen-associated metabolome changes in tomato plants, we performed untargeted metabolomics analysis in tomato leaves treated with *A. alternata*, *A. solani*, chitin and water (mock) at two different times (3 and 24 hpi). While the positive ionisation mode returned a total of 4494 mass spectrometric features, the negative ionisation mode returned a total of 2669 mass spectrometric features for analysis (Supporting Information: Tables 2 and 3). We conducted a multivariate analysis to compare the changes in the metabolite profiles for the different treatments. The principal component analysis (PCA) score plot for all the treatments revealed two separated groups corresponding to samples treated with *A. alternata*, *A. solani*, chitin and samples treated with water (mock), suggesting a possible large generic 'stress' response during treatment (Supporting Information: Figure 2). To visualise the smaller differences, we created PLS-DA score plots for the plants treated with *A. alternata*, chitin and *A. solani* compared with mock, at two different time points (3 and 24 hpi). These show that component 1 accounts for 20.5%, 22.7%, 17.6% and component 2 accounts for 6.4%, 3.5%, 9.2% of the variance, respectively (Figure 3a–c). The samples that overlapped in the PCA score plot (*A. alternata*, chitin and *A. solani* treatments) are clearly differentiated in the PLS-DA score. The differentiation of 3 and 24 hpi samples is also apparent. The PLS-DA had a variance of the response $R^2 = 0.99$, 0.99 and 0.98 for *A. alternata*, chitin and *A. solani*, respectively. And a predictive ability $Q^2 = 0.89$, 0.96 and 0.90. The model was validated using a permutation test statistic as shown in Supporting Information: Figure 3. R^2 explains the calibration of model samples. However, Q^2 described an estimate of the predictive ability of the model. Based on a study by (Bevilacqua & Bro, 2020), when Q^2 is very close to R^2, the score plot models displayed a meaningful result. Therefore, it is clear that the result implied a good model. We conclude that there is a significant reprogramming of the metabolome in the leaves following the different treatments.

3.4 | *A. alternata*, *A. solani* and chitin treatments induced a SM response

To look for the number of features that significantly accumulated or were reduced in each treatment at 3 and 24 hpi, we performed volcano diagrams analysis (FDR adjusted $p < 0.05$ and FC > 1). *A. alternata*,

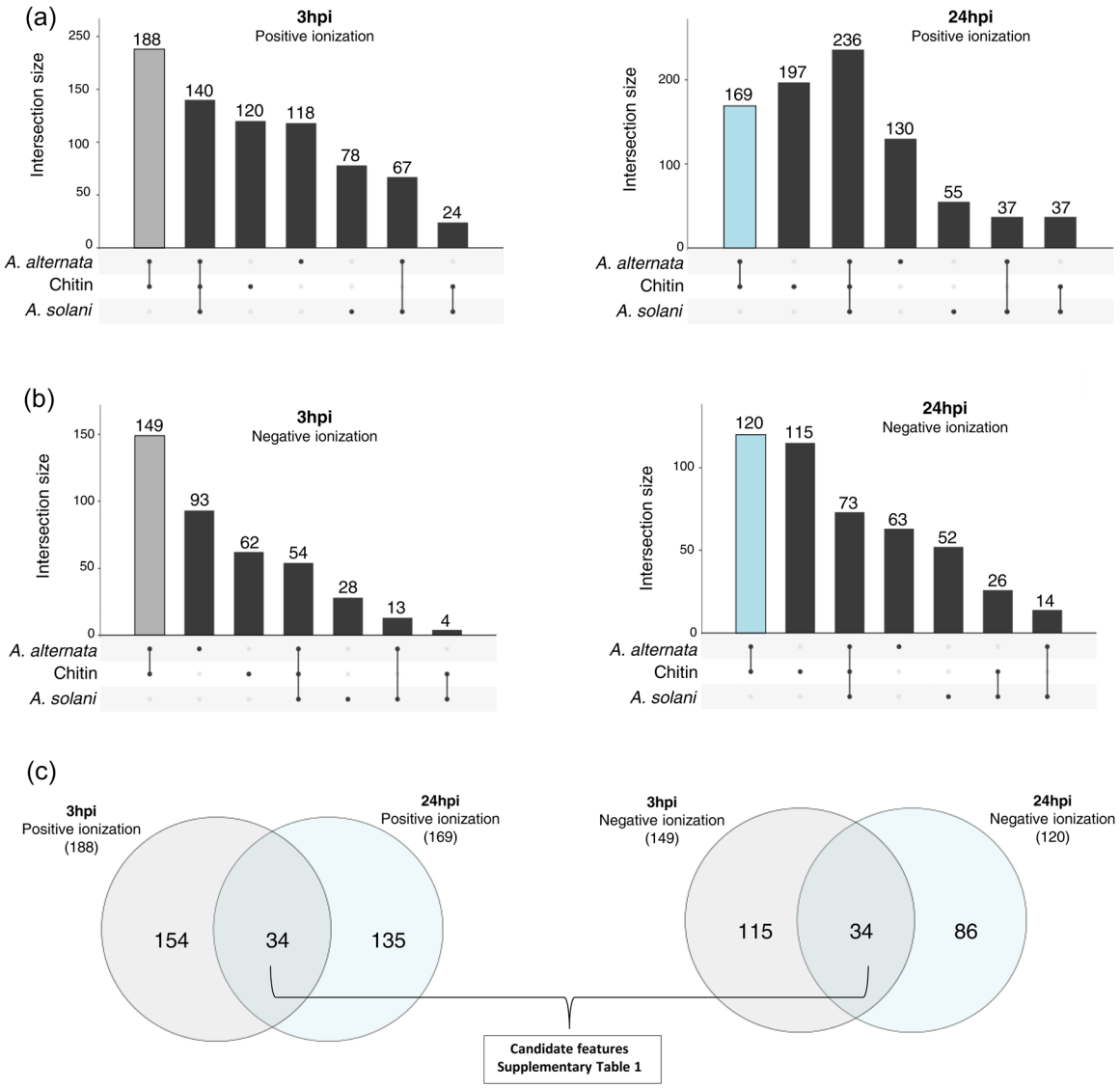

FIGURE 4 (a) Upset plot representing the number of significant differentially abundant features from each of the pairwise comparisons with mock treatment after treatment with *Alternaria alternata*, chitin and *Alternaria solani* at 3 and 24 h postinoculation and the numbers of overlapping features between each of them (intersection size, *y*-axis) for positive ionisation. (b) Differentially abundant features from each of the pairwise comparisons with mock treatment after treatment with *A. alternata*, chitin and *A. solani* at 3 and 24 h postinoculation and the numbers of overlapping features between each of them (intersection size, *y*-axis) for negative ionisation. (c) Venn diagram showing the shared features between *A. alternata* and chitin treatments at 3 and 24 h postinoculation for positive and negative ionisation. [Color figure can be viewed at wileyonlinelibrary.com]

