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Phenolic characterization and variability in leaves, stems and roots of Micro-Tom and patio tomatoes, in response to nitrogen limitation

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Abbreviations

1 AR: Apio-furanosyl-rutin

2 BR: Brassinosteroid

3 C: Carbon

4 CCoRG: Cyanidin-3(coumaroyl)rutinoside-5-O-glucoside

5 CGT: Chlorogenate:glucarate caffeoyl transferase

6 CHA1-5: Caffeoyl hexaric acid isomers 1 to 5

7 CMA: Caffeoyl malic acid

8 CNB: Carbon nutrient balance

9 CP: Caffeoyl putrescine

10 CQA1-3: Caffeoyl quinic acid isomers 1 to 3

11 DCoRG1-2: Delphinidin-3(coumaroyl)rutinoside-5-O-glucoside isomers 1 and 2

12 DCoS: Di-coumaroyl spermidine

13 DNP: Dictionary of natural product

14 DW: Dry weight

15 ESI^{+/-}: Electro-spray ionization in positive/negative mode

16 FAH1-2: Feruloyl hexaric acid isomers 1 and 2

17 FDA: Factorial discriminant analysis

18 FQA: Feruloyl quinic acid

19 GDBH: Growth differentiation balance hypothesis

20 JA: Jasmonic acid

21 JA-Ile: Jasmonic acid isoleucine conjugate

22 FT: Feruloyl tyramine

23 HN: High nitrogen

24 HPLC: High performance liquid chromatography

25 KR: Kaempferol rutinoside

26 KRG: Kaempferol-3-O-rutinoside-7-O-glucoside

27 LC-MS: Liquid chromatography coupled to mass spectrometry

28 LN: Low nitrogen

29 MS: Mass spectrometry

30 MetOH: Methanol

31 N: Nitrogen

32 NFT: Nutrient film technique

33 NMR: Nuclear magnetic resonance

34 *p*CoQA1-2: *para*-coumaroyl quinic acid isomers 1 and 2

35 *p*CoT: *para*-coumaroyl tyramine

PCoRG: Petunidin-3(coumaroyl)rutinoside-5-O-glucoside

PCRG: Petunidin-3(caffeoyl)rutinoside-5-O-glucoside

PHT : Putrescine hydroxycinnamoyl transferase

QRG: Quercetin-3-O-rutinoside-7-O-glucoside

R: Rutin

SG: Sinapoyl glucose

TCoS1-2: Tri-coumaroyl spermidine isomers 1 and 2

THT: Tyramine hydroxycinnamoyl transferase

UPLC: Ultra performance liquid chromatography

UV: Ultra-Violet

[X]: Concentration of X

1
2
3 **Abstract:**
4

5 Phenolics are implicated in the defence strategies of many plant species rendering their
6 concentration increase of putative practical interest in the field of crop protection. Little attention
7 has been given to the nature, concentration and distribution of phenolics within vegetative organs of
8 tomato (*Solanum lycopersicum*. L) as compared to fruits. In this study, we extensively characterized
9 the phenolics in leaves, stems and roots of nine tomato cultivars using high-performance liquid
10 chromatography coupled to electrospray ionization tandem mass spectrometry (LC-MSⁿ) and
11 assessed the impact of low nitrogen (LN) availability on their accumulation. Thirty-one phenolics
12 from the four sub-classes, hydroxycinnamoyl esters, flavonoids, anthocyanins and phenolamides
13 were identified, five of which had not previously been reported in these tomato organs. A higher
14 diversity and concentration of phenolics was found in leaves than in stems and roots. The qualitative
15 distribution of these compounds between plant organs was similar for the nine cultivars with the
16 exception of Micro-Tom because of its significantly higher phenolic concentrations in leaves and
17 stems as compared to roots. With few exceptions, the influence of the LN treatment on the three
18 organs of all cultivars was to increase the concentrations of hydroxycinnamoyl esters, flavonoids and
19 anthocyanins and to decrease those of phenolamides. This impact of LN was greater in roots than in
20 leaves and stems. Nitrogen nutrition thus appears as a means of modulating the concentration and
21 composition of organ phenolics and their distribution within the whole plant.
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27 **Keywords:** *Solanum lycopersicum* L., Micro-Tom, nitrogen limitation, LC-MSⁿ, phenolic compounds,
28 phenolamides, flavonoids
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1. Introduction

1 Phenolic compounds (phenolics), together with terpenoids and alkaloids, represent one of the three
2 main groups of plant secondary metabolites. In dicots, all phenolics are derived from the sole amino
3 acid phenylalanine but the subsequent complex metabolic network leads to several phenolic sub-
4 families, among them, flavonoids, anthocyanins, hydroxycinnamoyl esters, phenolamides and
5 monolignols.
6

7 Phenolics contribute largely to plant-environment interactions. They are implicated in symbiotic
8 relationships (pollination, nitrogen fixation in Fabaceas) as well as in the defence of plants against
9 biotic and abiotic constraints. Several studies have reported that high phenolic concentrations
10 increase plant resistance to pathogens [1-4]. Moreover, several phenolics have been identified as
11 resistance factors to specific pests. For example chlorogenic and feruloyl quinic acids in
12 *Chrysanthemum* and kaempferol glucoside in *Senecio* hybrids increase resistance to thrips [5,6] while
13 chlorogenic acid and kaempferol rutinoside enhance that of potato tubers to *Pectobacterium* [7].
14 Phenolics consequently represent a potential means of enhancing crop protection.
15

16 The accumulation of phenolics is controlled by genetic factors [8], the type of plant organ and its
17 developmental stage [9-12] and responds to environmental factors, notably temperature, light and
18 nitrogen (N) availability [13-15]. N deficiency or limitation leads to phenolic accumulation in different
19 plant parts [14, 16-18] though it alters plant growth and primary metabolism [14, 16-19]. In cropping
20 systems not seeking to maximize growth and yield, it is therefore to be expected that controlling N
21 supply combined with low pesticide input could represent a means of agronomic leverage in the
22 prospect of designing environmentally friendly practices. A promising approach to enhance plant
23 resistance may therefore be to control phenolic accumulation by rational N fertilization. Such
24 investigation necessitates extensive characterization of the phenolic composition of different plant
25 organs and the assessment of the impact of N limitation on the accumulation of individual
26 compounds. A recent work on tomato plants highlighted the accumulation of chlorogenic acid in all
27 vegetative organs in response to N limitation, roots being more responsive than leaves and stems
28 [17]. Although chlorogenic acid has been reported as the main phenolic in tomato vegetative organs,
29 the presence of minor phenolics might also be important for plant defence. There is therefore a need
30 to investigate their response to N limitation.
31

32 Metabolomics, using mass spectrometry (MS) and/or nuclear magnetic resonance (NMR), allow the
33 extensive characterization of phenolic composition and content in specific plant organs or tissues. It
34 has been widely used to characterize in detail the phenolic composition and content of edible organs
35 in crops like grapevine [20, 21], potato [22, 23], cabbage [24], sweet potato [25] or medicinal plants
36 [26-28], since it is proposed that phenolics provide a health protective effect because of their
37 antioxidant properties [29]. Much less is known, however, about non-edible plant organs, despite the
38 fact that (i) they are potential sites for pathogen infection or parasite attack and (ii) they constitute
39 valuable by-products for the cosmetic and pharmaceutical industries [30]. This is especially the case
40 for tomato, the third most important vegetable grown in the world. For this crop, the phenolic
41 composition of the fruit and its distribution within this organ, have been extensively documented
42 [31-35]. Most studies on vegetative organs (leaves, stems and roots), however, have only been
43 concerned with major compounds such as chlorogenic acid, rutin and kaempferol rutinoside [36, 37]
44 or a limited subset of molecules responding to specific treatments [38, 39]. As a result, the phenolic
45 composition of these organs remains largely unknown.
46

47 The objectives of the present work were, firstly, to characterize the phenolic composition of the
48 vegetative organs (leaves, stems and roots) of tomato plants and, secondly, to assess the impact of
49 nitrogen limitation on the accumulation of the characterized compounds in the different organs. The
50 study took into account the genetic variability of the phenolic composition and its response to N
51 limitation, by comparing six determinate and three indeterminate tomato genotypes (Table 1). The
52 plants were grown hydroponically in a greenhouse under two contrasting nitrogen fertilization
53 regimes, one limiting for plant growth (Low Nitrogen: LN), while the other adequate (High Nitrogen:
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HN) [17]. Phenolic composition and concentration in vegetative organs of 28-day-old plants were determined using liquid chromatography combined with mass spectrometry analyses.

2. Materials and methods

2.1. Standards and chemicals

Chlorogenic acid, rutin, kaempferol rutinoside, ferulic, *p*-coumaric, sinapic and caffeic acids were purchased from Sigma (Steinheim, Germany). Malvidin-3glucoside was purchased from Extrasynthese (Genay, France). Caffeoyl putrescine, dicoumaroyl spermidine and tricoumaroyl spermidine were kindly provided by Dr. Werck-Reichhart (IBMP, Strasbourg, France).

