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Metabolic Profile Changes and Early Detection of Nitrogen Deficiency in Sweet Corn

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Abstract: In this study, we investigate the nutrient supply of young corn (*Zea mays* var. rugosa) plants using conventional biological and chemical methods, as well as the N-glycan and metabolic profile of the plant sap using the MALDI-MS. Corn plants with three different nutrient supplies were grown on sandy soil for 45 days after emergence. In treatment 1 (T1), used as a control, the plants did not receive any nutrient supplementation. Plants in treatment 2 (T2) received "ideal" N-, P-, and K-nutrient supplementation in the form of inorganic fertilizers. In treatment 3 (T3), the plants were provided with the ideal amount of P and K, and a reduced amount of N fertilizer. In addition to the amount of biomass of the young plants, macronutrient content (N, P, K, Ca, and Mg) was measured in the dry matter. We examined the amount of 0.01 M $CaCl_2$-soluble N fractions of the experimental soils, and the P, K, Ca, and Mg content of their ammonium lactate (AL)-soluble fractions. We were unable to statistically distinguish between T1, T2, and T3 treatments using conventional methods (in this phenophase). Metabolic profiles in the sap from young plants, captured by MALDI-MS, showed significant differences between the control, "ideal", and N-deficient treatments. This method may also be suitable for early detection of N-deficient conditions in other plants.

Keywords: corn; nutrient deficiency; nutrient concentration; MALDI-MS; N-glycan

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1. Introduction

In Hungary, corn is a widely cultivated arable crop. Its yield is highly dependent on environmental factors (precipitation, atmospheric drought). These stress factors can be sustainably reduced by applying appropriate agronomic practices [1]. Water and nutrients have great interactions. Good nutrient supply results in favorable water use, increasing water use efficiency (WUE) [2].

Plants absorb nutrients from the soil for their vital activity, to produce crops and by-products. Therefore, to maintain the fertility of the soil, it is necessary to replenish the nutrients they extract. The quantity and quality of crops produced by plants (in arable and horticultural crops) are largely determined by their (macro- and microelements) nutrient supply [3–6]. This is also the case with corn [7].

Nitrogen is the macronutrient that plants generally require in large quantities and has the greatest impact on crop yield. However, its excessive application can increase fungal

infection in corn [8], lead to an imbalance in the plant's metabolic processes [9], and affect the uptake of other nutrients [10,11].

Early detection of plant nutrient deficiencies (in particular N deficiency) in a sustainable manner (with preferably few laboratory measurements) is an important challenge from both a crop safety and economic perspective. Biological and chemical methods are used to assess the nutrient supply of soils. Biological methods include traditional laboratory leaf and plant analyses, whereas chemical methods determine the amounts of different nutrient forms in soils [12].

One of the early indicators of non-harmonious nutrient supply and nutrient deficiency in plants can be the changes in the quality and quantity of free-type, N-linked glycans [13,14].

There is increasing use of metabolomics directed at detecting the effect of nutritional changes on plant metabolism. In particular, the metabolic fingerprint of nitrogen deficiency has been studied in *Arabidopsis thaliana* [15], tea plant (*Camellia sinensis* L.) [16], and rice [17]. These studies relied on Fourier transform ion cyclotron resonance MS of plant extracts, UPLC-MS, and HPLC-MS, respectively, resulting in low-sample throughput. With large-scale applications in mind, we opted to use a high-throughput MALDI-MS approach for sap analysis.

Growing *A. thaliana* under nitrogen-deficient conditions was found to modulate secondary metabolism, in particular glucosinolate production. The tea study was focused on the changes in flavonoid profile under different nitrogen supplementation conditions. Comparing metabolite profiles under normal and nitrogen-deficient growth conditions for rice revealed that the latter led to the increased production of stress response compounds, e.g., carbohydrates, and an increase in the level of amino sugars and nucleotide sugars.

With our experimental results, we aim to prove that nutrient deficiencies in the early phenophase of plants, which cannot yet be detected by traditional methods, induce changes in the quality and quantity of free N-glycans found in plant sap.

The main roles of N-glycans in plant cells are in the folding of proteins, and in the recognition and degradation of proteins with incorrect spatial structures [18–20]. They are also important in the biosynthesis of plant cellulose and in plant cell-wall formation [21,22]. N-glycans are important for the regulation of cytokinins, as in the absence of N-glycans, errors may occur in the folding of the polypeptide chain of cytokinin receptors [23]. Inhibited N-glycan formation leads to root and shoot development problems [24]. Mutant plants producing reduced N-glycans are characterized by poorer stress tolerance and reduced immune response [25].

Previous studies indicated a correlation between nutrient deficiency and changes in oligosaccharide, free N-glycan, and metabolite profiles in young tomato plants [14]. In this work, we expanded these investigations to the potential detection of nutrient deficiency in the early phenophase of corn plants grown on sandy soil deficient in soluble nitrogen compounds. Beyond the conventional characterization of soil and young plant material, high-performance mass spectrometry-based metabolomics was implemented to compare the effect of "ideal" and nitrogen-deficient nutrient supplementation on the small molecule composition of plant sap. If successful, this method can be applied for the early detection of nitrogen deficiency on a large scale.

2. Materials and Methods

The experiment was set up with sweet corn (*Zea mays* var. rugosa) test plants in the greenhouse of the Institute of Agrochemistry and Soil Science at the University of Debrecen, on sandy soil, with three treatments and three replications. The most important physical and chemical characteristics of the soil are listed in Table 1.

Table 1. Physical properties and chemical composition of sandy soil used as control (treatment 1, T1) [26].

K_A (Arany Plasticity Index)	<30
pH (in H_2O)	7.00
Humus %	0.6
Species soluble in AL [27]	mg/kg
AL-P_2O_5 (mg/kg)	88.1
AL-K_2O (mg/kg)	218.3
AL-Ca (mg/kg)	612.0
AL-Mg (mg/kg)	80.6
Species soluble in 0.01 M $CaCl_2$ [28]	mg/kg
0.01 M $CaCl_2$ total-N	6.5
0.01 M $CaCl_2$ nitrate-N	2.9
0.01 M $CaCl_2$ ammonium-N	2.5
0.01 M $CaCl_2$ organic-N	1.1

Based on the nutrient supply limit values of the MÉM NAK Hungarian fertilizer recommendation system, the soil of the experiment showed a poor supply of nitrogen, medium supply of phosphorus, and good supply of potassium [12,29].

We weighed 3.5 kg of air-dried sandy soil into the culture pots (diameter: 18.5 cm; depth: 14 cm). When calculating the nutrient supply, we used the specific nutrient requirement of sweet corn (N: 25 kg/t, P_2O_5: 13 kg/t, K_2O: 22 kg/t yield) and calculated with a medium (9–10 t/ha) average yield. We also took into account the nutrient supply level of the soils. ($M = Q \times f_{kor}$ (M: Fertilizer active ingredient requirement (kg ha^{-1}), Q: Expected crop, f_{kor}: corrected specific active ingredient requirement.))