A. solani and chitin treated samples were compared with mock samples independently to determine the differentially abundant features between all the treatments. This yields a total of 622 and 748, 606 and 618, 413 and 502 mass spectrometric features that were significantly higher accumulated for *A. alternata*, chitin and *A. solani* at 3 and 24 hpi, respectively. Similar numbers of features showed significantly lower accumulation in the treated samples (Supporting Information: Figure 4A,B). In most cases, the number of features that accumulated to significantly higher levels were higher than those that were reduced in abundance. For all the different treatments, a greater number of features changed in relative abundance at 24 hpi compared to 3 hpi, indicating a clear initial response and a change of the metabolome after all the treatments. We observed the highest number of differential features in *A. alternata* treated plants, implying that a successful defence leads to a stronger reprogramming towards the accumulation of SMs than chitin treatment or successful infection.

Our hypothesis is that the resistance of tomato to *A. alternata* may be PTI and that this can be seen in the accumulation of SM that are active against the pathogen. If accurate, *A. alternata*-triggered SM alterations in defence should overlap with those caused by the chitin treatment. We looked for the significantly accumulated features that both treatments shared. The number of unique differentially abundant mass spectrometric features observed for each treatment after 3 and 24 hpi was higher in plants treated with chitin compared to plants treated with *A. alternata* and *A. solani* suggesting that chitin triggers a more generic response (Figure 4a,b). The amount of

features that overlap between chitin and *A. alternata* treatments is higher compared to that shared between chitin and *A. solani* treatments at 3 and 24 hpi for positive and negative ionisation. Finally, despite successful infection, the samples treated with *A. solani* exhibited the lowest amount of features compared to the other treatments. These results suggest that, indeed, resistance to *A. alternata* is largely pattern-triggered and furthermore that the successful infection of *A. solani* in *S. lycopersicum* limits the differential accumulation of metabolites.

3.5 | Candidate metabolites associated with chitin and *A. alternata* resistance in *S. lycopersicum*

To look for specific candidate metabolites that can be pattern-triggered and related to *A. alternata* resistance, we filtered the compounds shared between chitin and *A. alternata* treatments at 3 and 24 hpi for positive (34 features) and negative (34 features) ionisation (Figure 4c). A total of 68 candidate features are shared between both treatments and detectable at both times. In most cases, the compounds significantly accumulated at 3 and 24 hpi after treatment with both chitin and *A. alternata* and less abundance was observed in mock and *A. solani* samples. After filtering the features detected in both ionisation modes, a total of 65 features were considered as candidate features associated with resistance in *S. lycopersicum* (Supporting Information: Table 1). Most of the candidate features show higher abundance after *A. alternata* and chitin treatments at 3 and 24 hpi compared with the lower abundance in *A. solani* and mock treatments (Figure 5, Supporting Information: Figure 5). Of the remaining features, eight are annotated based on MS1 and MS/MS. These compounds included primary metabolites: amino acids and derivatives, nucleotides and derivatives, sulphur-containing nucleosides, and SM: alkaloids, and aromatic acids (Table 1, Supporting Information: Figure 6). Some of these compounds are mentioned to be involved in defence in plants. While L-threonine itself is not a direct defence compound, the enzyme threonine deaminase converts threonine to α-ketobutyrate and ammonia as the committed step in isoleucine (Ile) biosynthesis and contributes to JA responses by producing the Ile needed to make the bioactivate JA-Ile conjugate, important for defence against necrotrophs (Gonzales-Vigil et al., 2011; Yeo et al., 2023). 1-Aminocyclopropanecarboxylic acid (ACC) acts as the direct precursor of the plant hormone ET, which regulates plant growth and biotic and abiotic stress responses (Van de Poel & Van Der Straeten, 2014; Zaynab et al., 2018). Trigonelline has been shown to accumulate in nonleguminous plants in response to stress, suggesting that it may play a role in stress adaptation (Tyihák et al., 1988). Additionally, trigonelline has been shown to have antioxidant properties, which may help protect plants from oxidative stress caused by biotic and abiotic stressors. Thus, we find several defence-associated compounds that are pattern-triggered and can be associated with *A. alternata* resistance, but not *A. solani* susceptibility.

3.6 | Physiological concentrations of trigonelline show antifungal activity

Because of the assumed high relevance and commercial availability of trigonelline, it was purchased and measured with the method described above to compare the retention time and MS2-fragmentation. After remeasuring and confirmation, trigonelline was selected for a bioassay to test its antifungal activity effect at physiological concentrations on *A. solani* and *A. alternara* isolates. We observed a significant reduction in the growth of the *A. solani* and *A. alternata* isolates after supplementing SNA growth medium with trigonelline (Figure 6a). After 14 days, the fungal plate growth diameter for both isolates showed a clear concentration-dependent reduction. For both isolates, trigonelline concentrations corresponding to the level found in tomato leaves (328 ppm) significantly inhibited fungal growth. Nicotinic acid, a precursor for biosynthesis of trigonelline was also tested and showed marked lower efficacy (Figure 6b). While fungal growth was completely inhibited for concentrations above the physiological levels on the tomato leaves for trigonelline, higher concentrations of nicotinic acid still allowed for some fungal growth. The spores' germination success was also tested on SNA medium supplemented with trigonelline (Figure 6c). An inhibition of the germination was observed in both fungal cultures at 3.28 mg/mL. Surprisingly, we already observed an inhibition of *A. solani* spores at 0.328 mg/mL, suggesting that trigonelline is indeed a potent antifungal compound.

