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Tomato response traits to pathogenic *Pseudomonas* species. Does nitrogen limitation matter?

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Abstract

Induced chemical defence is a cost-efficient protective strategy, whereby plants induce the biosynthesis of defence-related compounds only in the case of pest attack. Plant responses that are pathogen specific lower the cost of defence, compared to constitutive defence. As nitrogen availability (N) in the root zone is one of the levers mediating the concentration of

defence-related compounds in plants, we investigated its influence on response traits of tomato to two pathogenic bacteria, growing plants hydroponically at low or high N supply. Using two sets of plants for each level of N supply, we inoculated one leaf of one set of plants with *P. syringae*, and inoculated the stem of other set of plants with *P. corrugata*. Tomato response traits (growth, metabolites) were investigated one and twelve days after inoculation. In infected areas, *P. syringae* decreased carbohydrate concentrations whereas they were increased by *P. corrugata*. *P. syringae* mediated a redistribution of carbon within the phenylpropanoid pathway, regardless of N supply: phenolamides, especially caffeoylputrescine, were stimulated, impairing defence-related compounds such as chlorogenic acid. Inoculation of *P. syringae* produced strong and sustainable systemic responses. By contrast, inoculation of *P. corrugata* induced local and transient responses. The effects of pathogens on plant growth and leaf gas exchanges appeared to be independent of N supply. This work shows that the same genus of plant pathogens with different infection strategies can mediate contrasted plant responses.

Key words: Tomato-*Pseudomonas* pathogenic interactions; N limitation; carbohydrates; phenolics; elicitation; systemic response

Highlights

- Inoculation of *P. syringae* produced strong and sustainable systemic responses
- By contrast, inoculation of *P. corrugata* induced local and transient responses
- *P. syringae* mediated a redistribution of carbon within the phenylpropanoid pathway
- The study provides novel assessments of *P. corrugata*-tomato pathogenic interaction

Abbreviations

CO₂. carbon dioxide

DAI. days after inoculation

DAS. days after sowing

DW. dry weight

HN. high nitrogen

LN. low nitrogen

PPFD. photosynthetic photon flux density

[X]. concentration of X

1. Introduction

During the process of evolution, plants have acquired several natural defence mechanisms and specific barriers that act as protection against pests. This inherent protection, however, does not provide adequate defence in field crop production, as can be inferred from the regular practice of pesticide application required in conventional agriculture. In the current context of "green" agriculture, there is therefore an important challenge for agronomists to strengthen natural defence mechanisms of crop plants. Lowering the use of nitrogen (N) fertilisers and pesticides by crops forms a major part of the emerging concept of ecological intensification, which seeks to maximise primary production per unit area without compromising the ability of the system to sustain its productive capacity [1, 2]. It is well established that nitrogen availability influences the resistance of plants to pathogens [3-6] and that N limitation or deficiency enhances the concentration of certain defence-related compounds in plant tissues [7-11]. It is reasonable to postulate, therefore, that controlling N fertilisation may offer some practical agronomic leverage to strengthen plant defence by enhancing the composition and concentrations of defensive compounds in plant tissues. This approach accords with hypotheses that secondary metabolism is stimulated when nutrient resources in the environment are limited, thereby raising plant defence at the expense of plant growth [12]. For example, it has been reported that the susceptibility of tomato plants to *Pseudomonas syringae* decreases under low N fertilisation rates as compared to high rates [4] although the opposite effect has also been reported for other pathogens. This is the case for the susceptibility of tomato to *Botrytis cinerea*, which has been shown to decrease under increasing N fertilisation rates [3, 13, 14]. The effect of N on plant defence, therefore, appears to be pathogen-dependent, illustrating the complexity of plant-pathogen interactions. Plant defence involves a great diversity of mechanisms operating at molecular, cellular and whole-plant levels. Untangling this complexity of plant-enemy interactions is thus a major

challenge for research. Work in this area, is often focused on restricted aspects of plant defence. However, additional studies are required to identify the traits of plant response to pathogens. These include, for example, changes in plant architecture and in plant metabolism such as the balance between primary and secondary pathways. A greater knowledge of these traits would be expected to allow mediation of plant defence through crop/plant management or plant breeding.

In this study we investigated the response of adequately N-supplied vs N-limited tomato plants to two bacteria of the genus *Pseudomonas* (*P. syringae* pv. *tomato* and *P. corrugata*), that are capable of depressing crop yields of tomatoes produced either in open fields or greenhouses. *P. syringae* pv. *tomato* is the causal agent of bacterial speck (dark brown necrotic spots surrounded by a yellow halo) which preferentially attacks the leaves [15]. *P. corrugata* is the causal agent of pith necrosis (brown to black necrotic areas along the stem that may extend over several centimetres), which exclusively attacks the stems. On a longitudinal stem section, the pith appears brown, necrotic and ultimately decomposes [16, 17]. Very few studies have described the responses of both primary and secondary metabolism of tomato to *Pseudomonas* infection [18, 19]. Some studies have focused on the plant growth response and concentrations of primary metabolites (amino acids, carbohydrates) [15, 20]. Others have reported on defence mechanisms, including synthesis of defence metabolites [21-23]. As far as we are aware, nothing is known concerning changes in the metabolic profile of tomato tissues in response to the pathogen *P. corrugata*.

The main objective of this work was to assess the importance of N nutrition on chemical plant defence in the context of healthy versus pseudomonas-attacked tomato plants. In this respect we investigated the metabolic and ecophysiological changes of young hydroponically grown plants occurring during the first few hours and days following infection by these two *Pseudomonas* species. We tested the hypothesis that N limitation restricts the development of

symptoms by strengthening the tomato defensive responses. We also tested the hypothesis that tomato responses to *P. syringae* and to *P. corrugata* differ both as a consequence of elicitation of different metabolic pathways and differences in pathogen behaviour towards available resources (carbohydrates, nitrogen). The main traits monitored included net CO₂ assimilation rate, non-structural carbohydrates, and organ concentrations of phenolic compounds and tomatine.

The specialized metabolites targeted correspond to two major metabolic pathways - the ubiquitous phenylpropanoid pathway derived from phenylalanine (rutin, chlorogenic acid, kaempferol rutinoside, caffeoylputrescine...) and the glycoalkaloid pathway, which typically produces tomatine in tomato. All these metabolites are present in tomato [11] where they are considered as putative defence-related compounds [23, 24] and their concentrations are mediated by N availability [7, 9, 11].

We investigated these plant traits 24 h and 12 days after infection, in order to identify short-term responses, to evaluate their sustainability and eventually to assess longer-term processes. We considered that after 12 days there was a settlement in the reaction cascade following infection, including impacts on the carbon economy (C assimilation, regulation of source-sink interaction, partitioning of C to reserve compounds, [25]), on the concentration of specific metabolites elicited and probably on the dry matter partitioning.

2. Materials and methods

2.1 Plant material, growth conditions and experimental design

Tomato plants *Solanum lycopersicum* L. cv Better Bush (Park Seed Co., Hodges, SC, USA - resistant to Verticillium wilt and Fusarium wilt) were raised on a hydroponic system set up in

a growth chamber maintained at 25°C and 85% humidity. Germinating seeds were retained in the dark for the first three days then illuminated with a 16h photoperiod at 60% humidity.

Ten days after sowing (DAS), seedlings were transferred to two independent Nutrient Film Technique (NFT) systems set up in twin growth chambers (16h photoperiod, 25°C in air and nutrient solutions, 60% air humidity). Nineteen DAS (i.e. seven days before *Pseudomonas* inoculation), the ambient conditions were changed to 21°C and 90% humidity. All temperature sensors were calibrated in a water bath with 0.01°C precision (Haake model C35/F6, Karlsruhe, Germany).

Two contrasting N nutrition regimes were imposed. Half of the plants were fed with a N supply previously shown to be non-limiting for tomato growth [10, 24, 26] and presently referred to as "High N" (HN). This consisted of a complete nutrient solution described below, with N provided as NO_3^- at a concentration continuously regulated at 1.5 mM N. The second regime, previously shown to limit plant growth [9, 27] and referred to as "Low N" (LN), contained 0.15 mM N at the start of the experiment. It was adjusted every day using an automatic system that limited the daily N uptake of the LN plants to only 30% of that of HN plants [10, 24, 28]. The nitrate uptake rate calculations followed the unbiased procedure described by Adamowicz and Le Bot [29].

The complete HN nutrient solution was prepared with demineralised water and analytically pure salts at the following concentrations: KNO_3 (0.6 mM), KH_2PO_4 (1 mM), K_2SO_4 (0.7 mM), $\text{Ca}(\text{NO}_3)_2$ (0.3 mM), CaSO_4 (3.2 mM), $\text{Mg}(\text{NO}_3)_2$ (0.15 mM) and MgSO_4 (1.4 mM). The LN solution at 0.15 mM NO_3^- was composed of KNO_3 (0.06 mM), KH_2PO_4 (1 mM), K_2SO_4 (0.97 mM), $\text{Ca}(\text{NO}_3)_2$ (0.03 mM), CaSO_4 (3.47 mM), $\text{Mg}(\text{NO}_3)_2$ (0.13 mM) and MgSO_4 (1.49 mM). Sulphate was chosen as the compensating anion for nitrate variation so that in both solutions the concentrations of cations and H_2PO_4^- were the same and the anionic charge $[\text{NO}_3^-] + 2 \times [\text{SO}_4^{2-}]$ remained constant and equal to 12 mM.

Micronutrients were provided as a "ready to use" commercial mixture of Kanieltra (Hydro Azote, France) formula 6 Fe (0.1 l m^{-3}) and EDTA-Fe ($43 \text{ }\mu\text{M}$). Solution volumes, pH and NO_3^- concentration were measured, monitored and regulated automatically by the Totomatix system [28]. Nitrate adjustments were made by automatic additions of 1mM stock solution of NO_3 containing (in mol l^{-1}) 0.408 KNO_3 , 0.204 $\text{Ca}(\text{NO}_3)_2$ and 0.092 $\text{Mg}(\text{NO}_3)_2$ [28].

The experimental design comprised six randomised blocks equally distributed between the twin growth rooms. In each block the plants were fed with the complete nutrient solutions containing the HN and the LN regimes. For each N regime, the plants were inoculated with either water (as a mock inoculation) or a suspension of the bacterium (*P. syringae* or *P. corrugata*).

2.2 Plant inoculation procedure using pathogenic *Pseudomonas*

We used strain DC3000 of *P. syringae*, initially isolated in 1960 in Guernsey [30] and strain CFBP 5307 of *P. corrugata*, initially isolated in 1980 in France and kindly provided by Dr. Cindy Morris (INRA, Avignon).