2.2. Plant growth and harvest

Ten-day-old plantlets from six determinate (coded A–F, Table 1) and three indeterminate (coded G–I, Table 1) patio tomato (*Solanum lycopersicum* L.) cultivars were grown in a glasshouse in Avignon (43°56'58" N, 4°48'32" E) from April 17th to May 5th 2009. Details of the growth conditions and harvests are provided in detail in [17]. In summary, tomato plants were grown in fully randomized blocks with full nutrient solutions imposing two N regimes, one limiting plant growth (LN) and the other with high N availability (HN). Plants were grown using an NFT system with a [NO₃⁻] concentration of 3 mM for the HN treatment whereas [NO₃⁻] supply to the LN plants was periodically modified (from 10 to 30 μM) in order to maintain a NO₃⁻ uptake corresponding to 1/3 of that of the HN uptake. [NO₃⁻] and pH were corrected hourly using a computer-controlled setup (the Totomatix system) described previously [40]. Determinate and indeterminate cultivars were grown in eight and four separate blocks, respectively. In total, 120 plants were analysed, comprising eight and four replicates respectively for determinate and indeterminate cultivars, in each of the two N regimes.

The plants were harvested after 28-days of growth. Morphological traits were measured prior to separating leaves, stems and roots. Roots were rinsed in deionised water and spin-dried (2 min at 2800 g). The plant organs were weighed, frozen in liquid N₂ and stored at -80 °C until freeze-drying. Dry samples were weighed, ground to a fine powder and stored under dry air in a desiccator at room temperature.

2.3. Extraction procedure

Phenolics were extracted from dried leaf, stem and root powder as described in [17].

2.4. Qualitative HPLC-DAD-MSⁿ analysis of phenolics

Qualitative analysis of phenolics from the cv A was carried out using a HPLC-MS system (ThermoFisher Scientific, San Jose, CA, USA) consisting in a binary solvent delivery pump connected to a photodiode array detector (PDA) and a LTQ Orbitrap hybrid mass spectrometer equipped with an atmospheric pressure ionization interface operating in electrospray mode (ESI). Twenty μL of extracts were separated on a C18 LichroCART (250 mm × 4.6 mm) column (Merck, Germany). The flow rate was set at 700 μL.min⁻¹ and mobile phases consisted of water modified with formic acid (0.1%) for A and methanol modified with formic acid (0.1%) for B. Phenolics were eluted using a first isocratic step at 1% of B for 2 min, then a linear gradient from 1% to 50% of B for 50 min and finally a linear gradient from 50% to 90% of B for 20 min. Mass analysis was first carried out in ESI positive ion mode (ESI⁺), and secondly in ESI negative ion mode (ESI⁻). Mass spectrometric conditions were as follows for ESI⁺ mode: spray voltage was set at 5 kV; source gases were set (in arbitrary units min⁻¹) for sheath gas, auxiliary gas and sweep gas at 40, 10 and 10, respectively; capillary temperature was set at 300 °C; capillary voltage at 48 V; tube lens, split lens and front lens voltages at 138 V, -38 V and -4.25 V, respectively. For ESI⁻ mode, MS conditions were unchanged except ion optic parameters which were automatically adapted as follows: capillary voltage at -48 V; tube lens, split lens and front lens voltages at -138 V, 38 V and 4.25 V, respectively. Ion optic parameters were optimized in ESI⁺ mode by automatic tuning using a standard solution of rutin at 0.1 g.L⁻¹ infused in mobile phase (A/B:

50/50) at a flow rate of 5 $\mu\text{L}\cdot\text{min}^{-1}$. Full scan MS spectra were performed at high resolution ($R=30000$) on Orbitrap analyser from 120 to 2000 m/z to obtain exact masses. Data dependent MS^2 scans were automatically made on the LTQ analyser (Linear Trap Quadrupole) for structural investigation.

Raw data were processed using the XCALIBUR software program (version 2.1, <http://www.thermoscientific.com>). Experimental exact masses and MS^2 fragmentation data were compared to metabolomics data banks (DNP: <http://dnp.chemnetbase.com>, Pubchem Compound: <http://pubchem.ncbi.nlm.nih.gov/>, ReSpec: <http://spectra.psc.riken.jp/>, Mass Bank: <http://www.massbank.jp>) and other available data from the literature in order to identify the nature of the metabolites.

2.5. Quantitative UPLC-DAD-MS

For all the cultivars, the phenolics were separated on a U-HPLC system (Shimadzu, Japan) consisting of a binary solvent delivery pump connected to a diode array detector. One microliter of phenolic extract was separated on a C18 Kinetex (100 mm \times 2.1 mm) column (Phenomenex, USA) by using a gradient elution from 1 to 60% MetOH for 6.8 min, then 90 % MetOH for 2.7 min with a flow rate of 300 $\mu\text{L}\cdot\text{min}^{-1}$. The column was rinsed for 1 min with 90% MetOH and reequilibrated to 1% MetOH for 2 min prior to the next run. Compound quantification was based on measurement of area under each peak determined at 300 nm and expressed relative to calibration curves with ferulic acid (for FHA1-2, FQA, FT and feruloyl derivatives), chlorogenic acid (for CQA1-3), coumaric acid (for pCoQA1-2 and CoT), caffeic acid (for CHA1-5), sinapic acid (for SG), caffeoyl putrescine (for CP), di- and tri-coumaroyl spermidine (for DCoS and TCoS1-2 respectively), malvidin-3-glucoside (for PCRG, PCoRG and DCoRG1), rutin (for R, AR) and kaempferol rutinoside (for KR and KRG). All the standard calibration curves were made using a concentration range from 0.1 to 50 μM .

2.6. Statistics

For each statistical analysis, determinate and indeterminate cultivars were treated separately. Cultivar comparison under HN was carried out by (i) a factorial discriminant analysis (FDA) using the package ade4 in R software and (ii) a two-way analysis of variance (cultivar as fixed factor, block as random). The respective degrees of freedom for determinate and indeterminate cultivars were: cultivar = 5 and 2, blocks = 7 and 3, residuals = 40 and 6, respectively. Box-plots, Q-Q plots and correlation between variance and mean assessed the data distribution and homoscedasticity.

The impact of N treatment was assessed by a three-way analysis of variance (cultivar and nutrition as fixed factors, block as random). The respective degrees of freedom for determinate and indeterminate cultivars were: cultivar = 5 and 2, nutrition = 1 and 1, blocks = 7 and 3, cultivar \times nutrition = 5 and 2 residuals = 40 and 15, respectively. Data distribution and homoscedasticity were assessed as described above. When the cultivar \times nutrition interaction was not significant, Tukey's test was used for mean comparisons; otherwise, Student's t-test assessed the nutrition effect on each cultivar. Analyses of variance were performed using the procedure lm in R software (R Project for Statistical Computing, Available from <http://www.R-project.org>) and statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Identification of phenolics in tomato leaves, stems and roots

Our previous study based on HPLC-DAD analyses recorded at 300 nm [17] revealed that although the same phenolics were found in each of the 9 cultivars, the individual organs of each cultivar showed a specific profile. Consequently, phenolic identification was made on the vegetative organs of only one cultivar (A; Micro-Tom). The methanolic extracts from leaves, stems and roots were analysed on HPLC-DAD-ESI- MS^n in negative and positive ion modes. A characteristic UV chromatogram recorded at 300 nm for each organ is shown in figure 1. Table 2 summarizes the UV, mass and MS/MS fragmentation characteristics of the chromatographic peaks together with their proposed chemical formulae and structures. Up to 39 phenolic compounds were detected in the extracts of the three

vegetative organs. The phenolics identified could be divided into five groups according to their structures.

3.1.1 Hydroxycinnamoyl esters

Compounds 1, 2, 3, 4 and 7 showed the same molecular mass indicating that they were isomers. Based on the deduced formula $C_{15}H_{16}O_{11}$ and the fragmentation pattern, these compounds were designated as caffeoyl hexaric acid [41] and ranked from 1 to 5 (CHA1-5) according to their retention time. All five isomers were present in leaf extracts, whereas stem extracts contained only CHA3-4 with traces of CHA5 and root extracts, only trace amounts of CHA3-4. Compounds 9 and 10 exhibited a $[M-H]^-$ at m/z of 385.0767 and major $[M-H]^-$ fragmentation peaks at m/z 177 and 209 attesting the presence of feruloyl and hexaric acid moieties, respectively. These compounds were assigned as feruloyl hexaric acid isomers 1 and 2 (FHA1-2). FHA1 and 2 were detected only in the leaf extracts. Hydroxycinnamoyl hexaric acid derivatives have been described only in a few reports on Poaceae [42], Asteraceae [43, 44] and Rutaceae [45] and Solanaceae [46]. In tomato, caffeoyl esters of hexaric acid, *i.e.*, glucaric and galactaric acids, have been reported previously in leaves and seedlings [46] but not in stems and roots. To our knowledge, feruloyl hexaric acid has never been detected in tomato plants. The function of these compounds is poorly understood, however, it can be inferred at least, that they take part in plant defence. Indeed, caffeoyl glucaric acids have been shown to inhibit growth of the tomato fruit worm (*Helicoverpa zea* Boddie) [46]. The biosynthesis of these caffeoyl hexaric esters in tomato involves an enzyme (CGT; Chlorogenate:glucarate caffeoyl transferase) that transfers the caffeoyl moiety of chlorogenic acid (5 caffeoyl quinic acid) to glucaric or galactaric acids [47, 48].