4 | DISCUSSION

A. alternata and *A. solani* s.l. are two closely related species of plant pathogens that can cause EB. While both pathogens can potentially infect tomatoes and potatoes, we identified an *A. alternata* isolate (CS046) that was not able to infect Heinz 1706 tomato plants, whereas *A. solani* (1117-1) caused clear infection symptoms. To understand the mechanisms underlying this effect, we set out to determine which metabolic host factors can be associated with *A. alternata* resistance. Tomato plants have developed elaborate defence mechanisms against plant pathogens to survive. These mechanisms include both constitutive and induced defences. Constitutive defences are always present and include physical barriers and potential toxins such as glycoalkaloids (Nakayasu et al., 2021; Zhao et al., 2022). Induced defences are activated in response to pathogen attacks and include the production of pathogenesis-related proteins, phytohormones and SM such as flavonoids and terpenoids (Treutter, 2006; Wink, 2008).

One of the well-known glycoalkaloids produced by tomato plants as defence mechanisms against pathogens is α-tomatine (Pegg & Woodward, 1986). This compound is toxic to many pathogens, including fungi, bacteria, viruses and predatory insects (Duffey & Stout, 1996; Nakayasu et al., 2021). The toxicity mechanism of α-tomatine occurs by disrupting cellular membranes by binding to its

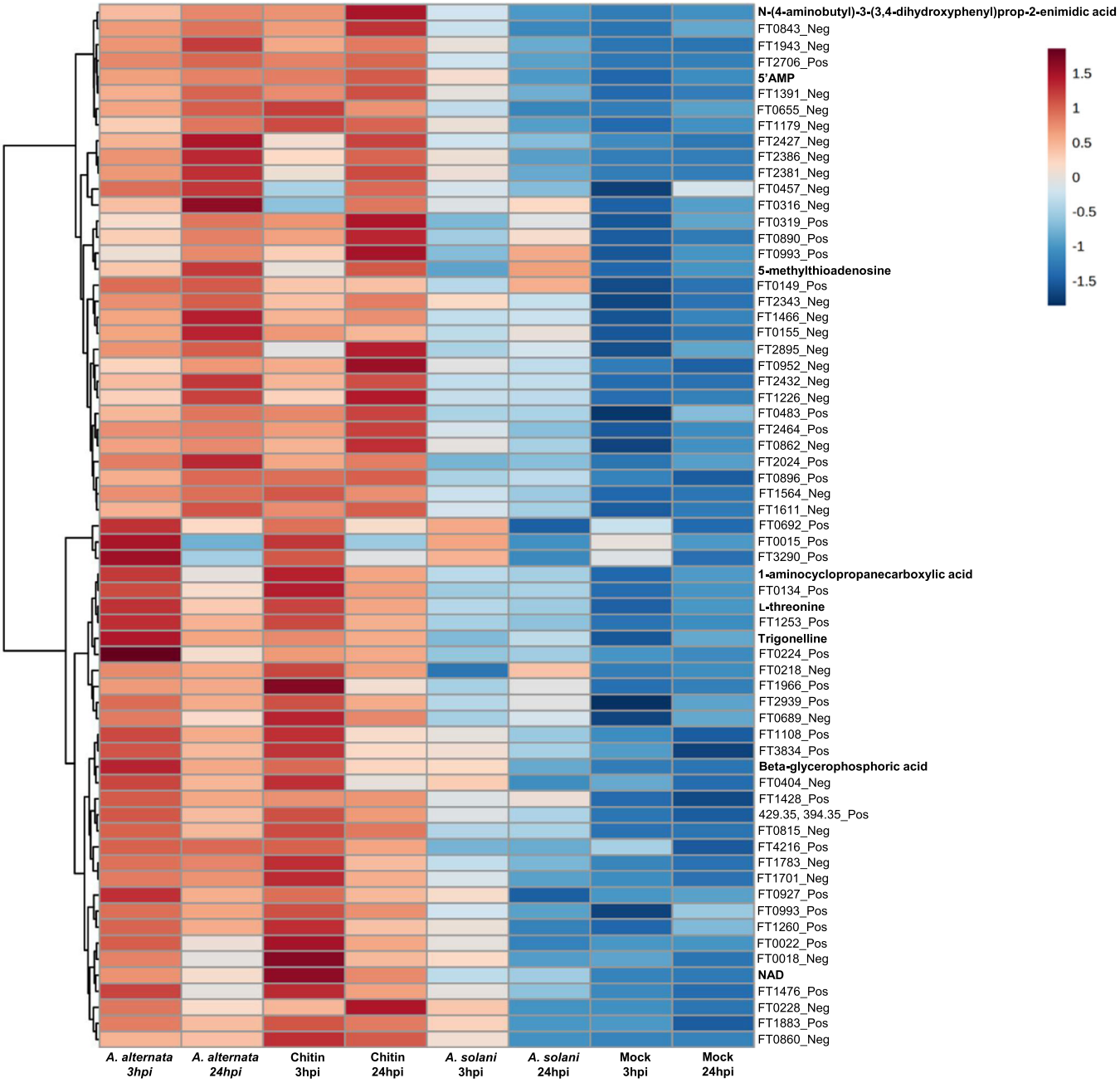

FIGURE 5 Heatmap of the total (65) features of interest (positive '_Pos' and negative '_Neg' ionisation) that overlap between *Alternaria alternata* and chitin treatments at 3 and 24 h postinoculation (Distance measure using Euclidean, and clustering algorithm using ward.D). [Color figure can be viewed at wileyonlinelibrary.com]

