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Title:

Characterization of physiological and molecular responses of *Zea mays* seedlings to different urea-ammonium ratios

Short running title: **urea and ammonium acquisition in maize**

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HIGHLIGHT

The simultaneous presence of urea and ammonium in the nutrient solution promotes the acquisition of ammonium

ABSTRACT

Despite the wide use of urea and ammonium as N-fertilizers, no information is available about the proper ratio useful to maximize the efficiency of their acquisition by crops. Ionomic analyses of maize seedlings fed with five different mixes of urea and ammonium indicated that after 7 days of treatment, the elemental composition of plant tissues was more influenced by ammonium in the nutrient solution than by urea.

Within 24 hours, similar high affinity influx rates of ammonium were measured in ammonium-treated seedlings, independently from the amount of the cation present in the nutrient solution (from 0.5 to 2.0 mM N), and it was confirmed by the similar accumulation of ^{15}N derived from ammonium source. After 7 days, some changes in ammonium acquisition occurred among treatments, with the highest ammonium uptake efficiency when the urea-to-ammonium ratio was 3:1.

Gene expression analyses of enzymes and transporters involved in N nutrition highlight a preferential induction of the cytosolic N-assimilatory pathway (*via* GS, ASNS) when both urea and ammonium were supplied in conjunction, this response might explain the higher N-acquisition efficiency when both sources are applied.

In conclusion, this study provides new insights on plant responses to mixes of N sources that maximize the N-uptake efficiency by crops and thus could allow to adapt agronomic practices in order to limit the economic and environmental impact of N-fertilization.

KEYWORDS: AMT, DUR3, mixture of N sources, nitrogen transporters, NRT, root uptake.

INTRODUCTION

Nitrogen (N) is a macronutrient for plants and its bioavailability in the soil is strictly correlated to the plant productivity (Gojon, 2017; Li et al., 2017). Ammonium and nitrate are the two main N forms that plants preferentially acquire to sustain their N needs (Hachiya and Sakakibara, 2017). On the other hand, organic N is the most abundant form of N in the soil that contributes to N bioavailability during organic matter decomposition through the release of amino acids and little peptides. Together with the urea, these organic N-sources can be acquired by roots and partially sustain plant N nutrition (Kojima et al., 2007; Tegeder and Rentsch, 2010; Forde, 2014). Urea in the soil can either have natural occurrence deriving from the catabolism of living organisms or have an anthropogenic origin deriving from fertilization events. Despite the worldwide diffusion of urea as N source in agriculture, little is yet known on the molecular mechanisms involved in its use by plants (Liu et al., 2003; Wang et al., 2012; Zanin et al., 2015a; Zanin et al., 2015b; Zanin et al., 2016). It is generally assumed that the soil application of urea determines an increase of N-bioavailability in soil mainly through its hydrolysis into ammonium. This latter form can be converted into nitrate by nitrification process, and both these inorganic forms mainly contribute to plant N nutrition.

Ammonium can be taken up by root cells through transporters located on plasma membranes (Ghiel et al., 2017); in maize, Gu et al. (2013) have identified two AMT1-type homologues to arabidopsis transporters (ZmAMT1;1a and ZmAMT1;3), which are localized in the rhizodermal cells. These transporters are probably responsible of the major acquisition of ammonium in the high-affinity range, are inducible by substrate rather than by N deficiency (Gu et al., 2013) and this response is dependent on genotype (Mascia et al., 2019).

Urea can, at least in part, be taken up by root cells directly as intact molecule through different mechanisms of acquisition, and their relative contribution depends on the external concentration of urea (Liu et al., 2003; Gu et al., 2012; Zanin et al., 2014). At low external concentration, this molecule can be taken up by a high-affinity transporter called DUR3 located on the plasma membrane of root cells, while, at high external concentration, urea might pass through plasma membrane by simple diffusion or be acquired by passive transport mediated by aquaporins (Kojima et al., 2006; Wang et al., 2008; Zanin et al., 2014).

In the soil, the stability of ammonium-based fertilizers varies with soil and environmental conditions (Cantarella et al., 2018). Especially in soil with high pH and low cation exchange capacity, N can be easily volatilized in form of ammonia into the atmosphere. Despite being more stable than ammonium, urea can be rapidly hydrolysed to ammonium/ammonia through the activity of soil microbial ureases (Sigurdarson et al., 2018). Therefore, in soil urea might suffer a destiny

similar to ammonium sources, and in turn it compromises the efficiency of urea-based fertilization (Nannipieri et al., 1990; Houdusse et al., 2005; Souza et al., 2016). The use of mixed-N forms as fertilizer takes the advantage to have both N sources, urea and ammonium, simultaneously bioavailable in the rhizosphere for root acquisition and therefore allow a simultaneous acquisition of both N-forms. A reciprocal interaction among two or three N sources, as urea, ammonium and nitrate, was previously reported on maize plants (Zanin et al., 2015b), wheat (Garnica et al., 2009) and oilseed rape (Arkoun et al. 2012). In some cases, a synergistic action between N sources was reported as showed by Garnica et al. (2009), who observed a significant increase of ammonium and urea uptake in wheat in the presence of nitrate, albeit the entity of this action might be influenced by the nitrophilic character of the studied plant species (Arkoun et al. 2012). In wheat, the co-presence of nitrate with ammonium and/or urea in the nutrient solution was associated with significant improvements in plant growth and N assimilation, maybe due to a rapid and transient stimulation of assimilatory pathway (glutamine synthetase, GS, and urease activity; Garnica et al., 2009; Garnica et al., 2010). Similar effects were also observed in maize where the simultaneous presence of urea and nitrate in the nutrient solution stimulated, at the transcriptional level, the concomitant activation of more pathways for N assimilation located in different cellular compartments, the plastidial GS2/GOGAT cycle and the cytosolic pathway involving GS1 and ASNS (Zanin et al., 2015b). However, the mechanism responsible for the beneficial effect of nitrate on urea or ammonium nutrition remains unclear (Houdusse et al., 2005). In maize, the use of mixed N-sources, nitrate:ammonium, improves plant growth (higher leaf area, shoot and root biomass) and photosynthetic rate in comparison to sole nitrate or sole ammonium (Wang et al., 2019). Some pieces of evidence suggest that this synergistic action between two N sources (as nitrate and ammonium) might contribute to prevent cytotoxic effect of ammonium (for review see Britto and Kronzucker, 2002) through a rapid N assimilation, a pH intracellular regulation or maybe favouring a hormonal balance in root cells (Gerendás et al., 1997; Chen et al., 1998; Zanin et al., 2015b). Based on these considerations, also urea might contribute to alleviate the toxicity of ammonium in root cells, as urea nutrition promotes root growth and stimulates cytosolic pathway for N assimilation (Mérigout et al., 2008b; Zanin et al., 2015b). Despite the wide relevance as nitrogen fertilizers, little is known about the interactions between urea and ammonium, and no studies have investigated before the proper urea:ammonium ratios useful to maximize the N acquisition in plants. Present work aims to evaluate the occurrence of a reciprocal influence on N-acquisition depending on type and availability of two N forms. Therefore, N-acquisition, ionomic profile and transcriptional pattern of most relevant genes for N nutrition were analysed when maize roots were simultaneously exposed to urea and ammonium applied in conjunction to nutrient solution (five

different urea-to-ammonium ratios). We speculated that fertilization with a mixture of urea-ammonium can promote N acquisition in maize.