Compounds 5, 13 and 16 displayed a $[M-H]^-$ of 353.0867 with fragments at m/z 191, 179 and 135 characteristic of caffeoyl quinic acids. The relative proportion of daughter ions has already been used to distinguish between the isomers [49]. Hence, compound 5 was assigned as 3-caffeoyl quinic acid, also named neochlorogenic acid (CQA1). Compound 13 was identified without doubts as 5-caffeoyl quinic acid, known as chlorogenic acid (CQA2) because of the relative abundance of MS/MS daughter ions in ESI⁻ and its exact comparison with a commercial standard. The fragmentation patterns in ESI⁻ and ESI⁺ for compound 16 were identical with chlorogenic acid implying that this compound is a stereoisomer of chlorogenic acid (CQA3). Chlorogenic acid and its putative stereoisomer were detected in all vegetative organs, whereas neochlorogenic acid was absent from roots.

Compounds 18 and 21 displayed $[M-H]^-$ parent ions at m/z 337.0926 corresponding to the chemical formula $C_{16}H_{18}O_8$. Compound 20 displayed $[M-H]^-$ at m/z 367.1025 corresponding to $C_{17}H_{20}O_9$. These three compounds exhibited a m/z $[M-H]^-$ fragment at 191 indicative of a quinic acid moiety. Thus, compounds 18 and 21 were assigned as coumaroyl quinic acid isomers (*p*CoQA1 and 2) and compound 20 as feruloyl quinic acid (FQA). FQA was detected in the three extracts whereas *p*CoQA1-2 were restricted to leaf and stem extracts. These compounds have previously been identified in tomato leaves [30, 38]. Hydroxycinnamoyl quinic acid derivatives are widely distributed in the plant kingdom. They constitute a group of biologically active compounds with antioxidant and antibiotic properties, making them important for plant response to biotic and abiotic stresses. Indeed, in tomato, chlorogenic acid concentration is positively correlated to resistance to *Pseudomonas syringae* and protection against oxidative stress [4]. Chlorogenic acid is also an intermediate in the lignin biosynthesis pathway and is considered as a storage form of hydroxycinnamic acid, notably in coffee [50, 51].

Compound 15 exhibited an expected exact mass at 386.1203 and major $[M+H]^+$ fragments at m/z 225 and 207 that were characteristic of sinapoyl-glycoside (SG, [52]). This compound was detected in both leaf and stem extracts. Sinapoyl glycoside has been characterized already in leaf extracts of "Bull's Heart" tomato cultivars [29, 37].

Compound 19 had a $[M-H]^-$ m/z at 295.0457 and deprotonated fragments at m/z 133 and 179 indicating malic and caffeic acid moieties [53], respectively. These data strongly suggest that compound 19 is caffeoyl malic acid (CMA) and to our knowledge, this is the first report of its detection in a tomato vegetative organ and specifically in the leaf extracts. The 2-caffeoyl-malic acid,

also named phasic acid, constitutes a major phenolic acid in some Fabaceae species, such as red clover, in which it participates in plant defence against pathogens and protection to UV light [54, 55].

3.1.2 Phenolamides

Compound 6 had a $[M+H]^+$ at m/z 251.1382 with fragments at m/z 234, 163 and 89, which correspond to the previously identified caffeoyl putrescine (CP) [56, 57]. CP was detected in leaves, stems and roots. Compound 24 exhibited a $[M+H]^+$ m/z at 438.2377 corresponding to the chemical formula $C_{25}H_{31}N_3O_4$. Its fragmentation profile in positive mode matched that of di-coumaroyl spermidine (DCoS) [58]. Accordingly, compounds 36 and 37 shared the same $[M+H]^+$ m/z at 584.2748, as compound 24, but in addition to this, contained a coumaroyl moiety (+146). From this information, compounds 36 and 37 were assigned as two isomers of tricoumaroyl spermidine (TCoS1 and 2) [59]. DCoS, TCoS1 and TCoS2 were detected only in stems. Compounds 28 and 30 displayed a $[M+H]^+$ at m/z 284.1271 and 314.1377 and showed fragmentation patterns that corresponded to coumaroyl tyramine (CoT) and feruloyl tyramine (FT), respectively [39]. CoT and FT were detected only in the tomato root extract. It has been previously reported that Solanaceous plants and particularly tomato leaves, may store several phenolamide derivatives such as hydroxycinnamoyl derivatives of octopamine, noradrenaline [60], tyramine or dopamine [39]. These compounds are all strongly induced in tomato leaves infected with the bacteria *Pseudomonas syringae* while present at low concentrations in healthy plants. Caffeoyl putrescine has already been detected in leaves of the tomato transformants down-regulated for Cinnamoyl-coA Reductase activity [61], however, to our knowledge, this is the first time that it has been detected in non-stressed wild type tomato plants. This is also the first time that the occurrence of coumaroyl and feruloyl tyramine has been reported in tomato roots, although these compounds have already been detected in the roots of tobacco [62]. Tri- and di-coumaroyl spermidines are usually detected in floral organs [63]. Their presence in our stem extract may have resulted from our sampling procedure, which bulked both stems and first flower buds in the stem extract of the 3-week-old plants. Phenolamides are involved in a large array of proven and putative functions in plant physiology, extensively reviewed [64]. For instance, phenolamides are associated with floral induction and are major pollen constituents. In addition, they participate in plant defence through direct antibacterial properties and also reinforcement of the cell wall.

3.1.3 Flavonoids

In addition to rutin (R, compound 27) and kaempferol rutinoside (KR, compound 32) whose identities were confirmed by comparison with standards, other molecules showed UV profiles with λ_{max} around 260 and 350nm, typical of flavonoids. Compounds 17 and 22 displayed a $[M-H]^-$ m/z at 771.1975 and 755.2027 corresponding to R and KR, respectively, plus one hexose (+162). These compounds were putatively assigned to quercetin-3-O-rutinoside-7-O-glucoside (QRG) and kaempferol-3-O-rutinoside-7-O-glucoside (KRG), based on their $[M-H]^-$ and $[M+H]^+$ fragmentation patterns [65, 66] and their previous identification in tomato fruits [32]. Compound 23 showed a $[M-H]^-$ m/z at 741.1864 with a major daughter ion at m/z 609 corresponding to the loss of a pentosyl moiety from the parent ion and at m/z 300 corresponding to $[quercetin-2H]^-$ [38]. Compound 23 is thus composed of one quercetin with pentosyl and rutinoside moieties, and should correspond to quercetin 3-O-(2-O- β -apiofuranosyl-6-O- α -rhamnopyranosyl)- β -glucopyranoside (AR) already characterized in tomato fruits [67]. All the flavonoids were detected only in the aerial organs, which is in accord with their major function as a UV-protectant. Flavonoids, notably R and KR are associated with plant defence [7, 68, 69].

3.1.4 Anthocyanins

Compounds 29, 31, 33, 34 and 35 showed similar UV profiles with a peak around 330 nm and a shoulder at 310 nm, easily distinguishable from the hydroxycinnamoyl esters which are characterized by a peak around 325 nm and a shoulder at 300 nm. Compound 29 exhibited a $[M-H]^-$ m/z at 947.2446 which corresponds to Petunidin-3-(caffeoyl)rutinoside-5-O-glucoside (PCR) previously

1 identified in tomato fruits [32]. Accordingly, compound 33 showed a $[M-H]^-$ m/z at 931.2495, which is
2 indicative of the loss of one hydroxyl group from PCRG (16). Compound 33 should therefore
3 correspond to Petunidin-3-(coumaroyl)rutinoside-5-O-glucoside (PCoRG), which has previously been
4 detected in tomato fruits [32]. Compounds 31 and 35 with a $[M-H]^-$ m/z at 917.2336 are two isomers
5 of delphinidin-3-O-(coumaroyl)rutinoside-5-O-glucoside (DCoRG1-2) [32, 70]. Compound 34, with an
6 experimental mass of 902.2470, corresponds to the chemical formula $C_{42}H_{46}O_{22}$ already assigned to
7 cyanidin-3-O-(coumaroyl)rutinoside-5-O-glucoside (CCoRG) [71]. CCoRG was detected at trace levels
8 in leaves and stems. PCRG and PCoRG were detected both in leaf and stem extracts, whereas
9 DCoRG1 and 2 were found only in leaf extracts. These compounds have been previously reported in
10 leaves of light-stressed wild-type tomatoes and also in tomatoes overexpressing the anthocyanin
11 specific transcription factor ANT1 [70]. Similarly to flavonoids, these compounds are associated with
12 UV-protection.
13

