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Transcriptomic and metabolomic profiles of *Zea mays* fed with urea and ammonium

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Abstract

The simultaneous presence of different N-forms in the rhizosphere leads to beneficial effects on nitrogen (N) nutrition in plants. Although widely used as fertilizers, the occurrence of cross connection between urea and ammonium nutrition has been scarcely studied in plants. Maize fed with a mixture of urea and ammonium displayed a better N-uptake efficiency than ammonium- or urea-fed plants (Buoso et al., Plant Physiol Biochem, 2021a; 162: 613–623). Through multiomic approaches, we provide the molecular characterization of maize response to urea and ammonium nutrition. Several transporters and enzymes involved in N-nutrition were upregulated by all three N-treatments (urea, ammonium, or urea and ammonium). Already after 1 day of treatment, the availability of different N-forms induced specific transcriptomic and metabolomic responses. The combination of urea and ammonium induced a prompt assimilation of N, characterized by high levels of some amino acids in shoots. Moreover, *ZmAMT1.1a*, *ZmGLN1;2*, *ZmGLN1;5*, *ZmGOT1*, and *ZmGOT3*, as well transcripts involved in glycolysis-TCA cycle were induced in roots by urea and ammonium mixture. Depending on N-form, even changes in the composition of phytohormones were observed in maize. This study paves the way to formulate guidelines for the optimization of N fertilization to improve N-use efficiency in maize and therefore limit N-losses in the environment.

KEYWORDS
AMT, DUR3, gene expression, nitrogen transporters, NRT, plant nutrition, RNA-seq, root uptake

1 | INTRODUCTION

As the main component of some molecules (i.e., amino acids, nucleic acids, and chlorophyll), nitrogen (N) plays a central role in plant metabolism and, therefore, its low availability greatly compromises plant growth and productivity (Hachiya & Sakakibara 2017; Hawkesford et al. 2012; Xu et al. 2012). A common agronomic practice to increase N availability in the soil, and therefore to sustain plant yield, is based on the use of N fertilizers. Among N-forms available as fertilizers, urea is the most widespread form followed by inorganic N-forms, nitrate and ammonium (Heffer & Prud'homme 2016). It is well known that plants preferentially sustain their N requirements through the acquisition of inorganic N (as ammonium and nitrate), nevertheless, the wide use in agriculture has contributed to the great relevance of urea among N-forms. Organic N-forms such as amino acids and small peptides become available for root uptake during the decomposition of organic matter, but in agricultural systems their contribution to plant nutrition is considered to be limited in comparison to inorganic N-sources (Xu et al. 2012).

In most intensive agricultural production systems, it has been estimated that over 50% of applied N is lost by leaching into the soil or by volatilization into the atmosphere, contributing to greenhouse gas, salinization in soil and eutrophication in aquatic systems (Cantarella et al. 2018; Raun & Johnson 1999; Sutton et al. 2011). In order to reduce N pollution, it is urgent to define new guidelines of N-fertilization practices acting to improve N-uptake efficiency (NUpE) in crops. In cereals, the N-use efficiency averages around 33%, indicating that there is still extensive room for improvement in the sustainability of agricultural management (Raun & Johnson 1999).

Extensive knowledge has been gained about nitrate and ammonium uptake, assimilation and signaling pathways (Hachiya & Sakakibara 2017; Kiba & Krapp 2016; Ravazzolo et al. 2020), whereas little information is available about urea nutrition in plants. In the soil, ureic-N is subject to rapid microbial conversion in ammonium and nitrate (Cantarella et al. 2018), two forms that, despite their instability in the soil, sustain greatly plant N requirement. However, in the last decades the direct acquisition of urea in roots operated by dedicated urea transporters has been characterized, demonstrating plant's ability to use urea as a direct N-source (Gu et al. 2012; Kojima et al. 2006; Liu et al. 2003; Zanin et al. 2014). Nevertheless, information on the molecular mechanisms involved in the use of this N source by plants is in large missing (Liu et al. 2003; Wang et al. 2012; Zanin et al. 2015a; Zanin et al. 2015b; Zanin et al. 2016). The deep comprehension of urea acquisition and the interplay of urea pathway with those of other nutrients or other N-forms would greatly contribute to increasing the agronomical efficiency of urea fertilization (Pinton et al. 2016).

Several studies report beneficial effects on plant growth when a mixture of more N-sources is used, mainly due to better N-use efficiency in plants (Arkoun et al. 2012; Britto & Kronzucker 2002; Buoso et al. 2021a; Garnica et al. 2009; Houdusse et al. 2005; Zanin et al. 2015b). In Arabidopsis, ammonium or nitrate uptake was not repressed by urea when inorganic N-forms were applied along with urea (Mérigout et al. 2008b). In maize, we recently found that urea did not interfere with the ammonium uptake rate in roots resulting in a significant increase in uptake efficiency of ammonium when both N-forms were applied to the nutrient solution (Buoso et al. 2021a). This may suggest that the use of mixed N sources, such as urea and ammonium, may represent a valid strategy to increase fertilizer use efficiency in crops. Moreover, to maximize the N use in crops, also the assimilation processes should be considered. It is well known that N and phytohormone signaling pathways are closely interconnected, although many aspects remain to be understood (Kiba et al. 2011; Krouk 2016; Krouk et al. 2011; Ristova et al. 2016; Vega et al. 2019). It is known that the biosynthesis, degradation, transport, and signaling of different phytohormones are regulated by nitrate, adjusting N availability and plant growth and development (Kiba et al. 2011; Ristova et al. 2016; Tian et al. 2008), whereas hormonal signaling feedback controls nitrate regulatory networks and metabolism (Krouk 2016; Krouk et al. 2011). In the last years, the relationship between N nutrition and phytohormones has mainly been studied in plants exposed to nitrate as the sole N-source, conversely, the information concerning the interaction of phytohormones with other N-sources are basically missing (Di et al. 2018; Kamada-Nobusada et al. 2013; Tamura et al. 2010). In recent study, Bauer and von Wirén (2020) provide evidence of a link between ammonium and cytokinin mediated signaling pathway suggesting that in wheat, the tillering can be influenced by the N-source applied.

In previous studies (Buoso et al. 2021a, 2021b), we observed that the concomitant presence of two N-sources in the nutrient solution (urea and ammonium) ameliorated plant growth, and the plants showed higher ammonium uptake efficiency than those treated with ammonium as a single N-source. At the morphological level, the use of urea and ammonium mixture led to beneficial effects on the development of roots and partially reduced the extracellular acidification. In the present study, to reveal the molecular basis of this response, transcriptomic and metabolomic changes of maize plants have been analyzed when urea and ammonium were simultaneously added to the nutrient solution, and this response was compared to urea- or ammonium-treated plants. The deep investigation of molecular responses to urea and ammonium mixture will provide useful bases to direct fertilizer management aiming to improve the N-use efficiency in maize.

2 | MATERIALS AND METHODS

2.1 | Plant growth

Maize plants (*Zea mays* L., P0423, Pioneer Hybrid Italia S.p.A.) were germinated over aerated 0.5 mM $CaSO_4$ solution. After 3 days, the seedlings were transferred into an aerated hydroponic system and under controlled conditions (16/8 h light/dark cycle, 220 μmol m^{-2} s^{-1} light intensity, 25/20°C temperature, 70–80% relative humidity). After 2 days, maize plants (5-day-old) were transferred to a N-free nutrient solution (μM: $CaSO_4$ 250; K_2SO_4 200; KH_2PO_4 175; $MgSO_4$

100; NaFe-EDTA 40; KCl 5; H_3BO_3 2.5; $MnSO_4$ 0.2; $ZnSO_4$ 0.2; $CuSO_4$ 0.05; Na_2MoO_4 0.05). Urea and/or ammonium were added to the N-free nutrient solution, hence three N-nutritional treatments were tested (2 mM total N): 1.00 mM CH_4N_2O (100U); 0.50 mM CH_4N_2O and 0.50 mM $(NH_4)_2SO_4$ (50U:50A); 1.00 mM $(NH_4)_2SO_4$ (100A). As a control, some plants were grown in the N-free nutrient solution (-N, Figure S1). Calcium sulphate ($CaSO_4$) was added to the N-free nutrient solution in variable amounts (1.00 mM $CaSO_4$ in -N and 100U; 0.50 mM $CaSO_4$ in 50U:50A) to compensate the sulfur amount deriving from ammonium sulphate treatment. The pH of the solution was buffered using 1 mM MES-BTP at pH 6.0. After 1 h from the beginning of the light phase (8:00 a.m.), the N sources have been added to the nutrient solution. The nutrient solution was renewed every 48 h, avoiding urea degradation. During this period, the hydrolysis of urea was unlikely in hydroponic conditions (Buoso et al. 2021a; Mérigout et al. 2008a; Zanin et al. 2015b). To evaluate the occurrence of nitrification processes, nitrate concentration in nutrient solution was evaluated after 48 h by spectrophotometer assay at 410 nm (Cataldo et al. 1975; Zanin et al. 2018), no detectable nitrate was present in all nutrient solutions. After 1 day (24 hours) and 7 days of treatment, shoots and roots were collected and frozen in liquid N for following analyses. Transcriptomic analyses were performed in roots after 1 day of treatment; metabolomic analyses were performed in root and shoot collected after 1 and 7 days of treatment.

2.2 | RNA extraction

Transcriptomic analyses were performed on different nutritional conditions, and three independent biological replicates were used for each condition. For each biological replicate, four plants of maize were pooled together, and total RNA was isolated. One gram of maize tissue was homogenized in liquid N, and total RNA was extracted from approximately 60 mg of powder with the Spectrum Plant Total RNA Kit (Sigma–Aldrich) according to the manufacturer's instructions. To verify the absence of genomic contamination, 1 μg of total RNA was analyzed electrophoretically, running on 1% agarose gel. The concentration and integrity of RNA were checked on the Qubit 2.0 Fluorometer (Life Technologies) and on the Agilent 2100 Bioanalyzer system following the manufacturer's protocol (Agilent Technologies). RIN scores ranged from 7.9 to 8.9.

2.3 | RNA-sequencing and bioinformatics analysis

The preparation of the cDNA library and following RNA sequencing reactions were performed by IGA Technology Services s.r.l. (Udine). Library preparation was performed following the Illumina protocol TrueSeq 2.0 using 2 μg of total RNA for each sample (Venuti et al. 2019). The 75 bp single-end reads were obtained using an Illumina NextSeq 500 platform. Reads were aligned to the B73 RefGen 4 reference genome and corresponding transcriptome (available at: ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/plant/Zea_

mays/latest_assembly_versions/GCA_000005005.6_B73_RefGen_v4) using hisat2 (Kim et al. 2019) with default parameters. Differential expression was assessed with cuffdiff (Trapnell et al. 2012) with default parameters, including the geometric method for library size normalization (Anders & Huber 2010). Four transcriptomic profiles were obtained (-N, 100U, 50U:50A, 100A) by averaging expression across replicates and displaying expression levels (Figure S2).