MATERIALS AND METHODS

Plant growth

Maize seeds (*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 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 seedlings (5-day-old) were transferred to a N-free nutrient solution (μM: 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 N-free nutrient solution, hence five nutritional treatments have been tested (2 mM total N): *100U*, 1.00 mM CH_4N_2O; *75U:25A*, 0.75 mM CH_4N_2O and 0.25 mM $(NH_4)_2SO_4$; *50U:50A*, 0.50 mM CH_4N_2O and 0.50 mM $(NH_4)_2SO_4$; *25U:75A*, 0.25 mM CH_4N_2O and 0.75 mM $(NH_4)_2SO_4$; *100A*, 1.00 mM $(NH_4)_2SO_4$.

As controls, some seedlings were grown in N-free nutrient solution (*-N*) or in -N nutrient solution containing nitrate (1 mM $Ca(NO_3)_2$, *Nitrate*). Sulphate was added to nutrient solution in variable amounts to compensate the sulphur amount deriving from ammonium sulphate. The pH of solution was buffered using 1 mM MES-BTP at pH 6.0. After 1 h from the beginning of the light phase (8:00 AM), the N sources have been added to nutrient solution. To avoid urea degradation, nutrient solution was renewed every 48 hours, therefore during this period, the hydrolysis of urea or nitrification processes are unlikely under hydroponic conditions (Zanin et al., 2015b; Mérigout et al., 2008a). At the end of the experiment, the light transmittance of leaves was monitored (SPAD-502, Minolta, Osaka, Japan).

The capability of maize to use N sources was evaluated through [^{15}N]-tracer experiments after 24 hours and 7 days of treatments. Therefore seedlings were exposed to nutrient solution containing labelling nitrogen as $CO([^{15}N]NH_2)_2$, $([^{15}N]NH_4)_2SO_4$ or $Ca[^{15}N]NO_3$ (10 atom% ^{15}N). Only one N-source was labelled when urea and ammonium were applied in conjunction in nutrient solution.

Root external acidification

The capability of roots to acidify the external media was performed after 6 hours of treatment with N sources and visualized on agar gel (0.9% w/v agar layer containing 0.04% w/v bromocresol purple, as pH indicator) as previously described by Zanin et al. (2017).

Elemental analysis

Elemental composition of roots and shoots was analysed as previously described (Zanin et al., 2017). Briefly, oven-dried samples (60°C) of shoots and roots (collected 24 hours and 7 days after treatments) were acid digested with concentrated ultrapure HNO_3 (650 mL L^{-1}; Carlo Erba, Milano, Italy) using a microwave oven (CEM Mars Xpress Matthews, NC, USA), according to the USEPA 3052 method "Plant Xpress" (USEPA, 1995). Element concentrations (calcium, Ca; copper, Cu; iron, Fe; potassium, K; magnesium, Mg; manganese Mn; molybdenum, Mo; sodium, Na; nickel, Ni; phosphorus, P; sulphur, S; zinc, Zn) were then determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS NexION 300, Perkin Elmer Inc., Shelton, CT USA) or Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES 5800, Agilent Technologies, Santa Clara, USA). Element quantifications were carried out using certified multi-element standards.

After 24 h and 7 days of treatment, shoots and roots of maize were dried and their total N, C content and ^{15}N enrichment were determined by EA-IRMS (Vario Isotope Select and Isoprime 100, Elementar Analysensysteme GmbH, Hanau, Germany). ^{15}N-fertilizer uptake efficiency of maize seedlings was calculated after 24 hours or 7 days of treatment and refers to the N-uptake efficiency (NUpE) of the labelling ^{15}N-source (calculated as: ^{15}N uptake (nmol) / ^{15}N applied (nmol) × 100).

Ammonium uptake rate

The uptake rate of ammonium was measured by accumulation of ^{15}N-labeled source into roots of maize seedlings after rinsing the roots of hydroponically-grown seedlings in 0.5 mM $CaSO_4$ solution for 1 min, followed by an incubation for 6 min containing ^{15}N-labeled ammonium sulphate (98 atom% ^{15}N, 0.1 mM $[^{15}N](NH_4)_2SO_4$ in 0.5 mM $CaSO_4$) and then rinsing roots in ice-cold ^{15}N-free solution (0.5 mM $CaSO_4$) for 1 min. Roots were then harvested and freeze dried. An aliquot of 2 mg of ground sample was used for ^{15}N analysis by elemental analyser/isotope ratio mass spectrometry (EA-IRMS, Vario Isotope Select and Isoprime 100, Elementar Analysensysteme GmbH, Hanau, Germany).

RNA Extraction and reverse transcription for Real time RT-PCR analyses

Real-time RT–PCR analyses were performed on maize roots as described by Venuti et al. (2019). Maize roots were sampled, and total RNA was extracted using Invisorb© Spin Plant RNA kit (Invitek Molecular, Berlin, Germany) following manufacturer's instructions. The quality and concentration of 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,

Milano, Italy), 20 U Prime RNase Inhibitor (Sigma Aldrich), 200 U of RNase H derivative of Moloney murine leukaemia virus (M-MLV reverse transcriptase, Sigma Aldrich), according to the manufacturer's protocol. Using Primer3 software (Koressaar and Remm, 2007; Untergrasser et al., 2012), primers were designed and synthesized by Sigma Aldrich (**Supplementary Table S1**). 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 averaged expression of two housekeeping genes *ZmGAPDH* and *ZmTUA* (**Supplementary Table S1**). Data were normalized using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analyses and data elaboration

Physiological and molecular analyses were performed on three independent biological replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test for multiple comparisons (p-value <0.05, N = 3). PCA analyses were performed with ClustVis web tool (https://biit.cs.ut.ee/clustvis/; Metsalu and Vilo, 2015) applying unit variance scaling to elements; singular value decomposition with imputation is used to calculate principal components, in heatmaps, elements were centred, unit variance scaling is applied to elements (both elements and samples were clustered using correlation distance and average linkage).