14 3.1.5 Partially identified compounds

15 Eight compounds were detected and analysed but neither their UV profile and exact mass nor their
16 fragmentation pattern in positive and negative modes allowed us to propose a relevant structure.
17 Among them, four could be partially characterized. Compounds 8 and 14 showed UV profiles typical
18 of hydroxycinnamoyl derivatives. Their exact masses $M=308.1365$ (compound 8) and $M=322.1522$
19 (compound 14) led to the respective formulae of $C_{15}H_{20}N_2O_5$ and $C_{16}H_{22}N_2O_5$. Moreover, the
20 fragmentation pattern in ESI^+ indicated the presence of a caffeoyl moiety ($[M+H]^+$ at m/z 163) and a
21 feruloyl moiety ($[M+H]^+$ at m/z 177) in compounds 8 and 14, respectively. Based on this evidence, the
22 formula of the other moiety could be $C_6H_{14}N_2O_2$, which corresponds to the amino acid lysine.
23 Compound 8 and 14 could thus be caffeoyl- and feruloyl-lysine, respectively. Confirmation by
24 complementary structural analyses, however, is required. Compounds 11 and 12, detected in root
25 extracts, showed a $[M-H]^-$ m/z at 952.3276 and 982.3376, suggesting that they might contain at least
26 one atom of N. These two compounds appear to differ by one methoxy group (30 atomic mass unity).
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31 3.2 Quantification and distribution in vegetative parts under HN

32 Quantification was achieved on 27 of the 31 identified molecules because CMA, QRG, CCoRG,
33 DCoRG2 were present only at trace levels. For the same reason, we did not quantify DCoRG1 in
34 leaves, *p*CoQA2 and CHA5 in stems or CHA3-4 and KR in roots.
35
36

37 The three vegetative organs showed specific phenolic patterns (Fig. 1), which were qualitatively
38 shared by the six determinate and the three indeterminate cultivars (Supplementary Table 1). Only 4
39 of the quantified molecules were found in the extracts of all three organs (leaf, stem and root), *i.e.*
40 CQA2-3, FQA and CP (Fig. 2).
41

42 Leaves contained high concentrations of hydroxycinnamoyl esters, mainly as chlorogenic acid (CQA2;
43 $829-2237 \mu\text{g}\cdot\text{g}^{-1}$ DW; Fig. 2) and the isomers 3 and 4 of caffeoyl hexaric acids (CHA3-4; $180-532$ and
44 $661-1850 \mu\text{g}\cdot\text{g}^{-1}$ DW respectively). Other hydroxycinnamoyl esters were below $300 \mu\text{g}\cdot\text{g}^{-1}$ DW. Leaves
45 were also rich in flavonoids. Rutin (R) was the most abundant in all cvs ($639-1831 \mu\text{g}\cdot\text{g}^{-1}$ DW), being
46 greater than KR (from 67 to $187 \mu\text{g}\cdot\text{g}^{-1}$ DW) and AR ($62-171 \mu\text{g}\cdot\text{g}^{-1}$ DW). Anthocyanins were
47 exclusively petunidin derivatives. The flavonoid concentrations measured in the nine cultivars were
48 similar to those found in "Cherry" and "Bull's Heart" tomato cvs, whereas the concentrations of
49 hydroxycinnamoyl esters were far higher [30].
50

51 In stems, hydroxycinnamoyl esters consisted mainly in CQA isomers, chlorogenic acid being the most
52 important (CQA2, $576-3315 \mu\text{g}\cdot\text{g}^{-1}$ DW), whereas (CHA) and feruloyl hexaric (FHA) acids were almost
53 absent (Fig. 2). Rutin was the dominant flavonoid (R, $151-1563 \mu\text{g}\cdot\text{g}^{-1}$ DW) and anthocyanins were
54 presented in the form of delphinidin (DCoRG1) and petunidin (PCRG) derivatives. Of the three
55 organs, the stem contained the highest concentration of caffeoyl putrescine (CP, $118-679 \mu\text{g}\cdot\text{g}^{-1}$ DW).
56 Di and tri-coumaroyl spermidine derivatives were confined to the stem, at a relatively low
57 concentration ($< 100 \mu\text{g}\cdot\text{g}^{-1}$ DW each).
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60

1 In roots, the two major phenolics were chlorogenic acid (CQA2, 318-1272 $\mu\text{g}\cdot\text{g}^{-1}$ DW) and caffeoyl
2 putrescine (CP, 56-219 $\mu\text{g}\cdot\text{g}^{-1}$ DW). Other hydroxycinnamoyl esters (CQA3, FQA) and phenolamides
3 (FT and CoT) were present at concentrations below 20 $\mu\text{g}\cdot\text{g}^{-1}$ DW (Fig. 2).

4 3.2.1 *Micro-Tom* exhibited strong differences with the other cvs for phenolic accumulation

5
6 A factorial discriminant analysis was conducted in order to compare the cvs from the viewpoint of
7 their phenolic concentrations in the vegetative organs. The analysis revealed a first factor explaining
8 32.8% of the total variance that correlated positively with CQA2-3, CP, FQA, AR, R and DCoRG1. The
9 second factor, representing 20.6% of the data variability, correlated positively with *p*CoQA1 and
10 *p*CoQA2. The distribution of the cvs on the plane defined by both factors discriminated two cvs, Red
11 Robin (B) and Micro-Tom (A), from the others (Fig. 3). Red Robin emerged in the upper part of the
12 plane (Fig. 3), indicating higher values for factor 2. This cv exhibited notably high leaf concentrations
13 of *p*CoQA1, *p*CoQA2 and PCRG that were significantly different from all determinate cvs other than
14 cv C ($p < 0.001$, Supplementary Table 1). Micro-Tom was clearly evident at the right side of the plane
15 due to its higher values for factor 1 (Fig. 3). Indeed, the leaves of Micro-Tom exhibited striking
16 characteristics of higher metabolite concentrations of [CQA1], [SG], [FQA], [R], [KRG], [PCRG],
17 [PCoRG] and [CP] compared with the other cvs ($p < 0.001$, Supplementary Table 1). In addition,
18 Micro-Tom accumulated high phenolic stem concentrations of [CQA1-3], [FQA], [AR], [R], [PCRG],
19 [DCoRG], [CP], [TCoS2] and [DCoS] these values also being significantly greater than the
20 corresponding concentrations in the other determinate cvs (2-20 fold, depending on the molecule
21 and the cv; $p < 0.001$, Supplementary Table 1). Furthermore, Micro-Tom differed from the other cvs
22 regarding the quantitative distribution of phenolics between leaves and stems. Indeed, the
23 hydroxycinnamoyl esters CQA2 and CQA3 and the flavonoid AR were found in higher concentrations
24 in Micro-Tom stems than in leaves, while the opposite was the case in all other cultivars
25 (Supplementary Table 1). Micro-Tom has emerged over the past years as a tomato plant model and
26 more recently as a fleshy-fruit plant model for physiology and molecular biology studies, because of
27 its small size and rapid growth [72]. The Micro-Tom phenotype is induced by at least two mutations,
28 *selfpruning*, conferring the determinate growth and *dwarf*, reducing the internode growth through a
29 perturbation in brassinosteroid (BR) metabolism [73]. Some recent studies on BR synthesis deficient
30 or signalling mutants have highlighted a potential role of BR in herbivore-induced accumulation of
31 defensive compounds in tomato [74] and *Nicotiana attenuata* [75]. In the latter, indeed, the silencing
32 of the BR receptor *BRI1* increases the plant's sensitivity to the insect herbivore *Manduca sexta*. This
33 behaviour has been correlated with a decrease in the level of herbivore-induced terpenes and
34 phenolics (chlorogenic acid and rutin), caused by a lower level of jasmonic acid isoleucine conjugate
35 (JA-Ile), an important mediator of the induced resistance to herbivores [75]. In tomato, however, the
36 *dpy* mutant lacking in BR biosynthesis has a higher trichome density and accumulates the defensive
37 compound zingiberene. A recent comparative characterization of the JA defective mutant *jai1-1* and
38 the double mutant *dpy/jai1-1*, suggests that BR could act as a negative regulator upstream of the JA
39 signalling pathway [74]. Moreover, other studies using Arabidopsis BR mutants also propose a more
40 direct link between BR and the flavonoid pathway. These proposals are based on the following
41 evidence: (i) the expression of flavonoid biosynthesis genes is perturbed in BR mutants and (ii) a
42 mutation in a dihydroflavonol-4-reductase like protein controls the brassinosteroid levels [76, 77].
43 From these considerations, it is tempting to hypothesize that the atypical concentrations of phenolics
44 and their distribution in the vegetative parts of Micro-Tom could relate to a reduced level of BR. A
45 specific study of BR and phenolic contents in Near Isogenic Lines of Micro-Tom harbouring wild type
46 allele of *dwarf* [78] would be necessary to test this hypothesis in order to contribute to a better
47 understanding of the link between brassinosteroid and phenolic accumulations. Apart from these
48 considerations, phenolics are well-known defensive compounds against pathogens. It should be
49 considered, however, that the observed differences in phenolic concentration and distribution might
50 render Micro-Tom atypical of other cvs in its sensitivity to pests. Although few data are available for
51 herbivores, the responses of Micro-Tom to several pathogens have been established, which indicate

1 sensitivity to the main tomato pathogens except *Fusarium oxysporum* for which resistance is
2 conferred by the *Immunity to Fusarium Wilt* mutation [79]. In this study, however, the kinetics of the
3 disease development was not compared with those of other cvs. Hence, the natural high
4 concentration of phenolics in Micro-Tom (in leaves and stems) might have influenced the sensitivity
5 threshold or disease development. From the current evidence available, researchers should resist the
6 temptation of simply extrapolating the plant-pathogen results obtained from Micro-Tom to
7 commercial cultivars.

