Metabolomics approach based on NMR spectroscopy and multivariate data analysis to explore the interaction between the leafminer *Tuta absoluta* and tomato (*Solanum lycopersicum)*

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Running title: Metabolomics to explore interaction between Tuta absoluta and tomato

Abstract

Introduction – *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most devastating and harmful pests of tomato (*S. lycopersicum)* crops causing up to 80–100% yield losses. A large arsenal of plant metabolites is induced by the leafminer feeding including defense compounds that could differ among varieties.

Objective – To compare the metabolomic changes of different genotypes of tomato (tolerant "T", susceptible "S" and F1 hybrid obtained between T and S) after exposition to *T. absoluta*.

Methodology – Nuclear magnetic resonance spectroscopy followed by multivariate data analysis were performed to analyse the metabolic profiles of control and infested samples on three different tomato genotypes.

Results – Signals related to GABA (γ-aminobutyric acid) were relatively much higher in all infested samples compared to the non-infested plants used as control. Infested T genotype samples were the most abundant in organic acids, including fatty acids (FA) and acyl sugars (AS), chlorogenic acid, neochlorogenic acid and feruloyl quinic acid, indicating a clear link between the exposure to leafminer. Results also showed an increase of trigonelline in all tomato varieties after exposition to *T. absoluta*.

Conclusion – Metabolomics approach based on NMR spectroscopy followed by multivariate data analysis allowed for a detailed metabolite profile of plant defences, providing fundamental information for breeding programmes in plant crops.

Keywords: NMR, Chemometrics, *Tuta absoluta*, *Solanum lycopersicum*, Plant-Pathogen Interaction.

Short Abstract

Tuta absoluta (Lepidoptera: Gelechiidae) is one of the most devastating and harmful pests of tomato crops. To promote the development of new tomato varieties resistant to this leafminer, a metabolomics analysis followed by chemometrics on plant-pest interaction have been carried out on three tomato genotypes. This study allowed to obtain a detailed metabolite profile of plant defences providing fundamental information for improving plant crops.

Introduction

Tomato crop (*Solanum lycopersicum*) is one of the most economically important vegetable worldwide with a very low-fat content and excellent source of antioxidants, dietary fibres, minerals and vitamins[1]. This crop is susceptible to a whole plethora of abiotic and biotic stress, translated in the most threatening and yield-loss damages[2]. Phytophagous insects represent a huge problem in global crop cultivation causing yield reductions and considerable costs in control measures. Among pathogens, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most devastating and harmful pests of Solanaceous crops. If no control measures are taken, then the pest can cause up to 80–100% yield losses in tomato crops[3]. Larval feeding activity reduces the plants photosynthetically active surface and, consequently, growth and yields[4]. Control of *T. absoluta* is a worldwide necessity but the efficacy of foliar insecticides is inconsistent, as it may require many applications with undesirable effects (residues, damage to natural enemies, resistance to chemicals, etc). Chemical control to combat such threats is often too expensive for growers and, in some cases, ineffective[5]. Moreover, the use of pesticides has been reduced due to environmental and consumer constraints. Hence, the identification of resistant cultivars results to be one of the most important research goals for promoting a sustainable agriculture. In the last years, with the development of analytical instrumentations, data processing and chemometric tools, many studies have been performed to analyse plant–pathogen interactions[1]. Among the mechanisms by which plants can control the biotic or abiotic stress, the production of secondary metabolites as defensive response is the most common feature. In this framework a key role could be played by the development of new tomato varieties resistant to the leafminer. Resistance to *T. absoluta* has been found in several wild tomato accessions, such as *Solanum pennellii* (*Lycopersicon pennellii*) LA716[6] and *S. peruvianum* (*L. peruvianum*) NAV29 and NAV115[7]. Several studies have enlightened that different compounds such as Zingiberene[8], Acylsugars[9] and 2-Tridecanone[10] are able to

confer resistance to *T. absoluta*. Metabolites production is the result of biochemical dynamics of living organisms starting with gene expression and affected by environmental conditions. Metabolomics can define the biochemical phenotype of the studied subject[11]. The aim of this research is to investigate the metabolic changes of different genotypes of tomato (tolerant "T", susceptible "S" and F1 hybrid obtained between T and S) after exposition to *T. absoluta*, in order to provide information about the chemical diversity of the signalling compounds involved in the defence response in plant–pest interaction.

