Nitrogen nutrition and xylem sap composition in *Zea mays:* effect of urea, ammonium and nitrate on ionomic and metabolic profiles

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ABSTRACT

In plants the communication between organs is mainly carried out via the xylem and phloem. The concentration and the molecular species of some phytohormones, assimilates and inorganic ions that are translocated in the xylem vessel play a key role in the systemic nutritional signaling in plants. In this work the composition of the xylem sap of maize was investigated at the metabolic and ionomic level depending on the N form available in the nutrient solution. Plants were grown up to 7 days in hydroponic system under N-free nutrient solution or nutrient solution containing N in form of nitrate, urea, ammonium or a combination of urea and ammonium.

For the first time this work provides evidence that the ureic nutrition reduced the water translocation in maize plants more than mineral N forms. This result correlates with those obtained from the analyses of photosynthetic parameters (stomatal conductance and transpiration rate) suggesting a parsimonious use of water by maize plants under urea nutrition. A peculiar composition in amino acids and phytohormones (i.e. S, Gln, Pro, ABA) of the xylem sap under urea nutrition could explain differences in xylem sap exudation in comparison to plants treated with mineral N forms. The knowledge improvement of urea nutrition will allow to further perform good agronomic strategies to improve the resilience of maize crop to water stress.

1. Introduction

Nitrogen (N) is a component of amino acids, proteins, cell walls, membranes and nucleic acids, and therefore is one of the most important elements for plant growth and development (Marschner, 2011). To increase the N availability in the soil and sustain plant yield, N fertilizers are widely used in agriculture. Plants preferentially sustain their N requirements through the acquisition of inorganic N-forms, nitrate and ammonium. However, mainly due to the high N content and low transport costs *per* unit of N, and safe handling, urea is broadly used as N-fertilizers worldwide (IFA, 2017; Heffer and Prudhomme, 2016). The information gained about the uptake, assimilation and signaling pathway of nitrate and ammonium is extensive (Kiba and Krapp, 2016; Hachiya and Sakakibara, 2017; Ravazzolo et al., 2020), while little knowledge has been acquired about urea nutrition in plants. The conversion of urea in ammonium and nitrate in soil allows the use of these

forms by plants to support their growth (Cantarella et al., 2018). Besides, it has been demonstrated that plants can also use urea as a N-source through its direct acquisition by urea transporters (Liu et al., 2003; Wang et al., 2012; Zanin et al., 2014).

A beneficial effect on plant growth was reported when a mixture of N-sources is used (Arkoun et al., 2012; Britto and Kronzucker, 2002; Buoso et al., 2021a; Buoso et al., 2021b; Houdusse et al., 2005; Garnica et al., 2009; Zanin et al., 2015). In maize, the use of urea and ammonium mixture improved plant growth leading to beneficial effects on the development of roots and decreasing the extracellular acidification that characterizes plant grown with ammonium as a single N-source (Buoso et al., 2021a, b). At transcriptomic level, plants grown in presence of urea and ammonium mixture showed the highest ammonium uptake efficiency, probably due to the highly expressed genes coding for transporters and enzymes involved in N uptake and assimilation (Buoso et al., 2021a, c). Moreover, the overaccumulation in maize of amino

acids after 1 day of treatment with urea and ammonium mixture indicated a prompt assimilation of N in this condition compared with plants grown in the presence of the single N-forms (urea or ammonium, Buoso et al., 2021c). The translocation of metabolites and ions through the xylem vessels is a key point to allocate the resources to the leaves. In a recent paper the xylem sap composition was found to be highly responsive to the N form applied (ammonium or nitrate) leading to peculiar proteomic, metabolomic and ionomic profile in poplar (Kasper et al., 2022).

Several studies provide evidence on the interconnections between N nutrition and phytohormones signaling pathway (Kiba et al., 2011; Krouk et al., 2011; Krouk, 2016; Ristova et al., 2016; Vega et al., 2019; Buoso et al., 2021c). This relationship was mainly characterized in plants grown with nitrate as sole N-form, therefore information on the link between phytohormones and other N-forms are lacking (Di et al., 2018; Buoso et al., 2021c; Kamada-Nobusada et al., 2013; Bauer and von Wirén, 2020). In a recent study, we observed differences in phytohormones concentration in maize roots and shoots, emphasizing the occurrence of a connection between phytohormones and N status in plants (Buoso et al., 2021c).

Aim of this work was to analyse the plant responses to different N-forms (nitrate, urea and ammonium) on the composition and translocation of nutrients, amino acids and phytohormones in the xylem sap. This data was integrated with ionomic and metabolomic data previously collected in roots and leaves of maize plants (Buoso et al., 2021a-c).

2. Materials and methods

2.1. Plant growth

Maize seeds (*Zea mays* L., P0423, Pioneer Hybrid Italia S.p.A.) were germinated over aerated 0.5 mM $CaSO_4$ solution for 3 days. Seedlings were subsequently transferred into aerated hydroponic system and grown under the following controlled conditions for 1 day: 16/8 h light/dark cycle, a PPFD of 220 μmol m^{-2} s^{-1}, 25/20 °C temperature, 70–80 % relative humidity, and then transferred into 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). Different ratios of urea and/or ammonium (2 mM total N) were added separately to N-free nutrient solution in order to obtain three different experimental conditions (*100U*, 1.00 mM CH_4N_2O; *50U:50A*, 0.50 mM CH_4N_2O and 0.50 mM $(NH_4)_2SO_4$; *100A*, 1.00 mM $(NH_4)_2SO_4$) as previously described by Buoso et al. (2021a). As controls, some seedlings were grown in nutrient solution containing nitrate [1 mM $Ca(NO_3)_2$, *Nitrate* treatment] or in N-free nutrient solution (-*N* treatment). Additionally, in all treatments, the sulfur content in the nutrient solution was equalized by further supplementing with calcium sulfate ($CaSO4$): 1.00 mM CaSO4 in -N, Nitrate, and 100U; 0.50 mM CaSO4 in 50U:50A. The pH of solution was buffered using 1 mM MES-BTP (pH 6.0). Nutrient solution was renewed every 48 h.

2.2. Xylem sap collection and exudate flux determination

Xylem exudates were collected by root pressure method (Alexou and Peuke, 2013), which has also been recently employed in several studies on crop plants to assess the composition of xylem sap (Paponov et al., 2021; Zhang et al., 2022). Briefly, maize seedlings were cut at 2 cm above the root–shoot interface. The liquid on the cut surface was absorbed and removed using a clean paper and then a filter tip (5.7 mm diameter ultra-slim, Rizla+, Terzia S.p.A., Italia) was placed on the top of the cut site in order to collect the exudates. After 2 h, the filter tips were placed inside 1.5 mL plastic tubes and then centrifuged for 3 min at 13 000 g in order to separate the collected exudate from the filter tip. Samples were collected after 1 day and 7 days of treatments. The volume of the exudates was quantified and stored at − 80 °C until analysed.

