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Genetic variation in eggplant (*Solanum melongena* L.) for Nitrogen Use Efficiency (NUE) under contrasting NO_3^- supply

Running title

Nitrogen Use Efficiency variation in eggplant

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Abstract:

Eggplant (Solanum melongena L.) yield is highly sensitive to N fertilization, the excessive use of which is responsible for environmental and human health damage. Lowering N input together with the selection of improved Nitrogen-Use-Efficiency (NUE) genotypes, more able to uptake, utilize, and remobilize N available in soils, can be challenging to maintain high crop yields in a sustainable agriculture. The aim of this study was to explore the natural variation among eggplant accessions from different origins, in response to Low (LN) and High (HN) Nitrate (NO3-) supply, to identify NUE-contrasting genotypes and their NUE-related traits, in hydroponic and greenhouse pot experiments. Two eggplants, AM222 and AM22, were identified as N-use efficient and inefficient respectively, in hydroponic, and these results were confirmed in a pot experiment, when crop yield was also evaluated. Overall, our results indicated the key role of Nutilization component (NUtE) to confer high NUE. The remobilization of N from leaves to fruits may be a strategy to enhance NUtE, suggesting glutamate synthase as a key enzyme. Further, omics technologies will be used for focusing on C-N metabolism interacting networks. The availability of RILs from two other selected NUE-contrasting genotypes will allow us to detect major genes/quantitative trait loci related to NUE.

Keywords: Nitrogen Uptake Efficiency (NUpE); Nitrogen Utilization Efficiency (NUtE); root morphology; plasticity; heritability; nitrogen balance index (NBI).

1. Introduction

Nitrogen (N) is a major limiting factor for plant growth and productivity in both natural and agricultural environments, being an essential component of proteins, secondary metabolites, and nucleic acids (Brady 1999). As a result, over the past decades, N fertilization has increased more than 20-fold, in excess compared to crop requirement (35%) (Glass 2003; Shen et al. 2003; Good et al. 2004; Sarasketa et al. 2014; Wang et al. 2014), causing massive environment and human health damages (Good and Beatty 2011). Lowering N fertilizer input together with the selection of genotypes more capable to uptake, utilize, and remobilize N available in soil (McAllister et al. 2012), could be an important challenge on plant nutrition research (Hirel et al. 2007) and pivotal to sustain high crop yields reducing environmental, economic, and health costs (Good et al. 2004; Sebilo et al. 2013). Nitrogen Use Efficiency (NUE), generally referred to as "grain yield production per unit of N available in soils" (Moll et al. 1982), is a complex genetic trait influenced by several environmental factors. As a rule, NUE has two main components: Nitrogen Uptake Efficiency (NUpE), which describes the ability of a plant to take up N from soil, and Nitrogen Utilization Efficiency (NUtE), which indicates the ability of a plant to convert in grain the assimilated/remobilized N (Good et al. 2004; Xu et al. 2012). Among N forms present in soil solutions, nitrate (NO3-) is the primary N-source in aerobic soils, distributed unevenly both in space and time (Crawford 1995; Miller and Cramer 2004; Sorgonà et al. 2011), whose uptake by roots is the first step for N metabolism in plant. However, the root ability to use NO3- efficiently depends on morphological and physiological features underlying a complex genetic control (Lynch 1995; Sorgonà et al. 2006; Ruffel et al. 2011). In particular, an efficient NO3- uptake, especially at low concentrations in soils, depends on root length and surface area, required to exploit a larger total soil volume, together with a higher number of nitrate transporters per unit of root surface (Lawlor and Cornic 2002). Plants have developed three NO3- uptake systems, a low-affinity transport system (LATS) and two high-affinity transport systems, a constitutive (cHATS) and an inducible (iHATS) (Forde and Clarkson 1999; Forde 2000; Glass et al. 2002). LATS is constitutively expressed, operating at high external NO3- concentrations

(>1 mM), and its uptake activity is linear depending on the external NO3- level (Glass 2009). Conversely, both the HATS operate at low NO3- concentrations (<0.5 mM), however, while cHATS is active in plants never provided with NO3- having a greater affinity for this anion, the iHATS is induced by NO3- supply (Glass et al. 2002; Glass 2009). NUpE performance is associated with root growth and ability to increase NO3- uptake rates, thus several strategies to improve root traits were developed (Lynch 2007; Smith and De Smet 2012; Gregory et al. 2013). However, since NO3- concentration in soils is often at low millimolar ranges (Miller et al. 2007), the selection for a more efficient HATS system could be of a particular importance for plant nutrient uptake. Several reports showed that NRT2.1 expression, A member of NRT2 nitrate transporter family, is highly synchronized with iHATS regulation in different plant species (Filleur and Daniel-Vedele 1999; Fraisier et al. 2000; Orsel et al. 2006; Cai et al., 2008; Sorgonà et al. 2011). Another member of the HATS, displaying the same pattern of NRT2.1 and belonging to NAR2 family (annotated also as NRT3 according to Léran et al. 2014), was identified in different plant species (Tong et al. 2005; Okamoto et al. 2006; Orsel et al. 2006). In Arabidopsis, a co-expression of both NRT2.1 and NAR2.1 highly sustains the activation of the HATS (Okamoto et al. 2006; Orsel et al. 2006).

Once NO3- has been absorbed, it is reduced to ammonium, through nitrate and nitrite reductase (NR and NiR, respectively), in leaves or roots, and then assimilated into amino acids through glutamine synthetase/glutamate synthase (GS/GOGAT) enzymes and successively remobilized, used and/or stored in vacuolar system (McAllister et al. 2012). These activities contribute to the NUtE physiological component in crops (Kant et al. 2011; Xu et al. 2012). Eggplant (Solanum melongena L.) is the third most important Solanaceous vegetable crop (Barchi et al. 2011), cultivated and consumed worldwide, especially in India and China (Hazra et al. 2003). Despite the relevance and complexity of plant NUE, essential for the development of a sustainable agriculture, limited information on genetic variation for this trait is available for eggplant, whose productivity is highly sensitive to N fertilizer (Pal et al. 2002). Several studies on model and crop species highlighted a large natural variation for quantitative traits such as root morphology, nitrogen uptake and

assimilation, related to both genetic control and environmental adaptation (Walch-Liu et al. 2008; Chardon et al. 2010, 2012; De Pessemier et al. 2013). Beyond the dissection of the phenotypic variability, these genetic resources could represent a pivotal tool for selecting high-NUE genotypes (Bi et al. 2007; Coque et al. 2008; Chardon et al. 2010; Han et al. 2015a). The aim of this study was to investigate the natural variation for NUE-related traits in several eggplant accessions from different geographic origins, grown for 18 days in a hydroponic system under low and high NO3- levels, to identify contrasting NUE genotypes through a morphological, physiological, and molecular approach. Root morphology and plant biomass (root and shoot dry weight) were measured and considering the two physiological components, N-uptake efficiency (NUpE) and N-utilization efficiency (NUtE), NUE was calculated. Afterwards, four NUE-contrasting eggplants, selected in hydroponic system, were grown in soil pots under greenhouse till fruit harvests to confirm their different N-use efficiency.

2. Results

2.1.Growth chamber experiments

2.1.1. Eggplant natural variation in response to nitrate

Eggplant biomass, in terms of SDW and RDW, among 19 eggplant accessions in response to low (0.5 mM, LN) and high (10 mM, HN) NO3- supplies, was firstly investigated. For each trait, biplot graphs showed the average values of each accession obtained at LN (plotted along the vertical axis) compared to those at HN (plotted along the horizontal axis) levels (Figure 1). The biplot analyses allowed us to understand which trait was mostly involved in response to NO3- supply. Indeed, the genetic variation among genotypes was explained by their distribution along the diagonal bisector of biplots, while the distance from the bisector indicated the adaptive response to the anion of each genotype. RDW showed a higher coefficient of variation (CV) compared to SDW (0.44 vs 0.37) depending on both N supply (especially at LN) and genotype (Figure 1). In particular, RDW showed a higher average (0.012 g) at LN compared to HN (0.0026 g), on the contrary, SDW average was higher (0.067 g) at HN (Table S1). The deviation from the bisector of genotypes for RDW appeared

to be more variable at LN (from 0.004 to 0.025 g), while it was more marked at HN (from 0.02 to 0.13) for SDW parameter (Figure 1; Table S1). The high variability highlighted in RDW suggested investigating more deeply on the other root morphological parameters (Figure 2). In general, the traits associated to root length had higher values under LN supply. For example, TRL (0.49 CV) and LRL (0.54 CV) showed the higher variation compared to the others, due to both N supply and genotype (Figure 2). A similar trend was observed in RLR, LRN, and SRL, which showed lower CV values (0.41, 0.33, and 0.35, respectively). Conversely, both RMR and RTD were strongly influenced by both LN and HN supplies, respectively (Figure 2). Finally, RF showed a low variability due to both N supplies (mainly at high HN) and genotype (Figure 2). Among the physiological traits, NUpE showed the highest coefficient of variation (CV = 0.47), while NUE and NUtE exhibited a lower CV, highlighting a similar distribution along the bisector, and their scarce response to nitrate supply compared to NUpE (Figure 3). In addition, NUpE average was significantly higher at HN respect to LN (4.12 vs 3.02 mg N) (Table S1). NUE average was 0.011 (g SDW N%−1) at LN, ranging from 0.0043 to 0.019, while at HN, no significant differences were recorded (0.010). A similar trend was observed for NUtE, which exhibited average values of 0.0011 and 0.0010 (g SDW2 mg N−1) at LN and HN, respectively (Table S1). Overall, the accessions showed highly significant ($P < 0.0001$) differences in all the traits at both nitrate supply, except NUE and RTD (Table S1).

2.1.2. Global ANOVA, plasticity, genetic variation and heritability

To determine the percentage of explained variance due to genetic, environment and their interaction, global ANOVA was performed for all the traits under both N supplies (Figure 4). The genotype resulted in the higher cause of variation, reaching values overcoming 50% in RF, NUtE, and SDW, while the lowest values were found in root traits (RLR, RMR, and SRL) (Figure 4). The eggplant collection showed a high level of plasticity (PL) for many traits, ranging from 0.382 to 0.807 (Table 1). RMR, SRL, LRL exhibited the highest PL values, while the lowest ones were observed in RF, followed by NUtE and SDW. Conversely, broad sense heritability (h2 B) ranged from 0.376 to 0.771 and 0.242 to 0.791 at LN and HN, respectively (Table 1). At LN, the highest values of h2 B were

detected in TRL, LRL, RF, and NUtE; by contrast, RLR, RMR, and SRL exhibited the lowest ones. Lowest h2B values for the same traits were also observed at HN, while NUpE and NUtE showed high h2B values and RF maintained the highest one (Table 1). Finally, the plastic heritability (h2PL), was calculated according to Scheiner and Lyman (1989) and showed a range from 0.085 (NUtE) to 0.227 (RTD) (Table 1).