Functional annotation of the reconstructed transcriptome was performed relying on Gene Ontology (Ashburner et al. 2000) and KEGG (Kanehisa et al. 2016). Gene ontology (GO) analysis and GO enrichment was performed using the Singular Enrichment Analysis (SEA) of AgriGO v2.0 (Tian et al. 2017) with Maize AGPv4 (Maize-GAMER) as custom reference background. Enrichment was assessed using hypergeometric tests with Yekutieli adjustment for multiple comparisons (p value ≤0.05). For KEGG annotation, the R package biomaRt (Durinck et al. 2009) was used to map KEGG terms to the transcripts, and the R package clusterProfiler (Yu et al. 2012) was used to perform enrichment analysis of KEGG terms. Clustering of genes based on expression across different N-treatments was performed using a fuzzy clustering approach with the R package mfuzz (Kumar & Futschik 2007). Shell scripts and R functions used for the present study are made freely available on GitHub (https://github.com/fabiomarroni/mais_DE_2020).

2.4 | Reverse transcription and real-time RT-PCR analyses

Real-time RT–PCR analyses were performed on maize roots as described by Buoso et al. (2021a). Maize roots were sampled, and total RNA was extracted using Invisorb© Spin Plant RNA kit (Invitek Molecular) following manufacturer's instructions. The quality and concentration of the RNA was checked by gel electrophoresis and by Nanodrop, respectively. Total RNA (1 μg) was reverse-transcribed in cDNA using 100 pmol of Oligo-d(T)$_{23}$ (Sigma–Aldrich), 20U Prime RNase Inhibitor (Sigma–Aldrich), 200U of RNase H derivative of Moloney murine leukemia virus (M-MLV reverse transcriptase, Sigma Aldrich), according to the manufacturer's protocol. Using Primer3 software (Koressaar & Remm 2007; Untergrasser et al. 2012), primers were designed and synthesized by Sigma Aldrich. Primers sequences were reported in Buoso et al. (2021a) and Table S2. The analyses were performed using CFX96 Real-Time RT-PCR Detection (Biorad) and qPCR package for statistical R software (R version 3.5.1, www.dr-spiess.de/qpcR.html). For each set of primers, the efficiencies of amplification were determined as indicated by Ritz and Spiess (2008). Data were referred to the expression of two housekeeping genes ZmGAPDH or ZmTUA. Data were normalized using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

2.5 | Metabolic analyses

Metabolomic analyses were performed on three independent biological replicates, and for each biological replicate, four maize plants were

pooled together. The shoot and root samples were extracted with 70% H_2O, 30% MeOH and centrifuged to collect the supernatant. The pellet was extracted again with H_2O, centrifuged, and the supernatant was pooled with the previous collection. For the UPLC-MS (Ultra performance liquid chromatography-Mass spectrometry) analysis, the separation and the detection were accomplished using an Acquity UPLC system coupled to a Xevo G2-S QTof mass spectrometer (Waters) equipped with a LockSpray electrospray ionization (ESI) source. Sample separation was carried out by injecting 10 µl into an HSS T3 C18, 2.1 × 100 mm, 1.8 µm column (Waters) at a flow rate of 0.4 ml min^{-1}, and the column oven was maintained at 30°C. The mobile phases were composed of solvent A Milli-Q water containing 0.1% formic acid (LCMS grade, Fluka analytics) and solvent B 50% MeOH, 50% acetonitrile (Fisher Optima) containing 0.1% formic acid. The MS acquisition was carried out in positive, sensitive ion mode with the following parameters: source voltage 0.5 kV; cone voltage 40 V; source temperature 120°C; desolvation gas temperature 550°C and desolvation gas flow 800 l h^{-1}.

Phytohormones (Abscisic acid, ABA; salicylic acid, SA; 12-oxo-phytodienoic acid, OPDA; jasmonic acid, JA; jasmonoyl-Isoleucine, JA-Ile; 1-amino-1-cyclopropane-carboxylic acid, ACC; indole-3-acetic acid, IAA) standards were purchased from Sigma and OlchemIn. Phytohormones were determined by a UHPLC–MS/MS system. Ten milligrams FW (Fresh Weight) samples were extracted with 70% methanol containing isotope-labeled internal standards and centrifuged at 17927 g to collect the supernatant. After evaporation (SPE Dry 96, Biotage), the extract was resuspended in 2% formic acid solution and purified thanks to an SPE ABN express column of 1 ml (Biotage). The phytohormones were eluted with methanol, and samples were evaporated and

resuspended in a 0.1% formic acid solution before injection into the system. The separation and detection were accomplished using a Nexera X2 UHPLC system (Shimadzu) coupled to a QTrap 6500+ mass spectrometer (Sciex) equipped with an IonDrive turbo V electrospray source. Phytohormones separation was carried out by injecting 2 µl into a Kinetex Evo C18 core-shell column (100 × 2.1 mm, 2.6 µm, Phenomenex) at a flow rate of 0.7 ml min^{-1}, and the column oven was maintained at 40°C. The mobile phases were composed of solvent A Milli-Q water containing 0.1% formic acid and solvent B acetonitrile LCMS grade containing 0.1% formic acid. The analysis was done in scheduled MRM (Multiple reaction monitoring) mode in positive and negative mode simultaneously with a polarity switching of 5 ms. The MS acquisition was carried out with the following parameters: Ion spray voltage 5500 V in positive mode and −4500 V in negative mode; Source temperature 600°C; Curtain gas 35 psi; Nebulizer gas 50 psi; Heater gas 60 psi; Collision gas medium; Entrance potential ±10 V; MRM detection window 30 s; Target scan time 0.075 s.

Cytokinins (Isopentenyladenine, IP; isopentenyladenosine, IPA; trans zeatin, TZ; cis zeatin, CZ; cis-zeatin riboside, CZR) standards were purchased from OlchemIn. Cytokinins were analyzed by a UHPLC–MS/MS system. Twenty milligrams FW samples were extracted with 70% methanol, 29% H_2O, 1% formic acid containing isotope-labeled internal standards and centrifuged at 17 927 g to collect the supernatant. After evaporation (SPE Dry 96, Biotage), the extract was resuspended in 2% formic acid solution and purified using an SPE CX express column of 1 ml (Biotage). The cytokinins were eluted with 5% ammonium hydroxide methanolic solution, and samples were evaporated and resuspended in 100 µl of 0.1% formic acid solution before injection into the system.
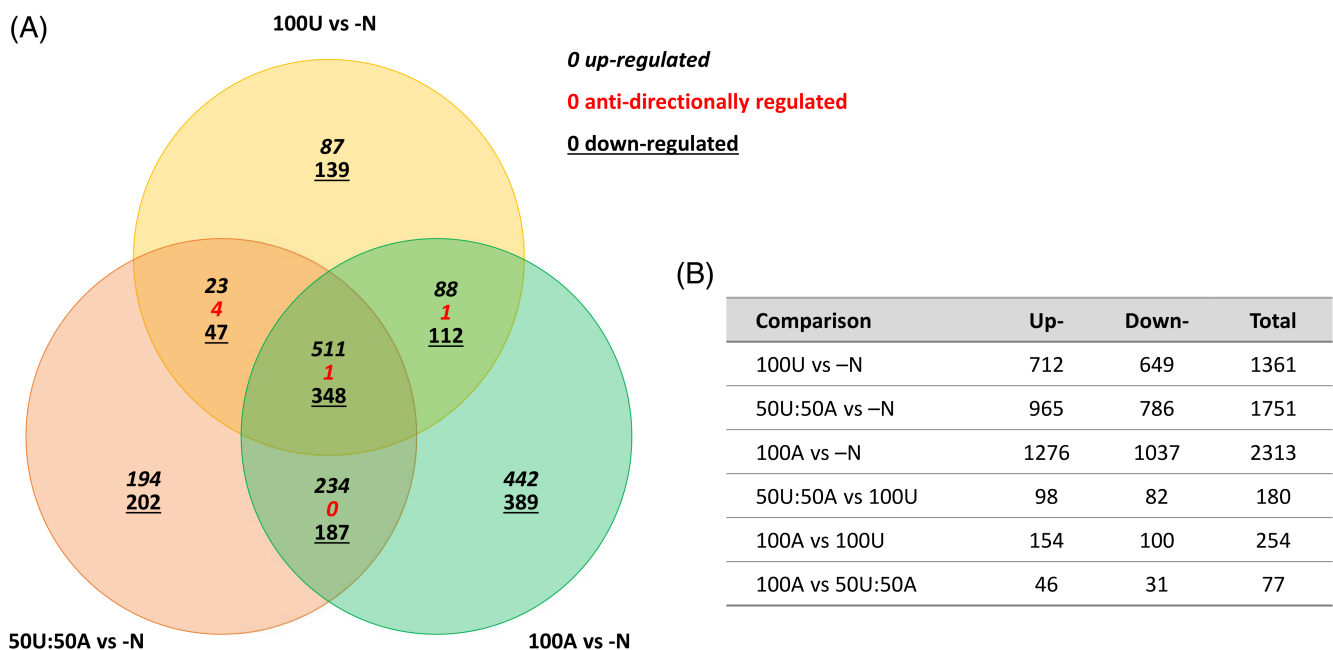
FIGURE 1   RNA sequencing results: (A) Venn diagram of DEGs transcriptionally modulated in three comparison urea (100U vs. -N), urea and ammonium (50U:50A vs. -N), ammonium (100A vs. -N). (B) Number of DEGs transcriptionally modulated in six comparison (N = 3, *q*-value ≤0.05)

TABLE 1 List of DEGs most involved in N acquisition and identified by transcriptomic analyses on maize roots after 1 day of treatment with urea (100U), urea and ammonium (50U:50A) or ammonium (100A)