RESULTS

Morphological observations

In order to verify the capability of maize to use urea and/or ammonium as N sources, 5-day-old maize seedlings were grown further on nutrient solution containing N sources for up to 7 days. In our experimental set up, no urease inhibitor was added to nutrient solution to avoid interference with the urea acquisition by plants (Krogmeier et al., 1989; Bremner, 1995; Watson, 2000; Zanin et al., 2015a; Zanin et al., 2016). Moreover, under hydroponic condition, the frequent renew of nutrient solution was sufficient to avoid urea hydrolysis (Mérigout et al., 2008a).

Despite no significant changes in maize weights were measured (Buoso et al., 2021), the presence of ammonium in the nutrient solution modified root architecture, through the elongation of lateral roots and a concomitant reduction of primary and seminal root lengths (well visible after 7 days of treatment, **Figs. 1A, 2**). Comparing to urea or nitrate, the presence of ammonium in nutrient solution induced a higher acidification activity in maize roots, already visible after 6 hours of

treatment (**Fig. 1B**). The presence of 0.5 mM NH_4^+ in the treatment 75U:25A was sufficient to stimulate the acidification of root external media and this effect was mainly localized around the primary roots. In 50U:50A, 25U:75A and 100A treatments, the acidification effect was induced around both primary and seminal roots. These effects (root external acidification and shortening of the primary root) were less visible when ammonium was provided in conjunction with urea (**Figs. 1, 2**).

Elemental analyses

Elemental composition of roots and shoots was analysed after 24 hours and 7 days of treatment (Buoso et al., 2021). Depending on N treatments, elemental distribution between shoots and roots showed differences among treatments and between the two sampling times (24 hours and 7 days).

Multivariate analyses (PCA) were carried out on the whole elemental dataset in order to highlight possible differences and similarities among the samples (**Fig. 3, Supplementary Fig. S1**). After 7 days of treatment, the PCA generated a six-component model accounting for a total variance of 96% in both shoots and in roots. The first two components (PC1 and PC2) explaining 70% of the total variance have been chosen to show sample distribution. In both shoots and roots, the PCA analyses discriminate four groups along PC1, where all samples deriving from ammonium-containing treatments clustered together (75U:25A, 50U:50A, 25U:75A and 100A, **Fig. 3**). This clear separation was visible only after 7 days of treatment (**Fig. 3)**. Conversely, after 24 hours of treatment with different N-sources, samples are clustered together (Buoso et al., 2021).

The root exposure to N sources determined a significant increase of N concentration in maize seedlings, and this effect was already evident after 24 hours of treatment when inorganic N sources (ammonium and nitrate) were applied to nutrient solution (Buoso et al., 2021).

Regarding the other nutrients, after 24 hours slight differences were observed among treatments, with the exception of K which concentration in shoots and roots of maize seedlings was already reduced in comparison to -N seedlings when ammonium was applied to nutrient solution (75U:25A, 50U:50A, 25U:75A and 100A, Buoso et al., 2021).

Prolonging the treatments to 7 days, visible changes in nutrient concentrations of shoots and roots occurred among treatments (Buoso et al., 2021). The element composition of ammonium-treated seedlings (100A) indicates an increase of S, P, Cu in roots and shoots, and an increase of Mn, Fe, Zn in shoots in comparison to -N seedlings. On the other hand, a reduction of K concentration was observed in shoots and roots of 100A.

Besides, for the first time, we provide the ionomic profile of seedlings fed with urea as sole N source (100U). It is interesting to note that, after 7 days of treatment, urea nutrition led to

characterize maize seedlings with a different ionomic pattern in comparison to N-deficient ones (–N) or seedlings fed with inorganic N-sources (as ammonium, 100A; or Nitrate; **Fig. 3**). In particular, the presence of urea as sole N source determined higher concentrations of: P, S, Fe in roots and shoots; Mg, Cu and Zn in shoots; and Mn in roots (Buoso et al., 2021).

Under our experimental conditions, when urea and ammonium were applied in conjunction to nutrient solution (75U:25A, 50U:50A, 25U:75A), intermediate values between 100 U and 100A in elemental concentrations of Mg, Cu, Mn, were observed in roots and shoots, and S in shoots, while the K and P concentrations were comparable to those detected in ammonium treated seedlings (100A) than to urea ones (100U; Buoso et al., 2021).

Ammonium influenced the internal redistribution of Mg in maize since ammonium treatments led to a reduction of Mg concentration in roots but increased its concentration in shoots.

^{15}N-labelling experiments

The capability of maize seedlings to acquire the different N sources was evaluated through ^{15}N-labelling experiments. Maize seedlings were fed with [^{15}N]-nitrate, [^{15}N]-urea or [^{15}N]-ammonium and the amount of ^{15}N taken up by roots was evaluated through EA-IRMS. Depending on time (24 hours or 7 days) and type of treatment, seedlings showed a different use of N sources (**Fig. 4**). The highest values of ^{15}N-concentration were detected in seedlings treated with inorganic N sources, nitrate and ammonium (75U:25A, 50U:50A, 25U:75A and 100A), both at 24 hours and 7 days.

Within 24 hours, ureic-^{15}N was proportional to the amount of urea available in the external media, while ^{15}N derived from ammonium contributed in a similar way among ammonium containing treatments. Conversely, after 7 days of treatment, urea source significantly contributed to N nutrition of maize increasing linearly with the amount of urea available in the external media (**Fig. 4**). At 24 hours, the N-uptake efficiency (NUpE) of ammonium fertilizer was higher in treatment 75U:25A. Moreover, also treatments 50U:50A and 25U:75A promoted ammonium uptake efficiency, with values higher than those recorded under treatment 100A (**Fig. 5**).

In terms of NUpE-^{15}N parameter, data indicated that after 7 days no significant changes in the uptake efficiency of ^{15}N-urea among urea:ammonium treatments were detected, while a high NUpE of ^{15}N-ammonium was observed for treatment 75U:25A (**Fig. 5**).

Ammonium uptake rate

The ^{15}N-ammonium influx experiments allowed to monitor the dynamic of ammonium influx in maize roots through the high-affinity components of ammonium transport system (HATS).