sterols, leading to membrane rupture and the leakage of cellular contents (Bailly, 2021). Pathogens have evolved mechanisms to detoxify α-tomatine, which allows them to infect tomato plants (Oka et al., 2006). We observed that both fungi were able to detoxify α-tomatine in tomato broth media, converting this compound into other molecules. Whereas both isolates were capable of detoxifying α-tomatine, it seems that they use different mechanisms for this. It has been described that an active way of dealing with α-tomatine is to secrete enzymes that degrade this compound to reduce its toxicity (Osbourn, 1996b; Sandrock & VanEtten, 1998). The degradation process can be categorised into three main actions, based on the hydrolysis products β_2-tomatine, β_1-tomatine and the aglycon tomatidine (You & van Kan, 2021). In our experiment, tomatidine, as well as β_1-tomatine appeared as final breakdown products with higher accumulation in the medium treated with *A. alternata*. We also detected (23*R*)-23-acetoxytomatidine, which is probably a derivate of α-tomatine (You & van Kan, 2021) was found in the roots of tomato stock and has a fungi-toxic activity, inhibiting the growth of *F. oxysporum* f. sp. *radices-lycopersici* (Nagaoka et al., 1995). Whereas the compound can be detected in control samples, yet it appears to

TABLE 1 List of candidate metabolites associated with immune response after treatment with *Alternaria alternata* and *Alternaria solani* s.l.

Compound identity	Class	Formula	Ionisation mode	*m/z*	RT (min)	Estimated coefficient	*p* Value (FDR)
L-Threonine	Amino acids	C4H9NO3	Positive ionisation	120.0639623	416.049	0.4983	1.47E−05
1-Aminocyclopropanecarboxylic acid	Alpha amino acids	C4H7NO2	Positive ionisation	102.0541726	449.4758	0.2628	1.52E−05
Trigonelline	Alkaloids	C7H7NO2	Positive ionisation	176.0088684	359.1731	0.2194	0.00059264
N-(4-aminobutyl)-3-(3,4-dihydroxyphenyl) prop-2-enimidic acid	Aromatic acids	C13H18N2O3	Positive ionisation	251.1376813	397.3705	0.756	0.003238927
5′-methylthioadenosine	Sulphur-containing nucleoside	C11H15N5O3S	Negative ionisation	296.081059	117.3577	0.377	0.00036463
Beta-glycerophosphoric acid	Glycerophosphates	C3H9O6P	Negative ionisation	171.0052829	445.5023	1.0569	7.24E−07
5′AMP	Nucleosides, nucleotides and analogues	C10H14N5O7P	Positive ionisation	346.0541336	440.0565	0.5124	0.000986548
NAD	Nucleosides, nucleotides and analogues	C21H27N7O14P2	Positive ionisation	664.1153632	471.4895	0.3640	5.80739E−06

not affect or can also be degraded by *A. alternata* or *A. solani*. The detected detoxification products of α-tomatine explain why both fungi were able to grow in the host broth media and suggest that induced defence responses, rather than phytoanticipins, explain the difference in resistance of the tomato plants against our *A. solani* and *A. alternata isolates*.

Untargeted metabolomics is a powerful tool that has been used recently for the annotation of antifungal compounds in plants. For example, one study used untargeted metabolomics to identify steroidal saponins in the foliage and tubers of potato plants as anti-oomycete compounds effective against *Phytophthora infestans* (Baur et al., 2022). Another study used an untargeted metabolite profiling approach on the leaf surface of the susceptible cultivated potato *Solanum tuberosum* and the resistant wild potato species *Solanum bulbocastanum* to the pathogen *P. infestans*. They found that lysophosphatidylcholine (LPC17:1) was accumulating on the surface of the wild potato, but not on *S. bulbocastanum* and in vitro assays revealed an antifungal activity against *P. infestans* (Gorzolka et al., 2021). SM associated with resistance were also the focus of another recent study in *Solanum commersonii*; the differences between accessions that are resistant against *A. solani* and those that are susceptible can be explained by differences in glycosyltransferase genes that are involved in the production of tetraose steroidal glycoalkaloids that are toxic to *A. solani* (Wolters et al., 2023). In this study, we investigate the potential SMs that may play a role in the resistance of cultivated tomato plants against *A. alternata*. We found a clear reprogramming of metabolomic profiles of tomato leaves after pathogen and chitin treatment. Differential compounds included primary metabolites such as amino acids, lipids and carbohydrates and SM such as glucosinolates, phenylpropanoids and organic acids. Although differences existed between metabolomics profiles of *A. alternata* and chitin, many commonly upregulated metabolites were detected. Small signalling molecules like ACC accumulated in samples after the treatments mentioned above. ACC is a precursor of ET which is a plant hormone involved in various physiological processes, including plant defence against necrotrophic pathogens (Zaynab et al., 2018). Enhanced ET production is an early, active response of plants to the perception of pathogen attack and is associated with the induction of disease resistance in plants (Van Loon et al., 2006). ACC has also been proposed to regulate plant development and growth independent of ET, and it can be easily transported over short and long distances, providing the plant with an elaborate system to control local and remote ET responses (Van de Poel & Van Der Straeten, 2014). Seeing the involvement of ACC, we suppose that ET signalling plays an important role in resistance against *A. alternata* and that *A. solani* might suppress the ET signalling for a successful infection.

We also observed the accumulation of trigonelline after the treatment with *A. alternata* and chitin. This alkaloid is associated with many processes occurring in plants, such as cell cycle regulation, plant growth and defence (Minorsky, 2002). It should be noted that higher levels of trigonelline might be associated with higher levels of NAD (which we also detected) as trigonelline synthesis is described