Gene_ID	100U vs. -N		50U:50A vs. -N		100A vs. -N		Description	Symbol (Zm)
	Log$_2$FC	q value	Log$_2$FC	q value	Log$_2$FC	q value		
N-transport								
Zm00001d017249	**1.917**	0.002	**1.915**	0.001	**1.992**	0.001	Ammonium transporter	AMT1;3
Zm00001d025831	0.603	0.070	**0.784**	0.030	0.548	0.080	Ammonium transporter	AMT1;1a
Zm00001d034782	−0.356	0.300	**−1.392**	0.001	**−1.088**	0.001	Ammonium transporter	AMT8
Zm00001d016771	−0.426	0.217	−0.281	0.533	**−0.670**	0.021	Ammonium transporter	AMT9
Zm00001d037242	**−1.504**	0.006	−0.996	0.098	**−1.720**	0.001	Urea transporter	DUR3
Zm00001d052261	0.413	0.090	0.084	0.849	**0.673**	0.001	Major Intrinsic Proteins.NIP	NIP1;2
Zm00001d002690	0.196	0.348	0.196	0.300	**0.308**	0.039	Major Intrinsic Proteins.PIP	PIP1a
Zm00001d051174	**0.392**	0.004	0.269	0.093	0.205	0.201	Major Intrinsic Proteins.PIP	PIP2c
Zm00001d019565	**0.391**	0.003	**0.503**	0.001	**0.429**	0.001	Major Intrinsic Proteins.PIP	PIP2f
Zm00001d011778	**0.717**	0.011	0.578	0.120	**0.722**	0.008	Major Intrinsic Proteins.PIP	TIP2
Zm00001d002738	−0.116	0.826	−0.180	0.727	**−0.656**	0.036	Major Intrinsic Proteins.PIP	TIP2;3
Zm00001d026177	**0.312**	0.036	0.213	0.214	0.173	0.297	Major Intrinsic Proteins.PIP	POR2
Zm00001d027652	**0.676**	0.002	**0.730**	0.001	**0.744**	0.001	Major Intrinsic Proteins.PIP	TIP1
Zm00001d037228	0.032	0.964	**−1.102**	0.031	−0.642	0.094	Major Intrinsic Proteins. unspecified	NIP2b
Zm00001d029932	**1.732**	0.002	**2.159**	0.001	**2.249**	0.001	Nitrate transporter	NRT1.1
Zm00001d054060	**1.547**	0.002	**1.695**	0.001	**1.524**	0.001	Nitrate transporter	NRT2.2
Zm00001d054057	**1.056**	0.002	0.287	0.533	0.292	0.396	Nitrate transporter	NRT2.1
Zm00001d024587	**0.577**	0.002	**0.893**	0.001	**0.914**	0.001	Nitrate transporter	NPF6.4
Zm00001d017666	**−0.520**	0.002	**−0.990**	0.001	**−0.855**	0.001	Nitrate transporter	NRT1.5
Zm00001d031213	−0.102	1.000	**1.581**	0.029	0.918	0.113	Nitrate transporter	NRT1.7
Zm00001d018799	0.085	0.738	**0.531**	0.001	**0.678**	0.001	CBL-interacting protein kinase 23	CIPK23
N-metabolism								
Zm00001d049995	**−0.804**	0.019	**−0.988**	0.014	**−0.945**	0.001	Nitrate reductase	NR
Zm00001d031769	−0.928	0.135	−1.179	0.131	**−1.343**	0.043	Nitrate reductase	NR4
Zm00001d052164	0.329	0.525	**0.860**	0.043	0.186	0.700	Nitrite reductase	NIR2
Zm00001d048050	**2.177**	0.002	**2.236**	0.001	**2.025**	0.001	Glutamine synthetase	GS3
Zm00001d051804	**−0.797**	0.002	**−0.981**	0.001	**−1.328**	0.001	Glutamine synthetase	GLN1;4
Zm00001d017958	−0.324	0.056	−0.295	0.059	**−0.430**	0.001	Glutamine synthetase	GLN1;3
Zm00001d033747	0.047	0.891	**0.387**	0.040	0.247	0.186	Glutamine synthetase	GLN1;2
Zm00001d034420	**0.693**	0.002	**0.987**	0.001	**0.871**	0.001	Glutamate dehydrogenase	GDH1
Zm00001d025984	**2.101**	0.002	**2.597**	0.001	**2.628**	0.001	Glutamate dehydrogenase	GDH2
Zm00001d022388	**0.554**	0.002	**0.757**	0.001	**0.586**	0.001	Glutamate synthase Fd-dependent	Fd-GOGAT
Zm00001d011610	**2.439**	0.002	**2.502**	0.001	**2.361**	0.001	Glutamate synthase NADH-dependent	GLT1
Zm00001d043845	**1.085**	0.002	**1.103**	0.001	**0.886**	0.001	Glutamate synthase NADH-dependent	GLT1
Zm00001d028750	**4.191**	0.002	**5.067**	0.001	**5.070**	0.001	Asparagine synthetase	ASNS3
Zm00001d047736	**4.537**	0.002	**5.194**	0.001	**5.253**	0.001	Asparagine synthetase	ASNS4
Zm00001d016198	**0.495**	0.002	**0.558**	0.001	**0.484**	0.001	Aspartate aminotransferase	GOT3
Zm00001d018386	−0.079	0.775	−0.218	0.286	**−0.336**	0.023	Aspartate aminotransferase	GOT2
Zm00001d043382	0.231	0.271	**0.341**	0.038	**0.355**	0.022	Aspartate aminotransferase	GOT1

Note: The expression data are shown as Log$_2$FC values and refers to -N treatment (-N). In bold, the statistically significant values are shown (N = 3, q value ≤ 0.05). *Symbol (Zm)* refers to the symbol gene name in *Zea mays*.

The separation and detection were accomplished using a Nexera X2 UHPLC system coupled to a QTrap 6500+ mass spectrometer equipped with an IonDrive turbo V electrospray source. Cytokinins separation was carried out by injecting 2 μl into a Kinetex Evo C18 core-shell column (100 × 2.1 mm, 2.6 μm, Phenomenex) at a flow rate of 0.7 ml min^{-1}, and the column oven was maintained at 40°C. The

mobile phases were composed of solvent A Milli-Q water containing 0.1% formic acid and solvent B acetonitrile containing 0.1% formic acid. The analysis was done in scheduled MRM mode in positive mode. The MS acquisition was carried out with the following parameters: Ion spray voltage 5500 V; Source temperature 650°C; Curtain gas 45 psi; Nebulizer gas 70 psi; Heater gas 70 psi; Collision gas medium; Entrance potential 10 V; MRM detection window 60 s; Target scan time 0.21 s.

2.6 | Statistical analyses

For each thesis, all analyses were performed on three independent biological replicates, and for each biological replicate, four plants of maize were pooled together. Transcriptomic data were analyzed using cuffdiff (N = 3, q-value ≤ 0.05; Trapnell et al. 2012) with the geometric method for library size normalization (Anders & Huber 2010). All statistically significant transcripts are expressed as positive or negative Log_2 (Fold Change, FC) values (corresponding to up-or down-regulated transcripts, respectively). GO enrichment, KEGG and clustering analyses were performed as described above (N = 3, q-value ≤0.05). Regarding metabolomic data, volcano plots comparing samples were generated by computing the Fisher p-value, the FC and then plotted using Matplotlib Python library (N = 3, p- value ≤0.05). Real-time RT-PCR data are provided as Log_2FC values, and their statistical significance was calculated by one-way ANOVA (Holm-Sidak test; N = 3, p-value ≤ 0.05).

To integrate information of gene expression and amino acids or phytohormones levels, an exploratory correlation analysis was conducted as follows. Gene expression and metabolite levels were averaged across conditions. Analyses were performed for metabolite levels measured at 1 day (both for root and shoot), and the Spearman correlation coefficient was measured across the four experimental conditions. Genes showing perfect positive or negative correlation with metabolic data were tested for KEGG enrichment as described above.

3 | RESULTS

3.1 | Transcriptomic analyses

Root RNA-seq analyses indicated that in comparison to -N plants, the treatment with urea (100U), urea and ammonium (50U:50A) or ammonium (100A) resulted in 1361, 1751, and 2313 Differentially Expressed Genes (DEGs), respectively (Figure 1). More than 850 DEGs were commonly modulated in all three treatments, whereas some genes were specifically modulated by each N-treatment: 226 (87 upregulated and 139 downregulated), 396 (194 and 202), and 831 (442 and 389) DEGs were exclusively modulated by 100U vs. -N, 50U:50A vs. -N and 100A vs. -N, respectively (Figure 1A). Seventy-four genes were modulated by urea and were also modulated when urea and ammonium were simultaneously present in nutrient solution

(50U:50A vs. -N; Figure 1A). On the other hand, 421 DEGs were modulated by ammonium (100A vs. -N) and by urea and ammonium (50U:50A vs. -N) but not by urea alone (100U vs. -N; Figure 1A). In comparison to -N roots, all three treatments upregulated the expression of genes involved in N-acquisition: nitrate and ammonium transporters (*ZmNRT1.1*, *ZmNRT2.2*, *ZmNPF6.4*, *ZmAMT1;3*), glutamine synthetase and glutamate synthases (*ZmGLN1;5*, *ZmGLU*, and *ZmGLT1*), glutamate dehydrogenases (*ZmGDH1* and *ZmGDH2*), asparagine synthetases (*ZmASNS3*, *ZmASNS4*), aspartate aminotransferase (*ZmGOT3*) and aquaporins (*ZmPIP2f*, *ZmTIP1*). On the other hand, all three comparisons shared a common downregulation of genes coding for a nitrate transporter, nitrate reductase and an isoform of glutamine synthetase (*ZmNRT1.5*, *ZmNR*, and *ZmGLN1;4*, respectively; Tables 1 and S1). Moreover, depending on the N source applied, a specific modulation of genes related to N acquisition was observed among comparisons. A specific upregulation of the following genes was observed by urea treatment (in 100U vs. -N): *ZmPIP2c*, *ZmPOR2*, and *ZmNRT2.1*. The presence of urea and ammonium in nutrient solution upregulated *ZmAMT1;1a*, *ZmNRT1.7*, *ZmNIR2*, *ZmGLN1;2* and downregulated *ZmNIP2b* (in 50U:50A vs. -N). Ammonium treatment (100A vs. -N) modulated the expression of the following genes: *ZmNIP1;2*, *ZmPIP1a*, were found upregulated; *ZmAMT9*, *ZmTIP2;3*, *ZmNR4*, *ZmGLN1;3*, and *ZmGOT2* were found downregulated. A downregulation of *ZmDUR3* was observed in the urea or in ammonium
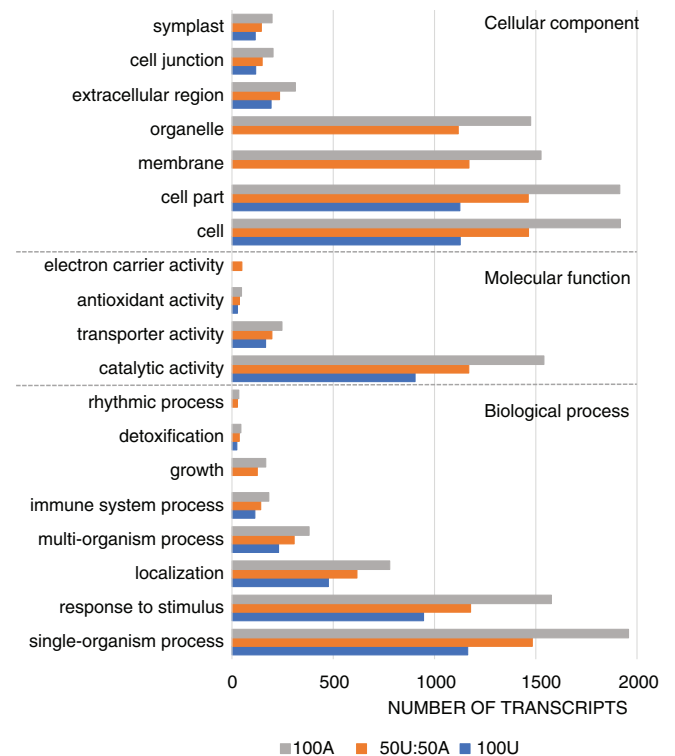
FIGURE 2 Gene ontology (GO) enrichment analyses (cross comparison of singular enrichment analysis) of transcriptomic profile of maize roots treated with urea (100U), urea and ammonium (50U:50A), ammonium (100A) in comparison to -N plants (-N; N = 3, q-value ≤0.05)

