Untargeted UHPLC-HR-QTOF-MS metabolomics study to unravel metabolites controlling wood formation in aspen trees

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

UNIVERSITY OF COPENHAGEN

ASMS 2020, WP 444

Magdalene Reinkensmeier[1], Ilara G.F. Budzinski[2], Thomas Moritz[3,4]

[1]Bruker Daltonics, Bremen, Germany
[2]Umeå Plant Science Centre, Department of Plant Physiology, Swedish University of Agricultural Sciences, Umeå, Sweden
[3]Swedish Metabolomics Centre, Swedish University of Agricultural Sciences, Umeå, Sweden
[4]Novo Nordisk Foundation Centre for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark

Introduction

Plants as sessile organisms have to cope with the environmental conditions in their inhabited soil, e.g. the existing amount of water and nutrients. The available resources must be allocated to growth and the handling of biotic challenges, such as the defense against predators.

Secondary growth in trees is of great economic importance as it results in the production of wood. The developmental process of wood takes place in the vascular cambium. Cambium activity results in the regular production of xylem and phloem, in opposite directions, where xylem having a role in water transport and tree stability, phloem has a role in sugar transport. Wood formation is a dynamic and continuous process, strongly affected by environmental factors such as water, nutrient, and light availability.

We investigated the impact of variations in nitrogen fertilization on the growth and the metabolite profiles of xylem and phloem of aspen trees using untargeted LC-HR-QTOF-MS metabolomics.

Within the untargeted approach we have put a focus on phenolic glucosides, the salicylates, which are major defense secondary metabolites of aspen and other *Salicaceae* species.

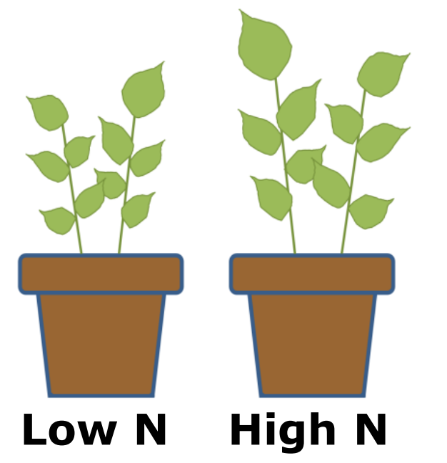
Methods

Cuttings of hybrid aspen *(Populus tremula* x *P. tremuloides)* were cultivated in a greenhouse. After two months the trees were treated with low or high nitrogen fertilizer three times per week for two months. At the harvest stem segments were sampled, flash-frozen in liquid nitrogen, divided into xylem and phloem samples with scraping, and thereafter stored at -80℃. Samples (10 mg each) were extracted according to Gullberg et al. 2004 [1].

Samples were analyzed with the Elute UHPLC (ACQUITY UPLC HSS T3, 2.1 x 50 mm, 1.8 µm, C18 column and 2.1 mm x 5 mm, 1.8 µm, VanGuard Pre-column (Waters, MA, USA) coupled to the impact II HR-QTOF-MS (Bruker Daltonics, MA, USA). Default metabolomics acquisition methods were used to collect full scan and fragment data in electrospray positive and negative ion mode. MetaboScape® 5.0 was used for comprehensive feature finding with the T-ReX® 3D algorithm, feature annotation, statistical data evaluation, and pathway mapping. Analyte lists created from in-house databases were used to annotate target compounds (including e.g. phenolic glucosides, oligolignols, amino acids). Pathway maps were prepared in PathVisio 3.3.0.

Additional measurements with the timsTOF Pro (Bruker Daltonics, MA, USA) were done to collect ion mobility data.