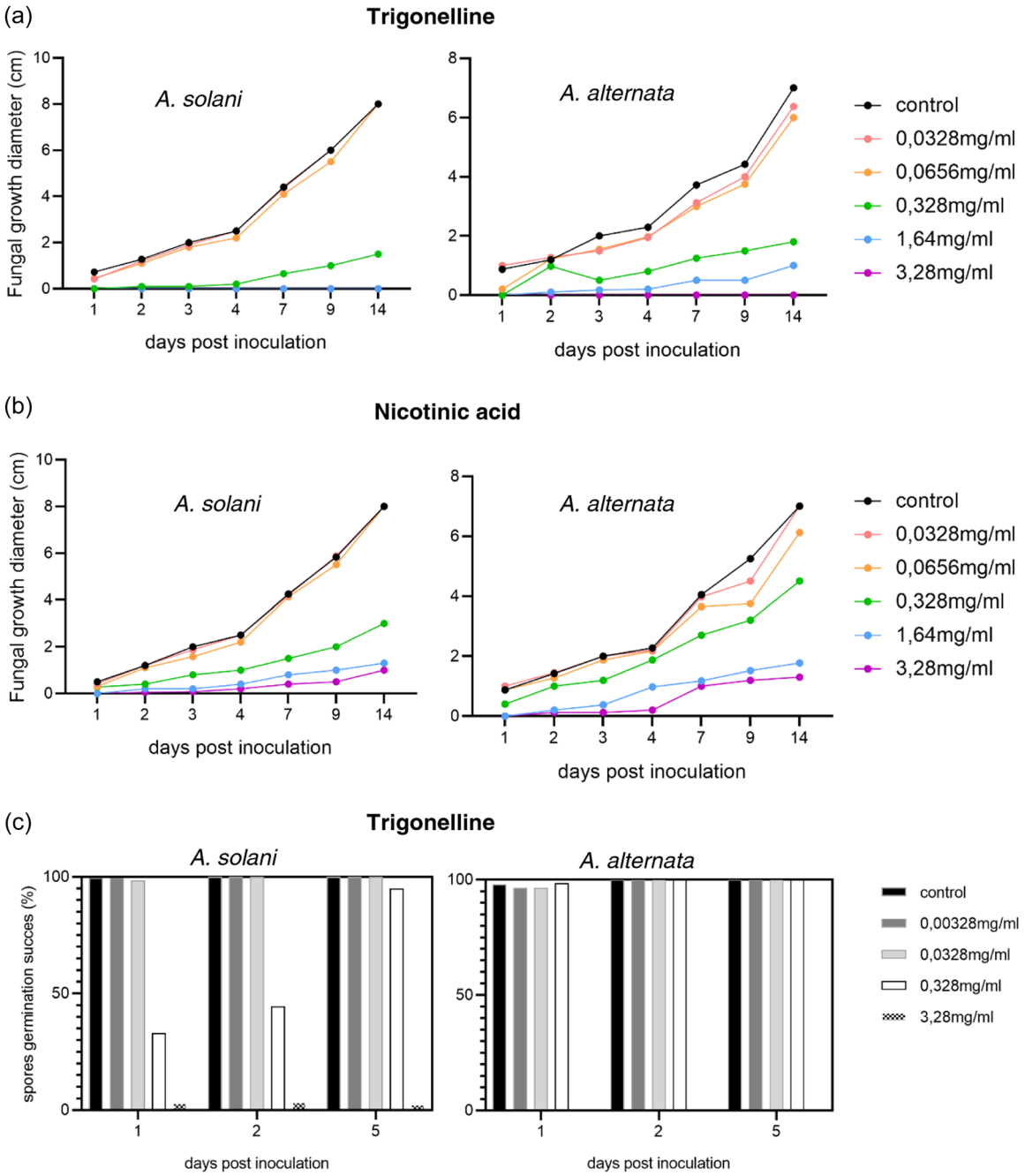

FIGURE 6 Antifungal activity of trigonelline on Alternaria strains. (a) *Alternaria solani* and *Alternaria alternata* growth in cm (*y*-axis) measured over 14 days after inoculation (*x*-axis) on agar plates using different concentrations of trigonelline. (b) *A. solani* and *A. alternata* growth in cm (*y*-axis) measured over 14 days after inoculation (*x*-axis) on agar plates using different concentrations of nicotinic acid. (c) Spore germination success of *A. solani* and *A. alternata* after 1, 2 and 5 days of inoculation using different concentrations of trigonelline in agar plates. [Color figure can be viewed at wileyonlinelibrary.com]

to happen with a single step-reaction from nicotinic acid, which is part of the NAD-cycle (Ashihara, 2006). In terms of pathogen defence, NAD is implicated in plant–pathogen interactions. Although the exact role of NAD during pathogen infection is not fully understood, it is clear that NAD is involved in vital defence mechanisms (Eastman et al., 2022). Inducible NAD overproduction in Arabidopsis has been shown to increase resistance to Pst-AvrRpm1, a bacterial pathogen, and is correlated with increased salicylate content, which is known to play a role in plant defence (Pétriacq et al., 2012). Trigonelline, which was extracted for the first time from fenugreek seeds, has a well-described biosynthetic pathway and is relatively well-studied for pharmacological activities (Mohamadi et al., 2018). However, there is a limited number of studies that specifically investigate the role of trigonelline in plant defence (Ashihara, 2006; De-la-Cruz Chacón et al., 2013; Sabino et al., 2019).

We validated the role of trigonelline in two bioassays. We show that the addition of this compound to SNA medium resulted in an inhibition of fungal growth of isolates from two *Alternaria* spp. We also tested nicotinic acid, the immediate precursor of trigonelline (Ashihara, 2006), which did not show complete inhibition of the fungal growth at similar concentrations, thus corroborating the antifungal activity of trigonelline. We used concentrations calculated from leaf extract concentrations but also speculate that at the fungal interface, defending cells might accumulate even higher levels of trigonelline.

Our data also suggests that *A. solani* suppresses PTI for successful infection in tomato plants. The samples with this treatment showed the least amount of compounds accumulated after 24 hpi, and many candidate defence-response compounds, shared between *A. alternata* and chitin treatments, did not significantly accumulate in tomato leaves treated with *A. solani*. To date, the number of reported functional effectors in *A. solani* has been limited and most of them seem to be associated with induction of cell death (Wang et al., 2022). Besides peptide effectors, *Alternaria* species can synthesise phytotoxic metabolites, such as AAL toxins and other host-specific or host-nonspecific toxins. Such toxins serve as chemical inhibition of defence compounds produced by the host or the induction of cell death (Wang et al., 2023). There is limited information on the specific role of toxins of *A. solani* in pathogen infection. However, a recent study showed the effect of alternaric acid, a toxin of *A. solani*, on the hypersensitive response of potato to *P. infestans* (Wang et al., 2023). Besides deeper investigations in other host defence mechanisms, follow-up studies on the identification of effectors or possible other small molecules that are responsible for PTI suppression by *A. solani* during infection of cultivated tomato plants could be of interest for further understanding of this host–pathogen interactions.