2.3. Xylem sap multielemental analyses

Multielemental concentrations (calcium, Ca; copper, Cu; iron, Fe; potassium, K; magnesium, Mg; manganese, Mn; sodium, Na; phosphorus, P; sulphur, S; zinc, Zn) of xylem sap samples were determined by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES 5800, Agilent Technologies, Santa Clara, USA) as previously described (Buoso et al., 2021a). The xylem sap was diluted in ultrapure 5 mL HNO_3 (1 % v/v). Element quantifications were carried out using certified multi-element standards.

2.4. Xylem sap metabolomic analyses

Amino acids and phytohormones concentrations in xylem sap were analysed as reported in Buoso et al. (2021c). The Ultra performance liquid chromatography-Mass spectrometry (UPLC-MS) analysis was performed for amino acids determination on xylem sap samples injected directly. Phytohormones determination was carried out by an Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) system.

2.5. Leaf gas exchange parameters: carbon assimilation, evapotranspiration and stomatal conductance licor

Gas exchange parameters were recorded with a portable infrared gas analyser LI-6400 XT (LI-COR, Inc., Lincoln, NE, USA), eight days after treatment, on the terminal leaflet of the more expanded leaf. Measurements were made at constant photon flux density (220 μmol m^{-2} s^{-1}) during the morning (from 11:00 AM to 2:00 PM). Area-based maximum net CO_2 assimilation (A; μmol m^{-2} s^{-1}), stomatal conductance (g_s; mol m^{-2} s^{-1}), and leaf transpiration rate (E; mol m^{-2} s^{-1}) were measured after a stabilization period of about 10 min under ambient concentration of CO_2 (400 ppm). Plants leaf area were measured by using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

2.6. Statistical analyses

All the analyses were performed on three independent biological replicates. For each biological replicate, xylem sap collected from four maize plants were pooled together. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test for multiple comparisons (p-value <0.05, N = 3). Principle Component Analyses (PCA) were performed using ClustVis web tool (https://biit.cs.ut.ee/clustvis/; Metsalu and Vilo, 2015). The significance of the clustering observed in the PCA was assessed by PERMANOVA test using 5000 permutations performed with R version 4.3.0 (package vegan, Oksanen et al., 2014).

Correlation analyses were performed using the R package corrplot (https://github.com/taiyun/corrplot); correlation is expressed as Spearman's correlation coefficient, p-value was corrected for multiple testing and reported as false discovery rate if not otherwise specified.

3. Results

3.1. Xylem sap flux rate and photosynthesis gas exchange

The xylem sap flux rate was determined after 1 day and 7 days of treatment (Supplementary Fig. 1, Fig. 1A). After 1 day, only plants grown in presence of Nitrate showed a significant increase in xylem sap flux rate in comparison to the other treatments (Supplementary Fig. 1). After 7 days, all the N treated plants enhanced the xylem sap flux rate in comparison to -N plants. In particular, plants grown in the presence of Nitrate displayed the maximum flux rate, lowest values were observed under urea nutrition, whereas intermediate values were observed depending on the ammonium availability in the nutrient solution (Fig. 1A).
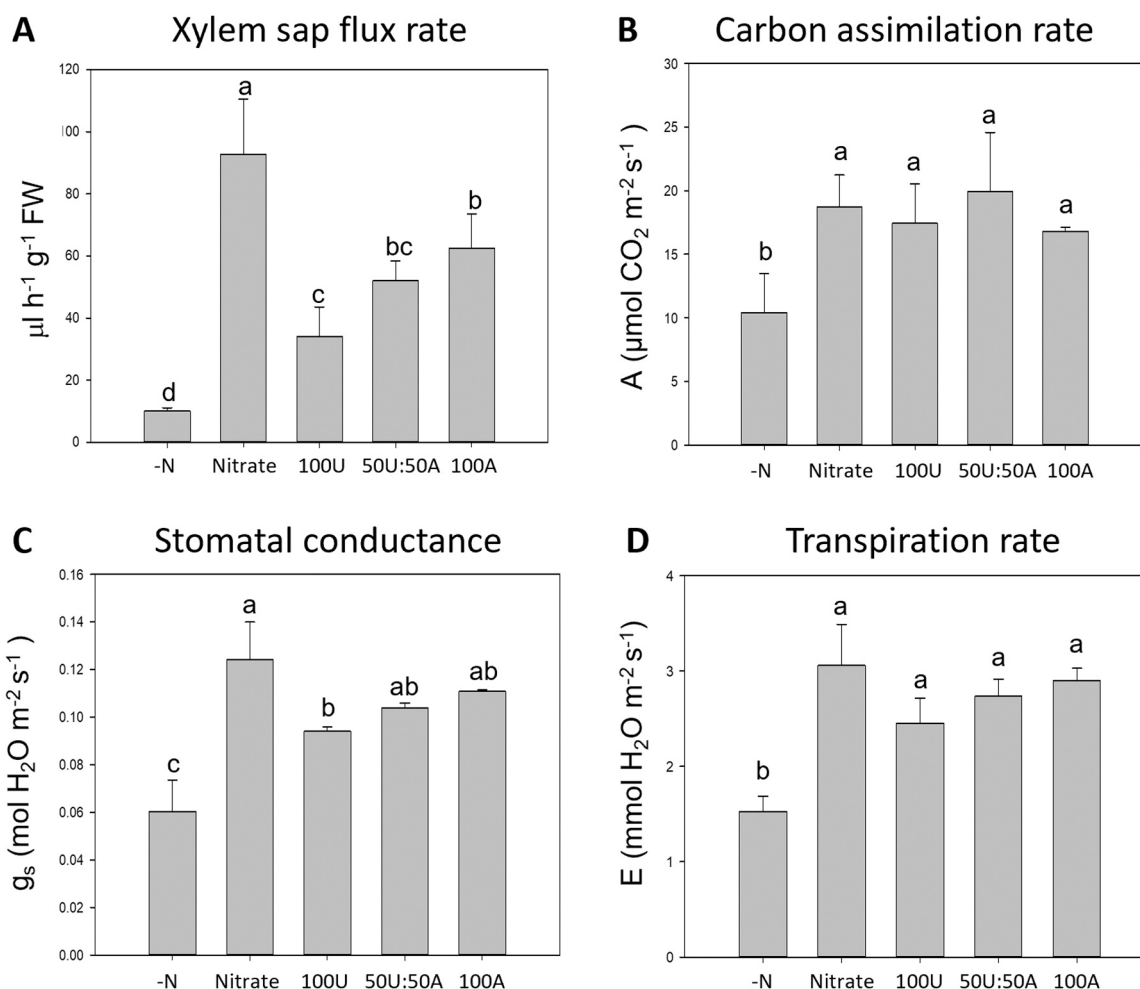
A Xylem sap flux rate

B Carbon assimilation rate

C Stomatal conductance

D Transpiration rate

Fig. 1. Xylem sap exudation rate (panel **A**), net CO_2 assimilation rate (A, panel **B**), stomatal conductance (g_s, panel **C**), and transpiration rate (E, panel **D**) measured in maize plants after 7 days of treatment (Student-Newman-Keuls test ANOVA, N = 3, p-value < 0.05).