2.1.3. Trait correlation and cluster analysis

Pearson's correlation analysis among morpho-physiological traits was carried out at LN and HN (Table 2). At LN supply, NUE resulted highly correlated with NutE (0.9723) and NUpE (0.6201) components together with LRN (0.8396) and SDW (0.6267); NUpE exhibited correlation coefficients >0.7 with SDW and RDW but also with TRL, LRL, and LRN, while NUtE showed the highest values of correlation for LRN (0.7954) (Table 2). At HN, NUE and its components showed the highest correlation with SDW (0.9564, 0.8091, and 0.8610, respectively), TRL and LRL (Table 2). Further, an ascendant hierarchical cluster analysis was performed to identify contrasting NUE-genotypes taking into account all the morpho-physiological traits. Three clusters were defined by using Ward's method (Ward 1963) (Figure 5). Cluster 1 contained the accessions AM22, AM194, 67-3, and AM151, showing the lowest SDW at HN compared to cluster 3 (Figure S1; Table S1). Interestingly, at LN, these genotypes exhibited a marked reduction for several root traits, such as TRL, LRL, and NLR, as well as for NUE and its components, compared to the other clusters (Figure S1; Table S2). Cluster 2, including eight accessions, exhibited intermediate values for NUE and its components as well as for several root traits (Figures 5, S1; Table S1). Additionally, cluster 3, composed by seven accessions, including AM222 and AM241, was characterized by the highest SDW and RDW values, NUE and its components and root traits such as TRL, LRL, NLR, and RLR, particularly at LN (Figure S1; Table S1). The N-use efficient contrasting genotypes were selected, considering at least one representative genotype from clusters 1 and 3, which showed the highest phenotypic diversity. In particular, in cluster 3, characterized by accessions with similar high NUE, AM222 was chosen as N-

efficient genotype for its highest values in root traits such as TRL and LRL compared to AM241 (Table S1). Among the genotypes included in cluster 1, AM22 was chosen as the N-inefficient for its lowest values in NUE and for its different geographic origin respect to AM222 (Table S1). Finally, 67-3 (cluster 1) and 305E40 (cluster 3) accessions were also selected for NUE-contrasting performance and because they are parents of a RIL segregant population recently developed from a F2 population (Toppino et al. 2016). Moreover, the genotype 67-3 was also sequenced (Rotino et al. 2014; http://www.eggplantgenome.org/). The genotype 67-3 showed a rather similar phenotype to AM22, while 305E40 showed a root morphology similar to AM222.

2.1.4. Enzyme activities

The activity of close related NUtE enzymes NR, GS, and GOGAT were determined in roots and shoots of the selected genotypes, at both LN and HN levels (Figure 6). At HN, all the accessions showed a higher NR activity in shoots without significant differences among them; while, at LN, 67-3 exhibited a significant higher activity compared to the others and to its own activity at the HN level (Figure 6A). Conversely, in roots, NR activity was influenced only by nitrate supply in all the accessions (Figure 6B). Different responses for GS activity were observed in shoots, among genotypes (Figure 6C, D). In particular, at LN, 67-3, and AM222 showed a significant higher GS activity compared to the others, whereas AM22 and 305E40 showed a significant higher activity at HN (Figure 6C). In roots, AM222 exhibited a strong GS activity at HN, while it was higher in 67-3 at LN; AM22 and 305E40 did not show any difference between N levels (Figure 6D). Finally, in both plant tissues, GOGAT activity pointed out a higher activity in all the accessions at HN, showing the higher activity in 305E40 shoots. No difference was evident among the accessions at LN (Figure 6E, F).

2.1.5. Expression analysis of candidate genes for nitrate uptake and assimilation in eggplant

Gene expression data were analyzed by two-way ANOVA (Table S3). Distinct patterns of nitrate transport and N metabolism key genes were observed in both roots and shoots in NUE contrasting eggplants (Figure 7). In roots, the transcription levels of N uptake and assimilation related genes

underlined different responses of genotypes to LN and HN. In particular, at LN, 67-3 showed a different gene expression pattern compared to AM22, AM222, and 305E40, with a significant higher transcript level of SmCLCa, SmGS2, SmNR, and SmNiR (Figure 7A). Furthermore, AM222 displayed a distinguishable pattern due to higher expression of SmNRT2.1, SmNRT3.1, SmGS1, and SmGOGAT, compared to AM22 and 305E40 (Figure 7A). At HN, 67-3 confirmed its different behavior compared to the others, due to the higher and lower expression levels of SmCLCa and SmNRT1.1, SmNRT2.1, SmNRT3.1, respectively. AM222 exhibited a high expression level of SmGS1 and SmGOGAT, while AM22 showed a marked expression of SmNRT1.1, SmNRT3.2, SmNR, and SmNiR genes (Figure 7A). In shoots, at LN, AM22 was characterized by the lowest expression level in all the genes (Figure 7B). By contrast, 67-3 showed a significant higher expression of SmNR, SmNiR, SmGS2, and SmCLCa; while SmGOGAT and SmGS1 expressions were higher in AM222 and 305E40, respectively (Figure 7B). Interestingly, considering the shoots at HN, 67-3 and AM22 were grouped in the same cluster as a consequence of a higher expression level in all the genes. In addition, SmCLCa appeared significantly higher expressed in AM22 respect to the other genotypes (Figure 7B). Remarkably, AM222 showed a higher expression of both SmGS1 and SmGOGAT compared to AM22 and 67-3 (Figure 7B).

2.2.Greenhouse pot experiment

2.2.1. Biomass and yield production

AM22, 67-3, AM222, and 305E40 eggplant genotypes were grown in a greenhouse pots experiment until berries reached commercial ripening. Genotypes exhibited different responses to HN and LN levels in terms of biomass production. Fruit, leaf and stem dry weights (FDW, LDW, and SDW, respectively) showed a significant increase in all the genotypes grown at HN compared to LN (Figure 8; Table S2). At HN, AM222, 67-3, and 305E40 exhibited similar performances for FDW, but significantly higher than AM22. However, AM22 appeared more sensitive to nitrate, increasing its FDW by six-fold from LN to HN treatment (Figure 8A). By contrast, AM22 displayed the highest LDW value at HN, whereas no significant differences were observed among the other accessions.

Interestingly, no differences among accessions were observed at LN (Figure 8B). Finally, 305E40 showed the highest SDW compared to the other accessions at HN, while at LN no significant differences were observed (Figure 8C).

2.2.2. Nitrogen content

The N content in fruits, leaves and stems was significantly increased in all the accessions at HN compared to LN treatment. In detail, AM222 showed the higher fruit N content at HN compared to the other accessions, which in contrast, did not exhibit significant differences among them (Figure 9A). At LN, a similar trend was observed among genotypes. Interestingly, a significant difference was evident between AM22, the N-inefficient genotype, having a lower N content than AM222, the N-efficient one (Figure 9A). By contrast, AM22 showed a significant higher leaf N content at HN, whereas at LN the accessions did not show any significant differences (Figure 9B). Moreover, 305E40 showed the highest stem N content at HN, while at LN no differences among accessions were exhibited (Figure 9C).

2.2.3. Nitrogen balance index

Leaf chlorophyll content index, estimated with the DUALEX instrument, was higher at HN compared to LN, regardless genotypes, which did not significantly differ among them (Figure 10A). Conversely, flavonoids content index in leaf epidermal showed an inverse trend, resulting higher at LN (Figure 10B). Therefore, Nitrogen Balance Index (NBI) showed a similar pattern to the chlorophyll index (Figure 10C). Correlations between Genotype x N-level, C and N leaf contents highlighted significant values between chlorophyll and N content ($r = 0.85$, $P < 0.008$), flavonoids and N content ($r = -0.74$, $P < 0.04$) as well as NBI and N content ($r = 0.80$, $P < 0.02$). Significant correlations resulted also for chlorophyll, flavonoids and NBI related to the leaf N/C ratio, $r = 0.85$, ($P < 0.008$), $r = -0.75$, ($P < 0.04$) and $r = 0.81$ ($P < 0.02$), respectively. Although not significantly different, chlorophyll content index at HN tended to be lower in 67-3 and AM222 genotypes, while NBI was significantly lower in 67-3 compared to the other genotypes. At LN, flavonoid content index was significantly lower in AM222 compared to 305E40 (Figure 10C).

2.2.4. Nitrate use efficiency in pot experiment

NUE calculation confirmed significant differences among contrasting genotypes, as already observed in the hydroponic experiment. At LN, AM222 was characterized by a significant highest NUE compared to the other accessions, whereas, at HN, AM22 showed the lowest NUE compared to AM222, 67-3, and 305E40, among which differences were not detected (Figure 11A). Thus, at LN, AM22 together with 305E40 confirmed their N-use inefficiency; at HN, only AM22 showed the lowest performance compared to the other genotypes. Furthermore, at LN, AM222, and 67-3 pointed out significant higher NUtE compared to AM22 and 305E40, while, at HN, no differences among the accessions were evident (Figure 11B). Finally, NUpE was strongly dependent on nitrate level, being highest at HN in all the genotypes. As a rule, no significant differences among the genotypes within each N level were observed (Figure 11C).

3. Discussion

Eggplant yield is toughly related to N fertilizer input, but to date the adaptive plant responses to N-limited fertilization are still poorly understood and disregarded to genetic mechanisms underlying a complex trait like NUE. Given the spatial and temporal soil heterogeneity for nutrients, a significant amount of genetic variation and phenotypic plasticity for NUE appeared not so unexpected (Byers 2005; Han et al. 2015b) and consequently needs to be explored. Therefore, to improve NUE in crops became pivotal to estimate the genetic variability related to this complex trait. Further, to select for high NUE two more key issues should be addressed: i) to operate at limited N supply; (ii) to detect flag traits highly correlated to yield and NUE. In this paper, for the first time, the genetic variation in response to nitrate supply was assessed in eggplant (Solanum melongena L.). A comprehensive framework, through a morphophysiological and molecular approach, was carried out, highlighting the differences in N-use efficiency (NUE) among genotypes. Nineteen eggplants, with different geographical origin and morphological features, were grown under low (LN) and high nitrate (HN) levels in hydroponic system. Morphological and physiological traits were analyzed allowing us to identify NUE contrasting eggplant accessions in response to LN and HN supply. Finally, the selected

genotypes were grown in greenhouse pots experiment up to berries ripening and harvesting, analyzing NUE and some related traits to confirm hydroponic data and NUE-contrasting genotypes. In the last decades, NUE-related traits were isolated and mapped by Quantitative Trait Loci (QTL) analysis (Xu et al. 2012). Thus, 67-3 and 305-E40 accessions were included in our collection, being the parents of a RIL segregant population already available, which may be employed in future experiments for isolating molecular markers linked to QTL of NUE interest. In addition, the line 67-3 was subjected to genome sequencing by the "Italian Eggplant Consortium".

