Differential induction of *Medicago truncatula* defence metabolites in response to rhizobial symbiosis and pea aphid infestation

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Figures: 6
Tables: 1
Total word count: 6250
Introduction: 680
Materials and Methods: 2039
Results: 1590
Discussion: 1941

Summary

- Legumes symbiosis with rhizobial nitrogen-fixing bacteria enable them to grow in nitrate-depleted soils. Rhizobial symbioses also induces systemic plant defence against bioagressors.
- We investigate how nitrogen-fixing symbiosis (NFS) in the legume *Medicago truncatula* can prime plant defence against the pea aphid *Acyrthosiphon pisum*. We analysed metabolite modification both by LC-MS and GC-MS and defence pathway gene expression by qPCR in leaves of both NFS and nitrate-fed (non-inoculated; NI) plants after aphid infestation (Amp).
- The accumulation of primary and secondary metabolites was modulated by both NFS and aphid infestation. 62 defense-related metabolites such as salicylate, pipecolate, gentisic acid and several soluble sugars were differentially regulated by aphid infestation in both NFS and NI conditions. 19 metabolites, including triterpenoid saponins, accumulated specifically in NFS_Amp conditions. Gene expression analysis showed that aphid-infested plants exhibited significantly higher expression of *Chalcone isomerase*, *flavonol synthase*, *hydroxyisoflavone-O-methyl transferase* and *Pterocarpan synthase*, while *D-pinitol dehydrogenase* was only significantly induced in NI infested leaves.
- Our data suggest that NFS, in addition to being a plant nitrogen provider, stimulates specific legume defenses upon pest attack and should also be considered as a potential tool in Integrated Pest Management strategy.

Key words: *Medicago truncatula*, *Sinorhizobium meliloti*, nitrogen-fixing symbiosis, *Acyrthosiphon pisum*, metabolomics, plant defence priming.

Introduction

Plants are under constant threat from pathogens and insect pests such as sap-feeding aphids (National Research Council *et al.*, 1985). Aphids are deleterious agronomic pests, not only because they feed on phloem sap and therefore weaken the plant, but also because they are vectors for various plant viruses (Ng & Perry, 2004). More than 5000 species of aphids are known today, a diversity that is partly due to sympatric speciation initiated by individuals adapting to new host plants (Diehl & Bush, 1984; Drès & Mallet, 2002). During feeding, the aphid stylet injures plant cells, inject saliva, and sucks up tiny amounts of the cell content to decide on plant acceptance (Martin *et al.*, 1997; Lu *et al.*, 2016). Previous studies have revealed that the secreted proteins present in saliva trigger plant responses (Pitino & Hogenhout, 2013; Rodriguez & Bos, 2013). These saliva proteins act as herbivore-associated molecular patterns (HAMPs) (Kaloshian & Walling, 2016) that bind to host plant pattern recognition receptors (PRRs) and trigger an immune response associated with the production of reactive oxygen species (ROS) and defence hormones Salicylic Acid (SA) and Jasmonic Acid (JA) pathways (Wu & Baldwin, 2010; Herrera-Vásquez *et al.*, 2015). The increase of SA and JA regulate the accumulation of various primary and secondary metabolites that play a role in plant defence (Isah, 2019) as feeding deterrents or toxins that decrease food intake or food use efficiency, decrease survival and reproduction of the pest, or indirectly act as attractants for its natural enemies (War *et al.*, 2012).The secondary metabolites involved in this process include terpenoids, phenolics, cyanogenic glycosides, glucosinolates, and alkaloids (Maag *et al.*, 2015; Züst & Agrawal, 2016). For example, high levels of saponins and phenolic compounds increase aphid mortality and result in reduced pest population (Züst & Agrawal, 2016), flavonoid glycosides also reduce aphid fecundity, while nitrogen-containing compounds cause host plant rejection (Kordan *et al.*, 2012).

One property of legume plants is their ability to associate with rhizobia to perform nitrogen fixation symbiosis (NFS), i.e., the conversion of atmospheric N_2 into plant-usable ammonium. The microbial partner supplies assimilable nitrogen to the plant in exchange for carbon resources and a protective environment in the root nodules (Lee & Hirsch, 2006). NFS has also been reported to potentially provide defence priming to plants against bio-aggressors (Benjamin *et al.*, 2022). For example, in pea (*Pisum sativum*), rhizobium inoculation decreased *Didymella pinodes* disease severity and significantly reduced the seed infection level (Desalegn *et al.*, 2016; Ranjbar Sistani *et al.*, 2017). NFS has also been reported to modulate resistance to biotrophic pathogens in both *Medicago truncatula* and pea by reducing penetration and sporulation of the powdery mildew fungus *Erysiphe pisi* (Smigielski *et al.*, 2019).

104 We have previously demonstrated that NFS also influences the *Medicago truncatula*–pea aphid
105 interaction and general plant defence response (Pandharikar *et al.*, 2020). A detrimental effect
106 of rhizobia-inoculated plants on aphid development was observed, with lower adult weights
107 compared to aphids from nitrate-fed plants. *Pathogenesis Related 1* (*PR1*) gene expression was
108 upregulated in aphid-infested shoots, indicating the activation of SA-dependent defence.
109 Moreover, a significantly higher expression of *Proteinase Inhibitor (PI)* gene, a marker for the
110 JA transduction pathway, was observed in NFS plants compared to nitrate-fed plants
111 (Pandharikar *et al.*, 2020).
112 Due to the observations from our preliminary studies, we have analysed by metabolomics, the
113 impact of the nitrogen source (KNO_3 *vs* NFS) on *M. truncatula* infestation by pea aphid. To
114 this end we analysed leaf metabolite profiles of NFS plants with and without aphid infestation
115 and compared them to KNO_3-fed plants (Non Inoculated; NI) and to obtain a maximum
116 coverage of the leaf metabolites, untargeted metabolomic was done both by gas
117 chromatography/mass spectrometry (GC-MS) and liquid chromatography/mass spectrometry
118 (LC-MS). Our results showed that both primary and secondary metabolisms are significantly
119 modulated by nitrogen source and aphid infestation. Gene expression analysis of enzymes
120 involved in the secondary metabolite synthesis pathways showed that regulation of secondary
121 metabolism is partially mediated by the modulation of gene expression. We identified specific
122 metabolites which are differentially regulated in NFS plants compared to NI plants under aphid
123 infestation.
124

125 **Materials and Methods**

126 Biological materials and experimental design

127 The pea aphid *Acyrtosiphon pisum* clone, YR2-amp, further named YR2, is derived from a
128 clover biotype line collected in England that was freed from the secondary symbiont *Regiella*
129 *insecticola* by ampicillin treatment (Simon *et al.*, 2011). This aphid line is stable (more than
130 15 years old) and was maintained on fava bean, 20°C, 16:8h light/dark cycle. To synchronize
131 aphid, 20-40 apterous female adults were placed in a petri dish containing a fava bean leaf and
132 let to reproduce for 24hrs. Then, 10 nymphs (L1) were collected and used for infestation.
133 For one experiment, four batches of 5 pots containing each six *Medicago truncatula* A17 plants
134 were grown as previously described (Pandharikar *et al.*, 2020) (Fig. S1). After 12 days, two
135 groups were inoculated with the nitrogen-fixing bacteria *Sinorhizobium meliloti* 2011
136 (nitrogen-fixing symbiosis, NFS condition), and two were supplemented once with 10 ml of 5

137 mM KNO_3 solution (non-inoculated, NI condition). Seven days after (the time to NFS plants
138 to develop nodules), for both NFS and NI conditions, one batch of NI plants and one batch of
139 NFS plants were infested with aphids (10 L1/pots). The aphid nymphs were then let to develop
140 into adult during 12 days. All pots were individually isolated in a ventilated plastic box and
141 maintained at 20°C under 16:8h light/dark photoperiod. For each condition, control pots were
142 treated and maintained under the same conditions except that no aphids were introduced in the
143 plastic box. At the end of the 12 days, harvested leaves were used immediately or frozen in
144 liquid nitrogen and stored at -80°C.
145 Rhizobium inoculation was done with a streptomycin-resistant strain of *Sinorhizobium meliloti*
146 2011. It was cultured on Luria-Bertani medium supplemented with 2.5mM $CaCl_2$ and $MgSO_4$
147 (LBMC) and streptomycin at 200µg ml^{-1} for 3 days at 30°C, then transferred and grown in
148 LBMC liquid medium for 24h, pelleted at 5,000g, washed twice with sterile distilled water,
149 and resuspended in sterile distilled water to a final optical density of 0.05 (OD_{600}). Each NFS
150 plant was inoculated with 10 mL of this *S. meliloti* suspension.
151 A total of 4 biological replicates (i.e. 4 times 4 pots of six plants for each condition), produced
152 as described above were used for analysis carried out in this study.
153
154 Analysis of metabolites using gas chromatography/mass spectrometry (GC-MS)
155 Leaves were ground in liquid nitrogen to obtain a fine powder and 50 mg was resuspended in
156 1ml of cold (-20°C) Water:Acetonitrile:Isopropanol (2:3:3 in volume) containing Ribitol
157 4µg/ml as internal standard. After extraction under shaking (10 min, 4°C), insoluble material
158 was removed by centrifugation at 20,000g for 5min and 50µl were collected and dried
159 overnight (SpeedVac™) and used immediately or stored at -80°C. Three blank tubes underwent
160 the same treatments to estimate possible contamination. A quality control was made by pooling
161 an equal volume of each condition.
162 Samples were warmed 15 min before opening and dried again for 1.5h at 35°C before addition
163 of 10µl of 20 mg ml^{-1} methoxyamine in pyridine and the reaction was performed for 90 min at
164 28°C under continuous shaking. 90µl of N-methyl-N-trimethylsilyltrifluoroacetamide
165 (MSTFA) were then added and the reaction continued for 30 min at 37°C. After cooling, 45µl
166 were taken for injection. 1µl of derivatized sample was injected in splitless and split (1:30)
167 modes on a gas chromatograph (Agilent 7890A; Santa Clara, USA) coupled to a mass
168 spectrometer (Agilent 5977B) with a heated separation column (Rxi-5SilMS; Restek, Lisses,
169 France) (temperature ramp: 70°C for 7 min then 10°C/min to 330°C for 5 min; run length 38
170 min). Helium flow was constant at 0.7 mL/min. 5 scans per second were acquired spanning a

