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Primary metabolism changes triggered in soybean leaves by *Fusarium tucumaniae* infection

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ABSTRACT

Sudden death syndrome (SDS) of soybean can be caused by at least four distinct *Fusarium* species, with *F. tucumaniae* being the main causal agent in Argentina. The fungus is a soil-borne pathogen that is largely confined to the roots, but damage also reaches aerial part of the plant and interveinal chlorosis and necrosis, followed by premature defoliation can be observed. In this study, two genetically diverse soybean cultivars, one susceptible (NA 4613) and one partially resistant (DM 4670) to SDS infection, were inoculated with *F. tucumaniae* or kept uninoculated. Leaf samples at 7, 10, 14 and 25 days post-inoculation (dpi) were chosen for analysis. With the aim of detecting early markers that could potentially discriminate the cultivar response to SDS, gas chromatography–mass spectrometry (GC–MS) analyses and biochemical studies were performed. Metabolic analyses show higher levels of several amino acids in the inoculated than in the uninoculated susceptible cultivar starting at 10 dpi. Biochemical studies indicate that pigment contents and Rubisco level were reduced while class III peroxidase activity was increased in the inoculated susceptible plant at 10 dpi. Taken together, our results indicate that the pathogen induced an accumulation of amino acids, a decrease of the photosynthetic activity, and an increase of plant-specific peroxidase activity in the susceptible cultivar before differences of visible foliar symptoms between genotypes could be observed, thus suggesting that metabolic and biochemical approaches may contribute to a rapid characterization of the cultivar response to SDS.

1. Introduction

Sudden death syndrome (SDS) is one of the most yield-limiting diseases of soybean. The disease is caused by at least four soil-borne *Fusarium* species: *F. tucumaniae*, *F. virguliforme*, *F. brasiliense*, and *F. crassistipitatum* [1–4]. Fungal colonization is restricted to the roots and cause root necrosis, but damage also reaches the above ground part of the plant and a sudden development of foliar chlorosis and necrosis, followed by premature defoliation can be observed [5–8]. Foliar symptoms are thought to be triggered by one or more toxins released by the pathogen into the roots followed by an internal transport system to the leaves. This was revealed for the *F. virguliforme* pathogen [9]. Several compounds isolated from fungus culture filtrates have been suggested as putative candidates for foliar SDS symptoms [9–14]. One of these compounds, the protein FvTox1, has been reported as an important virulence factor [11,12,15]. The toxicity effect of FvTox1 could be suppressed *in vitro* by several synthetic interacting peptides [16,17].

Molecular studies have also been performed to characterize the early response of stem-cut soybean plants exposed to *F. virguliforme* culture filtrates [18]. Comparing the gene expression profiles of three soybean genotypes with various levels of resistance to SDS, a number of genes related to plant antioxidant defense were differentially expressed [18]. Among these genes are those that codify for peroxidases and glutathione S-transferases [18]. Recent advances in understanding the genetic basis of SDS resistance have also been made. More than 50 quantitative trait loci (QTL) associated with the resistance response of various cultivars to SDS have been detected [19–21]. However, development of a management strategy through genetic resistance is

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challenging due to the quantitative nature of the trait and the influence of environment on disease progress [22,23]. Recently, the effect of a succinate dehydrogenase inhibitor on severity of SDS was evaluated. Results indicated that resistant cultivars in combination with the fluopyram fungicide seed treatment could provide an effective management of SDS caused by *F. virguliforme* [24]. In fact, *F. virguliforme* population appears to be sensitive to the fluopyram fungicide [25].

In addition to molecular studies, a biochemical analysis was also conducted with samples obtained from a susceptible soybean cultivar of between 14 and 21 days after emergence inoculated with a mixed inoculum from two *F. virguliforme* isolates [26]. This report suggests a possible involvement of free radicals and lipoxygenases in the development of foliar symptoms.

In Argentina, all four mentioned *Fusarium* species are present, with *F. tucumaniae* being the dominant species. One key difference between the four SDS pathogens is the ability of *F. tucumaniae* to generate genetic diversity through sexual reproduction both *in vitro* [27] and in field conditions [28]. This is a challenge for developing a practical and sustainable disease management. Thus, a suitable approach for a rapid screening of the cultivar response to SDS is needed. We have previously monitored the pathogenic responses to *F. tucumaniae* in two soybean genotypes with different levels of resistance to SDS at 7, 10, 14, and 25 dpi [29]. At 7 dpi, there were no visible foliar symptoms on either cultivar inoculated with the pathogen. By 10 dpi, foliar disease severity of inoculated susceptible plants did not differ significantly from data of the inoculated partially resistant cultivar. However, foliar disease severities between inoculated cultivars began to differ at 14 dpi. Then, in order to identify early specific responses of soybean roots to *F. tucumaniae* infection, gas chromatography–mass spectrometry (GC–MS) analyses were performed [29]. The study showed a differential accumulation of amino acids in inoculated susceptible plants during early stages of leaf tissue infection. Of the 13 amino acids identified, Ala, Asn, Asp, Glu, Leu, Pro, 5-oxoPro, Ser, Thr and Val displayed higher levels in inoculated roots of the susceptible cultivar compared with the uninoculated control at 7 dpi [29]. These primary metabolic changes that occurred in soybean roots were originated by the pathogen itself.

Here, we provide a better understanding of the response of above ground parts of soybean to one or more toxins that are translocated from infected roots to leaves. The experimental design involved the use of a single strain of *F. tucumaniae*, two genetically diverse soybean cultivars with contrasting resistance to SDS, one susceptible (Nidera A 4613, hereinafter referred to as NA 4613) and one partially resistant (Don Mario 4670, hereinafter referred to as DM 4670), and sampling at 7, 10, 14 and 25 days post-inoculation (dpi). The partially resistant cultivar showed minimal disease symptoms throughout the time course of *F. tucumaniae* infection.

A total of 52 analytes were detected, consisting of 42 known metabolites including amino acids, organic acids, soluble sugars, alcohols, fatty acids, and a miscellaneous group, three unidentified sugars (S) and seven unidentified analytes (UAs). In addition, we have also examined the effect *of F. tucumaniae* infection on photosynthesis and peroxidase activity at 10 dpi. These findings along with evidence from our previous work [29] provide a complete panorama of primary metabolic changes that are triggered in soybean by *F. tucumaniae* infection and could be used for a rapid characterization of the cultivar response to SDS. Even more, consistent data from both root and leaf tissues may be of great help to securely differentiate between susceptible and resistant genotypes.