3.1.Characterization of eggplants in hydroponic system

Biomass allocation (SDW and RDW) among accessions showed a more marked variability in root than shoot (CV 0.44 vs. 0.37), suggesting a different adaptive NO3- dependent response in root traits. These results confirmed the role of nitrate as either nutrient or signal also in eggplant root development, as previously reported in other species (Crawford 1995; Zhang and Forde 2000; De Pessemier et al. 2013). As root system is devoted to nutrient exploration and acquisition from soils (Lynch 2013; Li et al. 2015; Mu et al. 2015), mainly in starved conditions, morphological root traits were investigated. Contrasting response for root traits could depend on nutrient availability in soil and source allocation between roots and shoots (Ikram et al. 2012). Thus, the selection for high "root foraging" may represent a key point or flag trait to breed eggplants for higher NUE, based on the correlation between N uptake and QTLs for root morphological traits (Coque et al. 2008). Undeniably, eggplant roots exhibited a marked plasticity (phenotypic variation), mainly in lateral root length (LRL) and their number (LRN), characterized by higher coefficient of variation (CV), compared to other traits, mainly at LN. Indeed, root growth increased along with lateral root branching to improve the supplying ability of the root system at N starved condition (Ikram et al. 2012). Among physiological traits, NUtE was more influenced by genotype than NUE and NUpE. According to these results, NUtE was recently demonstrated more determinant than NUpE in NUE features in tomato (Abenavoli et al. 2016; Lupini et al. 2017), representing a useful trait to improve

the utilization efficiency in Solanaceae. Therefore, it is noteworthy that the ability to utilize nitrate (NUtE) showed a significant phenotypic variation due to the genetic variance, underlined a rather high heritability (Table 1). Conversely, NUE appeared poor affected by the nutrient conditions (N level) as already reported in other species, such as Arabidopsis and maize (Bertin and Gallais 2001; Coque et al. 2008; Chardon et al. 2010). Through the hierarchical clustering, the pair of Nuse efficient/inefficient genotypes AM222/AM22 based on their different origin (Turkey vs. China), genetic distance (Cericola et al. 2013) and their extreme contrasting root traits, were selected. In particular, AM222 exhibited high NUE, Lateral Root Length (LRL) as well as RLR and LRN, while AM22 showed low NUE and its components (NUpE and NUtE), associated with a reduced root system, low TNA and high root mass ratio. Further, other two contrasting lines, 305E40 and 67-3, parents of an already available RIL population, with a similar behavior to AM222 and AM22, respectively, were also included. Thus, the selected four genotypes were further analyzed for different N metabolism enzymes as well as gene expressions. Differential gene expression levels among the contrasting genotypes on the nitrate transporters SmNPF6.3 and SmNRT2.1 involved in root formation and acquisition (Garnet et al. 2009), confirmed the role of root system to confer N-use efficiency (Figure 7). Indeed, root size plays a critical role in nutrient uptake in tomato (Abenavoli et al. 2016; Lupini et al. 2017), maize (Li et al. 2015) and rapeseed (Wang et al. 2017). In eggplants, the N-key gene expression and enzyme activities highlighted a strong correlation with N-efficiency. In particular, the efficient genotype (AM222) showed favorable features related to NUpE (transporter gene expressions) and NUtE (enzyme activities). Indeed, the efficient genotype AM222 showed a marked higher expression of genes belonging to the high affinity transport, SmNRT2.1 together with SmNAR2.1 (also named NRT3.1; Léran et al. 2014), which encodes a protein partner needed for transporting nitrate (Quesada et al. 1994; Okamoto et al. 2006; Orsel et al. 2006; Lupini et al. 2016). In Arabidopsis, AtNAR2.1 and AtNRT2.1 resulted partner in HATS activity (Orsel et al. 2006; Okamoto et al. 2006) and Yong et al. (2010) later demonstrated the specific role of AtNAR2.1. Recently, the pivotal role of NAR2 for HATS activity was confirmed by an improved yield and NUE

in transgenic rice harboring the construct OsNAR2.1:OsNRT2.1 where OsNAR2.1 promoter was able to enhance OsNRT2.1 expression level, compared to its native promoter (Chen et al. 2017). In eggplants, for the first time, a co-functionality of SmNRT3.1 (=SmNAR2.1) together with SmNRT2.1 was highlighted, according to gene expressions. Indeed, SmNRT3.1 was co-expressed with SmNRT2.1, and both genes were more expressed in AM222 when compared to the other accessions, resulting in a consistent increasing of shoot biomass (SDW; Table S1). Interestingly, the functionality of SmNRT3.2 (another member of NRT3 gene family), which was upregulated at HN yet remaining unclear. Focusing on NUtE, our result indicated that AM222 (together with 67-3) was also characterized by higher GS enzyme activity at N-limiting condition, as well as SmGS2 transcript level in shoots. Functional genomics and QTL approaches have already showed a correlation between GS enzyme activity and N use efficiency (Bernard and Habash 2009). Moreover, the variation of GS (either GS1 or GS2) expression, as well as the enzyme activity were demonstrated to affect nitrate metabolism and NUE in several plants (Eckes et al. 1989; Miao et al. 1991; Fei et al. 2003; Brauer et al. 2011). However, further genomics studies may be useful to identify allelic variation or transcription factor to better understand the primary role of GS in NUtE.

3.2.Plasticity, genetic variation and heritability

Since the beginning of last century, Wright (1931) suggested that, within a population, individual phenotypicplasticity could be “perhaps the chief object of selection”. More recently, the increasing attention to plant plasticity responses to complex environments was paid for deeper understanding the residual quote of genetic variability (G × E) in plasticity useful for selection, as complementary to genetic heritability (e.g., Hedrick 1986; Schlichting 1986). Thus, the development of methods for quantifying he genetic component of phenotypic plasticity became a precious key-point. Scheiner and Lyman (1989) indicated phenotypic plasticity and its heritability calculation, considering their variation among populations and environments. Knowing the features of a specific trait, it was important to make a prediction about population response to selection (selection gain that is direct function of heritability) or to environmental variation (plant plasticity and its genetic component).

Here we reported broad sense heritability, plasticity and its genetic component related to shoot and root dry weights, root traits as well as N-use efficiency and its components (Table 1). Interestingly, our results underlined a high G × E interaction of target traits. By contrast, Han et al. (2015b) recording a limited G ×E interaction, obtained a weak selection gain. Frequently, high differences between h2 estimated at different conditions (here LN and HN) indicated traits with lower heritability and higher plasticity and vice versa. These contrasting features were already discussed between plasticity and its heritability (Scheiner and Lyman 1989), leaving out the huge amount of genetic variability that is considered here. Among traits, root mass ratio (RMR), root length ratio (RLR) and specific root length (SRL) showed the highest values of plasticity (PL). By contrast, root fineness (RF) and NUtE showed h2B values >0.7 together with the lowest PL values. Interestingly, traits with higher plasticity showed frequently high plastic heritability, which being due to a high G × N variance underlined an interesting residual quote of genetic variability useful for selection (Table 1). Furthermore, it is noteworthy that total root length (TRL), lateral root length (LRL), RF and NUtE, exhibiting h2B values >0.6, appeared significantly correlated with NUpE and NUE (Table 2). These results agreed with the statement that root system architecture (RSA) is related to plant ability to uptake N from soils and might affect NUpE (Foulkes et al. 2009). Although RSA was a target trait involved in NUpE/ NUE in model plant and crops (Zhang and Forde 1998; Garnett et al. 2009), the manipulation of RSA-related genes affecting N-uptake and NUE in crops remain a challenge (McAllister et al. 2012). As alternative, the sole upregulation of key N-transporters encoding genes would increase NUpE, as stated by Heidlebaugh et al. (2008).

3.3.Greenhouse experiment

The four selected eggplant accessions were grown in plastic pots in a greenhouse until yield production to calculate the agronomic NUE. First, the optimization of nitrogen (N) fertilization rate was deeply studied to avoid overfertilization, together with the monitoring of plant N status (Tremblay et al. 2012). Several strategies based on plant sensor diagnostic were proposed to define nondestructive methods. Fluorescence-based technologies provide new N status indicators by using

direct measurements (chlorophyll and flavonoids) or its derived index (NBI; Cartelat et al. 2005). NBI in eggplant genotypes at different N supply was monitored by DUALEX, and at the same time, C and N were analyzed by destructive method. Although a perfect correlation between DUALEX indexes and leaf C and N contents (C-N analyzer) were found, no significant differences at each N level (LN and HN) among genotypes were highlighted. However, AM222 showed the highest NBI at both N levels (Figure 10C), indicating the best N status compared to the others. Based on N fruit content, NUE and NUtE component, AM222 and AM22 were confirmed as NUE efficient and inefficient genotypes, respectively. Unexpectedly, similar performance to AM222 was observed in 67-3 that, in contrast with the hydroponic experiment, can be considered as N-use efficient genotype compared to 305E40 that appeared as N-use inefficient. The variations observed in fruit production among accessions may be due to a different source-sink balance, determined by different N translocation and remobilization, which strongly affected NUtE. Differences in NUpE were nitrate dosage-dependent, regardless genotypes. By contrast, genotypic variations were displayed for NUtE, where AM222 appeared more efficient to utilize N, compared to the others. NUtE is highly related to N remobilization ability, and leaves are the organ more active for this physiological process (Masclaux-Daubresse et al. 2010). Our results displayed a lower N leaves content in the efficient genotype AM222, thereby indicating a high N-utilization and re-mobilization efficiency, compared to the others. By contrast, a high N allocation in leaves and stem of AM22 and 305E40, respectively, which confirmed their N-use inefficiency (Figures 9, 11). Furthermore, the inefficient genotype AM22 showed a significant higher leaf dry weight (LDW), while AM222 produced a limited vegetative biomass (leaf and stem) addressing the resources to the fruits (Figure 8). Our results confirmed that differences in the nitrogen remobilization during the life cycle among the accessions might explain NUE different performances, mainly at N-limiting condition. Different enzymes involved in N-translocation and remobilization could determine the differences in NUtE observed in both experiments. QTL analysis in maize revealed a co-segregation between GS genes and physiological traits related to productivity (Hirel et al. 2001), and more recently a linkage between

NUE and GS/GOGAT was reported in wheat (Quraishi et al. 2011). AM222 exhibited higher GS activity and genes related expression, which might be responsible for high NUtE, confirming the results obtained in hydroponic system. Thus, NUtE, fruit dry weight and GS could be considered the best flag traits to identify rapidly high NUE eggplant genotypes, as observed in AM222. In conclusion, two NUE-contrasting genotypes (AM222/AM22) were selected in growth chamber/hydroponic experiment considering simple key traits, confirmed in greenhouse pots experiment by crop yield. Noteworthy, hydroponic 305E40 accession showed a higher NUE and NUE-related traits values, such as SDW, TRL, LRL, LRN, and NUtE, while these results appeared reversed in the greenhouse experiment, where 67-3 showed NUE performances similar to AM222, considered a high NUE genotype in both experiments. Based on NUE-contrasting performances, RILs might be phenotyped and genotyped to detect molecular markers (such as SSR and SNP) linked to major genes/QTL related to RSA and NUE. Interestingly, a high heritability (h2B) and G× E interaction of target traits, such as LRL, RF and NUtE, were observed that may avoid a weak selection gain. Moreover, the greenhouse pots experiment demonstrated that the remobilization of N stored from leaves to the fruits could be the key to enhancing NUE in eggplant. Then, molecular and physiological mechanisms indicated GS as a key enzyme influencing NUE, mainly its NutE component. Further omics studies (transcriptomic and metabolomics) focused on C-N metabolism networks may pave the way towards the selection of more resilient eggplant with regard to N-requirement.

