Physiological and molecular responses in tomato under different forms of N nutrition

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Abstract

Urea is the most common nitrogen (N) fertilizer in agriculture, due to its cheaper price and high N content. Although the reciprocal influence between NO3− and NH4+ nutrition are well known, urea (U) interactions with these N-inorganic forms are poorly studied. Here, the responses of two tomato genotypes to ammonium nitrate (AN), U alone or in combination were investigated. Significant differences in root and shoot biomass between genotypes were observed. Under AN + U supply, Linosa showed higher biomass compared to UC82, exhibiting also higher values for many root architectural traits. Linosa showed higher Nitrogen Uptake (NUpE) and Utilization Efficiency (NUtE) compared to UC82, under AN + U nutrition. Interestingly, Linosa exhibited also a significantly higher DUR3 transcript abundance. These results underline the beneficial effect of AN + U nutrition, highlighting new molecular and physiological strategies for selecting crops that can be used for more sustainable agriculture. The data suggest that translocation and utilization (NUtE) might be a more important component of NUE than uptake (NUpE) in tomato. Genetic variation could be a source for useful NUE traits in tomato; further experiments are needed to dissect the NUtE components that confer a higher ability to utilize N in Linosa

Keywords: Ammonium, Gene expression, Ion fluxes, nitrate, Tomato, Urea

1. Introduction

Nitrogen (N) is an essential constituent of many macromolecules, secondary metabolites and signaling compounds, it is required for plant growth and development. Sub-optimal N supply is frequently a major constraint for crop production, causing up to 50% yield loss (Jones et al., 2013; Iqbal et al., 2015). For this reason, large amounts of N fertilizer is applied to improve plant growth and yield (Glass, 2003; Good et al., 2004; Saraskeda et al., 2014) with an expected three-fold increase in application rate in the future (Good et al., 2004) and a negative impact on the environment as some is wasted. Ammonium (NH4+), nitrate (NO3−) and urea [CO(NH2)2] are the main N forms supplied to plant roots in fertilizers. However, urea is the most common N fertilizer used in agriculture worldwide, accounting for about 50% of the total world N fertilizer consumption (fao.org), due to its cheaper price and high N content (46% of mass). To optimize root uptake capacity of different N-forms from soils, plants have developed sophisticated mechanisms and strategies. A complex network, combining High- and Low-Affinity Transport Systems (HATS and LATS, respectively)

belonging to multigene families that operate over different concentration ranges, allows plants to maximize acquisition. In particular, NH4+ uptake by roots involves the Ammonium Transporter/ Methylammonium Permease/Rhesus (AMT/MEP/Rh) family (von Wirén and Merrick, 2004). Six AMT-type NH4+ transporters, from AMT1.1 to AMT1.5 and AMT2.1, belonging to AMT1 clade and MEP/ AMTB subfamily, respectively, were identified in Arabidopsis thaliana (Ludewig et al., 2001). The transporters, AMT1.1 and AMT1.3 operate for HATS and are localized in root epidermal and cortical cells (Kaiser et al., 2002; Loqué et al., 2006), whereas AMT1.2, which operates for LATS is localized in the root endodermal and cortical cells (Yuan et al., 2007). Nitrate, the main N form in many agricultural soils, is actively absorbed by specific transporters belonging to NPF/NRT1 and NRT2 families (Nacry et al., 2013; Léran et al., 2014). In Arabidopsis, transporters of NPF family have a low affinity for NO3−, except for NPF6.3 which displays dual affinity for NO3− in high and low affinity ranges (Liu and Tsay, 2003), showing also a role in NO3− sensing (Ho and Tsay, 2010). Among the seven genes of the Arabidopsis NRT2 family (Okamoto et al., 2003), NRT2.1 provides a major contribution to total HATS activity (Li et al., 2007) and it needs a partner protein called NAR2.1 for function (Tong et al., 2005; Okamoto et al., 2006; Orsel et al., 2006). Beside the inorganic N forms, plants are able to take up urea from the soil (Nacry et al., 2013; Mérigout et al., 2008). Transport systems in plant root cells for urea have been identified, and can be mediated by a DUR3 transporter and aquaporins (Kojima et al., 2007; Gu et al., 2012; Zanin et al., 2015; Liu et al., 2015; Yang et al., 2015). Uptake of some nutrients by roots requires energy to overcome the negative electrical potential across plasma membrane of root epidermal and cortical cells (Zanin et al., 2015; Miller et al., 2001; Ludewig et al., 2002), which is provided by the activity of plasma membrane H+-ATPase (PM H+-ATPase), a key enzyme in plant nutrition (Palmgren, 2001). Under urea fertilizer treatment, plant roots are concurrently exposed for a short period to urea, NH4+ and then NO3− (Mérigout et al., 2008). Urea is hydrolyzed into NH4+ by urease and both plants and microorganisms have this enzyme (Sirko and Brodzik, 2000; Witte et al., 2002; Watson et al., 1994), with subsequent microbial nitrifica- tion into NO3−. Although the interactions between NO3− and NH4+ in uptake and assimilation are well known, urea interactions with these inorganic N forms are poorly studied. Urea inhibits NH4+ and NO3− uptake in wheat, while NO3− can induced urea acquisition (Criddle et al., 1988). On the other hand, Mérigout et al. (2008) demonstrated that urea exerted a repressive effect on NO3− influx, while enhancing NH4+ uptake in Arabidopsis plants, interfering also with genes-related to the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. More recently, an up-regulation of some genes related to NO3− transport (NRT2) was observed in maize, when roots were exposed concurrently to urea and NO3−, thereby increasing NUE (Zanin et al., 2015). Unraveling the physiological and molecular basis of how plants sense and respond to changes in the availability of different N forms should enable the development of new strategies to increase NUE.