Overall, our findings show the presence of pattern-triggered chemical defence barriers against *A. alternata*, which likely contribute to the resistance of cultivated tomato plants. They also illustrate how untargeted metabolomics can help in the elucidation of new antifungal compounds that may be of interest for future crop protection strategies.

ACKNOWLEDGEMENTS

We acknowledge Nicole Bellé (Chair of Phytopathology, TUM) for providing the *Alternaria solani* s.l. (1117-1) isolate. This work was supported by the German Research Foundation (SFB924, projects: B8, B12 and B13). Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in MassIVE at https://massive.ucsd.edu/, reference number MSV000092161.

ORCID

Remco Stam http://orcid.org/0000-0002-3444-6954

REFERENCES

Adhikari, P., Oh, Y. & Panthee, D. (2017) Current status of early blight resistance in tomato: an update. *International Journal of Molecular Sciences*, 18(10), 2019. Available from: https://doi.org/10.3390/ijms18102019

Ahmad, F., Raziq, F., Ullah, N., Khan, H. & Din, N. (2017) In vitro and in vivo bio-assay of phytobiocidal effect of plant extracts on *Alternaria solani* causing agent of early blight disease in tomato. *Archives of Phytopathology and Plant Protection*, 50(11–12), 568–583. Available from: https://doi.org/10.1080/03235408.2017.1352247

Akhtar, K.P., Ullah, N., Saleem, M.Y., Iqbal, Q., Asghar, M. & Khan, A.R. (2019) Evaluation of tomato genotypes for early blight disease resistance caused by *Alternaria solani* in Pakistan. *Journal of Plant Pathology*, 101(4), 1159–1170.

Alizadeh-Moghaddam, G., Rezayatmand, Z., Nasr-Esfahani, M. & Khozaei, M. (2020) Bio-genetic analysis of resistance in tomato to early blight disease, *Alternaria alternata*. *Phytochemistry*, 179, 112486. Available from: https://doi.org/10.1016/j.phytochem.2020.112486

Allwood, J.W., Williams, A., Uthe, H., van Dam, N.M., Mur, L.A.J., Grant, M.R. et al. (2021) Unravelling plant responses to stress—the importance of targeted and untargeted metabolomics. *Metabolites*, 11(8), 558. Available from: https://doi.org/10.3390/metabo11080558

Ashihara, H. (2006) Metabolism of alkaloids in coffee plants. *Brazilian Journal of Plant Physiology*, 18, 1–8. Available from: https://doi.org/10.1590/S1677-04202006000100001

Bailly, C. (2021) The steroidal alkaloids α-tomatine and tomatidine: panorama of their mode of action and pharmacological properties. *Steroids*, 176, 108933. Available from: https://doi.org/10.1016/j.steroids.2021.108933

Baur, S., Bellé, N., Frank, O., Wurzer, S., Pieczonka, S.A., Fromme, T. et al. (2022) Steroidal saponins—new sources to develop potato (*Solanum tuberosum* L.) genotypes resistant against certain *Phytophthora infestans* strains. *Journal of Agricultural and Food Chemistry*, 70(24), 7447–7459. Available from: https://doi.org/10.1021/acs.jafc.2c02575

Bevilacqua, M. & Bro, R. (2020) Can we trust score plots? *Metabolites*, 10(7), Article 278. Available from: https://doi.org/10.3390/metabo10070278

Bozbuga, R., Ates, S.Y., Guler, P.G., Yildiz, H.N., Kara, P.A., Arpaci, B.B. et al. (2022) Host-pathogen and pest interactions: virus, nematode, viroid, bacteria, and pests in tomato cultivation. In: Viškelis, P., Urbonavičienė, D. & Viškelis, J. (Eds.), *Tomato—from cultivation to processing technology*. IntechOpen. https://doi.org/10.5772/intechopen.106064

Lê Cao, K.-A., Boitard, S. & Besse, P. (2011) Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. *BMC Bioinformatics*, 12(1), 253. Available from: https://doi.org/10.1186/1471-2105-12-253

Castro-Moretti, F.R., Gentzel, I.N., Mackey, D. & Alonso, A.P. (2020) Metabolomics as an emerging tool for the study of plant–pathogen interactions. *Metabolites*, 10(2), 52. Available from: https://doi.org/10.3390/metabo10020052

Chaerani, R., Groenwold, R., Stam, P. & Voorrips, R.E. (2007) Assessment of early blight (*Alternaria solani*) resistance in tomato using a droplet inoculation method. *Journal of General Plant Pathology*, 73(2), 96–103. Available from: https://doi.org/10.1007/s10327-006-0337-1

Chaerani, R. & Voorrips, R.E. (2006) Tomato early blight (*Alternaria solani*): the pathogen, genetics, and breeding for resistance. *Journal of General Plant Pathology*, 72(6), 335–347. Available from: https://doi.org/10.1007/s10327-006-0299-3

De-la-Cruz Chacón, I., Riley-Saldaña, C.A. & González-Esquinca, A.R. (2013) Secondary metabolites during early development in plants.