171 50 to 600Da range. Instrument was tuned with PFTBA with the *m/z* 69 and *m/z* 219 of equal
172 intensities. Samples were randomized. Three independent quality controls were injected at the
173 beginning, middle and end of the analysis for monitoring the derivatization stability. An alkane
174 mix (C10, C12, C15, C19, C22, C28, C32, C36) was injected during the run for external
175 calibration. Three independent derivatizations of the quality control were injected at the
176 beginning, in the middle and at the end of the series. A response coefficient was determined
177 for 4ng each of a set of 103 metabolites, respectively to the same amount of ribitol. This
178 compound was used to give an estimation of the absolute concentration of the metabolite in
179 what we may call a "one point calibration" (Fiehn, 2006, 2008).
180
181 Analysis of metabolites using liquid chromatography/mass spectrometry (LC-MS)
182 Metabolites were extracted from 6 mg of fresh weight ground sample using a protocol adapted
183 from the literature (Kim *et al.*, 2008). Briefly, 1.6ml of a mix of Methanol/H2O/Acetone/TFA
184 (40/32/28/0.05, v:v:v:v) and 300ng of Apigenin (used as internal standard) were added to each
185 sample, which was then stirred at 4°C for 30 minutes. After centrifugation (10 min, 20,000 g,
186 4°C), the supernatant was collected, and the pellet was extracted again by stirring with 1.6ml
187 of the previous solvent mix for 30 minutes. After centrifugation the two supernatants were
188 pooled, dried and resuspended in 200µl of water (ULC/MS grade)/Acetonitrile (90/10)
189 (Biosolve Chimie, Dieuze, France) and filtered (filter paper grade GF/A Whatman®).
190 Metabolomics data were acquired using a UHPLC system (Ultimate 3000, Thermo Scientific,
191 Waltham, USA) coupled to quadrupole time of flight mass spectrometer (Q-Tof Impact II
192 Bruker Daltonics, Bremen, Germany). A Nucleoshell RP18 plus reversed-phase column (2 x
193 100 mm, 2.7 µm; Macherey-Nagel, Hoerdt, France) was used for chromatographic separation.
194 The mobile phases used for the chromatographic separation were (A) 0.1% formic acid in H_2O
195 and (B) 0.1% formic acid in acetonitrile. The flow rate was of 400µl min^{-1} and the following
196 gradient was used: 95% of A for 1-min, followed by a linear gradient from 95% A to 80% A
197 from 1 to 3-min, then a linear gradient from 80% A to 75% A from 3 to 8-min, a linear gradient
198 from 75% A to 40% A from 8 to 20-min. 0% of A was hold until 24-min, followed by a linear
199 gradient from 0% A to 95% A from 24 to 27-min. Finally, the column was washed by 30% A
200 for 3.5min then re-equilibrated for 3.5-min (35-min total run time). Data-dependent acquisition
201 (DDA) methods were used for mass spectrometer data in positive and negative ESI modes
202 using the following parameters: capillary voltage, 4.5kV; nebuliser gas flow, 2.1 bar; dry gas
203 flow, 6L min^{-1}; drying gas in the heated electrospray source temperature, 140ºC. Samples were
204 analysed at 8Hz with a mass range of 100 to 1500 m/z. Stepping acquisition parameters were

205 created to improve the fragmentation profile with a collision RF from 200 to 700 Vpp, a transfer
206 time from 20 to 70 µs and collision energy from 20 to 40eV. Each cycle included an MS full-
207 scan and 5 MS/MS CID on the 5 main ions of the previous MS spectrum.
208
209 Soluble sugars and starch analysis
210 Soluble sugars (sucrose, glucose, and fructose) were extracted from 150 mg of frozen tissue
211 powder (both roots and leaves separately) with ethanol and water solution (800µl at 80% of
212 ethanol) by incubation in a water bath at 80°C for 15 min, with shaking every 5-minute. The
213 sample was centrifuged (5 min, 5,000 rpm) and the supernatant collected. The extraction was
214 repeated using the same conditions with 800µl of a 50% ethanol solution, 800µl of 100% water
215 and then 800µl 80% ethanol solution. All supernatants were mixed and then evaporated. The
216 dried sample was resuspended in 1ml of water and kept in the dark at -20°C until soluble sugar
217 analysis.
218 The residual pellet from sugar extraction was used for starch analysis. Immediately after
219 removal of the last supernatant, the pellet was resuspended in 3ml of thermostable α-amylase.
220 The tube was plunged in a boiling water bath and mixed every 2 mins. After 6 min, the sample
221 was transferred to 50°C bath and 0.1ml of amyloglucosidase (20U) was added, the tube was
222 mixed and incubated for 30 min. The entire content of the tube was then transferred to a larger
223 tube and volume adjusted to 20ml with distilled water, mixed thoroughly, and centrifuged at
224 5,000 rpm for 5 min. The clear, undiluted supernatant was used for the determination of glucose
225 issued from starch hydrolysis.
226 Sugar analyses were performed using Sucrose, D-Fructose and D-Glucose assay kit
227 (Megazyme K-SUFRG; Neogene, Mi, USA) and Total starch HK assay kit (Megazyme K-
228 TSHK), respectively, as described by the provider. Spectrophotometric measurements were
229 conducted at 340nm in cuvettes with a 1 cm optical path.
230
231 Gene Expression Analysis
232 For RNA extraction, plant material was ground in liquid nitrogen. Total RNAs from 100mg
233 was then isolated using RNAzol® RT (Sigma), quantified, and analysed on nanodrop and 1.5%
234 agarose gel electrophoresis to assess the purity. DNA digestion (RQ1 RNAse-free DNAse) and
235 reverse transcription (GoScript™ Reverse Transcription) were performed as described by the
236 manufacturer (Promega, Madison, USA). qPCR was performed (qPCR Master Mix plus CXR;
237 Promega) using cDNA template and each set of primers. *Pathogenesis Related 1* (*PR1*;
238 MtrunA17_Chr2g0295371) was used as SA defence gene marker and the *Proteinase Inhibitor*

239 (*PI*; PSI-1.2; MtrunA17_Chr4g0014461) as JA pathway activation gene (Pandharikar *et al.*,
240 2020). Other genes of interest analysed were *Chalcone Isomerase* (*CHI*;
241 MtrunA17_Chr1g0213011), *Flavanol Synthase/Flavanone 3-Hydroxylase* (*FLS/F3H*;
242 MtrunA17_Chr3g0092531), *Isoflavone 4'-O-methyltransferase* (*HI4'O-MT*;
243 MtrunA17_Chr4g0046341), *D-pinitol Dehydrogenase* (*OEPB*; MtrunA17_Chr6g0480011),
244 *Phenylalanine Ammonia-lyase* (*PAL*; MtrunA17_Chr1g0181091), *Pterocarpan Synthase 1*
245 (*PTS*; MtrunA17_Chr7g0259091) and *SAR Deficient 4* (*SARD4*; MtrunA17_Chr1g0202471) .
246 Real-time qPCR was performed with specific primers (Table S1) using 95°C for 3 min
247 followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec, and melting curves from 65°C to
248 95°C in increments of 0.5°C (AriaMx Real-time PCR machine, Agilent). Cycle threshold
249 values (Ct) were normalized to the average Ct of two housekeeping genes *MtC27*
250 (MtrunA17_Chr2g0295871) and *a38* (MtrunA17_Chr4g0061551) genes (Del Giudice *et al.*,
251 2011). The expression of these two genes was not affected by the treatments. The original Ct
252 obtained (Ariamix software; Agilent) (Dataset S1) were further used in the R qPCRBASE
253 package (Hilliou, 2013). For each gene, the expression level of the aphid infested plants was
254 compared with that of the non-infested control plants. The results of the qPCR analysis were
255 generated from four independent biological repeats.
256
257 Metabolomics Data Processing
258 For GC-MS, raw datafiles were converted in NetCDF format and analysed with AMDIS
259 software (http://chemdata.nist.gov/mass-spc/amdis/). A home retention indices/mass spectra
260 library built from the NIST, Golm (http://gmd.mpimp-golm.mpg.de/) and Fiehn databases
261 (https://fiehnlab.ucdavis.edu), and standard compounds was used for metabolites
262 identification. Peak areas were also determined with the Targetlynx software (Waters) after
263 conversion of the NetCDF file in masslynx format. AMDIS, Target Lynx in split-less and split
264 30 modes were compiled in one single Excel file for comparison. After blank mean subtraction
265 peak areas were normalized to Ribitol and leaves fresh weight.
266 For LC-MS, data files were converted and treated as previously described (Boutet *et al.*, 2022).
267 A first search was done in library with open-source software MZmine2 (Pluskal *et al.*, 2010)
268 with identification module and "custom database search" to begin the annotation with our
269 library, currently containing 159 annotations (RT and *m/z*) in positive mode and 61 in negative
270 mode, with RT tolerance of 0.3 min and m/z tolerance of 0.0025 Da or 6 ppm. Molecular
271 networks were generated with MetGem software (Olivon *et al.*, 2018)
272 (https://metgem.github.io) using the .mgf and .csv files obtained with MZmine2 analysis. ESI-

273 and ESI+ molecular networks were generated using cosine score thresholds of 0.8 in two
274 modes. Metabolites annotation was performed as described (Boutet *et al.*, 2022).
275
276 Statistical Analysis
277 Statistical analysis was performed with TMEV (https://sourceforge.net/projects/mev-tm4/) for
278 GC-MS. Univariate analyses by permutation (1-way-anova and 2-way anova) were firstly used
279 to select the significant metabolites (P-value < 0.01). Multivariate analyses (hierarchical
280 clustering an principal component analysis) were then made on both LC-MS and GC-MS data.
281 Mapman (http://www.gabipd.org/projects/MapMan/) was used for graphical representation of
282 the metabolic changes after Log2 transformation of the mean of the 3 replicates (Fiehn, 2006,
283 2008).
284 Tukey's multiple comparison tests were also performed on independent metabolites to identify
285 statistical differences between the various conditions for both primary and secondary
286 metabolites. Statistical analysis of individual metabolites was done using Prism v9.1.1
287 (GraphPad software, USA). All experimental data are expressed as mean ± standard error (SE).
288 Venn diagram was created based on results of Tukey's tests, clustering metabolites based on
289 statistical significance (P-value ≤ 0.05) using InteractiVenn (Heberle *et al.*, 2015).
290
291 **Results**
292 **Aphid infestation, more than nitrogen source, significantly alters leaf metabolite profile.**
293 *M. trunculata* leaves metabolites were analysed in four experimental conditions: nitrate-fed
294 plants (NI), nitrate-fed plants infested with aphids (NI_Amp), nitrogen-fixing symbiotic plants
295 (NFS) and nitrogen-fixing symbiotic plants infested with aphids (NFS_Amp) (Fig. S2). The
296 leave extracts were analysed by GC-MS, to obtain mainly primary metabolites, and by LC-MS,
297 to have access to the secondary metabolites. After GC-MS, 237 compounds were retained and
298 126 could be identified with confidence (Dataset S2), and among them the five main
299 quantitative compounds were sucrose, phosphate, malate, citrate, and glutamate (Fig. S3). By
300 LC-MS, 2627 compounds (in positive and negative modes) were obtained and 213 could be
301 identified (Dataset S2). We observed a strong effect of aphid infestation and nitrogen source in
302 the accumulation of metabolites and the hierarchical clustering of these compounds separates
303 both nitrogen source and plant infestation status (see Fig. 1A, S4 and S5 for GC-MS and Fig.
304 S6 for LC-MS, respectively). From the four experimental conditions, a total of 194 unique
305 metabolites, 126 from GC-MS and 69 from the LC-MS, were found significantly different

according in their accumulation (Dataset S2). Only sucrose was found significantly in both analysis. Among the 194 identified the most represented classes were flavonoids with 40 different compounds, followed by 30 carboxylic acids, 26 amino acids and 19 sugars (Fig. 1b).