The *P. syringae* complex is composed of numerous genetic lineages of strains from both agricultural and environmental habitats (including habitats closely linked to the water cycle). DC3000 strain is part of Phylogroup 1 which contains many isolates from diseased plants [31].

P. corrugata has the capacity to adapt to a large diversity of habitats (soil, plants). This bacterium is pathogenic on tomato (pith necrosis) but not of other plants such alfalfa [32]. It is more closely related to *Pseudomonas fluorescens* and it is outside the *P. syringae* species complex [33].

Inoculations were performed 26 DAS, either on the leaves or on the stems, depending on the pathogen. Inoculations of *P. syringae* consisted in infiltrating 100 μl aliquots of bacterial

suspension within the terminal leaflet of the fourth leaf (L4 - numbered from the cotyledon leaves) with a needleless syringe. Mock inoculations were carried out similarly with sterilised ultrapure water on control plants. The contours of infiltrated areas were traced with a felt pen. Inoculations with *P. corrugata* consisted in injecting with 10 µl aliquots of a bacterial suspension into the internode between leaves L4 and L5. The inoculum was set at the centre of the stem, using a 20 µl pipette tip. The same procedure was applied to control plants with the same volume of sterilised ultrapure water.

Both bacterial inocula (at a final concentration of approximately 10^8 cells ml⁻¹) were prepared from 48h cultures on KBC medium at 22-25°C [34]. We deliberately applied a high-titer inoculation (as made in other studies, for instance [35]), in order to maximize the chances of induction of a defence reaction from the plant, although such a high dose does not always induce a response ([30, 36]). Our objective was not to assess the evolution of the symptoms but to induce the hypersensitive-like reaction allowing ecophysiological and phytochemical investigations of plant responses.

2.3 Net CO₂ exchange rates

The CO₂ exchange rates (CER) were measured 27 DAS (*i.e.* one day after inoculation, DAI) on leaves of plants, which were later to be sampled at 38 DAS, using a portable photosynthesis system (Li-Cor 6400, Li-Cor, Lincoln Nebraska, USA) equipped with a LED light source (6400-02B red/blue diodes, Li-Cor, Lincoln Nebraska, USA). Temperature, [CO₂] and humidity were set at growth chamber ambience. Each measurement comprised two steps. Firstly, the LED illuminator was switched on and adjusted at the growth chamber PPFD (190 µmol photons m⁻² s⁻¹) in order to measure net photosynthesis. Secondly, the illuminator was switched off in order to measure respiration in the dark. The measurements were made on the terminal leaflet of the fourth true leaf (L4) using 6 plants per treatment. For

plants inoculated at leaf L4 with *P. syringae*, measurements were made on tissue close to (but not including) the inoculated area.

2.4 Plant sampling and sample preparation

To minimise the risks of mechanical stress and putative elicitation of metabolic pathways, plants were handled with caution throughout the experiment. Plant samples were collected 27 and 38 DAS (i.e. 24 hours and 12 days after inoculation, DAI). The inoculated organs and those adjacent (not in contact with *Pseudomonas*) were sampled separately to assess whether the chemical responses to the pathogen were localised or systemic. For plants inoculated with *P. syringae* and for the water-inoculated control plants, the inoculated leaf (L4) was sampled separately. The two adjacent leaves (L3 and L5) were combined and stems were also sampled. For plants inoculated with *P. corrugata* and the corresponding control plants, the inoculated internode was sampled separately and the two adjacent leaves (L4 and L5) were combined. In all cases, the remaining leaves and stems were pooled for shoot biomass determination. The whole inoculated organ (L4, internode) was sampled for analyses, the inoculated area representing only a small fraction of the whole organ (see Fig. 2).

At sampling dates, the leaf area of each plant was measured (Li-3100 C area meter, Li-Cor, Lincoln Nebraska, USA). The sampled organs were immediately frozen in liquid nitrogen, then stored at -20°C until freeze-drying. The dry samples were weighed, ground to a fine powder and further stored at -20°C.

2.5 Assessment of plant reaction

At the second sampling (12 DAI and 38 DAS), photographs were taken of the inoculated and mock-inoculated leaves and stems (cut in half lengthwise) in order to assess whether a hypersensitive-like response or an invasive infection had been established (Fig. 2 a & b).

2.6 Chemical analyses

2.6.1 Non-structural carbohydrates

Soluble carbohydrates were extracted (10 mg dry weight (DW) / 1 ml 80% ethanol) for 30 min at 80°C and centrifuged (10 min, 12100 g). This operation was repeated twice and the supernatant was collected and evaporated to dryness (Speedvac, Savant, USA). The residue was dissolved in 1.5 ml of ultrapure water. About 10 mg of polyvinylpolypyrrolidone were added to the extract and blended every 5 min for 15 min at 4°C in order to eliminate phenols and proteins. After centrifugation at 16100 g for 10 min at 4°C, the soluble proteins were eliminated by adding 450 µl of Courtonne reagent. After mixing, the extract was allowed to stand for 5 min at 4°C. A spatula tip of Na₂HPO₄ (*c.a.* 4 to 5 mg) was added to precipitate lead in excess. After centrifugation (10 min, 7500 g), the extract was filtered with a 0.22 µm mesh and frozen until analysis [37].

An internal standard of rhamnose solution (20 µl at 10 mg l⁻¹, Sigma, Saint Quentin Fallavier, France) was added to 10-fold diluted samples. Glucose, fructose and sucrose were separated and detected on a HPLC-MS (LTQ-MS, Thermo Scientific, San Jose, CA, USA). Separation was performed on a Polyamine II column (250 × 4.6 mm, YMC, Dinslaken, Germany) fitted with a guard column (7.5 × 4.6 mm, YMC, Dinslaken, Germany) following the protocol of Ricochon *et al.* [38].

The pellet from the extraction of soluble carbohydrates was vacuum dried for 10 min, dissolved in 0.5 or 1 ml of 0.02N NaOH (depending on the considered organ), autoclaved and centrifuged for 2 min at 12600 g. Starch was determined enzymatically on 100 to 400 µl of samples depending on expected concentration [39] (Enzytec Starch Ref. 1002942, R-Biopharm, Darmstadt, Germany)

2.6.2 Phenolics and tomatine

The extraction procedure was performed on 20 mg of dry powder and followed by separation and quantification of soluble phenolics as described in Royer *et al.* [11].

Phenolics contained in the infected tissues were separated on an U-HPLC system (Shimadzu, Japan) equipped with a binary solvent delivery pump and a diode array detector. Three microliters of extract were separated on a C18 Zorbax Eclipse Plus (150 × 2.1 mm, 1.8µm) column (Agilent, USA) by using a gradient elution from 1 to 50% MeOH 0.1% formic acid (FA) in 7.1 min, then 99% MeOH 0.1% FA in 0.8 min with a flow rate of 430 µl min⁻¹. The column was rinsed for 2 min with 99% MeOH 0.1% FA and re-equilibrated to the initial conditions for 2 min prior to the next run. Phenolic quantification was based on the area under peak determined at 320 nm and expressed relative to calibration curves with chlorogenic acid, caffeic acid, ferulic acid, caffeoylputrescine, rutin and kaempferol rutinoside (Sigma, Saint Quentin Fallavier, France).

Concentrations of chlorogenic acid, rutin and kaempferol rutinoside in the tissue adjacent to the infection site were measured using HPLC (model LC-20AD, Shimadzu Corp., Kyoto, Japan) on a RP-18 column (4 × 250 mm, 5 µm, Merck, Darmstadt, Germany) fitted with a guard column (Lichrospher RP - 18.5 µm, Merck). The mobile phase consisted of 0.1% formic acid in ultrapure water (A solvent) and 0.1% formic acid in methanol (B solvent). Detection was performed with a diode array detector (200-400 nm) and quantification from peak area was made on the basis of standard curves of rutin, chlorogenic acid and kaempferol-rutinoside (Sigma, Saint Quentin Fallavier, France). The presence of co-eluting peaks made it difficult to quantify caffeoylputrescine. This problem was resolved using an MS detector (LTQ Orbitrap, Thermofisher Scientific, San José, CA, USA) operating in electrospray mode (ESI). Caffeoylputrescine was quantified by following the amount of the

[M+H]⁺ peak at m/z 251 and using a range of known concentrations of caffeoylputrescine standard (provided by Dr. Werck-Reichhart from IBMP, Strasbourg).

Tomatine from 2 μ l samples previously diluted 50-fold in 70% MeOH with solanine as internal standard (50 μ M, Extrasynthese) was separated and detected on an HPLC-MS (LTQ-MS, Thermo Scientific, San Jose, CA, USA) with the same settings as those in Royer *et al.* [11].

2.7 Data analysis

All data were subjected to mixed two-way analyses of variance ([N] and *Pseudomonas* inoculation as fixed factors and blocks as random) using the lm procedure in R software (R project for Statistical computing, <http://www.r-project.org>). The statistical significance was set at $p < 0.05$ and denoted * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Normal Q-Q plots and correlation between variance and means were examined to assess the data distribution and homoscedasticity. When necessary, the data were Log or power transformed.

3. Results

The results described in detail below consider the effects of *Pseudomonas* infection (either *P. syringae* or *P. corrugata*) of tomato plants supplied with adequate (1.5 mM, HN) versus low N (0.15 mM, LN) nitrate-N supply. The experiment compares mock inoculated (i.e. control) with bacteria-inoculated plants. The data characterise these effects on stems and leaves sampled outside (adjacent organs) or within the inoculated zone of tomato plants harvested 1 or 12 days after inoculation (DAI).

The development of necrotic areas induced by both *Pseudomonas* species indicated the development of a reaction by the plant. Twelve DAI, necrotic areas typical of a hypersensitive-like reaction on the leaves inoculated with *P. syringae* (Fig. 1a). On plants

inoculated with *P. corrugata* in their stem, necrotic areas expanded internally in the pith around the inoculation point. Mock-inoculated control stems developed small necrotic areas around the point of water inoculation limited to the zone penetrated by the micropipette tip (Fig. 1b).

3.1 Plant growth and CO₂ exchange rates

Over the experimental period (1-12 DAI), shoot dry weights increased 4-6 fold (Fig. 2a,b). LN treatment reduced growth rapidly and consistently with time. Shoot dry weight was roughly halved in the LN plants compared to the HN plants (1 DAI: 1.5g for LN vs. 2.5g for HN; 12 DAI: 5g for LN vs. 15g for HN ***). No difference was observed between the two regimes of N nutrition on net photosynthesis (Fig. 2c) or raw photosynthesis (data not shown). Low N fertilisation, however, significantly reduced leaf respiration rate in the dark (Fig. 2d). There was no visual evidence of N deficiency in plants low in N supply during the experiment.