To date, there is limited information concerning the physiological and molecular responses of tomato plants and the reciprocal influence of these different N-forms reported. In this study, the responses of two tomato genotypes exposed to different N-forms (NH4+- NO3−, urea and their combination) has been investigated using morphological, electro- physiological and molecular approaches. Urea is already used in combination with other N forms in fertilizer and is commonly called UAN (Urea Ammonium Nitrate) fertilization; for this work, we investigate if it may be useful to improve NUE in tomato crops.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato seeds of UC82 and Linosa genotypes (kindly supplied by the Tomato Genetics Resource Center-Department of Plant Sciences, University of California Davis and the University of Palermo, Italy, respectively) were sterilized and then germinated as reported by Abenavoli et al. (2016). After 7 days, seedlings with uniform size were selected and transferred to pots (4.3 L, four seedlings per pot) contain- ing a following aerated hydroponic solution: 1 mM NH_4NO_3, 0.5 mM $CaSO_4$, 0.2 mM KH_2PO_4, 0.3 mM $MgSO_4$, 13.3 μM H_3BO_3, 3 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.1 μM Na_2MoO_4, 2 μM NaCl, 0.01 μM $CoCl_2$, 0.1 μM $NiSO_4$, 20 μM Fe-EDTA. The pH of the nutrient solution was adjusted to 5.8 with 1 M KOH. Tomato seedlings were then placed in a growth chamber maintained at 25 °C, 70% RH and 16 h photo- period with a light intensity of 350 μmol $m^{-2}s^{-1}$ for a further week. Tomato seedlings (14 d-old) were then transferred to the same nutrient solution containing 1 mM NH_4NO_3 (AN), 1 mM Urea (U) or 0.5 mM NH_4NO_3 plus 0.5 mM Urea (AN + U) for further 7 days. Nutrient solution was renewed every two days, thereby the same nutrient supply concentration was maintained throughout the experiment.

2.2. Root morphology measurements

Five tomato seedlings (21-d old), for each treatment and genotype, were collected and divided into roots and shoots. Roots were then stained using 0.1% (w/v) toluidine blue (Sigma-Aldrich, #89640) to improve the contrast during the scanner data acquisition. Then, stained roots were placed on the scanner, and an image was captured at 1200 dots per inch (dpi) of resolution. The total root length (TRL, cm), root volume (cm^3), root area (cm^2) were measured using WinRhizo Pro system v. 2002a software (Instruments Régent Inc., Quebec, Canada), and lateral root number was counted manually from the image (Lupini et al., 2014). Furthermore, roots and shoots were dried at 72 °C for 48 h to determine their dry weights (RDW, g and SDW, g). Finally, root length ratio (RLR, root length/whole dry weight, cm g^{-1}), root mass ratio (RMR, root dry weight/whole dry weight, g g^{-1}), specific root length (SRL, root length/root dry weight, cm g^{-1}), root fineness (RF, root length/root volume, cm cm^{-3}), and root tissue density (RTD, root dry weight/root volume, g cm^{-3}) were calculated as reported pre- viously (Lupini et al., 2016).

2.3. Nitrogen content and nitrogen use efficiency calculation

At the end of the treatments with different N-forms, UC82 and Linosa seedlings were collected and divided into roots and shoots to determine N content by dry combustion. Briefly, plant material (0.25 g) was maintained in oven at 72 °C for 4 days, to obtain a homogenized powder. Finally, N determination (mg kg^{-1} dry matter) was performed using LECO CN628 instrument (LECO Corporation). N values were used to estimate NUE based on different definitions as reported by Abenavoli et al. (2016). In particular, Total N Accumulation (TNA), calculated as the N concentration x total plant dry weight (mg N) (Lawlor, 2002); Nitrogen Efficiency Ratio (NER), calculated as the total plant dry weight divided by TNA (g TDW mg^{-1} N) (Gabelman and Gerloff, 1983); Nitrogen Utilization Efficiency (NUtE), calculated as the total plant dry weight divided by N concentration (g^2 TDW mg^{-1} N) (Siddiqi and Glass, 1981) and Nitrogen Uptake Efficiency (NUpE), calculated as TNA divided by root dry weight (mg N g^{-1} RDW) (Elliot and Lauchli, 1985), were determined.

2.4. Membrane potential measurements

All the electrophysiological measurements were performed on intact primary root cells of tomato seedlings (21-d old) at 1 cm from the tip, and previously grown in hydroponic nutrient solution containing 1 mM NH4NO. Tomato seedling were placed in a Plexiglass chamber and perfused with a basic buffer solution containing 0.5 mM CaCl2, 0.2 mM KCl, 1 mM MES-NaOH (pH 6), before performing the electrode impale- ment of roots as previously described (Miller et al., 2001; Abenavoli et al., 2016; Lupini et al., 2010). Membrane electrical potentials were measured with glass single-barreled microelectrodes back-filled with 200 mM KCl using a 70 mm long Microfil needle (World Precision Instruments Inc., Hitchin, UK). The reference salt bridge was filled with 200 mM KCl in 2% agar and placed in the perfusion chamber close to the root. During each measurement, the perfusion solution was a unbuffer solution containing 0.5 mM CaCl2, 0.2 mM KCl, and 0.5 mM NH4NO3 (pH 6 with NaOH), for 15 min. Thereafter, 0.5 mM urea was added and the cell membrane potential was recorded for further 15 min.

2.5. Ion fluxes measurements

In tomato seedlings (21 days old), previously grown in hydroponic nutrient solution containing 1 mM NH4NO3, net fluxes of NH4+, NO3− and H+ were concurrently measured by using a non-invasively vibrat- ing ion-selective electrode or MIFE technique (University of Tasmania, Hobart, Australia), according to Shabala et al. (1997), Shabala (2000). Briefly, borosilicate glass capillaries were pulled and dried in oven at 220°C overnight to dehydrate and render them hydrophobic by addition of silinization solution I (85126, Sigma). Cooled microelec- trodes were backfilled with 200 mM NH4Cl, 500 mM KNO3 plus100 mM KCl or 15 mM NaCl plus 40 mM KH2PO4 (adjusted to pH 6.0 using 0.1 M NaOH) for NH4+, NO3− or H+, respectively. Electrode tips were then filled with commercial H+ (Fluka n. 95297) or NO3− (Miller et al., 2001) or NH4+ (Wells and Miller, 2000) cocktail solutions. The reference electrode was a plastic tube containing 1 M KCl in 2% (w/v) agar. Before using, electrodes were calibrated against a range of standards (pNO3 or pNH4 from 1 to 5 and pH from 5 to 7.2). Electrodes with responses less than 50 mV/pIon were discarded. Tomato seedlings were equilibrated in the measuring solution for 10 min in a Plexiglas chamber containing 4 mL of 0.5 mM CaCl2, 0.2 mM KCl, 1 mM MES- NaOH (pH 6) plus 0.5 mM NH4NO3. Electrode tips were positioned close to the apical root tip (10 mm from tip) and 10 μm above the root surface and connected to a computer-controlled stepper motor which gently moved between two positions (with a distance of 30 μm) at a frequency of 0.1 Hz. The CHART software (Shabala et al., 1997; Newman, 2001) recorded the potential differences between the two positions and converted them into electrochemical potential differences using the calibrated Nernst slope of the electrodes. Ion fluxes were then calculated by using the MIFEFLUX software for cylindrical diffusion geometry (Newman, 2001). NH4+, NO3− and H+ fluxes were recorded before and after 0.5 mM urea supply for 15 min.