Experimental

Plant material

Three tomato (*Solanum lycopersicum*) genotypes were provided by FARAO seed company (Sarno, Italy). A tolerant/partial resistant cherry type tomato BR221 (named as 'T') and a susceptible variety, PS650 (named as 'S') were used in the experiment. These two genotypes were furthermore used as parental lines (Tolerant x Susceptible) to obtain an F1 hybrid CS823 (named as 'F1'), also used in the experiment.[12]

Growth condition

A special tunnel (500 x 60 x 90 cm) protected by anti-insect net (25 mesh, 0.72 x 0.97 mm) was build up in a greenhouse to perform the infestation trials on 20 cm high tomato plants. A total of 120 plants were used for the experiment. The tunnel was divided by a septum in two adjacent cages. Each cage contained the three genotypes under study in a randomized complete block design consisting of 20 plants/replica for each genotype and for each condition. Half of the plants were exposed to infestation of 320 adults of *T. absoluta* and remained in the cage for at least 45 days, when an overall plant damage was visually assessed. Leaves with and without mines from each plant were collected and immediately frozen in liquid nitrogen.

Solvent and chemicals

Chemicals First-grade dichloromethane and methanol were purchased from Delchimica Scientific Laboratories Glassware (Naples, Italy). Deuterium oxide (99.8 atom %D) and dimethyl-4-silapentanesodium sulfonate (DSS) were obtained from ARMAR Chemicals (Switzerland). Chloroform-d (99.8 atom %D) containing 0.03% (v/v) TMS, pure standard amino acids, organic acids, sugars, chlorogenic acid and its derivatives were purchased bySigma-Aldrich, Italy.

Extraction procedure

Leaves were collected in triplicates from each tomato variety, dried with liquid nitrogen and powdered finely with a pestle and mortar. Three hundred milligrams of each sample were dissolved in 5 mL of CH_2Cl_2/MeOH/H_2O in ratio of 2:1:1, mixed by vortex and incubated 15 min at room temperature. To ensure efficient lysis of cell membranes and to promote the escape of all metabolites, solution was sonicated for 1 min at 25°C with a Bandelin Sonoplus HD 2070. Each mixture was centrifuged at 3000 rpm for 30 minutes at room temperature and then the aqueous and the organic fractions were accurately separated. The extraction was repeated twice. The solvent of each extract was evaporated to dryness under vacuum at 30°C (Rotavapor R-114, Büchi, Switzerland and Edwards Rotary Vane Pump) and the dry residues were kept at 4°C until NMR analysis.

NMR experiments

Dried aqueous fractions were diluted in 600 µl of deuterium oxide (99.8 % D_2O) while dried organic fractions were dissolved in 600 µl of chloroform-d (99.8% $CDCl_3$) and transferred into a 5 mm NMR tubes. DSS and TMS, both 0.03% (v/v) in D_2O and $CDCl_3$, respectively, were used as an internal standard for aqueous and organic fractions, respectively. The pH of aqueous fractions was adjusted to 6.0 by using KH_2PO_4 as a buffering agent and 1N NaOD[13,14]. The NMR spectra were recorded at 298 K with Varian Unity Inova spectrometer operating at

400.422 MHz. For each sample 200 transients were recorded using a spectral width of 12 ppm on 32K data points and relaxation delay = 0.04 sec. Chemical shifts were referred to DSS and TMS signals (both 0.00 ppm). All spectra were processed using iNMR program (www.inmr.net), phased and baseline corrected manually. Quantification was performed by signal integration relative to the internal standard, DSS and TMS. The region of the solvent peaks was excluded from the analysis. Spectral peak assignments of organic acids, amino acids, carbohydrates, chlorogenic acid and its derivatives were obtained on the basis of pure standards purchased by Sigma-Aldrich. Spectral peak assignments of these and the other detected metabolites were obtained by two-dimensional (2D) NMR experiments, including $^{1}H–^{1}H$ correlation spectroscopy (COSY) and $^{1}H–^{13}C$ heteronuclear single-quantum correlation (HSQC) and comparison with data reported in the literature[15-18]. The COSY spectra were acquired with a spectral width of 6130 Hz in both dimensions, 8 K data points, and 512 increments with 32 transients per increment. The HSQC spectra were acquired with spectral widths of 8000 Hz in the F2 dimension and 25000 Hz in the F1 dimension, a data matrix with a size of 1K × 256 data points, and 64 transients per increment. The obtained values showed a very good repeatability, with coefficient of variation among replicates < 2.5% for all signals.

Multivariate Data Analyses

Multivariate analyses were applied to ^{1}H NMR spectral data from both aqueous and organic fractions of leaves extracts. ^{1}H NMR spectra were preliminarily normalized and reduced to integrated regions of equal widths (bins = 0.01 ppm), corresponding to 0 – 10 ppm and subsequently reduced to ASCII files using iNMR.[11,17-19,] Matrices were submitted to Principal Component Analysis (PCA) ordination using the STATISTICA 7 Software (StatSoft Inc., Tulsa, Oklahoma, USA). In a more detailed analysis on spectral data from the polar fraction, a submatrix limited to the spectral data was considered and submitted to PCA.