KEGG enrichment analyses of transcriptomic response of maize roots treated with urea (100U), urea and ammonium (50U:50A), ammonium (100A) in comparison to -N plants (-N; N = 3, q-value ≤0.05)
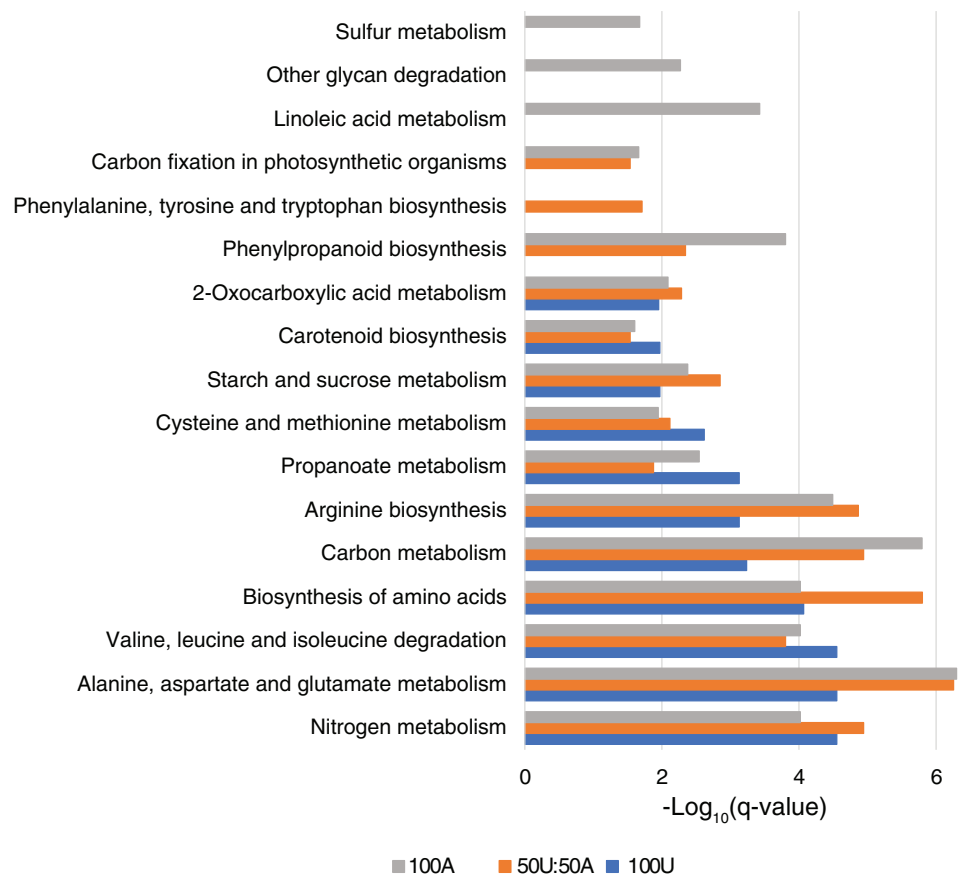

treated roots; on the contrary, *ZmTIP2* was found upregulated (100U vs. -N and 100A vs. -N). In presence of ammonium plants upregulated *ZmGOT1* and *ZmCIPK23* and downregulated *ZmAMT8* (50U:50A vs. -N and 100A vs. -N; gene descriptions are reported in Tables 1 and S1).

Gene ontology (GO) enrichment analyses allowed to identify over-represented classes by differentially modulated transcripts (Figures 2, S3, and S4). Analyses related to *cellular component* indicated an enrichment of DEGs involved in "organelle", "membrane", "cell part" and "cell" ("organelle" and "membrane" were not enriched by 100U vs. -N); *molecular function* indicated an enrichment of DEGs involved in "transporter activity" and "catalytic activity"; *biological process* indicated an enrichment of DEGs involved in "localization", "response to stimulus", "single-organism process". Among the "localization" class, several genes coding for N transporters were found differentially modulated by treatments, as nitrate transporters (*ZmNRT1.7*, *ZmNRT2.2*, *and ZmNRT2.1*), ammonium transporters (*ZmAMT1.1a*, *ZmAMT1.3*, and *ZmAMT9*) and urea transporter (*ZmDUR3*; Tables 1 and S1).

GO analyses showed that a relevant percentage of upregulated genes coded for proteins with catalytic functions, therefore KEGG enrichment analyses were performed in order to evaluate changes in the metabolic pathways (Figure 3). The presence of ammonium as the sole N source in the external solution (100A vs. -N) significantly modulated pathways involved in "sulfur metabolism", "other glycan degradation" and "linoleic acid metabolism". The pathway "phenylalanine,

tyrosine and tryptophan biosynthesis" was enriched only in response to urea and ammonium treatment (50U:50A vs. -N). In the presence of ammonium (100A vs. -N and 50U:50A vs. -N) significant enrichment of "carbon fixation in photosynthetic organism" and "phenylpropanoid biosynthesis" was observed. All three comparisons (100U vs. -N, 50U:50A vs. -N and 100A vs. -N) showed significant enrichment of the following metabolic pathways: "2-oxocarboxylic acid metabolism", "carotenoid biosynthesis", "starch and sucrose metabolism", "cysteine and methionine metabolism", "propanoate metabolism", "arginine biosynthesis", "carbon metabolism", "biosynthesis of amino acids", "valine, leucine and isoleucine degradation", "alanine aspartate and glutamate metabolism", "nitrogen metabolism". This latter class and "biosynthesis of amino acids" were two pathways mainly enriched by urea and ammonium mixture (50U:50A vs. -N; Figure 3). Overall, our results indicate that N treatments induced changes in primary and secondary metabolism and modulated genes involved in the acquisition and remobilization of N in plants.

On the basis of their expression values, DEGs were divided into eight clusters. Two clusters involved transcripts mainly responsive to urea (100U, clusters #1–2, Figure 4), other two clusters included transcripts upregulated mainly by ammonium (100A, clusters #3–4, Figure 4), and four clusters were referred to those transcripts that specifically were responsive to the mixture of two N-sources (urea and ammonium, 50U:50A, clusters #5–8, Figure 4). In Figure 4, DEGs mainly related to N-acquisition are listed for each cluster. Concerning N-acquisition, urea in nutrient solution induced the overall
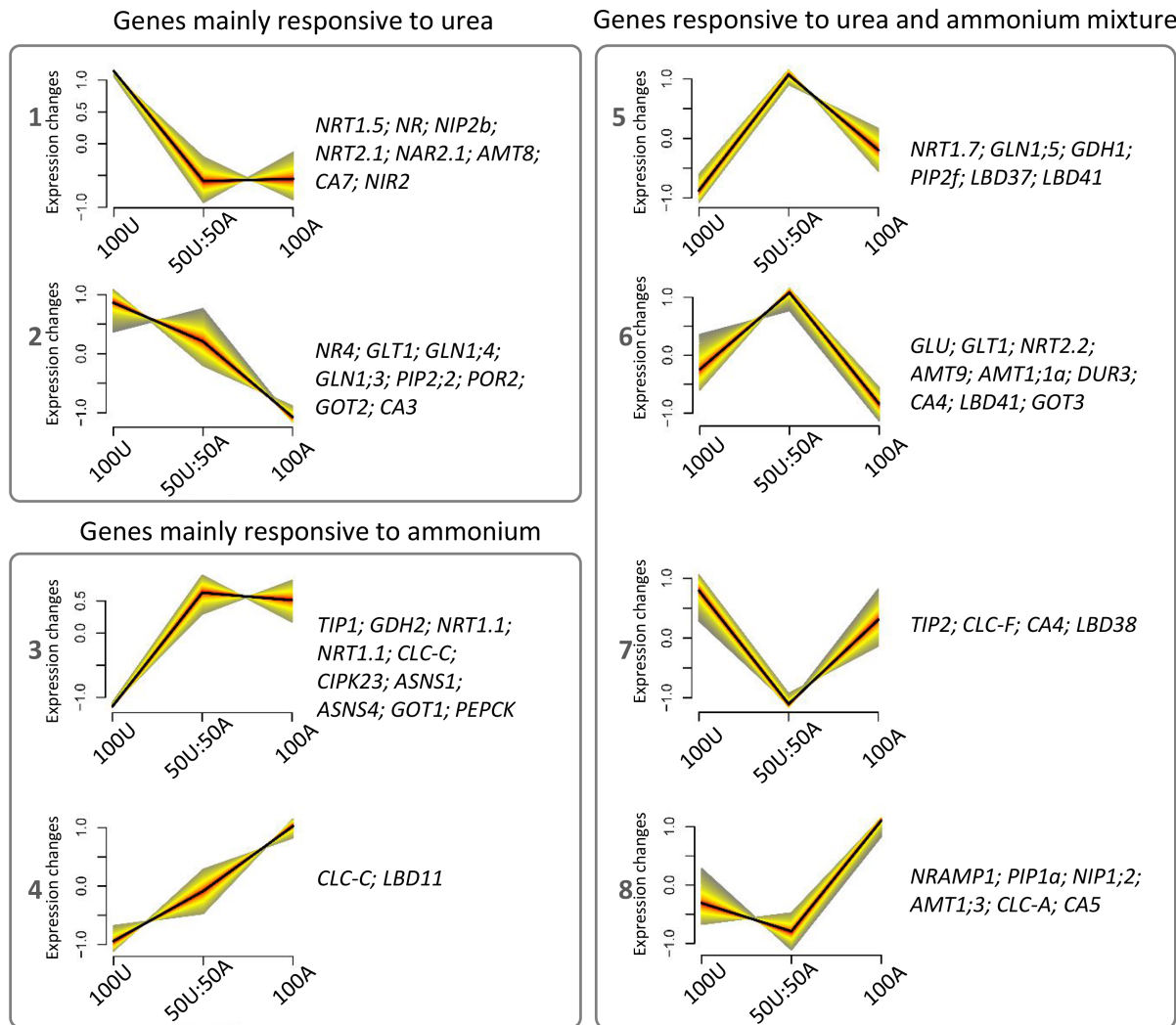
FIGURE 4 Clustering analyses of transcriptomic response of maize roots treated with urea (100U), urea and ammonium (50U:50A), ammonium (100A) in comparison to -N plants (-N; N = 3, q-value ≤ 0.05)

upregulation of the following genes coding for: ammonium transporter (ZmAMT8), several aquaporins (ZmNIP2b; ZmPIP2c; ZmPOR2), nitrate transporter and accessory protein (ZmNRT1.5, ZmNRT2.1, ZmNAR2.1), nitrate reductase (ZmNR and ZmNR4), nitrite reductase (ZmNIR2), glutamine and glutamate synthases (ZmGLN1;4, ZmGLT1), aspartate aminotransferase (ZmGOT2) and carbonic anhydrase (ZmCA7, ZmCA3; clusters #1–2, Figure 4). The presence of ammonium in the nutrient solution induced the expression of genes encoding dual-affinity nitrate transporters (ZmNRT1.1), regulative proteins (ZmCIPK23), transcription factors (ZmLBD11), aquaporins (ZmTIP1); chloride channel (ZmCLC-c), and other genes coding for enzymes involved in N-assimilation (ZmGDH2, ZmASNS1, ZmASNS4, ZmGOT1; clusters #3–4, Figure 4). The root exposure to a mixture of two N-sources, urea and ammonium, determined high expression values of the following genes involved in N-acquisition and encoding: nitrate transporter (ZmNRT1.7, ZmNRT2.2), ammonium transporter (ZmAMT9, ZmAMT1;1a), urea transporter (ZmDUR3), glutamine synthetase and glutamate synthases (ZmGLN1;5, ZmGLT1, ZmGLU), glutamate dehydrogenase (ZmGDH1), aspartate aminotransferase (ZmGOT3), aquaporins (ZmPIP2f), transcription factors (ZmLBD37,

ZmLBD41), carbonic anhydrase (ZmCA4; clusters #5–6, Figure 4). Two clusters (clusters #7–8, Figure 4) grouped genes which expression values were lower when two sources were applied together than when one single N source was used. In particular, the simultaneous use of urea and ammonium determined low expression values for genes encoding: aquaporins (ZmTIP2, ZmPIP1a, ZmNIP1;2), chloride channel (ZmCLC-F, ZmCLC-A), ammonium transporter (ZmAMT1;3), carbonic anhydrase (ZmCA4, ZmCA5), transcription factor (ZmLBD38).