2.6. Gene expression analysis

Total RNA was isolated from roots of UC82 and Linosa genotypes (21-d old) grown in hydroponic culture and exposed to 1 mM NH4NO3, 1 mM urea or 0.5 mM urea and 0.5 mM NH4NO3− for 7 days. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Milano, Italy) according to the manufacturer's protocol and its quality and quantification were assayed using a NanoDrop 2000 (Thermo Scientific).

A first-strand cDNA was synthesized from 2 μg of total RNA (Tetro cDNA synthesis kit), using oligo-dT primers as suggested by the Bioline manufacturer. The real-time PCR (qPCR) was performed on DNA Engine Opticon2 (Bio Rad) using SYBR Green master mix kit (Sigma- Aldrich) according to the manufacturer's instructions. The qPCR were carried out starting from 2 min at 95 °C (initial denaturation), then for 40 cycles consisting of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. Three biological replicates were performed for each N-form and genotype. Specific primers for SlNPF6.3 (formerly SlNRT1.1 accession number: X92853), SlNTR2.1 (accession number: NM001279334), SlNAR2.1 (XM004236225), SlAMT1.1 (X92854), SlAMT1.2 (2065194), SlDUR3 (XM004245951), SlLHA1 (NM001247846), and SlLHA8 (AF263917), tomato ubiquitin and actin genes (accession numbers: TC193502 and TC194780, respectively), the latter two were used as internal standards and were designed to amplify the expected size fragments (Supplemental Table 1). The qPCR results were analysed by the $2^{-\Delta Ct}$ comparative method as previously described (BioRad Real-time PCR Application guide) (Livak and Schmittgen, 2001). Based on the logarithmic fluorescence graph, the fitting threshold was chosen by calculating Ct using the 7000 System SDS software (RQ Study Application, Applied Biosystems). This method can detect relative changes in gene expression, where ΔCt is the difference in threshold cycles for target (Ct sample) and reference (Ct ubiquitin) genes. The Ct of each sample was normalized to ubiquitin for accounting the variability in the original concentration and quality of the total RNA, and the conversion efficiency of the reverse transcription reaction.

2.7. Statistical analysis

All the tomato experiments were set up in a completely randomized design with at least five replications for each. All data were checked for normality (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Leven median test). The data were analyzed by one-way ANOVA, and means were separated by Tukey's honest significant difference (HSD) test ($p < 0.05$), using Systat software (Systat Software Inc., Chicago, IL, USA).

3. Results

3.1. Plant growth and NUE definitions

As biomass production represents the summary response in a plant's capacity to transform minerals to organic compounds, it was measured as a first approach. Root and shoot biomass, expressed as RDW and SDW, were affected by different N supply forms and genotypes (Fig. 1). In particular, Linosa under AN or AN + U supplies exhibited either higher RDW by 56% and 222%, respectively, or higher SDW by 58% and 216%, respectively, when compared to UC82. The cultivar UC82 did not show any significant differences in root or shoot biomass when supplied with the different N supply forms (Fig. 1A–B). Both genotypes exposed to U did not show any significant differences in both root and shoot dry weights (Fig. 1A–B).

Applying different common definitions, NUE in UC82 and Linosa genotypes supplied with different N-forms was calculated (Fig. 2). The TNA did not show any significant differences between genotypes in all N-treatments ($p = 0.97$), although under AN + U supply, both geno- types exhibited significantly higher TNA values ($p < 0.001$) compared to AN and U (Fig. 2A). The NER was similar

in both genotypes under all N-forms supply (Fig. 2B). Conversely, NUtE was significantly increased in Linosa by 90% and 36% under AN and AN + U treatments, respectively compared to UC82 ($p = 0.046$) (Fig. 2C). Finally, Linosa showed a significantly higher NUpE values under AN + U supply compared to UC82 (Fig. 2D), whereas no differences between genotypes were observed in U or AN supplies (Fig. 2D).

3.2. Root morphology

Root morphological analysis was performed for both genotypes grown with different forms of N supply (Fig. 3). Total Root Length (TRL, cm) did not show any significant difference in AN and U supplies between genotypes, by contrast, under AN + U supply, Linosa exhib- ited higher TRL by 343% compared to UC82 (Fig. 3A). A similar trend was observed in lateral root length between genotypes under different treatments (Fig. 3B). In addition, U supply significantly increased lateral root number and lateral root density in both tomato genotypes (Supplemental Fig. 1).

The RLR, RMR, RF and RTD components were also examined (Fig. 3C–F). UC82 was characterized by a significant increase in RLR and RMR under AN and U supplies when compared to Linosa (Fig. 3C,D). Root finesses (RF) was strongly affected by the N supply form (Fig. 3E). In particular, UC82 exhibited higher RF compared to Linosa (102%) under AN supply, whereas the presence of urea (U), alone or in combination to AN, overturned this pattern increasing RF by 136% and 114%, respectively, in Linosa compared to UC82 (Fig. 3E). Conversely, RTD did not show any significant difference among treatments and genotypes (Fig. 3D).

3.3. Membrane potential and ion flux measurements

Electrical membrane potential responses to different N-forms supplies were measured in primary roots of intact UC82 and Linosa seedlings (Fig. 4). In all treatments, a rapid depolarization (less negative membrane potential) followed by a hyperpolarization (more negative membrane potential) was recorded. After AN perfusion for 15 min, both genotypes did not show any significant difference in depolarization (12 vs. 13 mV, in Linosa and UC82, respectively) (Fig. 4A). The addition of U in the perfusion solution generated a similar further increase in membrane depolarization in both genotypes (Fig. 4A). By contrast, Linosa exhibited higher hyperpolarization under AN and AN + U treatments compared to UC82 (Fig. 4B).

Nitrate, NH_4^+ and H^+ fluxes were simultaneously measured around primary roots (10 mm from the tip) of tomato genotypes for 15 min under AN condition, and for further 15 min after U supply (AN + U) (Fig. 5). Similar NO_3^- and NH_4^+ influxes were observed in both genotypes under AN supply, whereas a significant increase in NO_3^- and NH_4^+ influxes was recorded in Linosa compared to UC82 after the U addition into perfusion solution (Fig. 5A–B). No significant difference in H^+ efflux between genotypes was observed (Fig. 5C), but the U addition caused an increased H^+ efflux in Linosa (59 vs. 21 mmol m^{-2} s^{-1}), compared to UC82 (Fig. 5C).