Results and discussion

To investigate the metabolites involved in the tomato-pest interaction, three different genotypes of *S. lycopersicum* were infested with *T. absoluta* and their metabolomic profiles were analysed by NMR spectroscopy followed by chemometrics. ^{1}H NMR analysis of the aqueous and organic extracts showed detailed metabolite profiles of Tolerant (BR221), Susceptible (PS650) and F1 hybrid (CS823) genotypes infested with *T. absoluta* (Figures 1, S1 and S2). Both primary and secondary metabolites were identified through NMR spectroscopy. While the organic extracts contained mainly fatty acids (FA) as the major compounds (Figure S2), the aqueous extracts were shown to contain metabolites belonging to different classes of compounds. A representative model of each ^{1}H NMR Spectrum of the infested tomato plant extracts (T, F1 and S) is showed in Figure 1 in comparison with the corresponding non-infested tomato used as control. Triplicates of ^{1}H NMR spectra of the aqueous fractions for T, S and F1 infested with *T. absoluta* and the corresponding controls and reported in Figure S1. In particular, Figure 1 reports the indication of peaks related to the major metabolites identified in the spectra while full ^{1}H NMR assignments (chemical shifts and coupling constants) of the identified compounds are reported in Table 1. In particular, the presence of sucrose (Sucr) was observed by the appearance in the spectra of the characteristic anomeric signals at δ 5.25-5.29, whose assignment was confirmed by the correlation peaks in the 2D NMR COSY spectra. In Figure 2 the COSY spectrum of the infested T variety is reported. In addition, signals for α- and β-glucose (αGlc and βGlc) and for the related glucuronic acids (αGlcU and βGlcU) were also observed (Table 1 and Figure 1). Organic acids such as malic (MA), shikimic (SHA) acid and GABA (γ-aminobutyric acid) have been identified in the spectra with their related chemical shifts reported in Table 1. Further signals in the spectra were those related to complex fatty acids (cFA) attached to sugar residues in the acyl sugars (AS). In the low-field region of ^{1}H NMR spectra most of the signals belong to secondary metabolites, such as the aromatics

chlorogenic acid (cGA) and its derivatives, neo-chlorogenic acid (ncGA) and 5-O-feruloyl quinic acid (FQA). Diagnostic peaks of the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr) were also observed in this region of the spectra. All ^{1}H NMR data were then integrated using iNMR programme and subjected to a detailed Principal Component Analysis (PCA), in order to assess metabolomic differences among samples related to plant genotype and/or exposure to *T. absoluta*. Concerning the non-polar fraction, PCA of all spectral signals from $CDCl_3$ extracts of tomato genotypes is shown in the right part of Figure 3. In particular, the samples from unexposed leaf (indicated in the figure with Ctrl), irrespective of the plant variety, were consistently grouped together, at a short distance, in the topmost quadrant of the bi-dimensional space defined by the first two principal components, associated to signals resonating at 0.9-1.4 ppm. Moreover, samples exposed to *T. absoluta* (indicated in the figure with R) were arranged along a spatially ordered curve trajectory, but with no recognizable pattern related to plant variety. Interestingly, the sequence of exposed samples along the trajectory in the PC space corresponded to a progressively higher intensity of spectral signals resonating at 2.8-2.9, 5.4, and 2.1 ppm. These signals that are typical of unsaturated functionality on alkyl chain signals should be related with the plant exposure to the micro-moth. Further analyses could help to clarify if such compounds are involved as by-products of the pest attack, or as active molecules playing a role in the defence mechanisms of *tomato* against *T. absoluta*. On the contrary, the bi-dimensional PCA plot of the ^{1}H NMR spectral data from the aqueous fraction (Figure 3, left) clearly separated the samples based on plant genotypes, with leaf materials from T, F1 hybrid and S lines being selectively distributed in the bottom-left, top, and bottom-right quadrants, respectively. However, a higher dispersion was observed for F1 samples, indicating a higher heterogeneity of their spectra compared to Tolerant and especially Susceptible samples. In addition, replicates exposed to the leaf herbivore *T. absoluta* were not well separated from the unexposed controls, with the latter