2. Material and methods

2.1. Plant and pathogenic material

Leaf samples were from experiments previously performed [29]. Briefly, two commercial soybean cultivars of the same maturity group and similar growth habit, one susceptible (NA 4613) and one partially resistant (DM 4670) to SDS, were used in the experiments. Fungal infection was performed using sorghum infested with the isolate of *F. tucumaniae* CCC130-11 [CCC = Culture Collection of the CEREMIC (Centro de Referencia de Micología), Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR]. Each biological replicate consisted of a pot with 4–5 plants. All plants were cultivated in parallel in a greenhouse under a natural photoperiod of spring (14 h light:10 h dark) at 25° ± 3 °C for 7, 10, 14 and 25 days. This experimental design allowed us to discriminate effects that were due to progression of the plant-fungal interaction from those related to either genotype or to developmental effects of the plant.

2.2. Metabolomic analysis

Metabolites were extracted as described previously [30]. Briefly, leaf powder was mixed with methanol containing ribitol as an internal quantitative standard. The mixture was shaken at 70 °C and then centrifuged. The supernatant was transferred to a new tube followed by the addition of chloroform and water. After centrifugation, the upper phase was transferred to a new tube and dried under vacuum. Samples were then derivatized using methoxyamine hydrochloride dissolved in pyridine and *N*-methyl-*N*-trifluoroacetamide (MSTFA). Derivatized samples were then analyzed by GC–MS as described previously [29], except that an Agilent 7890B gas chromatograph coupled to an Agilent 5977 A mass spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA) and a 30 m HP-5 ms Ultra Inert with a 0.25 mm inner diameter and 0.25 µm film thicknesses (Agilent Technologies Inc., Palo Alto, CA, USA) were used. The sample was injected at 250 °C with a gas flow rate of 1 ml/min. The temperature program was isothermal for 5 min at 70 °C, followed by a 5 °C/min ramp to 310 °C and a hold at 310 °C for 1 min. Ions were generated by a ionization voltage of 70 eV with a scan range of 70 to 600 Da. Metabolites were identified by comparison of retention indexes and mass spectra data in the NIST 2011 Mass Spectral Library and an in-house database. Metabolite contents were normalized by both the sample fresh weight and the peak area of the internal ribitol standard, and expressed as relative response ratios. Original data from the experiments are shown in Supplementary Table S1 as recommended for reporting metabolite data [31]. Ratios of metabolite levels expressed as $\log_2$-fold changes are visualized in a heatmap performed using the MultiExperiment Viewer software (MeV v4.9, http://www.tm4.org/; [32]).

2.3. Protein extracts

Leaves (0.2 mg) were ground under liquid nitrogen and suspended in 1 ml of extraction buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$, 2% v/v glycerol, 1 mM PMSF). Suspensions were stirred at 4 °C for 30 min and separated by centrifugation at 13,000 *xg* for 10 min. Supernatants were desalted according to Penefsky [33] using a Sephadex G-25 column preequilibrated with extraction buffer. These crude extracts were used for enzyme activity, protein and immunological analyses.

2.4. Peroxidase enzyme activity assays

The activity of guaiacol peroxidase (GPX, EC 1.11.1.7) was determined spectrophotometrically in 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 25 mM H_2O_2 and 22.25 mM guaiacol in a final volume of 1.0 ml at 30 °C. Oxidation of guaiacol to tetraguaiacol was determined by the increase in absorbance at 470 nm using an extinction coefficient of 25.5 mM^{-1} cm^{-1} as described previously [34].

2.5. SDS-PAGE and immunological analyses

Proteins (8 µg) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) under denaturing conditions using 12% (w/v)

polyacrylamide/bisacrylamide gels according to Laemmli [35]. Proteins were then stained with Coomassie brilliant blue (R250) or electrotransferred to nitrocellulose membranes [36]. Polyclonal rabbit anti-Rubisco large subunit (LSU) antibody was used for detection. Bound primary antibody was recognized by goat anti-rabbit IgG polyclonal antibody conjugated to alkaline phosphatase and subsequently developed with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium [36]. Western blot signals were quantified by densitometric analysis using image analysis software in at least four independent blots.

2.6. Protein quantification

Total soluble proteins were determined by the method of Bradford [37] using the Bio-Rad protein assay reagent and bovine serum albumin as a standard.

2.7. Chlorophyll and carotenoid assays

Total chlorophylls and carotenoids were measured in leaf extracts by standard procedures [38].

2.8. Data analysis

Data comprise two biological and at least three technical replicates, except for the relative abundance of Rubisco LSU, where four biological and two technical replicates were used. Each biological replicate consisted of a pot with 4–5 plants. Data was subjected to a two-factorial analysis of variance (ANOVA) for a completely randomized design, with two levels for the genotype factor (NA 4613, susceptible; and DM 4670, partially resistant) and four for the dpi factor (7, 10, 14, and 25). Statistical analysis of the data was obtained using Infostat software v. 2017 [39].

Before performing multivariate analyses, the data was mean-centered and Pareto-scaled. For principal component analysis (PCA), covariance similarity indices were used. The first two PCs of each sample were drawn in a two-dimensional plot. Symbols indicate different genotypes and treatments: circles and triangles correspond to data obtained from susceptible and partially resistant cultivars, respectively, and filled and open symbols correspond to data from inoculated and uninoculated control plants, respectively. For the hierarchical cluster analysis (HCA), samples are grouped according to their dissimilarity by Chi-square distance. For agglomeration methods, the method of Ward was performed. The results are visualized as dendrograms with the branches representing the clustering of samples.

3. Results

3.1. Metabolic profiles of leaves from susceptible and partially resistant soybean cultivars in response to F. tucumaniae infection

To establish whether pathogen-derived toxins triggered metabolic shifts in soybean leaves, a time-course analysis was performed by GC–MS. Samples were obtained at four time points after inoculation from two soybean cultivars displaying different resistance to fungal infection. A total of 52 analytes were detected, consisting of 42 known metabolites identified in the NIST 2011 (National Institute of Standards and Technology) mass spectral library, three unidentified sugars (US) and seven unidentified analytes (UAs) (Supplementary Table S1). Each metabolite was classified into amino acids, organic acids, soluble sugars, alcohols, fatty acids, miscellaneous and unidentified analytes. Changes in metabolite levels expressed as $\log_2$ ratios were visualized using a heatmap (Fig. 1). Changes are shown in red or green when the levels of each metabolite was higher or lower, respectively, in the infected versus uninfected leaf tissues from the susceptible cultivar at each time point (Fig. 1A), in the infected versus uninfected leaf tissues from the partially resistant cultivar at each time point (Fig. 1B), in uninfected leaves from the susceptible versus the partially resistant cultivar (Fig. 1C), in infected leaves from the susceptible versus the partially resistant cultivar (Fig. 1D), in uninfected leaves from the susceptible soybean cultivar after different experimental time points versus the first time point (Fig. 1E), or in uninfected leaves from the partially resistant soybean cultivar after different experimental time points versus the first time point (Fig. 1F). Fig. 1A and B indicate metabolic changes due to progression of the plant–fungal interaction, Fig. 1C shows metabolic changes related to the genotype, Fig. 1D shows metabolic changes due to both the progression of the plant–fungal interaction and the genotype and Fig. 1E and F represent metabolic changes produced as a consequence of plant development.