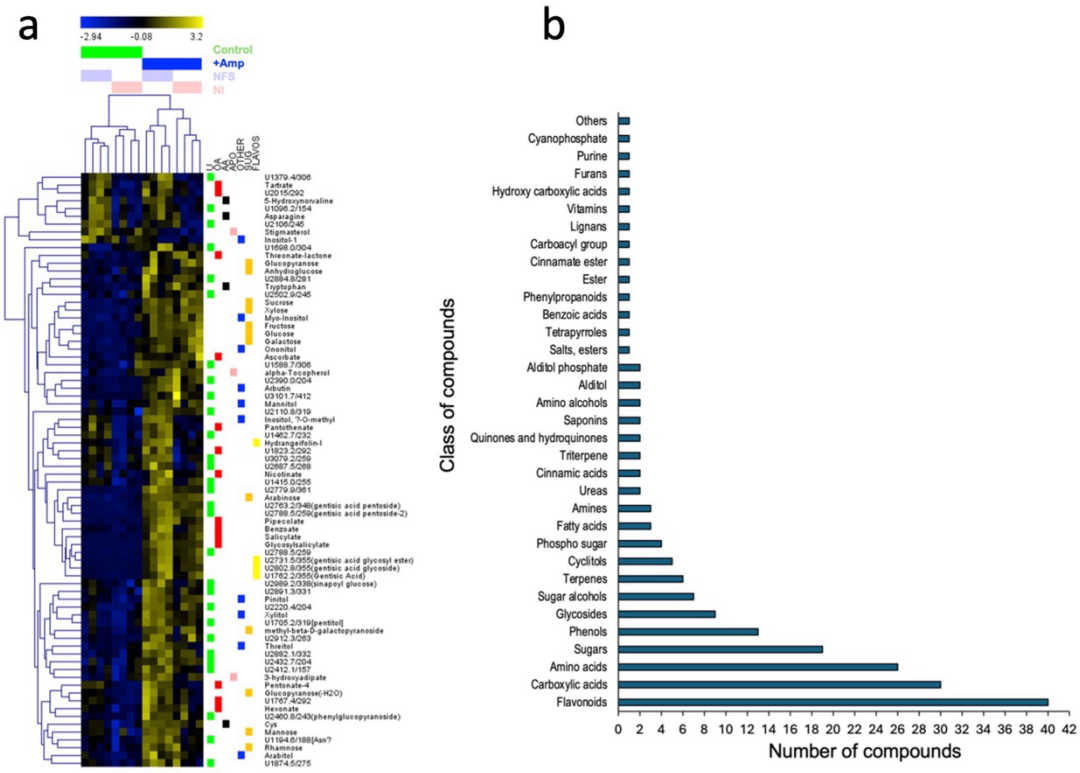


Fig. 1: Aphid infestation, more than nitrogen source, significantly alters leaf metabolite profile. a) Heatmap from GC-MS analysis showing unsupervised hierarchical clustering of compounds separated by both infestation and nitrogen source. b) Class abundance of metabolites from both GC-MS and LC-MS analyses.

We then identified statistical differences for the 194 unique metabolites between the four experimental conditions using multiple comparison tests (Fig 2; Dataset S3). 86 compounds were not found significatively accumulated in one specific condition, including many of the amino acids. 108 compounds were found to be differential accumulated in the different conditions. Amongst them, aphid infestation increased significantly the accumulation of 62 metabolites. 18 metabolites accumulated in control NFS conditions and in infected conditions, suggesting they are linked to a general infection/infestation response. 2 compounds are specifically found in NFS conditions and 19 compounds were significantly accumulated in NFS_Amp conditions.

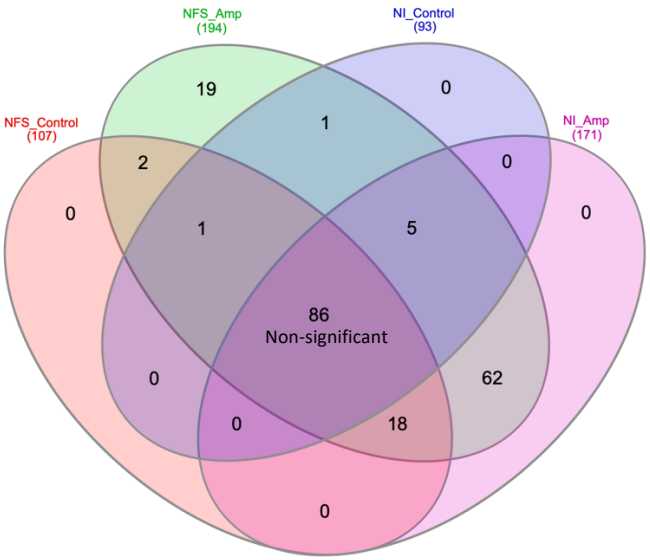


Fig. 2: Metabolite distribution according to nitrogen source and aphid infestation affects the metabolite profile of plants. Venn diagram showing distribution of statistically significant metabolites across the various experimental conditions. The name of the compounds and the statistic can be found in Dataset S3.

Aphid infestation triggers a greater accumulation of sugars than amino acids in both NFS and NI Plants.

Amino Acids

The majority of the amino acids (20) were not significantly accumulated in any specific condition. In contrast, tryptophan, cysteine, asparagine were significantly accumulated in NFS control, NFS and NI Amp. 5-Hydroxynorvaline was significantly accumulated in NFS control and NFS Amp compared to NI control and NI Amp. Finally, the putative asparagine or asparagine derivative (U1194.6/188[Asn?) is significantly more accumulated in NFS and NI Amp than in NFS control and NI control (Dataset S3).

Sugars

Among the 62 metabolites significantly accumulated under the aphid-infested conditions, glucose, sucrose and fructose contents were increased between three to five-fold compared to control conditions in leaves (Fig. 3). To investigate whether this leaf increase in sugar was associated with either a reduction in sugar transport from leaf to root or the results of the mobilization of leaf starch, sugar and starch contents were measured using biochemical assays in the leaves and roots of plants under the different conditions (Fig. 4). The increase in glucose, sucrose and fructose in infested leaves was confirmed (Fig. 4), but no significant accumulation

of starch in leaves and no change in root sugar concentration were observed, whatever the plant's growth conditions (Fig. 4), suggesting an alteration of the leaf sugar metabolism in response to aphid infestation.

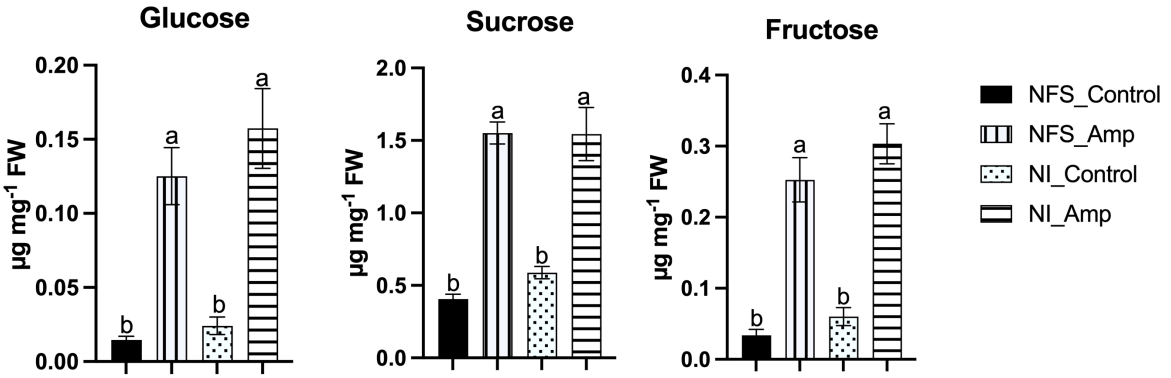


Fig. 3: Aphid infestation triggers accumulation of soluble sugars in both NFS and NI plant leaves. Bar graphs from GCMS analysis showing the significant accumulation of glucose sucrose and fructose in NFS and NI plants upon aphid infestation. Metabolites analysed and quantified in µg mg^{-1} of fresh weight (FW) of leaves of NFS and NI plants 12 days after infestation by the aphids (control = no aphid infestation). Mean ± SE (n=4): different letters indicate a $p \leq 0.05$ (Tukey's test).