closely grouped around the PC space centre. This means that, in general, the spectral contributions from the different reference compounds, corresponding to the selected spectral signals [i.e. δ_H 0.4-0.6, 2.3-2.4, 2.7-2.8, 4.3-4.5, 5.0-9.0], were differently distributed among and within genotypes and, moreover, consistent differences can be observed between exposed samples and controls. In other words, existing metabolic differences among non-infested genotypes (i.e. control samples) were amplified after the exposure to the leaf herbivore. The corresponding bi-dimensional plot of signal loadings (Figure 4) has allowed to discuss more in detail the general trend of the association between the analyzed samples and the axis of the PC space. In particular, the first PC axis was positively associated to the signals resonating at δ_H 2.7-2.8 and δ_H 7.3-7.4, diagnostic of malic acid and phenylalanine, respectively, and negatively to a rather wide spectral region including signals resonating at δ_H 0.4-0.6, 6.2-6.4, 6.7-7.1, 7.5-7.6, 8.6-8.7, and 8.9-9.0. Such signals are diagnostic of fatty acids (δ_H 0.39-0.65), chlorogenic and neochlorogenic acids (δ_H 6.19-6.27, 7.00-7.10, 7.42-7.67), 5-O-feruloyl quinic acid (δ_H 6.27-6.36, 7.00-7.10, 7.55-7.62) and trigonelline (δ_H 8.96-9.03, 8.62-8.75). The second PC axis was related to carbohydrate content, being positively associated with the signals resonating at δ_H 5.0-5.6, characteristic of sugars such as α–glucose (δ_H 5.07-5.09) and sucrose (δ_H 5.25-5.29), and negatively with the signals resonating at δ_H 4.3-4.5, characteristic of β-glucose (δ_H 4.48-4.51), and α- and β-glucuronic acid (δ_H 4.37-4.44). A more detailed characterization of metabolites elucidated with the ^{1}H NMR analysis has been carried out, by comparing the association between the PC axis and the spectral signal loadings (i.e. coloured arrows in the graph) with the samples scores in the PC space (i.e. sample locations in the graph) (Figure 4). In this way, both metabolomics of the three genotypes and the chemical changes (Figure 5) produced after the *T. absoluta* exposure, have been evaluated. First, S samples showed a higher content of malic acid (MA) and phenylalanine (Phe), which also increased after the exposure to the herbivore. Also, the T genotype showed MA production, but in smaller amounts

compared to S. On the contrary, the T genotype samples were the most abundant in organic acids, including Fatty Acids (FA), both free and as Acylsugars (AS), Chlorogenic acid (cGA), neochlorogenic acid (ncGA) and feruloyl quinic acid (FQA), detected in very small amounts in the Susceptible genotype. The content of these organic compounds was very low in control samples, indicating a clear link between the exposure and the metabolic pathways related to such specific organic molecules. Previously, it has been demonstrated that these compounds have negative effect on caterpillars[20,21] as well as for different leaf beetles[22-24]. Content of the pyridinic alkaloid Trigonelline (TG) was also detected in all the three genotypes, with the T line showing the highest change of abundance. Trigonelline is an alkaloid with multiple regulatory functions in plants, such as cell cycle, nodulation, oxidative, UV and salt stress response, and DNA methylation[25]. Mirnezhad and colleagues (2010)[16] also identified very low amounts of trigonelline in some tomato varieties resistant to *Frankliniella occidentalis*, hypothesizing that this observation may be the result of a metabolic trade-off favouring the production of acylsugars. Results also showed an increase of TG after exposition of tomato to *T. absoluta* (Figure 5, bottom). The role of this alkaloid could be considered for further investigation in plant-herbivores interactions. The F1 genotype was distinctively different from the other two genotypes since it showed higher amounts of α-glucose and sucrose and lower content in β-glucose and α- and β-glucuronic acids, whereas both T and S genotypes showed similar amounts of these carbohydrates. Furthermore, carbohydrates contents were always higher in infested samples than the non-infested, for all the three genotypes, indicating some connections between this aspect and the response to *T. absoluta*. Interestingly, signals related to GABA (γ-aminobutyric acid) (δ_H 2.3-2.4) were relatively much higher in infested samples of all genotypes compared to the corresponding non-infested controls. Consistently with our results, a physiological role of stress mitigation for GABA has been suggested, consistent with a stress-specific pattern of accumulation in plants[26]. Also, transgenic tobacco plants containing

elevated GABA levels were resistant to root-knot nematodes[27] and tobacco budworm larvae[28]. Since GABA is a neurotransmitter in vertebrates and invertebrates, it could be produced by plants to deter insect feeding, hypothesizing that its ingestion interferes with the normal development of insects[29]. All these findings corroborate our assumption of a leading role of GABA in the interaction between tomato and *T. absoluta*, even if no particular differences could be detected between Tolerant and Susceptible genotypes. In this study, a direct defence has been well elucidated by the metabolome analysis, revealing an involvement of compounds such as chlorogenic and neo-chlorogenic acids, GABA and pyridinic alkaloid trigonelline. NMR spectroscopy coupled with multivariate data analyses demonstrated to be a very successful tool to investigate plant-pathogen interaction. The F1 derived from the cross between the Tolerant and Susceptible lines, is a commercialized variety that showed good agronomic performance and tolerance to *T. absoluta*. These findings could be very useful for better direct future tomato breeding in agricultural and horticultural crops.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