8 *3.3 N limitation affects the accumulation of phenolics differently*

9
10 For the 9 cultivars tested, the N limitation treatment (LN) led to growth limitation and morphological
11 modification. These observations conformed to those of the literature [19, 36, 80] and have been
12 already extensively discussed in [17]. Briefly, LN led to a reduction in leaf and stem biomass, whereas
13 root biomass was unaffected. Total leaf area, leaf area ratio and specific leaf area were also reduced.
14 The number of flower was not affected.

15
16 Regarding phenolics, LN significantly increased the leaf concentration of almost all hydroxycinnamoyl
17 esters in both determinate and indeterminate cultivars (p from 0.05 to 10^{-15} ; Fig. 4a, Supplementary
18 Table 1). The concentrations of these compounds were increased by 1.2 to 4 fold (mean value $1.9 \pm$
19 0.7), depending on the cvs and molecules. In some cases this increase was not significant (FHA1 in cvs
20 B and C, CHA2 in indeterminate cvs and CQA3 in cv A) due to large standard deviations in the
21 analytical data. SG was the only phenolic whose concentration was not affected by N availability ($p >$
22 0.2).

23
24 In stems, the inducible effect of LN on the hydroxycinnamoyl ester concentrations was not so clear-
25 cut (Fig. 4b, Supplementary Table 1). Indeed, for most determinate cvs, LN was without significant
26 impact on the concentrations of SG, CQA1, CHA4 and pCoQA1. By contrast, the concentrations of
27 CHA3, CQA2, CQA3 and FQA increased under LN to the same degree as in leaves.

28
29 In roots, the hydroxycinnamoyl ester concentrations were increased by 1.2 to 6 fold (mean value 2.6
30 ± 1.1) under LN, rendering roots the most sensitive organ to LN in respect of inducing accumulation
31 of this phenolic family (Fig. 4c; Supplementary Table 1). This observation confirms our previous
32 report initially restricted to CQA2 [17].

33
34 Leaf flavonoid concentrations were increased under LN on average by 2.5 ± 0.6 fold (Fig. 4a), except
35 for KRG, which was unaffected. In stems, however, LN significantly increased only the concentration
36 of quercetin derivatives (AR and R; p from 0.05 to 10^{-15}) whereas kaempferol derivatives (KR, KRG)
37 were unaffected.

38
39 LN also increased the anthocyanin concentrations in leaves and stems (Fig. 4ab). This effects was
40 significant in the stems of all cvs and in the leaves of determinate cvs (except PCRG in cv A) but in the
41 leaves of the indeterminate cvs this increase was below the level of significance.

42
43 For all cvs, the LN treatment decreased CP concentrations in the roots and stems by a factor of $2.6 \pm$
44 0.5 and 2.5 ± 0.7 , respectively (Fig. 4bc; Supplementary Table 1). The decrease was always significant
45 except for the stems of indeterminate cvs where measurement yielded high standard deviations,
46 which may have hidden a significant effect. LN also significantly decreased CP concentrations in the
47 leaves of cvs A, B, C, E and H, on average by a factor of 2.2 ± 0.2 ($p < 0.05$). CoT and FT concentrations
48 were also significantly reduced in the roots of all cvs ($p < 0.05$). The flower-specific phenolamides
49 DCoS and TCoS1-2, which were found in the stem extracts, were not affected by LN.
50 Hydroxycinnamic spermidine derivatives are essential pollen constituents [63] and as we reported
51 earlier, LN did not affect the number of flower produced [17]. This result therefore supports the
52 concept that plants maintain their reproductive capacity even under abiotic constrains such as
53 nitrogen limitation. Similar behaviour has been demonstrated for spermidine derivatives in the
54 flowers of grape in response to N deficiency [81].

1 Most changes of phenolic concentrations reported in the vegetative organs of the nine tomato cvs in
2 response to LN are in accordance with the main trophic theories notably the growth differentiation
3 balance hypothesis (GDBH) and the carbon nutrient balance (CNB), which postulate that under
4 growth limiting conditions, C fixed in excess of growth requirement is directed toward C-rich
5 secondary metabolism [82, 83]. Indeed, under LN the C-rich compounds (hydroxycinnamoyl esters,
6 flavonoids and anthocyanins) accumulated in all the cultivars (Fig. 5). The response of phenolamides
7 to N availability, however, is more complex since these molecules contain a C-rich moiety originating
8 from the phenylpropanoid pathway and a N-rich part, i.e. a polyamine (putrescine, tyramine) from
9 amino acid degradation (arginine, tyrosine). The LN treatment undoubtedly increased the
10 concentration of caffeoyl substrates (Fig.4, CHA1-5 and CQA1-3) but the question remains as to
11 whether it decreased that of polyamines. These compounds were not analysed in this experiment
12 and there are contradictory reports in the literature concerning their response to N [81, 84-86]. It
13 would be worth investigating whether any polyamine concentration changes result from mere
14 changes in C- and N- based substrate concentrations or from specific regulation in the pathways
15 leading to phenolamide syntheses. For instance it could be possible to follow the expression level of
16 phenolamide specific genes (Fig. 5). The enzymatic steps leading to the synthesis of caffeoyl
17 putrescine and caffeoyl tyramine have previously been described in tomato. They involve enzymes,
18 belonging to the transferase family, that are able to transfer the acyl moiety from caffeoylCoA to
19 putrescine (putrescine/hydroxycinnamoyl transferase, PHT) or tyramine (tyramine hydroxycinnamoyl
20 transferase, THT). Four different THT cDNA have been isolated from tomato [87]. To date, however,
21 no PHT cDNA has been identified in tomato, even though a first PHT cDNA has recently been
22 identified in *N. attenuata* [88].

26 In brief, the LN treatment resulted in a global increase in the concentration of phenolics, but with
27 large composition changes, notably a decrease of the phenolamide proportion (Fig. 5). This phenolic
28 class was previously shown to participate in plant defence and especially to be part of the inducible
29 response to pathogens [60, 39]. From a plant-defence viewpoint, an interesting perspective of this
30 work would be to establish whether, and if so to what extent, such a modification of the phenolic
31 concentration and composition in the tomato vegetative organs could influence plant susceptibility
32 to a range of diverse pathogens.

35 **4. Conclusion**

36 The present study led to the identification of 31 phenolics, 5 of which are described for the first time
37 in the vegetative organs of tomato. Comparative analysis highlighted that even though the nine
38 cultivars shared similar phenolic composition in the three vegetative organs, Micro-Tom differed
39 markedly in its phenolic concentrations and their distribution in leaves and stems. With few
40 exceptions, the impact of the LN treatment on the three organs of all cultivars was to increase the
41 concentrations of hydroxycinnamoyl esters, flavonoids and anthocyanins and to decrease the
42 concentrations of phenolamides. The benefit of such phenolic redistribution within organs and the
43 whole plant in relation to plant resistance to predators needs to be evaluated in order to assess the
44 importance of N fertilization as an efficient agronomic leverage in the control of pest infestation.

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Figure Legends

Figure 1: **Typical HPLC chromatograms of soluble phenolics from vegetative organs of tomato (cv. Micro-Tom) grown under HN.** The profiles were recorded at 300 nm and expressed as relative absorbance to the highest peak of the chromatogram which was peak 13 in leaves and stems and peak T in roots. Peak numbers match those of Table 2.

Figure 2: **Concentrations ($\mu\text{g}\cdot\text{g}^{-1}$ DW, log scale) of the identified phenolics in vegetative organs of HN tomatoes.** Horizontal bars represent the range of mean values between the lowest and the highest concentrated cultivars for each molecule and each organ. Metabolite abbreviations are summarized in table 2. Notice that a few compounds were not plotted because they were detected only at trace levels: CMA, QRG, CCoRG, DCoRG2 in addition to DCoRG1 in leaves, CHA3-4 and KR in roots, CHA5 and pCoQA2 in stems.

Figure 3: **Canonical scores of the first canonical discriminant functions for organ phenolic concentrations in nine tomato cultivars coded with letters, as follows :** A Micro-Tom; B Red Robin; C Tiny Tim; D Florida Basket; E Pixie II; F Totem; G Husky Cherry Gold; H Husky Cherry Red; I Better Bush.

Figure 4: **Phenolic concentrations ($\mu\text{g}\cdot\text{g}^{-1}$ DW) of HN tomato cultivars plotted against LN, in leaves (a), stems (b) and roots (c).** Metabolite abbreviations are summarized in table 2. The diagonals (solid lines) indicate where LN = HN. It follows that compounds above the diagonal have lower concentration under LN than under HN.

Figure 5: **Proposed scheme of the impact of HN and LN on the concentrations of C-based and C/N-based phenolics and their precursors in plant organs.** A Decrease in amino acid contents and an induction of the biosynthetic pathway to C-based phenolics under LN condition has already been described [16]. The present paper demonstrates that C-based phenolics *i.e.* hydroxycinnamoyl esters, flavonoids and anthocyanins accumulate under LN conditions whereas C/N-based phenolics *i.e.* phenolamides are depleted at the same time. Two non-exclusive hypotheses are proposed to explain this effect. First (h1), LN conditions result in a decrease of putrescine and tyramine contents, two precursors of phenolamides, second (h2) the final biosynthetic step leading to phenolamide is down-regulated. THT, Tyramine hydroxycinnamoyl transferase; PHT, Putrescine hydroxycinnamoyl transferase. Metabolite pools under HN are presented in dotted circles with solid lines. Metabolite pools under LN are in white circles with dashed lines.