Present data confirmed ammonium influx is stimulated by its substrate, while N deficient seedlings (-N) and nitrate treated seedlings showed only a little stimulation of ammonium acquisition after 24 hours of treatment (**Fig. 6A, B**). Moreover, data indicated that treatments 75U:25A, 50U:50A, 25U:75A and 100A showed similar high-affinity transport activity within 24 hours of treatment suggesting that the presence of urea did not interfere with the high-affinity ammonium influx (**Fig. 6D, E, F**). In the presence of urea as sole N-source (100U), seedlings showed a gradual increase during 24 hours of ammonium acquisition, reaching the highest influx rate after 24 hours of treatment (**Fig. 6C**). However, the simultaneous exposure of maize seedlings to both N sources did not induce an over stimulation of ammonium acquisition: the influx rate observed under urea:ammonium mix (treatment 75U:25A, 50U:50A, 25U:75A) was comparable to the influx pattern observed under ammonium alone (100A). The only exception was observed after 2 hours of 50U:50A treatment, since the ratio 1:1 urea:ammonium determined higher ammonium uptake rate in roots in comparison to ammonium alone (100A, **Fig. 6E**).

Gene expression analyses

To characterize the expression profile of genes involved in N transport and assimilation, gene expression analyses were performed in maize roots after 24 hours and 7 days of treatment (**Fig. 7**). The expression of twenty-one genes coding for different isoforms of enzymes and transporters involved in N nutrition highlightened a different induction of N-assimilatory pathways (Buoso et al., 2021**)**. Main responsive genes by urea and ammonium treatment were: *ZmNRT1.1*, *ZmDUR3*, *ZmAMT1;1a*, *ZmAMT1;3*, *ZmGS2*, *ZmGS3*, *ZmASNS3* and *ZmASNS4* (**Fig. 7**). After 24 hours (**Fig. 7A;** Buoso et al., 2021), the expression of the low-affinity nitrate transporter *ZmNRT1.1* was induced by the presence of N in the nutrient solution (regardless of the nitrogen source) compared to -N treatment, while urea transporter *ZmDUR3* was downregulated by all N-treatments. The expression of the ammonium transporters *ZmAMT1;1a* and *ZmAMT1;3* were induced by ammonium and were responsive to the amount of ammonium available in the external media. When urea was added in the external media as the only N-source (100U), a strong induction of ammonium transporter *ZmAMT1;3* gene was observed at 24 hours in comparison to the other treatments. Moreover, in comparison to -N and nitrate treatment, urea (100U) induced also genes involved in the ammonium assimilation *via* cytosolic pathway, as demonstrated by the high transcript levels of *ZmGS3*, *ZmASNS3* and *ZmASNS4*. This induction characterized also urea:ammonium mix (treatments 75U:25A, 50U:50A, 25U:75A) and ammonium (100A) treatments. Conversely, *ZmGS2*, the plastidial isoform of glutamine synthetase, was up-regulated only by nitrate treatment. After 7 days of treatment (**Fig. 7B**), the expression of *ZmNRT1.1* was induced by the presence of N in the

nutrient solution in comparison to N-deficient maize. On the contrary, *ZmDUR3* was downregulated by N treatments regardless to N form applied in nutrient solution. The expression of *ZmAMT1;1a* and *ZmAMT1;3* was induced by urea and ammonium treatment (100U, 75U:25A, 50U:50A, 25U:75A and 100A) compared to -N and nitrate seedlings. These treatments (100U, 75U:25A, 50U:50A, 25U:75A and 100A) also induce the expression of *ZmGS3*, *ZmASNS3* and *ZmASNS4*. *ZmGS2* gene appears to be induced only by the presence of nitrate. The expression of *ZmUrease* was not altered by the different treatments (Buoso et al., 2021).

DISCUSSION

Several studies provide evidence that a combination of different N sources leads to positive effects on the nutritional status of crops and therefore the combine use of more N forms might contribute to increase the N use efficiency (Kronzucker et al., 1999; Mérigout et al., 2008a; Garnica et al., 2009; Arkoun et al. 2012; Zanin et al., 2015b). Deep investigations have been performed to study the interaction between inorganic N sources (ammonium and nitrate; Kronzucker et al., 1999; Yang et al., 2017), while the combinatory effects of urea and ammonium on plant nutrition has been less studied. The use of urea along with ammonium might provide an advantage for N nutrition: urea does not undergo to direct volatilization as intact molecule, and, at the same time, ammonium (applied or released by urea hydrolysis) can sustain plant N requirements since the inorganic forms are preferentially taken up by plants (Harrison et al., 2007; Ashton et al., 2008). Moreover, as urea and ammonium share key-point of N assimilatory process in plants, the simultaneous availability of both N forms in the external solution might exert a reciprocal interaction on their acquisition in plants.

Plant responses to ammonium

When ammonium was applied as sole N source, no cytotoxic effects were visible on maize seedlings and on fresh and dry weights, being similar among N-treatments (Buoso et al., 2021). Depending on ammonium availability in nutrient solution, a shortening of root length and an increase of root external acidification occurred (**Figs. 1, 2**). These morphological changes were previously described in plants (Meier et al., 2020; Liu et al., 2013; Anderson et al., 1991). Ammonium acquisition is linked to an increase of root external acidification due to a strong extrusion of protons as possible consequence of pH drop in the cytosol of root cells due to ammonium assimilation (Meier et al., 2020; Gerendás et al., 1997; Taylor and Bloom, 1998).

Extensive studies have been performed to study ammonium transport system in plants (von Wirén et al., 2000; Gu et al., 2013; Giehl et al., 2017; Duan et al., 2018). The ^{15}N-ammonium influx experiment confirmed that in maize, ammonium HATS is stimulated by its substrate (**Fig. 6**). Two transporters have been characterized to be responsible of substrate-inducible HATS for ammonium uptake in roots of maize, ZmAMT1;1a and ZmAMT1;3 (Gu et al., 2013). At molecular level, gene expression analyses of maize roots confirmed that *ZmAMT1;1a* and *ZmAMT1;3* were induced by ammonium (**Fig. 7**).

The nitrate transporter NRT1.1 (Tsay et al., 1993) is involved in multiple physiological processes, which provide plant resistance to unfavourable environment such as ammonium excess and acidic toxicity (Fang et al., 2016; Jian et al., 2018). In ammonium-fed seedlings, AMT genes induction is partially dependent on NRT1.1 and, at physiological level, the absence of a functional *NRT1.1* gene led to a decrease of ammonium uptake into roots. Interestingly, the presence of ammonium (with or without urea) in the external media induced the expression of *ZmNRT1.1* alongside the overexpression of the previously reported *ZmAMT* genes (**Fig. 7,** Buoso et al., 2021). However, the rationale behind the role of this gene on the regulation and in general on the ammonium uptake still need to be elucidated (Jian et al., 2018).

Plant response to urea

Urea promoted a good development of maize roots with an extensive proliferation and elongation of the roots (Zanin et al., 2015b) and no visible changes in the root external pH occurred when maize seedlings were supplied with urea as sole N source (**Figs. 1, 2**).