Workflow



Low N **High N**
fertilization

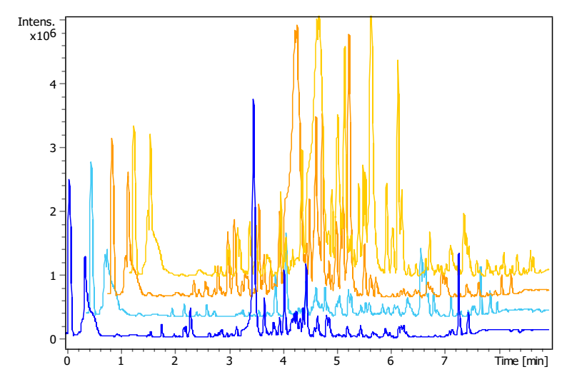
Harvest

Phloem

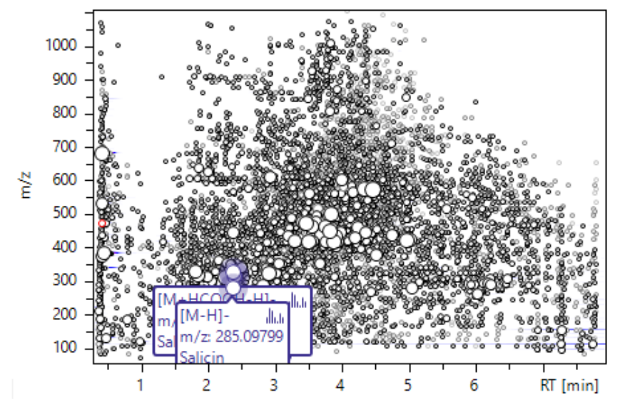
Xylem

Sample prep

Data acquisition



Chromatograms of the four groups (negative ion mode).



Features picked with TReX® 3D (negative ion mode).

LC-MS/MS

- C18 column
- ESI, +/- ion mode
- Default Metabolomics methods

Data pre-processing in MetaboScape®

- Feature finding (T-ReX® 3D)
- Recursive feature extraction
- Automatic recalibration
- Non-linear RT-alignment
- Isotope and adduct clustering
- Merge of pos & neg ion mode date