4. Material and Methods

4.1.Plant material

Nineteen eggplant (Solanum melongena L.) accessions were selected from a large germplasm collection (Table S4, Cericola et al. 2013) which included genotypes of different geographic origin from Mediterranean Basin (Turkey (5), Italy (3), Spain (1)) and Asia (Thailand (4), China (2), India (2)), and a wide variability for fruit size, shape, color and plant growth habit. Furthermore, two breeding lines were included (67-3 and 305E40), parents (male and female, respectively) of a

segregant population already established and characterized as F2 (Toppino et al. 2016), which nowadays become a recombinant inbred lines (RIL) population.

4.2.Hydroponic system growth conditions

Seeds, surface sterilized with 5% (v/v) NaClO for 15 minutes and rinsed with deionized water, were then germinated in Petri dishes (Ø 90 mm) on filter paper with 0.1 mM CaSO4. After 10 days, seedlings with fully expanded cotyledons, were selected by uniform size and transferred in hydroponic tanks (4 L, ten seedlings for tank) containing 2.5mM K2SO4, 2mM MgSO4, 1mM KH2PO4, 46 µM H3BO3, 9µM MnCl2 , 0.76µM ZnSO4, 0.32µM CuSO4, 0.11µM Na2 MoO4, and 20µM Fe-EDTA. Nitrate was added to solution as Ca(NO3)2 at 0.5mM (low nitrate, LN) or 10mM (high nitrate, HN) concentrations. Furthermore, in order to balance Ca2+, 4.75mM CaSO4 were added in the LN solution. Then, the growing units were transferred to a growth chamber at 24°C, 65% relative humidity and 14 h photoperiod with 350 µmol m2 s−1 light intensity for 18 days. The nutrient solution was renewed every three days and the pH was adjusted to 5.8with 1 N KOH.

4.3.Morphological root analysis

Five plants (28 days old) for each genotype, exposed to LN and HN for 18 days, were collected and divided into shoots and roots. Roots were dipped in 0.1% (w/v) toluidine blue (Sigma Aldrich, #89160) for 5min and then scanned at 300 dpi resolution (WinRhizo STD 1600, Instruments Règent Inc., Quebec, Canada) to determine primary (PRL; cm), lateral (LRL; cm) and total root length (TRL; cm), and volume (cm3) using WinRhizo Pro System v. 2002a software (Lupini et al. 2016, 2017). Lateral root number (LRN, #) was manually counted as reported by Lupini et al. (2014). Shoots and roots were then dried at 72°C for 48 h to determine their dry weight (SDW and RDW, respectively, g). Based on the above measurements, 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 also calculated (Lupini et al. 2016, 2017).

4.4.Nitrogen concentration and Nitrogen Use Efficiency

Total nitrogen content (Nc, mg N) was determined by combustion method through a LECO-CNS-1000 analyzer (LECO Instruments Ltd., Mississauga, ON, Canada) as reported by Lupini et al. (2017). Nitrogen Use Efficiency (NUE, SDW N%-1, where N% is the g N (100 g DW)-1) (Chardon et al. 2010) and Nitrogen Utilization Efficiency (NUtE, SDW2 Nc-1) (Siddiqi and Glass 1981) were calculated. Nitrogen Uptake Efficiency (NUpE) was also estimated as total (shoot+root) dry weight (TDW) x N concentration (g N g TDW-1) (Chardon et al. 2010). The mean is the average value of five plants.

4.5.Enzyme activities

Enzyme activities of related-nitrogen metabolism (Nitrate Reductase, NR; Glutamine Synthetase, GS; and Glutamine Oxoglutarate Aminotransferase, GOGAT) were assayed on root and shoot of the selected genotypes grown at both N levels. Nitrate reductase (NR) enzyme was extracted using 50mM MOPS-KOH buffer (pH 7.8) containing PVP 0.5% (w/v), 5mM NaF, 1 μM Na2MoO4, 10μM FAD, 1 μM leupeptin, 2 mMβ-mercaptoethanol, 5mM EDTA. Then, the homogenate was centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatant was immediately incubated at 30°C for 35 min in a solution containing 50mM MOPS-KOH buffer (pH 7.6), supplemented with 5mM NaF, 10mM KNO3, 155 μM NADH, and 5mM EDTA. After, the reaction was stopped with 5.8mM sulfanilamide and 0.8 mM N-naphthyl-ethylene-diamine-dichloride (NNED). The absorbance was measured at 540 nm (Foyer et al. 1998). Glutamine synthetase (GS) enzyme was extracted using 25mM Tris-HCl buffer (pH 7.6), 1mM MgCl2, 1 mM EDTA-Na2, 14mM β-mercaptoethanol and PVP 1% (w/v), and the activity was measured according to Debouba et al. (2007), using hydroxylamine as substrate (Wallsgrove et al. 1979). Finally, GOGAT activity was measured by the decrease in absorbance at 340 nm of the NADH oxidation according to Groat and Vance (1982). All procedures of enzymes extraction were carried out at 4°C. Total soluble protein was estimated using bovine serum albumin as standard, according to Bradford (1976). The measurement of absorbance was performed using a UV-Vis spectrophotometer (Perkin Elmer Lambda 35, Walthman, MA, USA). Enzyme activities were performed with eight biological replicates and each replicate was apool of three plants.

4.6.RNA isolation and qRT-PCR

Shoots and roots of selected genotypes (28 days old), grown in hydroponic and exposed to LN and HN for 18 days, were collected (three independent biological replicates for each sample) and immediately frozen in liquid nitrogen. Total RNA was extracted from 100mg of eggplant roots and shoots using “RNeasy Plant Mini Kit” (Qiagen) according to the technical bulletin. RNA was subjected to DNase treatment using the “Precision DNase Kit” (Primer Design) and then reverse transcribed into cDNA with “QuantiTect Reverse Transcription Kit” (Qiagen) following the manufacturer’s protocol. Gene expression was analyzed using quantitative realtime polymerase chain reaction (qRT-PCR), which was performed with a Rotor-Gene RG-6000 thermal cycler (Corbett Research). To amplify the gene fragments, cDNA template diluted 1:10 was used in a 15 µL reaction with 7.5 ul of SYBR Green (IQTM Supermix Master Bio-Rad) and 0.5–1.2 uM of gene-specific primer listed in Table S5. The amplification reactions were carried out as follows: 95°C for 5 min, followed by incubation for 15 s at 95°C and denaturation for 15 s at 95°C, annealing for 60 s at 59°C for 40 cycles, followed by elongation at 72°C for 20 s. Specificity of amplification was assessed by melt curves analyzed for the presence of a single peak. The analysis was done on three biological replicates and in technical triplicate. A relative standard curve for each gene was generated using a two-fold serial dilution of pooled cDNA (obtained mixing equal proportion of all cDNA samples) using the Rotor gene software (Q-Rex Software version 1.0, QIAGEN). PCR efficiency of primer pairs was optimized to be in the range 92%–100% with R2 values of 0.996. The DNA sequences of candidate NUE-related genes were kindly provided by the Italian Eggplant Genome Consortium (Rotino et al. 2014). Adenine phosphoribosyl transferase (SmAPRT) and Glyceraldehyde 3-phosphate dehydrogenase (SmGAPDH) from eggplant were used as reference genes (Gantasala et al. 2013; Barbierato et al. 2017). The relative quantification of each gene in different samples was performed using the geometric averaging method (geNorm) (Vandesompele et al. 2002). Gene expression heatmap was obtained by using gplots Rpackage version 3.5.0 (Warnes et al. 2005).

4.7.Eggplant yield and Nitrogen Use Efficiency under greenhouse condition

The performance of four NUE-contrasting eggplant accessions was evaluated in greenhouse experiment at the CREA-Center for Genomics and Bioinformatics Research in Montanaso Lombardo (45° 20′N, 9° 26′E, Italy) from 15th June to 26th October 2017. The AM22, AM222, 67-3, and 305E40 accessions were sown in a 54-hole tray filled with peat (Technic N° 3, from Free peat B.V. Sluiskade NZ 79/80 Vriezenveen, Holland). Plantlets, at the third leaves, were transplanted in plastic pots (13 L) containing 7 kg of sterilized soil: peat: perlite (1:1:1, v:v:v) mixture. The soil was a medium-silty soil collected at the experimental CREA center, and perlite was Agrilit® (Perlite Italiana srl, Corsico, Milano, Italy). Beyond N concentration in soil that was 1.39%, after 28 days 0 and 0 kg ha-1 of calcium nitrate were added (LN and HN treatment, respectively), dissolved in irrigation water, split in six sub-applications taking care to maintain the field capacity. Two additional fertilization with liquid Specialfos from Alfe srl (Pomponesco, MN, Italy), which contains 30% P and 20% K as potassium phosphonate and potassium phosphite was performed by using 1mL/pot dissolved in 500mL of water, after 69 and 116 days. Fruits were harvested when they reached the commercial ripeness stage; 15 harvests were performed and number, fresh and dry weight determined. At the end of the pot experiment (120 days), leaves and stems were separately collected to determine fresh and dry weight as well as nitrogen content as reported above. NUE and its components were calculated as Yield (Dry fruit weight)/Nitrogen supply – Ns (NUE), Nitrogen content/Nitrogen supply (NUpE) and Yield/N content (NUtE), according to Moll et al. (1982).

4.8.Nitrogen Balance Index

A DUALEX instrument (Force A, Orsay, France) was used for the determination of index values for leaf chlorophyll and epidermal flavonoid contents (Goulas et al. 2004; Cartelat et al. 2005). The same day for each plant (at first fruit cluster when the first fruit has reached typical size – BBCH-scale 71–701; Feller et al. 1995), measurements were taken on the central parts of the last fully developed leaf avoiding major veins, abaxially and adaxially. The chlorophyll index used subsequently was based on the measurement on the adaxial leaf surface, the flavonoid index was calculated as the sum of the

index values from measurements on the abaxial and adaxial surface. After the DUALEX reading, a sample from leaf surrounding the measurement site was extracted with a borer, immediately transferred to liquid nitrogen and later on used for determination of C and N contents. The values of leaf chlorophyll index were linearly correlated with leaf chlorophyll content (Cerovic et al. 2012). The ratio of chlorophyll to flavonoid index values was defined as nitrogen balance index (NBI), an indicator of C/N allocation by Cartelat et al. (2005), following the theory that flavonoid synthesis increases when the use of carbon assimilates for growing new organs was limited by nitrogen availability.

