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New insights into N-utilization efficiency in tomato (*Solanum lycopersicum* L.) under N limiting condition

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Abstract

Understanding Nitrogen Use Efficiency (NUE) physiological and molecular mechanisms in high N demanding crops has become decisive for improving NUE in sustainable cropping systems. How the Nitrogen Utilization Efficiency (NUtE) component contributes to the NUE enhancement under nitrate limiting conditions in tomato remains to be elucidated. This study deals with the changes in several important nitrate metabolism related gene expressions (nitrate assimilation, transport, remobilization and storage/sequestration) engendered by short and long-term limiting nitrate exposure in two selected NUE-contrasting genotypes, Regina Ostuni (RO) and UC82, efficient and inefficient, respectively. At short-term, nitrate limiting supply triggered higher *SlCLCa* and *SlNRT1.7* expressions in RO root and shoot, respectively, suggesting a higher nitrate storage and remobilization compared to UC82, explaining how RO withstood the nitrate deficiency better than UC82. At long-term, nitrate reductase (*SlNR*) and nitrite reductase (*SlNIR*) expression were not significantly different between nitrate treatments in RO, while significantly down-regulated under nitrate limiting treatment in UC82. In addition, *SlCLCa* and *SlNRT1.8* transcript levels were significantly lower in RO, while those of *SlNRT1.5* and *SlNR* appeared significantly higher. This suggested that the efficient genotype stored less nitrate, which was allocated and assimilated to the shoot compared to

UC82. More interestingly, the expression of *SlNRT2.7* was significantly higher in RO shoot compared to UC82 and strongly correlated to RO higher growth as well as to NUE and NUtE component. Our findings underlined the differential regulation of N-metabolism genes that may confer to NUtE component a pivotal role in NUE enhancement in tomato.
Keywords: Nitrogen deficiency, NUtE, nitrate transport, nitrate assimilation, nitrate remobilization, nitrate storage.

1. Introduction

Nitrogen (N) fertilizers have largely contributed to vegetable crops high yield worldwide to meet the increasing food demands (Robertson and Vitousek; 2009). Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops with 4.7 million cultivated hectares and 182 million producted tonnes in 2018 (FAOstat, 2020). The lowest N supply recommended for a high tomato yielding is about 100-150 kg ha^{-1} (Doorenbos and Kassam, 1986), but doses more than two fold higher are usually applied (Scholberg et al., 2000). However, crops utilize less than half of the applied N-fertilizer and the remaining amount is lost into the environment causing dangerous pollution as well as reducing nitrogen use effieciency (NUE) in crops (Socolow, 1999; Garnett et al., 2009; Xu et al., 2012). So, improving crop NUE, exploiting the genetic diversity for this trait, is considered one of the most promising strategy to enhance crop production sustainability (Lammerts van Bueren and Struik, 2017), minimizing the impact of high N-fertilization (Gutiérrez, 2012, Xu et al., 2012). In this context, long storage tomato ecotypes cultivated in the Mediterranean basin are of great interest being resilient to drougth and N-limited conditions, typical of this area (Abenavoli et al., 2016). Besides, deep insights on both physiological and molecular mechanisms to cope low-N are required for an effective use of genetic and genomic approaches when improving NUE.

NUE is a complex trait in which physiological, developmental and environmental factors are involved; it encompasses the plant efficiency to absorbe (NUpE component), assimilate, transport and remobilize the available N into the soil (NUtE component) (Jackson et al., 2008, Xu et al., 2012). In tomato, physiological and molecular NUE-related traits were studied under contrasting N-supply, focusing mostly on root morphology, nitrogen uptake and transport systems (Abenavoli et al., 2016, Lupini et al., 2017), albeit further efforts should be addressed to nitrogen utilization efficiency (NUtE).

Nitrate (NO_3^-) is the major N source in well-aerated soil (Crawford and Glass, 1998). Once uptaken into the root cell, NO_3^- is either assimilated to organic nitrogen or stored/sequestred

in root cell tonoplasts (Orsel et al., 2002); otherwise, it is loaded into the xylem vessels and then transported to above-ground organs (Marschner et al., 1997). In *Arabidopsis,* a greater NO_3^- allocation to the shoot was correlated with higher NUE (Lin et al., 2008). Tang et al. (2012 and 2013) suggested that the promotion of NO_3^- transport from root to shoot represents an "adventagous physiological adaptation", which allows the utilization of solar energy for NO_3^- assimilation contributing to higher NUE. According to Hirose and Bazzaz (1998), high-NUtE seems to be related to the ability of efficient genotypes to reallocate N to the best lighted leaves with an efficient photosynthetic activity useful for a more cost-effective assimiliation. Thus, NUtE is considered positively correlated to the photosynthetic activity (Smirnoff and Stewart, 1985; Tang et al., 2013).

The NO_3^- long and short-distance transport mechanisms are as well significantly involved in NUtE. In particular, the *NRT1.5* and *NRT1.8* genes regulate nitrate long-distance transport and its distribution between roots and shoots. In roots of *Brassica napus* and *Arabidopsis*, *NRT1.5* is responsible for xylem NO_3^- loading, whereas *NRT1.8* mediates xylem NO_3^- unloading (Lin et al., 2008, Li et al., 2010, Han et al., 2016). Their regulation is controlled by cytosolic NO_3^- concentration, which in turn depends on NO_3^- short-distance transport between cytosol and vacuole, mediated by chlorid channel protein (CLCa) in the tonoplast membrane (De Angeli et al. 2006, Han et al., 2016). Indeed, the NO_3^- sequestration in root cells vacuole prevents its assimilation and allocation to the shoots for further utilization (Han et al., 2015).