Levels of the 17 amino acids identified were found in higher concentrations in the inoculated than in the uninoculated susceptible control plants at various time points (Fig. 1A). At 10 dpi, levels of Ala, β-Ala, Asn, Gly, Ile, Leu, 5-oxoPro, Ser, Thr, Trp, Tyr and Val were statistically higher in inoculated leaves from the susceptible cultivar compared with the uninoculated control; at 14 dpi, all the amino acids displayed higher levels in the inoculated than in the uninoculated susceptible cultivar while at 25 dpi, levels of β-Ala, Asn, Asp, Gln, Gly, Ser and Trp still remained significantly elevated in the inoculated susceptible plant in contrast to their controls. The levels of some amino acids whose carbon skeletons come from different intermediates of the glycolysis pathway (phosphoenolpyruvate or pyruvate) or the tricarboxylic acid (TCA) cycle (oxaloacetate) are shown in Fig. 2. Significantly high levels of Asn (Fig. 2A), Ile (Fig. 2B), Tyr (Fig. 2C) and Val (Fig. 2D) were detected in inoculated samples of the susceptible cultivar compared with the uninoculated control at 10 and 14 dpi. Asn also displayed higher levels in the inoculated than in the uninoculated partially resistant cultivar at 14 dpi, although levels did not reach those of the susceptible cultivar (Fig. 2A). Comparisons between genotypes at equivalent time points indicated that amino acid levels were only slightly affected by genotype (Fig. 1C). Infected leaves from the susceptible cultivar showed higher levels of some amino acids than infected leaves from the partially resistant cultivar starting at 10 dpi (Figs. 1D and 2). These amino acids include Ala, β-Ala, Asn (Fig. 2A), Asp, GABA, Gln, Glu, Gly, Leu, Ile (Fig. 2B), Ser, Thr, Trp, Tyr (Fig. 2C), and Val (Fig. 2D). Comparisons related to plant development showed a trend towards lower levels in leaves from older plants (Figs. Fig. 11E, F and 2).

The organic acid pattern was diverse. Fumarate and malonate exhibited the highest relative contents of organic acids. TCA cycle intermediates were slightly affected by genotypes and time post-inoculation (Fig. 1). At 10 dpi, levels of citrate, fumarate, and malate were statistically higher in inoculated leaves of the partially resistant cultivar compared with the uninoculated control (Fig. 1B). The response of the susceptible cultivar to the infection was slightly different. An increase in citrate and malate levels was observed in the inoculated susceptible cultivar compared with the uninoculated control at 14 dpi. Later, at 25 dpi, levels of all identified TCA cycle intermediates were statistically lower in the inoculated leaves from the susceptible cultivar than in leaves from the uninoculated control (Fig. 1A). Derivatives of intermediates produced by the glycolysis pathway were also found to be slightly altered by inoculation and genotypes (Fig. 1A–C). At 10 dpi, glycerate and lactate levels increased in inoculated leaves of the susceptible cultivar compared with the uninoculated control while malonate displayed higher levels in the inoculated than in the uninoculated partially resistant cultivar (Fig. 1A). At 14 dpi, saccharate, glycerate, malonate and ribonate levels showed an increase in the susceptible cultivar compared with the control (Fig. 1A). However, all the mentioned organic acids changed their levels less than 2-fold. Comparisons related to plant development showed that some organic acids increased their levels in leaves from older plants (Fig. 1E and F).

Levels of soluble sugars were significantly affected by infection and time post-inoculation. Levels of fructose, glucose, methyl