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375 **Tables**

376 **Table 1.** Full ^{1}H-NMR assignment with chemical shifts and multiplicity in 400 MHz spectrum

377 of tomato samples detected in D_2O (KH_2PO_4 buffer pH 6.0) [a]

Metabolites	**Assignment**	**δ_H (ppm)**	**multiplicity (J in Hz)**
complex fatty acids	-CH_3	0.39-0.65	
(cFA/AS)			
amino acids (AA)		0.94-2.10	
glutamic acid (Glu)	-$COCH_3$	2.10	
GABA	-$COCH_3$	2.36-2.42	t (7.0)
malic acid (MA)	dd (15.7, 3.7)	2-72-2.81	dd (15.7, 3.7)
	dd (8.9, 3.7)	4.40	dd (8.9, 3.7)
aspartic acid (Asp)		2.81	dd (17.4; 3.5)
		2.65	dd, (17.4; 9.3)
shikimic acid (SHA)	CH-4	4.30-4.44	dd
	CH-3	6.71-6.82	s
α-glucuronic acid (αGlcU)	CH-5	4.37-4.44	
β-glucuronic acid (βGlcU)	CH-5	4.37-4.44	
β-glucose (βGlc)	CH-1	4.48-4.51	d (7)
α-glucose (αGlc)	CH-1	5.07-5.09	d (3)
sucrose (Sucr)	CH-1	5.25-5.29	
chlorogenic acid (cGA)	CH-8’	6.19-6.27	d (16)
(IUPAC: 5-O-caffeoyl	CH-6’	7.00-7.10	bd (9)
quinic acid)	CH-7’	7.41-7.55	d (16)
neochlorogenic acid (ncGA)	CH-8’	6.19-6.27	d (16)

(IUPAC: 3-O-caffeoyl	CH-6’	7.00-7.10	bd (9)
quinic acid)	CH-7’	7.42-7.67	d (16)
tyrosine (Tyr)		6.90	d (8)
5-O-feruloyl quinic acid	CH-8’	6.27-6.36	d (16)
(FQA)	CH-6’	7.00-7.10	bd ()
	CH-7’	7.55-7.62	d (16)
phenylalanine (Phe)	CH-2-6	7.34-7.41	
trigonelline (TG)	CH-1	8.96-9.03	
	CH-3,5	8.62-8.75	

[a]Assignments were performed by analysis of 1D and 2D NMR spectra and comparison with pure standards (see Experimental) and reference data available in the literature.[15-19]

Figures

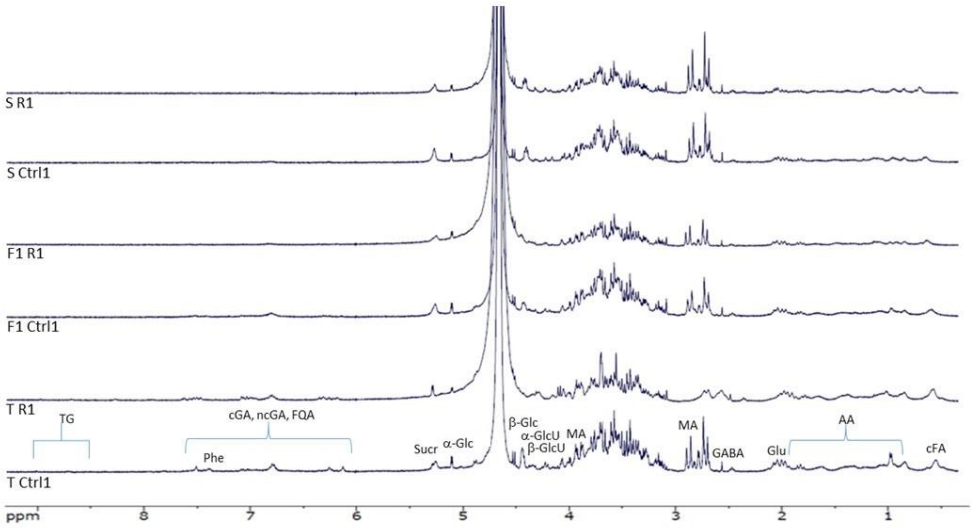
Figure 1. ^{1}H NMR representative spectra in D_2O at 400 MHz of three tomato genotypes: T, tolerant (BR221); F1, hybrid (CS823); S, susceptible (PS650), infested with *Tuta absoluta* (Tinf, F1inf, and Sinf) and non-infested control samples (Tctrl, F1crtl, and Sctrl) with identification of the major compounds detected.

Figure 2. 2D COSY NMR spectrum (D_2O, 400 MHz) of infested tolerant tomato (Tinf).