4.9.Statistical analysis

The hydroponic experiments were set up in a completely randomized design with at least five replications. All data were checked for normality (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Leven median test). The data were analyzed by two-way ANOVA (genotype and nitrate as main factors), and means were separated by Tukey's honest significant difference (HSD) test ($P<0.05$). In addition, hierarchical classification of accessions was carried out according to the Ward's method (Ward 1963). The coefficient of variation for each parameter was estimated as the ratio of standard deviation to the mean of the whole collection. Statistical analysis was employed using Systat software (Systat Software Inc., Chicago, IL, USA). Phenotypic plasticity (PL), defined as the change in phenotype caused by the environment variation (Bradshaw, 1965), was calculated as the plastic variance ($\sigma 2PL$), which is the sum of the environment and genotype-environment interaction variances: $\sigma 2E+ \sigma 2$ G x E. On the other hand, plasticity (pl) is a quote of phenotypic variance ($\sigma 2P$), calculated as $\sigma 2pl = \sigma 2PL/\sigma 2P$ (Scheiner and Lyman 1989). Finally, the heritable component of the plastic variation due to the genotype-environment interaction (h2PL), was estimated as $\sigma 2G$ x $E/\sigma 2P$ (Scheiner and Lyman 1989). The “‘lmer”’ function of the lme4 package (Bates et al. 2013) in Rv.2.15.1 (Development Core Team R, Vienna, Austria, 2012) was employed and fit a REML-based analysis of variance (ANOVA) model: Phenotype = Genotype + N level + Genotype-by-N Level + Residuals, where N level (low or high) was treated as a fixed effect, and

Genotype and Genotype-by-N level were treated as random effects (Corbeil and Searle 1976). Conversely, trait broad sense heritability (h2B) was calculated as σ2G/σ2P, where σ2G is the genetic variance component (attributable to variation among genotypes), while σ2P is the total phenotypic variance, as previously defined. The same function in R, as reported above, was used to fit a REML based ANOVA model: Phenotype = Genotype + Residuals, where Genotype was treated as a random effect. Greenhouse pot experiment was set up as completely randomized blocks with six replicates for each accession and N-treatment. After checking for normality and homogeneity of variance, the data were analyzed by two-way ANOVA (genotype and nitrate as main factors), and means separated by Tukey's HSD test ($P < 0.05$). Data of relative gene expression were analyzed by two-way ANOVA based on three biological replicates for each treatment by using R software version 3.5.0.

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Table 1

TRAIT	Broad sense heritability (h^2_B)[1] at LN/HN[2]	Plasticity (PL)[3]	Plastic heritability (h^2_{PL})[4]
Shoot dry weight (SDW, g)	0.480/0.565	0.472	0.114
Root dry weight (RDW, g)	0.509/0.489	0.713	0.217
Total root length (TRL, cm)	0.634/0.533	0.578	0.096
Primary root length (PRL, cm)	0.459/0.493	0.723	0.134
Lateral root length (LRL, cm)	0.643/0.542	0.569	0.099
Number lateral root (N#)	0.551/0.448	0.681	0.138
Root length ratio (RLR, root length/whole dry weight, cm g^{-1})	0.494/0.632	0.774	0.186
Root mass ratio (RMR, root dry weight/whole dry weight, g g^{-1})	0.376/0.242	0.807	0.122
Specific root length (SRL, root length/root dry weight, cm g−1)	0.425/0.300	0.750	0.118
Root fineness (RF, root length/root volume, cm cm^{-3})	0.771/0.791	0.382	0.156
Root tissue density (RTD, root dry weight/root volume, g cm^{-3})	0.520/0.409	0.721	0.227
Nitrogen uptake efficiency (NUpE, mg N)	0.517/0.751	0.547	0.181
Nitrogen utilization efficiency (NUtE, SDW^2 TNA^{-1})	0.620/0.706	0.442	0.085
Nitrogen use efficiency (NUE, SDW $N\%^{-1}$)	0.553/0.527	0.576	0.115

1 Heritability was calculated as σ^2_G/σ^2_P, where σ^2_G is the genetic variance component (attributable to variation among landraces), while σ^2_P is the total phenotypic variance.

2 Heritability for each trait were calculated at LN and HN; the first should be utilized for a further selection at limited N availability.

3 Plasticity was calculated as σ^2_{PL}/σ^2_P, where σ^2_{PL} is the plastic variance defined by $\sigma^2_E + \sigma^2_{G \times E}$, which are the environmental and genotype-environment interaction variances, respectively (Scheiner and Lyman, 1989).

4The heritable component of the plastic variation (h^2_{PL}) was finally calculated as $\sigma^2_{G \times E}/\sigma^2_P$ (Scheiner and Lyman, 1989).

Table 2.

Traits **LN**	RDW	SDW	TRL	PRL	LRL	LRN	NUpE	NUE	NUtE	RLR	RMR	SRL	RF	RTD
RDW	**1**	**0,5791**	**0,5396**	0,3542	**0,5206**	**0,5253**	**0,7355**	**0,5454**	**0,5077**	0,0955	0,4534	-0,4515	-0,2108	0,2732
SDW	**0,5791**	**1**	0,4345	0,1734	0,4340	**0,6734**	**0,7946**	**0,6267**	**0,5795**	-0,1483	-0,1081	**-0,4743**	-0,2299	0,4278
TRL	**0,5396**	0,4345	**1**	0,4237	**0,9942**	**0,5517**	**0,7090**	0,4137	0,4158	**0,7318**	0,2072	0,2788	0,4211	0,1791
PRL	0,3542	0,1734	0,4237	**1**	0,3313	0,1523	0,2482	0,0261	-0,0718	0,3061	-0,0616	0,0440	-0,1021	-0,2204
LRL	**0,5206**	0,4340	**0,9942**	0,3313	**1**	**0,5555**	**0,7105**	0,4166	0,4324	**0,7331**	0,2260	0,2814	0,4427	0,2074
NLR	**0,5253**	**0,6734**	**0,5517**	0,1523	**0,5555**	**1**	**0,6802**	**0,8396**	**0,7954**	0,1605	0,0215	-0,1472	-0,0097	0,3783
NUpE	**0,7355**	**0,7946**	**0,7090**	0,2482	**0,7105**	**0,7102**	**1**	**0,6201**	**0,6143**	0,1572	0,1115	-0,3174	-0,0391	0,4040
NUE	**0,5454**	**0,6267**	0,4137	0,0261	0,4166	**0,8396**	**0,6201**	**1**	**0,9723**	-0,0926	-0,1034	-0,3217	-0,1366	0,4376
NUtE	**0,5077**	**0,5795**	0,4158	-0,0718	0,4324	**0,7954**	**0,6143**	**0,9723**	**1**	-0,0317	-0,0713	-0,2487	-0,1157	0,4004
RLR	0,0955	-0,1483	**0,7318**	0,3061	**0,7331**	0,1605	0,1572	-0,0926	-0,0317	**1**	0,2547	**0,7545**	**0,5952**	-0,1894
RMR	0,4534	-0,1081	0,2072	-0,0616	0,2260	0,0215	0,1115	-0,1034	-0,0713	0,2547	**1**	-0,0177	-0,0370	-0,2230
SRL	-0,4515	**-0,4743**	0,2788	0,0440	0,2814	-0,1472	-0,3174	-0,3217	-0,2487	**0,7545**	-0,0177	**1**	**0,5972**	-0,4406
RF	-0,2108	-0,2299	0,4211	-0,1021	0,4427	-0,0097	-0,0391	-0,1366	-0,1157	**0,5952**	-0,0370	**0,5972**	**1**	0,3840
RTD	0,2732	0,4278	0,1791	-0,2204	0,2074	0,3783	0,4040	0,4376	0,4004	-0,1894	-0,2230	-0,4406	0,3840	**1**
Traits **HN**	RDW	SDW	TRL	PRL	LRL	LRN	NUpE	NUE	NUtE	RLR	RMR	SRL	RF	RTD
RDW	**1**	**0,7130**	**0,6794**	0,2359	**0,7275**	**0,6940**	**0,5496**	**0,6247**	**0,6335**	-0,3020	0,4530	**-0,7694**	-0,0325	**0,5297**
SDW	**0,7130**	**1**	**0,7651**	-0,0038	**0,7993**	**0,6029**	**0,8091**	**0,9564**	**0,8610**	-0,4360	-0,2099	**-0,4777**	0,3584	**0,5911**
TRL	**0,6794**	**0,7651**	**1**	0,4032	**0,9892**	**0,6951**	**0,7055**	**0,7623**	**0,7584**	-0,0114	-0,0654	-0,3137	0,3612	**0,5387**
PRL	0,2359	-0,0038	0,4032	**1**	0,3086	0,4226	0,0205	0,1105	0,2101	0,3443	0,2456	-0,0939	-0,1638	-0,1002
LRL	**0,7275**	**0,7993**	**0,9892**	0,3086	**1**	**0,6956**	**0,7240**	**0,7631**	**0,7530**	-0,0807	-0,0299	-0,3732	0,3547	**0,5952**
NLR	**0,6940**	**0,6029**	**0,6951**	0,4226	**0,6956**	**1**	0,3955	**0,6258**	**0,5705**	-0,2027	0,0806	**-0,5746**	0,1990	**0,7061**
NUpE	**0,5496**	**0,8091**	**0,7055**	0,0205	**0,7240**	0,3955	**1**	**0,7442**	**0,9134**	-0,3723	-0,3002	-0,2948	0,3387	0,3353
NUE	**0,6247**	**0,9564**	**0,7623**	0,1105	**0,7631**	**0,6258**	**0,7442**	**1**	**0,8869**	-0,4096	-0,3287	-0,3980	0,3530	**0,5170**
NUtE	**0,6335**	**0,8610**	**0,7584**	0,2101	**0,7530**	**0,5705**	**0,9134**	**0,8869**	**1**	-0,4509	-0,2922	-0,3785	0,2780	0,3605
RLR	-0,3020	-0,4360	-0,0114	0,3443	-0,0807	-0,2027	-0,3723	-0,4096	-0,4509	**1**	0,2806	0,3720	0,0447	-0,2355
RMR	0,4530	-0,2099	-0,0654	0,2456	-0,0299	0,0806	-0,3002	-0,3287	-0,2922	0,2806	**1**	-0,4479	-0,4131	0,0187
SRL	**-0,7694**	**-0,4777**	-0,3137	-0,0939	-0,3732	**-0,5746**	-0,2948	-0,3980	-0,3785	0,3720	-0,4479	**1**	0,2713	-0,4539
RF	-0,0325	0,3584	0,3612	-0,1638	0,3547	0,1990	0,3387	0,3530	0,2780	0,0447	-0,4131	0,2713	**1**	**0,5947**
RTD	**0,5297**	**0,5911**	**0,5387**	-0,1002	**0,5952**	**0,7061**	0,3353	**0,5170**	0,3605	-0,2355	0,0187	-0,4539	**0,5947**	**1**

Bold values indicated significance at $p<0.05$

Tables Legends

Table 1. Heritability (broad sense, h^2_B) of each trait calculated at LN and HN; plasticity (PL) and plastic heritability (h^2_{PL}) were also estimated taking in to account G x N interaction.

Table 2. Pearson's correlation between traits calculated at LN and HN.

Figure 1.