Tables

	Code	Cultivar	Average plant size (from breeder resources)	Breeder
Determinate	A	Micro-Tom	15-20 cm	University of Florida, Bradenton
	B	Red Robin	20-30 cm	Burpee/seminis
	C	Tiny Tim	25-35 cm	University of New Hampshire, Durham
	D	Florida Basket	30-45 cm	University of Florida, Bradenton
	E	Pixie II (F1)	45 cm	Burpee/seminis
	F	Totem (F1)	60 cm	Floranova/Vegetalis
Indeterminate	G	Husky Cherry Gold (F1)	90-120 cm	Petoseed
	H	Husky Cherry Red (F1)	90-120 cm	Petoseed
	I	Better Bush	90-120 cm	Park seed

Table 1: **Patio tomato cultivars with codes used in text and figures.** The coding matches that found in [17]. The alphabetical order denotes increasing whole plant dry biomass at harvest. The phenotypic characteristics are those of the breeders' technical resources.

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<i>Rt</i>	<i>Peak</i>	<i>λmax (nm)</i>	<i>[M+H]⁺</i>	<i>[M-H]</i>	<i>MS/MS [M+H]⁺</i>	<i>MS/MS [M-H]</i>	<i>Calculated Mass</i>	<i>Formula</i>	<i>Δppm</i>	<i>Proposed molecule</i>	<i>abbreviation</i>	<i>Organ</i>	<i>References</i>
6.05	1	300sh, 326	373.0753	371.0608	163 (100), 329 (7), 337 (5)	209(100), 191 (60)	372.0681	C ₁₅ H ₁₆ O ₁₁	3.121	Caffeoyl hexaric acid	CHA1	L	41, 46
8.73	2	300sh, 326	373.0753	371.0608	163 (100), 329 (7), 337 (5)	209(100), 191 (60)	372.0681	C ₁₅ H ₁₆ O ₁₁	3.121	Caffeoyl hexaric acid	CHA2	L	41, 46
13.62	3	300sh, 326	373.0753	371.0608	163 (100), 329 (7), 337 (5)	209(100), 191 (26)	372.0681	C ₁₅ H ₁₆ O ₁₁	3.121	Caffeoyl hexaric acid	CHA3	L, S, R	41, 46
31.32	4	300sh, 326	373.0753	371.0608	163 (100), 329 (7), 337 (5)	209(100), 191 (60)	372.0681	C ₁₅ H ₁₆ O ₁₁	3.121	Caffeoyl hexaric acid	CHA4	L, S, R	41, 46
17.13	5	300sh, 325	355.1013	353.0867	163 (100), 145 (5), 267 (8), 319 (8)	191 (100), 179 (65), 135 (26)	354.0940	C ₁₆ H ₁₈ O ₉	3.056	Neochlorogenic acid (3 caffeoyl quinic acid)	CQA1	L, S	49
17.91	6	300sh, 321	251.1382	249.124	163 (100), 234 (5), 89 (14)	155 (100), 205 (71), 135 (11)	250.1311	C ₁₃ H ₁₈ N ₂ O ₃	2.568	Caffeoyl putrescine (std)	CP	L, S, R	56
18.93	7	300sh, 326	373.0753	371.0608	163 (100), 177 (40), 307 (20), 337 (20)	285 (100), 209(60), 191 (40)	372.0681	C ₁₅ H ₁₆ O ₁₁	3.121	Caffeoyl hexaric acid	CHA5	L, S	41, 46
20.02	8	300sh, 321	309.1436	307.1294	147 (100), 130 (35), 163 (17), 246 (14)	135 (100), 262 (50), 71 (30)	308.1365	C ₁₅ H ₂₀ N ₂ O ₅	2.342	Caffeoyl derivative	Caf Der	S, R	
21.37	9	300sh, 327	387.0912	385.0767	177 (100), 225(90), 207 (80), 343 (50)	249 (100), 291 (70), 191 (71), 209 (50)	386.0840	C ₁₆ H ₁₈ O ₁₁	2.361	Feruloyl hexaric acid	FHA1	L	45
23.05	10	300sh, 327	387.0912	385.0767	177 (100), 225(90), 207 (80), 343 (50)	249 (100), 291 (70), 191 (71), 209 (50)	386.0840	C ₁₆ H ₁₈ O ₁₁	2.361	Feruloyl hexaric acid	FHA2	L	45
23.45	11	300sh, 313	954.3453	952.3276	322 (100), 794 (30), 303 4 (20), 920 (40)	812 (100), 692 (100), 589 (100), 487 (100)	953.3365					R	
25.23	12	300sh, 313	984.3569	982.3376	322 (100), 824 (50), 950 (50), 304 (20)	842 (50), 722 (90), 602 (100), 457 (60)	983.3473					R	
26.41	13	300sh, 324	355.1013	353.0867	163 (100), 145 (5)	191 (100), 179 (2)	354.094	C ₁₆ H ₁₈ O ₉	3.056	Chlorogenic acid (5 caffeoyl quinic acid) (std)	CQA2	L, S, R	49
28.63	14	300sh, 319	323.1592	321.1451	177 (100), 145 (8), 260 (8)	276(100), 175 (55)	322.1522	C ₁₆ H ₂₂ N ₂ O ₅	2.085	Feruloyl derivative	Fer Der	S, R	
28.71	15	300sh, 324	387.1275	385.1131	207 (100)	223 (100), 205 (83), 247 (56), 191 (46), 190 (26)	386.1203	C ₁₇ H ₂₂ O ₁₀	2.582	Sinapoyl glucoside	SG	L, S	52
30.08	16	300sh, 324	355.1013	353.0867	163 (100), 145 (4)	191 (100)	354.0940	C ₁₆ H ₁₈ O ₉	3.056	Chlorogenic acid isomer	CQA3	L, S, R	49

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6	32.84	17	257, 356	773.2139	771.1975	465 (100), 303 (53), 611 (35)	609 (100)	772.2057	C ₃₃ H ₄₀ O ₂₁	0.658	Quercetin-3-O-rutinoside-7-O-glucoside	QRG	L, S	66
8	33.1	18	300sh, 325	339.1065	337.0926	147 (100)	191 (100), 179 (10)	338.0996	C ₁₆ H ₁₈ O ₈	1.679	Coumaroyl quinic acid	pCoQA1	L, S	38
10	33.4	19	298, 330	297.0596	295.0457	163 (100), 265 (98), 251 (57)	133 (100), 179 (100), 250 (58)	296.0527	C ₁₃ H ₁₂ O ₈	1.748	Caffeoyl malic acid	CMA	L	53
12	35.16	20	300sh, 325	369.1172	367.1025	177 (100), 145 (10), 207 (2)	191 (100), 193 (5), 150 (3), 135 (2)	368.1099	C ₁₇ H ₂₀ O ₉	2.261	Feruloyl quinic acid	FQA	L, S, R	38
15	35.52	21	300sh, 325	339.1065	337.0926	147 (100)	191 (100), 179 (10)	338.0996	C ₁₆ H ₁₈ O ₈	1.679	Coumaroyl quinic acid	pCoQA2	L, S	38
18	37.03	22	266, 346	757.2185	755.2027	449 (100), 287 (62), 611 (31), 595 (31)	593 (100), 717 (66), 624 (50), 509 (40)	756.2106	C ₃₃ H ₄₀ O ₂₀	0.917	Kaempferol 3-O-rutinoside-7-O-glucoside	KRG	L	65
20	38.59	T	237/287	305.0648							Taxifoline internal standard			
23	44.88	23	257, 355	743.2023	741.1864	303 (100), 465 (15), 597 (7), 611 (14)	300 (100), 609 (60), 595 (25)	742.1944	C ₃₂ H ₃₈ O ₂₀	1.676	Quercetin 3-(2"-Apiosyl-6"-rhamnosylglucoside)	AR	L, S	38, 67
25	46.21	24	300sh, 326	337.1846	335.1698	210 (100), 175 (60)	173 (100)	336.1772					R	
28	46.38	25	299	438.2377	436.2231	147 (23), 204 (100), 218 (13), 275 (13), 292 (52)	316 (100), 249 (40)	437.2304	C ₂₅ H ₃₁ N ₃ O ₄	2.416	Dicoumaroyl spermidine (std)	DCoS	S	59
31	47.2	26	296	359.1592	357.1448	313 (100), 179 (30), 134 (9)	135 (100), 190 (20)	358.1520					S	
33	49.06	27	254, 352	611.1604	609.1436	303 (100), 465 (27)	454 (100), 300 (80)	610.1520	C ₂₇ H ₃₀ O ₁₆	2.27	Rutin (std)	R	L, S	67
35	50.24	28	295, 305	284.1271	282.1125	147 (100)	119 (100), 145 (70)	283.1198	C ₁₇ H ₁₇ NO ₃	3.685	Coumaroyl tyramine	CoT	R	39
38	51.62	29	310sh, 330	949.2614	947.2446	303 (100), 465 (20), 787 (10)	-	948.2530	C ₄₃ H ₄₈ O ₂₄	0.583	Petunidin-3-O-(caffeoyl)rutinoside-5-O-glucoside	PCRG	L, S	32, 70
40	51.83	30	285,315	314.1377	312.123	177 (100), 145 (15)	178 (100), 135 (30)	313.1304	C ₁₈ H ₁₈ NO ₄	3.219	Feruloyl tyramine	FT	R	39
42	53.39	31	310sh, 334	919.2509	917.2336	757 (100), 303 (75)	917 (100)	918.2423	C ₄₂ H ₄₆ O ₂₃	0.749	Delphinidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	DCoRG1	L, S	32, 70