The N remobilization also plays a key role in NUtE improvement (Mickelson et al., 2003; Masclaux-Daubresse et al., 2008). Indeed, the N remobilization from the older to the younger leaves results essential to sustain plant vigorous growth under N deficiency (Rossato et al., 2001; Schiltz et al., 2005; Fan et al., 2009). During leaf senescence, organic N is the major remobilized form (Good et al., 2004; Masclaux-Daubresse et al., 2008); although, the stored NO_3^- can also be remobilized from older leaves to N demanding tissues as in *Arabidopsis.* In this species, NO_3^- remobilization is mediated by *NRT1.7* gene, encoding a low affinity NO_3^- transporter, expressed in phloem source leaves minor veins and responsible for NO_3^- loading into the sink tissues (Fan et al., 2009; Chen et al., 2020). Another gene, *NRT2.7* could be involved in NUE improvement. This high affinity NO_3^- transporter is responsible for its storage in *Arabidopsis* seeds (Chopin et al., 2007), and seems to play a role in NO_3^- efflux regulation in leaves, balancing NO_3^-assimilable amount by transporting back into xylem vessels any NO_3^- excess (Orsel et al., 2002). The *NRT2.7* transcripts were also reported in N-stress tolerant sorghum genotype leaves (Gelli et al., 2014).

Recently, a genotypes pair contrasting for NUE was selected among some long-storage tomatoes, speculating about the key role which might play NUtE in NUE performance (Abenavoli et al., 2016). In the present work, NUE performance of both genotypes were confirmed by using different NUE definitions, in addition the NUtE component was deeply evaluated throughout the gene expression analysis of most NO_3^- metabolism related genes in both shoots and roots under NO_3^- limiting and non-limiting supply. In partcular, we focused on the ability of N-effcient and inefficient genotypes to modulate long-distance N transport, assimilation, remobilization and vacuolar sequestration based on the related genes expression. The correlation between NUE and its components and the N-metabolism-related gene expressions was highligthed.

2. Materials and Methods

2.1 Screening for NUE

2.1.1 Plant material and growth conditions

~~Seeds of t~~Three tomato landraces were chosen for our study, namely Linosa and Piriddu from Sicily (University of Palermo, Italy), Regina Ostuni (RO) from Apulia (University of Bari, Italy) and a North American cultivar, UC82, from the Department of Plant Sciences (University of California Davis)~~.~~, Seeds of the four genotypes were sterilized with 10% (v/v) NaClO for 15 min and then transferred in Petri dishes (Ø 90 mm) for 10 days as reported by Lupini et al. (2017). Seedlings of each genotype, with uniform size, were selected and transferred in an aerated hydroponic system containing a complete Hoagland solution supplied with 10 mM $Ca(NO_3)_2$, according to Abenavoli et al. (2016) with some modifications. Tomato seedlings were then placed in a growth chamber maintained at 25°C, 70% relative humidity and 16 h photoperiod with a light intensity of 350 µmol $m^{-2}s^{-1}$. The nutrient solution was renewed every two days and the pH was maintained at 5.8 with 1 M KOH. After 10 days, the half of each genotype was maintained in non-limiting N condition (10 Mm $Ca(NO_3)_2$) (control), while the remaining was transferred in N-limiting condition (0.5 mM $Ca(NO_3)_2$), for one week. These two NO_3^- concentrations (0.5 and 10 mM) were previously established (Abenavoli et al. 2016).

2.1.2 Root and shoot morphology and biomass evaluation

Ten plants (27-d old) from each genotype and treatment (10 mM and 0.5 mM) were collected, divided into shoot and root, and weighted. Roots were dipped in 0.1% (w/v) toluidine blue (Sigma Aldrich, #89160) for 5 min, rinsed in deionized water, and then scanned at 1200 dpi

resolution (WinRhizo STD 1600, Instruments Règent Inc., Quebec, Canada) to determine the total root length (TRL, cm) and root volume (RV, cm^3) using WinRhizo Pro System v. 2002a software, as reported by Lupini et al. (2016; 2017). Shoots were analyzed by IMAGE J software to measure plant height (cm), leaf number (#) and leaf area (cm^2). Then, shoots and roots were dried at 70°C for two days until their weight remained constant to determine their dry weight (SDW and RDW, respectively). Total dry weight (TDW, g) was calculated by adding SDW to RDW. Root length ratio, RLR (root length/whole plant dry weight, cm g^{-1}), root mass ratio, RMR (root dry weight/whole plant dry weight, g g^{-1}), root thickness or fineness, RF (root length/root volume, cm cm^{-3}) and root density, RD (root dry weight/root volume, g cm^{-3}) were calculated.

2.1.3 Chlorophyll content

Chlorophyll content was also evaluated by SPAD meter (Minolta). Ten measurements per plants (five) for each genotype and treatment were performed on the adaxial surface of leaves.

2.1.4 Nitrogen content

Total nitrogen content (mg N, Nc) was determined in both shoot and root of each genotype by combustion method through a LECO-CNS-1000 analyzer (LECO Instruments Ltd., Mississauga, ON) as reported by Lupini et al. (2017), root/shoot Nc ratio was then calculated. The mean is the average N-content of five plants for each genotype and treatment.