Treatment 1 (T1), the sandy soil with no nutrient supplementation, was used as a control. The plants relied on the nutrients originally found in the soil for their development. Plants in treatment 2 (T2) received N, P, and K nutrient supplementation in the form of an inorganic fertilizer to emulate "ideal" conditions. In treatment 3 (T3), the maize plants were supplied with the same amount of P and K as in T2, and a reduced amount of nitrogen fertilizer, thereby inducing an N-deficient state. Nitrogen and phosphorus were added as $NH_4H_2PO_4$ (0.288 g 3.5 kg^{-1} soil) and NH_4NO_3 (0.878 g 3.5 kg^{-1} soil) solutions, whereas potassium was supplied as KCl (0.477 g 3.5 kg^{-1} soil) solution. The treatment plan is presented in Table 2.

Table 2. Treatment plan of the experiment.

Treatments	N (kg/ha)	P_2O_5 (kg/ha)	K_2O (kg/ha)
Control (T1)	-	-	-
"Ideal" (T2)	225	117	198
Nitrogen-deficient (T3)	21.3	117	198

The soils were mixed, and the plants were sown on 15 May 2023 and emerged on 22 May. We started the development of four plants in each pot. The soil in the culture pots was watered daily to a water supply level of 50% of the maximum water retention capacity. The plants developed under the same temperature and light conditions as the external conditions. The experiment was terminated on 6 July (45 days after plant emergence, i.e., V4. stage). At that time, plant and soil samples were taken, as well as plant sap.

The total nitrogen content of dry sweet corn (leaf and stem) samples was determined using an elemental analyzer operating on the combustion principle ("dry combustion method") [30]. To determine the plant element content, the samples were decomposed at 220 °C (cc. H_2SO_4:$30_{W/W}\%H_2O_2$ = 1:1 volume). The P content of the decomposition product was determined photometrically using the molybdenum blue method [31], and

the K, Ca, and Mg contents were measured using an atomic absorption (AA) spectrometer (Varian AA10 Plus).

The amount of ammonium lactate (AL)-soluble K, P, Ca, and Mg (AL-soluble represents the fraction of the nutrient that is easily absorbed by plants) was determined in the soil of the treatments. The P content of the AL filtrates [27,32] was measured photometrically [33], and the amount of K, Ca, and Mg was measured with an AA spectrometer (Varian AA10 Plus). The amounts of 0.01M $CaCl_2$ soluble NO_3^--N, NH_4^+-N, and organic N-forms in the soils [28,34] were measured with a continuous flow device (Skalar, SA2000/4000 Segmented Flow Analyzer, HQ-Skalar Analytical BV, Breda, The Netherlands).

For the statistical evaluation of our results (plant and soil element contents), we used the one-way analysis of variance (Tukey test) of the SPSS (Version: 28.0.0.0.(190)) program. We performed correlation calculations between the examined parameters.

The saps were collected according to the protocol described by Tsujimori et al. [35] and then lyophilized. In the summer of 2023, the lyophilized samples were transported to the Vertes laboratory at the Department of Chemistry, George Washington University, Washington, DC, USA, where metabolites, including free oligosaccharides and N-glycans were analyzed in the samples by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).

From the lyophilized sap, a 1 mg sample was resuspended in 500 µL of HPLC-grade water (W5-4, Fisher Scientific, Waltham, MA, USA) and mixed for 20 min at room temperature. The resuspended lyophilate was centrifuged (Centrifuge 5430 R, Eppendorf AG, Hamburg, Germany) at 11,000 rpm for 20 min and the resulting supernatant was collected for MS measurements. The 2,5-dihydroxybenzoic acid (DHB) (Catalog no. 58707, Sigma Aldrich, St Louis, MO, USA) MALDI matrix was dissolved in methanol (A452-4, Fisher Scientific, Waltham, MA, USA) at 10 mg/mL and mixed with the supernatant at a 5:2 ratio.

Aliquots of 1 µL of the resulting mixture were deposited onto a stainless steel MALDI sample plate and left to air-dry. Measurements were conducted on a MALDI-LTQ-Orbitrap XL MS (Thermo Scientific, San Jose, CA, USA) with a resolving power set to 30,000 for an acquisition range of m/z 150 to 2000. The nitrogen laser used for ion production delivered 10 pulses with 4 µJ energy each for every sample spot. Calibration of the mass spectrometer was achieved by using a commercial kit (MSCAL4, Sigma-Aldrich, St Louise, MO, USA).

After selecting the significant peaks with S/N > 3 and deisotoping, the resulting m/z values were searched against the GlycoMod database in Expasy (https://web.expasy.org/glycomod/, accessed on 18 February 2025) for tentative assignments of possible oligosaccharides and N-glycans. Comparative statistical analysis of the T1, T2, and T3 dataset was accomplished by the MetaboAnalyst online resource (https://www.metaboanalyst.ca/MetaboAnalyst/, accessed on 18 February 2025). For the statistical models, peak intensities were normalized by the sum of sample-related ion abundances, log transformed, and subjected to Pareto scaling. Pareto scaling, often used in metabolomics, is based on normalization of a variable by the square root of its standard deviation. It ensures that ion intensities with large and small fold changes in a comparative analysis retain similar importance. Due to the robust signal in both the MALDI-MS and NAPA-LDI-MS measurements, there was no need for missing value imputation. Biomarker candidates were identified by univariate (volcano plot) and multivariate (orthogonal partial least squares-discriminant analysis (OPLS-DA)) statistical methods.

For the tentative identification of metabolites, the accurate m/z values for the spectral features of interest were searched against the organism-specific metabolite database within PlantCyc, i.e., the *Z. mays* database (https://pmn.plantcyc.org/organism-summary?object=CORN; last accessed on 22 February 2025). Based on the mass accuracy of the instrument, the searches were conducted with $\Delta m = \pm 20$ mDa mass tolerance. In some cases, quasi-

isobaric ions within this window were distinguished based on the comparison of the measured isotope distributions with patterns calculated by the enviPat online platform (https://www.envipat.eawag.ch/, accessed on 3 April 2025). Other cases resulting in alternative assignments stemmed from structural isomers. These could not be resolved without additional measurements, e.g., by separation techniques.

3. Results and Discussion

3.1. Plant Material Characterization

At this early phenophase of maize development, i.e., in the V4 stage, the dry matter yield of unfertilized plants (T1) at 0.867 ± 0.416 g was statistically lower (p = 0.003) than that of fertilized plants T2 at 2.300 ± 0.300 g and T3 at 1.800 ± 0.100 g (see Table 3). There was no significant difference (p = 0.186) in plant biomass between nitrogen-deficient (T3) and ideal (T2) treatments.

Table 3. Dry mass and nutrient concentrations per dry weight for three treatments of young corn plants.