After 7 days of treatment, photosynthetic traits were measured in leaves. The net CO_2 assimilation rate, the stomatal conductance and the transpiration rate were significantly higher in all N treated plants than in -N control plants (Fig. 1B–D). In particular, among N treatments a significant increase of stomatal conductance was observed in Nitrate plants in comparison to 100 U, whereas the two thesis with ammonium displayed intermediate values (following a similar pattern observed for the xylem sap flux rate: -N < 100 U < 50 U:50 A≤100 A<Nitrate, Fig. 1C). After 7 days, a significant correlation was observed among the datasets of physiological traits, with the exception of net CO_2 assimilation rate and stomatal conductance and of net CO_2 assimilation rate and xylem sap flux rate that did not correlate (Supplementary Fig. 2).

3.2. Multielemental concentrations in xylem sap

The multielemental concentrations were evaluated in xylem sap collected after 1 day and 7 days of treatment (Fig. 2). The multivariate analyses (PCA) were carried out on the whole elemental dataset in order to highlight possible differences and similarities among the samples after 1 day and after 7 days of treatment (Fig. 3). After 1 day of treatment with different N-sources, the samples were not well separated into distinct clusters (Fig. 3A). Conversely, after 7 days of treatment, the PCA generated a six component model accounting for a total variance of 99 %. The first two components (PC1 and PC2) explaining 84 % of the total variance have been chosen to show sample distribution. The PCA analysis discriminates four groups, with each group referring to one sample, except for 50 U:50 A and 100 U, which clustered together (Fig. 3C).

After 1 day of treatment (Fig. 2, Supplementary Fig. 3), P concentration increased in urea and/or ammonium treated plants (100 U, 50 U:50 A and 100 A) in comparison to -N and Nitrate. Cu concentration enhanced in ammonium treated plants (50 U:50 A and 100 A) in comparison to -N and Nitrate. Also S and Mg were concentrated in the xylem sap when ammonium was applied to nutrient solution. In particular both elements increased under 50 U:50 A and 100A-treatments in comparison to -N, 100 U or Nitrate treatment (following this trend 100 A = 50 U:50 A> 100 U = -N > Nitrate). Conversely, in all N treated plants in comparison to -N, the concentration of Fe and K in xylem sap was decreased. Nitrate reduced the concentration of Mg, S and Zn in the xylem sap in comparison to all the other treatment.

After 7 days of treatment (Fig. 2, Supplementary Fig. 4), Fe concentration in xylem sap decreased in Nitrate and 100 A treated plants in comparison to -N and urea treated plants (100 U and 50 U:50 A). Cu concentration increased in xylem sap of 50 U:50 A plants in comparison to -N and Nitrate. Ca, K and Mn concentration decreased in plants grown in presence of N, independently from the N source (nitrate, urea or ammonium). S concentration was enhanced in urea and/or ammonium treated plants (100 U, 50 U:50 A and 100 A) in comparison to -N and Nitrate, besides S and Zn concentrations decreased in Nitrate treated plants in comparison to all the other treatments. Mg and P increased in the xylem sap of plants grown with urea as sole N source (100 U) compared to all the other treatment, whereas decreased in Nitrate treated plants.

Considering the xylem sap exudation rate, the elaboration of ionic data provides an indication of the translocation rate of elements into
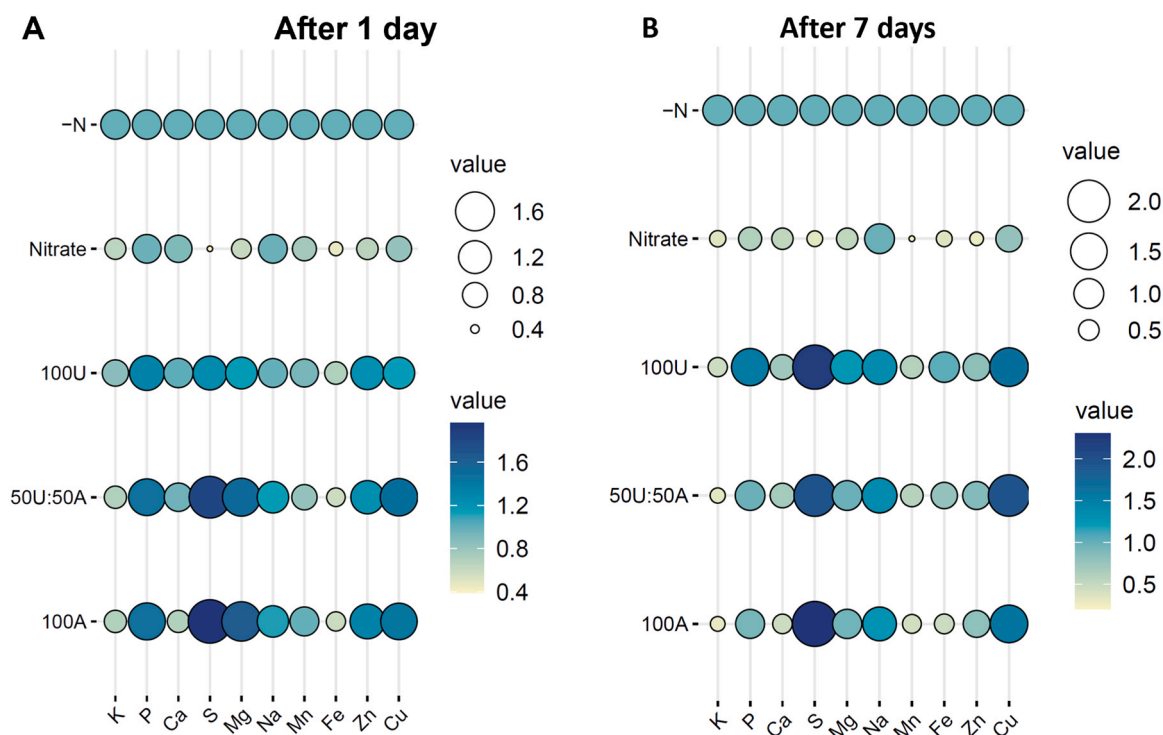
Fig. 2. Graphical matrix (ballon plot) where each cell contains a dot whose size reflects the relative magnitude of the corresponding element expressed as xylem sap concentration after 1 day (panel A) and after 7 days (panel B; ballon plot was plotted by https://www.bioinformatics.com.cn/en).

leaves (Supplementary Figs. 5–7). After 7 days of treatment plants increased the translocation rate of S, Zn and Mn depending on ammonium availability, whereas the translocation rate of K and Ca was higher in nitrate treated plants than in ammonium treated ones (Supplementary Fig. 7).