Figure 3. PCA of ^{1}H NMR spectral data for polar (left) and non-polar (right) extracts of tomatoes. Top: plot of sample scores. Symbol color and shape indicate plant variety (white, T; grey: F1; black: S) and treatment (triangles: replicates exposed to infestant *T. absoluta*, squares: unexposed controls), respectively. Bottom: plot of signal loadings. Labels in top and bottom panels indicate sample ID and signal resonance (ppm), respectively.

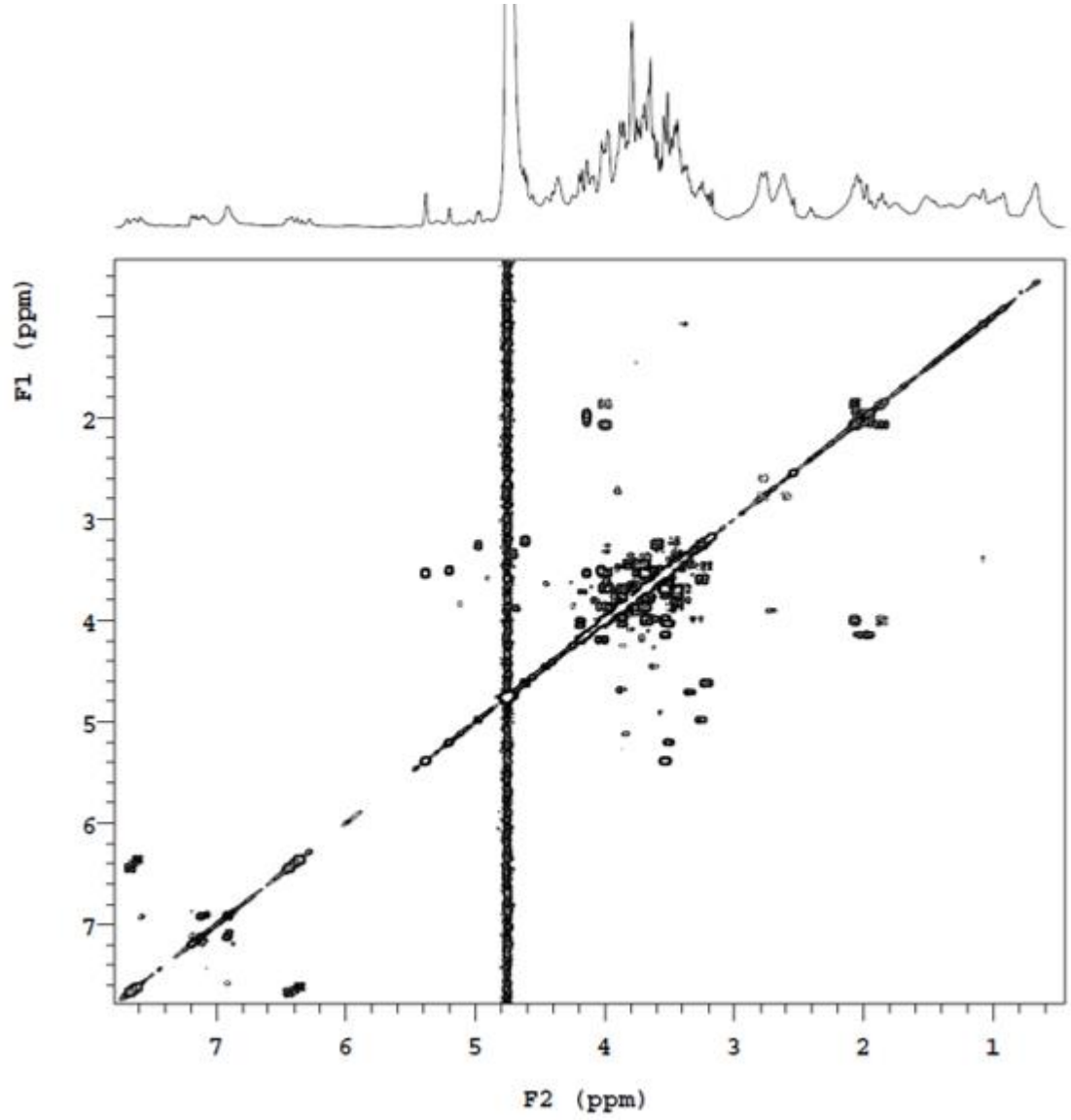
Figure 4. PCA of selected reference ^{1}H NMR spectral signals for polar extract of tomatoes. Left: plot of sample scores. Symbol color and shape indicate plant variety (white, T; grey: F1; black: S) and treatment (triangles: replicates exposed to infestant *T. absoluta*, squares: unexposed controls), respectively. Right: plot of signal loadings. Data labels indicate sample ID and signal resonance (ppm), respectively.