	Treatments		
Properties	**Control (T1)**	**"Ideal" (T2)**	**N Deficient (T3)**
Dry Mass (g/pot)	0.867 ± 0.416	2.300 ± 0.300	1.800 ± 0.100
N (g/kg)	10.62 ± 0.740	36.73 ± 1.749	10.48 ± 0.423
P (g/kg)	4.671 ± 0.206	4.980 ± 0.240	6.434 ± 0.244
K (g/kg)	19.40 ± 2.651	30.23 ± 2.151	31.78 ± 0.325
Ca (g/kg)	14.21 ± 1.422	19.04 ± 2.526	14.88 ± 2.295
Mg (g/kg)	4.958 ± 0.218	5.000 ± 0.636	2.642 ± 0.083

By examining the N concentrations measured in the dry matter of the plants (see Table 3), it can be established that the plants with ideal nutrient supply treatment (T2) had an exceptionally high N concentration (36.73 ± 1.749). Based on this property, they can be statistically separated from the plants with the other two treatments (p = 0.000). The N concentration (10.48 ± 0.423) of the plants fertilized with a reduced amount of N (T3) is almost the same as that of the unfertilized, control (T1) plants (10.62 ± 0.740), so the plants of these two treatments can be classified into the same group based on this property. The two N concentrations are not significantly different (p = 0.988). Plants in T3 treatments produced a higher biomass mass than plants in T1 treatment, so the N concentration decreased due to the dilution effect.

The P concentrations measured in the dry matter of young maize (see Table 3) indicate that the plants with treatments providing P supplementation (T3) but producing a smaller biomass mass have the highest value (6.434 ± 0.244) (p = 0.001). Only small differences can be observed between the P concentrations of the plants of the control (T1) (4.671 ± 0.206) and the ideal (T2) (4.980 ± 0.240) treatments, so they cannot be separated from each other based on this parameter (p = 0.301).

The lowest K concentration (19.40 ± 2.651) (see Table 3), was measured in the control (T1) plants not fertilized with potassium (p = 0.010). No significant difference (0.627) was observed in the K concentrations of the plants with T2 (30.23 ± 2.151) and T3 (31.78 ± 0.325) treatments that received the same K fertilizer dose but different N doses.

Intensive (large-scale) nutrient uptake by corn began in this phenophase, but their incorporation into organic matter had not yet occurred, so we do not experience large differences in the biomass mass produced.

Overall, nutrient deficiency cannot be clearly detected in this early phenophase of the corn plant by examining the concentrations of the fertilized nutrients. A large part of the nitrogen reservoir in plant tissue is present in the form of proteins and nucleic

acids. Measuring the total nitrogen in the plant does not separate these abundant nitrogen containing species; therefore, small changes in nitrogen levels due to altered metabolism are initially not noticeable.

We also measured the concentrations of two non-fertilized macronutrients (Ca and Mg) in the dry matter of the plants (see Table 3). The supply of N, P, and K nutrients did not significantly (p = 0.072) affect the Ca concentrations in the plants with the three treatments. The Mg concentration of the plants with T1 (4.958 ± 0.218) and T2 (5.000 ± 0.636) treatments was very similar, whereas that of the plants with T3 (2.642 ± 0.083) treatment was significantly lower (p = 0.001). Nevertheless, nutrient deficiencies cannot be detected based solely on the concentrations of the two unfertilized nutrients examined.

We also examined the correlations between the parameters measured in young maize plants. There was a positive correlation between the plant biomass mass and the nitrogen (r = 0.69, p = 0.04), calcium (r = 0.66, p = 0.05), and potassium (r = 0.70, p = 0.03) concentrations of the plant. A similar positive significant relationship was also found between the N concentration and the Ca content (r = 0.76, p = 0.02) of the maize grown in our experiment. We obtained a very strong negative correlation between plant P and Mg concentrations (r = −0.93, $p < 0.01$).

3.2. Plant-Accessible Nutrient Changes in Soil

The AL-soluble K concentrations (K_2O mg/kg) of soil samples taken at the termination of the experiment decreased during the growing season. It changed from 218.3 mg/kg at the beginning to 210.8 ± 3.136 mg/kg in the soil without K supplementation (T1) (p = 0.003). The K concentration of the soils with treatments with different N but the same K supplementation was almost the same, 220.6 ± 4.373 mg/kg for T2 and 220.5 ± 2.550 mg/kg for T3. We could not statistically distinguish the three treatments based on the AL-K concentration of the soil.

The AL-soluble P concentration (P_2O_5 mg/kg) showed a slight increase during the growing season, changing from the initial 88.1 mg/kg to 90.7 ± 0.294 mg/kg. According to the data, the transformation and release of phosphorus into an easily absorbable form occurred more intensively than its uptake by plants from the soil. As expected, significantly lower P concentrations (p = 0.001) were measured in the soils of the control (T1) treatment than in the soils of the other two treatments. Based on this parameter, we could not separate the soils of the T2 (117.1 ± 3.171 mg/kg) and T3 (122.0 ± 2.631 mg/kg) treatments (p = 0.154). The concentrations of the two non-fertilized macronutrients (Ca and Mg) in the AL-soluble fractions, 514.0 ± 50.71 mg/kg, 530.7 ± 42.16 mg/kg, and 602.5 ± 44.96 mg/kg for T1, T2, and T3, respectively (p = 0.083), for Ca, and 74.1 ± 1.973 mg/kg, 73.5 ± 8.164 mg/kg, and 73.7 ± 4.244 mg/kg for T1, T2, and T3, respectively, for Mg, showed no statistically significant (p = 0.989) differences between treatments. Compared to their presence in the soil, their concentrations decreased during the growing season (compare values to Table 1).

We also examined the amount of 0.01M $CaCl_2$-soluble total N, and the nitrate-, ammonium-, and organic-N fractions in the soil (Figure 1). The amount of 0.01M $CaCl_2$-soluble N forms in the soil for the "ideal" nutrient supply treatment (T2) showed statistically verifiable higher values than those measured in the soils of the control (T1) and reduced N-dose treatments (T3). High N-dose fertilization (T2) increased these parameters of the soils, whereas in the control (T1) and the N-deficient case (T3), the amount of $CaCl_2$-soluble total-N significantly decreased as a result of cultivation (compare values in Table 1 and Figure 1). Based solely on these soil parameters, the T1 and T3 treatments cannot be separated. By measuring the element content of different soil extractants, we can examine nutrient fractions that are bound to different strengths in the soil. With the methods we

used, we wanted to determine the soluble forms and the forms that are easily absorbed by plants. Plant nutrient uptake is influenced by many factors, so these methods are also somewhat uncertain in assessing nutrient supply.