The expression of several DEGs was validated through real-time RT-PCR (Table S2). Regarding tested genes, the expression values detected by the transcriptomic approach were confirmed by real-time RT-PCR.

3.2 | Amino acid concentration in maize shoots and roots

The amino acid concentrations were evaluated in shoots and roots after 1 day and 7 days of treatment (Figures 5 and 6, Table S3). After 1 day of treatment, the amino acid profile in the shoot indicated that the use

SHOOT
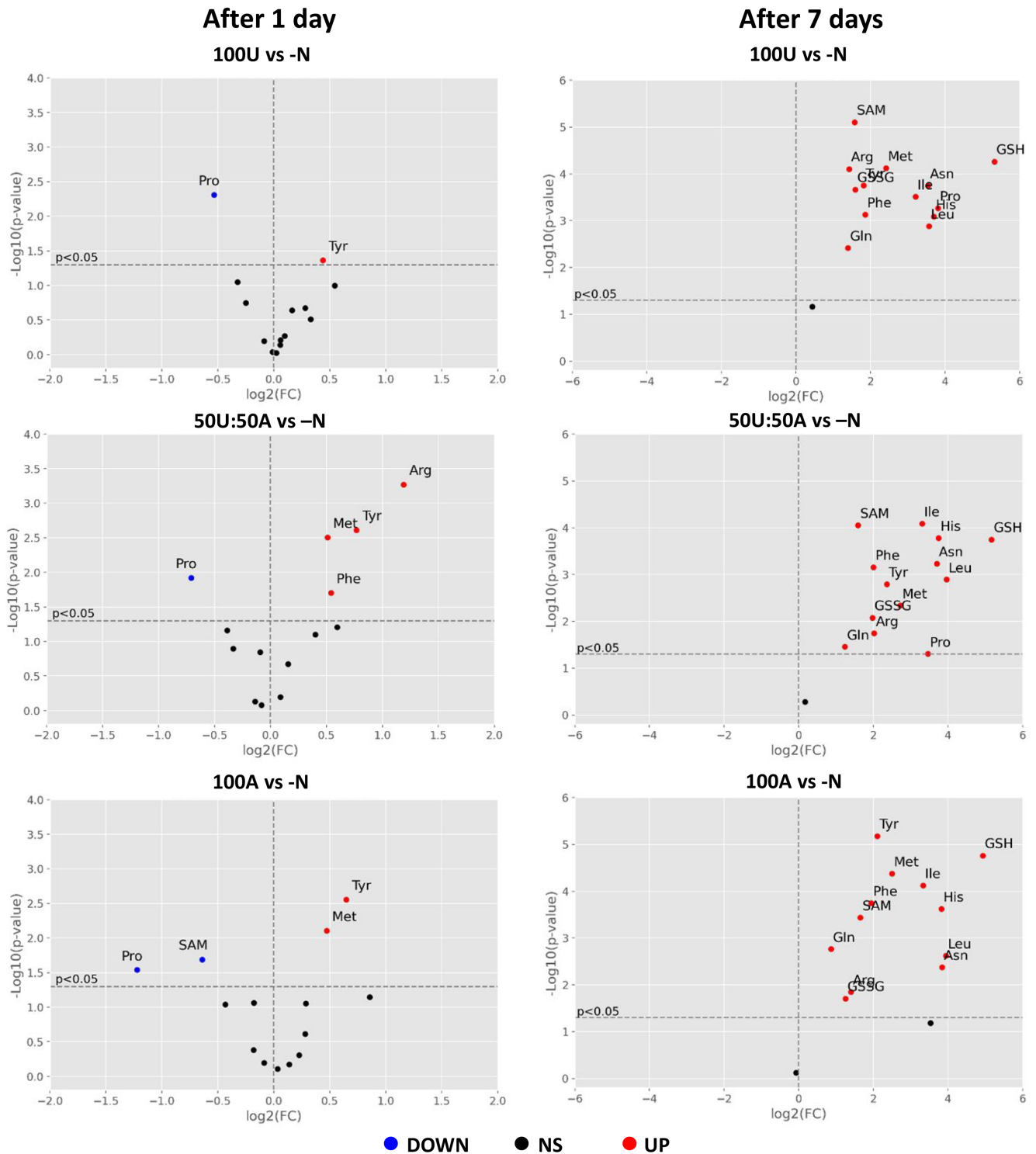
FIGURE 5   Volcano plots of amino acid profile in shoots of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downconcentrated metabolite (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upconcentrated metabolite (in red; Fisher's test, N = 3, *p*-value ≤ 0.05)

of urea and ammonium mixture led to a significant increase in the concentration of Met, Phe, Tyr, Arg. On the contrary, few amino acids were modulated by the other two N treatments (Tyr in 100U vs. -N; Tyr and Met in 100A vs. -N). A significant reduction of Pro (in all the three N-treatments) and SAM (in 100A vs. -N) was observed in the shoot. In root, an increase in the concentration of
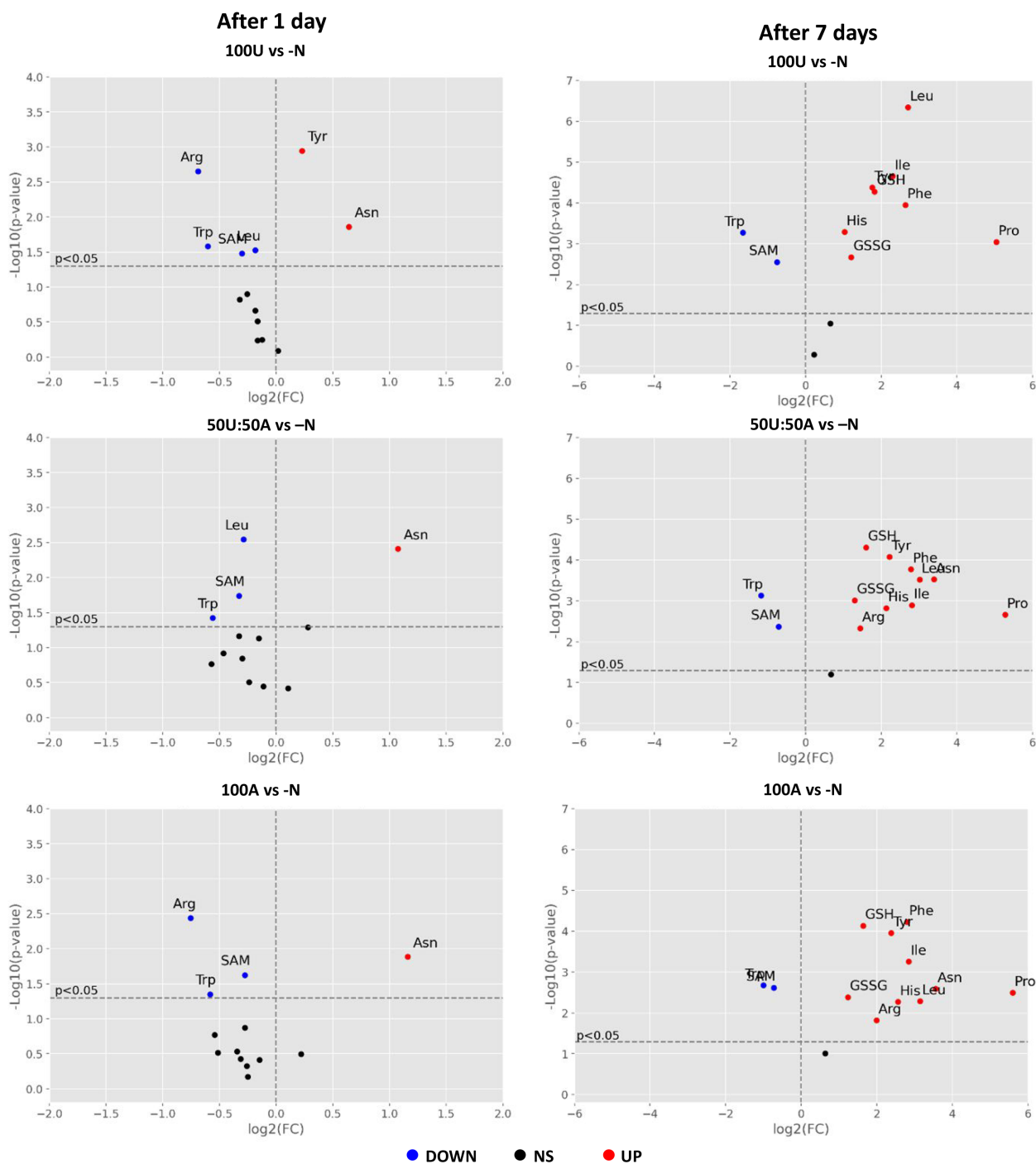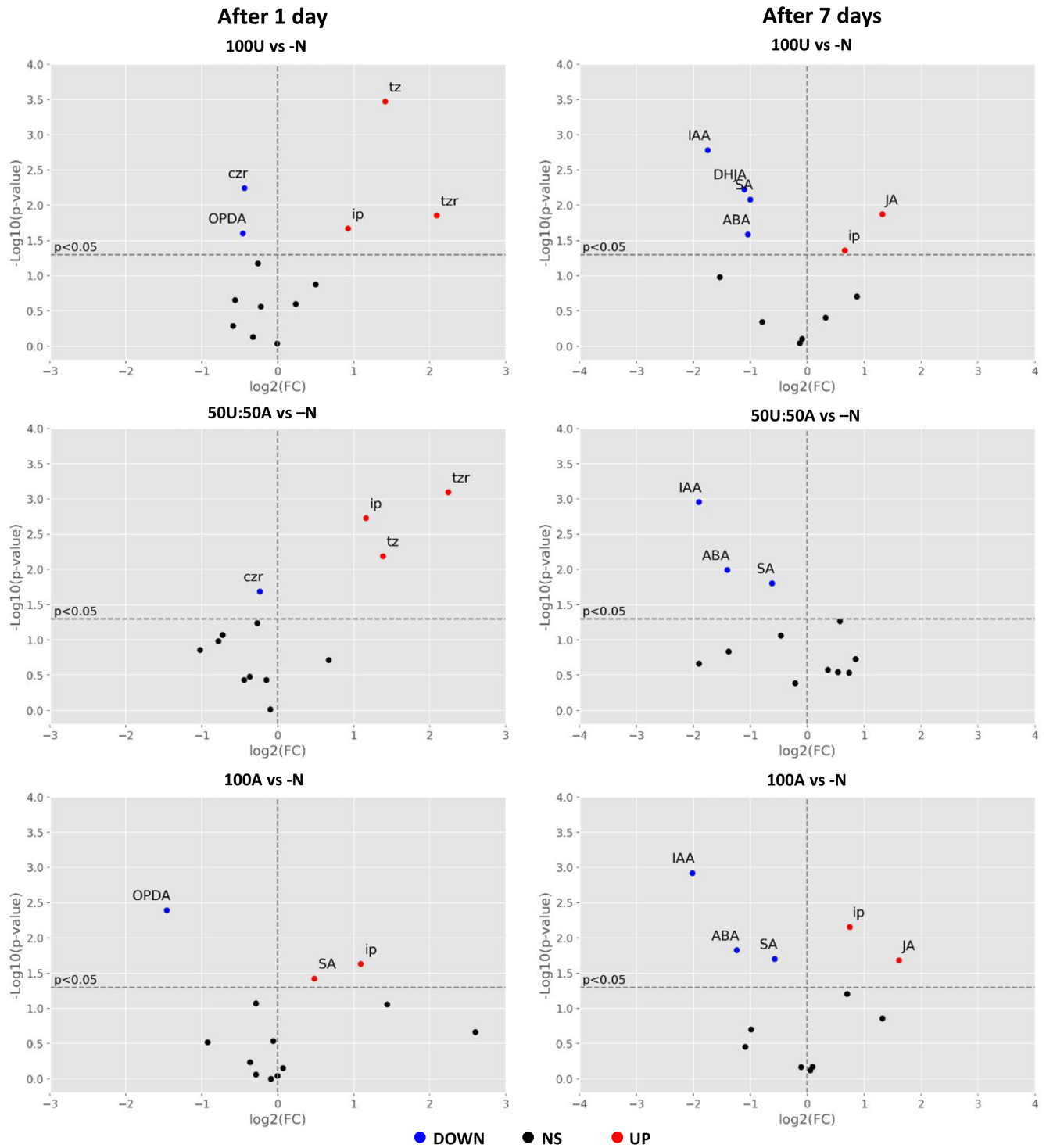
ROOT