Phytochemistry Reviews, 12(1), 47–64. Available from: https://doi.org/10.1007/s11101-012-9250-8

Duffey, S.S. & Stout, M.J. (1996) Antinutritive and toxic components of plant defense against insects. *Archives of Insect Biochemistry and Physiology*, 32(1), 3–37. Available from: https://doi.org/10.1002/(SICI)1520-6327(1996)32:1<3::AID-ARCH2>3.0.CO;2-1

Eastman, S., Smith, T., Zaydman, M.A., Kim, P., Martinez, S., Damaraju, N. et al. (2022) A phytobacterial TIR domain effector manipulates NAD+ to promote virulence. *New Phytologist*, 233(2), 890–904. Available from: https://doi.org/10.1111/nph.17805

Glas, J., Schimmel, B., Alba, J., Escobar-Bravo, R., Schuurink, R. & Kant, M. (2012) Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. *International Journal of Molecular Sciences*, 13(12), 17077–17103. Available from: https://doi.org/10.3390/ijms131217077

Gonzales-Vigil, E., Bianchetti, C.M., Phillips, G.N. & Howe, G.A. (2011) Adaptive evolution of threonine deaminase in plant defense against insect herbivores. *Proceedings of the National Academy of Sciences*, 108(14), 5897–5902. Available from: https://doi.org/10.1073/pnas.1016157108

Gorzolka, K., Perino, E.H.B., Lederer, S., Smolka, U. & Rosahl, S. (2021) Lysophosphatidylcholine 17:1 from the leaf surface of the wild potato species *Solanum bulbocastanum* inhibits *Phytophthora infestans*. *Journal of Agricultural and Food Chemistry*, 69(20), 5607–5617. Available from: https://doi.org/10.1021/acs.jafc.0c07199

Landeo Villanueva, S., Malvestiti, M.C., van Ieperen, W., Joosten, M.H.A.J. & van Kan, J.A.L. (2021) Red light imaging for programmed cell death visualization and quantification in plant–pathogen interactions. *Molecular Plant Pathology*, 22(3), 361–372. Available from: https://doi.org/10.1111/mpp.13027

Van Loon, L.C., Geraats, B.P.J. & Linthorst, H.J.M. (2006) Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science*, 11(4), 184–191. Available from: https://doi.org/10.1016/j.tplants.2006.02.005

Minorsky, P.V. (2002) The hot and the classic. *Plant Physiology*, 128(1), 7–8. Available from: https://doi.org/10.1104/pp.900014

Mohamadi, N., Sharififar, F., Pournamdari, M. & Ansari, M. (2018) A review on biosynthesis, analytical techniques, and pharmacological activities of trigonelline as a plant alkaloid. *Journal of Dietary Supplements*, 15(2), 207–222. Available from: https://doi.org/10.1080/19390211.2017.1329244

Muñoz Hoyos, L. & Stam, R. (2023) Metabolomics in plant pathogen defense: from single molecules to large-scale analysis. *Phytopathology®*, 113, 760–770. Available from: https://doi.org/10.1094/PHYTO-11-22-0415-FI

Nagaoka, T., Ohra, J., Yoshihara, T. & Sakamura, S. (1995) Fungitoxic compounds from the roots of tomato stock. *Japanese Journal of Phytopathology*, 61(2), 103–108. Available from: https://doi.org/10.3186/jjphytopath.61.103

Nakayasu, M., Ohno, K., Takamatsu, K., Aoki, Y., Yamazaki, S., Takase, H. et al. (2021) Tomato roots secrete tomatine to modulate the bacterial assemblage of the rhizosphere. *Plant Physiology*, 186(1), 270–284. Available from: https://doi.org/10.1093/plphys/kiab069

Oka, K., Okubo, A., Kodama, M. & Otani, H. (2006) Detoxification of α-tomatine by tomato pathogens *Alternaria alternata* tomato pathotype and *Corynespora cassiicola* and its role in infection. *Journal of General Plant Pathology*, 72(3), 152–158. Available from: https://doi.org/10.1007/s10327-005-0262-8

Ökmen, B., Etalo, D.W., Joosten, M.H.A.J., Bouwmeester, H.J., de Vos, R.C.H., Collemare, J. et al. (2013) Detoxification of α-tomatine by *Cladosporium fulvum* is required for full virulence on tomato. *New Phytologist*, 198(4), 1203–1214. Available from: https://doi.org/10.1111/nph.12208

Osbourn, A. (1996a) Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell*, 8(10), 1821–1831.

Osbourn, A. (1996b) Saponins and plant defence—a soap story. *Trends in Plant Science*, 1(1), 4–9. Available from: https://doi.org/10.1016/S1360-1385(96)80016-1

Özer, Ç. (2011) Genetic analysis of resistance to early blight disease in tomato. *African Journal of Biotechnology*, 10(79), 18071–18077. https://doi.org/10.5897/AJB11.1872

Pandey, D., Rajendran, S.R.C.K., Gaur, M., Sajeesh, P.K. & Kumar, A. (2016) Plant defense signaling and responses against necrotrophic fungal pathogens. *Journal of Plant Growth Regulation*, 35(4), 1159–1174. Available from: https://doi.org/10.1007/s00344-016-9600-7

Pegg, G.F. & Woodward, S. (1986) Synthesis and metabolism of α-tomatine in tomato isolines in relation to resistance to *Verticillium albo-atrum*. *Physiological and Molecular Plant Pathology*, 28(2), 187–201. Available from: https://doi.org/10.1016/S0048-4059(86)80063-7

Pétriacq, P., de Bont, L., Hager, J., Didierlaurent, L., Mauve, C., Guérard, F. et al. (2012) Inducible NAD overproduction in Arabidopsis alters metabolic pools and gene expression correlated with increased salicylate content and resistance to Pst-AvrRpm1. *The Plant Journal*, 70(4), 650–665. Available from: https://doi.org/10.1111/j.1365-313X.2012.04920.x

Van de Poel, B. & Van Der Straeten, D. (2014) 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: more than just the precursor of ethylene. *Frontiers in Plant Science*, 5, 640. Available from: https://doi.org/10.3389/fpls.2014.00640

Quidde, T., Osbourn, A.E. & Tudzynski, P. (1998) Detoxification of α-tomatine by *Botrytis cinerea*. *Physiological and Molecular Plant Pathology*, 52(3), 151–165. Available from: https://doi.org/10.1006/pmpp.1998.0142

Ray, S., Mondal, S., Chowdhury, S. & Kundu, S. (2015) Differential responses of resistant and susceptible tomato varieties to inoculation with *Alternaria solani*. *Physiological and Molecular Plant Pathology*, 90, 78–88. Available from: https://doi.org/10.1016/j.pmpp.2015.04.002