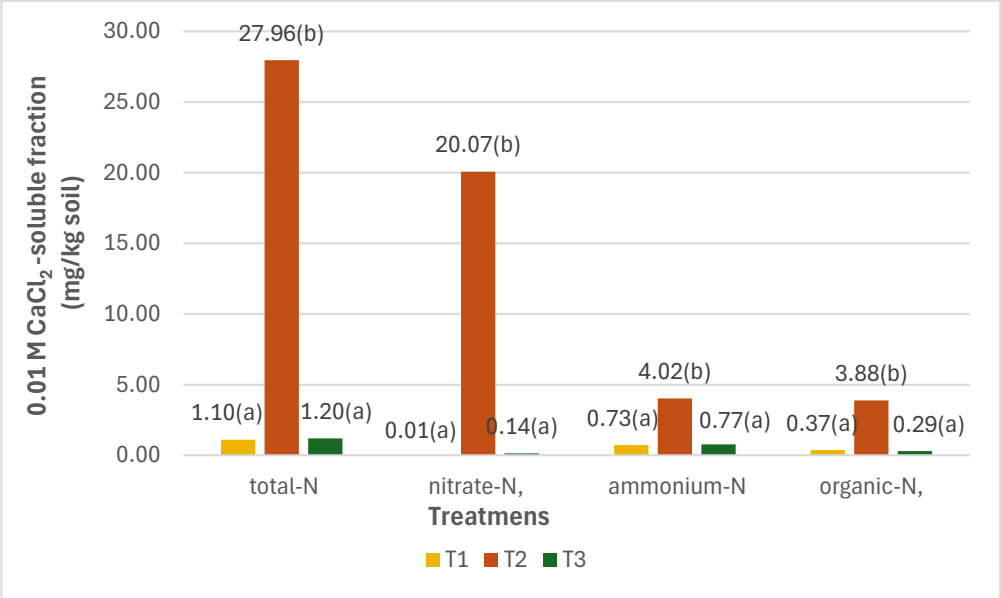

Figure 1. 0.01 M $CaCl_2$ soluble N fractions (mg/kg soil) in soil at end of experiment. Note: data marked with the same letter are not significantly different at the significant level of $p \leq 0.05$.

The correlations between the parameters measured in the soils indicate that there is a close positive relationship between the AL-soluble K and P concentrations in the soils (r = 0.80, $p = 0.01$). We also observed a very strong positive correlation between the 0.01 M $CaCl_2$-soluble N forms ($CaCl_{2\ total\text{-}N}$—$CaCl_{2\ nitrate\text{-}N}$ r = 1.00, $p < 0.01$; $CaCl_{2\ total\text{-}N}$—$CaCl_{2\ ammonium\text{-}N}$ r = 0.99, $p < 0.01$; $CaCl_{2\ total\text{-}N}$—$CaCl_{2\ organic\text{-}N}$ r = 0.99, $p < 0.01$).

Correlations between plant and soil properties are shown in Table 4. There is a significant positive effect between the plant product and the AL-soluble K and P concentrations of the soil, as well as the 0.01 M $CaCl_2$-soluble N forms. We observed a close correlation between the plant N concentration and the 0.01 M $CaCl_2$-soluble N forms. There is a statistically verifiable positive relationship between plant phosphorus and the AL-soluble P and Ca concentrations of the soil. In addition, there is a very close positive correlation between the plant P concentration and the AL-soluble K and P concentrations in the soil. There is also a very strong, statistically verifiable positive relationship between the plant Ca concentration and the 0.01 M $CaCl_2$-soluble N forms in the soil. We observed a significant negative relationship between the plant Mg concentration and the AL-soluble Ca concentration of the soil. Other researchers have also looked for a relationship between the element content of the plant and the 0.01M $CaCl_2$-soluble N forms of the soil [36].

Table 4. Correlations between properties of young corn plant and soil.

		Plant					
	r / p	Biomass	N	P	K	Ca	Mg
Soil	AL-K	0.689 *	0.313	0.613	0.888 **	0.2	−0.559
		0.04	0.412	0.079	0.001	0.606	0.118
	AL-P	0.794 *	0.368	0.710 *	0.940 **	0.451	−0.541
		0.011	0.33	0.032	0	0.223	0.133
	AL-Ca	0.135	−0.255	0.686 *	0.562	0.091	−0.723 *
		0.729	0.508	0.042	0.115	0.817	0.028
	AL-Mg	0.005	−0.038	0	−0.166	0.342	0.231
		0.99	0.924	1	0.67	0.368	0.55
	$CaCl_2$ total-N	0.698 *	0.934 **	−0.328	0.301	0.874 **	0.544
		0.037	0	0.389	0.432	0.002	0.13
	$CaCl_2$ nitrate-N	0.695 *	0.908 **	−0.328	0.303	0.867 **	0.545
		0.038	0.001	0.389	0.428	0.002	0.129
	$CaCl_2$ ammonium-N	0.716 *	0.945 **	−0.302	0.268	0.906 **	0.536
		0.03	0.001	0.43	0.485	0.001	0.137
	$CaCl_2$ organic-N	0.683 *	0.945 **	−0.348	0.311	0.868 **	0.542
		0.043	0	0.359	0.415	0.002	0.132

* Significant correlation at the 0.05 level; ** Significant correlation at the 0.01 level.

3.3. Nutritional Changes in Corn Sap Metabolites

Nutritional shortcomings and the application of fertilizers are known to alter plant metabolism. Modern methods of metabolomics give an in-depth insight into the nature and level of metabolites affected by nutritional supplementation [14,37]. To explore the changes in metabolite composition in young corn as a result of supplementing macronutrients and in the presence of nitrogen deficit, resuspended sap samples from the plants with control (T1), "ideal" (T2), and nitrogen-deficient (T3) treatments were studied by MALDI-MS. Representative positive ion mass spectra in the m/z 50 to 4000 range from the three sample types are shown in Figure 2. Hundreds of spectral features were observed, and their m/z values and intensities were extracted in the $50 < m/z < 2500$ range for the three treatment types.

As we were interested in the presence of oligosaccharides and N-glycans, the peak lists were searched against the GlycoMod database with $\Delta m = \pm 20$ mDa mass tolerance for tentative assignments. A selected list of potential structures is shown in Table 5. At this stage, no tandem mass spectra were collected, thus, structural isomers of the assigned species cannot be excluded. Also, in some cases, quasi-isobaric ions were consistent with a particular measured accurate mass. For example, for m/z 1011.185, two very different ionic species, sodiated $(Hex)_5$ $(Sulph)_2$ and potassiated $(Hex)_4$ $(Deoxyhexose)_1$ $(Phos)_2$, satisfied the search tolerance requirements. Further studies involving tandem mass spectrometry are needed to clarify if one, the other, or both species are present. Comparison of the oligosaccharide and N-glycan species present and their abundances for the studied treatment types revealed no major differences, so we shifted the focus to spectral features with more variations.

Numerous other peaks, corresponding to small metabolites, were observed. Attempting their identification is a monumental undertaking due to the need for extensive additional experiments. Therefore, a subset of peaks was selected that appeared both biologically and statistically significant for the different treatments. Biological significance was gauged by peak-intensity fold change (FC) values, e.g., I_{T1}/I_{T2}, to compare the control to the "ideal" fertilizer treatment, whereas statistical significance was measured by the p values for pairwise t tests. Conventional cutoff values for significance are FC < 0.5 or

FC > 2, and $p < 0.05$. The volcano plots in Figure 3 show the detected peaks in a -$\log_{10}$ p versus $\log_2$ FC space. The dashed lines indicate the cutoff values for both metrics. Points under the horizontal dashed line and between the vertical dashed lines, colored gray, lack statistical and biological significance, respectively.