2.1.5 Nitrogen Use Efficiency and its components

Nitrogen Use Efficiency (NUE, SDW $N\%^{-1}$, where N% is the g N (100 g TDW)$^{-1}$) (Chardon et al., 2010), Nitrogen Utilization Efficiency (NUtE, $SDW^2\ Nc^{-1}$) (Siddiqi and Glass, 1981) and Nitrogen Uptake Efficiency (NUpE) (TDW x N concentration (g N g TDW^{-1}) (Chardon et al., 2010) were calculated. The mean is the average value of five plants for each genotype and treatment.

2.2 Gene expression analysis

2.2.1 Growth conditions

Since the internal nitrate concentration modifies the N response and its regulatory mechanisms (Forde and Clarkson, 1999), an experiment was carried out to define the nitrate starvation time in the NUE contrasting genotypes, RO and UC82. Thus, tomato seedlings (10 days old) grown in hydroponic system in non-limiting NO_3^- supply for 10 days, were

transferred to N-free solution. Shoots and roots were sampled at 0, 1, 4 and 7 days of treatment, for Nc determination and results were evaluated by a non linear regression model. The recovery key time where the starvation was reached (minimum Nc value) was estimated at 5 days. The mean for each sampling time is the average of three plants (Figure S1).

2.2.2 RNA extractions and cDNA synthesis

Plants (20-d old) were starved in an N-free solution for 5 days and then exposed to 0.5 mM and 10 mM NO_3^- for 7 days. Shoots and roots were harvested separately after 0, 8, 24 h and one week N-treatment and immediately frozen in liquid nitrogen. Total RNA from shoots and roots of both genotypes was isolated and purified using RNeasy Plant Mini Kit (Qiagen, Milano, Italy) according to the manufacturer's protocol. The total RNA was quantified using a NanoDrop 2000 (Thermo Scientific), and its integrity was assayed on 2% agarose gel electrophoresis. A first-strand cDNA was synthesized from 2 µg of total RNA using Maxima First Stand cDNA Synthesis Kit (Thermo Fisher Scientific Baltics UBA) according to the manufacturer instructions.

The RT-PCR was used to detect the primer specificity of candidate reference genes, and the mixed cDNA was used as template. The PCR reaction mix included 12.5 µL 2×Dream Tap Green PCR Master Mix (Thermo Scientific), 1 µL forward/reverse primer (100 µM), 1ul cDNAs (50ng/µL), 9.5ul ddH_2O supplement. The RT-PCR reaction procedures were as follows: 35 cycles, 94° C for 3 min, 94° C for 30 sec, 59° C for 30 sec, followed by elongation at 72° C for 12 sec and extension for 5 min. At the end of the reaction, 1 % agarose gel electrophoresis was used to detect primers specificity.

2.2.3 Quantitative Real-Time PCR (qRT-PCR)

Specific primers for nitrate and nitrite reductase (*SlNR* and *SlNIR*, respectively), chloroplastic glutamate synthetase (*SlGS2*) and glutamine synthase (*SlGOGAT*), low and high affinity NO_3^- transporters (*SlNRT1.5*, *SlNRT1.8*, *SlNRT1.7* and *SlNRT2.7*) and chloride channel protein (*SlCLCa*) were designed together with the reference gene (*SlActin*) using primer 3 (http://primer3.u.ee/) (Table 2). The qRT-PCR was performed in 96-well plates on StepOne™ Real-Time PCR System (Applied Biosystems, foster, CA, USA) using PowerUp SYBR Green master mix (Applied Biosystems by Thermo Fisher Scientific) according to the manufacturer instructions. The qRT-PCR was carried out starting from 2 min at 50° C, 2 min 95° C (initial denaturation), then 40 cycles of 15 s at 95 °C, 1 min at 59° C and finally 15 s at 95° C, 1 min at 60° C and 15s at 95° C. Three biological and two technical replicates were performed for

each genotype and NO_3^- level. The PCR efficiency of primer pairs was optimized in the range 92-105% with R^2-values of 0.997. The qPCR results were normalized adopting the $2^{-\Delta\Delta Ct}$ comparative method (Livak and Schmittgen, 2001) considering time 0 for each target gene as calibrator and where $\Delta\Delta C_T = (C_{T,Target} - C_{,Actin})_{Time\ x} - (C_{T,Target} - C_{,Actin})_{Time\ 0}$. In the formula, "Time x" and "time 0" represent any time point and the 1X expression of the target gene normalized to the internal control gene (*SlActin*), respectively. The qPCR results at T0 are presented in the supplementary materials as the normalized relative quantity of each target gene's expression with respect to the reference gene *SlActin* ($2^{-\Delta Ct}$).

2.3 Statistical analysis

All the experiments were set up in a completely randomized design with at least five replications. The data were checked for normality (Kolmogorov–Smirnov test) and tested for the homogeneity of variance (Leven median test). The data were then analyzed by ANOVA, and the means were separated by Tukey's Honest Significant Difference (HSD) test ($p < 0.05$), using Systat software (Systat Software Inc., Chicago, IL, USA). The relative gene expressions ($2^{-\Delta\Delta Ct}$) within each time point were analyzed by ANOVA based on three biological replicates for each treatment by using R software version 3.5.0.