FIGURE 6 Volcano plots of amino acid profile in roots of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downconcentrated metabolite (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upconcentrated metabolite (in red; Fisher's test, N = 3, *p*-value ≤ 0.05)

Asn was detected in N-treated plants, whereas Tyr was significantly higher only in urea treated plants (100U vs. -N). In comparison to -N, Trp and SAM decreased in all N-treated roots, moreover a reduction in the concentration of Arg was measured in 100U and 100A-roots and Leu in 100U and 50U:50A-roots. The concentration profile of other amino acids did not change significantly comparing different N-treatments with -N plants.

After 7 days of treatment, the concentrations of all amino acids (except Trp) were significantly higher in N-treated plants than -N ones.

Glutathione (GSH; γ-glutamyl-cysteinyl-glycine) was measured in plant tissue, and the reduced form (GSH) over the oxidized one (GSSG) was mainly present. After 7 days of treatment, the N-treated plants showed higher concentration of GSH in comparison to -N plants. Comparing N treatments, at both sampling times (1 day

SHOOT

FIGURE 7 Volcano plots of phytohormones profile in shoots of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downconcentrated metabolite (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upconcentrated metabolite (in red; Fisher's test, N = 3, *p*-value ≤ 0.05)

and 7 days), the GSH concentration in shoots was affected by ammonium in nutrient solution, since low amount of GSH was present in 100A vs-N.

The correlation analyses showed that the concentration pattern of Met, Tyr, and Arg in shoots and Met, Tyr, GSH, and GSSG in roots among the thesis correlated with the expression
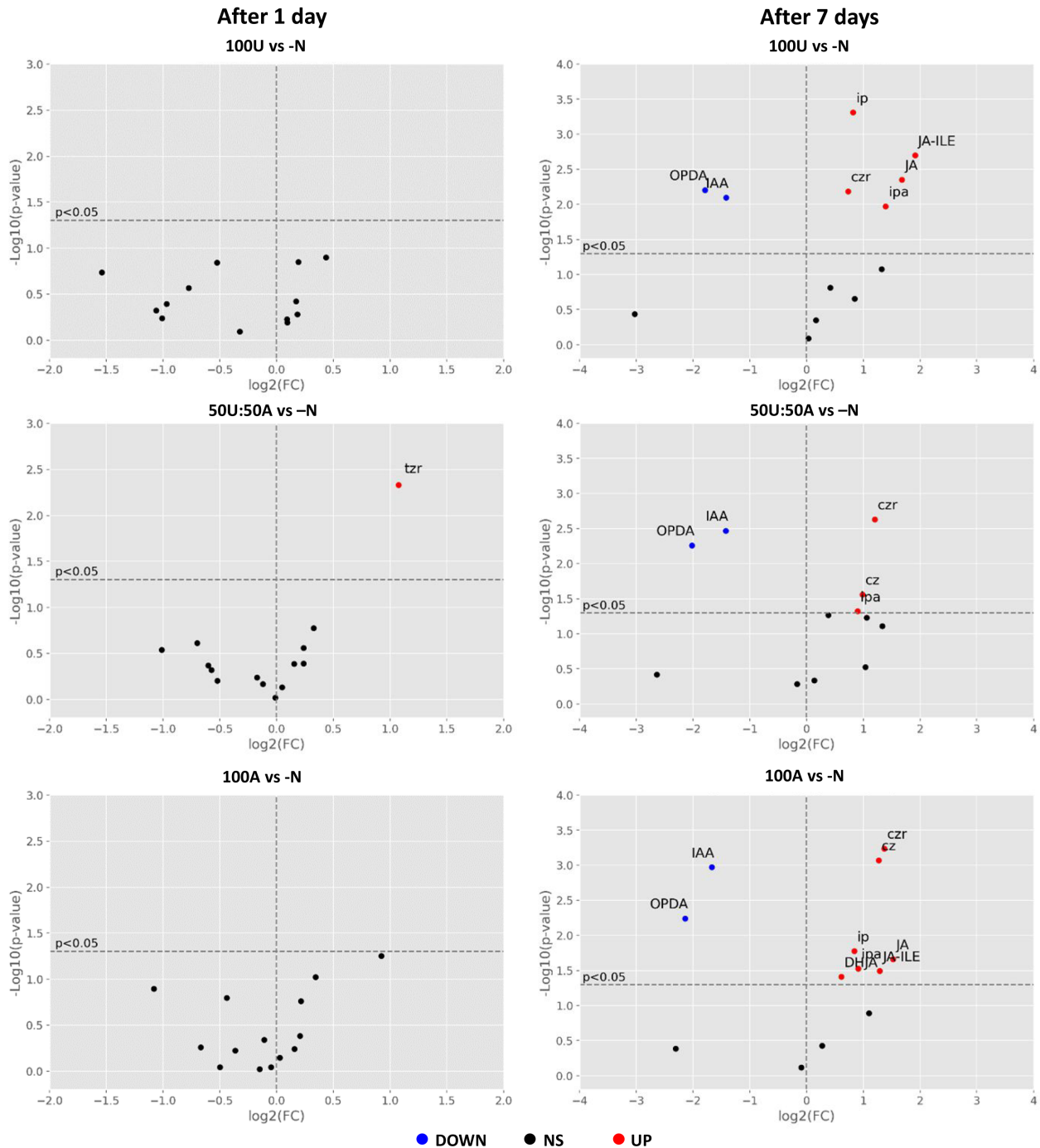
ROOT

FIGURE 8 Volcano plots of phytohormones profile in roots of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downconcentrated metabolite (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upconcentrated metabolite (in red; Fisher's test, N = 3, *p*-value ≤ 0.05)

profile displayed by those genes related to "biosynthesis of amino acids", "amino sugar and nucleotide sugar metabolism", "Starch and sucrose metabolism", "fructose and mannose metabolism", "carbon metabolism" as indicated by enrichment analyses (Table S4).

3.3 | Phytohormone concentration in maize shoots and roots

The phytohormones (abscisic acid, ABA; salicylic acid, SA; 12-oxo-phytodienoic acid, OPDA; jasmonic acid, JA; jasmonoyl-Isoleucine, JA-
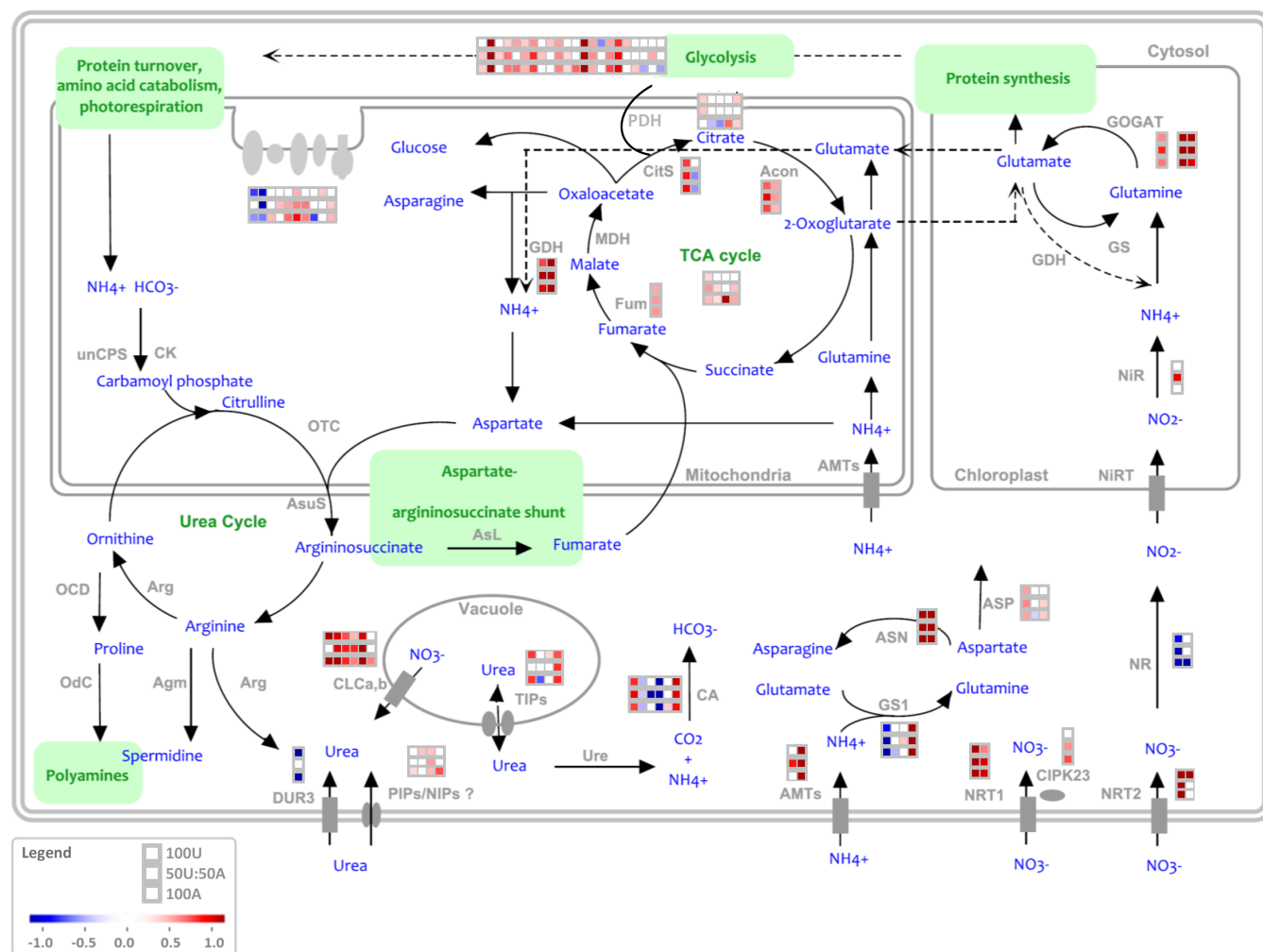
FIGURE 9 Gene expression modulation of genes most involved in primary N-assimilation. Color scale refers to up regulated genes (in red) and to down regulated genes (in blue), the color intensity refers to Log$_2$FC value of transcriptional modulation. For each mapping point three rows are shown and refers to transcriptional modulation induced by 100U vs. -N (row above), 50U:50A vs. -N (row in the middle), 100A vs. -N (row below); for each row, multiple squares refer to different gene isoforms. Gene description and expression values are reported in Table S1