Data evaluation in MetaboScape®

Feature annotation

- Target Analyte Lists
- Spectral library search
- CompoundCrawler+MetFrag

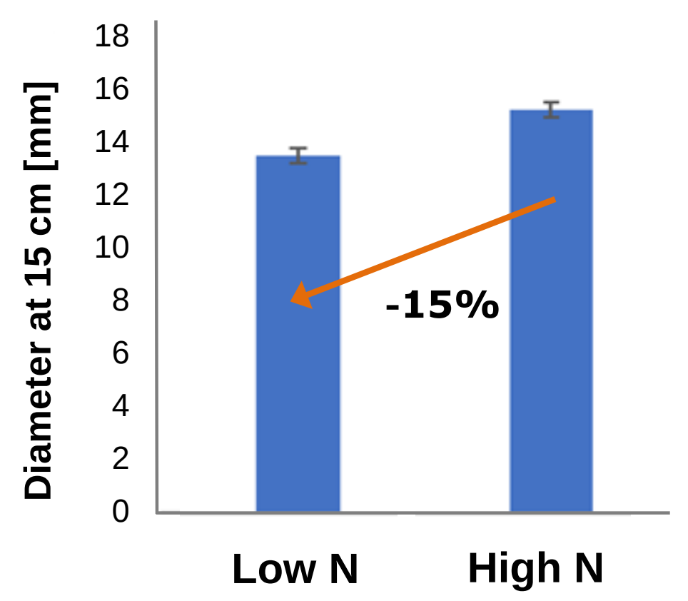
Statistical evaluation

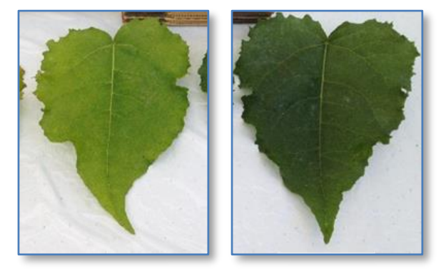
- PCA
- t-test

Pathway mapping

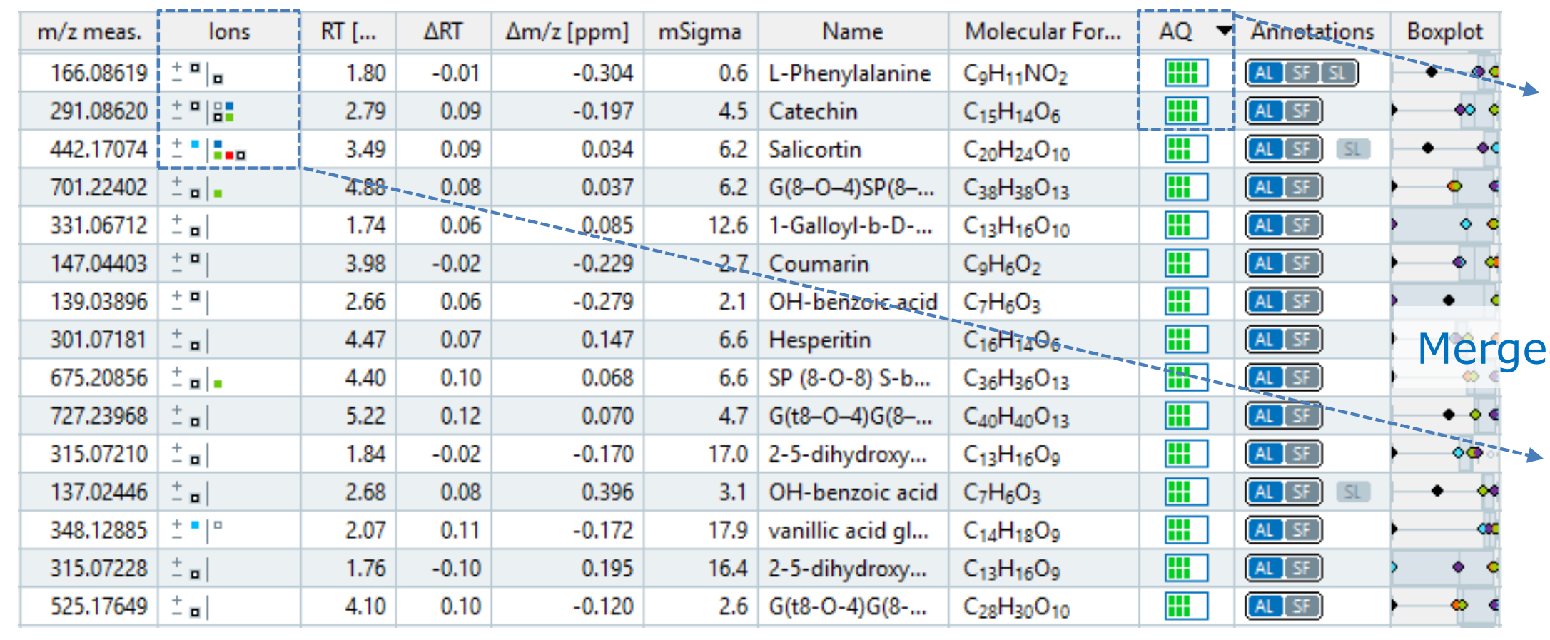
Results

→ Significant effects of different N fertilization on biomass



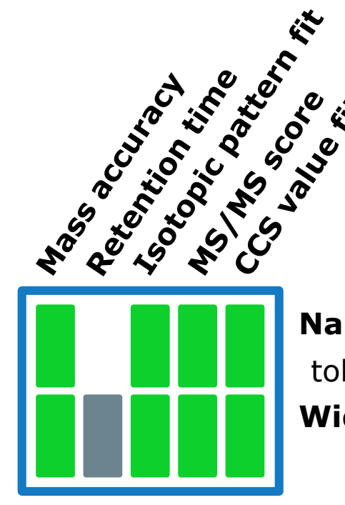


→ Reliable and comprehensible annotation



m/z meas.	Ions	RT [...	ΔRT	Δm/z [ppm]	mSigma	Name	Molecular For...	AQ	Annotations	Boxplot
166.08619		1.80	-0.01	-0.304	0.6	L-Phenylalanine	$C_9H_{11}NO_2$		AL SF SL	
291.08620		2.79	0.09	-0.197	4.5	Catechin	$C_{15}H_{14}O_6$		AL SF	
442.17074		3.49	0.09	0.034	6.2	Salicortin	$C_{20}H_{24}O_{10}$		AL SF SL	
701.22402		4.88	0.08	0.037	6.2	G(8–O–4)SP(8–...	$C_{38}H_{38}O_{13}$		AL SF	
331.06712		1.74	0.06	0.085	12.6	1-Galloyl-b-D-...	$C_{13}H_{16}O_{10}$		AL SF	
147.04403		3.98	-0.02	-0.229	2.7	Coumarin	$C_9H_6O_2$		AL SF	
139.03896		2.66	0.06	-0.279	2.1	OH-benzoic acid	$C_7H_6O_3$		AL SF	
301.07181		4.47	0.07	0.147	6.6	Hesperitin	$C_{16}H_{14}O_6$		AL SF	
675.20856		4.40	0.10	0.068	6.6	SP (8-O-8) S-b...	$C_{36}H_{36}O_{13}$		AL SF	
727.23968		5.22	0.12	0.070	4.7	G(t8–O–4)G(8–...	$C_{40}H_{40}O_{13}$		AL SF	
315.07210		1.84	-0.02	-0.170	17.0	2-5-dihydroxy...	$C_{13}H_{16}O_9$		AL SF	
137.02446		2.68	0.08	0.396	3.1	OH-benzoic acid	$C_7H_6O_3$		AL SF SL	
348.12885		2.07	0.11	-0.172	17.9	vanillic acid gl...	$C_{14}H_{18}O_9$		AL SF	
315.07228		1.76	-0.10	0.195	16.4	2-5-dihydroxy...	$C_{13}H_{16}O_9$		AL SF	
525.17649		4.10	0.10	-0.120	2.6	G(t8-O-4)G(8-...	$C_{28}H_{30}O_{10}$		AL SF	