3. Results

3.1 NUE evaluation

3.1.1 Biomass and morphological response to limiting NO_3^- supply

The two tomato genotypes were grown under limiting and non-limiting NO_3^- and analyzed for plant growth ~~and NUE parameters~~ (Figure S2). The biomass production, expressed as shoot dry weight (SDW), varied significantly between NO_3^- treatments and among tomato genotypes ($P < 0.05$) (Figure 1A). By contrast, root dry weight (RDW) did not differ significantly among genotypes and between N-treatments (Figure 1B). The SDW results indicated that RO was the less sensitive to NO_3^- limiting supply unlike UC82. ~~compared to the others.~~

All the genotypes did not show any significant variation in response to different NO_3^- supply in total root length (TRL), root tissue density (RTD), root fineness (RF) and shoot length (SL), whilst root length ratio (RLR), root mass ratio (RMR), leaf number (# L) and leaf area (LA) varied significantly except for RO ($P < 0.05$) (Table 2). The last four parameters (RLR,

RMR, #L and LA) ~~RLR, RMR, #L and LA results~~ indicated that RO was the less sensitive among genotypes to NO_3^- limiting supply ~~among genotypes~~ (Table 2).

3.1.2 Chlorophyll content (SPAD)

Chlorophyll content was measured in tomato leaves of each genotype and treatment, the SPAD values showed significant differences in response to limiting and non-limiting NO_3^- conditions in all the genotypes, except for RO that exhibited similar values at both N-treatments. ~~These results showed also that~~In addition, UC82 ~~was~~appeared the most sensitive genotype to N limitation (Figure 2).

3.1.3 Nitrogen content and nitrogen use efficiency

The N content (Nc) did not vary significantly in the root of all the genotypes, except for RO, which showed a significant lower Nc under NO_3^- limiting condition; by contrast, significant differences were observed in the shoot of all the genotypes (Figure S3~~2~~; Table S1). To further analyse N distribution in plant, the root/shoot Nc ratio (R/S Nc ratio) was also calculated. In NO_3^- limiting supply, RO exhibited the lowest R/S Nc ratio value indicating a lower N content in root compared to the other genotypes. By contrast, similar R/S Nc ratio values were observed under NO_3^- non-limiting condition among genotypes (Figure 3A). Under NO_3^- limiting supply, NUE increased significantly in RO respect to the control, while any significant difference was observed between treatments in the other genotypes. In the same condition, NUpE decrased in all the genotypes except for Linosa, while NUtE decreased significantly only in UC82, respect to the control. Overall, in NO_3^- limiting supply, RO showed significant higher NUE and NUtE ~~compared to the other genotypes~~while UC82 exhibited a critical NUtE decrease, compared to the other genotypes. (Figure 3B, C and D).

3.2 Gene expression analysis

Based on morphological and physiological traits, RO and UC82 were selected for their contrasting response in NO_3^- limiting conditions. The expression patterns of NO_3^- assimilation, allocation, remobilization and storage/sequestration related genes in root and shoot of both genotypes were analyzed at 0, 8 and 24 h (short-term response) and after one week (long term response) from NO_3^- recovery.

3.2.1 Short and long-term response to limited NO_3^- supply in shoot

The time-course of *SlNR, SlNIR, SlGS2, SlGOGAT, SlNRT1.7, SlNRT2.7* and *SlCLCa* expressions was assessed (Figure 4). Before NO_3^- recovery (0h), no significant differences

were observed in gene expressions ($2^{-\Delta Ct}$) between the two genotypes except for *SlNR* and *SlGS2*, which were significantly more expressed in UC82 respect to RO (P <0.05) (Figure S43A; Table S2).

After 8h from NO_3^- recovery, *SlNR* and *SlNRT1.7* expressions were significantly up-regulated in both genotypes (Figure 4A,E), while *SlNIR* and *SlGOGAT* were significantly down-regulated only in RO under NO_3^- limiting (0.5mM) compared to non-limiting (10mM) condition (Figure 4B,D). In turns, *SlCLCa* was significantly up- and down-regulated in RO and UC82, respectively, in NO_3^- limiting compared to the non-limiting condition (P <0.05) (Figure 4G; Table S3). Furthermore, *SlGS2* and *SlNRT2.7* did not show significant differences between genotypes and N treatments (Figure 4C,F). Interestingly, the expression levels of *SlNRT1.7* was significantly higher in RO compared to UC82, while *SlNIR*, *SlGOGAT* and *SlCLCa* were significantly more expressed in UC82 compared to RO in the NO_3^- limiting condition (P < 0.05) (Table S3).

After 24h, *SlNR* and *SlNIR* expressions did not show significant differences between genotypes and treatments (Figure 4A-B). The *SlGS2* and *SlCLCa* expressions were significantly down-regulated in RO and UC82, respectively, under NO_3^- limiting compared to non-limiting condition (P < 0.05) (Figure 4C,G; Table S3). Further, *SlGOGAT* expression was significantly down-regulated in RO and UC82 at 0.5mM (Figure 4D), *SlNRT1.7* expression was significantly up-regulated in RO (Figure 4E), while *SlNRT2.7* expression was significantly up and down-regulated in RO and UC82, respectively under NO_3^- limiting compared to non-limiting condition (P < 0.05) (Figure 4F; Table S3). At this recovery time, the *SlNRT1.7* and *SlNRT2.7* expression levels under NO_3^- limiting supply were significantly higher in RO compared to UC82 (P < 0.05) (Table S3).

After one week (7d) from NO_3^- recovery, the *SlNR* and *SlNIR* expression levels did not show any significant differences between NO_3^- treatments in RO, while both gene expressions were significantly down-regulated in UC82 at 0.5mM compared to non-limiting N-treatment (P < 0.05) (Figure 4A,B; Table S3). Furthermore, *SlGS2* did not show significant differences between genotypes and N treatments (Figure 4C), while *SlGOGAT* and *SlNRT1.7* expressions were significantly down and up-regulated, respectively, in both genotypes (Figure 4D-E). Finally, *SlNRT2.7* was significantly up-regulated only in RO in NO_3^- limiting condition respect to the control (P < 0.05) (Figure 4F, Table S3). In addition, the *SlNR* and *SlNRT2.7* transcripts abundance was significantly higher in RO compared to UC82, while *SlGOGAT* expression level was higher in UC82 compared to RO, in NO_3^- limiting condition (P < 0.05) (Table S3).