3.4. Gene expression analysis

The transcript levels of N-related genes in tomato seedlings growing under different N-forms (AN, U or AN + U) were measured (Fig. 6). In particular, the expression of genes for primary N acquisition,

such as NPF and NRT (NO_3^- transporter families), AMT (NH4+ transporter family), DUR (urea transporter family) and LHA (proton pumps) were examined. The transcript level of AMT1.1 gene was significantly higher in UC82 than Linosa, under AN treatment (Fig. 6A). By contrast, in presence of U a similar response between genotypes was observed, while the concurrent supply of AN + U severely reduced the transcript level in UC82, but not in Linosa (Fig. 6A). At all N-forms, AMT1.2 expression resulted significantly higher in UC82 compared to Linosa; the presence of U, alone or in combination, increased the AMT1.2 transcript levels in both genotypes (Fig. 6B). Transcript abundance of DUR3 was similar in both genotypes under AN or U, whereas in the simultaneous presence of AN + U there was a significantly higher transcript level in Linosa compared to UC82 (Fig. 6C). The NPF6.3, NRT2.1 and NAR2.1 transcript levels in response to different forms of N supply were also analyzed (Fig. 6D–F). Although under AN supply, the NPF6.3 transcripts were not different between genotypes, the U supply, alone or in combination, up-regulated gene expression in both genotypes, whereas Linosa exhibited a significant higher transcript levels compared to UC82 (Fig. 6D). A similar pattern was observed in NRT2.1 and NAR2.1 gene expression, except with AN treatment where UC82 showed higher NRT2.1 expression compared to Linosa (Fig. 6E–F).

Furthermore, under AN supply both tomato genotypes did not show any difference in LHA1 expression, while a higher LHA8 transcript abundance was observed in UC82 (Fig. 6G–H). With U supply, alone or in combination with AN, there was a marked down-regulation of both proton pump genes in UC82 compared to Linosa (Fig. 6G–H). It is noteworthy that Linosa did not show any significant difference in both LHA1 (Fig. 6G) and LHA8 transcripts abundance (Fig. 6H) when supplied with all of the different N forms.

4. Discussion

Crops growing under field conditions can be supplied with many different N-forms (NO3−, NH4+ and urea) and concentrations, which frequently limit plant growth and yield (Kant et al., 2011). The pathways by which the different N-supplies are transferred to the plant are complex. The excessive application of N fertilizers has led to increases in crop production, but causing at the same time environ- mental pollution (Ding et al., 2015). Indeed, crop plants are only able to acquire 30–40% of all the N fertilizer applied (Raun and Johnson, 1999), while the remaining N is immobilized in organic matter or adsorbed to the soil matrix, and/or lost by NO3− leaching, denitrifica- tion from the soil and loss of ammonia to the atmosphere, causing deleterious environmental effects (Glass, 2003; Vitousek et al., 1997). Hence, understanding how crops respond at physiological, morpholo- gical and molecular levels to different N-forms is important for breeding new cultivars with high nitrogen use efficiency (NUE) and minimizing the agriculture environmental impacts. Here, the responses to different N supply forms of two tomato genotypes, UC82 and Linosa, each having very different geographical origins was compared. The contrasting backgrounds of these two genotypes is likely to have resulted in different specific adaptation strategies to N-limited conditions (Mercati et al., 2015). The results indicated that the tomato genotypes showed contrasting behavior in terms of plant biomass allocation in response to different N- supply forms. An exception to this pattern occurs when supplied with U as the sole N source and then both genotypes behave similarly. Linosa exhibited more growth than UC82 when supplied with AN, which was further increased with U addition. Similar results were also reported in maize (Zanin et al., 2015; van Beusichem and Neeteson, 1982), wheat (Bradley et al., 1989), tomato (Kirkby and Mengel, 1967;

Ikeda and Tan, 1998) and Arabidopsis where the higher biomass under AN + U supply seemed to be correlated to a higher total nitrogen accumulation (TNA) (Mérigout et al., 2008). Conversely, comparing Linosa and UC82 genotypes no difference was observed in TNA. Since biomass production is closely correlated with NUE (Fan et al., 2007), other different NUE definitions can be compared, and in particular, N uptake (NUpE) and utilization efficiency (NUtE), and N efficiency ratio (NER) (Good et al., 2004; Xu et al., 2012). The results indicated that Linosa was characterized by both higher NUpE and NUtE than UC82, when exposed to AN + U with increased biomass allocation. Thus, root morphological, electrophysiological and molecular analysis in response to different N supply forms were assessed to unravel key traits responsible for the NUE differences between tomato genotypes. Linosa exhibited higher lateral root length and root finesses when supplied with AN + U, as already reported in tomato (Kirkby and Mengel, 1967). Recently, Esteban et al. (2016) demonstrated that both AN and U supplies severely affected the root architecture resulting in changes in the main root elongation rate, lateral root development, and position from the root base. The authors suggested that the indole-3-acetic acid pool is an important component of the root response of M. truncatula under AN or U as the sole N source. The involvement of various possible signals in N supply lateral root responses, including ABA, auxin and CKs in different species has been already reviewed (Kiba et al., 2011).

Although a full comparison of N supply forms, including U and linking these to specific hormonal effects on root architecture is needed. Zanin et al. (2015) observed that U promoted whole maize root development, showing a significant increase in the maximum number of roots, area, perimeter, surface area, and length. They further analysed plants fed with other N sources, such as AN, NO_3^-, AN + U or NO_3^- + U demonstrating that AN supply strongly reduced most root morphology parameters. On the other hand, NO_3^- slightly stimulated the develop- ment of the root system, when it was applied with U the highest morphometric changes were recorded (Zanin et al., 2015). The greatest increase in root length observed in Linosa was also accompanied by an increase in root fineness, a structural parameter, allowing an efficient soil exploitation and nutrient acquisition with subsequent higher crop productivity (Postma et al., 2014). Plants respond to uneven nutrient supply by modulating lateral root development as well as N uptake systems (Forde and Walch-Liu, 2009; Kiba and Krapp, 2016). For this reason, in the present paper, NO_3^-, NH_4^+ and proton fluxes were analysed, before and after U application in tomato roots exposed to AN. Before U application, tomato genotypes did not show any differences in NO_3^-, NH_4^+ and proton fluxes, but after U addition, Linosa increased NO_3^- and NH_4^+ influxes, accompanied by an increase of proton efflux compared to UC82. The increase in NO_3^- and NH_4^+ uptake observed here are in contrast with that observed in Arabidopsis (Mérigout et al., 2008). These contrasting results may be due to species differences or the use of the more sensitive ion-selective microelectrode technique that overcomes many limits for the measurements, such as low time resolution, sensitivity and spatial resolution (Shabala, 2006).