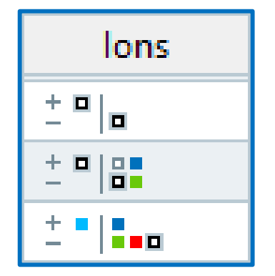
Excerpt of the annotated merged bucket table



Annotation Quality (AQ) symbol

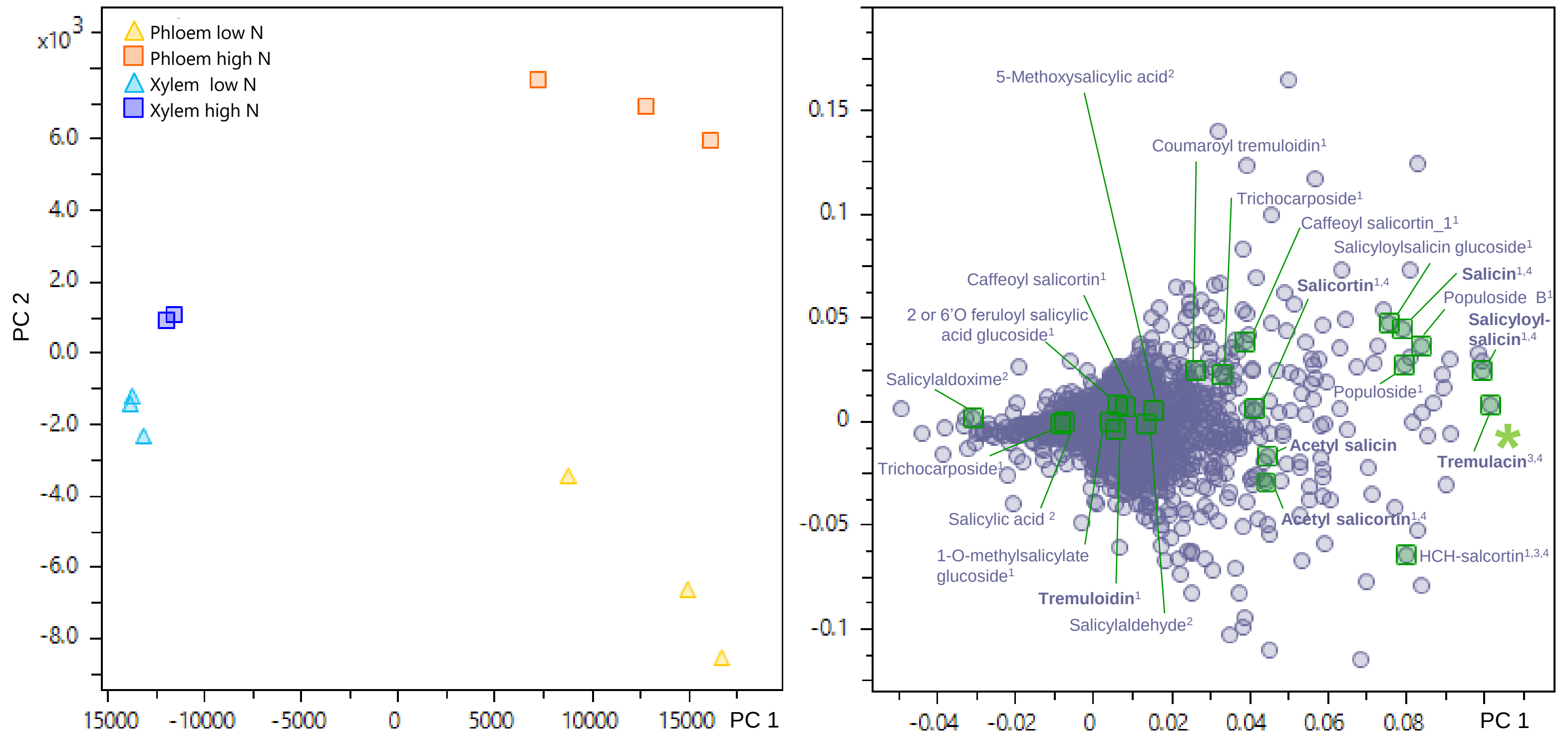
Narrow tolerances
Wide

Merged ion signals



- [M+H]+ or [M-H]-
- [M+Na]+
- [M+NH4]+
- [M+Cl]-
- [M+HCOOH-H]-
- [M+H-H₂O]+ or [M-H-H₂O]-