3.2.2 Short and long-term response to limited NO_3^- supply in root

The time course of *SlNR*, *SlCLCa, SlNRT1.5* and *SlNRT1.8* expressions was assessed (Figure 5). Before NO_3^- recovery (0h), all the gene expression levels ($2^{-\Delta Ct}$) were significantly different between genotypes; in particular, *SlNR, SlCLCa* and *SlNRT1.5* were significantly more expressed in RO, while *SlNRT1.8* was significantly more expressed in UC82 ($P < 0.05$) (Figure S43B, Table S2).

After 8h from NO_3^- recovery, *SlNR* and *SlNRT1.8* expressions were significantly down-regulated in UC82 (Figure 5A,D), while *SlCLCa* expression was significantly up-regulated in RO under NO_3^- limiting compared to non-limiting condition (Figure 5B). Further, *SlNR* and *SlNRT1.5* were significantly more expressed in UC82 compared to RO, while *SlCLCa* expression level was significantly higher in RO ($P < 0.05$) (Figure 5A,B,Table S3).

After 24h, *SlNR*, *SlCLCa* and *SlNRT1.5* expressions were significantly down-regulated in both genotypes (Figure 5A,B,C), while *SlNRT1.8* appeared significantly down-regulated only in RO (Figure 5D) under NO_3^- limiting compared to non-limiting condition ($P < 0.05$) (Table S3). In addition, all the analyzed gene expressions were significantly more expressed in UC82 compared to RO under NO_3^- limiting condition ($P < 0.05$) (Table S3).

After one week (7d) from NO_3^- recovery, all the gene expressions were significantly down-regulated in both genotypes under NO_3^-limiting condition compared to non-limiting ones, except for *SlNRT1.8* ($P < 0.05$) (Figure 5, Table S3). Furthermore, under NO_3^- limiting condition, *SlNR* expression was not significantly different between genotypes (Figure 5A), *SlCLCa* and *SlNRT1.8* expression levels were significantly higher in UC82 compared to RO (Figure 5B,D), while *SlNRT1.5* expression level was significantly higher in RO compared to UC82 (Figure 5C, Table S3).

Moreover, the results obtained after one week of NO_3^- treatments on NUE and its components together with the molecular responses observed in both genotypes as well in root and shoot were highlighted in a heatmap (Figure 6). The observed differences in N use efficiency could be explained by *SlNR, SlCLCa* and *SlNRT2.7* expressions in shoot, and *SlNRT1.5* and *SlNRT1.8* in root displaying contrasting expressions between tomato genotypes in NO_3^- limiting condition (Figure 6).

3.2.3 NO_3^- metabolism-related genes expressions and NUE parameters correlations

Pearson correlation between genes expression and morpho-physiological traits (including NUE and its components) in shoot and root of both tomato genotypes after one week under limiting NO_3^- treatment is presented in Figure 7.

According to the matrix visualization, NUE, NUtE and NUpE showed a significant and positive correlation with SDW, as expected, but also with NO_3^- assimilation and efflux related genes expression (*SlNR, SlNIR* and *SlNRT2.7*) in shoot, and NO_3^- long-distance transporter gene expression (*SlNRT1.5*) in root. Otherwise, NUE and its components exhibited a negative correlation with *SlCLCa* expression in both shoot and root, *SlNRT1.8* expression in root and R/S Nc ratio. Moreover, *SlGOGAT* and *SlGS2* showed a significant negative correlation with NUE and its components (NUpE and NUpE).

The results highlighted also some specific negative correlations; in detail: a) *SlNIR* and *SlNRT2.7* expression in shoot as well as *SlNRT1.5* expression in root with NO_3^- storage related gene (*SlCLCa*) and *SlNRT1.8* in root; b) the chlorophyll content (SPAD) with the *SlCLCa* expression in root; .c) the R/S Nc ratio with SPAD values and NO_3^- assimilation and transporter gene expressions in both shoot and root (*SlNRT2.7*, *SlNIR* and *SlNR*); d) the SDW with *SlCLC*a and *SlNRT1.8* expressions in root (Figure 7).

4. Discussion

Limiting nitrogen availability drives specific and complex physiological, morphological and developmental responses in plants (Yang et al., 2011). These can differ among cultivars of the same species due to the genetic variation for N uptake (Rodgers and Barneix, 1988) and utilization (Chardon et al., 2010; Coque and Galleis, 2007), laying the foundations for improving Nitrogen Use Efficiency (NUE). The present study further confirms the existing differences among genotypes for a complex trait like NUE and its components in tomato.

Our findings confirmed RO and UC82 as the best NUE contrasting genotypes, namely N-use efficient and inefficient, respectively (Abenavoli et al 2016). Previous researches showed that considerable variation in NUE occurs mainly for biomass production in tomato, barley, maize and cotton (Abenavoli et al, 2016; Lupini et al., 2017; Xu et al., 2016; Granato et al, 2014; Iqbal et al., 2020); in agreement, our results showed the highest RO biomass production (SDW) compared to the others, in turns it decreased considerably in UC82 mainly under limiting NO_3^- treatment. Accordingly, NUE appeared noticeably enhanced in RO under limiting NO_3^- supply, while UC82 exhibited the most significant decrease in NUtE level, compared to the other tomato genotypes.