For the first time in plant species, ionomic changes in response to urea have been characterized. The multielement profiling of urea-treated seedlings (100U) showed high contents of P, S, Mg, Mn, Fe and Zn in comparison to -N seedlings (**Fig. 3; Supplementary Fig. S1;** Buoso et al., 2021). Urea treatment characterized the elemental composition of maize seedlings with a defined pattern not overlapped to those of nitrate- or ammonium-fed maize (**Fig. 3)**.

Therefore, urea nutrition induces a plant response characterized by a peculiar physiological, ionomic and transcriptional modulations (Zanin et al., 2015b).

Confirming the evidence from the literature, maize seedlings use urea as the sole N-source as demonstrated by an increase of biomass and N concentration in comparison to -N maize (Buoso et al., 2021), although inorganic N sources determined an even higher N content in plants (Bradley et al., 1989; Tan et al., 2000; Houdusse et al., 2005; Mérigout et al., 2008a; Buoso et al., 2021). Over the time (from 24 hours to 7 day), an increase of urea NUpE was observed (**Fig. 5**). These data agree with previous observations in tomato, where the N-absorption of urea was shown to increase

more with the advancement along the plant growth stages than with inorganic N-forms (Tan et al., 2000). Therefore, agronomical practices that act to preserve the urea stability in the soil might take advantage of this dynamic response in plants.

In agreement with previous evidence (Zanin et al., 2015b), *ZmDUR3* was downregulated by urea or the other N forms in the media (**Fig. 7**) and *ZmUrease* expression was not responsive to urea or other N forms in the media (Buoso et al., 2021). Interestingly, urea treatment (100U) strongly induced the transcription of ammonium transporters: *ZmAMT1;1a* and *ZmAMT1;3* at 24 hours **(Fig. 7),** and the upregulation of AMT-transporters agrees with a concomitant induction of ammonium influx through HATS in maize roots (**Fig. 6**). The positive effect of urea on the expression of *AMT* was also reported in arabidopsis (Mérigout et al., 2008b).

The route of urea assimilation is supposed to be mostly compartmentalized in the cytosol. Molecular evidence (transcriptomic) might suggest that ureic-N undergoes metabolic reactions located in the cytosol, *via* transformation by urease, glutamine synthetase (GS)1 and asparagine synthetase (ASNS; Mérigout et al., 2008b; Zanin et al., 2015b). In the present work, urea induced genes involved in the ammonium assimilation *via* cytosolic pathway (GS, ASNS), as suggested by high transcript levels of *ZmGS3*, *ZmASNS3* and *ZmASNS4* (**Fig. 7**). Based on this evidence, the urea nutrition might also promote the assimilation of ammonium when the cation is directly taken up from root external solution.

Effect of urea and ammonium mix

Concerning ammonium acquisition, maize seedlings showed an increase of NUpE inversely related to ammonium availability in the nutrient solution. In particular, the treatment 75U:25A determined the highest NUpE of ammonium, and this effect was visible after both 24 hours and 7 days of treatment (**Fig. 5**). The presence of urea in the nutrient solution did not interfere with the ammonium uptake, especially considering the high affinity transport system (HATS, **Fig. 6**). The high efficiency of ammonium acquisition in presence of the urea and ammonium mix might be related to the inducible feature of the ammonium HATS, indeed, especially *ZmAMT1;3* was found to be induced not only by ammonium but also by urea, regardless of the ammonium concentration in the nutrient solution (after 7 days of treatment, **Fig. 7**). This transcriptional response might contribute to the high NUpE of ammonium when urea and ammonium are used in conjunction. On the other hand, data did not indicate the occurrence of a reciprocal interaction of ammonium on urea acquisition, as neither changes in the expression of urea transporter nor in NUpE of urea were observed among mix treatments (**Figs. 6, 8**).

Overall, data indicate that the application of urea:ammonium fertilizer in the ratio of 3:1 stimulated the ammonium uptake efficiency in plants and may contribute to limit N loss by ammonia volatilization as in this urea-to-ammonium combination most of N is applied in form of urea. A synergistic effect between the N sources urea and ammonium was also observed in wheat, where the presence of urea in the external media promoted the ammonium acquisition increasing the uptake rate of the cation form within 24 hours (Garnica et al., 2009). These data suggest that agronomical practices acting to preserve both the presence of urea and ammonium may help to promote an efficient N acquisition.

It has been hypothesized that depending on N-forms (nitrate, ammonium or urea), N assimilation might involve several molecular pathways or isoenzymes located in different compartments (Garnica et al., 2010). It is well reported that N deriving from the reductive steps of nitrate becomes substrate of the GS-GOGAT cycle located in the plastids (Li et al., 2017), while the molecular pathways directly involved in the assimilation of N deriving from urea or ammonium have been less investigated. Molecular analyses highlighted that urea and ammonium mix treatments induced an over-expression of those enzyme isoforms that are known to be localized in the cytosol (GS, ASNS) rather than in the plastid (GS-GOGAT cycle; **Fig. 7**, Buoso et al., 2021). These results indicate that ammonium, deriving from urea hydrolysis or directly taken up by roots, stimulates the N assimilation through cytosolic isoenzymes while the plastidic pathway is involved only when the assimilation of ammonium followed the nitrate-nitrite reduction (Lee et al., 1992; Lam et al., 1996; Ishiyama et al., 2004; Liu and von Wirén, 2017).

When urea was mixed with ammonium, the elemental analyses indicated that the ionomic composition of maize seedlings was mainly influenced by the presence of ammonium in the nutrient solution rather than by urea, and this effect was evident after 7 days of treatment (**Fig. 3**). This behaviour might be consequence of ammonium uptake on the acquisition of other macronutrients. Indeed, the acquisition of N in the cationic form (ammonium) can determine in plants a lower demand of cations for charge balance and therefore reduce the acquisition of other cations, such as K, Mg and Ca (Engels and Marschner, 1993; Rayar and van Hai, 1977).

It is interesting to note that the presence of urea and ammonium in the nutrient solution promoted S accumulation in maize. This behaviour might derive from a high acidification activity by ammonium-treated roots, as the transmembrane proton gradient is needed to energize sulphate acquisition by roots (Buchner et al., 2004). Moreover, conversely to nitrate nutrition, the assimilation of N deriving from urea- or ammonium-based fertilizers allows plants to save reducing power within cells as N occurs already in a reductive state. In this way, NAD(P)H and reduced ferredoxin are preserved for the assimilation of other nutrients (e.g., the reduction of sulphate to

sulphur). Therefore, these data sustain the hypothesis that the use of mixed N sources might be also advantageous for energetic reasons, since in maize, ammonium is readily assimilated in roots for local demand while nitrate or urea can be easily translocated into the shoots and assimilated there (Gerendás et al., 1997; Glass et al., 1997; Bloom et al., 1993).