→ Considerable differences in the metabolite profiles

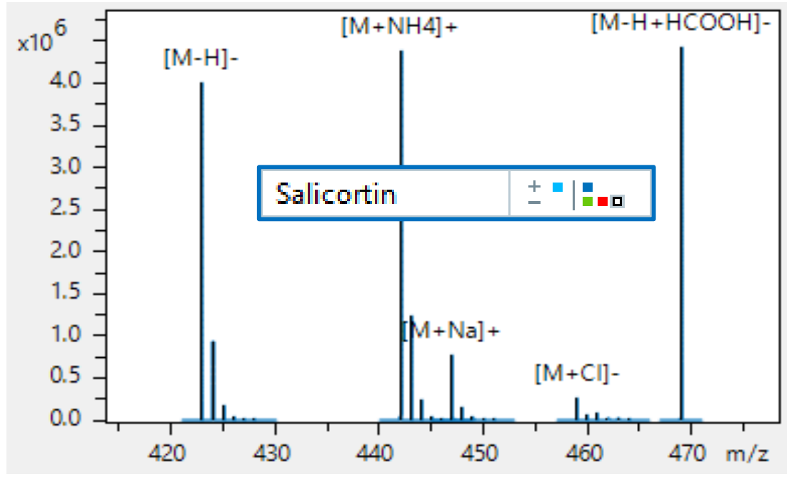


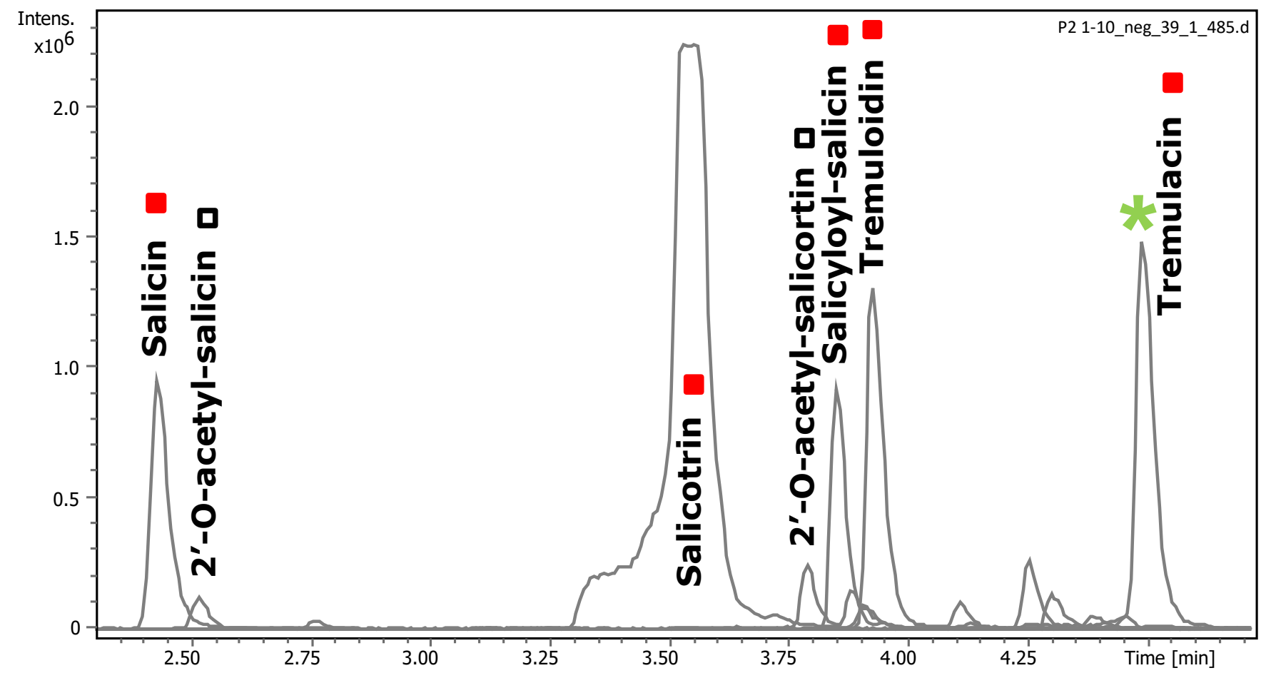
PCA scores (left) and loadings plot (right) for the merged metabolite profiling data (*N*=3 per group). Annotated salicylates and related compounds are named. Salicylates shown in the pathway maps are in bold.