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6	54.1	32	349	595.1653	593.1492	287 (100), 449 (18)	285 (100), 327 (10)	594.15725	C ₂₇ H ₃₀ O ₁₅	1.97	Kaempferol rutinoside (std)	KR	L, S, R	
7	54.6	33	300sh, 334	933.2667	931.2495	771 (100), 317 (65), 479 (60)	-	932.2581	C ₄₃ H ₄₈ O ₂₃	0.577	petunidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	PCoRG	L	32, 70
10	56.33	34	310sh, 327	903.2556	901.2384	741 (100), 725 (40)	694 (100), 771 (80)	902.2470	C ₄₂ H ₄₆ O ₂₂	1.189	Cyanidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	CCoRG	L, S	71
13	58.6	35	310sh, 330	919.2509	917.2336	303 (100), 611 (33), 757 (32), 465 (15)	-	918.2423	C ₄₂ H ₄₆ O ₂₃	0.749	Delphinidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	DCoRG2	L	32, 70
15	59.45	36	294, 310	584.2748	582.2597	420 (100), 438 (91), 275 (10), 204 (5)	462 (100), 549 (50), 356 (30)	583.2673	C ₃₄ H ₃₇ N ₃ O ₇	1.605	Tricoumaroyl spermidine (std)	TCoS1	S	59
18	60.92	37	294, 310	584.2748	582.2597	420 (100), 438 (91), 275 (10), 204 (5)	462 (100), 549 (50), 356 (30)	583.2673	C ₃₄ H ₃₇ N ₃ O ₇	1.605	Tricoumaroyl spermidine (std)	TCoS2	S	59
21	66.92	38	315	309.2053	307.1907	273 (100)	236 (100), 271 (50), 235 (30)	308.1980					L, S, R	
23	67.47	39	315	309.2053	307.1907	273 (100)	236 (100), 271 (50), 235 (30)	308.1980					L, S, R	

Table 2: Metabolites putatively identified by HPLC- MSⁿ Orbitrap analysis in tomato vegetative organs (Leaf, L; Stem, S; Root, R). Peak number as in Fig. 1. λ_{max} is the absorbance maxima in the UV/visible range. sh, shoulder; the reported fragments were observed in source and verified through MS/MS analysis of the parental ion in both negative and positive mode. Abundance of each fragment is indicated between brackets; Δ ppm, deviation of the observed ion mass from the corresponding calculated monoisotopic mass. (std) indicates identification based on standard compound. The reference column relates previous reports on metabolites in different plants.

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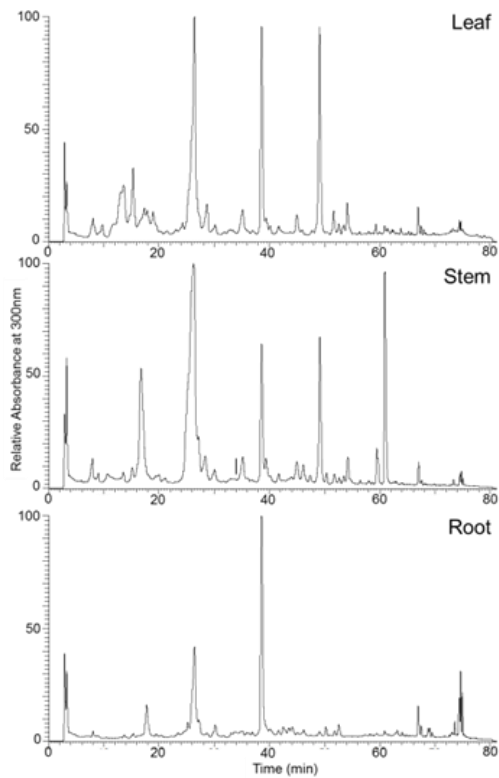
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Phenolic characterization and variability in leaves, stems and roots of Micro-Tom and patio tomatoes, in response to nitrogen limitation

Romain Larbat^{*}, Cédric Paris, Jacques Le Bot and Stéphane Adamowicz



Phenolics contribute to plant defence. By the measurement of phenolic composition in leaves, stems and roots of patio tomatoes, we show that nitrogen limitation increases the total concentration and modifies the phenolic composition in these organs.