CONCLUSIONS

In the present work, five urea to ammonium ratios were tested on maize seedlings and the plant response was characterized at physiological and molecular levels. The ionomic profile indicates that the elemental composition of maize is influenced by ammonium rather than by urea in the nutrient solution. Within 24 hours, maize seedlings showed similar acquisition of ammonium (^{15}N concentration and influx) irrespectively of the urea to ammonium ratio, while in the long term, the ammonium accumulation went along the availability of N source. Nevertheless, the highest ammonium uptake efficiency was observed when the N source was applied in the nutrient solution in conjunction with urea, at a 3:1 urea to ammonium ratio. Considering a slow-release urea-based fertilizers and conditions unfavourable to the nitrification of ammonium, this behaviour might allow plants to take up ammonium with a high efficiency as soon as it becomes available from urea hydrolysis. The activation of cytosolic pathway for the ammonium assimilation was induced by ammonium as well as by urea in the nutrient solution. Therefore, the plant nutrition might take advantage of fertilization with urea and ammonium when they are supplied in conjunction, as a beneficial action of urea on ammonium assimilation seems to occur in maize roots.

Aiming to mitigating N losses, this study provides guidelines for the development of cost-effective technologies and environmentally friendly solutions for a more sustainable fertilization practices in agriculture.

SUPPLEMETARY DATA

Supplementary Table S1. List of primers used for Real-time RT-PCR.

Supplementary Figure S1. Ionomic analysis of maize seedlings after 24 hours of treatment with different N-sources.

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AUTHOR CONTRIBUTION

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

DISCLOSURES

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Figure 1. A, representative pictures of maize seedlings after 24 hours of treatment with nitrogen sources. **B**, root acidification assay using a pH indicator (bromocresol purple) embedded in a thin layer of agar gel. The pH scale refers to the colour of bromocresol purple at different pH values.

Figure 2. Representative pictures of maize seedlings after 7 days of treatment with nitrogen sources (white arrows indicate primary roots; RL, primary root length: average ± standard deviation). In the box below a magnification of leaves and relative SPAD index values (average ± standard deviation) are shown (Holm–Sidak ANOVA, N=20, p-value < 0.05).

Figure 3. Ionomic analysis of maize seedlings after 7 days of treatment with different N-sources in roots (**A-C**) and in shoots (**D-F**). In radar plots, the concentration of each element in roots (**A)** and shoots (**D**) was scaled to average value of -N samples (value 1.0). PCA analyses show principal component 1 and principal component 2 that explain: 39% and 30.8% of the total variance in root (**B**) and 48.6% and 21.4% of the total variance in shoot (**E**). Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. In heatmaps, a clustering of elemental concentrations and samples in maize roots (**C)** and shoots (**F**) is shown.

Figure 4. ^{15}N-concentration in roots (**A, D**), shoots (**B, E**) and whole seedlings (**C, F**) of maize after 24 hours (**A-C**) or 7 days (**D-F**) of treatment with different N-sources. Letters refers to statistical significance (Holm–Sidak ANOVA, N=3, p-value < 0.05).

Figure 5. ^{15}N-fertilizer uptake efficiency of maize seedlings after 24 hours (left graph) or 7 days (right graph) of treatment with different N-sources, calculated as N-uptake efficiency (NUpE) of the labelling ^{15}N-source. Letters refers to statistical significance (Holm–Sidak ANOVA, N=3, p-value < 0.05).

Figure 6. ^{15}N-ammonium influx in maize roots up to 24 hours of treatment with N-sources. Asterisks refers to significancy within same sampling time in comparison to the reference (100A). The ^{15}N-ammonium influx in maize roots of 100A seedlings is compared to the influx observed in roots of: -N (**A**), Nitrate (**B**), 100U (**C**), 75U:25A (**D**), 50U:50A (**E**), 25U:75A (**F**) seedlings. Letters refers to statistical significance within same thesis during the experiment (Holm–Sidak ANOVA, N=3, p-value < 0.05).

Figure 7. Real-time RT-PCR analyses of gene transcript level in maize roots after 24 hours (**A**) and 7 days (**B**) of treatment with different N-sources. The mRNA levels were normalized with respect to the mean transcript level of the housekeeping genes *ZmTUA* and *ZmGAPDH*. Relative changes in gene transcript levels were referred to the average transcript level of housekeeping genes in -N roots

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FIGURES

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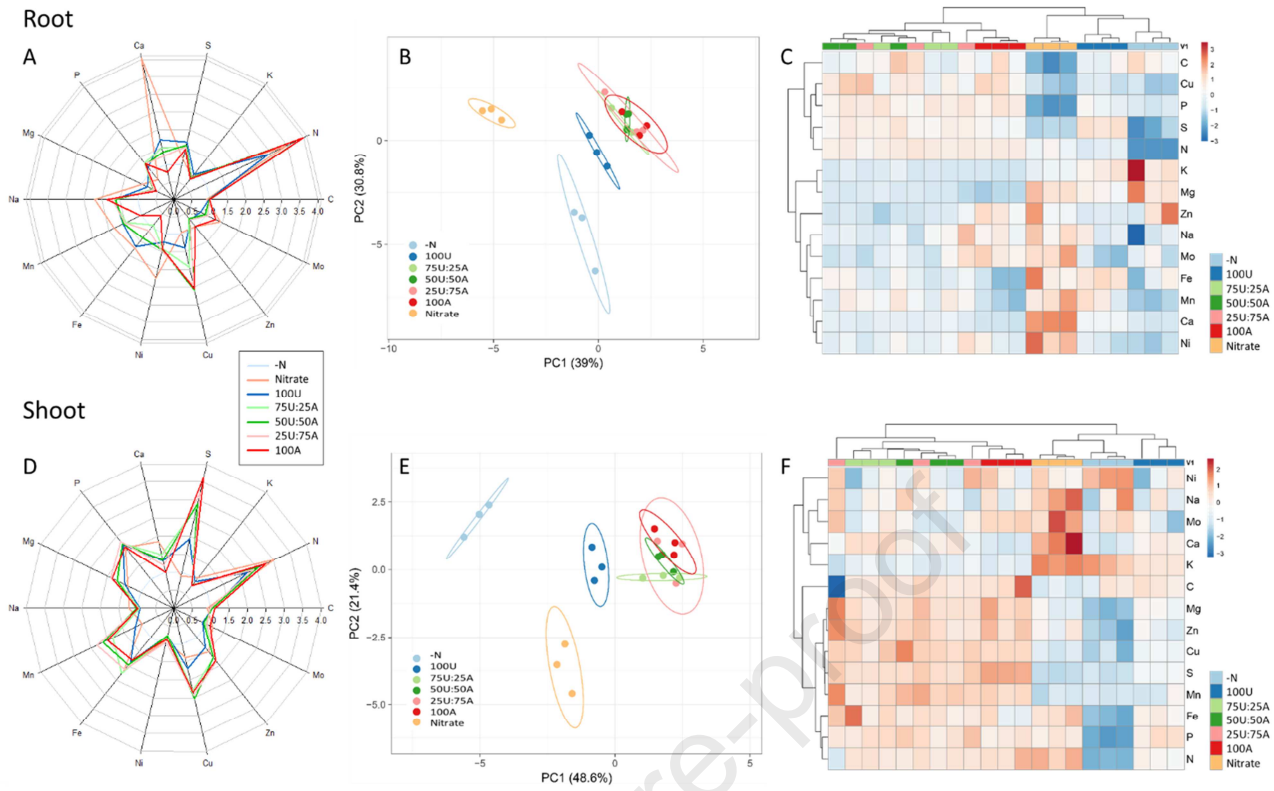