The annotation is based on [1]a target Analyte List, [2]Spectral library search in the Bruker MetaboBase® Personal Library 3.0 and Bruker HMDB Metabolite Library 2.0, [3]SmartFormula3D +CompoundCrawler+MetFrag [2,3], [4]Comparison with published MS/MS spectra [4,5].

→ A closer look on salicylates

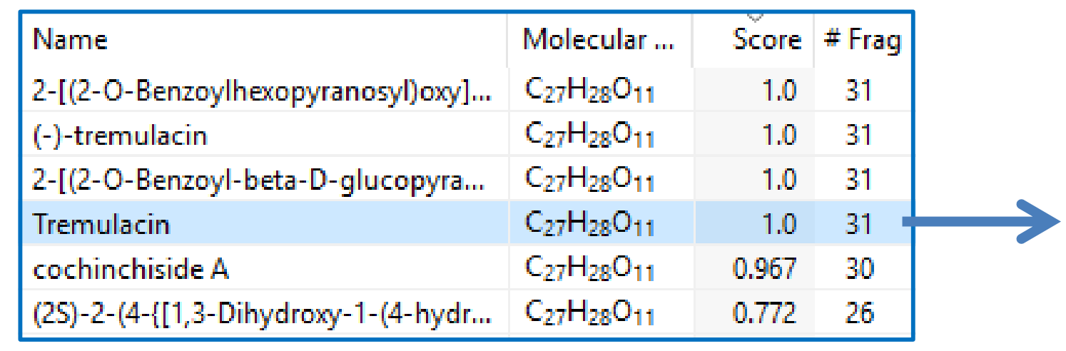
Salicylates were found in negative mode mainly as [M-H+HCOOH]- and [M-H]-. Some compounds like salicin or salicortin were also detected in positive ion mode. Below the full scan spectrum of salicortin is shown (merged positive and negative ion mode data) .





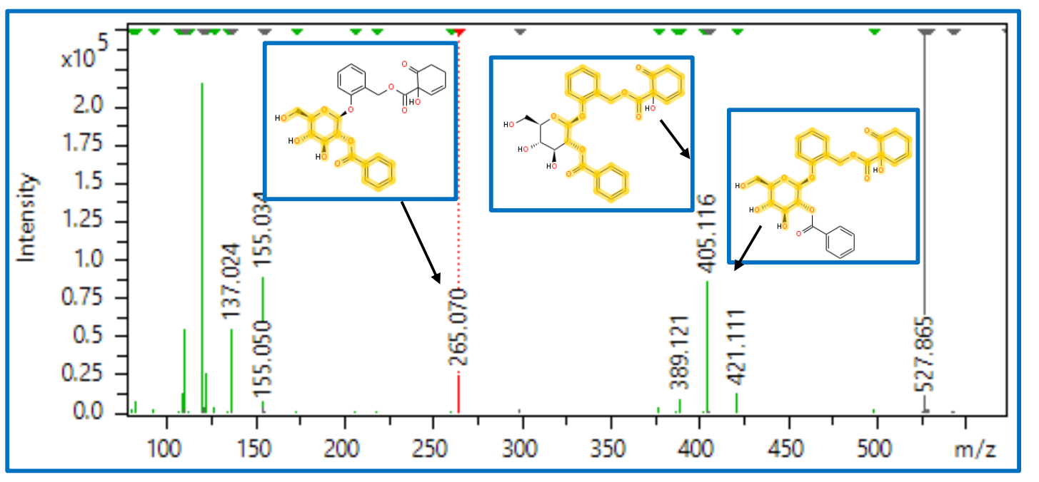
EICs (±0.005 Da) of several salicylates in negative ion mode (◘ [M-H]-, ■ [M+HCOOH-H]-).