To integrate the elemental concentration of root, shoot and xylem sap of maize plants grown under the different treatments, a correlation analysis was performed using data previously collected (Buoso et al., 2021b,c). The statistical analysis highlighted a positive correlation between the concentration of Fe in shoot and xylem sap at 1 day and same behaviour was observed for S concentration. Regarding K, its concentration positively correlated between the root and xylem sap after 7 days of treatment (Supplementary Table 1, Supplementary Fig. 8).

3.3. Amino acids concentrations in xylem sap

The amino acid concentrations were evaluated in xylem sap collected after 1 day and 7 days of treatment (Fig. 4, Supplementary Table 1, Supplementary Fig. 9). After 1 day of treatment, the amino acid profile indicated that the use of nitrate (Nitrate treatment) led to a significant increase in the concentration of Phe and a decrease of Pro and GSSG in comparison to -N. An increase in the concentration of Arg, Gln, Met and Phe was detected in the xylem sap of plants grown with urea as sole N form (100 U) compared to -N. Plants grown with ammonium (50 U:50 A and 100 A) showed an increase in the concentration of Arg, Gln, Met and Tyr and a decrease of Trp in comparison to -N. After 7 days of treatment, the use of Nitrate increased the concentration of Arg and Phe in the xylem sap in comparison to -N plants, whereas the use of urea or/and ammonium (100 U, 50 U:50 A and 100 A) increased the concentration of a large number of amino acids compared to -N plants (Arg, Gln, His, Met, Phe, Pro and Tyr in 100 U; Arg, His, Met, Phe and Pro in 50 A:50 U; Arg, His, Met and Phe in 100 A).

Regarding the glutathione, during the xylem sap collection this molecule is subjected to oxidation processes transforming the reducing form GSH into GSSG. Therefore the GSSG form detected in the xylem sap was referred also to the oxidized GSH molecules and for this reason the

GSSG form was more abundant than the GSH one. The presence of urea in the nutrient solution (100 U and 50 U:50 A) induced a significant increase of GSSG in the xylem sap in comparison to -N plants.

The statistical analysis showed a positive correlation between the concentration of Tyr in root and xylem sap after 1 day of treatment and a positive correlation between the concentration of Phe in root and xylem sap after 7 days of treatment (Supplementary Table 1, Supplementary Fig. 8). Moreover, the concentration of Met in xylem sap after 1 day positively correlate with its concentration after 7 days of treatment.