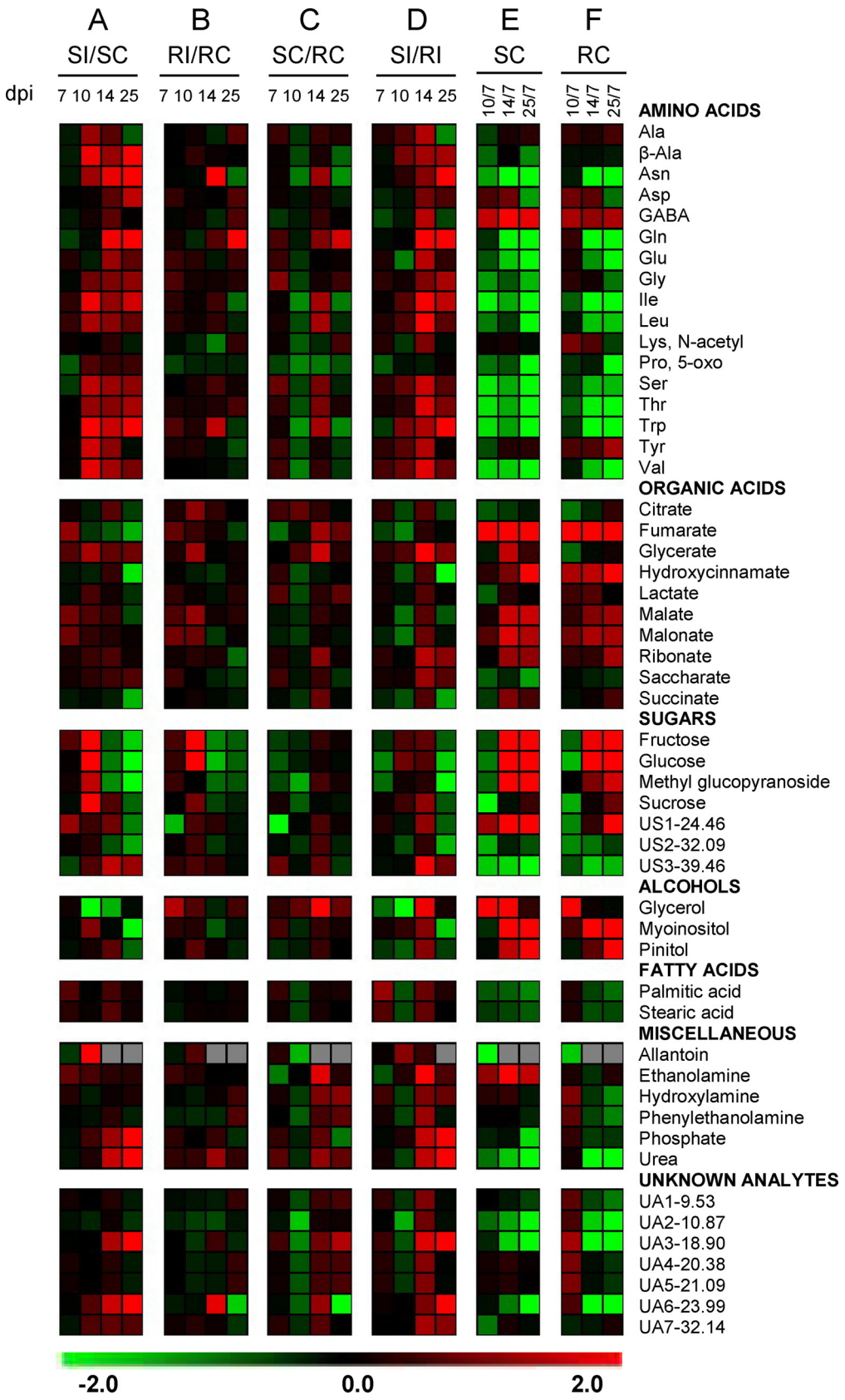

Fig. 1. Heatmap representation of metabolic response of soybean plants to *F. tucumaniae* infection. (A) Metabolite level changes (log_2) in the inoculated susceptible cultivar compared with the uninoculated control at 7, 10, 14, and 25 days post-inoculation (dpi). (B) Metabolite level changes (log_2) in the inoculated partially resistant cultivar compared with the uninoculated control at 7, 10, 14, and 25 dpi. (C) Metabolite level changes (log_2) of uninoculated susceptible over uninoculated partially resistant soybean cultivars at different time points (7, 10, 14, and 25 dpi). (D) Metabolite level changes (log_2) of inoculated susceptible over inoculated partially resistant soybean cultivars at different time points (7, 10, 14, and 25 dpi). (E) Metabolite level changes (log_2) of samples from the uninoculated susceptible cultivar at 10, 14, and 25 dpi over 7 dpi. (F) Metabolite level changes (log_2) of samples from the uninoculated partially resistant cultivar at 10, 14, and 25 dpi over 7 dpi. S, susceptible soybean cultivar; R, partially resistant soybean cultivar; I, inoculated with *F. tucumaniae* and C, uninoculated control. Red indicates increased metabolite levels and green represents decreased levels (see colour scale bar); grey indicates levels below the detection limit in either denominator or numerator values. Metabolites were classified in the following major classes: amino acids, organic acids, soluble sugars, alcohols, fatty acids, miscellaneous, and unknown analytes (UAs). The numbers at the right of the unidentified metabolites indicate the retention time (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

glucopyranoside and sucrose in infected versus uninfected leaf tissues peaked at 10 dpi in both cultivars, with the exception of methyl glucopyranoside in the partially resistant cultivar (Figs. Fig. 11A, B and 3). The increase due to the infection at 10 dpi showed a trend to higher values in the susceptible compared with the partially resistant cultivar (Fig. 3, Supplementary Table S1). Comparisons between genotypes at equivalent time points showed only slight differences in sugar levels between cultivars (Figs. Fig. 11C and 3). Comparisons related to plant

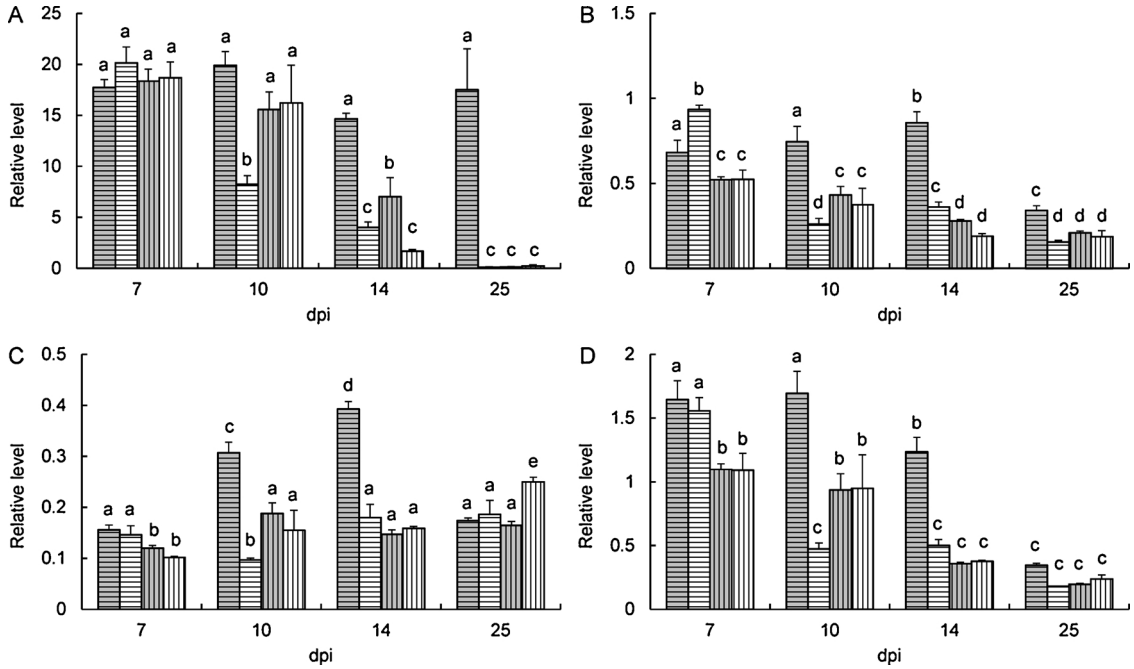

Fig. 2. Variation in amino acid content detected by GC–MS. (A) Asparagine, (B) isoleucine, (C) tyrosine, and (D) valine levels in leaf extracts of susceptible (horizontal lines) and partially resistant (vertical lines) soybean cultivars inoculated with *F. tucumaniae* (dark grey) or uninoculated (light grey) at different time points after inoculation. Means with the same letter are not significantly different ($P < 0.05$). dpi, days post-inoculation.

development indicate that leaves from older plants displayed higher levels of the identified sugars than leaves from 7-days-old plants (Figs. Fig.11E, F and 3). However, it should be mentioned that these increases were not observed in the monosaccharide levels from the inoculated cultivars (Fig. 3). Three unidentified sugar (US) compounds designated as US1, US2 and US3 were detected in all the leaf extracts. The five most abundant ion mass fragments of US1 have mass-to-charge (*m/z*) ratios of 147, 117, 205, 306 and 234; of US2, 204, 133, 217, 197and 189; and of US3, 245, 217, 147, 208 and 253. US1 showed a similar pattern to sucrose, although US1 levels at 7 and 10 dpi were lower than sucrose levels.