Ile; 1-amino-1-cyclopropane-carboxylic acid, ACC; indole-3-acetic acid, IAA; isopentenyladenine, ip; isopentenyladenosine, ipa; trans zeatin, tz; trans-zeatin riboside, tzr; cis zeatin, cz; cis-zeatin riboside, czr) concentrations were evaluated in shoot and root after 1 day and 7 days of treatment (Figures 7 and 8 and Table S5). After 1 day of treatment application, the content of tz, tzr and ip increased in the shoot of plants fed with urea (100U vs. -N and 50U:50A vs. -N), whereas plants grown with ammonium as sole N-source are characterized by an increase of SA and ip (100A vs. -N). Conversely, a decrease of czr in 100U vs. -N and 50U:50A vs. -N, and OPDA in 100U vs. -N and 100A vs. -N was detected. In root, a significative increase of tzr characterized plants grown in the presence of both urea and ammonium (50U:50A vs. -N). After 7 days, the analysis of phytohormone concentration highlights more differences among the treatments. In shoots, JA and ip concentration increased in urea or ammonium treated plants (100U vs. -N and 100A vs. -N). N-treatment decreased the content of ABA, SA and IAA, regardless of the N-source. In roots, N-nutrition decreased the level of OPDA and IAA compared to -N. The presence of urea or ammonium in

the nutrient solution (100U vs. -N and 100A vs. -N) increased the level of JA, JA-Ile, ip, czr and ipa, whereas urea and ammonium mixture (50U:50A vs. -N) enhanced the concentration of cz, czr and ipa.

The correlation analyses showed that the concentration pattern of ip and JA in shoots and ipa and tzr in roots among the thesis correlated with the expression profile displayed by those genes related to "biosynthesis of amino acids", "amino sugar and nucleotide sugar metabolism", "Starch and sucrose metabolism", "fructose and mannose metabolism", "carbon metabolism" as indicated by enrichment analyses (Table S5).

4 | DISCUSSION

Ammonium and urea are two N sources widely used in agriculture. However, the reciprocal interaction between these two sources for plant nutrition has been scarcely investigated. Previous physiological evidence highlighted the advantage of ammonium acquisition by
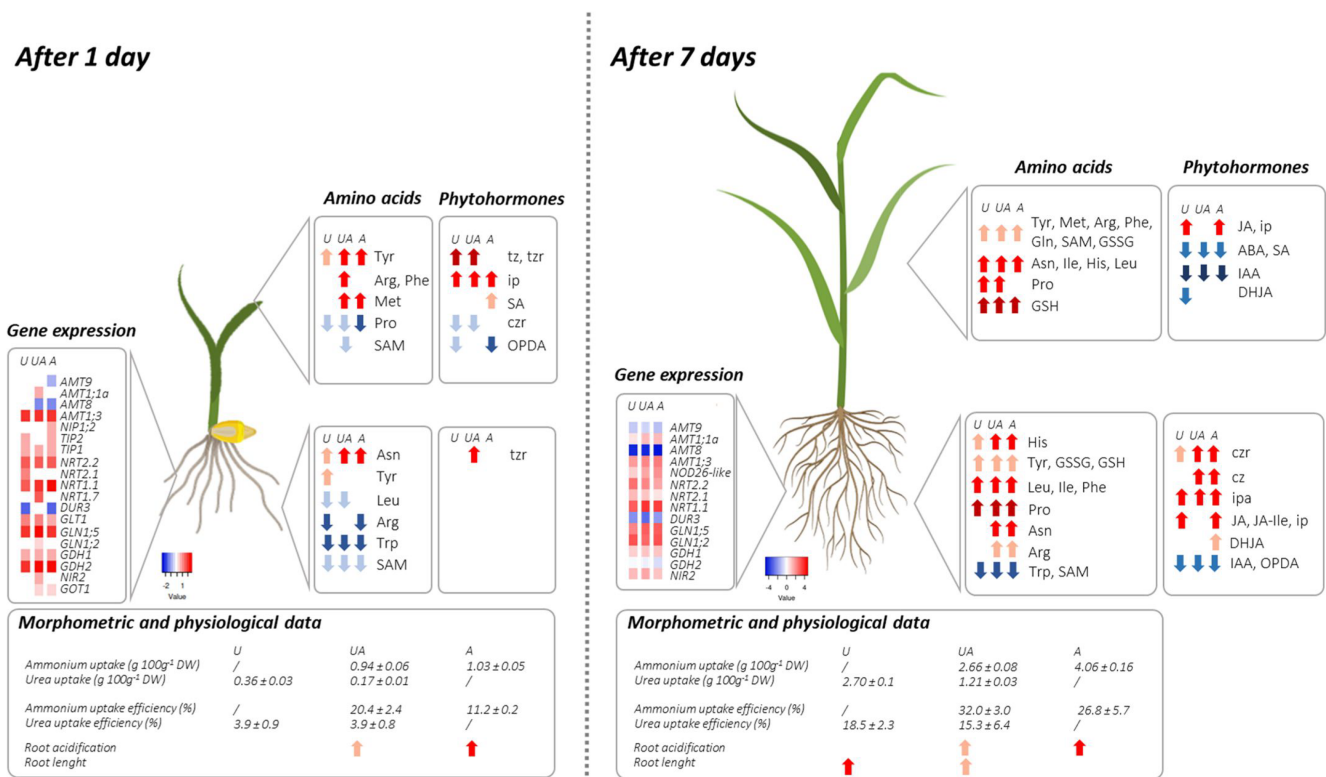
FIGURE 10 Schematic representation of gene expression, amino acid and phytohormones changes occurring in maize plants after 1 day and 7 days of treatment 100U vs. -N (U), 50U:50A vs. -N (UA), 100A vs. -N (A). The reported gene expression data for 7 days of treatment were obtained from Buoso et al. (2021a). Color scale refers to positive modulation (in red) and to negative modulation (in blue). Morphometric and physiological data refer to previous evidence using same experimental conditions (Buoso et al. 2021a)

the presence of urea in the external media (Buoso et al. 2021a). In the present study, transcriptomic and metabolomic approaches were used to deeply investigate plant response and reveal how urea and ammonium mixture might contribute to improving N-use in plants.

4.1 | The induction of N-transporters is dependent on the availability of N-source in nutrient solution

In roots all three N-treatments (100U, 50U:50A, 100A) induced the expression of genes involved in N-acquisition, suggesting that the relating metabolic pathway were stimulated by both reduced N-forms (Figure 9 and Table S1). Regarding ammonium, all N-treatments (100U, 50U:50A, 100A) upregulated *ZmAMT1;3* gene, which mainly contributes (along with *ZmAMT1;1a*) to the high-affinity transport system (Gu et al. 2013). The induction of *ZmAMT1;3* by urea supports previous physiological evidence indicating that the ammonium high-affinity influx in maize roots was not inhibited by urea but rather promoted (Buoso et al. 2021a). The induction of *AMT* gene by urea alone was also observed in Arabidopsis (Mérigout et al. 2008b). However, it should be considered that in Arabidopsis, the ammonium HATS displays an inducible feature by N deprivation, on the contrary, in maize ammonium HATS is stimulated by the substrate (Gu et al. 2013; Mérigout et al. 2008b). The upregulation of *AMT* gene by urea in maize as well in

Arabidopsis, indicates that urea *per se* might play a stimulatory action on *AMT* expression. It is interesting to note that plants fed with urea and ammonium mixture induced even *ZmAMT1;1a* (Figure 9 and Table 1). Beside this modulation, clustering analyses revealed that the expression of several genes involved in N acquisition were highly induced by 50U:50A treatment rather than by one N source (urea or ammonium, Figure 4). This behavior might give reason of the better uptake efficiency of ammonium-N source when plants were fed with the mixture of two N sources (50U:50A treatment, Buoso et al. 2021a).

The low affinity system mediates the acquisition of ammonium under high concentrations in the external media (in the millimolar range, Giehl et al. 2017). In Arabidopsis, AtAMT2;1 transporter moderately contributes to root uptake in the low-affinity range and functions in root-to-shoot translocation, as its coexpression along with AMT1-transporters promoted the ammonium translocation in shoots (Giehl et al. 2017). In maize, the expression of *ZmAMT8* (homologous to *AtAMT2;1*) was downregulated by ammonium (100A, and 50U:50A treatments) and not by urea alone (100U, Figure 9 and Table 1). This data agrees with the common idea that roots are the main organ in maize for the assimilation of ammonium when taken up from the external media (Hachiya & Sakakibara 2017), whereas urea can be transported by transpiration stream in leaves (Tan et al. 2000). This implies that under urea treatment (100U), maize plants operated a prompt redistribution of N, which does not request a negative regulation of *ZmAMT8* ammonium transporter.

The acquisition of urea in roots is attributed to DUR3 and to several aquaporins (belonging to superfamily of major intrinsic proteins, MIPs), and these latter might even mediate ammonium uptake (Beier & Kojima 2021; Wang et al. 2008). In the present study, the expression of high-affinity urea transporter *ZmDUR3* (Zanin et al. 2014) was downregulated by urea or ammonium, confirming that this transporter is not substrate-inducible at the transcriptional level, but rather it might be subject to a feedback regulation on transport systems operated by the substrate itself or by N-metabolic products (Figure 9 and Table 1; Zanin et al. 2015b; Pinton et al. 2016). Conversely, the combined use of urea and ammonium did not affect the expression of this transporter. Transcriptomic data indicated an overall upregulation of several aquaporins (*ZmPIPs*, *ZmTIPs*, *ZmNIPs*) by all three N treatments (Figure 9 and Table 1). All three N-treatments upregulated *ZmTIP1*, a transcript homolog to the Arabidopsis At2g36830. This latter codes for an aquaporin which expression complemented the phenotype of *dur3*-knockout yeast and might play a role to take up N from the external solution or storage the nutrient into vacuole (Liu et al. 2003). Other aquaporins (*ZmTIP2* and *ZmNIP1;2*) involved in the low affinity transport of urea in plants were found upregulated when a single N source (100U or 100A) was applied to nutrient solution rather than the combination of the two N sources. This data suggests that under urea and ammonium mixture a prompt metabolism and redistribution of N in the whole plants might prevent the overaccumulation of urea or ammonium in the cytosol and thus it is not requested the activation of MIPs or a negative feedback regulation on *ZmDUR3*.