Gene expression in tomato roots was investigated by RT-PCR to elucidate how N-related gene transcripts can differ between genotypes. The analysis of NH_4^+-related genes revealed that AMT1.2 was more expressed in UC82 compared to Linosa at all N-forms supplied. Therefore, the increase of NH_4^+ influx in Linosa exposed to AN + U was not correlated with AMT1.2 transcript level. Higher expression of AMT1.1 in UC82 compared to Linosa was confirmed only when AN was supplied; by contrast, a higher level of transcript was shown in Linosa when supplied with U, alone or in combination with AN. This inverted trend appeared to be correlated with the different NH_4^+ fluxes between genotypes, and was already reported in previous results, where different N-supply and/or N nutritional status affected NH_4^+ uptake in Arabidopsis (Gazzarrini et al., 1999) and tomato (von Wirén et al., 2000). Although AMT genes expression is up-regulated by N limitation (high affinity

system regulation) in Arabidopsis, our results confirmed the NH4+ −inducible AMT expression as already reported in tomato, rice, and maize (von Wirén et al., 2000; Sonoda et al., 2003; Gu et al., 2013). Hence, the addition of U supply and its interaction with NH4+ −uptake deserve further investigation, but as post-translational regula- tion of NH4+ uptake can be important (Jacquot et al., 2017) we may not expect to see a good correlation between the measured NH4+ uptake and AMT expression.

The expression of DUR3 was down-regulated by AN supply, as previously observed in oilseed rape and rice (Arkoun et al., 2012; Wang et al., 2012), in contrast an up-regulation was observed when U is the sole N-form in the nutrient solution in both tomato genotypes, as already observed in Arabidopsis (Kojima et al., 2007) and maize (Zanin et al., 2015), whereas a different response between genotypes was exhibited in the concurrent supply with AN + U indicating that there may be a role for cytokinin in this response as treatment with this hormone down-regulated the expression of DUR3 in Arabidopsis (Kiba et al., 2011).

For NO3− influx, the expression of the main genes related to NO3− uptake at low (NPF6.3) and high affinity (NRT2.1) and its related accessory protein (NAR2.1) were evaluated.

In the presence of AN, NPF6.3 showed a very low level of transcript abundance in both genotypes, while no significant differences between genotypes were observed for NAR2.1, and this result need further investigation, suggesting the NAR2 may have other functions. In presence of U, Linosa showed an up-regulation of all three genes in agreement with observations in maize, where U supply increased either NO3− uptake or gene-related expression (Zanin et al., 2015). By contrast, the same genes appeared much less up-regulated in UC82 compared to Linosa after U addition to AN, and this might be explained by a negative feedback mechanism, as already reported in oilseed rape (Arkoun et al., 2012). It is noteworthy that these differences between tomato genotypes could reflect the higher NUtE observed in Linosa. Indeed, the transcriptional up-regulation of the NH4+, NO3− and U transporter genes in Linosa when concurrently supplied with AN + U are likely to affect the glutamine pools or other organic N forms in the root that may be important signals for the N status of the plant (Rawat et al., 1999; Vidmar et al., 2000; Nazoa et al., 2003; Fan et al., 2006). Furthermore, Linosa may show a lower N concentration in the roots due to its higher ability to better assimilate these mixed forms of N supply, supported by a significantly higher NUtE when supplied with AN + U, compared to UC82 (see Fig. 2). Measurements of the activity of key assimilatory enzymes such as nitrate reductase and GS-GOGAT under AN + U nutrition may identify differences between Linosa and UC82 to support this idea.

Finally, taking into account the energy required for uptake of NH4+, NO3− and U, provided by pm H+-ATPase activity, we checked if the expression of LHA1 and LHA8, two genes involved in proton pumping activity (Ewing and Bennett, 1994; Kalampanayil and Wimmers, 2001) was changed. The results did not show any significant decrease in transcript abundance in Linosa, among treatments for both LHA isoforms, by contrast a significant down-regulation was observed in UC82 when U was added in the nutrient solution. The decreased expression of pm H+-ATPase in UC82 could result in less energy supply for uptake, and this will influence all pm transporters. A decreased proton flux was measured 15 min after U addition in UC82, but an increase was observed for Linosa (Fig. 5C). The 15 min time scale for these changes is fast for gene expression changes, but the proton pump activity may be regulated both post-translationally and at the transcript level (Haruta et al., 2015). Furthermore, UC82 exhibited a higher LHA8 transcript level than Linosa when AN was supplied, but the concurrent presence of U in the nutrient solution changed this pattern indicating that LHA8 expression is triggered specifically by U treatment in combination with AN. Thus, the gene

expression supported the main- tenance of a higher proton pump activity in Linosa after U or AN + U supply, confirmed also by a significant H+ extrusion (Fig. 5C) and a larger cell membrane hyperpolarization (Fig. 4B) resulted in a higher NO3− and NH4+ fluxes (Fig. 5A,B) as well as a more competitive ability to N utilization in Linosa compared to UC82. The interesting result is the contrasting behavior of the two genotypes and sequence compar- isons of the LHA isoforms and their promoters for the two tomato genotypes may identify some key regulatory regions.

In conclusion, morphological, electrophysiological and molecular analysis has shown different responses to AN and U supply, alone or in combination, in two tomato genotypes, underlying that several traits play important roles in NUE, including root morphology and gene expression changes. The beneficial effect of AN + U nutrition could provide a basis for better crop agronomy to improve the fertilization practice for more sustainable agriculture. The comparison between tomato genotypes from two contrasting origins highlighted genetic variation that could be a source of useful traits to improve NUE. In fact, Linosa belongs to long storage type of tomato, which is often cultivated in Mediterranean regions characterized by drought and N-limited conditions (Abenavoli et al., 2006). By contrast, the UC82 genotype was selected under non N-limited conditions (University of California) resulting in low NUE.

Despite the obvious importance for NUE to consider the whole life cycle including fruiting, the present work on vegetative growth could represent a starting point facilitating the selection of cultivars for improved NUE.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the

online version, at http://dx.doi.org/10.1016/j.jplph.2017.05.013.