Infection with *P. syringae* significantly restricted shoot dry weight (12 DAI *, Fig. 2a), net photosynthesis (***, Fig. 2c) but it stimulated leaf respiration rate in the dark (**, Fig. 2d), and reduced area of infected leaves (Fig. 1e, 1 DAI ** and 12 DAI **). By contrast, *P. corrugata* infection produced no significant effect on shoot dry weight (Fig. 2b), net photosynthesis (Fig. 2c) or leaf respiration rate in the dark (Fig. 2d), or adjacent leaf area (Fig. 2f).

Infection with *P. syringae* did not affect the leaf area adjacent to the inoculation point (Fig. 2g).

3.2 Metabolite concentrations in infected organs

3.2.1 Carbohydrates

Compared to mock inoculation, the presence of *P. syringae* decreased sharply both the glucose and fructose concentrations of inoculated leaves 1 DAI (***, Fig. 3a) and 12 DAI (**, Fig. 3b), the effect being more pronounced 1 DAI (5-13 fold) than 12 DAI (2-5 fold). One DAI, low N nutrition increased fructose concentration in mock inoculated but not infected leaves (*, Fig. 3a). One DAI, *P. syringae* had no significant effect on starch concentration (Fig. 3a) but 12 DAI its value decreased fourfold in response to inoculation, regardless of N treatment (***, Fig. 3b). Sucrose concentration was never affected by *P. syringae* (Fig. 3a,b).

One DAI, *P. corrugata* increased the stem concentrations of glucose (1.8-2.2 fold, ***), fructose (1.5-2 fold, **) and sucrose (1.1-1.3 fold, *), as compared to mock inoculated stems (Fig. 3c). Twelve DAI these concentration differences were no longer present between control and infected plants (Fig. 3d). Starch concentration in stems was low and not responsive to *P. corrugata* inoculation (Fig. 3c,d).

3.2.2 Defence-related compounds

According to Larbat *et al.*[24], the main phenolics found in healthy tomato leaf and stem extracts are isomers of caffeoyl hexaric acid (CHA1-5), chlorogenic acid (the main form CA and the minor isomers CA_a and b), quercetin derivatives (Rutin, R; Apio-rutin, AR), kaempferol derivatives (Kaempferol-rutinoside, KR; Unknown kaempferol derivative, K Der), caffeoylputrescine (CP; with 2 isoforms, one predominating), feruloylputrescine, coumaroyltyramine and two partially described phenolics assigned as putative phenolamides (Caf Der and Fer Der). Our study confirmed these findings, with 20 phenolic compounds detected (Supplementary Figs. 1, 2). We also observed that LN nutrition increased the

concentration of all phenolic compounds in healthy plant leaves and stems, with the exception of CP, Caf Der, Fer Der, and FP in stem, whose concentrations decreased under LN (Fig. 4, Supplementary Tables 1, 2). LN nutrition enhanced also the concentration of the glycoalkaloid tomatine (Fig. 4).

The presence of *P. syringae* caused large, rapid and long-lasting accumulation of caffeoylputrescine in inoculated leaves (1 DAI, 20-98 fold increase, ***, Fig. 4a and 12 DAI 33-100 fold increase, ***, Fig. 4b) that was amplified by the HN treatment (*). *P. syringae* also increased leaf Caf Der concentration (1 DAI, 2-2.8 fold increase, **; 12 DAI 1.4-2.3 fold increase, **; Supplementary Table 1). Inoculation with *P. syringae* immediately decreased (1 DAI) the leaf concentration of chlorogenic acid (1.8-3.1 fold, ***, Fig. 4a). However, this effect disappeared 12 DAI as the concentrations of all phenolic compounds (except caffeoylputrescine, Caf Der and tomatine) in inoculated and mock inoculated leaves were of similar values (Fig. 4a,b; Supplementary Table 1).

Inoculation with *P. corrugata* induced a rapid (1 DAI) and slight increase of stem chlorogenic acid concentration (1.2 fold, *; Fig. 4c,d, Supplementary Table 2) but caffeoylputrescine concentration was unchanged. This constituent increased rapidly (1 DAI) but to a small extent. Other constituents increasing in concentrations were: Caf Der (1.4 fold, *), Fer Der (1.4 fold, *), rutin (1.3 fold, *; Fig. 4c,d) and tomatine (1.2 fold, ***, insets of Fig. 4b,d). All these effects disappeared 12 DAI (Fig. 4c,d; Supplementary Fig. 2; Supplementary Table 2).

The overall comparison of U-HPLC chromatograms from organs obtained from healthy or inoculated plants with *P. syringae* or *P. corrugata*, revealed that of the two pathogens tested, *P. syringae* induced the most marked changes (Supplementary Figs. 1,2).

3.3 Metabolite concentrations in adjacent organs

3.3.1 Carbohydrates

Inoculation with *P. syringae* modified the concentrations of several metabolites in stems and leaves distant from the inoculation site. One DAI, a marked decrease in the concentrations of glucose (2.9 fold, *) and fructose (3.7 fold, **) occurred in the adjacent stem of the HN plants only (Fig. 5a). This significant effect eventually extended to both HN and LN regimes 12 DAI (glucose: 2-2.2 fold, ***; fructose: 1.8-2 fold, ***; Fig. 5b). Starch concentration in the adjacent stem decreased significantly 12 DAI only in LN inoculated plants (2.1 fold, **; Fig. 5b). In the leaves adjacent to those inoculated with *P. syringae*, sucrose concentration was slightly reduced 1 DAI (1.2 fold, *, Fig. 5c). Twelve DAI, both glucose and fructose concentrations tended to decrease in the adjacent leaves of *P. syringae* inoculated plants but the effect was not statistically significant (Fig. 5d).

By contrast, *P. corrugata* infection produced little effect on carbohydrate concentrations in leaves and stems adjacent to the infection site. Only glucose and fructose concentrations increased in adjacent leaves, but solely 1 DAI under HN (1.7 fold ** and 1.6 fold *, respectively; data not shown).

3.3.2 Defensive compounds

Chlorogenic acid, rutin, kaempferol rutinoside and caffeoylputrescine concentrations in adjacent tissues were used as marker molecules to assess the impacts of *P. syringae* and *P. corrugata* inoculation on the defensive tomato compounds. One DAI, caffeoylputrescine concentration increased markedly in the adjacent stem (2.2-3.4 fold, ***; Fig. 5a) and leaves (1.6-2.7 fold, **; Fig. 5b). These increases were amplified 12 DAI both in stems (2-9.5 fold, ***; Fig. 5b) and leaves (5.6-48.3 fold, ***; Fig. 5d). *P. syringae* decreased the concentration of chlorogenic acid in the leaves adjacent to the inoculation site 1 DAI (1.1-1.3 fold, *, Fig.

5c) and 12 DAI (1.6-1.9 fold, ***; Fig. 5d) but this was not the case in the adjacent stems (data not shown). Twelve DAI but only under LN supply, *P. syringae* inoculation reduced rutin concentration in adjacent leaves (1.5 fold, *; Fig. 5d) and adjacent stems (1.7 fold, ***; Fig. 5b). By contrast, *P. corrugata* infection produced no significant effect on the concentrations of defensive compounds in the adjacent leaves and stems (data not shown). Low N nutrition increased the concentration of all phenolics and tomatine in the adjacent leaves and stems while that of CP was decreased (Fig. 5).

4. Discussion

The present work reports on the effects of two pathogenic species of *Pseudomonas* on tomato growth and concentrations of primary and secondary metabolites in leaves and stems. It also attempts to assess whether or not N nutrition is able to modulate these responses. We inoculated plants with *P. syringae* or *P. corrugata* and used appropriate mock inoculations with sterile water as controls, then compared plant growth and composition responses one and twelve days after the inoculation. Those two sampling dates integrate the period during which the plant develops short-term metabolic responses and longer-term ones after infection, a common pattern observed with other pathosystems [40-42]. In order to investigate the influence of N in relation to the pathogens, the plants were either supplied adequately with N or with a closely regulated low rate of N supply. By this means, we obtained two groups of plants, one growing at the maximum rate allowed by the climatic conditions of the growth room while growth of the other was limited by plant N uptake. Growth of the low N plants lagged behind that of the high N plants by roughly 50% because of lack of N. Both groups of plants, however, maintained the same photosynthetic rate per unit area. These conditions therefore, fit those described in the framework of the growth-

differentiation balance (GDB) hypothesis [12, 43] since growth was restricted by a lack of N (Fig. 2a,b) without affecting net assimilation rate per unit leaf area (Fig. 2c). N limitation lowered carbon acquisition at plant level as a consequence of restricted leaf area but not of photosynthetic efficiency [26]. Such conditions are favourable to increases in secondary metabolite concentrations in plant tissues [9, 44]. These clear-cut experimental conditions were essential to study the tomato response traits to pathogenic pseudomonas species under conditions of N nutrition limitation.

4.1 Pseudomonas species differed in effect on carbohydrate metabolism during the early stage of infection

Sugars are a source of energy in plants but are also precursors in the biosynthesis of defence-related compounds [18]. While photosynthesis is clearly the major source of carbon, several sinks compete simultaneously for carbohydrates, such as synthesis of secondary metabolites, plant growth as well as pathogen development [20]. From these viewpoints *P. syringae* and *P. corrugata* behaved very differently.

P. syringae mediated changes in carbohydrate metabolism. A rapid and sustainable decrease in concentrations of glucose and fructose was observed in the infected leaves, as already described for another strain of *P. syringae* [15] and for the pathogenic fungus *B. cinerea* [20]. Starch, which is the main storage form of carbohydrate in tomato [27, 45, 46], was consequently depressed in the long term response to *P. syringae* (Fig. 3b). Contrary to a previous study with *B. cinerea* and tomato [20], the concentration of sucrose, a transport carbohydrate, did not drop after inoculation in our study. The decrease in carbohydrate concentration following *P. syringae* inoculation was likely the consequence of reduction in photosynthesis concomitant with changes in C allocation towards secondary metabolism. Indeed, the gas exchange rates measured in non-necrotic areas of the inoculated leaf revealed

reduced photosynthesis in daylight (Fig. 2c) and enhanced respiration in the dark (Fig. 2d), the latter revealing higher metabolic rates in non-infected zones. This pattern is consistent with the findings of Bonfig *et al.* [47] on *Arabidopsis thaliana* infected with the same strain of *P. syringae*. In tomato, *P. syringae* inoculation causes down-regulation of photosynthetic gene expression [20]. As plant growth was greatly reduced following *P. syringae* inoculation in the present study (Fig. 2a), the higher metabolic activity in non-inoculated zones revealed enhanced plant secondary metabolism. Infection by the tobacco mosaic virus in *Nicotiana tabaccum* leaves also mediates an alteration of the metabolic profile in infected leaves, with an increase of compounds belonging to terpenoids and phenylpropanoids such as 5-caffeoylquinic acid and flavonoids [42, 48].