Figure 5. Relative abundance (%) of main metabolites detected by ^{1}H NMR analysis in D_2O extracts of tomato genotypes, except for fatty acids acquired in $CDCl_3$ extract, as calculated from spectral peak intensity. For each metabolite, peaks reported in Table 1 were considered. Data refer to mean and standard deviation of 3 replicated spectra for each population.



403

404 **Figure 1.** ^{1}H NMR representative spectra in D_2O at 400 MHz of three tomato genotypes: T,
405 tolerant (BR221); F1, hybrid (CS823); S, susceptible (PS650), infested with *Tuta absoluta*
406 (Tinf, F1inf, and Sinf) and non-infested control samples (Tctrl, F1crtl, and Sctrl) with
407 identification of the major compounds detected.



408

409 **Figure 2.** 2D COSY NMR spectrum (D_2O, 400 MHz) of infested tolerant tomato (Tinf).

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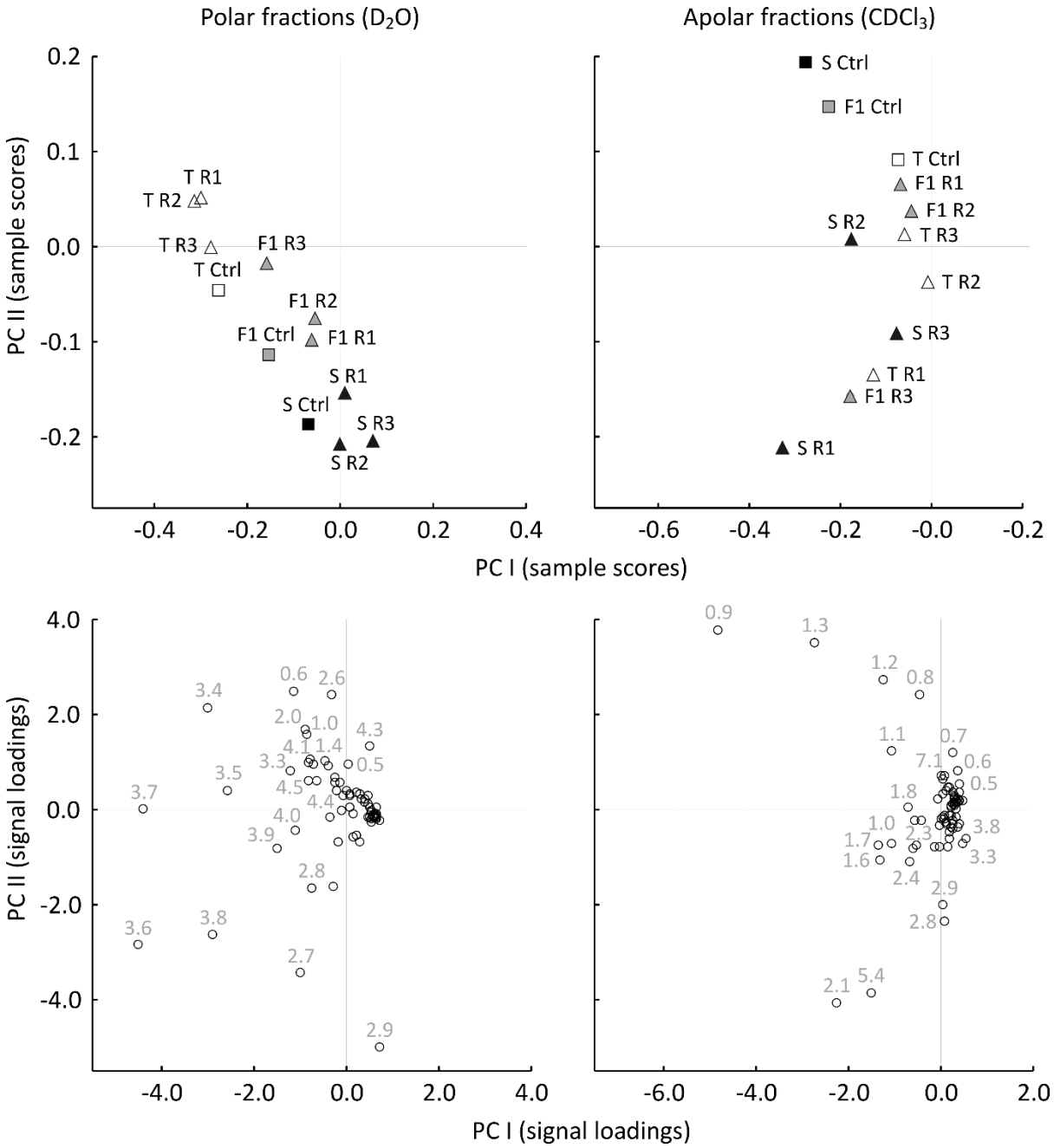