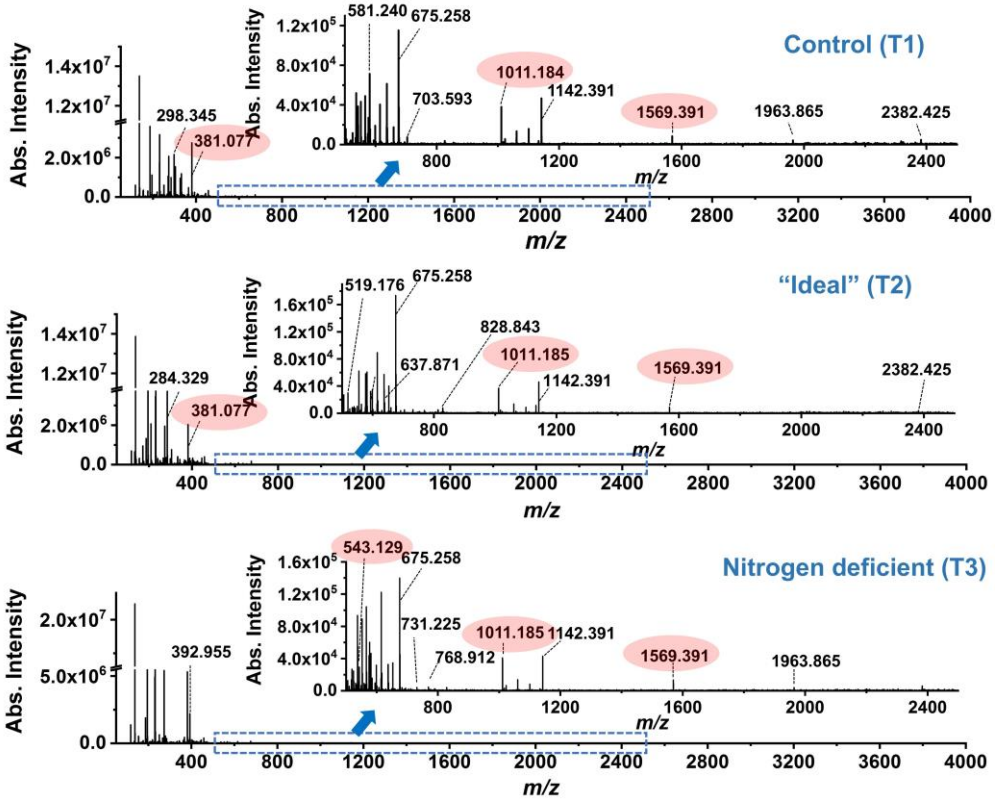

Figure 2. Mass spectra for corn sap samples from plants produced with different nutritional conditions: (**top panel**) control (T1), (**middle panel**) "ideal" N-, P-, and K-nutrient supplementation (T2), and (**bottom panel**) nitrogen-deficient nutrient supplementation (T3). Insets show peaks of lower intensity on an expanded vertical scale. Peaks with m/z values highlighted in pink potentially correspond to free oligosaccharides.

Table 5. Tentative assignments of non-conjugated oligosaccharides and N-glycans detected in corn sap samples.

m/z	Adduct	Glycoform Mass	Δ*m* (mDa)	Structure	Type
365.103	$[M+Na]^+$	324.106	−5	$(Hex)_2$	
381.077	$[M+K]^+$	324.106	−1	$(Hex)_2$	
393.084	$[M+H]^+$	374.061	1	$(Hex)_1$ $(Pent)_1$ $(Phos)_1$	
425.187	$[M+H]^+$	406.159	10	$(HexNAc)_2$	
543.130	$[M+K]^+$	486.158	−1	$(Hex)_3$	
607.224	$[M+H]^+$	588.190	10	$(Hex)_2$ $(Pent)_2$	
675.258	$[M+H]^+$	656.228	12	$(Hex)_1$ $(HexNAc)_1$ $(NeuAc)_1$	
1011.185	$[M+Na]^+$	970.178	2	$(Hex)_5$ $(Sulph)_2$	
	$[M+K]^+$	954.202	4	$(Hex)_4$ $(Deoxyhexose)_1$ $(Phos)_2$	
1569.391	$[M+K]^+$	1512.418	−1	$(Hex)_1$ $(HexNAc)_1$ $(Deoxyhexose)_1$ $(NeuGc)_3$ $(Sulph)_1$	Paucimannose
				$(Hex)_2$ $(HexNAc)_1$ $(NeuAc)_1$ $(NeuGc)_2$ $(Sulph)_1$	Paucimannose

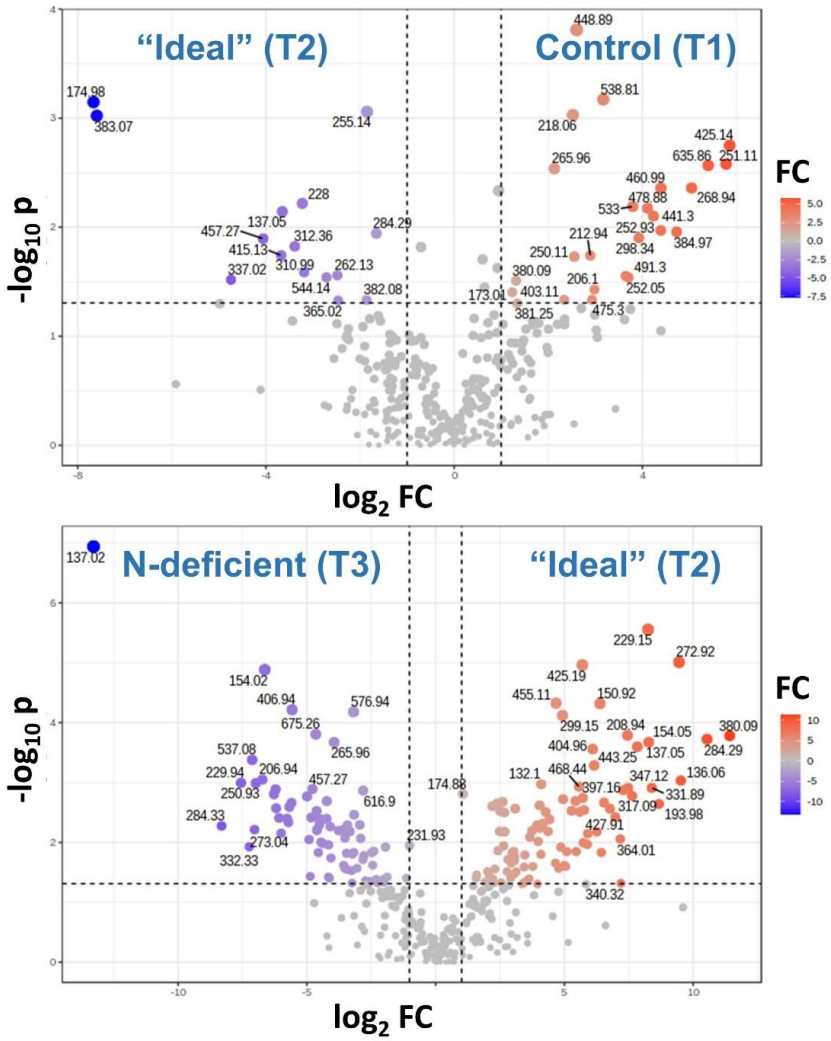

Figure 3. Volcano plots to detect up- and downregulated spectral features in T1, T2, and T3 samples. A total of 40 and 170 regulated species were found for T1 vs. T2 (**top panel**) and T2 vs. T3 (**bottom panel**) pairs, respectively. Points are labeled with the corresponding m/z values. For emphasis, false color scales encode the FC values.