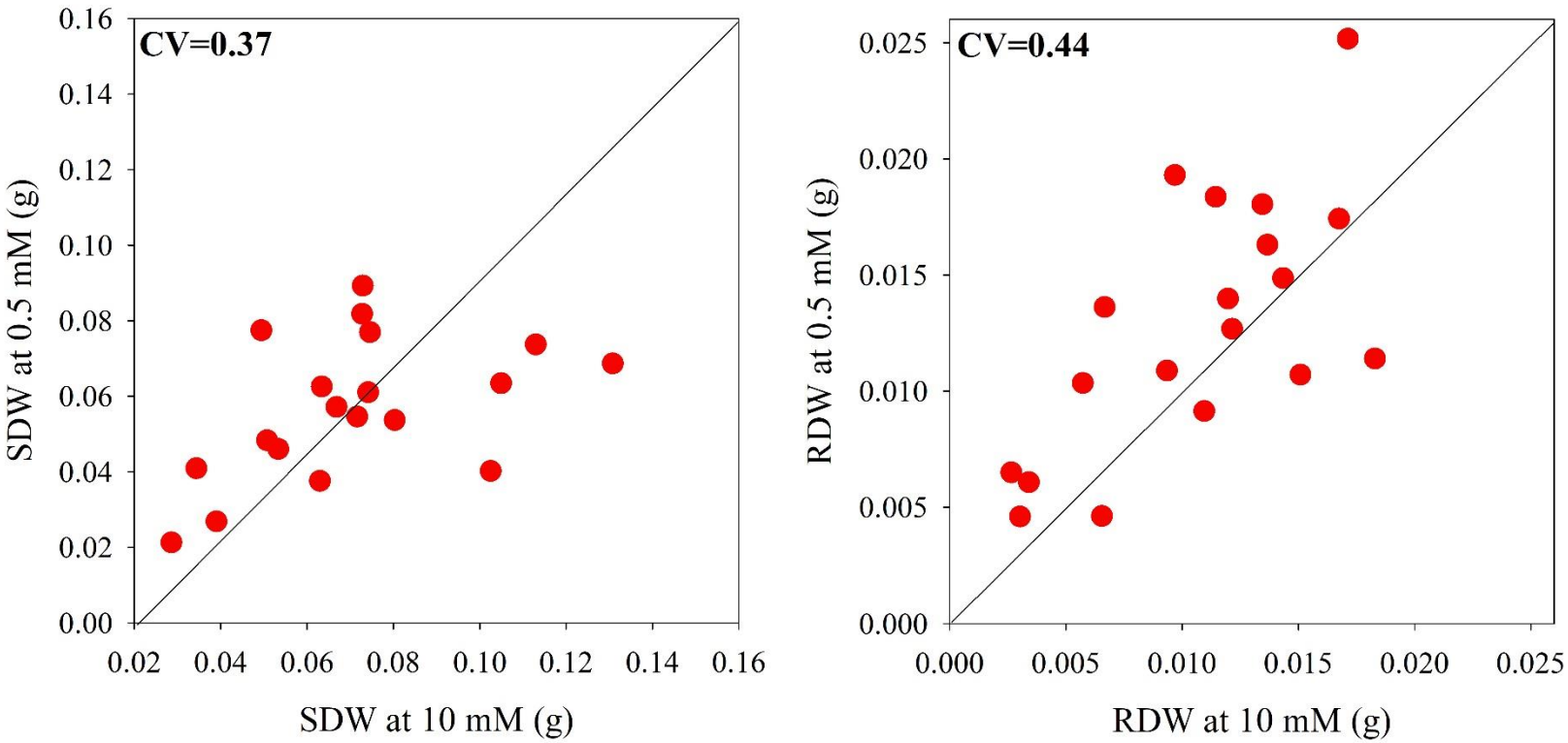
CV=0.37
SDW at 0.5 mM (g)
SDW at 10 mM (g)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
CV=0.44
RDW at 0.5 mM (g)
RDW at 10 mM (g)
0.000
0.005
0.010
0.015
0.020
0.025

Figure 2.

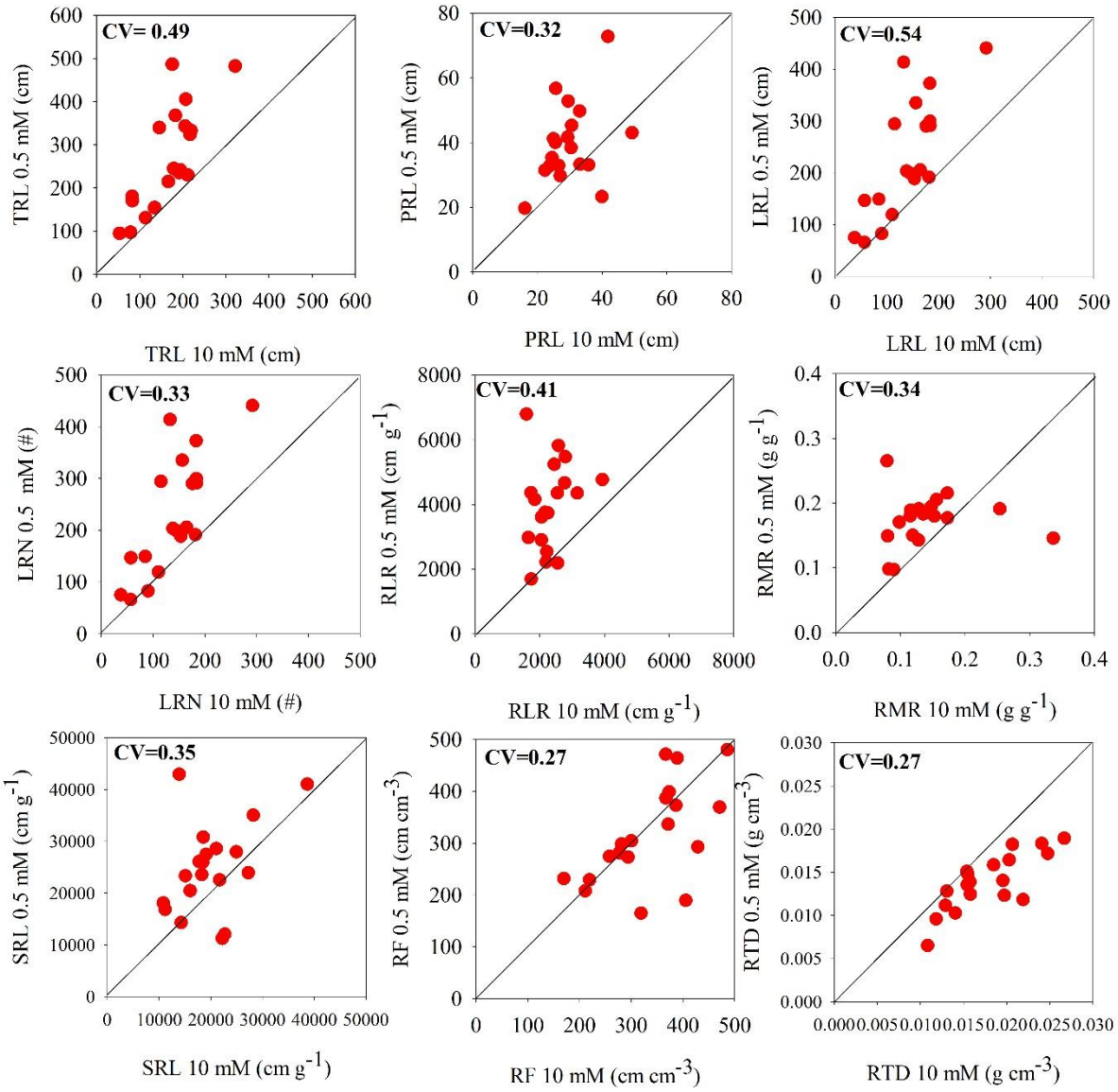
CV= 0.49
TRL 0.5 mM (cm)
TRL 10 mM (cm)
CV=0.32
PRL 0.5 mM (cm)
PRL 10 mM (cm)
CV=0.54
LRL 0.5 mM (cm)
LRL 10 mM (cm)
CV=0.33
LRN 0.5 mM (#)
LRN 10 mM (#)
CV=0.41
RLR 0.5 mM (cm g-1)
RLR 10 mM (cm g-1)
CV=0.34
RMR 0.5 mM (g g-1)
RMR 10 mM (g g-1)
CV=0.35
SRL 0.5 mM (cm g-1)
SRL 10 mM (cm g-1)
CV=0.27
RF 0.5 mM (cm cm-3)
RF 10 mM (cm cm-3)
CV=0.27
RTD 0.5 mM (g cm-3)
RTD 10 mM (g cm-3)

Figure 3.

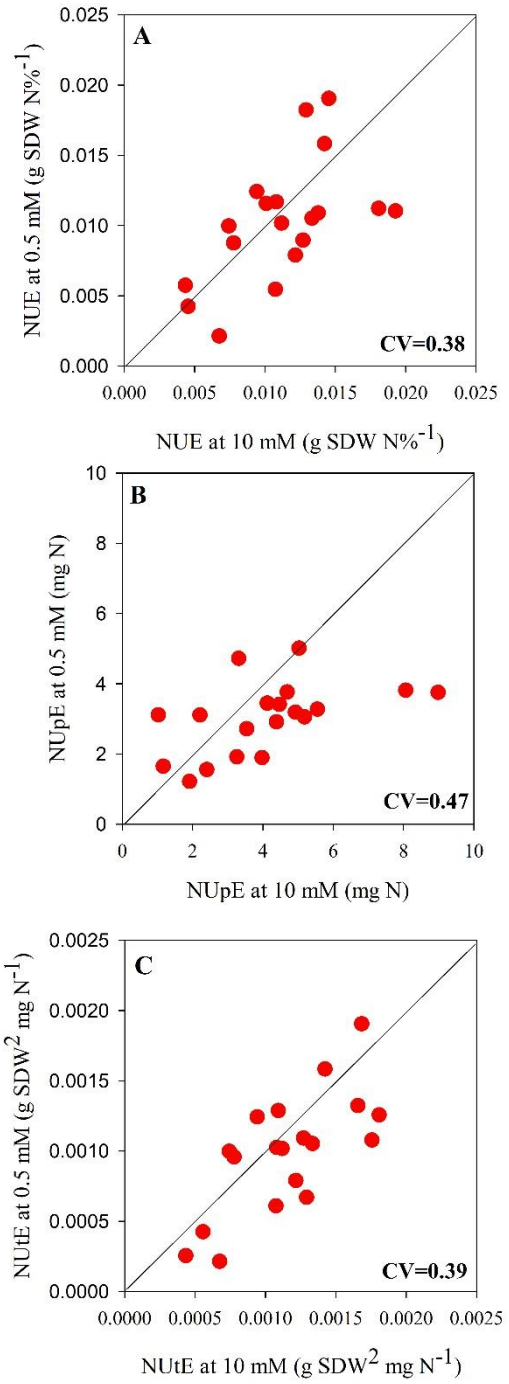
A
CV=0.38
NUE at 0.5 mM (g SDW N%-1)
NUE at 10 mM (g SDW N%-1)
0.000
0.005
0.010
0.015
0.020
0.025
B
CV=0.47
NUpE at 0.5 mM (mg N)
NUpE at 10 mM (mg N)
0
2
4
6
8
10
C
CV=0.39
NUtE at 0.5 mM (g SDW2 mg N-1)
NUtE at 10 mM (g SDW2 mg N-1)
0.0000
0.0005
0.0010
0.0015
0.0020
0.0025

Figure 4.