The SPAD values, that predict crop nitrogen deficiency and the photosynthetic rates (Debaeke et al, 2006; Reis et al., 2009), further emphasized the contrasting responses between RO and UC82 facing NO_3^- limitation, underlying the highest tolerance to N scarcity of RO throughout the photosynthetic efficiency and its positive correlation with higher biomass production (here measured as SDW) (Figure 7). Furthermore, under NO_3^- limiting condition, the R/S Nc ratio indicated that RO translocated more N to the shoot than the other genotypes supporting the positive correlation between NO_3^- shoot allocation (*SlNRT1.5*) and NUE (Figure7), as well as between NUtE and the photosynthetic efficiency (Smirnoff and Stewart, 1985; Lin et al., 2008; Tang et al., 2013). Overall, our preliminary results suggested that the strategy adopted by RO to perform a considerable NUE enhancement seemed to be due to a high NUtE, while NUpE was of less importance in NUE performance, as already reported by Abenavoli et al. (2016).

As a signal molecule, NO_3^- regulates several plant physiological processes by inducing or repressing the expression of its transport, assimilation and remobilization related genes (Kant, 2017; Hachiya and Sakakibara, 2017). The identification of key metabolic pathways in genotypes able to optimize NO_3^- utilization under N-stress is essential for crop NUE improvement (Lian et al., 2005). In the present study, all the genes related to NO_3^- translocation, assimilation and storage were up-regulated whereas those encoding for NO_3^- remobilization and efflux appeared down-regulated during the early hours after NO_3^- re-supply, regardless NO_3^- concentration and plant tissue. Interestingly, the chloroplastic glutamine synthetase (*SlGS2*) expression level was maintained in both RO and UC82 throughout time in shoot, suggesting that the constitutive *SlGS2* expression was enough to support nitrogen metabolism in tomato under NO_3^- stress. Similar expression patterns were already observed in *Thellungiella halophila* and barley (Kant et al., 2008; Chen et al., 2018).

It is well known that nitrate reductase (NR) and nitrite reductase (NIR) are the first enzymes that reduce NO_3^- to NH_4^+ for sustaining N assimilation (Meyer and Stitt, 2001). Nitrate limiting condition triggered differences in NO_3^- assimilation between the NUE contrasting genotypes after one week of treatment. In support, *SlNR* and *SlNIR* did not exhibit significant differences between NO_3^- treatments in RO, while they were significantly down-regulated under NO_3^- limiting condition in UC82, and *SlNR* transcripts abundance were significantly higher in RO compared to UC82 in shoot. Both gene expressions were significantly correlated among them and to NUE and its components (NUpE and NUtE) (Figure 7). These results could sustain the higher NUtE maintained by RO under low N supply compared to UC82. In

agreement, the same *NR* and *NIR* expression profiles in potato and barley under N-limiting condition were observed (Li et al. 2010; Chen et al. 2018; Kollaricsné Horvath et al. 2019).

Beside, the GS/GOGAT pathway is of critical importance for NO_3^- assimilation catalyzing the reactions that transform inorganic to organic nitrogen (Lea and Miflin, 1974). Therefore, the induction of both genes (*GS2* and *GOGAT*) was identified as the major effector for NUE under NO_3^- limiting supply in many crops species (Quraishi et al., 2011; Chen et al, 2018; Mauceri et al., 2020). Conversely, our findings correlated lower GS/GOGAT gene expression to higher NUE under NO_3^- limiting conditions, as recently reported in *Arabidopsis* by Meyer et al. (2019). They suggested that this result could be related to the fact that good NUE definition in their study was essentially based on good biomass production under NO_3^- deficiency, as considered in our study.

Nitrate remobilization was reported as another key factor for improving NUE (Masclaux-Daubresse et al., 2008). To discern NO_3^- remobilization role in tomato NUE, we evaluated the differential expression of *SlNRT1.7* between genotypes pair, based on its involvement in the stored NO_3^- remobilization from older leaves to N-demanding tissues through phloem (Fan et al., 2009; Chen et al., 2020). Nitrate limiting condition triggered an *SlNRT1.7* up-regulation in both genotypes compared to non-limiting N-supply, more interestingly RO mantained higher *SlNRT1.7* transcript abudance than UC82 mainly after 8 and 24h of N-stress ~~(Figure 8)~~. Hence, the adopted strategy by RO during the early hours under NO_3^- limiting supply appeared of crucial importance for facing the long-term NO_3^- stress. Chen et al. (2020) improved *Arabidopsis*, tobacco and rice NUE through a novel strategy aiming to specifically enhance *NRT1.7*-mediated NO_3^- remobilization.

Our results evidenced as well the strong correlation between both NUE and NUtE and *SlNRT2.7* expression level in the shoot of RO, the N-use efficient genotype (Figure 7~~8~~). Until now, limited information have been reported on this high affinity NO_3^- transporter in shoot tissues. However, Orsel et al. (2002) stated that *NRT2.7* was the only NRT2 family member not apparently involved in NO_3^- uptake from soil, showing a strong leaf tissue specific expression pattern in *Arabidopsis* under limiting NO_3^- supply, in agreement with our results. They suggested that, under N-starvation, NRT2.7 protein regulated NO_3^- efflux balancing the NO_3^- assimilable amount transporting back into the xylem any excess (Orsel et al., 2002).