In this univariate analysis, the top right quadrant in the top panel (T1 to T2 comparison) shows the species upregulated under control treatment, whereas the top left quadrant contains the species upregulated under "ideal" treatment. The bottom panel compares the "ideal" (T2) to the nitrogen-deficient (T3) treatments. In this panel, the top right quadrant shows the species upregulated under "ideal" treatment, whereas the top left quadrant contains the species upregulated under nitrogen-deficient treatment. Overall, the control to "ideal" comparison results in a total of 40 regulated species, whereas the "ideal" to nitrogen-deficient comparison yields 170 regulated species. This finding establishes the presence of major metabolic profile changes as a function of nutrient supply. Not all regulated species impact the spectra to the same degree. Identification of the species corresponding to points with the highest significance is much more manageable and meaningful than trying to develop assignments to all points in the volcano plot.

A deeper analysis of metabolomics data can be achieved by multivariate statistical analysis. This approach takes into account the simultaneous interactions between multiple components in a complex mass spectrum. A method offering specific advantages in metabolomics is OPLS-DA. In Figure 4, both the control to "ideal" treatment (left panel) and the "ideal" to nitrogen-deficient treatment (right panel) comparisons show good separation

between the treatments. The vertical separation of the points shows the differences in outcome for a specific treatment. To identify the spectral features responsible for most of the variance between the spectra, the correlation as a function of the covariance has to be plotted. In these S-plots (see Figure 5), the points with high absolute correlation and covariance, i.e., the points in the wings of the plot deserve close attention. The numerous points in between are typically ignored due to their much lower significance. It is worth noting that some of the prominent features flagged by the S-plot, e.g., m/z 174.98, 383.07, 635.86, and 251.11, have also been present in the volcano plots.

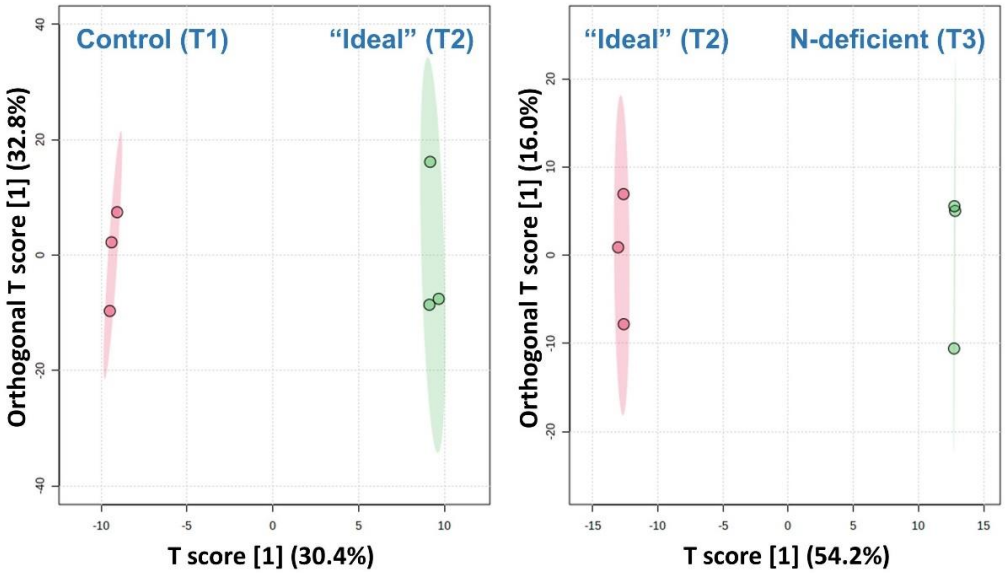

Figure 4. Score plots for OPLS-DA of MALDI-MS spectra. Score plots generated by OPLS-DA for the pairwise comparisons demonstrated clear distinctions between treatments.

To further identify the peaks with the most impact, the variable influence on projection (VIP) scores can be calculated. Figure 6 shows the VIP scores for the 15 most influential peaks for each of the control to "ideal" treatment (top panel) and the "ideal" to nitrogen-deficient treatment (bottom panel) comparisons.

Searching the PlantCyc Z. mays database (https://pmn.plantcyc.org/organism-summary?object=CORN; last accessed on 22 February 2025) for metabolites consistent with the ions showing high VIP scores, a list can be compiled for the control (T1) to "ideal" supplementation (T2) comparison (Table 6). The search considered protonated, sodiated, and potassiated ion adducts with a $\Delta m = \pm 20$ mDa mass tolerance. The regulated secondary metabolites in this treatment comparison belong to organonitrogen compounds (e.g., N-pyruvoyl-glutamate, hydroxymethyl-dihydropterin, $preQ_1$, and hexosamine), flavonoids (e.g., flavanone-O-glucoside, hydroxy-salvigenin, and nevadensin), and lipids (e.g., zealexin B1, traumatin, and hydroxylaurate). Hexosamine is a key metabolite in the biosynthesis of uridine diphosphate-N-acetyl glucosamine (UDP-GlcNAc) essential for protein glycosylation. Although there is no biosynthesis of $preQ_1$ in corn, it is perceivable that a structural isomer of this molecule with five nitrogen atoms is modulated by nitrogen-deficient nutrition.