Contrary to *P. syringae*, inoculation with *P. corrugata* mediated a rapid transient increase of all soluble carbohydrate concentrations in stems (Fig. 3c,d), which cannot be explained by changes in systemic leaf activities. Leaf necrosis was not observed; neither were net photosynthetic nor dark respiration rates affected by stem inoculation (Fig. 2c,d). *P. corrugata* infiltrates host tomatoes at stem or collar levels [49] and in our experiment, necrosis developed markedly in infected stems (Fig. 1). It can thus be postulated that *P. corrugata* impaired the performance of the vascular system, causing local sugar accumulation, particularly that of sucrose, the usual form of sugar transport.

4.2 Pseudomonas species induced different effects on phenolic profiles

P. syringae induced a large disturbance in the phenolic profile of infected leaves, the salient effect being a strong and durable induction of caffeoylputrescine and of two other putative phenolamides. Caffeoylputrescine has previously been reported in healthy leaves and stems of eight tomato cultivars, including Better Bush [24]. Its elicitation by a plant pathogen has only been demonstrated following *P. syringae* and *Phytophthora infestans* infections in

tobacco and potato cell cultures, respectively [50, 51]. Other tomato phenolamides, such as *p*-coumaroyldopamine, feruloyldopamine, *p*-coumaroyloctopamine, *p*-coumaroylnoradrenaline, *p*-coumaroyltyramine, feruloyloctopamine, feruloylnoradrenaline and feruloyltyramine, were previously shown to be induced by inoculation with *P. syringae* [22, 23, 52]. To our knowledge, this is the first evidence of caffeoylputrescine response to *P. syringae* inoculation in tomato and it is reasonable to postulate that discrepancies in phenolamide profiles may reflect cultivar specificity. Phenolamides are considered as antiviral, antibacterial, antifungal and repellent compounds taking part in chemical defence against pest attacks [53]. As for other molecules they can bind with and reinforce cell walls [54]. From a quantitative viewpoint, caffeoylputrescine accumulation in leaves was the major change induced by *P. syringae* in the phenolic pathway. Indeed, its 45-fold concentration rise rendered caffeoylputrescine the most concentrated leaf phenolic. The concurrent decline in chlorogenic acid concentration in infected leaves is consistent with a similar finding in Solanaceae and indicative that chlorogenic acid and caffeoylputrescine compete for the same substrate pool of hydroxycinnamoyl coA esters [55, 56]. Thus, it is likely that during our experiment, tomato responded to *Pseudomonas* inoculation by diverting the carbon flux towards caffeoylputrescine synthesis at the expense of chlorogenic acid synthesis. Comparatively, the flavonoid pathway was little affected by *P. syringae*, since 12 DAI only the kaempferol derivative was weakly enhanced.

P. corrugata induced only slight and transient rises in rutin, tomatine (Fig. 4c,d) and the two nitrogenous phenolic Caf Der and Fer Der stem concentrations. *P. syringae* and *P. corrugata* differed slightly regarding their impact on tomatine concentration, the former brought about no change while the latter produced a small but significant decrease 1 DAI. Interestingly, it has been reported that tomatine is induced by *Ralstonia solanacearum* (formerly *Pseudomonas solanacearum*) in tomato [57].

Thus, the metabolic profile of the infected organs is pathogen-specific. The specificity of the responses is also plant-species dependent, as demonstrated in a recent study in Brassicales; the metabolic profile induced by the attack of *Pieris rapae*, a leaf miner, differed in each of the 3 brassicale host-plant species studied [58].

4.3 Nitrogen supply influenced concentrations of secondary metabolites

N regime had a major impact on the concentrations of defence-related compounds, both in infected and healthy plants. Higher concentrations were nearly always observed under LN supply, even for the alkaloid tomatine (a N-containing molecule). The only exception to these findings concerned caffeoylputrescine (also a N-containing molecule) whose concentration was lower under LN than HN. These N effects are consistent with earlier observations made on tomato [3, 4, 10, 11].

4.4 *Pseudomonas syringae* had a systemic effect on metabolites

For *P. corrugata* chemical response was restricted locally near the point of inoculation for both carbohydrates and secondary metabolites whereas that induced by *P. syringae* inoculation extended to other plant organs. In particular, the elicitation of secondary compounds was clearly systemic for chlorogenic acid (in adjacent leaves) and caffeoylputrescine (in adjacent stems and leaves). There is one report in the literature, that chemical response to *P. syringae* inoculation remains local for *p*-coumaroyldopamine and feruloyldopamine production [22]. Our results (spread of symptoms and no systemic response of tomato plant) show that the interaction between tomato cv. Better Bush and *P. corrugata* was compatible. These results are consistent with findings on other tomato cultivars by Gustine *et al.* [59], Bellés *et al.* [60] and Desender *et al.* [61].

5. Conclusions

This study provides a clear illustration that tomato plant response is species-dependent within a same genus of plant pathogens.

The inoculation site at which each species develops its pathogenicity may also explain the difference observed. We report that the attack of *P. syringae* induced a large decrease in the hexose concentration of plant tissues while *P. corrugata* produced the opposite effect. *P. syringae* elicited the accumulation of caffeoylputrescine, a phenolamide. This result is consistent with previous studies showing that some phenolamides are elicited during *P. syringae* inoculation [22]. To our knowledge, our study shows for the first time in tomato, an elicitation of caffeoylputrescine *in planta*. We verify in this study that N limitation increased the concentration of most of the plant-defence related compounds investigated (except the N-containing phenolamides). Our study reveals no clear response pattern in secondary metabolite composition and concentration during the pseudomonas inoculation in response to N supply. During *P. syringae* attack, we demonstrated that organs outside the inoculation zone elicited secondary compounds, suggesting a systemic acquired resistance. Such a hypothesis remains to be tested, for example by following the concentration of salicylic acid in the plant. Our work also provides the first assessment on the impact of *P. corrugata* on carbohydrates and defence-related secondary compounds in tomato.

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7. References

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8. Figures Captions

Figure 1: a. Leaf pictures shot 12 days after infiltration of water or inoculation with *P. syringae* under HN or LN fertilization - b. Pictures of the stem longitudinal section shot 12 days after infiltration of water or inoculation with *P. corrugata* under HN or LN fertilization.

Figure 2: Response of tomato growth and leaf net CO₂ exchanges rates (CER, $\mu\text{mol m}^{-2} \text{s}^{-1}$) responses to N nutrition and infection by *P. syringae* (a, c, e, g) and by *P. corrugata* (b, d, f). Growth measurements were performed one and 12 days after inoculation: dry shoot weights (g per plant, a-b), area of inoculated leaves (cm², e) and of adjacent leaves (cm², f-g). Leaf net CO₂ exchange rates (CER, $\mu\text{mol m}^{-2} \text{s}^{-1}$) at light (190 $\mu\text{mol PPF}$) (c) and in the darkness (d) were performed on non-infected leaf zones, 24 hours after *Pseudomonas* inoculation. LN and HN denote low and high N nutrition offered to tomato plants during growth. Vertical bars denote SE (n=6)

Figure 3: Carbohydrate concentrations (glucose G, fructose F, sucrose Su and starch S, mg g⁻¹ DW) in organs infected by *P. syringae* (leaves: a, b) or *P. corrugata* (stems: c, d). Abscissa and ordinate concern organs from LN and HN tomato plants, respectively. The measurements were performed one (a, c) and twelve days (b, d) after *Pseudomonas* inoculation. Symbols ▲ and ■ denote mock inoculated and infected plants, respectively. The diagonals (straight lines) indicate where LN = HN. It follows that data above the diagonals are depressed by LN, whereas below the line, they are enhanced by LN. Vertical and horizontal bars denote SE (n=6).

Figure 4: Defence metabolite concentrations (chlorogenic acid CA, rutin R, tomatin T, kaempferol-rutinoside KR and caffeoylputrescine CP, mg g⁻¹ DW) in organs infected by *P. syringae* (leaves: a, b) or *P. corrugata* (stems: c, d). Abscissa and ordinate concern organs from LN and HN tomato plants, respectively. The measurements were performed one (a, c)

and twelve days (b, d) after *Pseudomonas* inoculation. Kaempferol-rutinoside was detected only in leaves. Symbols ▲ and ■ denote mock inoculated and infected plants, respectively. The diagonals (straight lines) indicate where LN = HN. It follows that data above the diagonals are depressed by LN, whereas below the line, they are enhanced by LN. Vertical and horizontal bars denote SE (n=6).

Figure 5: Primary and secondary metabolite concentrations (chlorogenic acid CA, rutin R, starch S, sucrose Su, glucose G, fructose F and caffeoylputrescine CP, mg g⁻¹ DW) in organs adjacent to those infected by *P. syringae* (adjacent stems: a, b ; adjacent leaves: c, d). Abscissa and ordinate concern organs from LN and HN tomato plants, respectively. The measurements were performed one (a, c) and twelve days (b, d) after *Pseudomonas* inoculation. Symbols ▲ and ■ denote mock inoculated and infected plants, respectively. The diagonals (straight lines) indicate where LN = HN. It follows that data above the diagonals are depressed by LN, whereas below the line, they are enhanced by LN. Vertical and horizontal bars denote SE (n=6).

Supplementary Figure 1: U-HPLC chromatograms of soluble phenolics from *P. syringae* infected (A) and control (B) tomato leaf recorded at 320nm. CHA1-5: Isomers of caffeoyl hexaric acid; CP: Caffeoyl putrescine; CP_a : minor isomer of caffeoyl putrescine; CA: Chlorogenic acid; CA_b Chlorogenic acid isomer; Caf Der.: caffeoyl derivative; Put CoHA: Putative Coumaroyl HydroxyAgmatine; SG: Sinapoyl glucose; FQA: Feruloyl quinic acid; Fer Der: Feruloyl derivative; AR: Apio-rutin ;T: taxifoline (used as external standard); R: Rutin; K Der: Kaempferol derivative; KR: Kaempferol rutinoside; CT: Coumaroyl tyramine.