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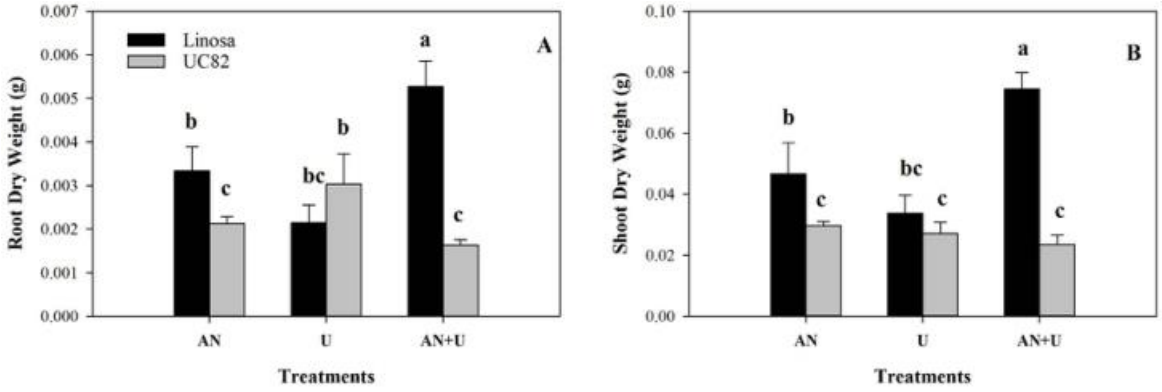

Fig. 1. Root (A) and Shoot Dry Weight (B) in two tomato genotypes (Linosa and UC82) exposed to different N-forms (AN = 1 mM NH4NO3; U = 1 mM Urea; AN + U = 0.5 mM NH4NO3 + 0.5 mM Urea). The values are presented as mean ± SE (n = 8). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$

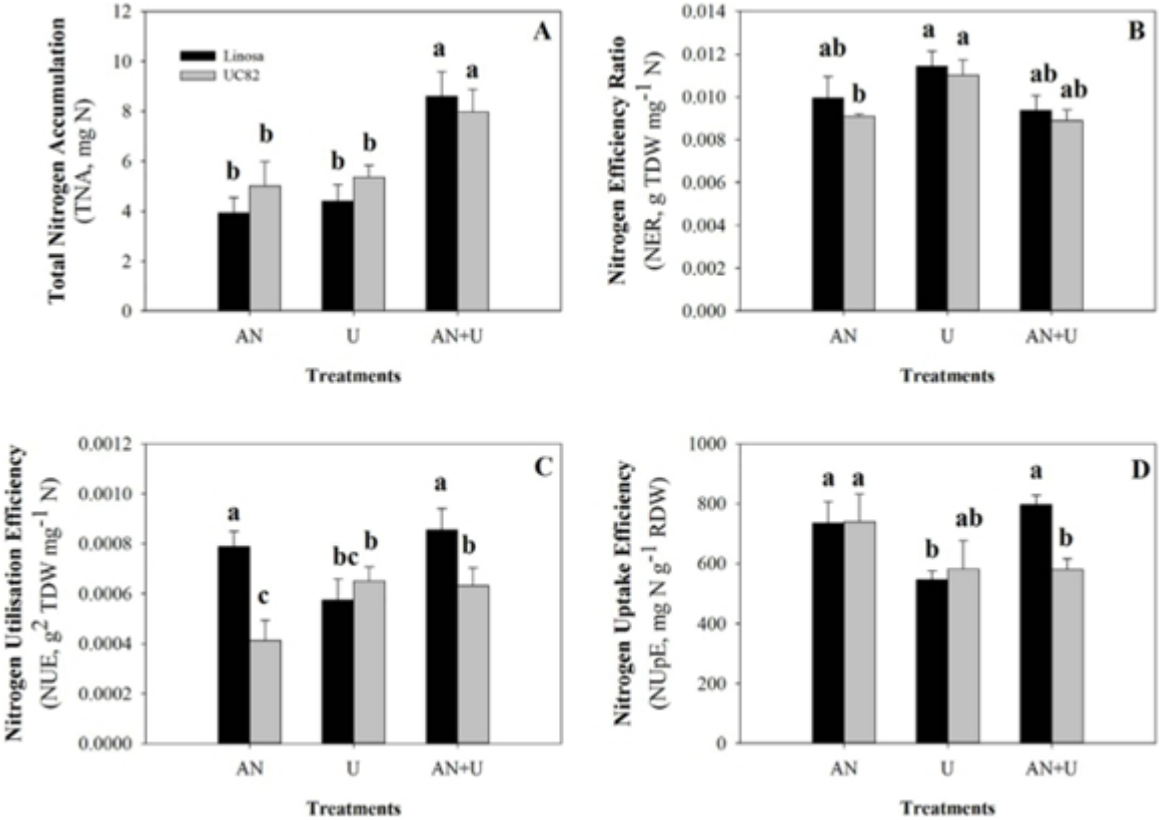

Fig. 2. (A) Total N accumulation (TNA), (B) N efficiency ratio (NER), (C) N utilization efficiency (NUtE) and (D) N uptake efficiency (NUpE) of two tomato genotypes (Linosa and UC82) exposed to different nitrogen form (AN = 1 mM NH4NO3; U = 1 mM Urea; AN + U = 0.5 mM NH4NO3 + 0.5 mM Urea). The values are presented as mean ± SE (n = 8). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$