Figure 1

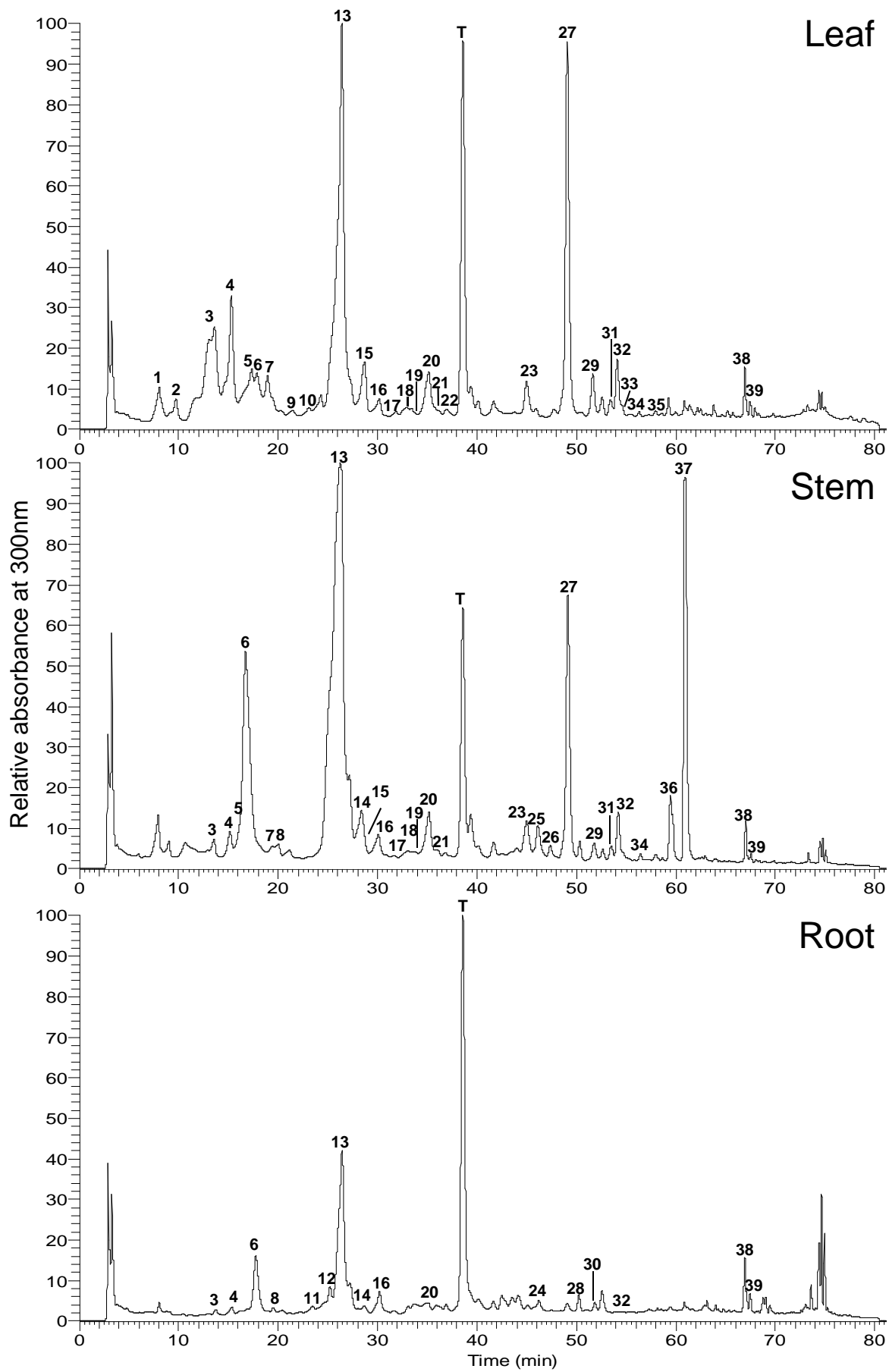


Figure 2

leaves
 stems
 roots

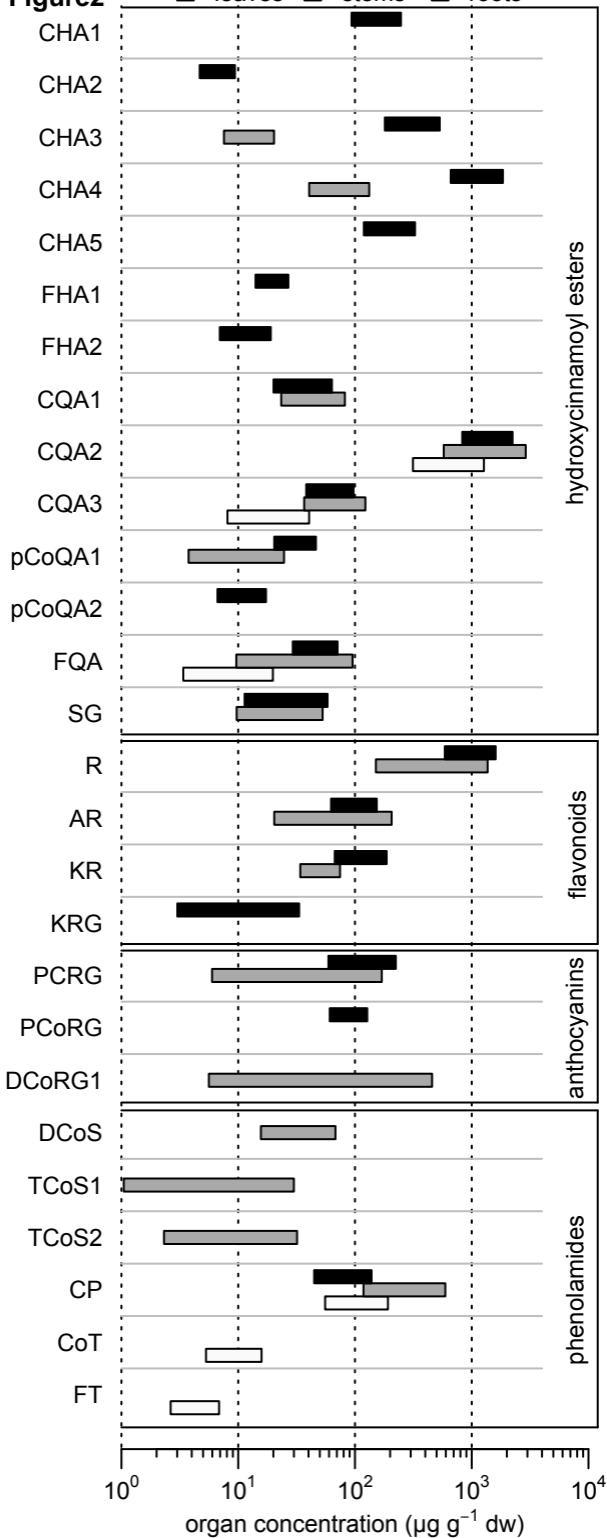


Figure3

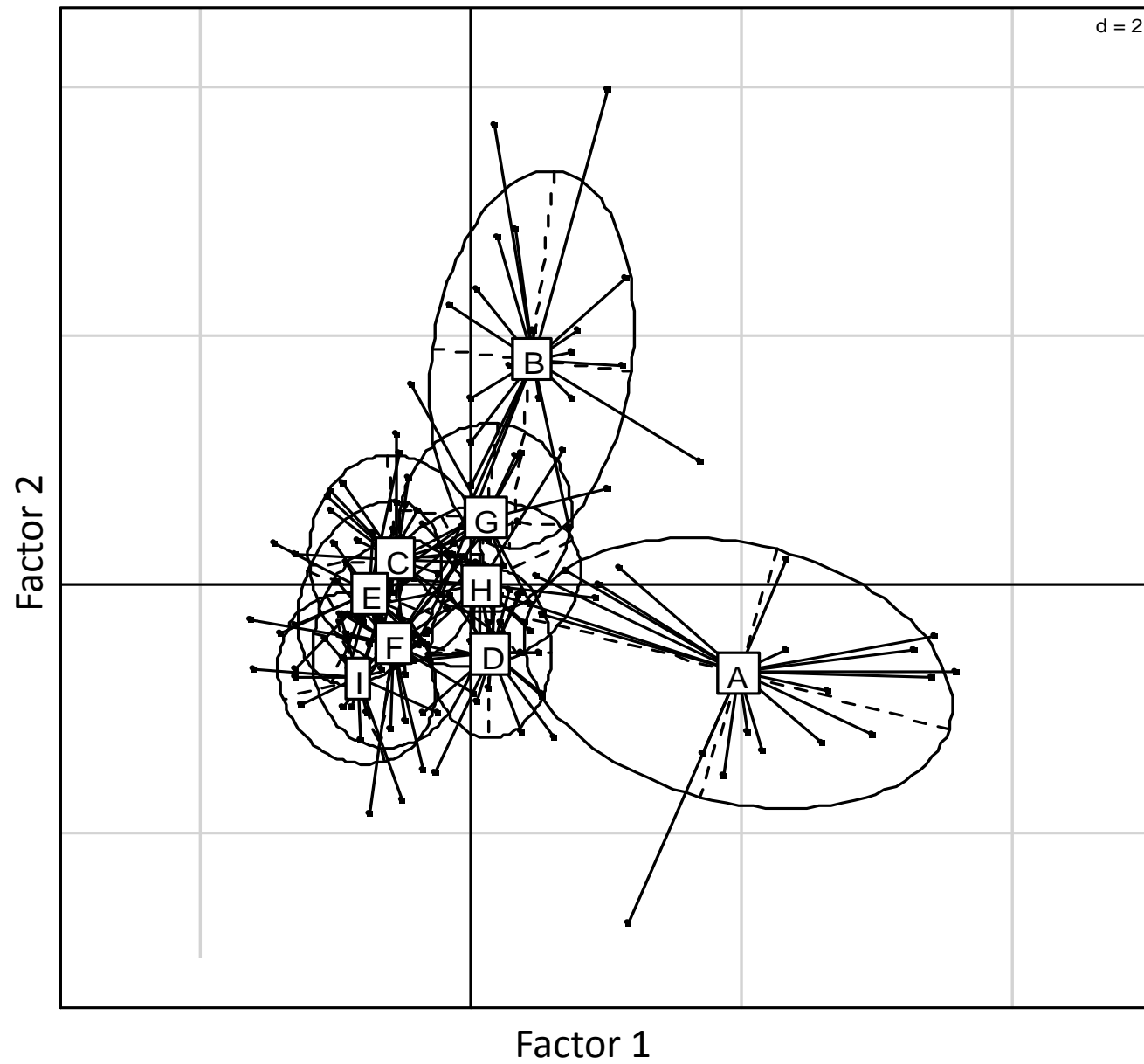


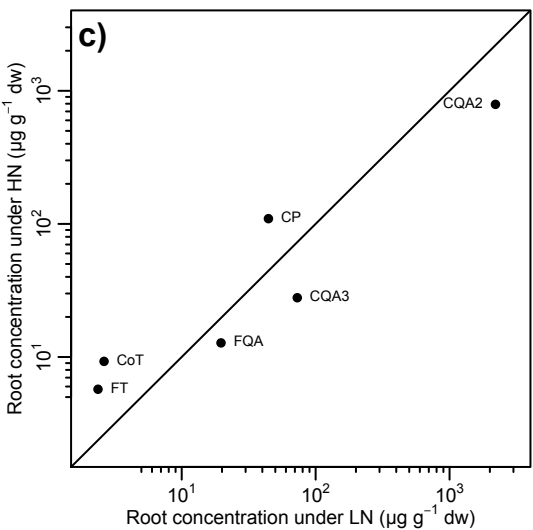
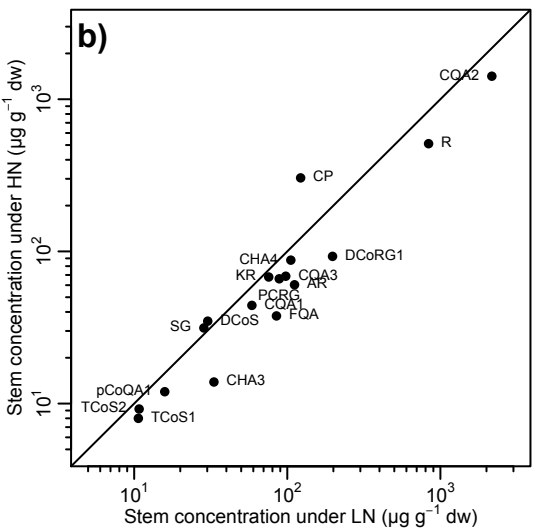
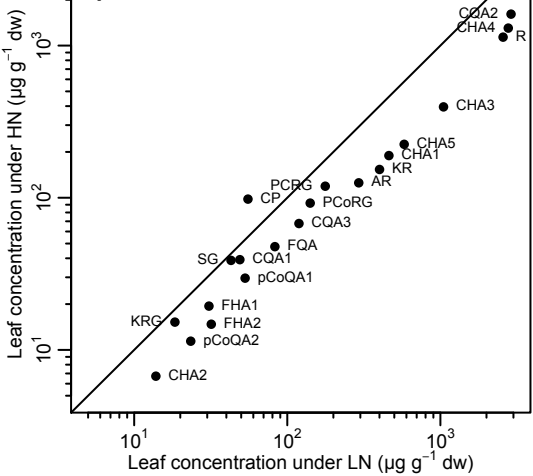
Figure 4

Figure 5