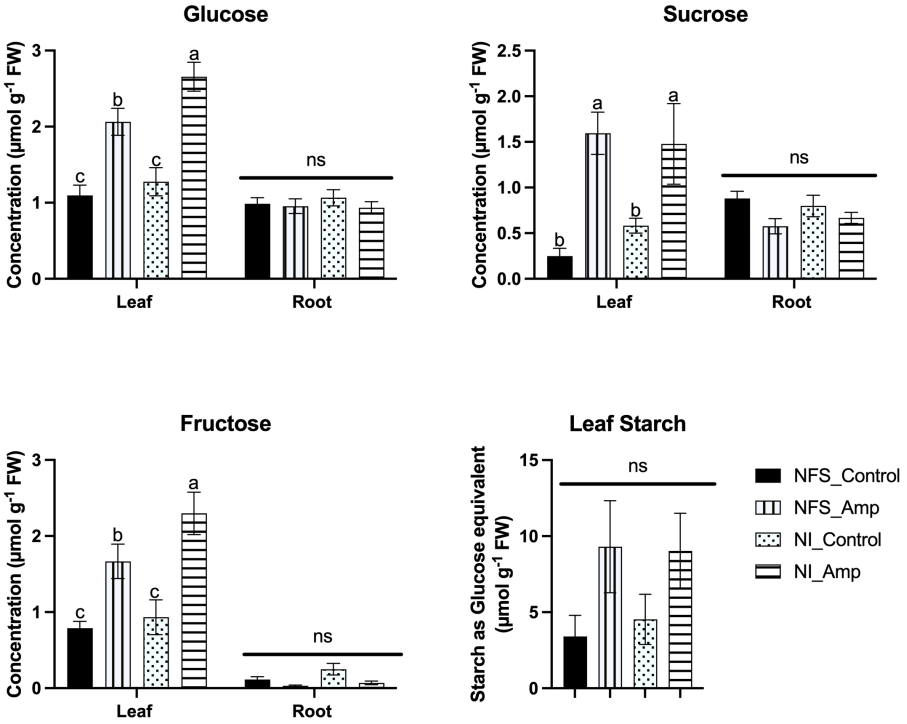
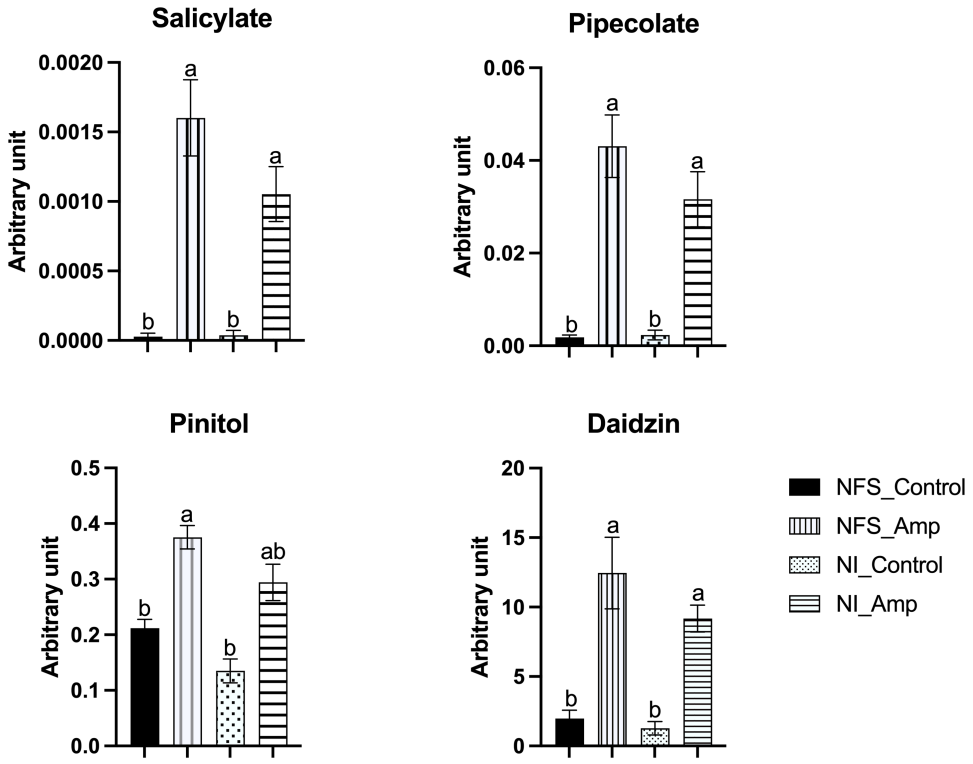


Fig. 4: Aphid infestation affects sugar concentration in leaves but not in roots. Biochemical analysis showing the significant accumulation of glucose, sucrose, and fructose in leaves of NFS and NI plants. No significant difference was found in roots upon aphid infestation. No statistical difference was observed in leaves starch, measured as glucose equivalent, between the different conditions. Metabolites analysed and quantified in µmol g^{-1} of fresh weight (FW) of leaves and roots from NFS and NI plants 12 days after infestation by the aphids (control indicates no aphids infestation). Mean± SE (n=4); different letters indicate a $p \leq 0.05$ (Tukey's test).

362 **Aphid infestation triggers accumulation of defence related metabolites in both NFS and**
363 **NI plants.**
364 Amongst the metabolites significantly accumulated during aphid infestation (Fig. S5; Dataset
365 S3), many are involved in the regulation of plant defence pathways such as salicylates,
366 pipecolate, an intermediate of the lysine catabolic pathway, pinitol, a cyclitol derived from
367 myo-inositol, as well as secondary metabolites with known defence activity such as the putative
368 daidzin, the glycoside form of the aglycone daidzein (Fig. 5).



369
370 **Fig. 5: Aphid infestation triggers accumulation of defence-related metabolites in both NFS and NI particularly from**
371 **the salicylic acid defence pathway.** Graphs show accumulation of metabolites expressed as arbitrary unit (in mg apigenin
372 equivalent mg^{-1}) of leaves fresh weight (FW) of NFS and NI plants 12 days after infestation by the aphids (control indicates
373 no aphids infestation). Mean± SE (n=4); different letters indicate a $p \leq 0.05$ (Tukey's test).
374
375 **Aphid infestation induces expression of genes involved in the flavonoid synthesis**
376 **pathway.**
377 In order to test whether pipecolate, pinitol, and diadzin accumulation resulted also from an
378 increase in expression of the gene(s) involved in their synthesis pathways, we have measured
379 the expression of *Chalcone isomerase (CHI)*, *flavonol synthase/ flavonone 3β-hydroxylase*
380 *(FLS/F3H)*, *hydroxyisoflavone-O-methyl transferase* (*HI4'O-MT)* and *Pterocarpan synthase*
381 (*PTS)*, *Phenylalanine Ammonia Lyase (PAL), SAR-DEFICIENT4 (SARD4)* and (*OEPB*) by
382 RT-qPCR (Table 1). Since we previously showed that *PR1,* a marker for SA defence pathway

383 and *PI*, a marker for jasmonic acid defence pathway were also differently induced in NFS and
384 NI plants after aphid infestation (Pandharikar *et al.*, 2020), we test these genes as the plant
385 condition controls. Here, RT-qPCR analysis showed that *PR1* expression increased 3.4 times
386 more in NI_Amp condition than in NFS_Amp condition. In contrast *PI* was 2.3 times more
387 expressed in NFS_Amp condition than in NI_Amp condition, in agreement with our previous
388 results. The analysis of the expression of genes involved in secondary metabolism pathway
389 showed that no significant change in *SARD4* and *PAL* expression was detected in aphid infected
390 compared to the control plants. *OEPB* expression was significantly induced in NI_Amp leaves
391 but not NSF_Amp leaves, although the amplitude of increase was similar. *CHI*, *FLS/F3H*,
392 *HI4'O-MT* and *PTS* showed significant induction upon aphid infestation in both NFS and NI
393 conditions; the induction being 2-fold for *FLS/F3H*, and reaching more than 50-fold for *PTS*
394 in NSF_Amp.
395 Taken together these results showed that the accumulation of secondary metabolites is at least
396 partially associated to an higher expression of enzymes involved in their synthesis pathways.
397
398 **Table 1: Gene expression analysis of NFS and NI plants.**

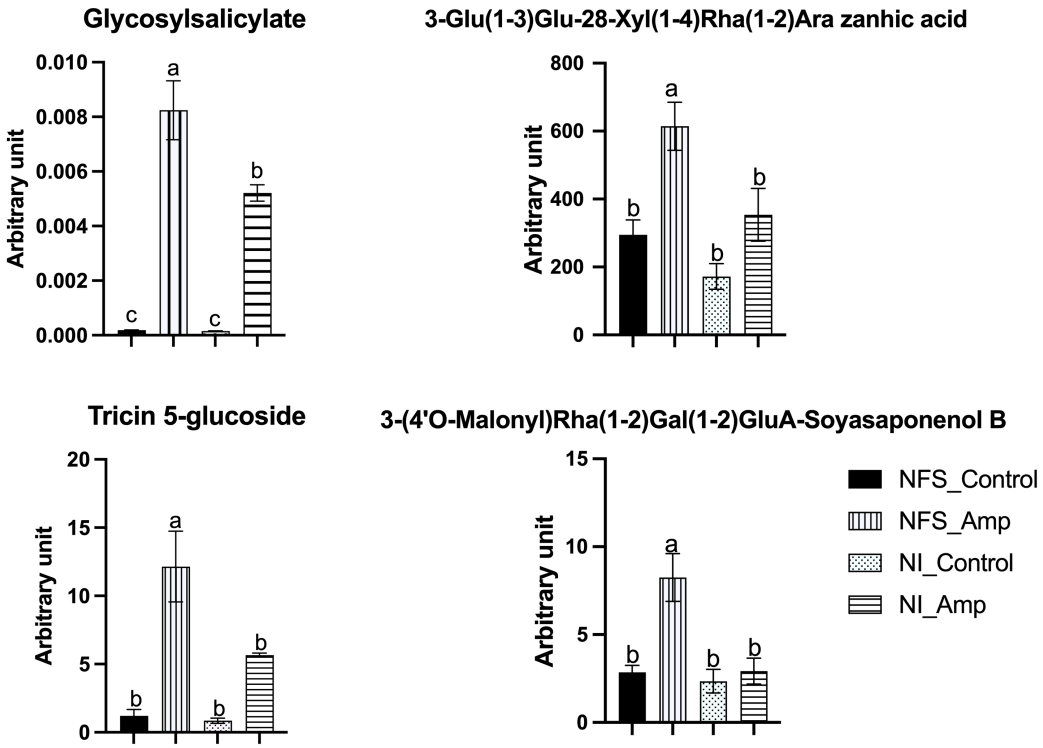
Gene	Pathway	Product	Fold Increase from Control (Mean ± SE)		*p* value	
			NFS_Amp/ NFS	NI_Amp/NI	NFS_Amp/ NFS	NI_Amp/NI
PR1	SA pathway	Salicylic acid	3.5 ± 0.32	11.99 ± 1.25	0.05	0.02
PI	JA pathway	Jasmonic acid	10.72 ± 0.26	4.73 ± 0.75	0.0006	0.002
PAL	Phenylpropanoid pathway	Phenylpropanoids	1.40 ± 0.02	1.35 ± 0.14	0.06	0.31
SARD4	Lysine Biosynthesis pathway	Pipecolate	1.16 ± 0.29	0.74 ± 0.09	0.73	0.88
OEPB	Pinitol Biosynthesis	Pinitol	1.75 ± 0.01	1.96 ± 0.31	0.07	0.01
FLS/F3H	Isoflavonoid pathway	Flavonoids	1.96 ± 0.05	2.5 ± 0.40	0.00002	0.01
HI4'O-MT	Isoflavonoid pathway	Formononetin	4.32 ± 0.10	3.37 ± 0.53	0.001	0.02
CHI	Isoflavonoid pathway	Flavonoids	4.58 ± 0.11	5.83 ± 0.93	0.006	0.004
PTS	Isoflavonoid pathway	Medicarpin	53.35 ± 1.27	24.66 ± 3.91	0.0001	0.001