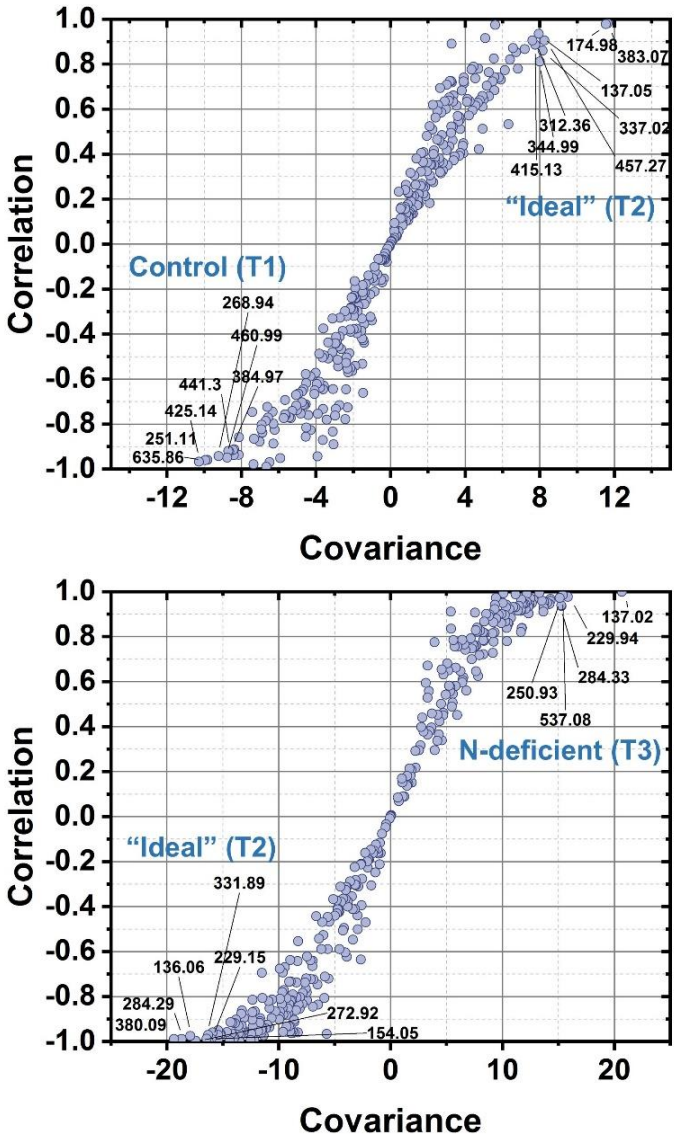

Figure 5. S-plots produced by OPLS-DA models. The most influential spectral features that distinguish the treatments are in the wings.

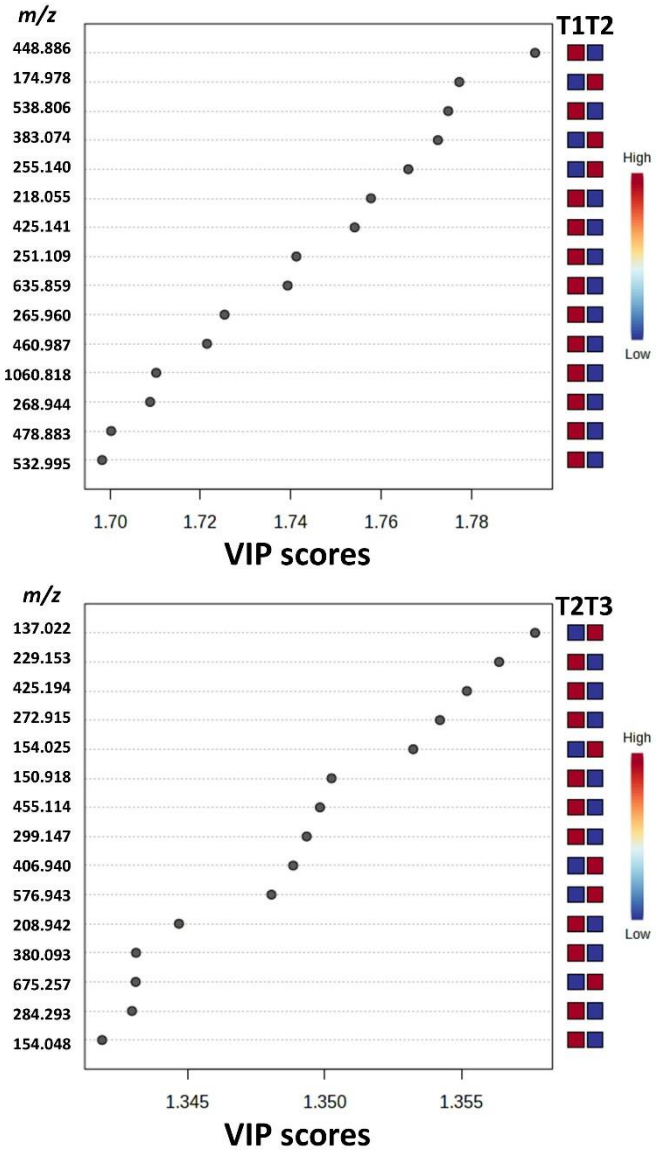

Figure 6. Spectral features with 15 as highest variable influence on projection (VIP) scores identifying species in order of their influence on the statistical model for T1 vs. T2 (**top panel**) and T2 vs. T3 (**bottom panel**).

A similar analysis was performed for the comparison of "ideal" nutrient supplementation (T2) and the nitrogen-deficient treatment (T3) (Table 7). The regulated secondary metabolites in this treatment comparison belong to organonitrogen compounds (e.g., sulfino-alanine, amino-hydroxybenzoate, and glutamate-semialdehyde), flavonoids (e.g., baicalein O-glucoside, and genistin), lipids (e.g., hydroxy traumatin, epoxy-oxododecanoate, and traumatate), amino acids and intermediates in amino acid metabolism (hydroxyproline, 5-aminolevulinate, proline, glutamate-semialdehyde, and hydroxyanthranilate), glutathione derivatives (S-lactoylglutathione hemithioacetal, and S-lactoylglutathione), and a glycan (N,N'-diacetylchitobiose). Nitrogen deficiency itself is a major stress, so separating a generic stress response from it is challenging. One can distinguish the reallocation of nitrogen to other metabolites, e.g., amino acids and other organonitrogen compounds, and a metabolic response that does not involve nitrogen, e.g., flavonoids and lipids.

Table 6. Tentative assignment of secondary metabolites regulated between T1 and T2 treatments.

Search MW	Adduct	Calculated MW	Δ*m* (mDa)	Compound Name	Link
217.047	H	217.059	−12	N-pyruvoyl-glutamate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-24913
250.101	H	250.087	14	[(methylsulfanyl)pentyl]malate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPDQT-38
195.065	Na	195.053	12	dopaquinone	https://pmn.plantcyc.org/compound?orgid=CORN&id=DOPAQUINONE
195.065	Na	195.076	−11	hydroxymethyl-dihydropterin	https://pmn.plantcyc.org/compound?orgid=CORN&id=AMINO-OH-HYDROXYMETHYL-DIHYDROPTERIDINE
232.150	Na	232.146	4	zealexin B1	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-13572
402.151	Na	402.131	20	flavanone-O-glucoside	https://pmn.plantcyc.org/compound?orgid=CORN&id=FLAVANONE-7-O-GLUCOSIDES
179.091	K	179.081	10	preQ1	https://pmn.plantcyc.org/compound?orgid=CORN&id=7-AMINOMETHYL-7-DEAZAGUANINE
179.091	K	179.079	12	hexosamine	https://pmn.plantcyc.org/compound?orgid=CORN&id=Hexosamines
212.145	K	212.141	4	traumatin	https://pmn.plantcyc.org/compound?orgid=CORN&id=12-OXO-TRANS-10-DODECENOATE
216.176	K	216.173	3	hydroxylaurate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-12282
344.110	K	344.090	20	hydroxy-salvigenin	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-15476
344.110	K	344.090	20	nevadensin	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-15468

Table 7. Tentative assignment of metabolites regulated between T2 and T3 treatments.