With respect to polyols, pinitol and myoinositol were the most abundant. Pinitol levels increased 1.6-fold in inoculated leaves of the partially resistant cultivar compared with the uninoculated control at 10 dpi, while a similar change level was observed later at 14 dpi in the susceptible inoculated plant compared with its control (Fig. 1A and B). At 25 dpi, under control conditions, leaves from both genotypes accumulated comparable pinitol levels, but this accumulation was affected by the infection. On the other hand, myoinositol decreased 1.6-fold in leaves from the inoculated partially resistant cultivar compared with the uninoculated control at 14 dpi and 4-fold in leaves from the inoculated susceptible cultivar compared with the uninoculated control at 25 dpi (Fig. 1A and B). Interestingly, glycerol levels displayed significant differences between genotypes. Glycerol levels were reduced by 80% and 60% in the susceptible cultivar at 10 and 14 dpi, respectively, in comparison with the uninoculated control plant, whereas no decrease in the content of glycerol was observed in the partially resistant cultivar (Fig. 1A and B).

Levels of palmitic and stearic fatty acids showed an increase, on average, of 50% in leaves of the susceptible cultivar at 7 and 14 dpi when compared with the uninoculated control (Fig. 1A and B). This increase was not observed in the inoculated partially resistant plant.

Among the miscellaneous group, levels of inorganic phosphate, urea and allantoin were the most affected. Inorganic phosphate statistically increased 2.4- and 5.8-fold in treated leaves of the susceptible cultivar compared with the uninoculated control at 14 and 25 dpi, respectively, while urea displayed statistically higher levels (16-fold) in the inoculated than in the uninoculated susceptible cultivar at 25 dpi (Fig. 1A). Allantoin showed a similar pattern to urea (Fig. 1A), although allantoin levels increased earlier (10 dpi) in both cultivars upon infection than urea levels (14 dpi).

Seven unidentified analytes, referred as UA1 to UA7, were also discovered. These analytes could not be matched to the NIST mass spectral library. The five most abundant ion mass fragments of UA1 have mass-to-charge (*m/z*) ratios of 131, 117, 184, 110 and 200; of UA2, 147, 204, 116, 59 and 149; of UA3, 141, 77, 149, 133 and 254; of UA4, 174, 228, 184, 217, 155; of UA5, 174, 154, 243, 86 and 82; of UA6, 218, 100, 261, 147 and 115; and of UA7, 275, 292, 219, 189 and 207. Significantly high levels of UA3, UA6, and UA7 were detected in inoculated samples of the susceptible cultivar at 14 and 25 dpi. Profiles of UA1, UA2, UA4, and UA5 were similar and showed small changes due to the genotypes at 10 dpi.

3.2. Multivariate statistical analysis of metabolic responses of leaves from susceptible and partially resistant soybean cultivars to F. tucumaniae infection

In order to investigate the global effect of *F. tucumaniae* infection on the metabolomic profiles of two soybean genotypes, multivariate non-parametric statistical tests were applied. Principal component (PC) analysis performed on a covariance matrix is shown in Fig. 4A. Results showed that the first four PCs explained 90.84% of the overall variance of metabolite profiles (57.26, 20.99, 8.55 and 4.03% for principal components 1–4, respectively). A clear separation between genotypes or infection was not observed at 7 dpi, but samples from the inoculated susceptible cultivar obtained at 10, 14 and 25 dpi could be separated from their corresponding controls (Fig. 4A). These data grouped together with the metabolic composition of the infected partially resistant cultivar obtained at 10 dpi. At later time points, data from both uninoculated cultivars clustered together with data from the infected partially resistant plant (Fig. 4A).

A hierarchical cluster analysis (HCA) was then constructed. The resulting HCA dendrogram calculated using the Ward linkage method is shown in Fig. 4B. Cluster 1 included samples from inoculated and

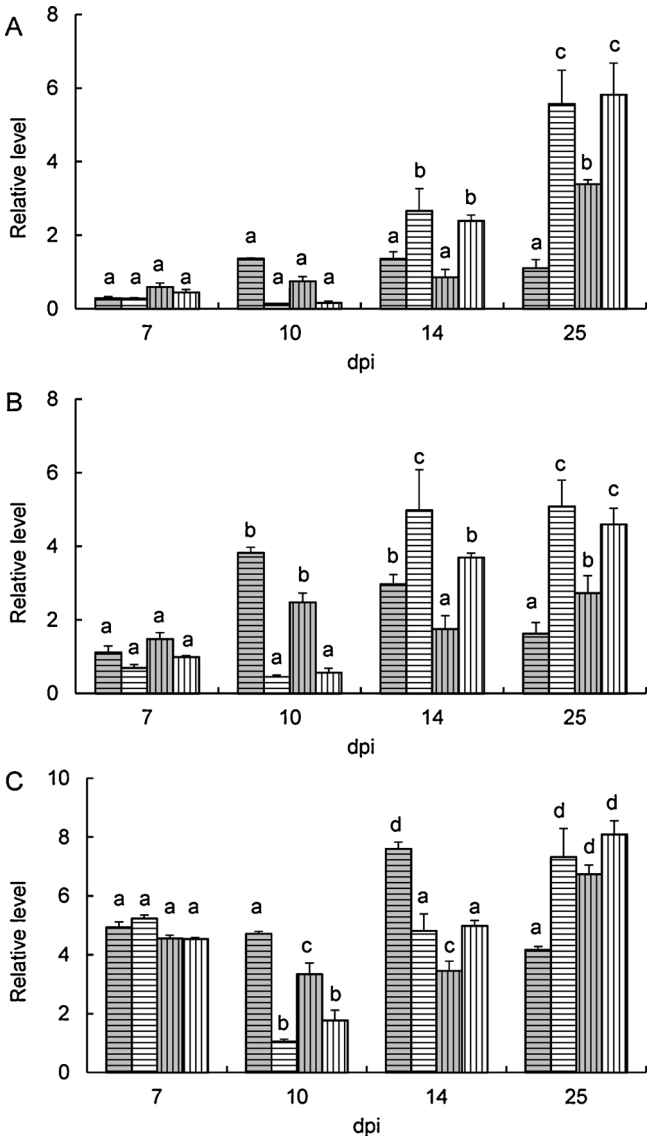

Fig. 3. Variation in soluble sugar content detected by GC–MS. (A) Glucose, (B) fructose, (C) sucrose levels in leaf extracts of susceptible (horizontal lines) and partially resistant (vertical lines) soybean cultivars inoculated with *F. tucumaniae* (dark grey) or uninoculated (light grey) at different time points after inoculation. Means with the same letter are not significantly different ($P < 0.05$). dpi, days post-inoculation.