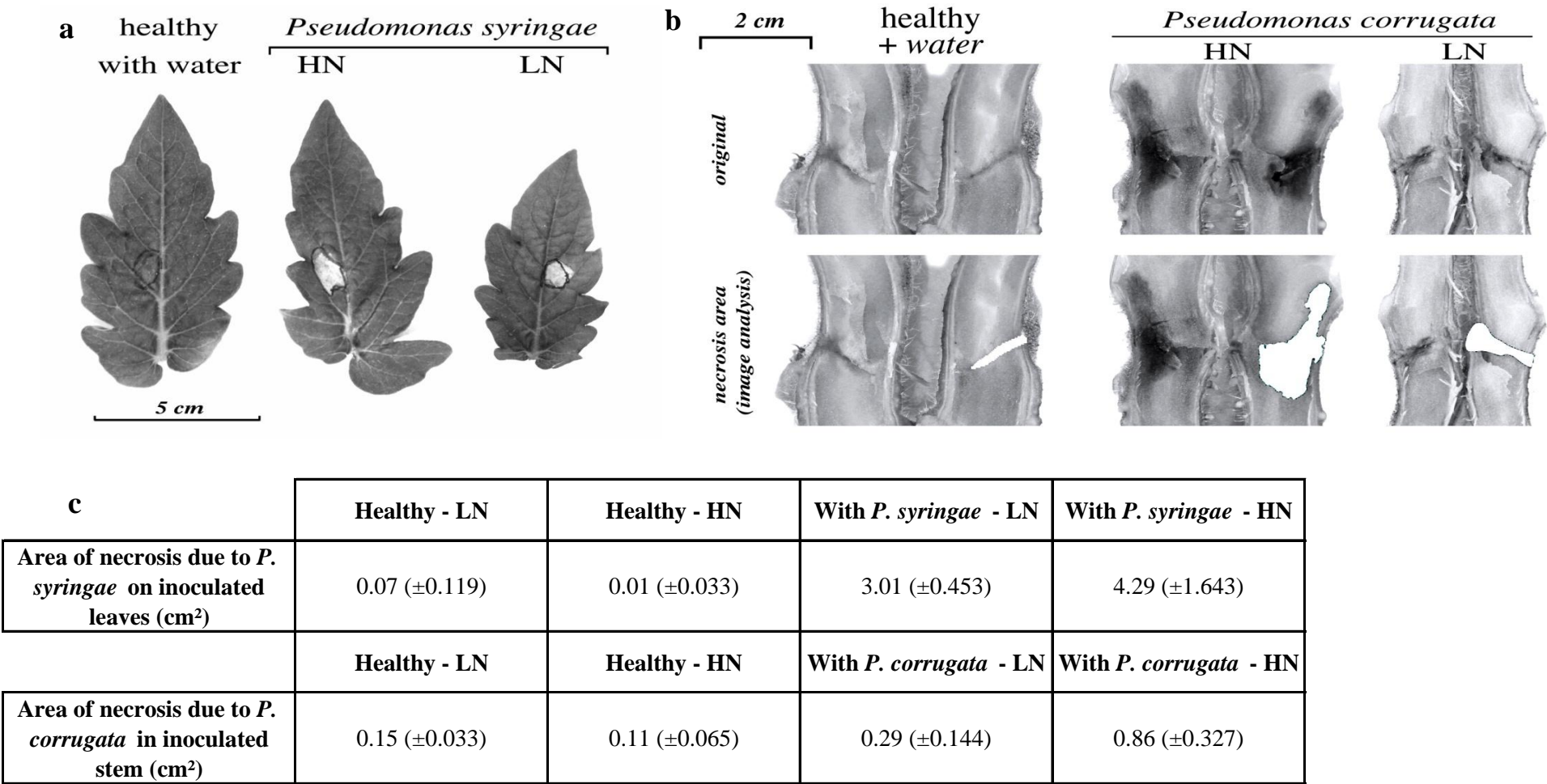
Supplementary Figure 2: U-HPLC chromatograms of soluble phenolics from *P. corrugata* infected (A) and control (B) tomato stem recorded at 320nm. CHA1-4: Isomers of caffeoyl hexaric acid; CP: Caffeoyl putrescine; CP_a minor isomer of caffeoyl putrescine ; CA: Chlorogenic acid; CA_{a-b} Chlorogenic acid isomers; Caf Der.: Caffeoyl derivative; FP:

Feruloyl putrescine; Put CoHA: Putative Coumaroyl HydroxyAgmatine; SG: Sinapoyl
glucose; FQA: Feruloyl quinic acid; T: taxifoline (used as external standard); Fer Der:
Feruloyl derivative; AR: Apio-rutin; R: Rutin; CT: Coumaroyl tyramine.

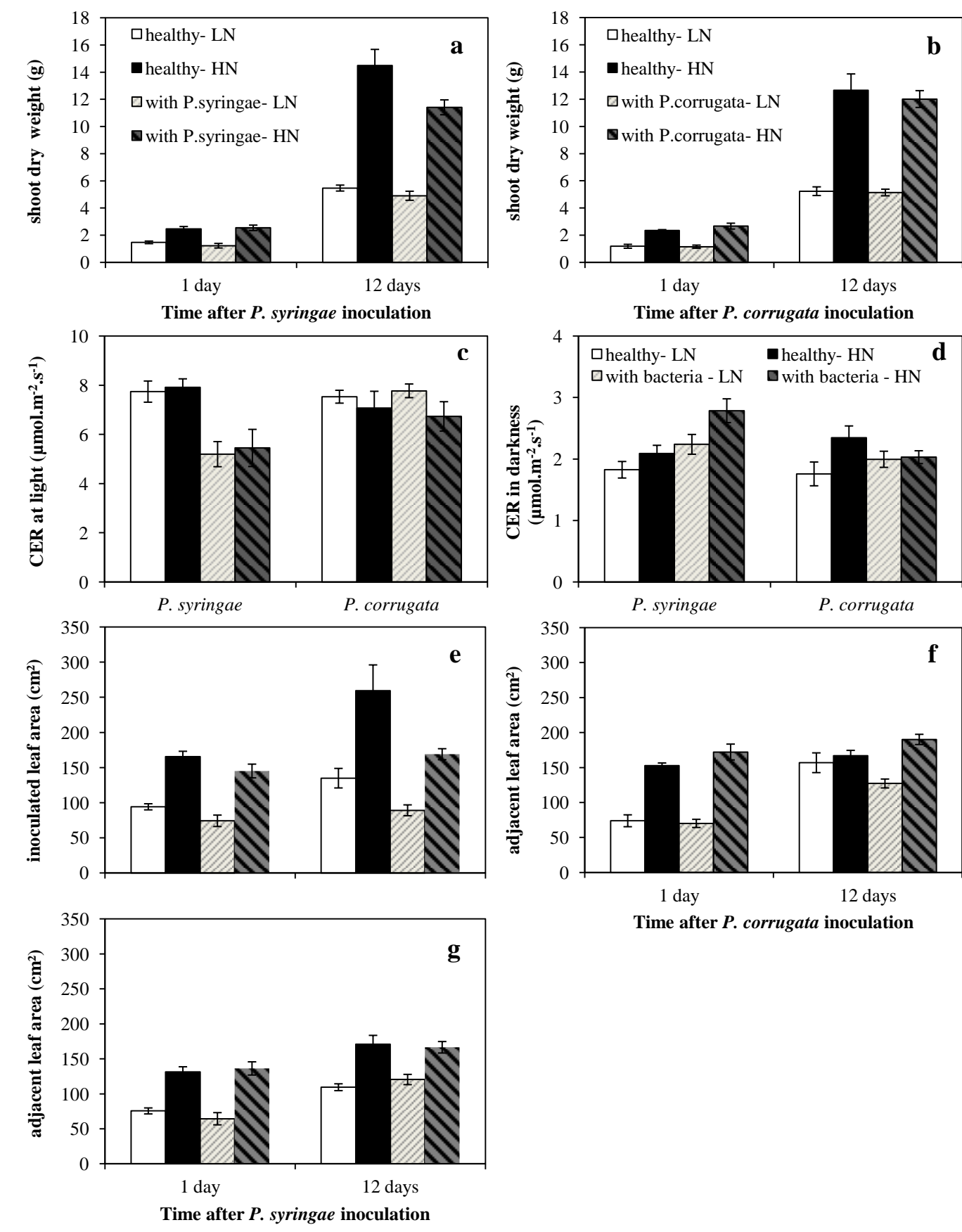
Supplementary Table 1: Concentration of phenolic compounds in *P. syringae* inoculated
and control leaf 1 and 12 DAI. All concentrations are given in $\mu\text{g g}^{-1}$ DW. CHA1-5: Isomers
of caffeoyl hexaric acid; CP: Caffeoyl putrescine; CP_a: minor isomer of caffeoyl putrescine;
CA: Chlorogenic acid; CA_b Chlorogenic acid isomer; Caf Der.: caffeoyl derivative; FP:
Feruloyl putrescine; Put CoHA: Putative Coumaroyl HydroxyAgmatine; SG: Sinapoyl
glucose; FQA: Feruloyl quinic acid; Fer Der: Feruloyl derivative; AR: Apio-rutin; T:
taxifoline (used as external standard); R: Rutin; K Der: Kaempferol derivative; KR:
Kaempferol rutinoside; CT: Coumaroyl tyramine. Values are means of 6 replicates and are
given +/- SE.

Supplementary Table 2: Concentration of phenolic compounds in *P. corrugata* inoculated
and control stem 1 and 12 DAI. All concentrations are given in $\mu\text{g g}^{-1}$ DW. Molecules
abbreviations are in supplementary Table 1 legend. Values are means of 6 replicates and are
given +/- SE.