399 Rescaled qPCR expression showing the level of gene induction upon aphid infestation in NFS and NI plants (NFS_Amp and
400 NI_Amp, respectively) compared to their control (NFS and NI, respectively). Data are expressed as mean ± standard error
401 (SE); t-test on all genes, $P \leq 0.05$, significant.
402

403 **NFS induces a differential defence mechanism upon aphid infestation**

404 Nineteen metabolites (10% of the identified metabolites) were significantly accumulated in
405 NSF_Amp plants compared to other conditions (Fig. S7; Dataset S3). Fifteen of them are

406 secondary metabolites from phenyl-propanoid (13) and terpene (2) synthesis pathways. For
407 example, amongst these metabolites, putative triterpenoid saponin 3-Glu(1-3)Glu-28-Xyl(1-
408 4)Rha(1-2)Ara zanhic acid is increased two-fold in NFS_Amp plants compared to NI-Amp
409 plants, putative 3-(4'O-Malonyl)Rha(1-2)Gal(1-2)GluA-Soyasaponenol B is 2.8 time more
410 accumulated in infested NFS-Amp plants than in the other three plant growth conditions,
411 flavonoid putative tricin 5-glucoside is about 2 times more accumulated in NFS-Amp
412 compared to NI-Amp while glycosylsalicylate is half a fold more in NFS-Amp than in NI-Amp
413 (Fig. 6).



414

415 **Fig. 6: NFS induces a differential defence mechanism upon aphid infestation.** Graph showing significantly accumulated
416 metabolites in NFS upon aphid infestation. Metabolites expressed as arbitrary unit (in mg apigenin equivalent mg^{-1}) of leaves
417 fresh weight (FW) of NFS and NI plants 12 days after infestation by aphids (control indicates no aphids infestation). Mean±
418 SE (n=4); different letters indicate a $p \leq 0.05$ (Tukey's test).

419

420 **Discussion**

421 There is a growing interest in better understanding the roles of symbiotic microbes in plant
422 defence and in a broader sense how this could influence plant interactions with bioagressors.
423 Beneficial microbes, in addition to enhancing plant growth and development have been
424 reported to induce defence reaction and confer protection on their host plants (Gopalakrishnan
425 *et al.*, 2015; Liu *et al.*, 2020; Benjamin *et al.*, 2022). Rhizobia are able to induce systemic

426 resistance in legumes such as pigeon pea against fusarial wilt as it was found that a combination
427 of rhizobial strains was better in inducing resistance (Dutta *et al.*, 2008).
428 Upon pea infection with the fungi Didymella, Turetschek and colleagues (2017) observed a
429 strong increase in sugars, sugar alcohols and glycolysis/TCA intermediates when studying
430 cultivars in symbiosis with rhizobia and mycorrhiza. They also observed the accumulation of
431 galactose, raffinose, maltose, threitol, melibiose, fructose and pyruvate in the pea Protecta
432 cultivar. Similar accumulation was also reported for amino acid pools, however, there was
433 significant depletion of phenylalanine in the pea cultivar Messire (Turetschek *et al.*, 2017).
434 Plants produce diverse primary and secondary metabolites that are involved in various
435 functions including development and defence. Previous work have demonstrated that aphids
436 are able to modify the overall metabolite profile of plants to establish feeding and that plants
437 may react by producing metabolites that have antifeeding or deterrent effects (Giordanengo *et*
438 *al.*, 2010; Kumar, 2017; Jakobs *et al.*, 2019; Shih *et al.*, 2023). Few metabolomic studies have
439 focused on pea aphids and legumes interaction (Sanchez-Arcos *et al.*, 2019) and none have
440 addressed the role of rhizobia bacteria on the plant metabolomic response during aphid attack.
441 Based on our previous observation that nitrogen-fixing symbiosis is detrimental to aphid fitness
442 in *M. truncatula* in association with potential changes in hormonal balance between SA and JA
443 (Pandharikar *et al.*, 2020), we performed an untargeted metabolomic analysis in *M. truncatula*
444 leaves 12 days after aphid infestation (chronic effect) in both NFS and NI plants.
445 In our study, among amino acids (Dataset S3), we observed an increase in asparagine content
446 in NFS plant leaves compared to NI control plants that could be associated with asparagine
447 formation in root nodule which account for 60% of nodule amino acid content (Sulieman &
448 Schulze, 2010). Similarly, cysteine was found to be more accumulated in symbiotic *Lotus*
449 *japonicus* plants as the nodule is an important source of reduced sulphur in the plant (Kalloniati
450 *et al.*, 2015). In contrast, tryptophan accumulation in the leaves of NFS plants has not yet been
451 observed, and a potential role in defence mechanism can be assumed (Hiruma *et al.*, 2013). In
452 NFS- and NI-infested plants, the increase of asparagine, cysteine and tryptophan content
453 compared to NI plants could be associated with the change of metabolism induced by aphids
454 for better nutritional content (Chiozza *et al.*, 2010; Tegeder, 2014) or by the plant through the
455 induction of defence reaction (Taylor & Ostrowsky, 2019). Citrate, fumarate, malate and
456 succinate accumulation were not significantly different among our four growth conditions,
457 suggesting that tricarboxylic acid (TCA) cycle is not changed by NFS or aphid infestation. In
458 contrast to the large number of metabolites that did accumulate differentially in the different
459 growth conditions, two (5-hydroxynorvaline and tartrate) were specifically accumulated in

460 NFS conditions. Whereas the significance of tartrate accumulation, which is and end point
461 product of the catabolism of ascorbic acid, is more difficult to analyse (Burbidge *et al.*, 2021),
462 unless it is used by the rhizobial bacteria (Ramachandran *et al.*, 2011), that of the 5-
463 hydroxynorvaline, a non-protein amino acid, may be related to its defensive functions against
464 insects (Huang *et al.*, 2011; Yan *et al.*, 2015). No metabolite was specifically more accumulated
465 in NI- and infested NI-conditions.
466 Our data show that the sugar metabolism is particularly affected by the aphid infestation (Fig.
467 3). Amongst the primary metabolites, 12 are sugars and represent 70% of the number of
468 primary metabolites significantly accumulated in infested plants (Dataset S3). In our
469 experimental conditions, we were interested by the sugar transport to root to feed the root
470 nodule involved in biological nitrogen fixation. Indeed, we previously showed that biological
471 nitrogen fixation is affected in the infested conditions (Pandharikar *et al.*, 2020) and we
472 hypothesized that the decrease in biological nitrogen fixation could be associated to a reduced
473 sugar transport from leaves to roots. However we did not observed a significant modification
474 in the accumulation of root glucose, fructose and sucrose between control and infested plants,
475 indicating that the modification of sugar metabolism is not associated with the sugar transport
476 to the root. We also observed that starch seemed more accumulated in infested leaves than in
477 control ones but this difference was not significant. In conclusion, the modification of sugar
478 metabolism does not seem to be associated to a modification of sugar storage through starch or
479 sugar transport to roots. Moreover, the modification of sugar metabolism by aphids seems not
480 dependent of the nitrogen source. The accumulation of sugars has been previously reported in
481 plant-aphid (Ponzio *et al.*, 2017) as well as in plant-pathogen interactions (Kanwar & Jha,
482 2019) as being involved in the coordination of plant defence signalling (Yamada & Mine,
483 2024), this perhaps could be an explanation for our observation.
484 A large number of significantly accumulated metabolites (62 metabolites, 31% of the total
485 metabolites) is present in NFS- and NI- infested plants compared to their control counterparts.
486 This results show that Medicago plants respond significantly to aphid infestation and that the
487 nitrogen source does not play a significant role in their accumulation. Amongst these secondary
488 metabolites, 72% are from 3 families, flavonoids (30), phenolics (11) and glycosides (4). These
489 different compounds are mainly associated with plant defence against pests such as biocide
490 activity (i.e. acacetin, chrysoeriol, daidzin), feeding deterring activity and defence signalling
491 activity (i.e. salicylate, pipecolate) (Stochmal *et al.*, 2001; Goławska *et al.*, 2012, 2024; Kim
492 *et al.*, 2022; Pawłowska & Stepczyńska, 2022). Pinitol has also been shown to participate to
493 the biological control of powdery mildew in cucumber (Chen *et al.*, 2014) and myoinositol