The peak marked with ✱ showed a clear prominence in the PCA loadings plot. It was putatively annotated as **tremulacin** using the SmartFormula3D tool (search for elemental composition), CompoundCrawler (tool for the search of candidate structures in online data bases, e.g. ChEBI and ChemSpider), and *in-silico* MetFrag fragmentation [2,3] in MetaboScape® (see below).

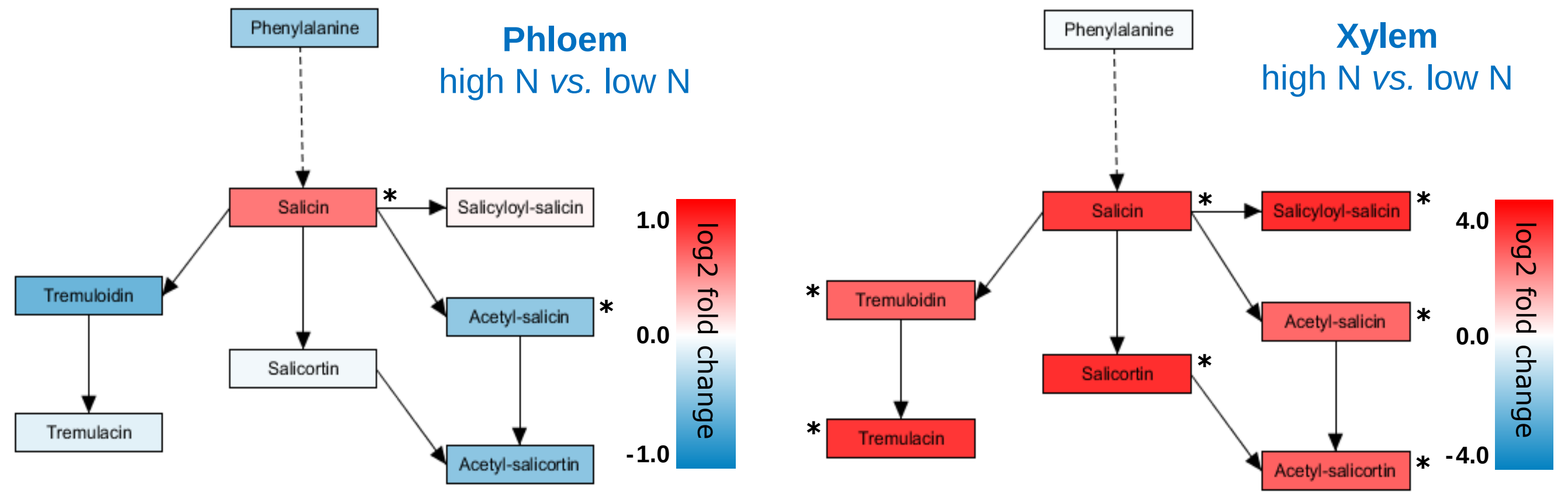


Name	Molecular ...	Score	# Frag
2-[(2-O-Benzoylhexopyranosyl)oxy]...	$C_{27}H_{28}O_{11}$	1.0	31
(-)-tremulacin	$C_{27}H_{28}O_{11}$	1.0	31
2-[(2-O-Benzoyl-beta-D-glucopyra...	$C_{27}H_{28}O_{11}$	1.0	31
Tremulacin	$C_{27}H_{28}O_{11}$	1.0	31
cochinchiside A	$C_{27}H_{28}O_{11}$	0.967	30
(2S)-2-(4-{[1,3-Dihydroxy-1-(4-hydr...	$C_{27}H_{28}O_{11}$	0.772	26

MetFrag result for the MS/MS spectrum on the right; candidates sorted by score.