Search MW	Adduct	Calculated MW	Δ*m* (mDa)	Compound Name	Link
153.017	H	153.010	7	sulfino-alanine	https://pmn.plantcyc.org/compound?orgid=CORN&id=3-SULFINOALANINE
153.040	H	153.043	−3	hydroxyanthranilate	https://pmn.plantcyc.org/compound?orgid=CORN&id=3-HYDROXY-ANTHRANILATE
153.040	H	153.043	−3	amino-hydroxybenzoate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-14873
228.145	H	228.136	9	hydroxy traumatin	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-19335
228.145	H	228.136	9	epoxy-oxododecanoate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-25935
228.145	H	228.136	9	traumatate	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-25932
379.085	H	379.089	−4	N-methylguanosine-phosphate	https://pmn.plantcyc.org/compound?orgid=CORN&id=7-METHYLGUANOSINE-5-PHOSPHATE
379.085	H	379.105	−20	S-lactoylglutathione hemithioacetal	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-26676
379.085	H	379.105	−20	S-lactoylglutathione	https://pmn.plantcyc.org/compound?orgid=CORN&id=S-LACTOYL-GLUTATHIONE
424.186	H	424.169	17	N,N′-diacetylchitobiose	https://pmn.plantcyc.org/compound?orgid=CORN&id=CHITOBIOSE

Table 7. *Cont.*

Search MW	Adduct	Calculated MW	Δ*m* (mDa)	Compound Name	Link
114.032	Na	114.032	0	oxopent-enoate	https://pmn.plantcyc.org/compound?orgid=CORN&id=OXOPENTENOATE
131.058	Na	131.058	0	glutamate-semialdehyde	https://pmn.plantcyc.org/compound?orgid=CORN&id=L-GLUTAMATE_GAMMA-SEMIALDEHYDE
131.058	Na	131.058	0	hydroxyproline	https://pmn.plantcyc.org/compound?orgid=CORN&id=4-HYDROXY-L-PROLINE
131.058	Na	131.058	0	aminolevulinate	https://pmn.plantcyc.org/compound?orgid=CORN&id=5-AMINO-LEVULINATE
276.157	Na	276.159	−2	coumaroylagmatine	https://pmn.plantcyc.org/compound?orgid=CORN&id=N-4-GUANIDINOBUTYL-4-HYDROXYCINNAMAMID
432.124	Na	432.106	18	baicalein O-glucoside	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-15176
432.124	Na	432.106	18	genistin	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-3421
652.267	Na	652.273	−6	bis-glucosyl crocetin	https://pmn.plantcyc.org/compound?orgid=CORN&id=CPD-8667
115.061	K	115.063	−2	proline	https://pmn.plantcyc.org/compound?orgid=CORN&id=PRO

In the iterative multistep process of metabolite identification, four levels of confidence are distinguished. These levels are defined by the Metabolomics Standard Initiative (MSI) [38]. The putative assignments in Tables 6 and 7 rely on accurate mass measurements and searches in relevant databases. Without tandem MS and/or another orthogonal measurement technique, structural isomers and quasi-isobaric species cannot be ruled out. Therefore, they correspond to level 2 or level 3 assignments according to the MSI classification.

Comparing VIP scores for the "ideal" nutrition and the nitrogen-deficient cases listed in Table 7, a tentatively identified metabolic marker profile can be assembled. Among the key markers are some organonitrogen compounds, sulfino-alanine, amino-hydroxybenzoate, and glutamate-semialdehyde), amino acids and intermediates in amino acid metabolism, hydroxyproline, 5-aminolevulinate, and hydroxyanthranilate, and a glycan, N,N′-diacetylchitobiose. Notably, consistent with the altered supply of nitrogen, all of these molecules carry one or more nitrogen atoms.

Overall, multivariate analysis of MALDI-MS spectra not only clearly distinguishes the three treatment types, but also gives information about the particular species, with a potential to uncover metabolic pathways up- or downregulated by them. Monitoring the metabolic profile of the developing plants can be used to titrate the amount and select the timing of nitrogen fertilizer supplementation. This prevents overfertilization and reduces GHGI (e.g., in the form of nitrous oxide) emission.

4. Conclusions

Plant and soil analytical experiments were carried out to discern metabolic markers of nutrient supplementation and deficient nitrogen supply for young corn plants. Monitoring solely the produced biomass and changes in inorganic macronutrients, N, P, K, Ca, and Mg, no differences were observed between the N concentrations measured in the dry matter from the control (T1) and low nitrogen supplementation (T3) treatments. The corresponding P concentrations did not show a difference between the plants produced by T1 and "ideal" supplementation (T2) treatments. The K concentrations for the plants with T2 and T3 treatments were almost identical. Similar results were found in the case of non-

fertilized nutrients (Ca and Mg). At this early phenophase (45 days after plant emergence corresponding to the V4 stage), we did not observe a statistically significant difference in the element content measured in the dry matter as a function of the nutrient supply.

For the "ideal" (T2) and nitrogen-deficient (T3) treatments, the AL-soluble K and P concentrations of the soils could not be distinguished. There were no significant differences between the AL-soluble Ca and Mg concentrations for any of the treatments. Based on the amount of 0.01M $CaCl_2$-soluble N fractions, the control and nitrogen-deficient treatments could not be distinguished from each other. The often-observed close correlations between plant and soil test results indicate that nutrient-deficiency states can be detected using traditional plant and soil analyses, but only in the later phenophases of plant development.

Metabolic profiles in the sap from young plants, captured by MALDI-MS, showed significant differences between the control, "ideal", and N-deficient treatments. Although a limited number of N-glycans and oligosaccharides were detected, their variety and abundances revealed no major differences for the treatment types. Surprisingly, mostly changes in secondary metabolites were detected, although for the T2 to T3 comparison, changes in the abundances of a few amino acids and intermediates in amino acid metabolism were also noted. Multivariate analysis clearly distinguishes the three treatment types and pinpoints the spectral features that account for most of the variance between the spectra. Current putative assignment of metabolites to the spectral features relies on accurate mass and the search for established database corn metabolomic databases. Going forward, the fidelity of these identifications has to be improved, e.g., by tandem MS measurements. Other orthogonal techniques, such as HPLC or NMR, can also significantly improve the assignments. In addition, multiomics approaches, based on transcriptomics and metabolomics in addition to metabolomics, can go a long way to uncover the up- or downregulated metabolic pathways.

Conventional nutrient analysis is based on measuring total nitrogen in the plant, including the nitrogen present in proteins, nucleic acids, metabolites, and nitrogen present in inorganic form. Plant development relies on the abundance of nitrogen containing building blocks, i.e., water-soluble metabolites, for the synthesis of plant material. Metabolomics selectively measures and identifies such metabolites; thus, it is more appropriate for the detection of nutrient deficiency than total nitrogen measurements.

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