Roddick, J.G. (1974) The steroidal glycoalkaloid α-tomatine. *Phytochemistry*, 13(1), 9–25. Available from: https://doi.org/10.1016/S0031-9422(00)91261-5

Roldán-Arjona, T., Pérez-Espinosa, A. & Ruiz-Rubio, M. (1999) Tomatinase from *Fusarium oxysporum* f. Sp. *lycopersici* defines a new class of saponinases. *Molecular Plant-Microbe Interactions®*, 12(10), 852–861. Available from: https://doi.org/10.1094/MPMI.1999.12.10.852

Sabino, A.R., Tavares, S.S., Riffel, A., Li, J.V., Oliveira, D.J.A., Feres, C.I.M.A. et al. (2019) 1H NMR metabolomic approach reveals chlorogenic acid as a response of sugarcane induced by exposure to *Diatraea saccharalis*. *Industrial Crops and Products*, 140, 111651. Available from: https://doi.org/10.1016/j.indcrop.2019.111651

Sadeghi, B., Mirzaei, S. & Fatehi, F. (2022) The proteomic analysis of the resistance responses in tomato during interaction with *Alternaria alternate*. *Scientia Horticulturae*, 304, 111295. Available from: https://doi.org/10.1016/j.scienta.2022.111295

Sandrock, R.W. & VanEtten, H.D. (1998) Fungal sensitivity to and enzymatic degradation of the phytoanticipin α-tomatine. *Phytopathology®*, 88(2), 137–143. Available from: https://doi.org/10.1094/PHYTO.1998.88.2.137

Schauer, N., Semel, Y., Balbo, I., Steinfath, M., Repsilber, D., Selbig, J. et al. (2008) Mode of inheritance of primary metabolic traits in tomato. *The Plant Cell*, 20(3), 509–523. Available from: https://doi.org/10.1105/tpc.107.056523

Schmey, T., Small, C., Muñoz Hoyoz, L.M., Ali, T., Gamboa, S., Mamami, B. et al. (2022) Small-spored *Alternaria* spp. (section *Alternaria*) are common pathogens on wild tomato species [preprint]. *Environmental Microbiology*, 25, 1830–1846. Available from:https://doi.org/10.1101/2022.12.08.519636

Simmons, E.G. (2007) *Alternaria: an identification manual: fully illustrated and with catalogue Raisonné 1796-2007*. CBS Fungal Biodiversity Centre. https://books.google.co.uk/books?id=F_AzKgAACAAJ

Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R. & Siuzdak, G. (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical Chemistry*, 78(3), 779–787. Available from: https://doi.org/10.1021/ac051437y

Taiz, L. & Zeiger, E. (1991) *Plant physiology*. Benjamin/Cummings Publishing Company. https://books.google.co.uk/books?id=KelUAQAAIAAJ

Treutter, D. (2006) Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*, 4(3), 147–157. Available from: https://doi.org/10.1007/s10311-006-0068-8

Tyihák, E., Sarhan, A.R.T., Cong, N.T., Barna, B. & Király, Z. (1988) The level of trigonelline and other quaternary ammonium compounds in tomato leaves in ratio to the changing nitrogen supply. *Plant and Soil*, 109(2), 285–287. Available from: https://doi.org/10.1007/BF02202097

Wang, C., Wang, J., Zhang, D., Cheng, J., Zhu, J. & Yang, Z. (2023) Identification and functional analysis of protein secreted by *Alternaria solani*. *PLoS One*, 18(3), e0281530. Available from: https://doi.org/10.1371/journal.pone.0281530

Wang, C., Zhang, D., Cheng, J., Zhao, D., Pan, Y., Li, Q. et al. (2022) Identification of effector CEP112 that promotes the infection of necrotrophic *Alternaria solani*. *BMC Plant Biology*, 22(1), 466. Available from: https://doi.org/10.1186/s12870-022-03845-w

Wink, M. (2008) Plant secondary metabolism: diversity, function and its evolution. *Natural Product Communications*, 3(8), 1934578X0800300. Available from: https://doi.org/10.1177/1934578X0800300801

Wolters, P.J., Wouters, D., Tikunov, Y.M., Ayilalath, S., Kodde, L.P., Strijker, M. et al. (2023) Tetraose steroidal glycoalkaloids from potato can provide complete protection against fungi and insects. *eLife*, 12, RP87135. Available from: https://doi.org/10.7554/eLife.87135

Xia, J., Psychogios, N., Young, N. & Wishart, D.S. (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*, 37(suppl_2), W652–W660. Available from: https://doi.org/10.1093/nar/gkp356

Yeo, I.-C., de Azevedo Manhaes, A.M.E., Liu, J., Avila, J., He, P. & Devarenne, T.P. (2023) An unexpected role for tomato threonine deaminase 2 in host defense against bacterial infection. *Plant Physiology*, 192(1), 527–545. Available from: https://doi.org/10.1093/plphys/kiac584

You, Y. & van Kan, J.A.L. (2021) Bitter and sweet make tomato hard to (b) eat. *New Phytologist*, 230(1), 90–100. Available from: https://doi.org/10.1111/nph.17104

Zaynab, M., Fatima, M., Abbas, S., Sharif, Y., Umair, M., Zafar, M.H. et al. (2018) Role of secondary metabolites in plant defense against pathogens. *Microbial Pathogenesis*, 124, 198–202. Available from: https://doi.org/10.1016/j.micpath.2018.08.034

Zhao, T., Pei, T., Jiang, J., Yang, H., Zhang, H., Li, J. et al. (2022) Understanding the mechanisms of resistance to tomato leaf mold: a review. *Horticultural Plant Journal*, 8(6), 667–675. Available from: https://doi.org/10.1016/j.hpj.2022.04.008

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Muñoz Hoyos, L., Anisha, W.P., Meng, C., Kleigrewe, K., Dawid, C., Hückelhoven, R. et al. (2024) Untargeted metabolomics reveals PTI-associated metabolites. *Plant, Cell & Environment*, 47, 1224–1237. https://doi.org/10.1111/pce.14794