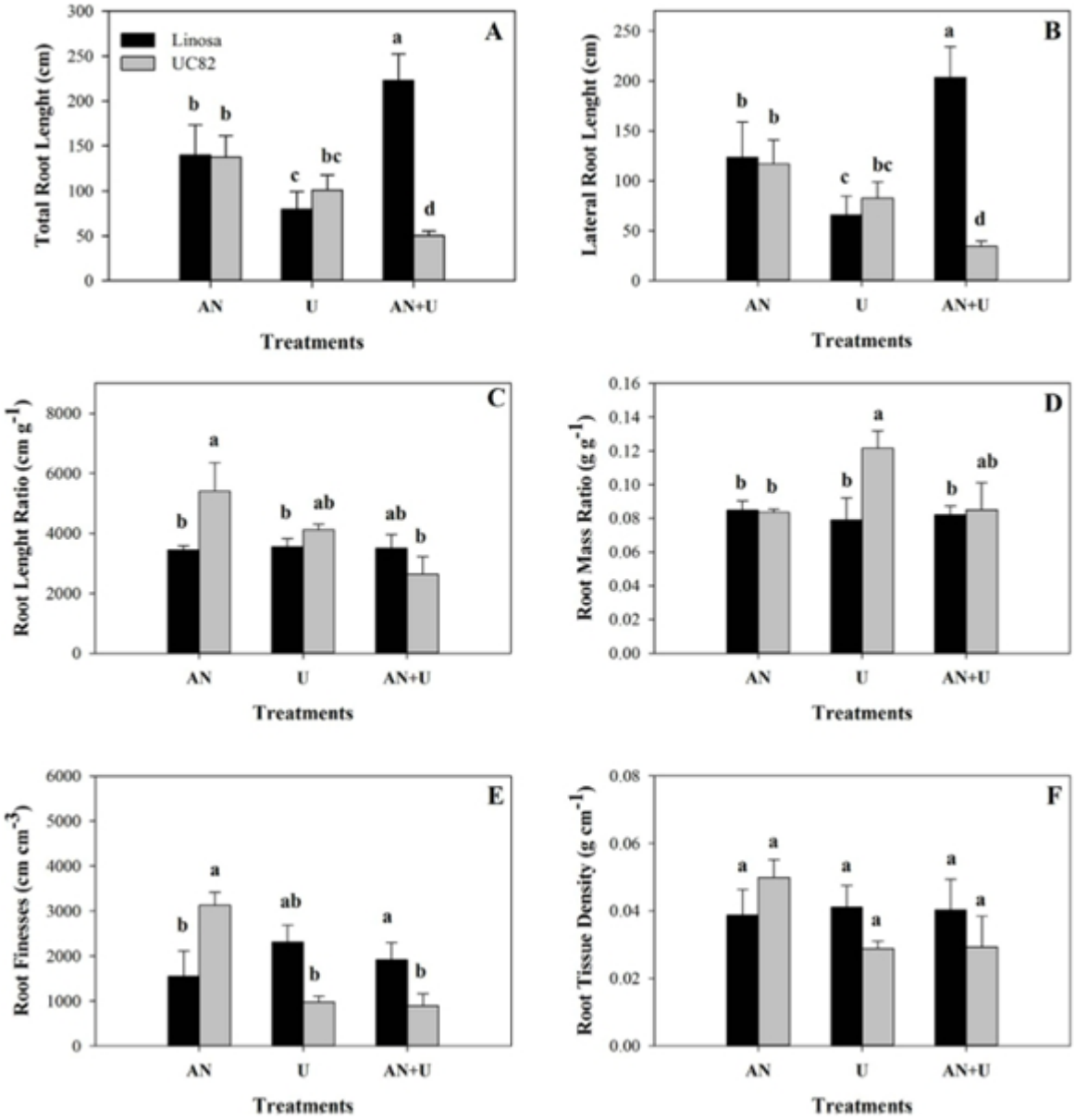

Fig. 3. Total root length (A), Lateral root length (B), Root length ratio (C), Root mass ratio (D), Root finesses (E) and Root tissue density (F) of two tomato genotypes (Linosa and UC82) exposed to different nitrogen form (AN = 1 mM NH4NO3; U = 1 mM Urea; AN + U = 0.5 mM NH4NO3 + 0.5 mM Urea). The values are presented as mean ± SE (n = 8). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$

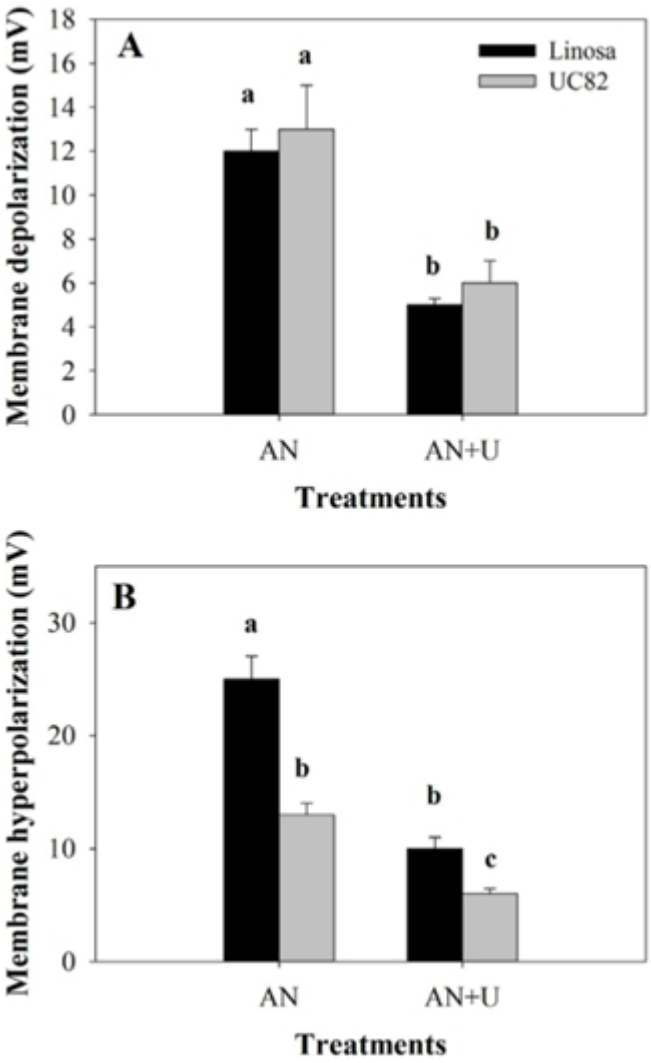

Fig. 4. Membrane depolarization (A) and hyperpolarization (B) in two tomato genotypes (Linosa and UC82) perfused with 0.5 mM NH4NO3 (AN) for 15 min. After, 0.5 mM Urea was added (AN + U) and the membrane potential was recorded for a further 15 min. The values are presented as mean ± SE (n = 15). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$.