494 influences the plant bacterial colonization (O'Banion *et al.*, 2023). Thus, in parallel with
495 secondary metabolism, the modification of inositol metabolism in NFS plant may be involved
496 in the differential defence process observed in infested NFS-plants compared to compared to
497 infested NI-plants. The significant differential accumulation of 3-Glu(1-3)Glu-28-Xyl(1-
498 4)Rha(1-2)Ara zanhic acid and 3-(4'O-Malonyl)Rha(1-2)Gal(1-2)GluA-Soyasaponenol B, two
499 terpene molecules are also a marker of both the symbiotic state and the infection by aphids.
500 Finally, 3-(4'O-Malonyl)Rha(1-2)Gal(1-2)GluA-Soyasaponenol B maybe one of the most
501 interesting and intriguing metabolite in our experiment. This triterpenoid saponin is a defensive
502 compound against pathogenic microbes and herbivores (Osbourn, 1996; Kuzina *et al.*, 2009;
503 Szakiel *et al.*, 2011) and may act as feeding deterrents for plant specialist herbivores (Cui *et*
504 *al.*, 2019) causing a cytotoxic effect in hindgut and fat body of insects (Adel & Sammour,
505 2012). Production of triterpenoids have also been observed as an effect of rhizobia on pea seeds
506 infected with Didymella. Accumulation of seed terpenoid Pisumoside B was observed in
507 uninfected rhizobial treated seeds, meanwhile, Soyasapogenol C, Api_Dai_Kae_Flavon and
508 6-Hydroxyapigenin 7-[6"-(3-hydroxy-3-methylglutaryl)glucoside] were significantly
509 enhanced in infected rhizobial treated seeds in Protecta cultivar (Ranjbar Sistani *et al.*, 2017).
510 It may represent a very interesting biological marker of the symbiosis induced defence priming.
511 The induction of defence mechanisms is associated to reprograming of gene expression in
512 plants (Aerts *et al.*, 2022). For example reciprocal interactions between a chewing herbivore,
513 *Sitona lineatus* (pea leaf weevil) and *Pisum sativum* (pea) plants grown with or without rhizobia
514 (*Rhizobium leguminosarum* biovar. *viciae)* revealed that plants grown with rhizobia had
515 increased gene transcript expression associated with hormone-related defence (jasmonic acid,
516 ethylene, abscisic acid) as well as physical and antioxidant-related defence, which may explain
517 reduced feeding by *S. lineatus* (Basu *et al.*, 2022).
518 We have also looked at the expression of some genes involved in the secondary metabolism
519 pathway. As expected from previous analysis, *PR1* and *PI* are differentially expressed in
520 infested NFS plants and NI plants suggesting that different defence transcriptional
521 reprograming occurs in the two conditions.
522 Looking at the genes involved in secondary metabolism synthesis pathway, transcript
523 accumulation of *SARD4*, that encodes a key enzyme for pipecolic acid biosynthesis, and *PAL*,
524 the first enzyme of the phenylpropanoid pathway, is not modified by aphid infestation neither
525 in NFS-plants nor in NI-plants. This was surprising since *SARD4* was shown to be required
526 for the establishment of systemic acquired resistance to pathogen infection in Arabidopsis
527 (Ding *et al.*, 2016). However, *SARD4* deleted plants are still able to biosynthesize pipecolate,

528 this pathway may involve thus other enzyme(s). PAL is also a member of a multigenic family
529 and other members of this family may be induced. In contrast the isoflavonoid pathway genes
530 *CHI, FLS/F3H, HI4'O-MT* and *PTS* expression was significantly increased in infested plants:
531 while the induction of *CHI, FLS/F3H, HI4'O-MT* does not vary more than two fold in the two
532 infested growth conditions, the *PTS* expression is induced 53- and 25-fold times by aphids in
533 NFS and NI plants, respectively. *PTS* is involved in synthesis of pterocarpans that constitute
534 the second largest group of natural isoflavonoids and play an important role as phytoalexins.
535 In Medicago, the pterocarpans medicarpin was shown to protect the plant from the powdered
536 mildew *Erysiphe pisi* and activates the SA pathway (Gupta *et al.*, 2022). Medicarpin was also
537 shown to be accumulated in Medicago leaves upon long term or strong attack by pea aphids
538 (Stewart *et al.*, 2016) and also in response of infection with the fungal pathogen *Phoma*
539 *medicaginis* (Jasiński *et al.*, 2009) suggesting some large spectrum defensive role. In contrast,
540 medicarpin was shown to be an antagonists of nod gene expression necessary for rhizobia to
541 form their association with the plant roots (Zuanazzi *et al.*, 1998). Thus an increase in
542 medicarpin synthesis during aphid infestation could also explain in part the effect on the root
543 nodules previously observed (Pandharikar *et al.*, 2020). *OEPB*, a gene involved in pinitol
544 synthesis (Pupel *et al.*, 2019), was significantly induced in infested-NI plants and not in
545 infested-NFS plants. Pinitol has been shown to prolonged the pea aphid probing behaviour but
546 did not prevent them from feeding (Campbell & Binder, 1984; Kordan *et al.*, 2011). Pinitol has
547 also been involved in maintenance of the nodule osmotic balance during development (Poole
548 & Ledermann, 2022) and *Sinorhizobium meliloti*, may catabolize pinitol to form nodule
549 (Kennedy-Mendez, 2018). Thus why *OEPB* increases only in NI plant remains to be
550 understood.
551 In aphid infestation, signal transduction pathway is activated to induce related transcription
552 factors that regulate the expression of downstream disease resistance genes (Gao *et al.*, 2021).
553 Taken together, our results show that, in our conditions, aphids are able to trigger accumulation
554 of various metabolites and expression of defence genes responsible for SAR and SA defence
555 pathway as well as modify the metabolism of primary sugars in legumes. Under infestation by
556 pea aphids, legumes in symbiosis are able to produce a differential defence reaction by
557 activating the JA defence pathway and production of defence metabolites such as triterpenoid
558 saponins (soyasapogenol). Here, we have chosen one aphid line that develops well on the A17
559 *Medicago truncatula* cultivar used, however we know that the metabolic profiles of plants
560 infested with their native or non-native aphid host race could vary (Sanchez-Arcos *et al.*, 2019).
561 Meanwhile in these interactions the potential change induced by the presence of the rhizobium

562 is not questioned since the symbiotic status of the plant used is generally unknown. Thus our
563 results provide a foundation for the development and inclusion of symbiosis in such studies
564 and in a more applied role as a potential stimulant of plant defence against herbivores that may
565 be a new form of biocontrol in integrated pest management.
566
567 **Acknowledgements**
568 We are highly grateful to S. Tares-Amichot and L. Arthaud for help in aphids manipulation
569 and rearing and for M. Bosseno for help in Medicago plants seedling. GB is supported by a
570 doctoral fellowship from the department "Santé des Plantes et Environnement" of Institut
571 National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) and
572 the Université Côte d'Azur (UniCA). The IJPB benefits from the support of Saclay Plant
573 Sciences-SPS (ANR-17-EUR-0007). This work has benefited from the support of IJPB's Plant
574 Observatory platform PO-Chem. This work was supported by UniCA, the SPE department of
575 INRAE and the French Government (National Research Agency, ANR) through the
576 "Investments for the Future" programs LABEX SIGNALIFE ANR-11-LABX-0028-01 and
577 IDEX UCAJedi ANR-15-IDEX-01.
578
579 **Competing Interests**
580 None declared.
581
582 **Author contributions**
583 GB, PF, MP, and JLG conceived the ideas. GB, PF, MP, and JLG planned and designed the
584 research. SB and GC performed the metabolomics analysis and analysed the data. GB and MP
585 performed qPCR analysis. GB and RB performed sugar and starch analysis. GB analysed all
586 other data. GB wrote the manuscript with inputs from PF, MP, and JLG. All authors contributed
587 and agreed to the final revisions.
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Data Availability

The metabolomics data that support the findings of this study are available in MassIVE server with the reference number MSV000095254; http://doi.org/10.25345/C5CV4C35C. Datasets S1, S2 and S3 are available on Recherche Data Gouv server at https://doi.org/10.57745/PCDG8H.

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859 **Supporting Information**
860 Datasets available as: Benjamin, Goodluck, 2024, "Supplementary Data for Differential
861 induction of Medicago truncatula defence metabolites in response to rhizobial symbiosis and
862 pea aphid infestation", https://doi.org/10.57745/PCDG8H, Recherche Data Gouv, V1,
863 UNF:6:A0SbGGbxQocqa6JsmMuoMA== [fileUNF]
864 -Dataset S1: File containing qPCR Data (qPCR CT Data.xlsx)
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Supplementary Data for

Differential induction of *Medicago truncatula* defence metabolites in response to rhizobial symbiosis and pea aphid infestation

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Supplementary Data

Datasets available as: Benjamin, Goodluck, 2024, "Supplementary Data for Differential induction of Medicago truncatula defence metabolites in response to rhizobial symbiosis and pea aphid infestation", https://doi.org/10.57745/PCDG8H, Recherche Data Gouv, V1, UNF:6:A0SbGGbxQocqa6JsmMuoMA== [fileUNF]

Dataset S1: File containing qPCR Data (qPCR CT Data.xlsx)

Dataset S2: File containing GC-MS and LC-MS metabolomics data (Metabolomics_data.xlsx)

Dataset S3: File containing Significant accumulated metabolites analysed by Anova and Venn Analysis (Significant metabolites_Venn.xlsx)

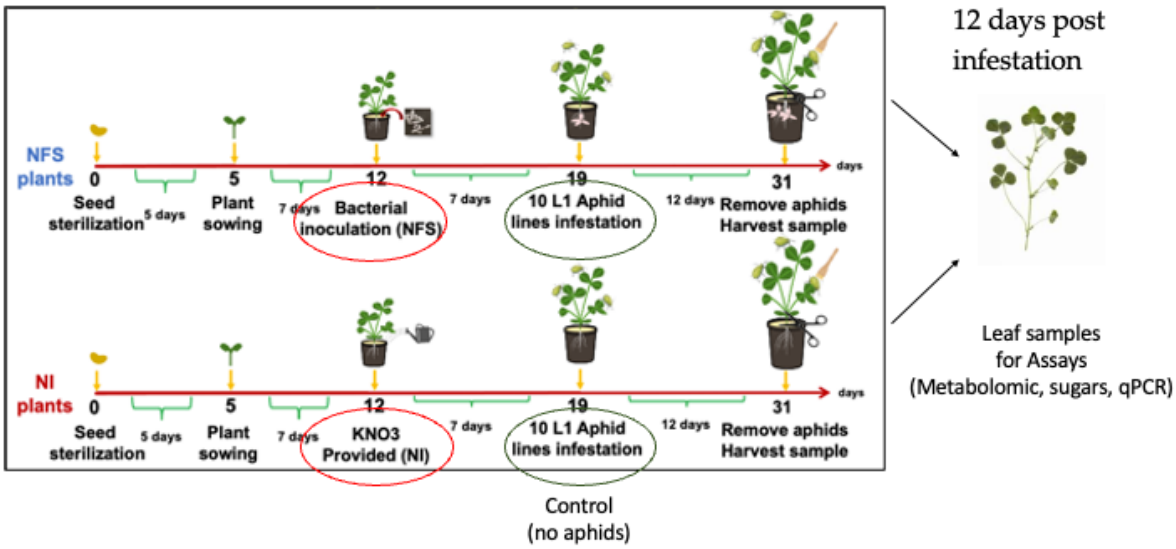

Fig. S1: Experimental setup. Diagram showing timeline leading up to assay with key date points for bacteria inoculation and aphid infestation marked up. Each pot contained six plants and 4 pots were made for each condition per biological replicate. Assay was performed using 4 biological replicates produced using the same timeline.