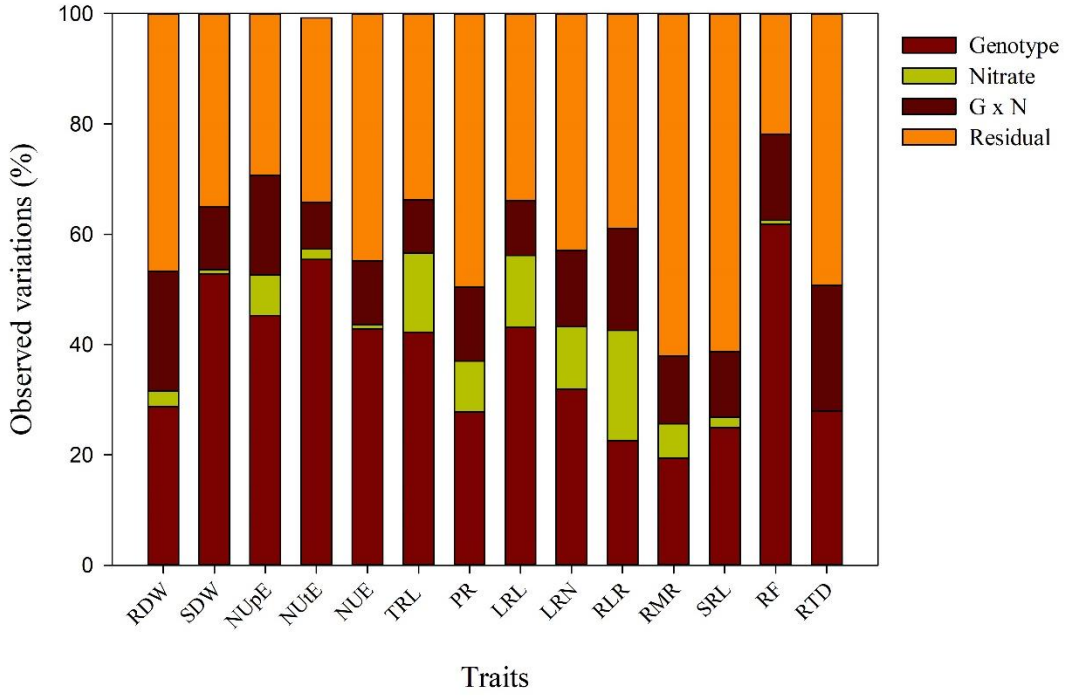
Observed variations (%)
100
80
60
40
20
0
RDW
SDW
NUpE
NUtE
NUE
TRL
PR
LRL
LRN
RLR
RMR
SRL
RF
RTD
Traits
Genotype
Nitrate
G x N
Residual

Figure 5.

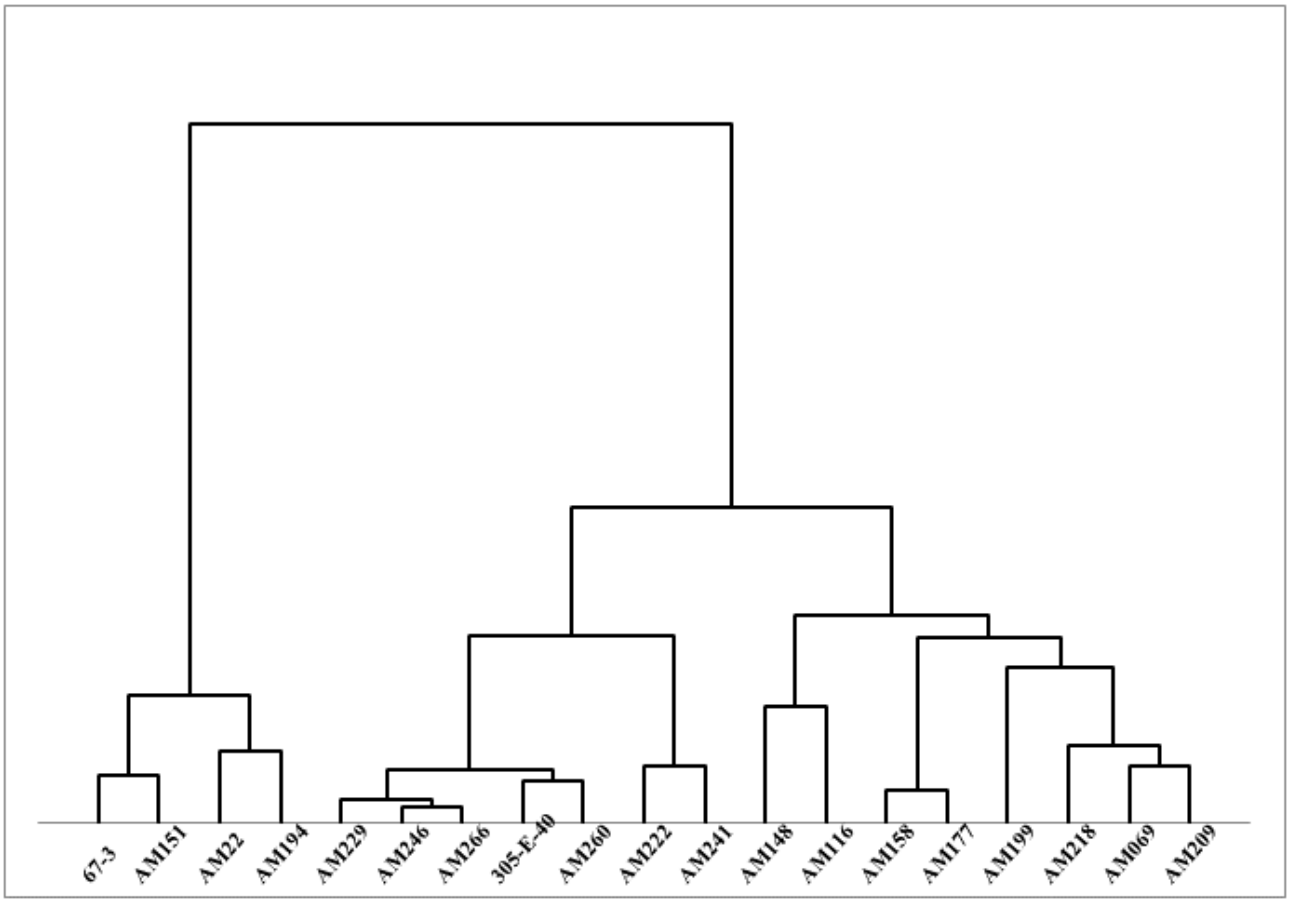
67-3
AM151
AM22
AM194
AM229
AM246
AM266
305-E-40
AM260
AM222
AM241
AM148
AM116
AM158
AM177
AM199
AM218
AM069
AM209

Figure 6.

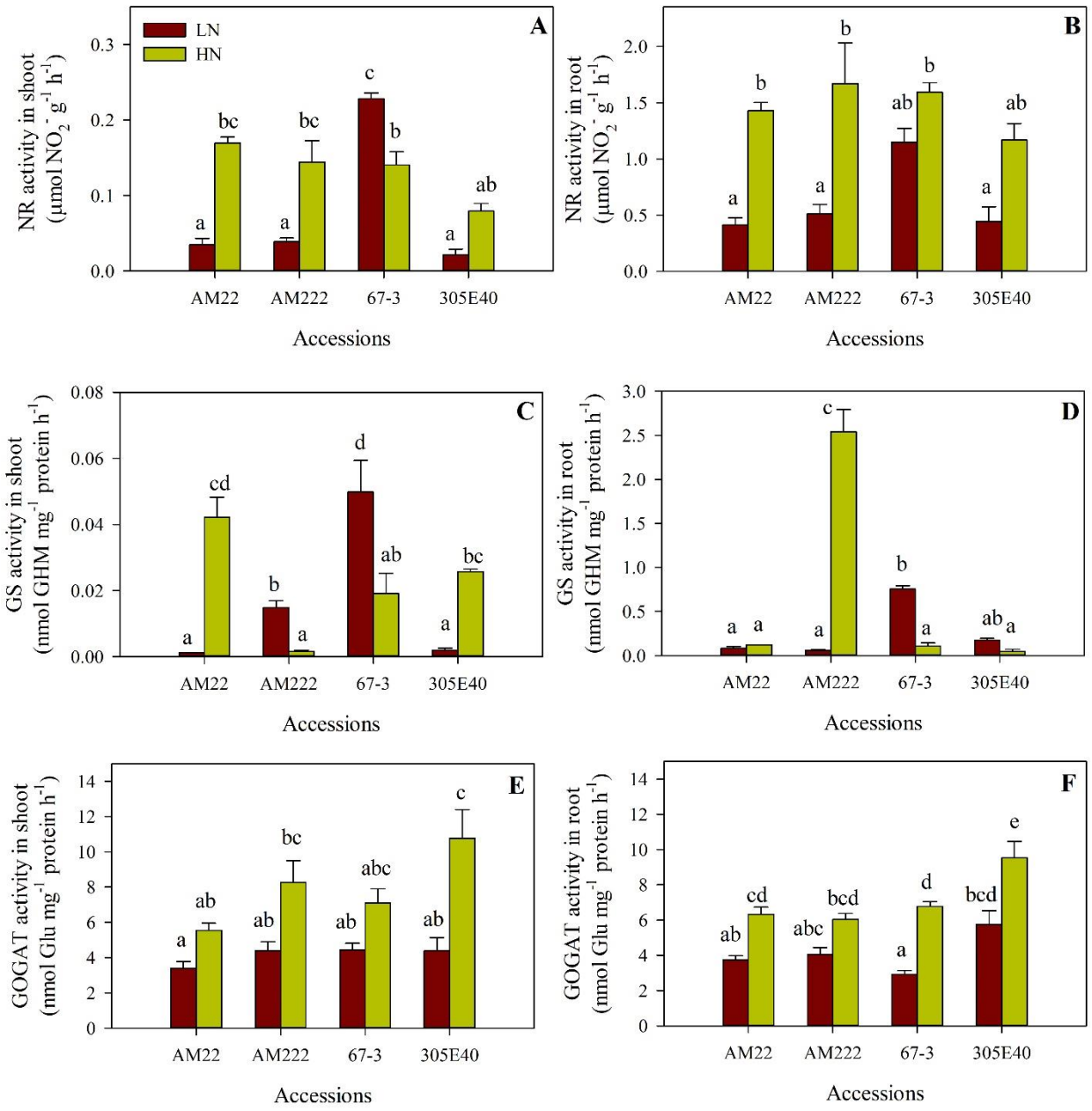
A
LN
HN
NR activity in shoot (μmol NO2- g-1 h-1)
0.0
0.1
0.2
0.3
a
bc
a
bc
c
b
a
ab
AM22
AM222
67-3
305E40
Accessions
B
NR activity in root (μmol NO2- g-1 h-1)
0.0
0.5
1.0
1.5
2.0
a
b
a
b
ab
b
a
ab
AM22
AM222
67-3
305E40
Accessions
C
GS activity in shoot (nmol GHM mg-1 protein h-1)
0.00
0.02
0.04
0.06
0.08
a
cd
b
a
d
ab
a
bc
AM22
AM222
67-3
305E40
Accessions
D
GS activity in root (nmol GHM mg-1 protein h-1)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
a
a
a
c
b
a
ab
a
AM22
AM222
67-3
305E40
Accessions
E
GOGAT activity in shoot (nmol Glu mg-1 protein h-1)
0
2
4
6
8
10
12
14
a
ab
ab
bc
ab
abc
ab
c
AM22
AM222
67-3
305E40
Accessions
F
GOGAT activity in root (nmol Glu mg-1 protein h-1)
0
2
4
6
8
10
12
14
ab
cd
abc
bcd
a
d
bcd
e
AM22
AM222
67-3
305E40
Accessions

Figure 7.