Nitrate long-distance transport from root to shoot likely contributes to plant growth and NUE enhancement (Andrews, 1986; Tang et al., 2012; Han et al., 2016) since higher NO_3^- assimilation efficiency occurred in shoot tissues (Smirnoff and Stewart, 1985; Tang et al., 2013). This transport is regulated by *NRT1.5* and *NRT1.8* genes, which control xylem NO_3^-

loading and unloading, respectively (Lin et al., 2008; Li et al., 2010; Han et al., 2016). However, NO_3^- long-distance transport was strongly affected by its storage/sequestration in vacuoles (Han et al., 2015; Han et al., 2016). This short-distance transport between cytosol and vacuole is mediated by CLCa, a NO_3^-/H^+ exchanger localized in the tonoplast and responsible for NO_3^- homeostasis maintenance in the cytosol (De Angelis et al., 2006; Wege et al., 2014).

In our study, after seven days of N-treatment high-NUE was significantly correlated with a higher NO_3^- xylem uploading gene expression (*SlNRT1.5*) and thereby to a major NO_3^- allocation to the shoot and a higher assimilation guided by *SlNR* and *SlNIR* expression (Figure 7~~8~~). By contrast, high-NUE was negatively correlated to both xylem NO_3^- unloading (*SlNRT1.8*) and its storage/sequestration in vacuoles (*SlCLCa*) in root (Figure 7). These results are in agreement with the statement that a higher CLCa activity in root induces a down- and up-regulation of *NRT1.5* and *NRT1.8*, respectively (Lin et al., 2008; Han et al., 2016). Noticeably, our N-use efficient genotype RO exhibited a significant higher *SlCLCa* transcript abundance in root compared to UC82 at the first 8h of N-stress. These differential gene expressions suggested that high-NUE in RO could occur~~red~~ for a higher NO_3^- accumulation in root cell vacuoles during ~~a~~ the short-term NO_3^- stress exposure, ~~and~~ followed by a significant higher NO_3^- translocation to the shoot (guided by *SlNRT1.5* higher expression)~~,~~ under ~~a~~ the long-term NO_3^- stress periode, compared to UC82 (Figure 8).

After long-term stress (7d), UC82 showed a higher NO_3^- storage/sequestration ~~in root~~ and NO_3^- xylem unloading in root (*SlNRT1.8* higher expression), thereby less NO_3^- amount was translocated to shoot and available for assimilation. ~~Finally~~Moreover, we observed a negative correlation between *SlCLCa* and *SlNR* expressions confirming that CLCa activity limits NO_3^- availability in the cytosol and reduces NR activity, as previously reported in *Brassica napus* (Han et al., 2015).

Finally, designing efficient breeding strategies for nitrogen use efficiency improvement requ~~a~~ier~~e~~s integration of agronomy, physiology and molecular insight (Lammerts van Bueren and Struik, 2017). The same authors concluded that discriminative traits related to NUE better express themselves under low N than under high N. This correlate well with our results which emphasized some tomato specific morpho-physiological and molecular markers that contributed to high NUE making RO a precious genetic resourse for future tomato NUE breeding programs.

5. Conclusion

Our experiment contributed to shed light on NUE enhancement mechanisms under limiting NO_3^- supply in tomato. The differential expressions of the most important nitrate metabolism related genes explained the RO higher N-utilization efficiency (NUtE) and consequently higher NUE, compared to UC82.

The results suggested a RO faster adaptation to NO_3^- limiting condition compared to the inefficient genotype, afterwards RO appears able to ensure high NO_3^- efficient storage in cell root vacuoles within the short term N-stress as well as a constant NO_3^- remobilization. RO high NUE seems to rely also on a more efficient NO_3^- translocation to the shoot for a higher assimilation efficiency, compared to UC82.

Overall, our results revealed some aspect of the molecular adaptation to NO_3^- deficiency and suggested that NUE in tomato could be mainly determined by the genotype ability to regulate long-distance N transport, assimilation, remobilization and storage/sequestration genes.

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.

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Table 1: Primers for qRT-PCR

Gene	Accession ID		Primer Sequences (5' to 3')	Amplicon length
SlNR	NM_001328498.1	Forward	5'-GGTGGATGGATGGCAAAGGA-3'	127
		Reverse	5'-TCCTCACCTCGGACATGGAA-3'	
SlGOGAT	XM_004234907.4	Forward	5'-GTGGTTTGGGCCATCTCTGA-3'	83
		Reverse	5'-CACGACTGTTGGCTGCTTTT-3'	
SlGS2cp	NM_001323669.1	Forward	5′-TGGAGTTGAGGTGTAATTGTTGG-3′	105
		Reverse	5′-CATTCGGAAAGAGCACACCA-3′	
SlNir	XM_004248688.4	Forward	5′-GGACAGGTTGCCCAAATACA-3′	67
		Reverse	5′-GTCAGGCATCCCATGAATCCG-3′	
SlNRT1.7	XM_004238712.2	Forward	5'- TCCCCGAAAACATGAGCAGT -3'	117
		Reverse	5'- GCCCATTTCCTCCCGTAGTG -3'	
SlNRT2.7	XM_004233279.4	Forward	5'-TCCTTCGTTCATTTCATGGCG-3'	101
		Reverse	5'-CATCAGGTAAGTCCTGGCCG-3'	
SlCLC-a	XM_004231738	Forward	5′-CGTCTCCCTTTTCACCTCCA-3′	93
		Reverse	5′-CCAGGACAGGACCCTTGAAT-3′	
SlNRT1.5	XM_004244498.4	Forward	5′-TCCTTAGTGTAGCAGGCGTC-3′	127
		Reverse	5′-ACCAGTCCAATACCCATCCG-3′	
SlNRT1.8	XM_010328990.3	Forward	5′-GCCTTTGTGCAGTGTCTCAA-3′	141
		Reverse	5′-CTGTTTTCATTGCAGCCCCT-3′	
SlActin[a)]	NM_001330119.1	Forward	5′AGGTATTGTGTTGGACTCTGGTGAT-3′	81
		Reverse	5′-ACGAGAATGGCATGTGGAA-3′	

a) Reference gene used as internal standard (Løvdal and Lillo, 2009)