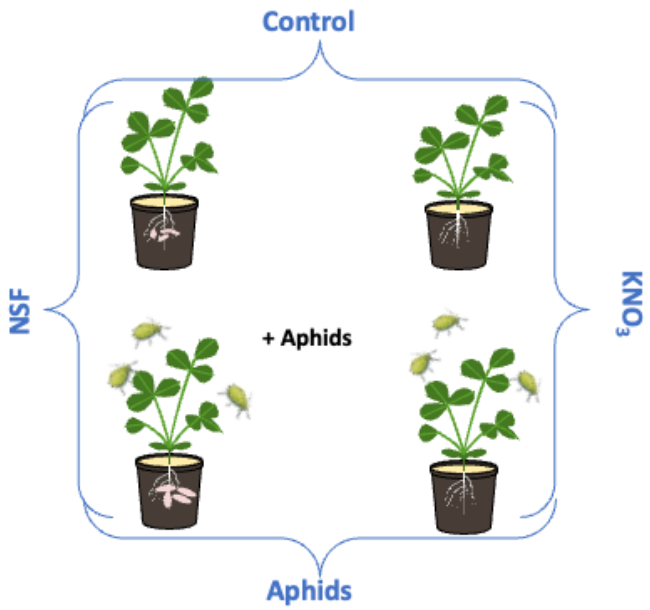

Fig. S2: Experiment treatment classification. Diagram showing the classification of studied treatments into 4 groups, NFS_Control, NFS_Amp, NI_Control (KNO_3) and NI_Amp (KNO_3).

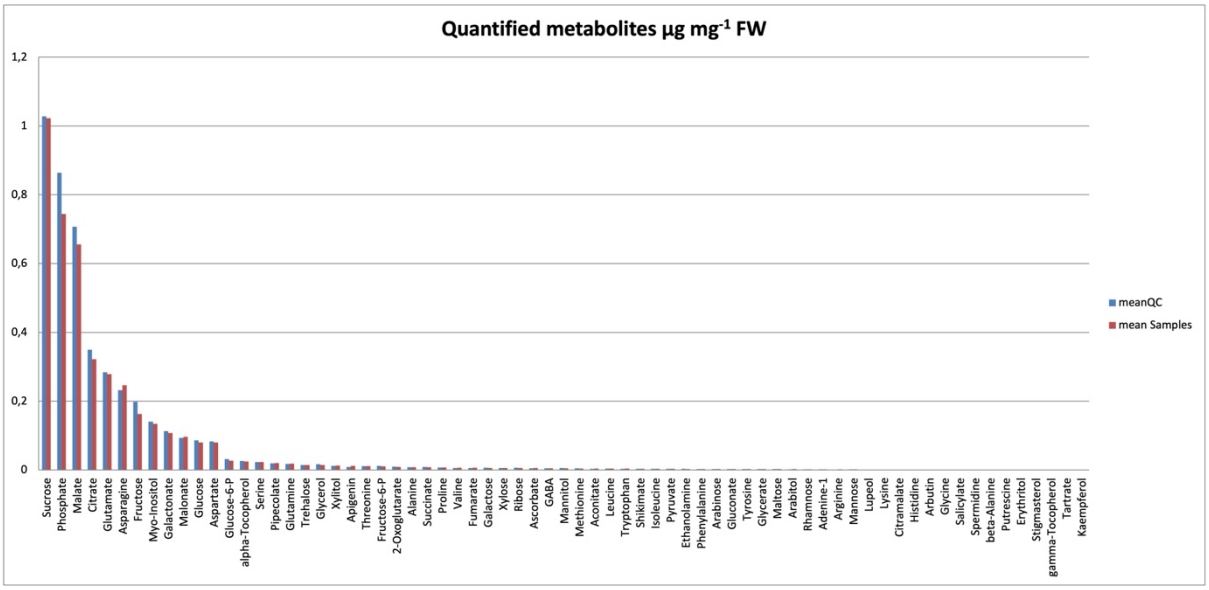

Fig. S3: Abundance of quantified metabolites. Graph of quantified metabolites from GC-MS analysis showing accuracy of abundance by comparing the mean obtained from the quality controls (QC, blue bars) and mean obtained from the samples (Samples, red bars).

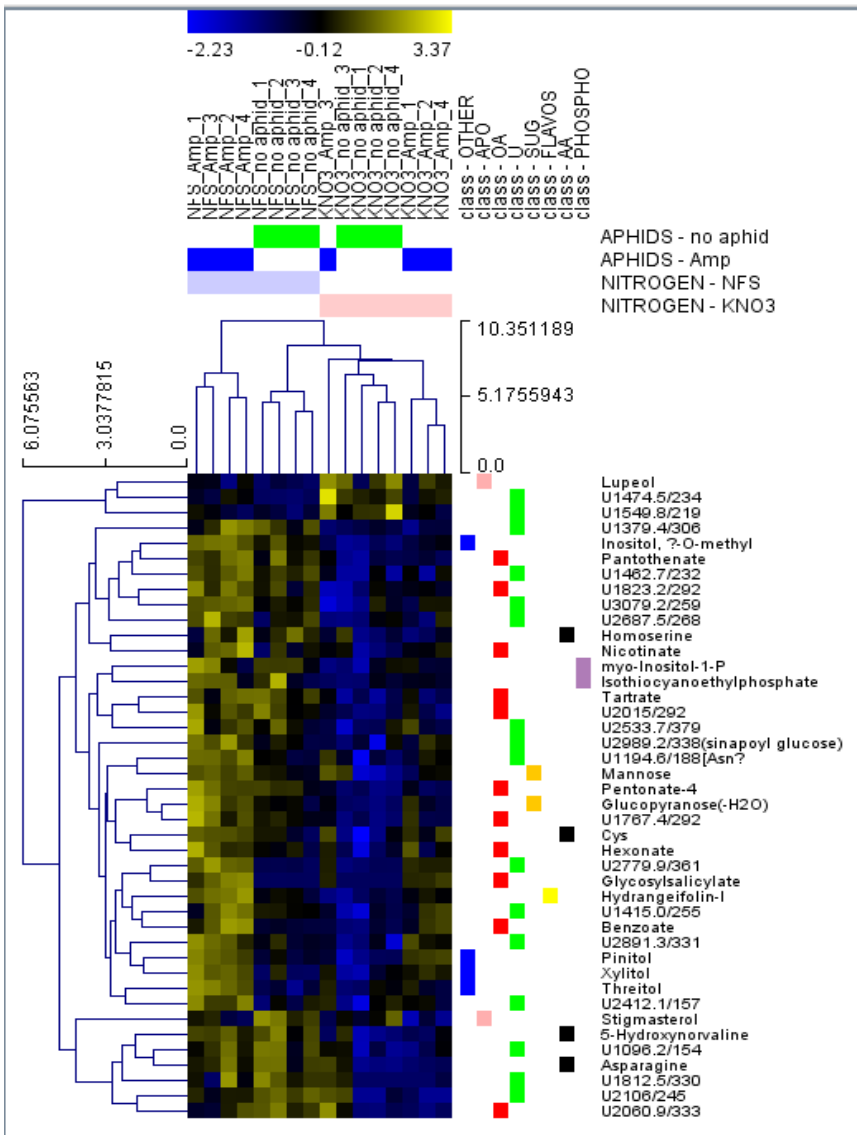

Fig. S4: Nitrogen source significantly modifies the leaf metabolite profile. Heatmap of 2-way Anova analysis from GC-MS showing unsupervised hierarchical clustering of compounds separated by nitrogen source. NFS = Nitrogen fixing symbiosis, KNO3 = Non inoculated, Amp = aphid infested, no aphids = Control.

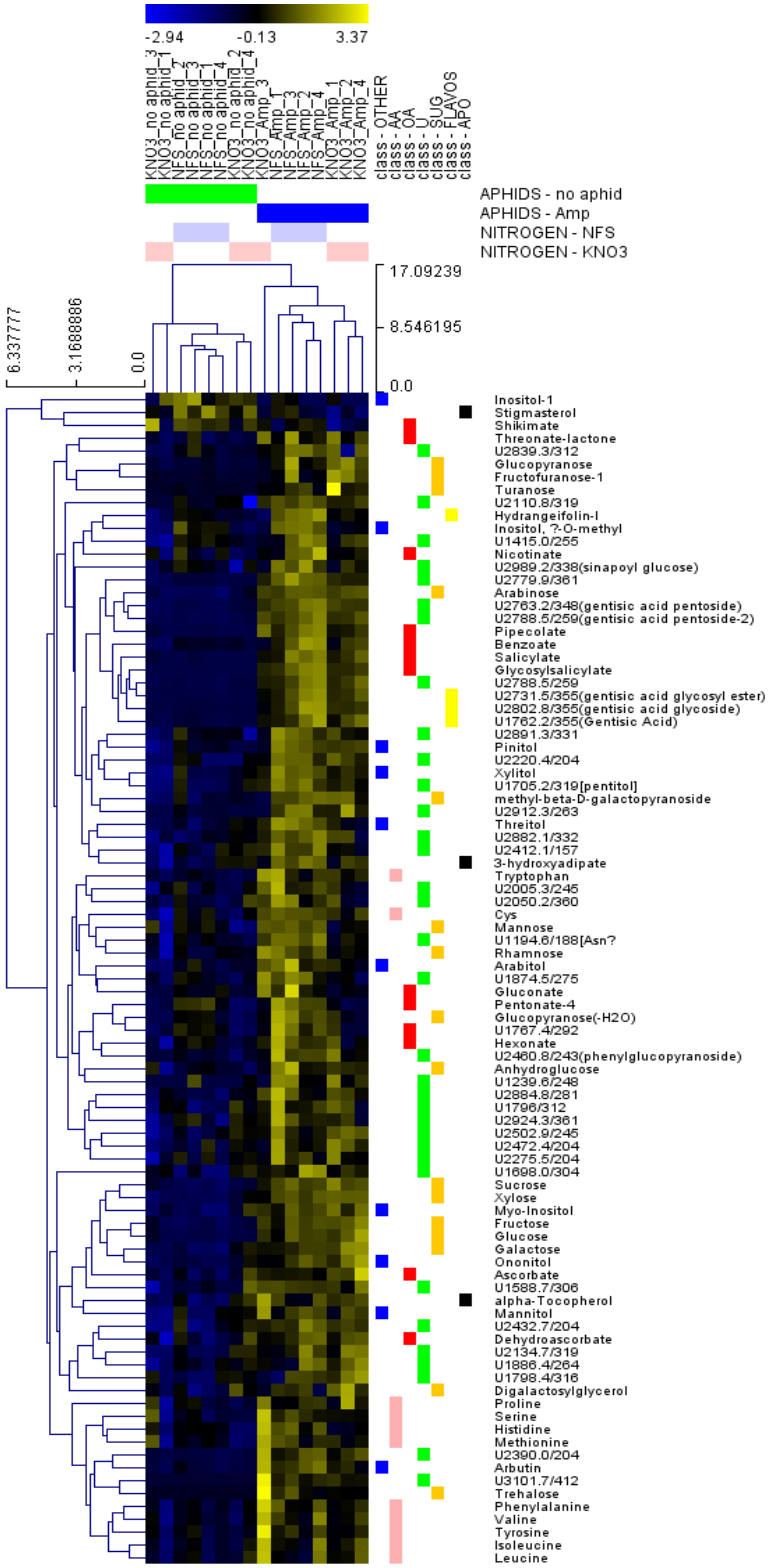

Fig. S5: Aphid infestation significantly modifies the leaf metabolite profile. Heatmap of 2-way Anova analysis from GC-MS showing unsupervised hierarchical clustering of compounds separated by aphid infestation. NFS = Nitrogen fixing symbiosis, KNO3 = Non inoculated, Amp = aphid infested, no aphids = Control.