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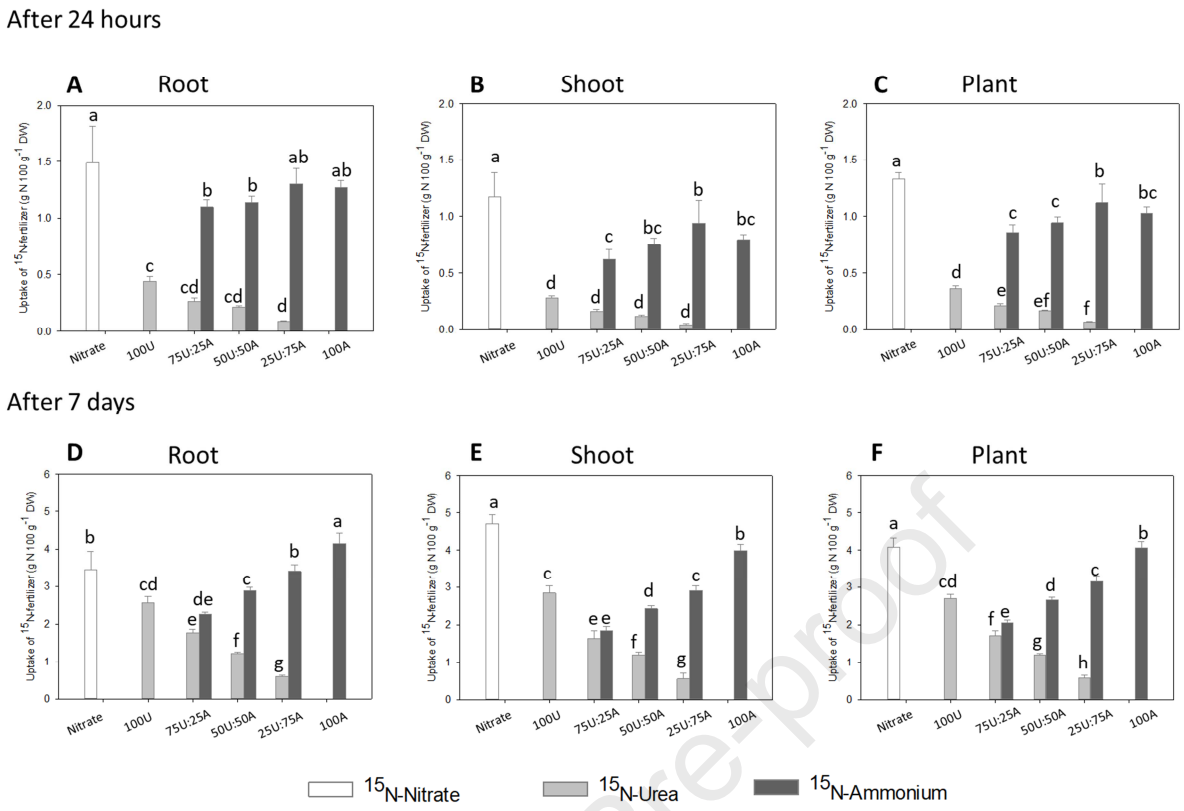

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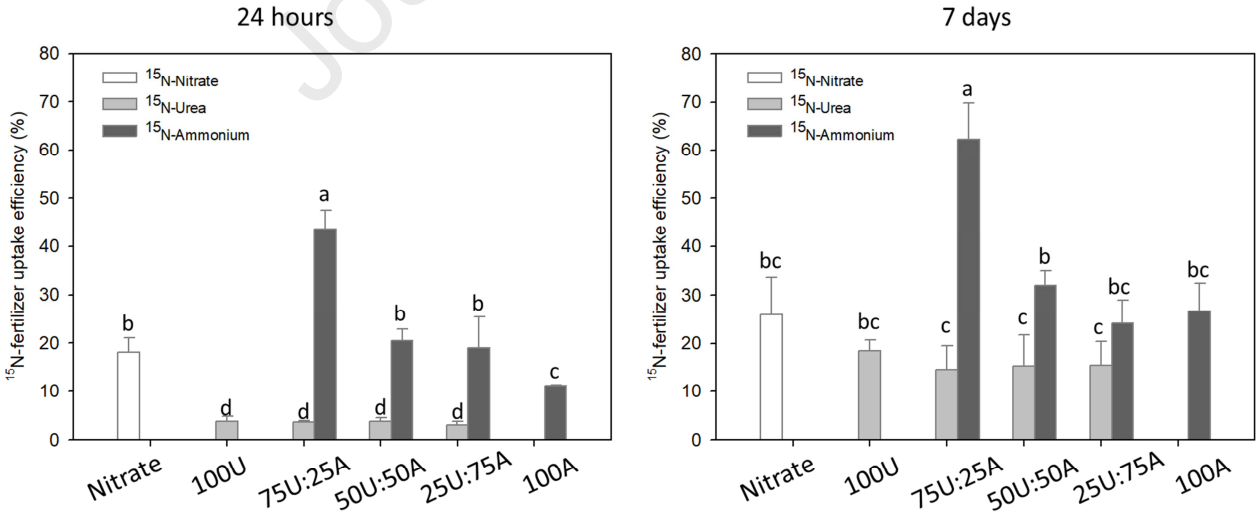