Besides ammonium and urea transporters, N-treatments modulated even some transporters involved in nitrate acquisition, such as *ZmNRT1.1*, *ZmNRT2.1*, and *ZmNRT2.2*. In plants, NRT1s and NRT2s are involved in nitrate acquisition through LATS and HATS, respectively, and *ZmNRT1.1* is responsible also for the constitutive HATS (Okamoto et al. 2003). Under our conditions, no nitrate was detectable in nutrient solution (as reported above). Nevertheless, the transporters *ZmNRT1.1* and *ZmNRT2.2* were both upregulated under all three N treatments (100U, 50U:50A, 100A), and the entity of *ZmNRT1.1* upregulation was dependent on the availability of ammonium in nutrient solution (Figure 9 and Table 1). Jian et al. (2018) suggested that NRT1.1, a nitrate transporter that functions in multiple physiological processes in plants (Bouguyon et al. 2015; Guo et al. 2003; Jian et al. 2018; Krouk et al. 2010; Tsay et al. 1993), might have a signaling role to regulate ammonium uptake in roots and mediate its assimilation into amino acid, preventing toxicity condition. Considering that nitrate is the main inorganic form of N in aerobic soils (Wolt 1994), the upregulation of these transporters might have great relevance in agricultural fields as it suggests that the use of urea and/or ammonium stimulates the root capability to nitrate recruitment once available for plants.

4.2 | Urea and ammonium mixture stimulates amino acid metabolism

At the transcriptional level, urea and ammonium mixture enriched metabolic pathways related to "biosynthesis of amino acids" and "nitrogen metabolism" (Figure 3). At the metabolic level, the Met, Tyr and Arg

profiles among thesis correlated with the transcriptional modulation related to the amino acid synthesis (Table S4). Interestingly, the concomitant use of urea and ammonium led to a significant increase in the concentration of Met, Phe, Tyr, Arg, and Asn in plants already after 1 day, on the contrary, the concentration of few amino acids were modulated by single N sources (Asn, Met and Tyr in 100A-treated plants; Tyr and Asn in 100U-treated plants; Figures 5 and 6 and Table S3). This observation indicates that the metabolism in 50U:50A fed plants was more active than in plants fed with one N source. In agreement with the modulation of aquaporins (as reported above), a better redistribution of N in the whole plant under 50U:50A treatment might promote the amino acid synthesis avoiding the occurrence of feedback control on the transporter and metabolic pathways. Maybe related to urea metabolism, the combined use of two N sources limited the root acidification process in comparison to ammonium fed plants, with positive effects on the growth and length of the root system. This behavior might contribute to explain the high uptake efficiency of ammonium acquisition in 50U:50A plants (Buoso et al. 2021a; Figure 10). After 7 days of treatment, an overall increase of amino acids' concentrations was observed in maize irrespectively to N treatment (Figures 5 and 6 and Table S3), and in particular, N-metabolites (as Gln, Asn, and Arg) were more concentrated in N-treated plants than N-deficient ones. Due to the low C: N ratio, these amino acids play important roles in N-storage and transport in plants (Gaufichon et al. 2010). The overlap in the amino acid profiles under different N treatments indicates that, at least after 7 days, plants are able to use urea in a similar way to ammonium. The ammonium released by urea hydrolysis might be promptly assimilated through a GS/ASNS pathway located in the cytoplasm and that might serve also for the assimilation of ammonium when it is directly taken up by roots (Liu & von Wirén 2017). Transcriptomic data confirms that the activation of a cytosolic pathway for the assimilation of the reductive forms of N seems to occur when plants are fed with urea and/or ammonium, whereas the plastidial pathway (involving GS/GOGAT cycle) seems to be mainly dedicated to the assimilation of N deriving from nitrate reduction (Buoso et al. 2021a). The positive effect of 50U:50A treatment might be linked to the concomitant upregulation of several GLN isoforms that speed up the N assimilation (Figure 9).

Regarding the phenylpropanoid synthesis, the gateway enzyme Phenylalanine Ammonia-Lyase (PAL) of maize also has Tyrosine Ammonia-Lyase (TAL) activity, which mediates the direct conversion of tyrosin in *p*-coumaric acid and ammonium (Rösler et al. 1997). The upregulation of this pathway is linked to the nutritional status of low N availability as the ammonium released by PAL/TAL activity may be recycled by plants. A significant overconcentration of Tyr was found after 1 day in urea-treated roots, suggesting that under urea treatment, the Tyr conversion in secondary metabolites (through phenylpropanoid pathway) might be slowed down.

4.3 | Phytohormonal profile is responsive to the type of N-source available in nutrient solution

Among phytohormones, auxin, CKs, and ABA are those that are mainly involved in the coordination of the demand and the acquisition

of N (Argueso et al. 2009; Ristova et al. 2016; Signora et al. 2001; Walch-Liu et al. 2006; Wilkinson & Davies 2002). Although a reciprocal influence between phytohormones and nitrate on plant growth and developmental processes has been extensively studied (Vega et al. 2019), information about cross-connection between phytohormones and other N-sources (i.e., urea and/or ammonium) is mostly missing.

The form and concentration of N have important influences on endogenous CK synthesis (Mercier et al. 1997; Neuberg et al. 2011; Sattelmacher & Marschner 1978; Smiciklas & Below 1992; Wagner & Beck 1993; Walch-Liu et al. 2000). In our data, the pattern of CKs concentrations (ip, ipa and tzr) among thesis correlated with the transcriptional changes related to primary metabolic pathways (such as "carbon metabolism" and "biosynthesis of amino acids", Table S4). The analyses of roots and shoots showed an overall increase of CKs in N-treated plants (Figures 7 and 8), supporting the role of this family of compounds to act as a root-to-shoot long-distance signal of N-status in plants (Sakakibara et al. 2006). Moreover, strong differences in CK composition and concentration were observed in maize depending on N-source (100U, 50U:50A, 100A) and on the duration of treatment (1 or 7 days; Figures 7 and 8). The active CK trans-zeatin (tz) was identified as a long-distance signal from root to shoots that triggers nitrate transcriptional responses in both roots and shoots, regulating root growth and nitrate transport (Poitout et al. 2018). Moreover, along with its precursor tzr, tz upregulated several genes involved in N assimilation (ASN, NR and GDH, Gu et al. 2018). After 1 day, tz and tzr were upconcentrated in shoots by urea-containing treatments (100U and 50U:50A) and tzr accumulated even in 50U:50A roots in comparison to -N plants. This data supports the hypothesis that these molecules are involved in N-status signaling when urea is used as N source, and their occurrence might confirm that in plants, ureic-N undergoes a prompt assimilation. The accumulation of tzr in the whole 50U:50A plants (especially in roots) may reinforce the role of this phytohormone on N nutrition and agrees with the upregulation of the N metabolic pathway by the combined use of two N sources ("biosynthesis of amino acids" and "N metabolism", Figure 3).

After 7 days of treatment, high CK concentrations occurred in roots for all three N-treatments, and the composition of CKs was related to the type of N-source applied (Figure 8). Gu et al. (2018) have hypothesized a negative effect of ip on NRTs and AMTs. The absence of ip in plants fed with urea and ammonium mixture agrees with the positive modulation on N-transporters by 50U:50A (*ZmAMT1;1a*, *ZmNRT1.7*).

A link between N nutrition and auxin signaling pathway was described in plants, revealing a role of auxin on root system architecture in response to N availability (Krouk et al. 2010; Krouk et al. 2011; Liu et al. 2010; Song et al. 2013; Sun et al. 2020; Xu et al. 2015). After 7 days, N-deficient plants showed high IAA concentration, conversely lower values were recorded in N-treated plants (Figures 7 and 8 and Table S5). The amino acid analyses highlighted an interesting increase of Trp concentration in N-deficient roots both at 1 day and 7 days (Figure 6), showing a higher availability of the substrate for IAA biosynthesis in this condition (Woodward & Bartel 2005). Besides local IAA

synthesis, Sun et al. (2020) reported that low N induces root elongation in maize by enhancing shoot-to-root auxin transport and increasing the auxin level in the root tip. The IAA accumulated in N-deficient plants might trigger the increase of root elongation (Buoso et al. 2021a). Maize plants subjected to different treatments (100U, 50U:50A, 100A) display a different root architecture, in particular, urea promoted a good development of roots, whereas ammonium treatment induces the elongation of lateral roots and a concomitant reduction of primary and seminal root lengths (Buoso et al. 2021a). However, the same IAA concentration detected in the whole root of plants subjected to the various N-treatments (100U, 50U:50A, 100A; Figure 8) led us to hypothesize that the development of root architecture might be linked to a peculiar distribution of auxin in roots more than to the whole IAA amount. In particular, the nitrate transport NRT1.1 displays auxin transport activity and regulates lateral root growth by modulating auxin transport activity in a nitrate-dependent manner (Krouk et al. 2010). Our data indicate an increase of *ZmNRT1.1* expression in N treated plants and in particular the highest levels were detected in roots under ammonium (100A) suggesting the occurrence of a localize transport of auxin to promote lateral root elongation (Figure 9 and Table 1).

A cross connection between ABA levels and N status has been reported in plant species, although several aspects of their reciprocal interaction are still unclear (Kiba et al. 2011). In Arabidopsis, a link between ABA and ammonium signaling pathways have been described, and it might enroll a plastidial metalloproteases acting to prevent leaves from chloroplast damages (Liu & von Wirén 2017). In our experiments, a significant decrease of ABA concentration was detected in shoots after 7 days regardless of the N-treatment applied (Figure 7). In other plant species, ricinus and rice, the ammonium nutrition increased ABA translocation and accumulation in shoots (Ding et al. 2016; Peuke et al. 1998). These contrasting results might indicate that the link between ammonium and ABA depends on multiple factors, including genotype, concentration and timing of treatment with ammonium.

When urea and ammonium were applied together, no changes in JA, JA-Ile and OPDA levels were observed in plants, whereas significant variations were induced by urea or by ammonium alone (Figures 7 and 8). Overall these changes in phytohormonal profiles (CKs, JA, ABA) highlighted the occurrence of a strong connection between phytohormones and N status in maize. Further investigations on their role on the assimilation of N might be of interest to improve the assimilation of N in plants.

A schematic representation of transcriptional and metabolomic changes of maize plants under urea and/or ammonium nutrition is provided in Figure 10. The modulation of transporters and enzymes involved in N uptake and assimilation as well the overaccumulation of amino acids already after 1 day exposure in maize plants indicates a prompt assimilation of N when plants are treated with urea and ammonium mixture. Here reported results suggest that the use of mixed N sources, urea and ammonium, can be employed as an efficient management tool to increase the use of N fertilization in crops.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors contributed to the study conception, design, data collection, analyses and manuscript preparation. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) under the accession GSE167027.

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SUPPORTING INFORMATION

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