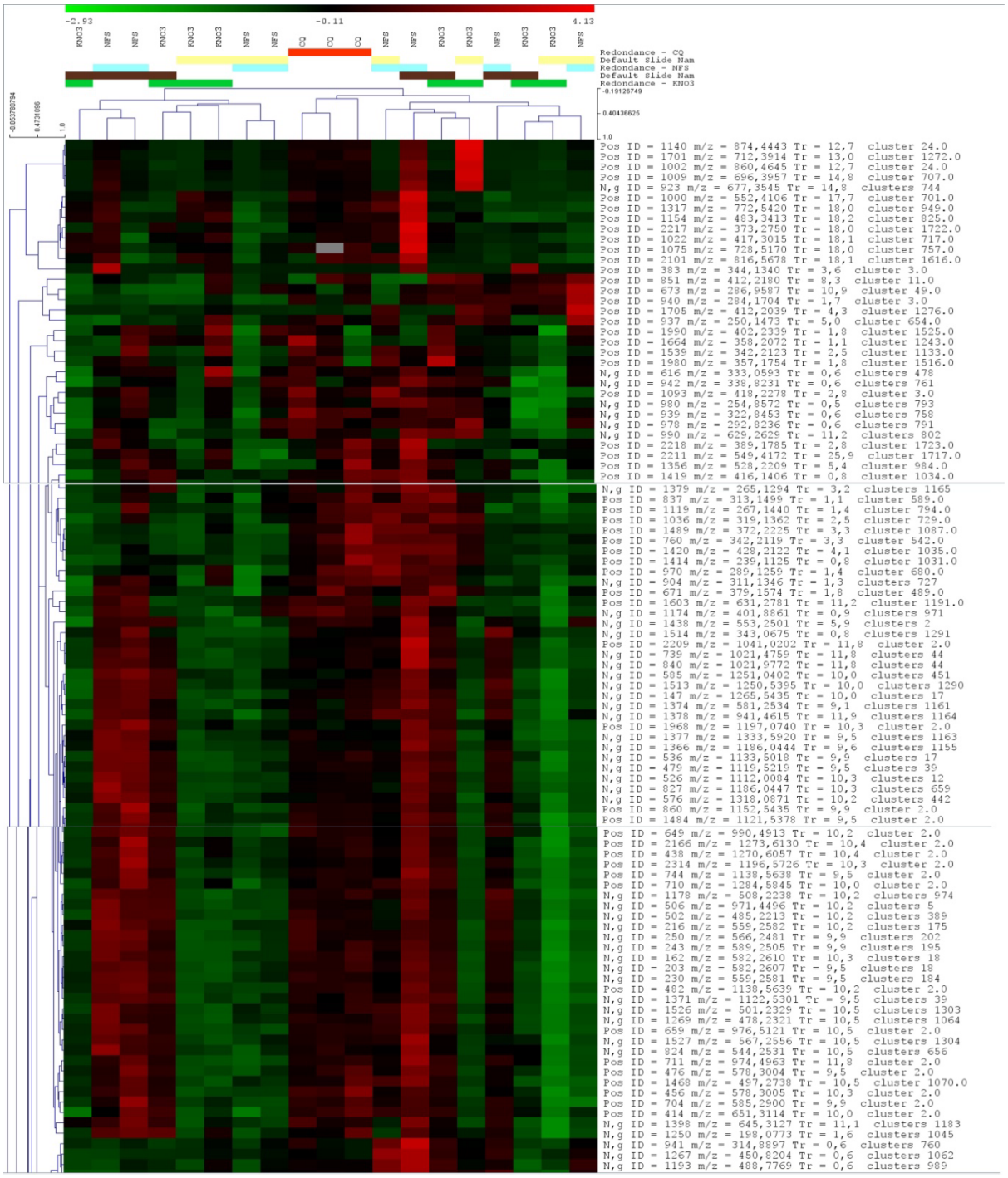

Fig. S6: Unsupervised clustering of LC-MS compounds. Heatmap showing the top 100 features from LC-MS analysis.

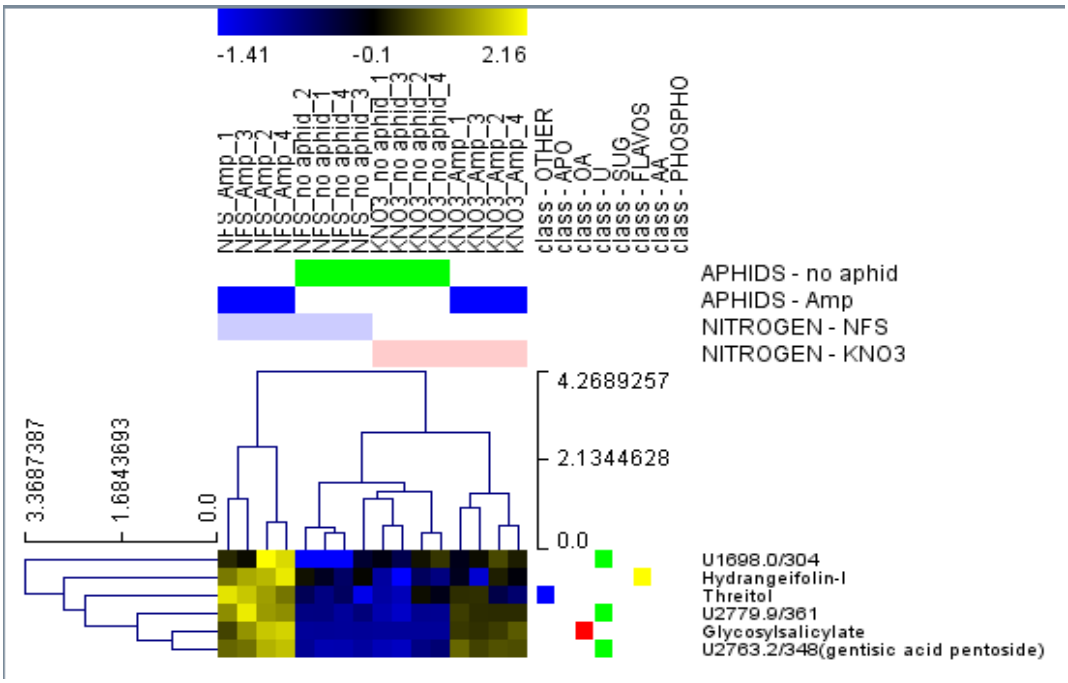

Fig. S7: NFS and Aphid infestation significantly modifies the leaf metabolite profile. Heatmap of 2-way Anova analysis from GC-MS showing unsupervised hierarchical clustering of compounds separated by the interaction of NFS and aphid infestation. NFS = Nitrogen fixing symbiosis, KNO3 = Non inoculated, Amp = aphid infested, no aphids = Control.

1093 **Table S1: List of genes and oligos sequences used for RT-qPCR analysis.**

Description	Name	Genomic ID	Forward primer	Reverse primer	References
Chalcone Isomerase	*CHI*	MtrunA17_Chr1g0213011	CCTGAAAAGGAGGCTGCACT	ACAGCGCTTAAGATCAGGGG	This work
Flavanol Synthase/Flavanone 3-Hydroxylase	*FLS/F3H*	MtrunA17_Chr3g0092531	CACAATTCACCAAAGAGATTGGGG	ATCCTCTGGCCTAGAAGGTGG	This work
Isoflavone 4'-O-methyltransferase	*HI4'O-MT*	MtrunA17_Chr4g0046341	GCAACAGGGGAGAGTTTTTGG	AGACTCCAAACCCTCGAAAACG	This work
D-pinitol Dehydrogenase	*OEPB*	MtrunA17_Chr6g0480011	GCACTCAGATGGGTGTACCA	GCACTCAGATGGGTGTACCA	This work
Phenylalanine Ammonia-lyase	*PAL*	MtrunA17_Chr1g0181091	AGCGCTTATGTTAAAGCCGC	AGCGCTTATGTTAAAGCCGC	This work
Pterocarpan Synthase 1	*PTS*	MtrunA17_Chr7g0259091	AAGGAGATGCCATTGTGGAG	CCTATTCGTACTATAGGTGAGAGTG	This work
SAR Deficient 4	*SARD4*	MtrunA17_Chr1g0202471	CCCCAATTCGCCAACACTAC	CAGGGAAATGGGTCACGAGT	This work
Pathogenesis Related Protein-1	*PR1*	MtrunA17_Chr2g0295371	TTCGGGTTGGATGTGCTAAG	GGTTGAAGCTCAATGGCACT	Pandharikar *et al*., 2020
Proteinase Inhibitor	*PI*	MtrunA17_Chr4g0014461	TGTGGTGCAATTCTTTCAGG	ATTTTGGGGTGAGGTGTTGA	Pandharikar *et al*., 2020
Housekeeping gene	*MtC27*	MtrunA17_Chr2g0295871	TGAGGGAGCAACCAAATACC	GCGAAAACCAAGCTACCATC	Del Guidice *et al*., 2011
Housekeeping gene	*a38*	MtrunA17_Chr4g0061551	TCGTGGTGGTGGTTATCAAA	TTCAGACCTTCCCATTGACA	Del Guidice *et al*., 2011

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