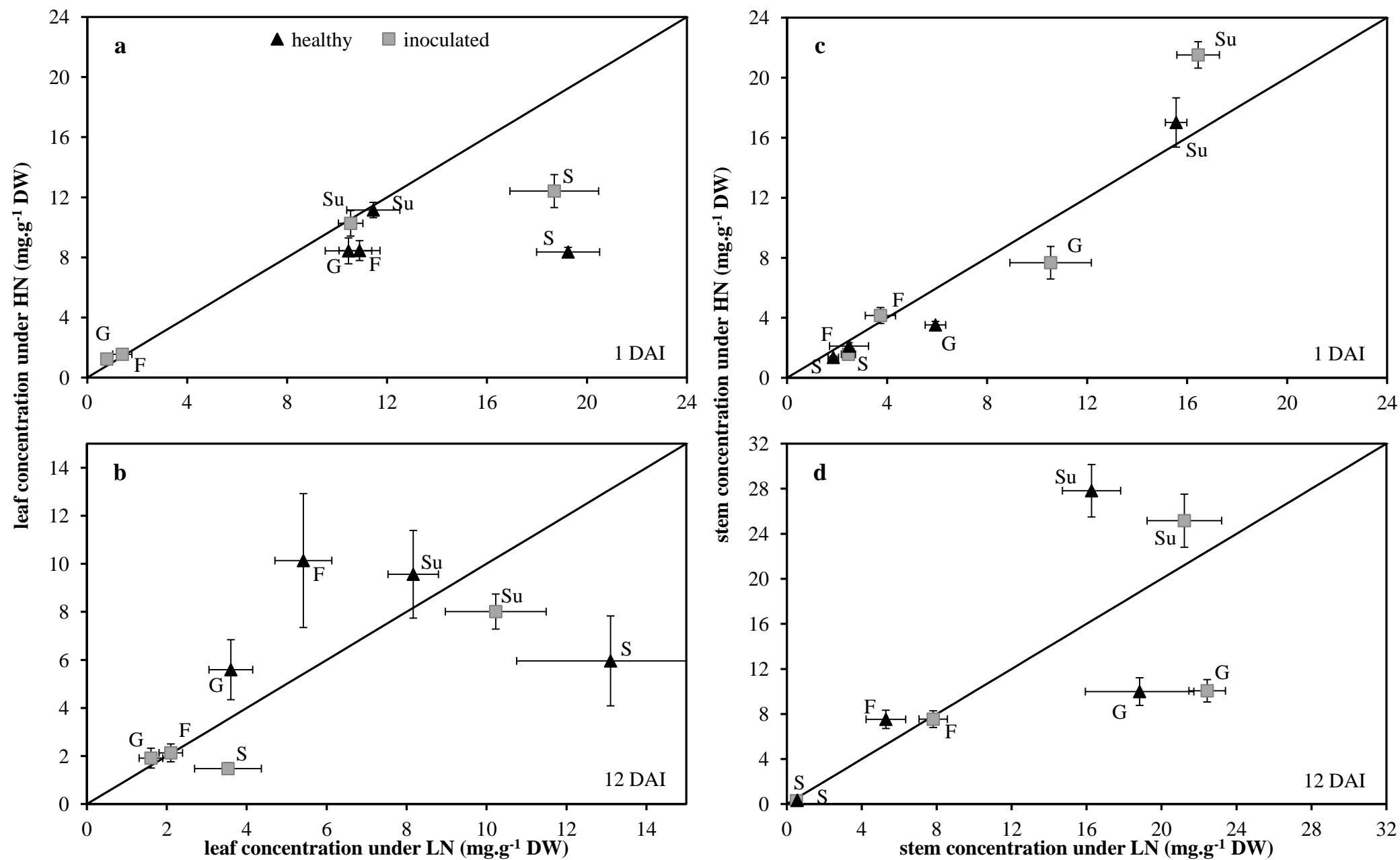
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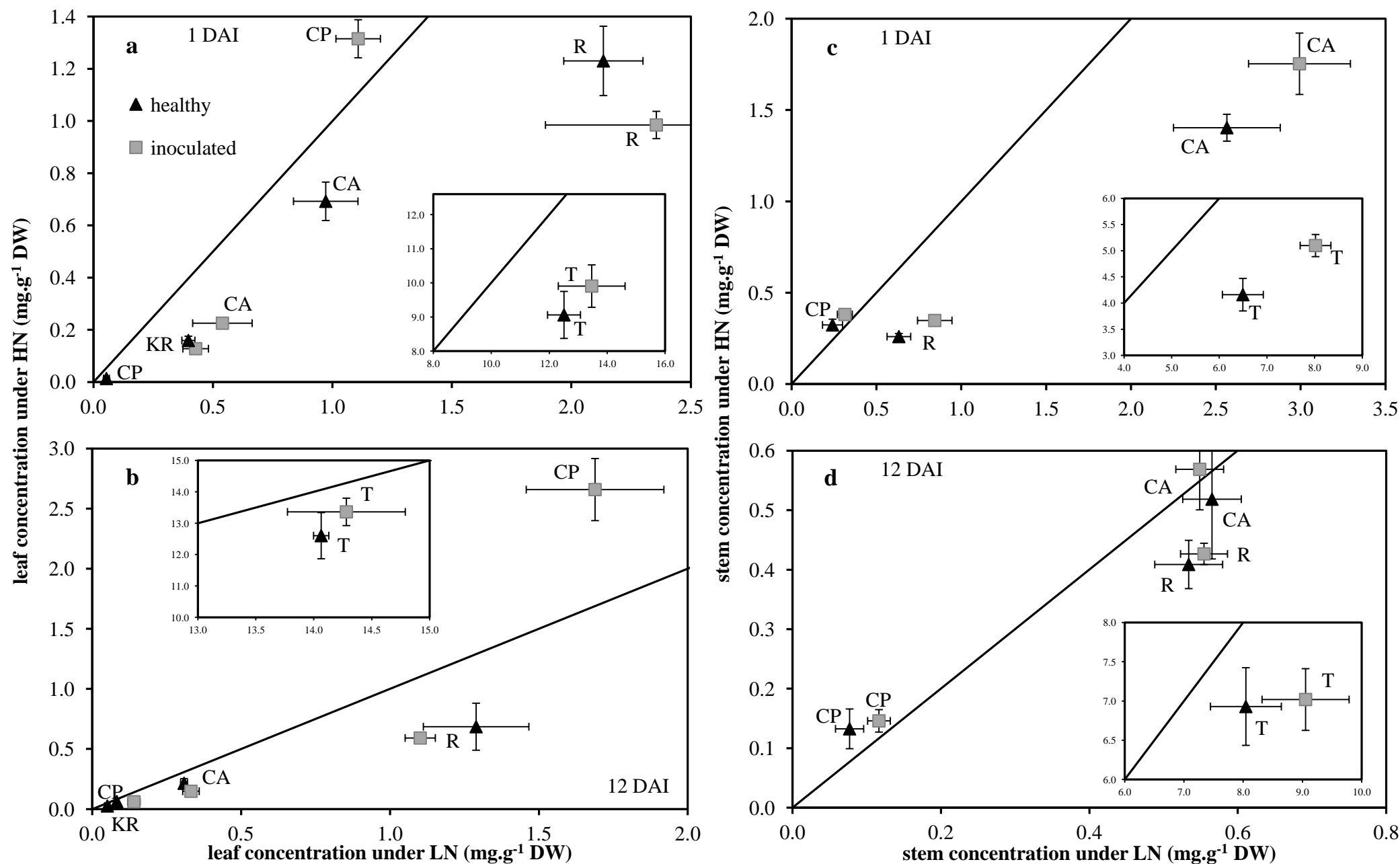
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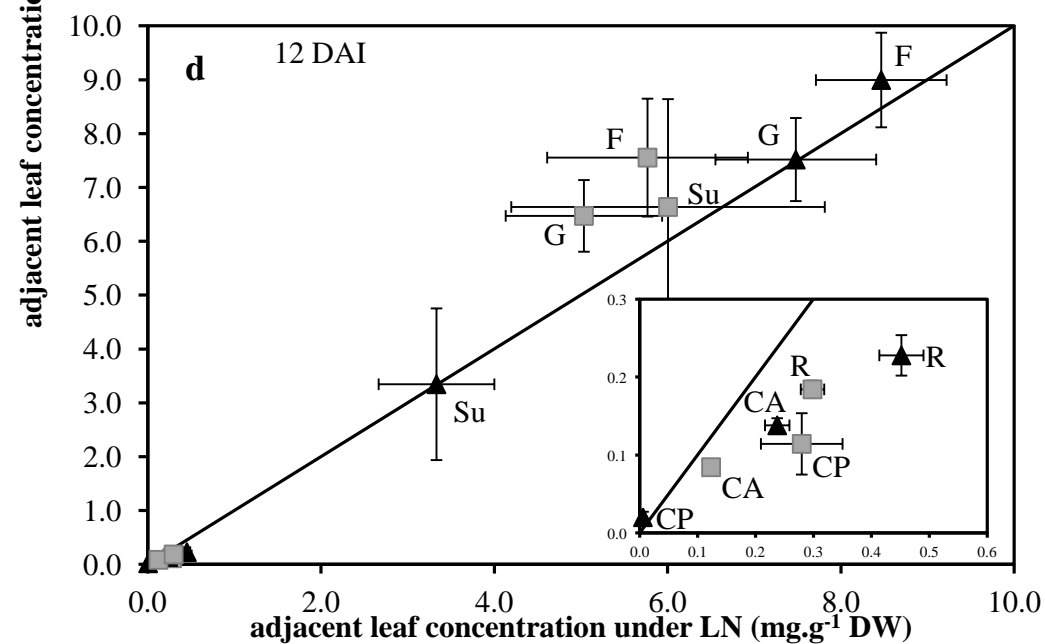
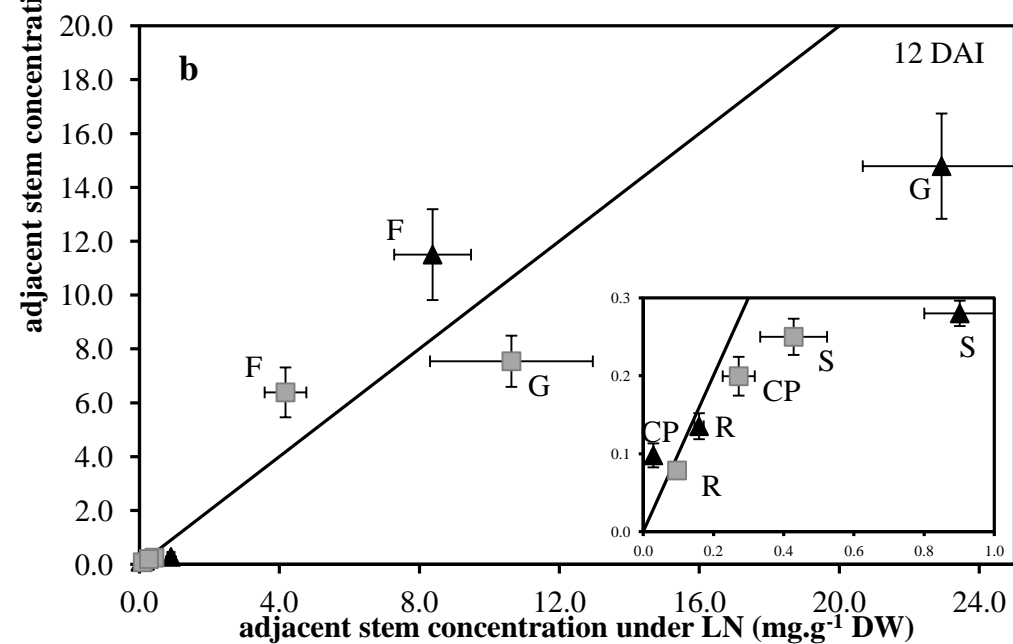
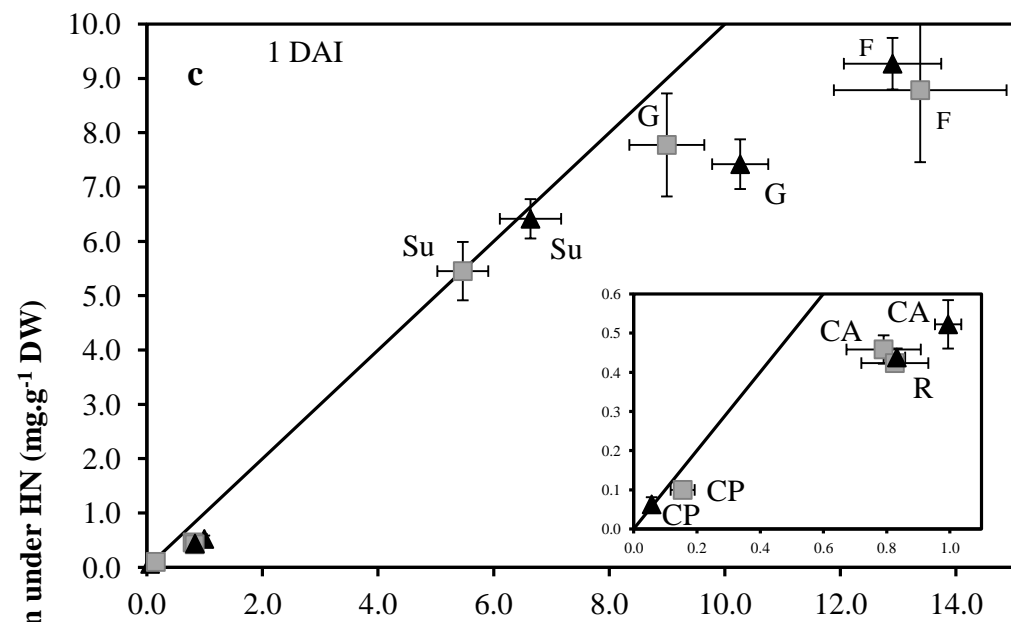
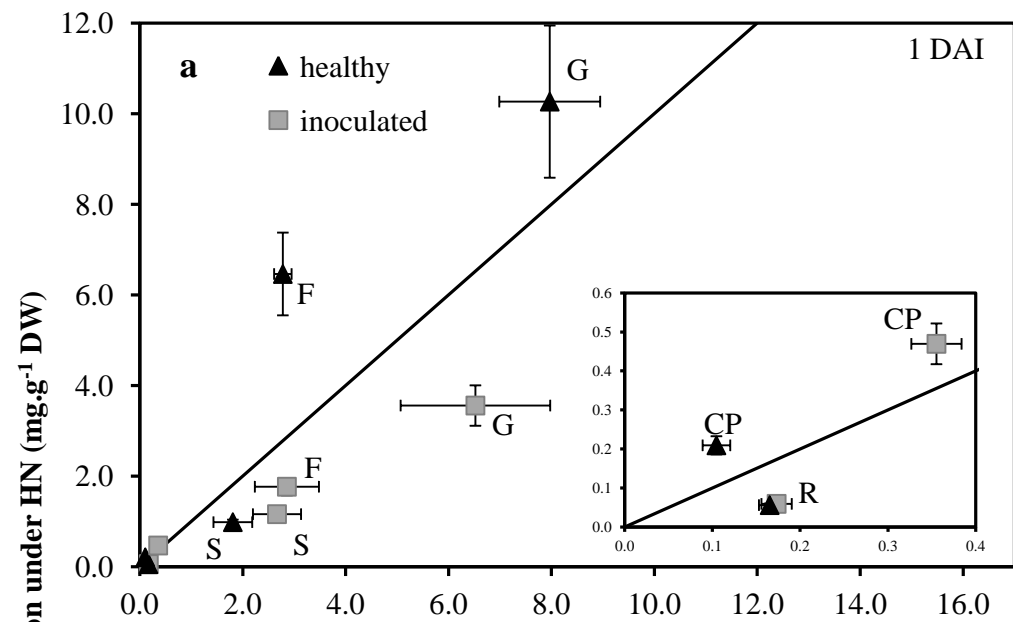