3.4. Phytohormones concentrations in xylem sap

The phytohormones (abscisic acid, ABA; salicylic acid, SA; 12-oxo-phytodienoic acid, OPDA; jasmonic acid, JA; jasmonoyl-Isoleucine, JA-Ile; dihydro-jasmonate, DHJA; 1-amino-1-cyclopropane-carboxylic acid, ACC; indole-3-acetic acid, IAA; isopentenyladenine, ip; iso-pentenyladenosine, ipa; trans zeatin, tz; trans-zeatin riboside, tzr; cis zeatin, cz; cis-zeatin riboside, czr) concentrations were evaluated in xylem sap collected after 1 day and 7 days of treatment (Fig. 5; Supplementary Table 2, Supplementary Fig. 10). After 1 day of treatment, the presence of Nitrate in the nutrient solution led to a modulation of some phytohormones in comparison to -N (increase of ACC, tz and tzr; decrease of ABA, cz, ip, OPDA, JA and JA-Ile). Urea (100 U) and ammonium (100 A) treatments enhanced the concentration of ACC and tzr in comparison to -N. The simultaneous presence of urea and ammonium (50 U:50 A) in the nutrient solution increased the concentration of ABA, ACC, czr, ipa, tz and tzr. After 7 days, Nitrate treatment increased the concentration of ACC, ip, tz and tzr and reduce the concentration of ABA, czr, JA and JA-Ile, compared to -N. Urea treated plants (100 U and 50 U:50 A) enhanced the concentration of ACC and tzr, whereas reduced JA concentration (compared with -N plants). The use of ammonium as the sole N source (100 A) increased the concentration of ACC, ip, ipa, DHJA and tzr and decreased the concentration of ABA, JA and JA-Ile in comparison to -N plants. The correlation analysis highlighted a positive correlation between the concentration of tz in root and xylem sap at 1 day (Supplementary Table 1, Supplementary Fig. 8).
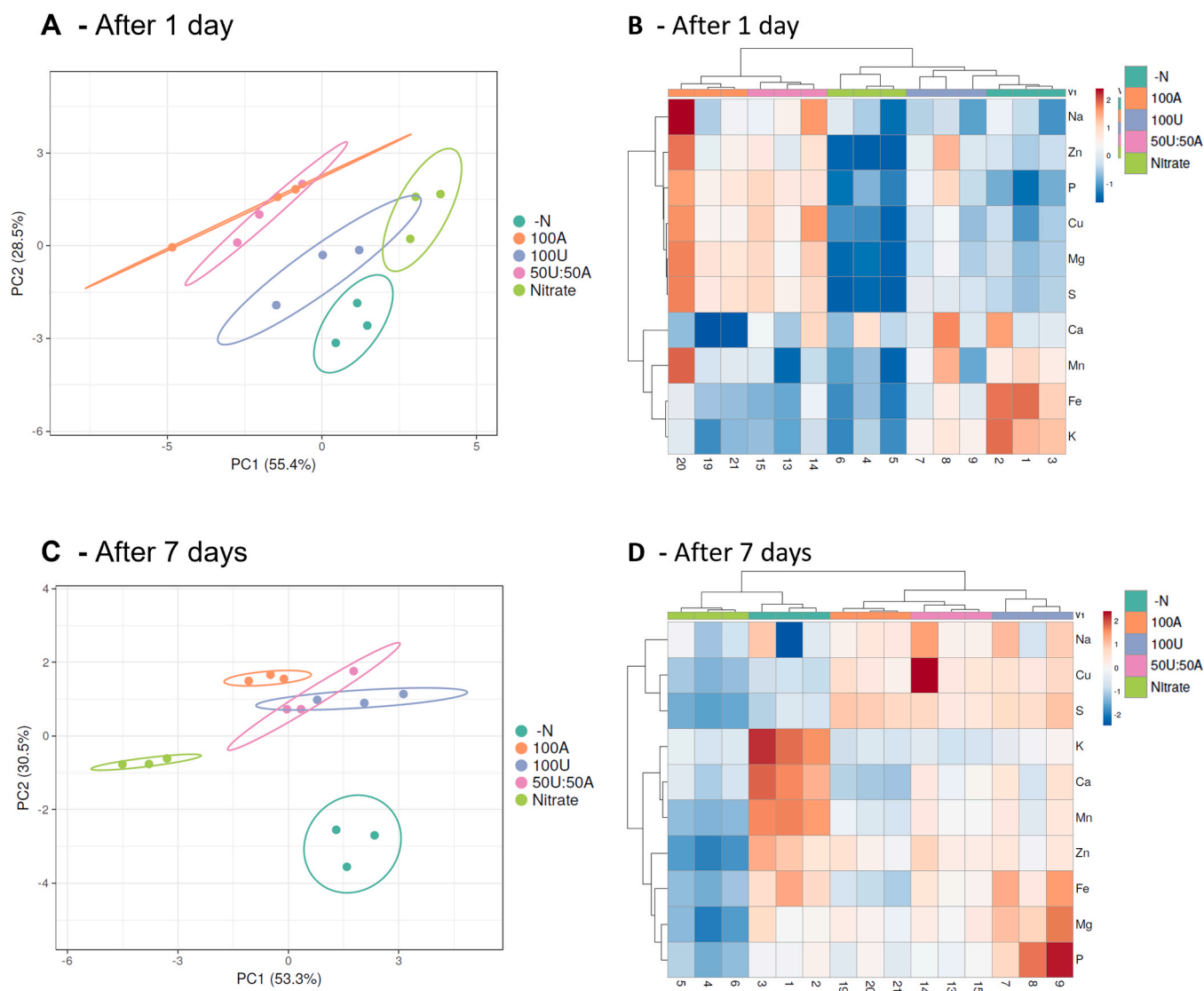
Fig. 3. PCA and Heatmaps of the ionomic analyses of xylem sap after 1 day of treatment (**A** and **B** panels, respectively) and 7 days of treatment (**C** and **D** panels, respectively) with different N-sources. PCA analyses were performed applying unit variance scaling to rows; SVD with imputation is used to calculate principal components. The clusters separation of both PCAs has been assessed by PERMANOVA analysis with 5000 permutations (p < 0.001). X and Y axis show principal component 1 and principal component 2 that explain 55.4 % and 28.5 % of the total variance, respectively, in xylem sap after 1 day of treatment (**A**) and principal component 1 and principal component 2 that explain 53.3 % and 30.5 % of the total variance, respectively, in the xylem sap after 7 days of treatment (**C**). Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. (**B, D**) In the heatmaps the elements were centered; unit variance scaling is applied to elements. Both elements and samples are clustered using correlation distance and average linkage.

4. Discussion

Nitrogen and C metabolism are strongly interconnected, as numerous C precursors are required for N-assimilation into amino acid and vice versa, reactions in photosynthesis or carbohydrate breakdown are crucial to generate C-skeletons and the reducing equivalents that are needed for the reduction of nitrate to ammonium (Kaiser and Zech, 2000; Foyer et al., 2001; Morot-Gaudry et al., (2001); Stitt et al., 2002). In agreement with literature data (Lu and Zhang, 2000; Lawlor et al., 1987), lower level of C assimilation rate was observed under N-deficient condition in comparison to N-sufficiency. Moreover, results of this study indicate that N-treated maize plants displayed similar capacity to assimilate C regardless to N-form applied to nutrient solution (Fig. 1B). After 7 days of urea treatment, maize plants exhibited a similar rate of C assimilation as those plants exposed to nitrate or ammonium. The comparable values of the C assimilation rate among all N treatments confirm that, in the short term, plants can use urea as a N source without

negatively impacting photosynthetic activity (Fig. 1).