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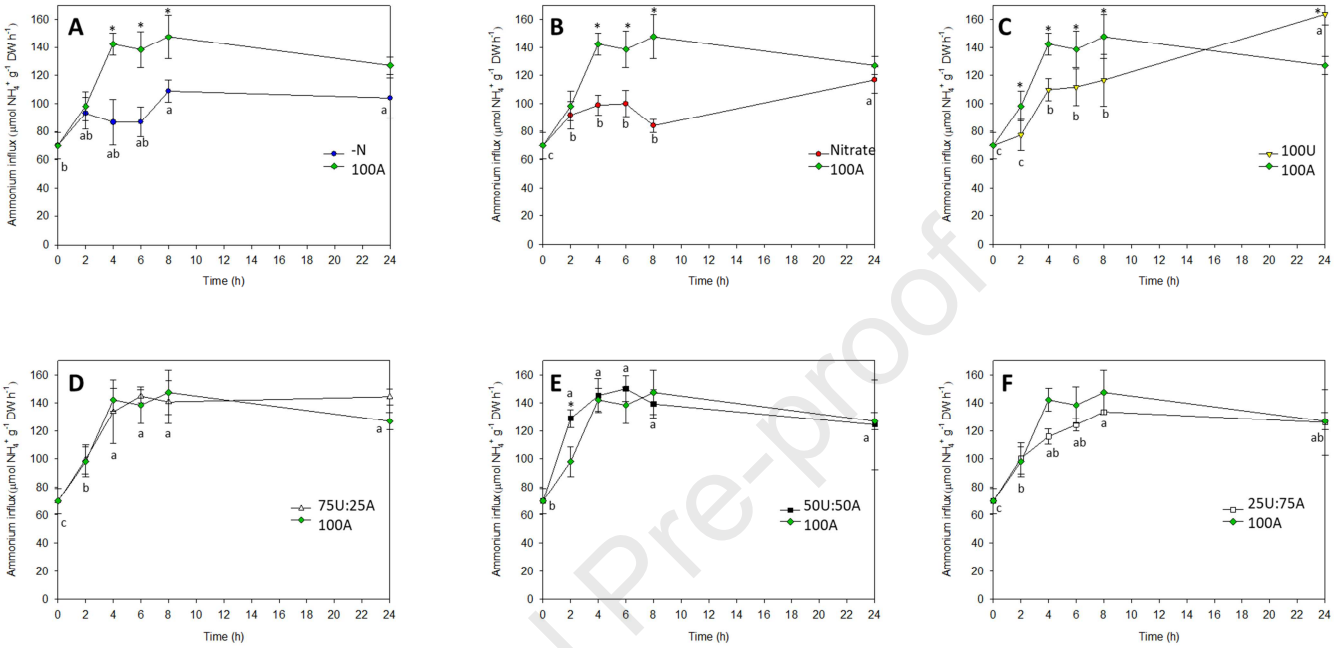

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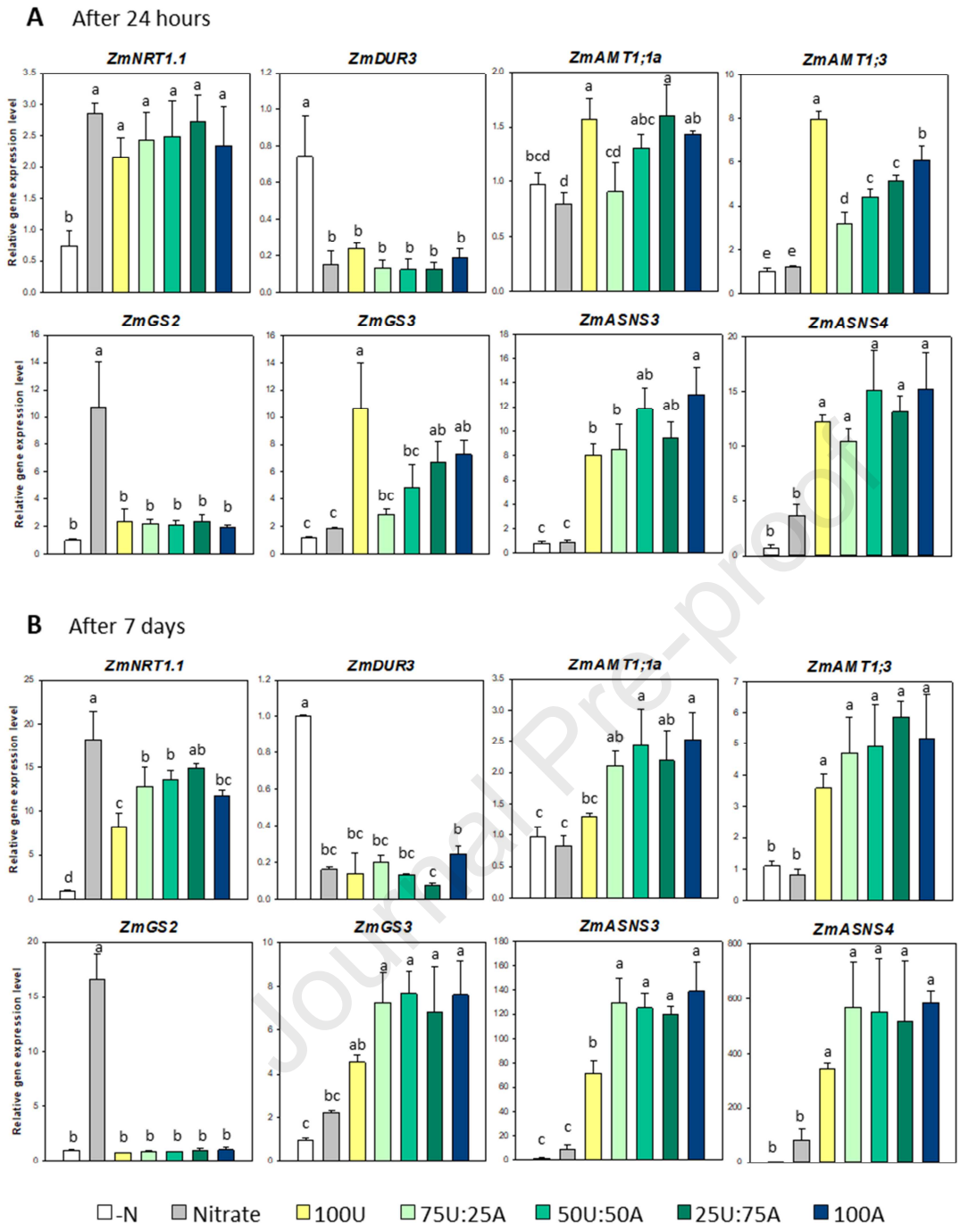

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DISCLOSURES

The authors have no conflict of interest to declare.