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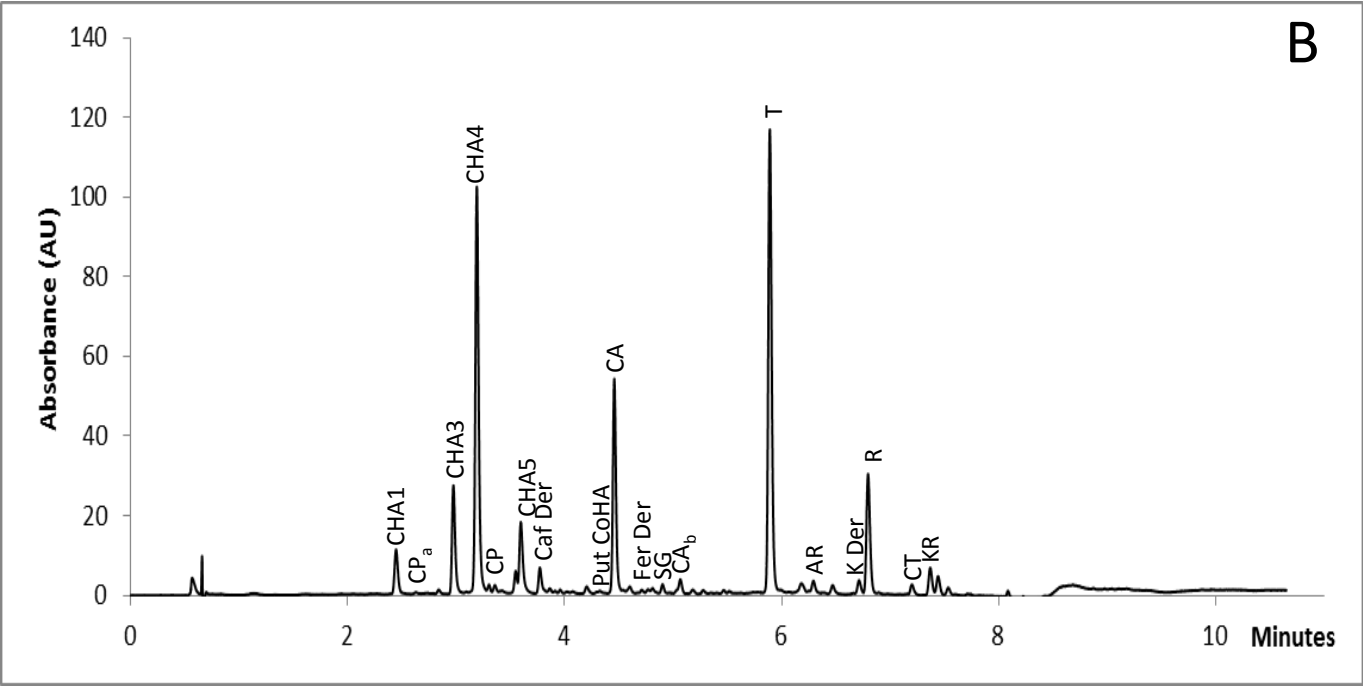
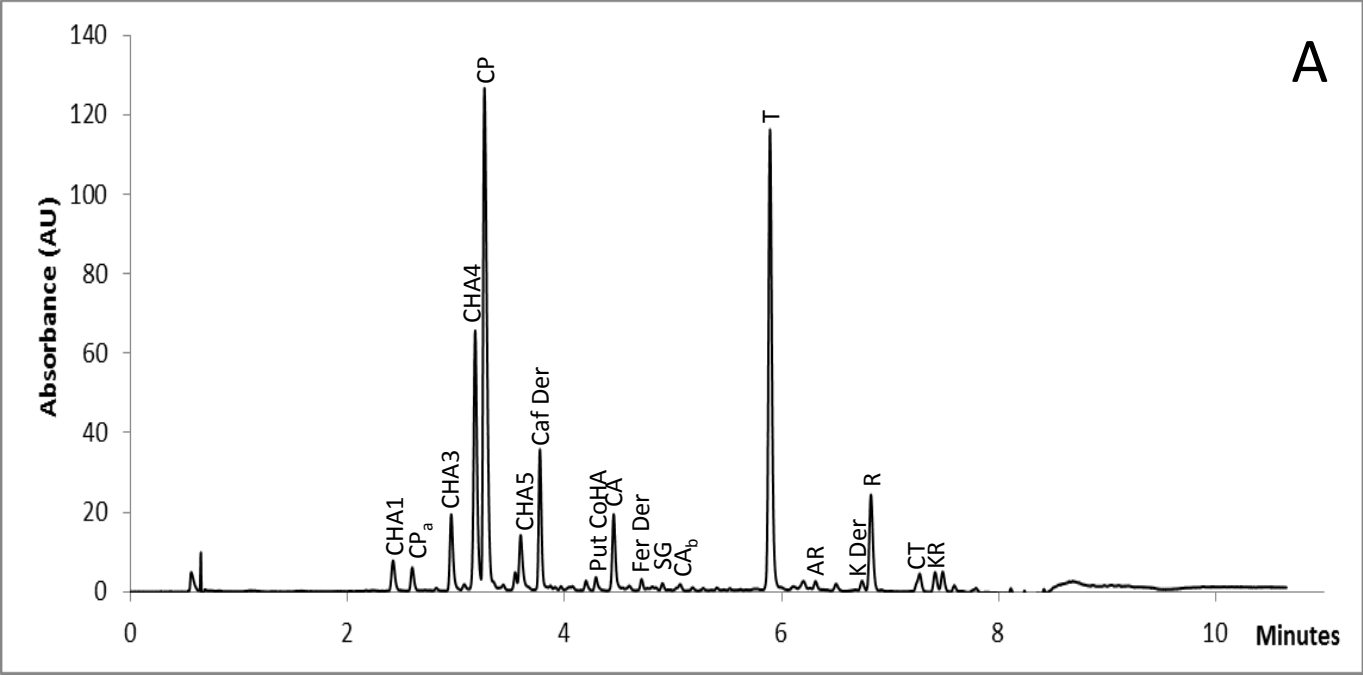
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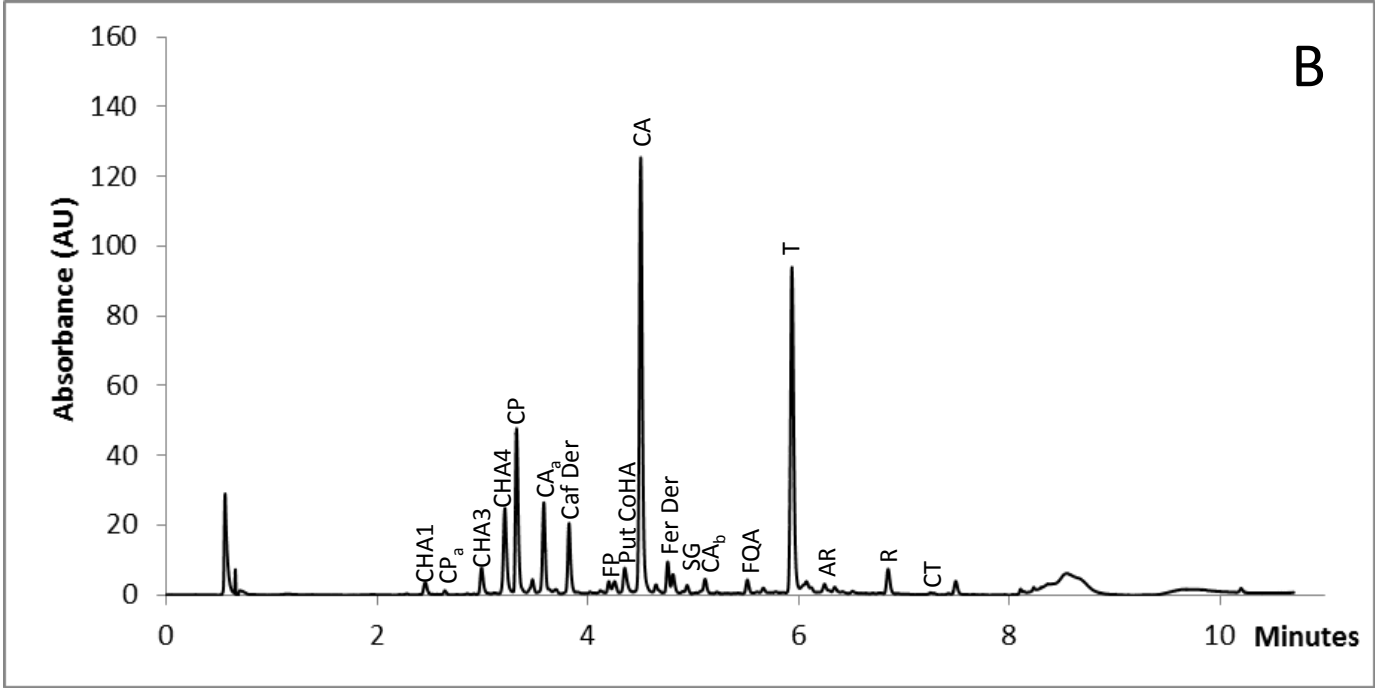
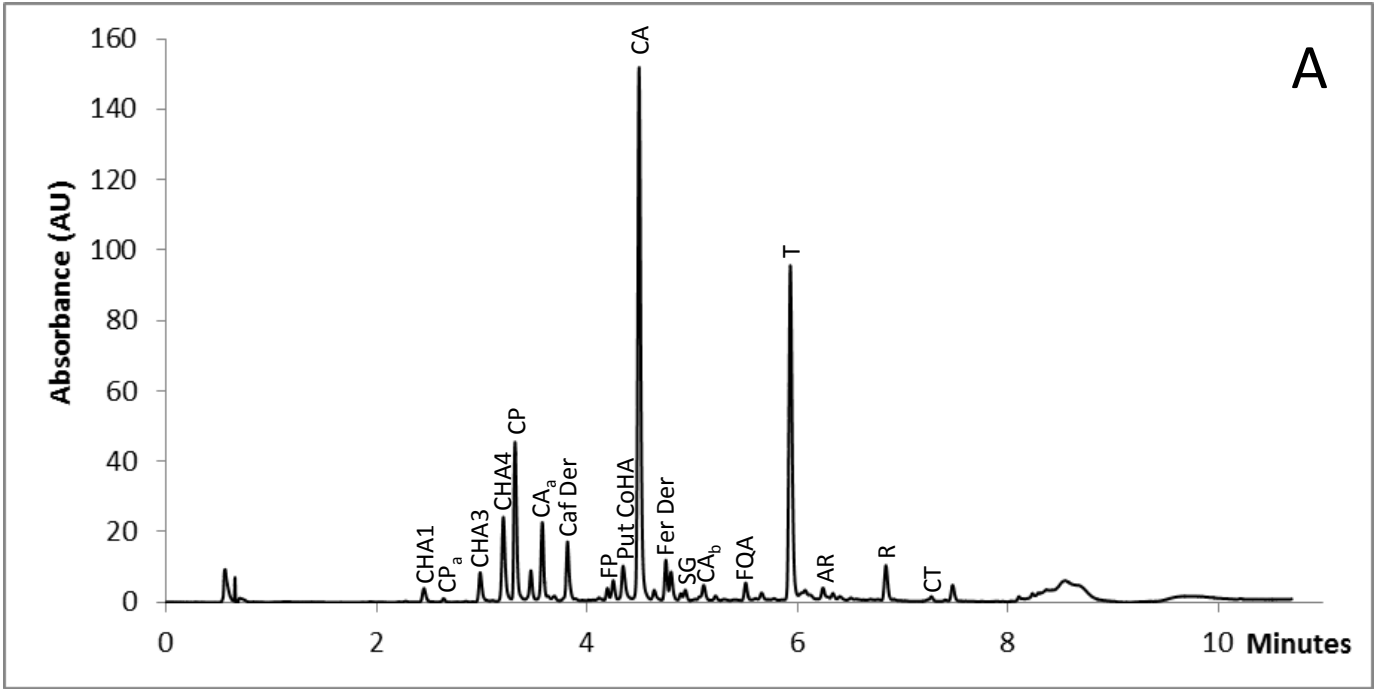
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Figure(s)