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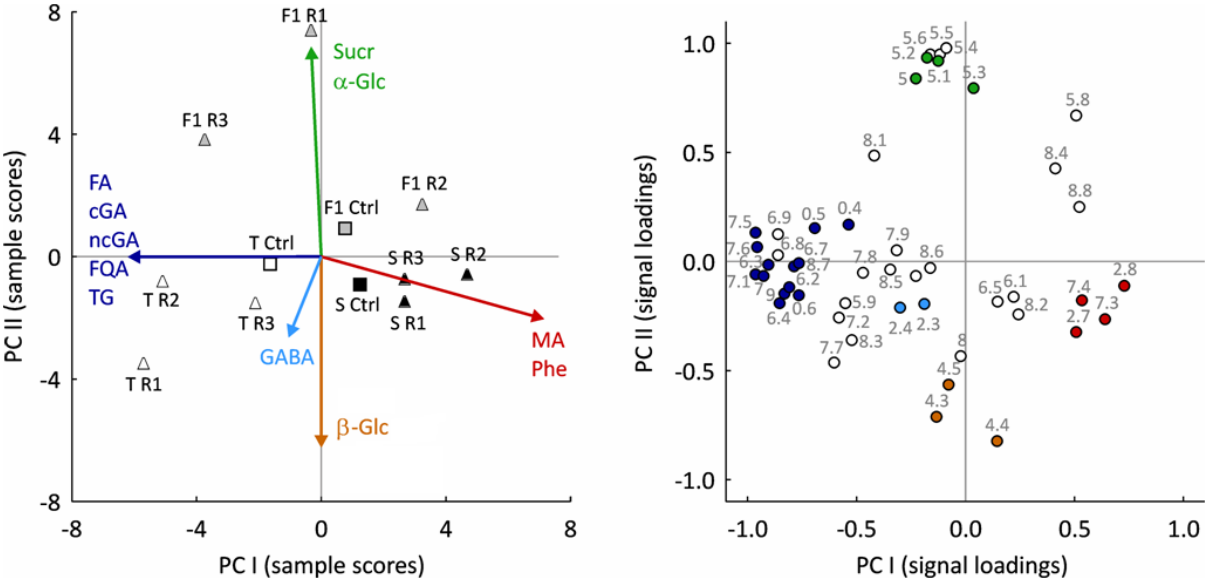
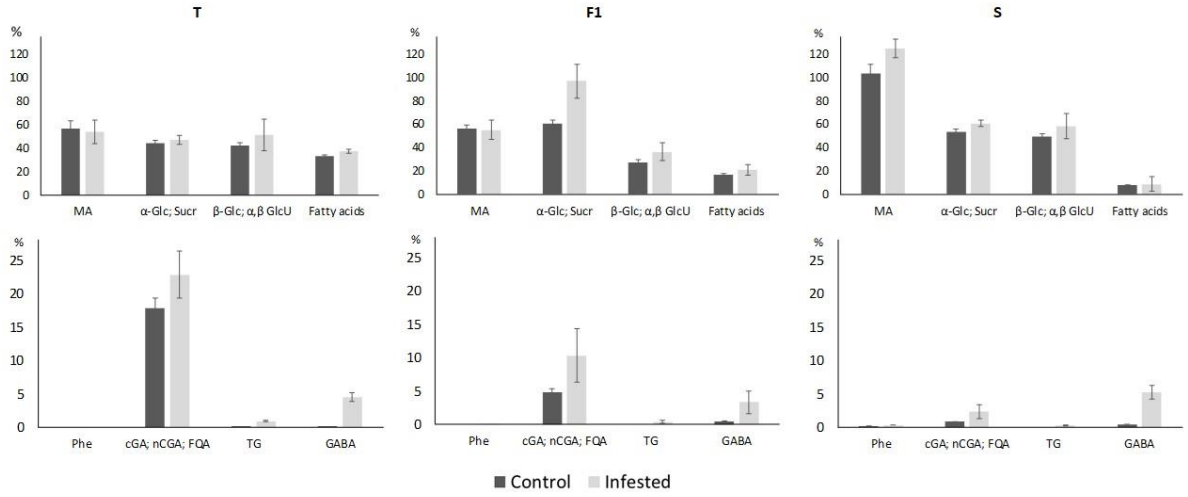


Figure 4 PCA of selected reference ^{1}H NMR spectral signals for polar extract of tomatoes. Left: plot of sample scores. Symbol color and shape indicate plant variety (white, T; grey: F1; black: S) and treatment (triangles: replicates exposed to infestant *T. absoluta*, squares: unexposed controls), respectively. Right: plot of signal loadings. Data labels indicate sample ID and signal resonance (ppm), respectively



428

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