Table 2: Comparison of root and shoot morphology among four tomato genotypes grown under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply. ~~Different letters along column indicate statistical significant differences ($P < 0.05$).~~ (**TRL,** Total Root Length; **RLR,** Root Length Ratio; **RMR,** Root Mass Ration**; RTD,** Root Tissu Density; **RF,** Root Fineness; **#L,** Leaves number; **LA,** Leaf Area; **SL,** Shoot Length) Different letters along column indicate statistical significant differences ($P < 0.05$).

Genotypes	[NO_3^-]	TRL (cm)	RLR (cm g^{-1})	RMR	RTD (g cm^{-3})	RF (cm cm^{-3})	# L	LA (cm^2)	SL (cm)
Linosa	0.5	845,74 ca	6192,55 ab	0,094 abbc	0,07 abc	3595,78 abc	19,66 ca	93,38 abbc	17,52 abdc
	10	1015,19 bcab	2302,42 ba	0,052 ca	0,08 abc	3508,05 abc	33,66 abbc	163,29 ca	18,97 ae
Piriddu	0.5	1006,94 bcab	6456,08 ab	0,101 abbc	0,065 bcab	4181,68 ae	26,67 bcab	83,72 ae	13,57 cdbc
	10	727,77 ca	2969,67 ba	0,051 ca	0,07 abc	4047,86 ae	34,66 ae	123,98 b	16,1 bced
R.O	0.5	1734,72 ae	4846,31 baab	0,091 abbc	0,081 abbc	4006,72 ae	36,66 ae	168.91 ca	10,20 ca
	10	1780,38 ae	3281,76 ba	0,074 bcab	0,092 ae	3640,12 abbc	39,66 ae	180.26 ca	10,61 ca
UC82	0.5	1453,20 abbc	5806,038 ab	0,117 ae	0,058 bcab	2835,59 ca	25,33 ca	67,13 ae	11,81 dcab
	10	1713,02 ae	3104,09 ba	0,057 ca	0,055 ca	3012,13 bcab	38,67 ae	126,72 b	14,96 bced

Figure 1. Shoot (A) and root (B) dry weight of four tomato genotypes grown under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply ($P < 0.05$, n=5).

Figure 2. Chlorophyll content (SPAD values) in four tomato genotypes grown under NO3- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply ($P < 0.05$, n=5).

Figure 3. (A) Root/shoot N content ratio (R/S ratio), (B) Nitrogen Use efficiency (NUE), (C) Nitrogen Utilization Efficiency (NUtE) and (D) Nitrogen Uptake efficiency (NUpE) of four tomato genotypes grown under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply ($P < 0.05$, n=5).

Figure 4. Differential relative expression of NO_3^- metabolism-related gene in shoot of RO and UC82 grown under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply. Shoots of 32d old plants were sampled at 0h, 8h and 24 h after NO_3^- recovery. The mean fold change in expression of the target gene at each time point was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta CT = (C_{T,Target} - C_{,Actin})_{Time\ x} - (C_{T,Target} - C_{,Actin})_{Time\ 0}$, *SlActin* was the internal control gene and time 0 was the calibrator. Means and standard errors are shown from the analysis of three biological replicates ($P < 0.05$, n=3) and different letters within each time point indicate significant differences at $P < 0.05$).

Figure 5. Differential relative expression of NO_3^- metabolism-related gene in root of RO and UC82 grown under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply. Roots of 32d old plants were sampled at 0h, 8h and 24 h after NO_3^- recovery. The mean fold change in expression of the target gene at each time point was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta CT = (C_{T,Target} - C_{,Actin})_{Time\ x} - (C_{T,Target} - C_{,Actin})_{Time\ 0}$, *SlActin* was the internal control gene and time 0 was the calibrator. Means and standard errors are shown from the analysis of three biological replicates ($P < 0.05$, n=3) and different letters within each time point indicate significant differences at $P < 0.05$).

Figure 6. Heatmap of NO_3^- metabolism-related gene expressions in shoot and root, physiological NUE-related parameters and NUE and its components of RO and UC82 after one week under NO_3^- limiting (0.5 mM $Ca(NO_3)_2$) and non-limiting (10 mM $Ca(NO_3)_2$) supply.

Figure 7. Correlation matrix visualization of the correlations between NUE related parameters and NO_3^- metabolism-related gene expressions of RO and UC82 after one week under NO_3^- limiting supply (0.5 mM $Ca(NO_3)_2$). * $0.01 < p \leq 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$

Figure 8. The proposed model describinges ~~the~~ Regina Ostuni high N-utilization efficiency (NUtE).

In black and bold the different expressed key ~~molecular~~ genes ~~making~~ that could be involved the high NUE tomato genotype able to cope N-limiting supply. The gene expression increase or decrease ~~of expressed genes~~ are indicated with a red (↑) and green (↓ arrow, respectively.