Table(s)

	1 DAI				12 DAI			
	Healthy		P. syringae inoculated		Healthy		P. syringae inoculated	
	LN	HN	LN	HN	LN	HN	LN	HN
CHA1	772 ± 96	203 ± 29	880 ± 108	135 ± 16	563 ± 21	193 ± 32	574 ± 32	225 ± 12
CP _a	2.4 ± 0.7	3.0 ± 0.1	30 ± 3	61 ± 5	5 ± 1	4 ± 1	213 ± 31	300 ± 43
CHA3	1721 ± 229	234 ± 68	1876 ± 248	307 ± 40	1275 ± 50	400 ± 63	1349 ± 74	505 ± 31
CHA4	5079 ± 535	1486 ± 231	5392 ± 834	916± 57	3198 ± 160	1322 ± 233	2916 ± 142	1220 ± 82
CP	54 ± 12	13 ± 3	1107 ± 93	1314 ± 72	51 ± 9	26 ± 7	1687 ± 231	2659 ± 258
CHA5	896 ± 76	332 ± 17	948 ± 134	238 ± 20	1491 ± 73	613 ± 102	1575 ± 40	821 ± 49
Caf Der	69 ± 9	89 ± 23	119 ± 32	252 ± 70	67 ± 3	89 ± 43	97 ± 6	202 ± 47
Put CoHA	14 ± 1	10 ± 1	19 ± 4	27 ± 7	22 ± 2	15 ± 3	21 ± 2	21 ± 5
CA	972 ± 135	692 ± 74	540 ± 124	225 ± 13	320 ± 12	223 ± 40	344 ± 29	154 ± 11
Fer Der	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.3	1.6 ± 0.4	4.0 ± 0.1	3.5 ± 0.3	2.9 ± 0.1	1.5 ± 0.1
SG	23 ± 3	25 ± 2	21 ± 4	20 ± 1	24 ± 3	32 ± 3	43 ± 3	29 ± 5
CA _b	46 ± 8	31 ± 5	57 ± 12	26 ± 1	31 ± 2	22 ± 2	32 ± 2	19 ± 1
FQA	13 ± 2	6 ± 1	8 ± 1	3 ± 1	22 ± 4	10 ± 3	17 ± 3	7 ± 2
AR	297 ± 29	132 ± 9	293 ± 31	101 ± 4	278 ± 23	119 ± 37	281 ± 12	166 ± 6
K Der	245 ± 18	82 ± 4	247 ± 24	66 ± 2	192 ± 11	77 ± 30	223 ± 12	90 ± 4
R	2134 ± 165	1230 ± 132	2356 ± 464	984 ± 52	1290 ± 177	685 ± 196	1102 ± 51	591 ± 22
CT	37 ± 3	27 ± 1	47 ± 5	39 ± 3	51 ± 2	29 ± 8	38 ± 4	20 ± 2
KR	397 ± 27	159 ± 17	428 ± 53	127 ± 12	83 ± 2	62 ± 3	140 ± 20	60 ± 4

Table(s)

	1 DAI				12 DAI			
	Healthy		<i>P. corrugata</i> inoculated		Healthy		<i>P. corrugata</i> inoculated	
	LN	HN	LN	HN	LN	HN	LN	HN
CHA1	91 ± 2	42 ± 2	102 ± 4	48 ± 6	45 ± 7	26 ± 3	43 ± 5	24 ± 2
CP _a	8 ± 1	15 ± 4	7 ± 2	14 ± 4	8 ± 3	8 ± 1	8 ± 2	8 ± 1
CHA3	170 ± 7	81 ± 4	169 ± 34	93 ± 12	87 ± 15	48 ± 6	83 ± 10	47 ± 5
CHA4	536 ± 42	307 ± 22	610 ± 38	331 ± 44	262 ± 29	142 ± 13	245 ± 21	144 ± 11
CP	241 ± 59	324 ± 32	314 ± 44	381 ± 25	77 ± 19	133 ± 33	116 ± 15	146 ± 19
CA _a	362 ± 38	177 ± 53	422 ± 47	261 ± 18	408 ± 20	149 ± 20	323 ± 31	161 ± 7
Caf der	96 ± 42	185 ± 31	140 ± 39	272 ± 27	213 ± 39	253 ± 61	216 ± 58	177 ± 20
FP	30 ± 4	28 ± 4	39 ± 3	37 ± 4	55 ± 4	94 ± 10	57 ± 12	80 ± 5
Put CoHA	72 ± 13	69 ± 4	73 ± 10	97 ± 15	80 ± 5	173 ± 15	86 ± 19	153 ± 9
CA	2565 ± 315	1403 ± 74	2993 ± 300	1753 ± 168	565 ± 39	518 ± 100	549 ± 32	569 ± 68
Fer der	60 ± 20	83 ± 12	94 ± 20	168 ± 17	115 ± 25	137 ± 22	124 ± 29	121 ± 12
SG	39 ± 5	40 ± 2	51 ± 9	48 ± 4	37 ± 2	32 ± 5	31 ± 2	33 ± 2
CA _b	99 ± 14	82 ± 16	81 ± 8	84 ± 9	36 ± 3	34 ± 4	36 ± 4	35 ± 5
FQA	95 ± 11	35 ± 2	118 ± 12	48 ± 7	3 ± 1	8 ± 3	2 ± 1	6 ± 1
AR	107 ± 14	55 ± 2	120 ± 15	57 ± 7	52 ± 3	11 ± 1	36 ± 3	11 ± 1
R	632 ± 70	260 ± 17	844 ± 102	348 ± 29	534 ± 46	409 ± 41	554 ± 32	426 ± 18
CT	12 ± 2	14 ± 5	17 ± 2	18 ± 3	35 ± 15	48 ± 15	64 ± 15	32 ± 12