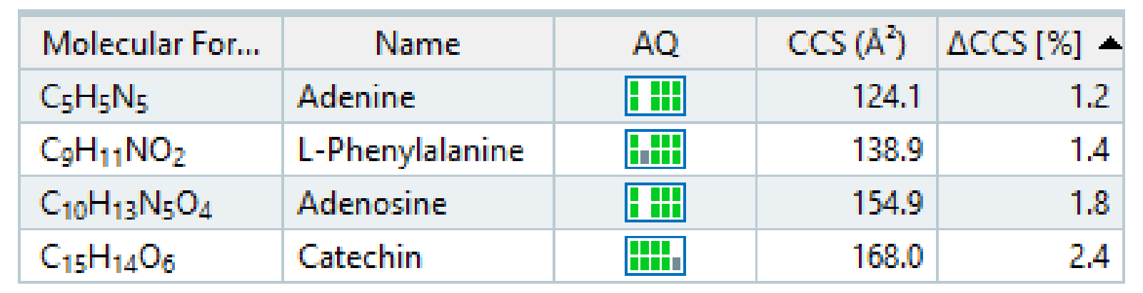
→ Pathway mapping illustrate cell type specific metabolic response



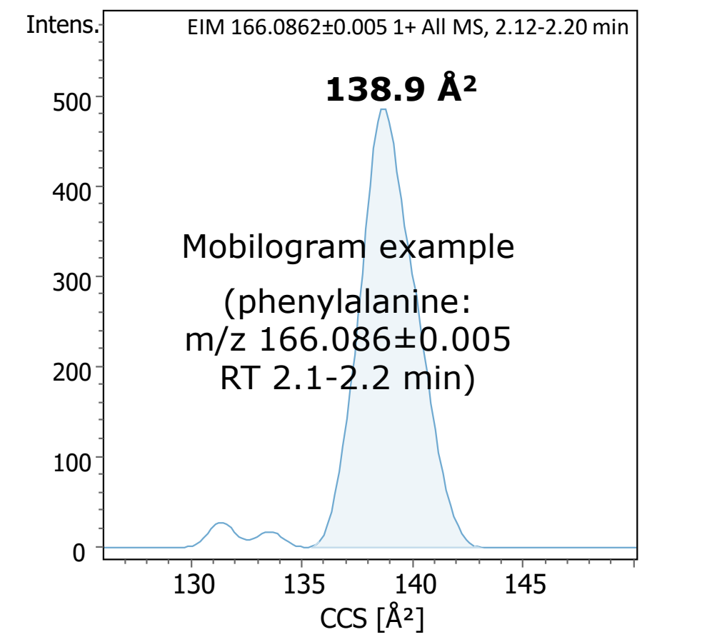
Simplified biochemical pathway for the salicylates grid and mapped results for phloem and xylem. p-value≥0.05: *, t-test.

→ Enhanced annotation certainty through inclusion of CCS values

The addition of CCS value into the annotation workflow in MetaboScape® led to an elevated annotation certainty for numerous features. Reference CCS values were extracted from the Unified CCS Compendium [6].



Molecular For...	Name	AQ	CCS (Å²)	ΔCCS [%]
$C_5H_5N_5$	Adenine		124.1	1.2
$C_9H_{11}NO_2$	L-Phenylalanine		138.9	1.4
$C_{10}H_{13}N_5O_4$	Adenosine		154.9	1.8
$C_{15}H_{14}O_6$	Catechin		168.0	2.4



Mobilogram example

(phenylalanine: m/z 166.086±0.005 RT 2.1-2.2 min)

References

[1] Gullberg J. *et al.*, Anal. Biochem. 2004, 331:283–295
[2] Wolf S. *et al.*, BMC Bioinformatics 2010, 201011:148
[3] Ruttkies C. *et al.*, J. Cheminformatics 2016, 8:3
[4] Abreu I.N. *et al.*, J. Chem. Ecol. 37(8):857–870
[5] Snyder D.T. *et al.*, Anal. Methods 2015, 7:870–876
[6] Picache, J. A. *et al.*, Chem. Sci. 2019, 10:983–993

Conclusions

- Different amounts of N fertilizer led to distinct changes in metabolite profiles for both phloem and xylem tissues.
- Salicylates were strongly affected especially in xylem.
- Other metabolite groups such as oligolignols also showed specific patterns in the different sample cell types and fertilizer groups (data not shown here).
- This pilot study shows that the experimental set-up, in combination with untargeted LC-MS metabolomics analysis, has potential to identify metabolite markers controlling important aspects of plant growth and development.
- Further experiments with TIMS technology will help to get a deeper insight into the complex xylem and phloem samples and lead to more comprehensive feature annotation by incorporating a further dimension of resolution.

UHR-QqTOF