The analyses of the other leaf physiological parameters indicated that the N form applied to nutrient solution changed the stomatal conductance of plants following the pattern -N < 100 U < 50 U:50 A= 100 A<Nitrate, and this pattern correlated to the tendency of the data of transpiration rate and with the xylem sap flux rate (Fig. 1, Supplementary Fig. 2). This behavior suggests that maize plants display a different water use capacity depending on the N form available for the root uptake. Previous works provided evidence of the effect of nitrate to increase the water uptake, translocation and amino acid composition in the xylem vessels (Barthes et al., 1996; Guo et al., 2002; Prinsi and Espen, 2015). Using enzymatic inhibitors of N assimilatory pathway, Barthes et al. (1996) indicated that the nitrate effect on xylem exudation was mainly linked to the composition of the sap that in turn changed its hydraulic conductance. Based on this consideration, we have characterized the composition of the xylem sap of maize plants depending on N form applied to nutrient solution. The differences in
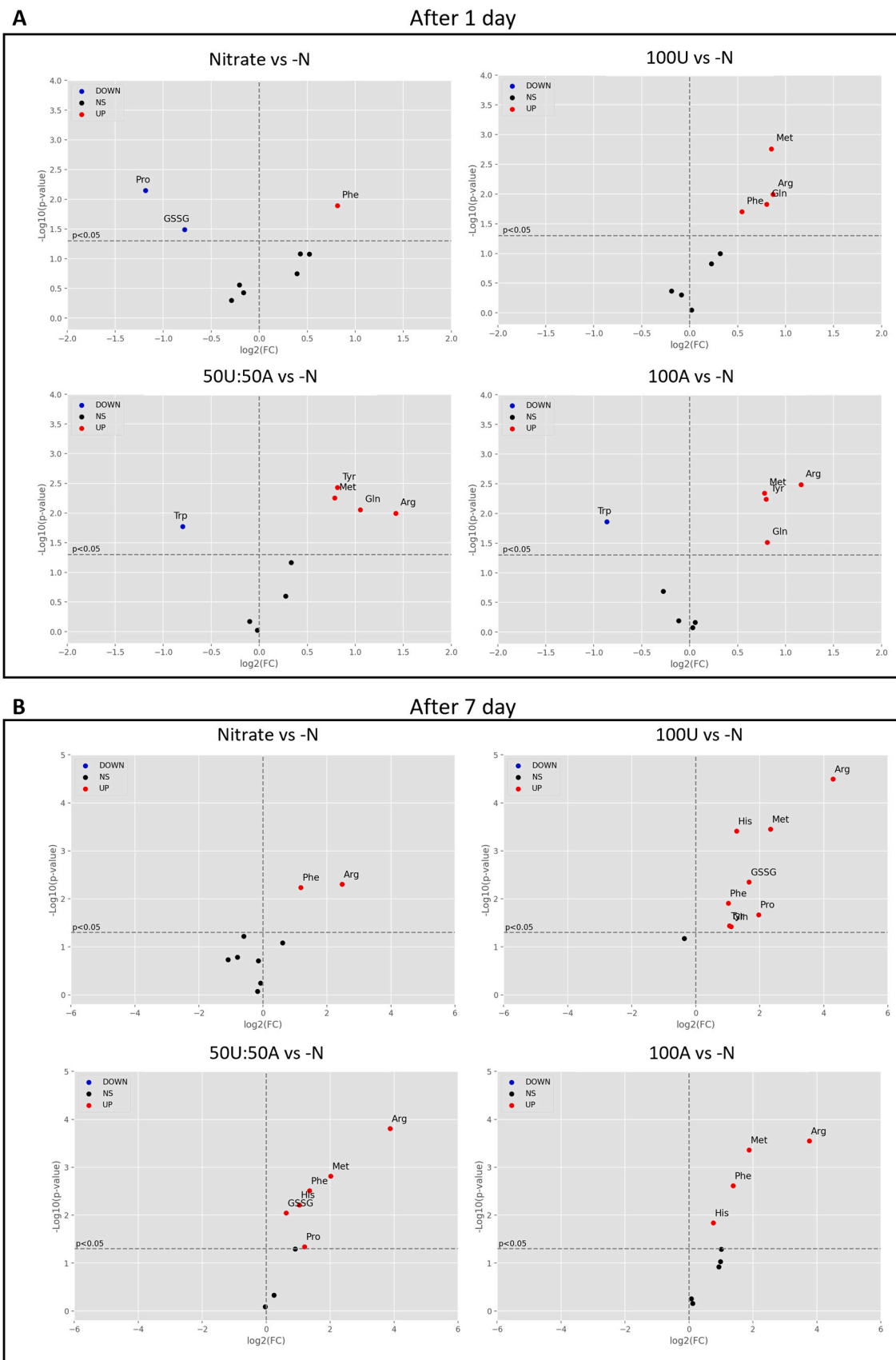
Fig. 4. Volcano plots of amino acid profile in xylem sap of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downregulation of metabolite concentration (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upregulation of metabolite concentration (in red; Fisher's test, N = 3, p-value ≤ 0.05).
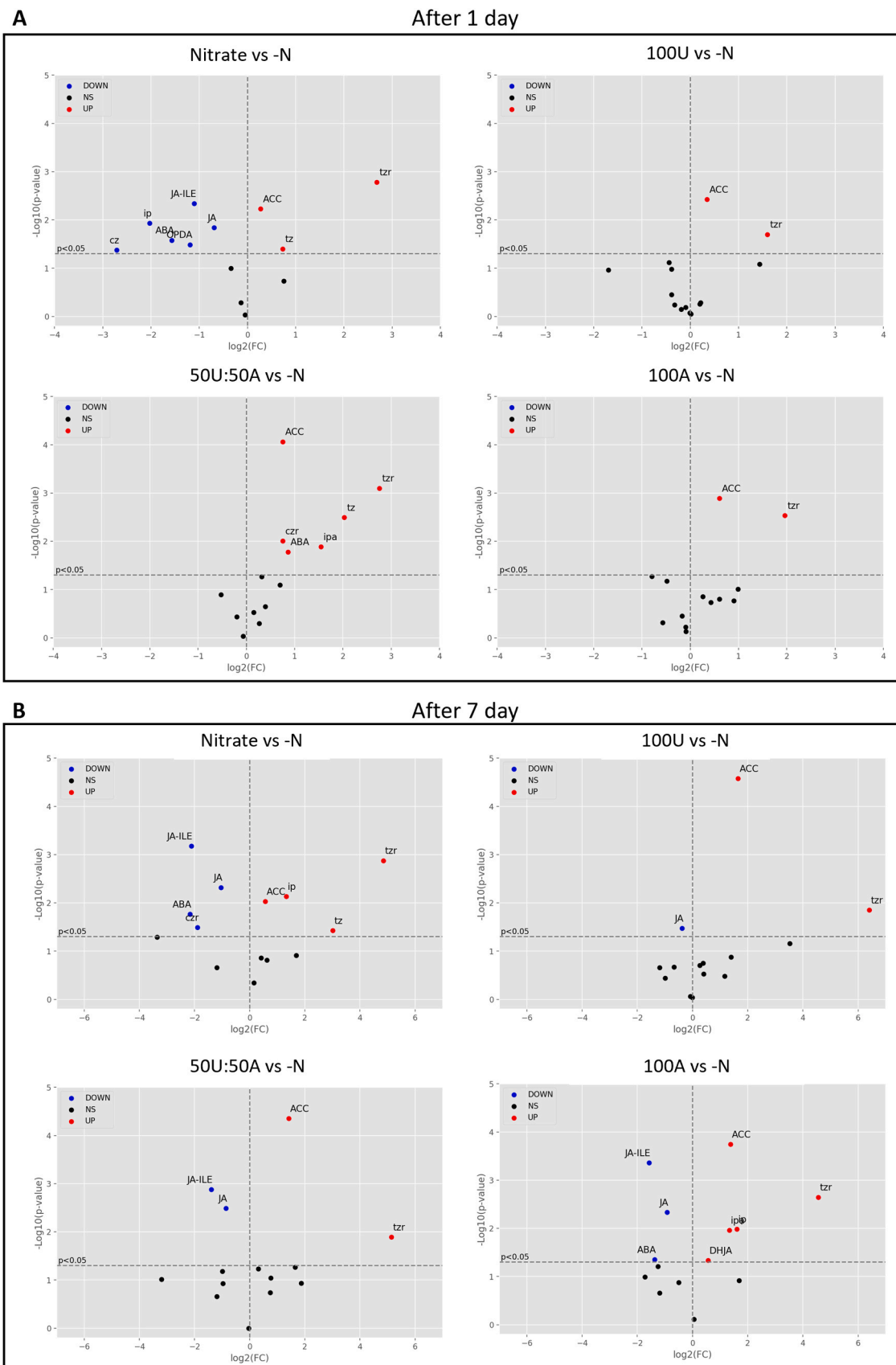
Fig. 5. Volcano plots of phytohormones profile in xylem sap of maize plants after 1 day or 7 days of treatment with the different N-sources. *DOWN*, downregulation of metabolite concentration (in blue); *NS*, not significant concentrated metabolite (in black); *UP*, upregulation of metabolite concentration (in red; Fisher's test, N = 3, p-value ≤ 0.05).

xylem sap exudation rate collected after 1 day or 7 days of treatment might be ascribable to a different capacity of maize plants to assimilate N depending on the provided forms (nitrate, urea and ammonium). Numerous amino acids are transported within the xylem and phloem, and the concentration of individual amino acids may vary, depending on plant species and environmental conditions (Yao et al., 2020; Peuke, 2010).