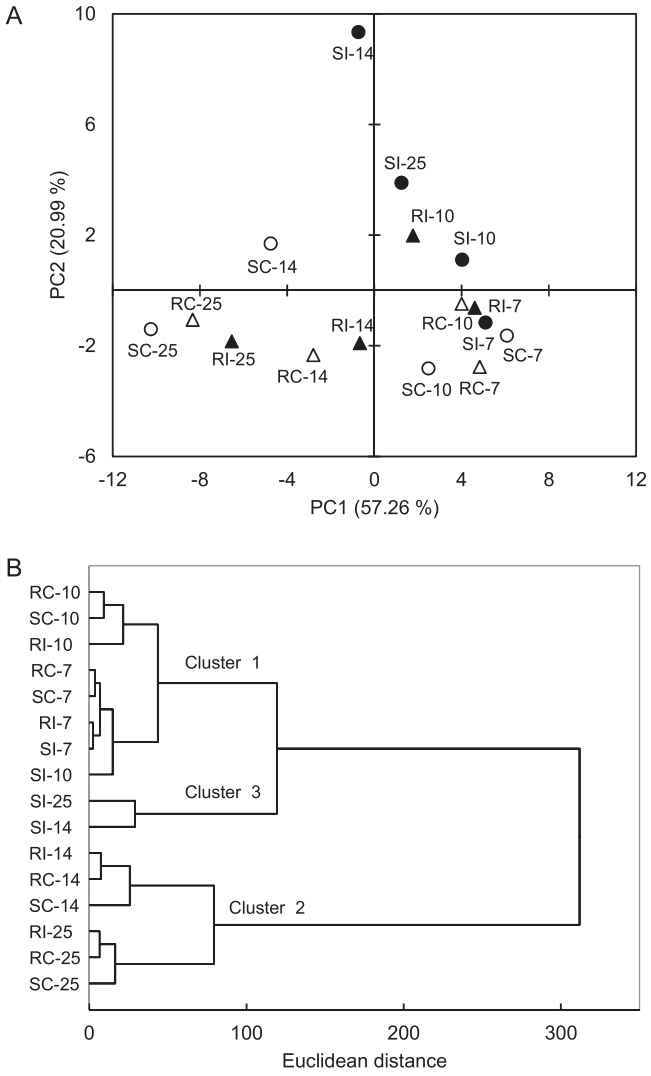

Fig. 4. (A) Principal component analysis of the metabolite profiles of inoculated and uninoculated control susceptible and partially resistant genotypes at different time points. The first (PC1) and second (PC2) principal components explain 78.26% of the variance. Circles and triangles correspond to data obtained from susceptible (S) and partially resistant (R) cultivars, respectively. Filled and open symbols correspond to data from inoculated (I) and uninoculated control (C) plants, respectively. Different time points are indicated by numbers (7, 10, 14, and 25). (B) Dendrogram generated using the method of Ward as the agglomeration method and the Euclidean distance as the dissimilarity coefficient.

uninoculated susceptible and partially resistant plants obtained at 7 and 10 dpi. Cluster 2 comprised samples from both uninoculated cultivars and from the infected partially resistant plant obtained at 14 and 25 dpi. Finally, samples from the inoculated susceptible cultivar obtained at 14 and 25 dpi clustered together.

3.3. Photosynthetic pigment and Rubisco contents in leaves from susceptible and partially resistant soybean cultivars in response to F. tucumaniae infection

To investigate whether pathogen-derived toxins induced other early biochemical responses in leaves, photosynthesis and antioxidant enzyme activities were studied. Concentrations of chlorophylls and carotenoids and the relative abundance of the ribulose-1,5-biphosphate carboxylase oxygenase large subunit (Rubisco LSU) were determined in samples obtained at 10 dpi. Results are shown in Fig. 5. No significant differences due to genotype were detected. Mean chlorophyll *a*, chlorophyll *b* and carotenoid contents were 5.79 ± 0.30 mg/g, 2.50 ± 0.10 mg/g and 0.84 ± 0.05 mg/g, respectively, for the susceptible control plant and 5.59 ± 0.19 mg/g, 2.37 ± 0.13 mg/g and 0.81 ± 0.02 mg/g, respectively, for the partially resistant control cultivar (Fig. 5A). However, chlorophyll *a*, chlorophyll *b* and carotenoid contents were significantly reduced in the inoculated susceptible cultivar by 43.1%, 43.7% and 37.6%, respectively (Fig. 5A). Only a slight reduction in chlorophyll contents was observed in the inoculated partially resistant plants compared with the uninoculated control (21.6% and 23.6% for chlorophyll *a* and chlorophyll *b*, respectively) (Fig. 5A). No changes in carotenoid contents were found in the partially resistant cultivar in response to the infection (Fig. 5A).

The relative abundance of Rubisco LSU was estimated using Western Blot analysis (Fig. 5B) and expressed in arbitrary units (Fig. 5C). Fig. 5D shows an SDS-PAGE of total leaf soluble proteins stained with Coomassie Blue R250 as a representative loading control. In leaves from control plants, the amount of Rubisco LSU protein was 98.9 ± 4.3 A.U and 94.5 ± 7.5 A.U for the susceptible and partially