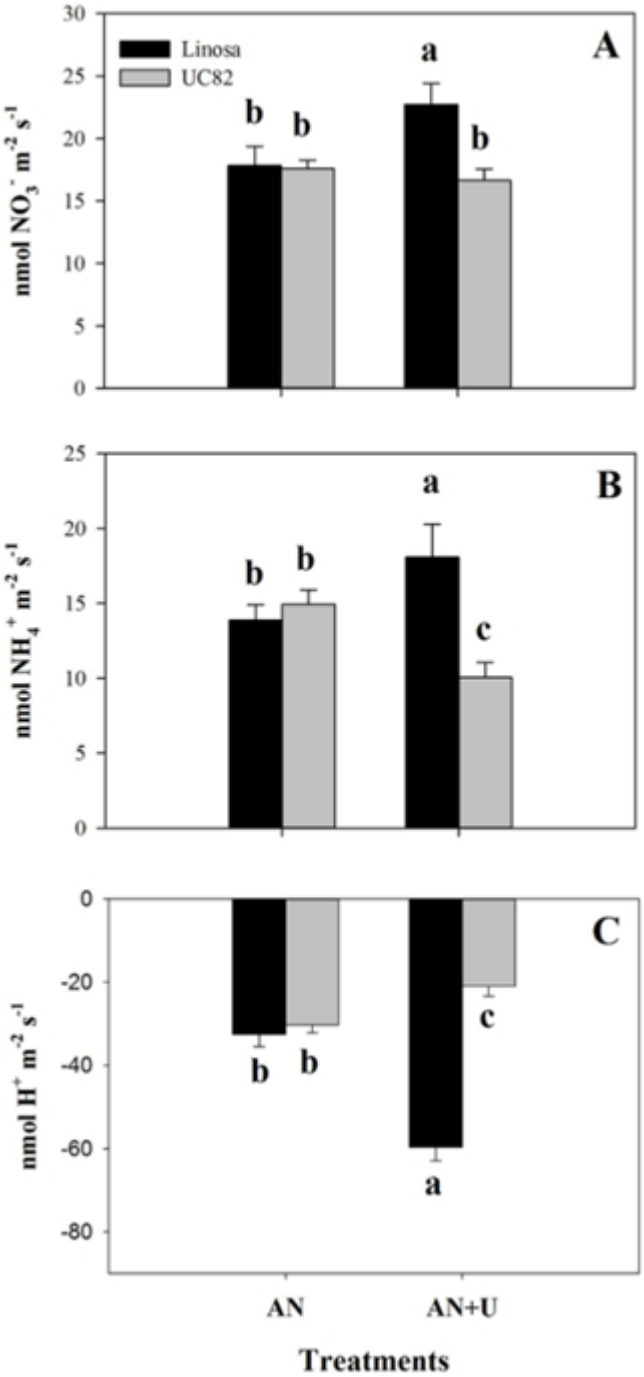

Fig. 5. Nitrate (A), ammonium (B) and proton fluxes (C) in two tomato genotypes (Linosa and UC82) perfused with 0.5 mM NH4NO3 (AN) for 15 min. After, 0.5 mM Urea was added (AN + U) and the ion fluxes were recorded for a further 15 min. The values are presented as mean of the 15 min SE (n = 15). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$.

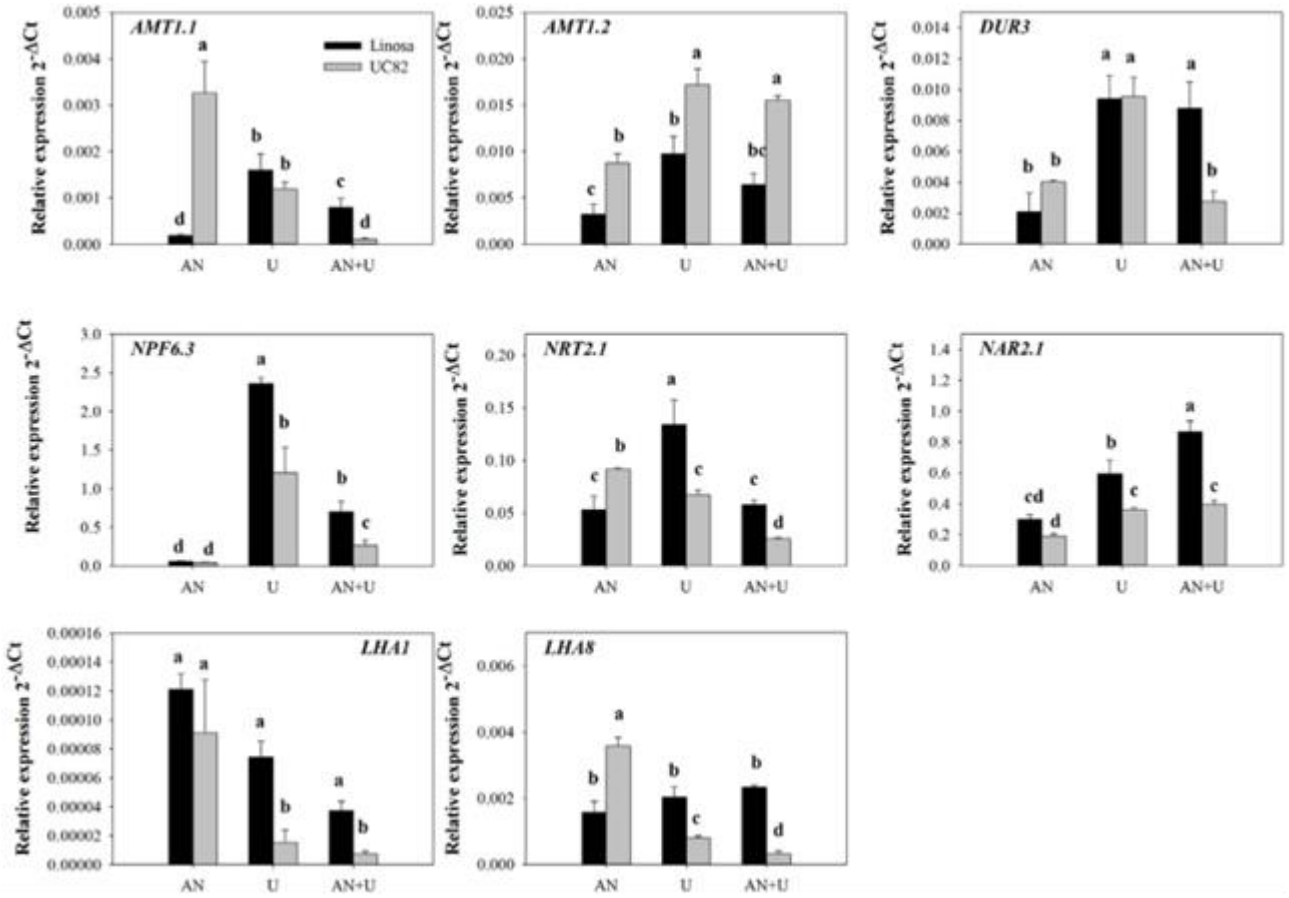

Fig. 6. Gene expression patterns of some N transport genes in two tomato genotypes (Linosa and UC82) exposed to different N form (AN = 1 mM NH4NO3; U = 1 mM Urea; AN + U = 0.5 mM NH4NO3 + 0.5 mM Urea). The values are presented as mean ± SE (n = 5). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p < 0.05$.