In this study, maize plants grown under different N treatments showed dissimilar amino acids concentration in xylem sap (Fig. 4), highlighting that within the xylem the concentration of individual amino acids is also dependent on the N form applied. After 7 days of treatment, the use of reduced N forms (urea and/or ammonium) upregulated the concentration of His and Met in the xylem vessels. Moreover, Gln, Pro and glutathione were significantly concentrated in the xylem only when urea was added to the nutrient solution. In the xylem sap Gln is known to be the main compound for N translocation and its concentration changes depending on the mineral N source applied to nutrient solution (Prinsi and Espen, 2015). Thus, the occurrence of Gln in the xylem sap of urea-treated plants suggests that the N pathway for ureic assimilation has been activated in roots and then the N assimilatory product are translocated to the shoot. Regarding Pro, this amino acid is considered to be a stress-related signal, nevertheless all functions of proline for the stress tolerance are still matter of debate (Kaur and Asthir, 2015). The Pro metabolism is related to several pathways, including urea cycle and glutamate-glutamine metabolism, and the accumulation of this amino acid increased the cellular osmolarity during water limitation (Verslues and Sharma, 2010). In our samples, the upregulation of proline levels in the xylem sap of urea treated-plants might be consequence of the activation of these metabolic pathways and might act as a molecular signal to reduce the xylem sap flux rate when urea was applied to nutrient solution (Fig. 1A). Further research is needed to clarify the role of urea as a N source in enhancing the resilience of crops to water stress.

Regarding the phytohormones, cytokinins (CKs) play an important role in the integration of nutrient signals transduction from roots to shoots at the whole plant level (Hwang and Sakakibara, 2006; Luo et al., 2020; Sakakibara, 2021). Lu et al. (2009) showed that the total concentration of CKs in xylem sap decreased in tomato plants grown in presence of nitrate and ammonium mixture depending on the increase of ammonium ratio. Nevertheless, other studies reported an increase in different individual CK concentration in presence of mixture on N-forms (Fetene and Beck, 1993; Smiciklas and Below, 1992), highlighting that N-forms might have different effect on CKs concentration depending on plant cultivars (Wang and Below, 1996). Under our condition, after 7 days of treatment, an increase in CK concentration was observed in the xylem sap depending on the available form of N in the nutrient solution (100 U and 50 U:50 A: tzr; 100 A: tzr, ip, ipa; Nitrate: tzr, tz, ip; Fig. 5). Previous studies indicated that in response to N status, plants have multiple ways to regulate the synthesis of new CKs in roots (Gu et al., 2018) and thus modulate the type and the concentration of these molecules in the xylem. One of these ways is mediated by a nitrate-specific signal, whereas another one is based on a glutamine-related signal. Both these ways are active in the regulation of the CKs biosynthesis and could represent the N availability and the assimilated N status in plants (Sakakibara et al., 2006). Thus, these results confirm the strong relationship between CKs and N nutrition in plants and suggest that the molecular pattern of CKs species transported from root-to-shoot may have an important role in systemic nutritional signaling as being responsive to the N form available in the nutrient solution.

Different effects of N-forms were also observed on abscisic acid (ABA) levels in plants depending on multiple factors (i.e. genotype, N concentration; Ding et al., 2016; Liu and von Wirén, 2021; Peuke et al., 1998; Buoso et al., 2021c). In *Commelina communis* nitrate treatment increase the stomatal response to ABA, probably due to a modulation of the pH of the xylem sap (Jia and Davies, 2007). The occurrence of a link between ammonium and ABA was corroborated by the increase of

root-to-shoot transport of ABA induced by ammonium treatment (Peuke et al., 1998). Under water stress the stomatal closure is mainly controlled by soil moisture level through biochemical signals as ABA rather than leaf water status (for review see Pirasteh-Anosheh et al., (2016)), and in this condition this phytohormone is synthetized in dehydrating roots and translocated to the shoot via xylem. After 7 days of treatment, the low levels of ABA in the xylem sap of nitrate- or ammonium-treated plants (Fig. 5) agrees with high values of xylem exudation rate and stomatal conductance. Considering the similar C assimilation rate among N treated plants, we can speculate that the use of urea as an N source may promote a parsimonious use of water within plants. The comprehension of the mechanism by which urea nutrition triggers with photosynthetic activity and transpiration process may be relevant to improve the resilience of plants to water stress.

Beside metabolic profile, even the ionomic composition of the xylem sap of urea-treated plants was different than the control (-N) and highlighted some specific features that were not observed under ammonium or nitrate. After 7 days, urea increased the concentration of some elements in the xylem sap, such as P, S and Fe which were less abundant under nitrate or ammonium nutrition solution (Fig. 2). Considering the translocation rate, similar amount of P and Fe should be translocated to the shoots of N treated plants independently to the N-source applied. On the other hand, S is more translocated to leaves when urea and/or ammonium were applied to nutrient solution than -N plants or than nitrate treated-plants (Supplementary Fig. 7). The interplay between N and S nutritional pathways is well known, as the biosynthesis of S containing amino acids like cysteine and methionine requires amino acids that in turn are produced by transaminase reactions. Moreover, the acquisition system of sulphate and nitrate is similar as both nutrients are taken up as anions in cotransport with protons against electrochemical gradient, thus both nutrients are acquired through secondary active transport based on a consumption of ATP energy power and are assimilated through reductive steps using reducing equivalents. Therefore, changes in the availability of one nutrient can affect the root uptake and the metabolism of the other (Courbet et al., 2019). Taking together our results suggest a cross link between urea nutrition and S translocation to the shoot as, after 7 days, urea treatment increased the Met, glutathione and S concentration in the xylem sap more than the other N forms or control plants. In the xylem sap the glutathione is translocated its concentration is related to the presence of ABA (Lee et al., 2022). In maize the ABA biosynthesis was demonstrated to be under control of sulphate in the xylem, as this latter acts as a xylem-born chemical signal for the expression of genes involved in the synthesis of the phytohormone in leaves (Ernst et al., 2010). The relevance of the interactive effect of ABA and sulphate in maize was also confirmed by a decrease of transpiration rate and stomatal closure (Ernst et al., 2010; Batool et al., 2018).