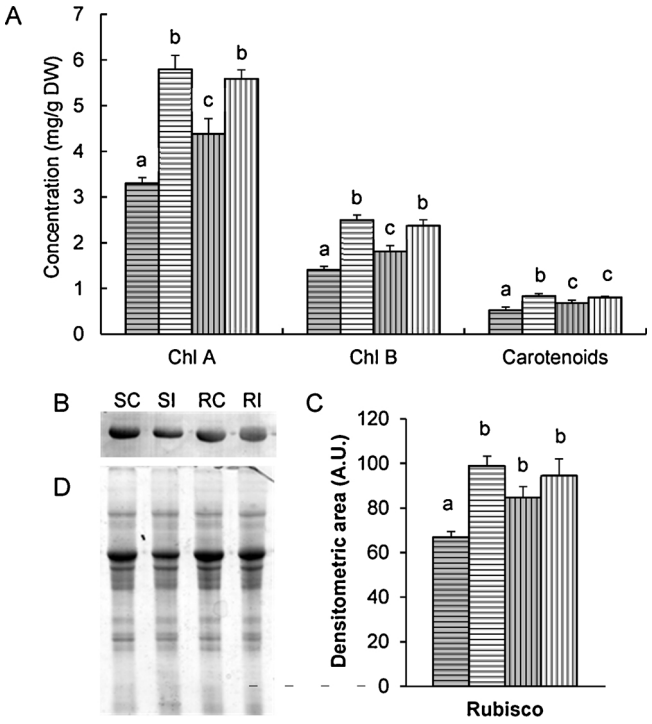

Fig. 5. (A) Variation in chlorophyll *A* (Chl A), chlorophyll *B* (Chl B) and carotenoid contents in leaves of susceptible (horizontal lines) and partially resistant (vertical lines) soybean cultivars inoculated with *F. tucumaniae* (dark grey) after 10 dpi or uninoculated (white). Means with the same letter were not significantly different ($P < 0.05$) among each pigment. (B) Representative Western blot of the abundance of the Rubisco large subunit in leaves of susceptible (S) and partially resistant (R) soybean cultivars inoculated with *F. tucumaniae* (I) after 10 dpi or uninoculated control (C). (C) Densitometric analysis of the abundance of the Rubisco large subunit in leaves of susceptible (horizontal lines) and partially resistant (vertical lines) soybean cultivars inoculated with *F. tucumaniae* (dark grey) after 10 dpi or uninoculated (white). Data comprise four biological and two technical replicates. Means with the same letter are not significantly different ($P < 0.05$). (D) SDS-PAGE of 8 u g of total leaf soluble proteins loaded per lane. Gel was stained with Coomassie Blue R250 and is included as a representative loading control. dpi, days post-inoculation.

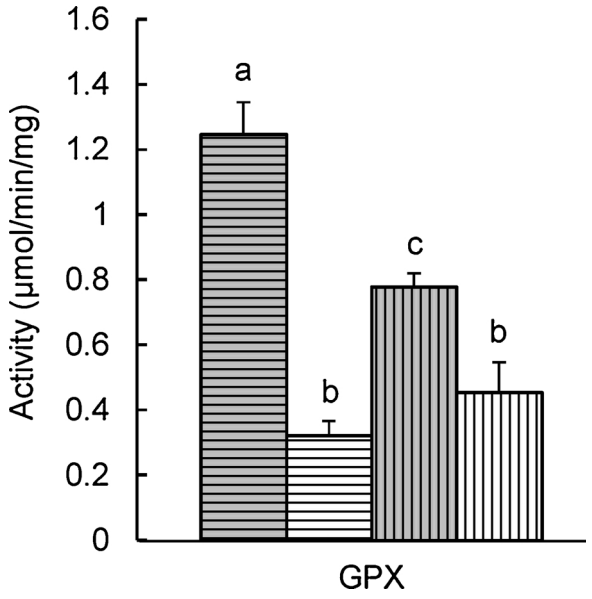

Fig. 6. Variation in guaiacol peroxidase (GPX) activity in leaves of susceptible (horizontal lines) and partially resistant (vertical lines) soybean cultivars inoculated with *F. tucumaniae* (dark grey) after 10 dpi or uninoculated (white). Means with the same letter are not significantly different ($P < 0.05$).

resistant genotypes, respectively. These values show a non-significantly difference neither between them nor with the protein amount in leaves from the partially resistant inoculated cultivar (84.7 ± 4.8 A.U; Fig. 5B and C). However, the Rubisco LSU protein level was 30% lower in leaves from the susceptible inoculated cultivar than in leaves from the partially resistant inoculated plant (Fig. 5B and C).

In addition, the activity of guaiacol peroxidase (GPX) was measured in samples obtained at 10 dpi (Fig. 6). GPX activity increased in both genotypes after the fungal infection. In the susceptible cultivar, the enzyme activity was 1.25 µmol/min/mg in leaves from the inoculated plant, 3.9-fold higher when compared with the uninoculated control (0.32 µmol/min/mg). In the partially resistant cultivar, the GPX activity was 0.78 µmol/min/mg in leaves from the inoculated plant and 0.45 µmol/min/mg in the uninoculated control (1.7-fold increase).

4. Discussion

To date no source of complete resistance to SDS is available for soybean breeders. However, various levels of SDS resistance to *F. tucumaniae* in Argentinean soybean cultivars have been described [40,41]. In order to evaluate the level of resistance of soybean genotypes to *F. tucumaniae* infection, the pathogen effect on leaf metabolism in pre-symptomatic plants was studied. Reports involving *F. tucumaniae* are scarce. We have previously characterized specific responses of soybean roots to *F. tucumaniae* infection [29]. Our results indicated that the pathogen induced amino acid accumulation at early time points after infection in roots from the susceptible cultivar [29].

In this study, a time-course analysis of the primary metabolic changes triggered in soybean leaves by fungal-derived toxins was performed. Regulation of amino acid metabolism appeared to be the most affected pathway in the infected susceptible cultivar. Accumulation of Ala, B-Ala, Asn, Gly, Ile, Leu, 5-oxo Pro, Ser, Thr, Trp, Tyr and Val occurred in leaves from the susceptible cultivar as early as 10 dpi; whereas all of the identified amino acids were detected four days later (at 14 dpi) and six amino acids remained high until 25 dpi. Our results agree with a previous report on the effect of *F. virguliforme* on the metabolism of a susceptible soybean cultivar [26]. These authors only monitored the accumulation pattern of metabolites after approximately between 14 and 21 days post-emergence. Comparison of their results with our data obtained at 14 dpi indicates similar changes on amino acid levels, with Asn being the most abundant while 5-oxo Pro being the least abundant amino acid after fungal infection. This is further supported by observations that indicate that more genes involved in nitrogen metabolism are regulated in response to necrotrophic pathogens than to biotrophic pathogens [42]. In particular, a rapid activation of Asn synthetase in susceptible tomato plants following infection with the necrotrophic fungus *Botrytis cinerea* fungus has been reported [43]. Asn may serve as a rich nitrogen source to support the growth of the fungi *in vitro* and possibly in the host [43]. In addition, a recent report indicated that Gln levels influenced the susceptibility of *Medicago truncatula* to the root hemibiotrophic oomycete *Aphanomyces euteiches*, with high Gln levels being correlated with enhanced plant susceptibility [44]. Even more, it has been reported that some amino acid-related metabolic pathways are also involved in defense responses [45] and that disease severity induced by fungal pathogens is modified following nitrogen fertilization [42,46–48].

Consistent with an accumulation of amino acids in leaves from the susceptible cultivar, we observed that urea has been highly enriched at later time points. Urea is a product of amino acid catabolism [49,50] and has been implicated as a marker for leaf senescence [51].

Organic acids provide redox equilibrium and energy balance in plants [52]. The most abundant organic acids were fumarate and malonate. Fumarate accumulates to high levels in soybeans and

increases with plant age in Arabidopsis [53], while malonate accumulates in plants belonging to the *Fabaceae* family [52]. Accordingly, we have also observed high levels of these organic acids in leaves from both genotypes and a trend towards increasing fumarate levels in leaves from older control plants. Interestingly, variation in malate and fumarate accumulation was found to alter pathogen susceptibility. It was reported that tomato fruits of malate dehydrogenase deficient genotypes showed higher malate and fumarate levels and more susceptibility to the infection by the fungal pathogen *Botrytis cinerea* than wild type plants, while fruits of fumarase deficient lines displayed lower malate and fumarate levels and less pathogen susceptibility than wild type plants [54]. Our results also reflect the genotype effect on fumarate accumulation and *F. tucumaniae* susceptibility.