Finally, the present study revealed that Tyr was the amino acid most abundant in the xylem sap and its levels were upregulated by urea. In maize Rösler et al. (1997) demonstrated that, in contrast to dicots, the upstream enzyme of the phenylpropanoid pathway has dual activity (PAL and TAL activity) as both L-phenylalanine and L-tyrosine can be substrate of the deamination reaction. Thus, the occurrence of Tyr in the xylem sap and its translocation in the shoot might be relevant as being substrate for the synthesis of phenolic compounds. In maize the water stress led to increase the Tyr concentration in the xylem sap (Alvarez et al., 2008). It is interesting note that after 7 days of treatment, the addition of urea as the sole N source induced an upregulation of this amino acid concentration supporting the idea that the urea nutritional pathway may activate some responses usually observed under water stress (low transpiration flux rate, Tyr and Pro increase).

5. Conclusions

This study presents novel evidence demonstrating that urea-based nutrition reduces the water translocation in maize plants compared to mineral N forms. This finding, along with the stomatal conductance

results, suggests that maize plants efficiently utilize water when supplied with urea. The distinct composition of amino acids and phytohormones (e.g., S, Gln, Pro, ABA) in the xylem sap under urea nutrition may account for the variations in xylem sap exudation compared to plants treated with mineral forms. Enhancing our understanding of urea

nutrition will facilitate the development of effective agronomic strategies to enhance the resilience of maize crops against water stress.

In a previous work (Buoso et al., 2021a) urea and ammonium mixture in nutrient solution allowed a higher N uptake efficiency in maize than the single N forms (urea or ammonium). In terms of xylem
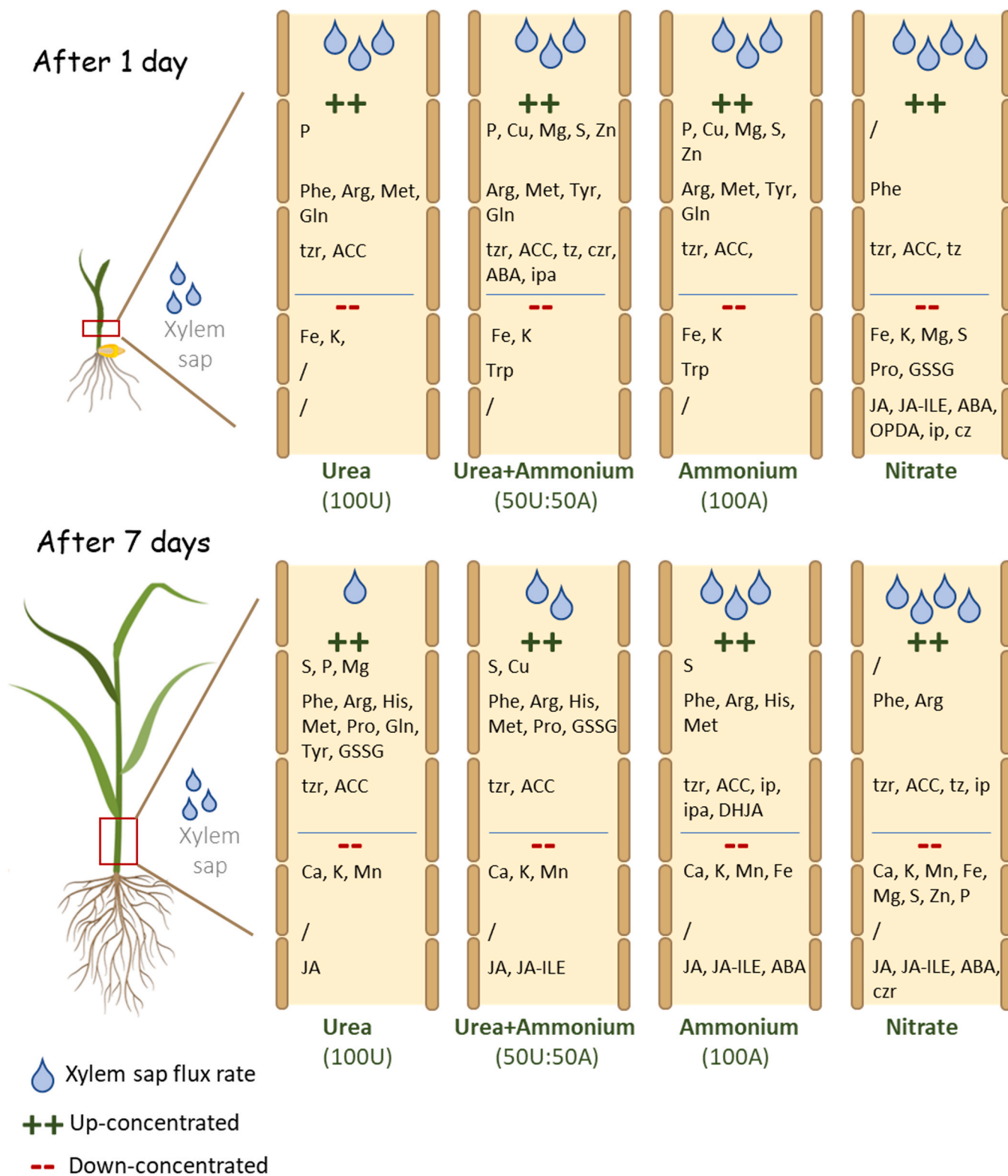

Fig. 6. Schematic representation of changes in the composition (amino acids, phytohormones, elements) and flux rate of xylem sap in maize plants after 7 days of treatment with nitrate (Nitrate), urea (100 U), urea and ammonium (50 U:50 A) or ammonium (100 A) in comparison to N deficient plants (-N). Color refers to a downregulation of metabolite concentration (– in red) and to an upregulation of metabolite concentration (++ in green).

sap composition, the occurrence of ammonium along with urea in nutrient solution determined an intermediate plant response in terms of physiological, ionomic and metabolic profiles than those registered under a single N source (Fig. 6). Thus, taking into account also the water availability, the application of both urea and ammonium to maize might be a valid strategy to promote an optimal use of nitrogen applied reducing the N emissions in the environment (i.e. ammonia volatilization and nitrate leaching).

Author contribution

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

I have shared all the data as supplementary material.

Acknowledgments

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111825.

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