Levels of fructose, glucose, and sucrose showed a trend to higher values in the inoculated compared with the uninoculated leaf tissues in both cultivars at 10 dpi. However, the increase due to the infection was two-fold higher in the susceptible compared with the partially resistant cultivar. Increases of the identified monosaccharides were not maintained at later times and leaves from control older cultivars displayed higher levels of sugars than leaves from 7-days-old plants. Several lines of evidence suggest that plants have evolved mechanisms to modulate their sugar pools in response to infections to act either as nutrient source for pathogens, as signals to induce defense responses or as priming agents to activate immune reactions (reviewed in [55–61]). The present results support these previous observations and suggest that the pathogen may differentially affect sugar metabolism, transport or/and partitioning of each genotype.

Polyols are a source of carbon and energy. We identified two cyclitols (pinitol and myoinositol) and an acyclic polyol (glycerol). It was noted that pinitol was the most abundant O-methylinositol in soybean plants [62] and to increase during plant development [63]. These reports are consistent with our results. In addition, we have also observed that pinitol accumulation was affected in both genotypes by the infection at later time points. The level of glycerol was significantly reduced in the susceptible soybean cultivar in response to pathogen infection. Accordingly, infection of *Arabidopsis* plants with the hemibiotrophic *Colletotrichum higginsianum* pathogen reduced the glycerol level while concomitantly accumulating the glycerol-3-phosphate content [64]. Levels of glycerol-3-phosphate were reported to be closely associated with the activation of systemic acquired resistance (SAR) in plants [65–67]. Even more, a mechanistic link between glycerol-3-phosphate, unsaturated fatty acids and azelaic acid has been reported [68]. Pathogen inoculation induced the release of unsaturated fatty acids. Unsaturated fatty acids are the precursors for the C9 fragment azelaic acid. Azelaic acid increased glycerol-3-phosphate levels and triggered SAR [68]. Consistent with this report, we have observed an increase in palmitic and stearic acids in response to pathogen infection only in the susceptible cultivar.

Levels of phosphoric acid showed a statistically increase in the infected susceptible cultivar at later time points (14 and 25 dpi). This result probably suggests a response to the biotic stress or cell damage.

Metabolic responses were also visualized by PCA and HCA. It is clear that at 7 dpi, aerial plant responses to *F. tucumaniae* infection are still minimal. However, metabolic profiles of leaves from the inoculated susceptible genotype obtained at later time points after infection were clearly differentiated from their controls.

Based on these results and considering that a characterization of cultivar response to SDS before the appearance of visible symptoms is needed, we also examined the effect of *F. tucumaniae* infection on photosynthesis and peroxidase activity at 10 dpi. The results presented here demonstrate that pigment contents and Rubisco level were reduced in leaves from infected plants, and the decrease was more pronounced in the susceptible cultivar. Consistent with this, a down-regulation of genes involved in chlorophyll biosynthesis and photosynthesis due to fungal pathogen attack have been reported [69–74]. Previous studies have also shown that application of the recombinant FvTox1 from *F. virguliforme* induced a higher loss of chlorophyll content in soybean leaf disks of susceptible cultivars compared with resistant cultivars [12] and that cell free *Fusarium solani* f. sp. *glycines* culture filtrates containing phytotoxins caused degradation of the Rubisco large subunit [14]. Another observation have indicated that a host-selective toxin from *Cochliobolus victoriae* produced chlorophyll loss and proteolytic cleavage of the Rubisco large subunit (LSU) of leaf slices from susceptible oat tissues, but no effect on the resistant tissues [75]. Interestingly, we observe that the photosynthesis reduction in the susceptible cultivar preceded differences in foliar disease severity between genotypes [29]. These results are also consistent with the finding that the host-specific toxin from the fungal pathogen *Pyrenophora tritici-repentis* induced declines in net photosynthetic rates in wheat prior to the development of any visible chlorosis [76].

In addition, the activity of class III peroxidase was analyzed using guaiacol as an artificial phenolic substrate [77]. This reaction is not specific and ascorbate peroxidase enzymes are also able to oxidize guaiacol [78–81]. The direct or indirect involvement of peroxidases in plant defense reactions has been reviewed [82,83]. We show that GPX activity was strongly induced in soybean leaves after *F. tucumaniae* infection. These results agree with those found during interaction between *Phaseolus vulgaris* and the necrotrophic fungus *Botrytis cinerea* [84]. An induction of GPX activity was also shown during interaction between *Solanum lycopersicum* and the necrotrophic fungus *Fusarium oxysporum* f. sp. *lycopersici* [85], *Dianthus caryophyllus* L and the necrotrophic fungus *Fusarium oxysporum f.* sp. *dianthi* [86], *Brassica napus* and the hemibiotrophic fungus *Leptosphaeria maculans* [87] and *Glycine max* and the biotrophic fungus *Phakopsora pachyrhizi* [88]. Furthermore, in the present study we found that the GPX activity has been differentially regulated between genotypes after 10 dpi. The increase in enzyme activity was 2-fold higher in the susceptible cultivar compared with the partially resistant soybean plant. Accordingly, previous reports indicate a differential gene expression of class III peroxidases in highly or intermediate susceptible soybean varieties compared to a partially resistant genotype in response to *F. virguliforme* phytotoxin [18]. These authors show an increased transcript level of class III peroxidase of 3.4, 4.9 or 1.8 between mock and treated leaf tissues from highly susceptible, intermediate susceptible or partially resistant soybean genotypes [18]. Other reports also indicate that peroxidase levels were differentially expressed in susceptible and resistant plants and these differences were dependent on the time after inoculation [86,88].

In summary, in this work, we show the impact of *F. tucumaniae*-derived toxin(s) on the primary metabolism of inoculated soybean leaves from two genotypes with contrasting resistance to fungal infection. The study indicated that inoculated susceptible plants accumulated amino acids as early as 10 dpi post-inoculation. At this time point, we also observed a photosynthesis reduction and a class III peroxidase activity induction in the inoculated susceptible cultivar. Interestingly, these changes preceded differences of visible foliar symptoms between genotypes and could thus help to rapidly characterize the cultivar response to SDS. These results require further validation using a large number of susceptible and partially resistant genotypes to determine that the screening method can efficiently predict field cultivar response to *F. tucumaniae* infection.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.05.013.

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