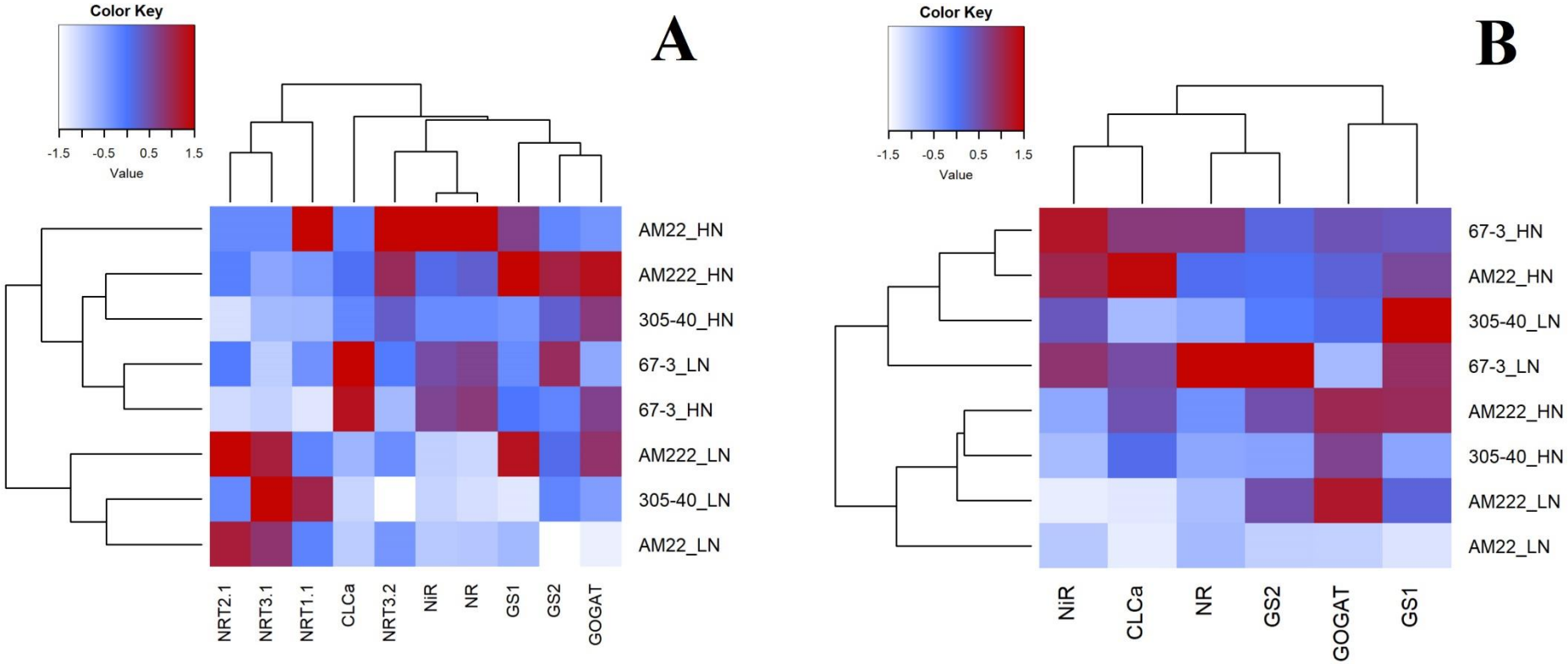
A
Color Key
-1.5 -0.5 0.5 1.5
Value
AM22_HN
AM222_HN
305-40_HN
67-3_LN
67-3_HN
AM222_LN
305-40_LN
AM22_LN
NRT2.1
NRT3.1
NRT1.1
CLCa
NRT3.2
NiR
NR
GS1
GS2
GOGAT
B
Color Key
-1.5 -0.5 0.5 1.5
Value
67-3_HN
AM22_HN
305-40_LN
67-3_LN
AM222_HN
305-40_HN
AM222_LN
AM22_LN
NiR
CLCa
NR
GS2
GOGAT
GS1

Figure 8.

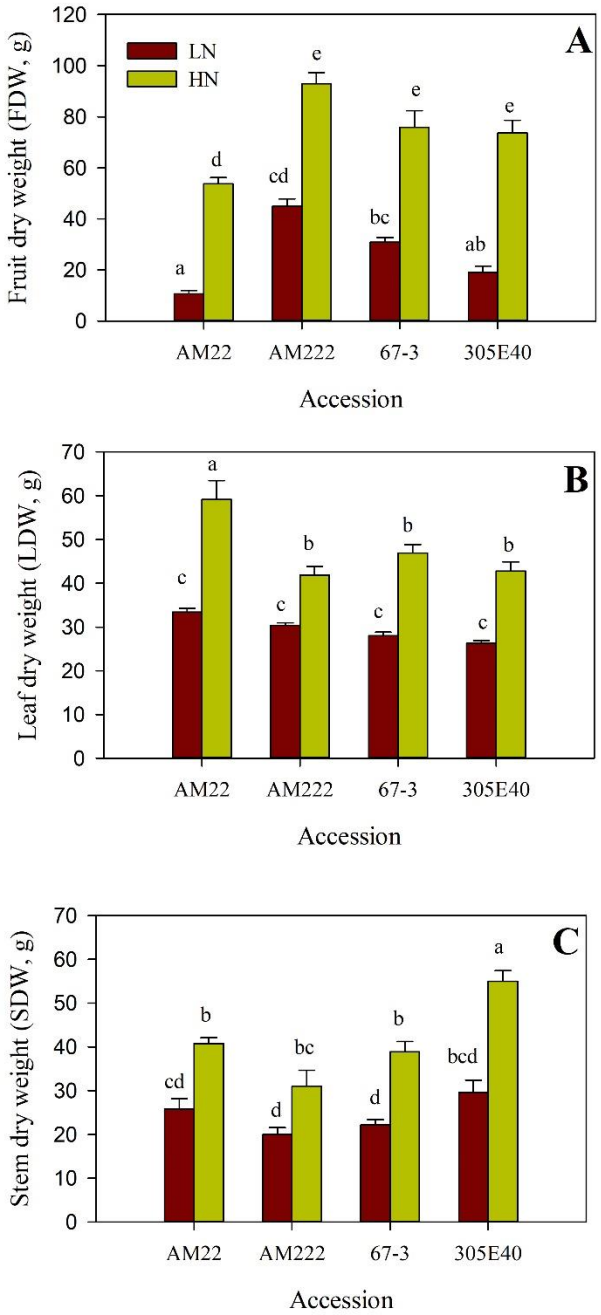
A
LN
HN
Fruit dry weight (FDW, g)
0
20
40
60
80
100
120
a
d
cd
e
bc
e
ab
e
AM22
AM222
67-3
305E40
Accession
B
Leaf dry weight (LDW, g)
0
10
20
30
40
50
60
70
c
a
c
b
c
b
c
b
AM22
AM222
67-3
305E40
Accession
C
Stem dry weight (SDW, g)
0
10
20
30
40
50
60
70
cd
b
d
bc
d
b
bcd
a
AM22
AM222
67-3
305E40
Accession

Figure 9.

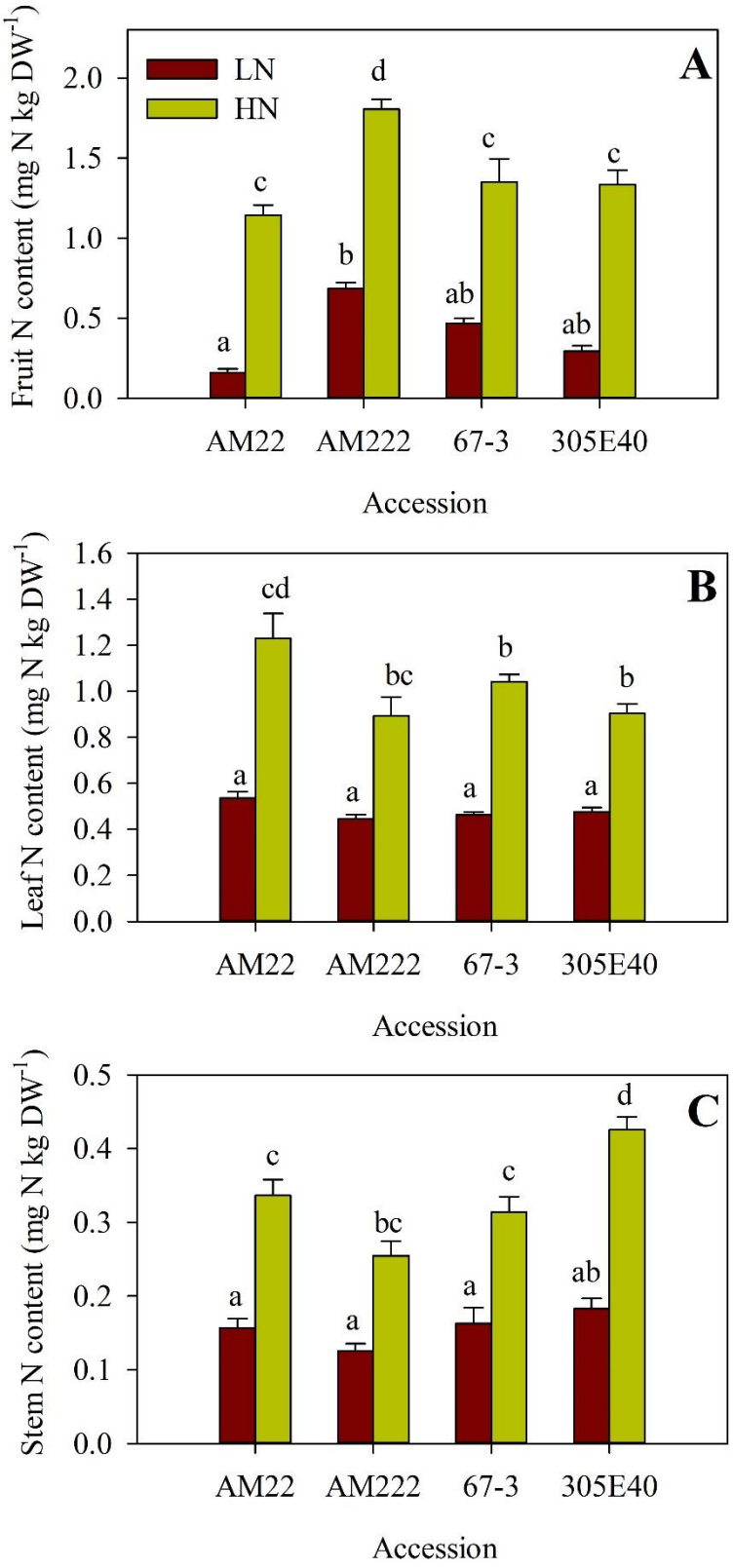
A
LN
HN
Fruit N content (mg N kg DW^{-1})
2.0
1.5
1.0
0.5
0.0
a
c
b
d
ab
c
ab
c
AM22
AM222
67-3
305E40
Accession
B
Leaf N content (mg N kg DW^{-1})
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
cd
a
bc
a
b
a
b
AM22
AM222
67-3
305E40
Accession
C
Stem N content (mg N kg DW^{-1})
0.5
0.4
0.3
0.2
0.1
0.0
a
c
a
bc
a
c
ab
d
AM22
AM222
67-3
305E40
Accession

Figure 10.

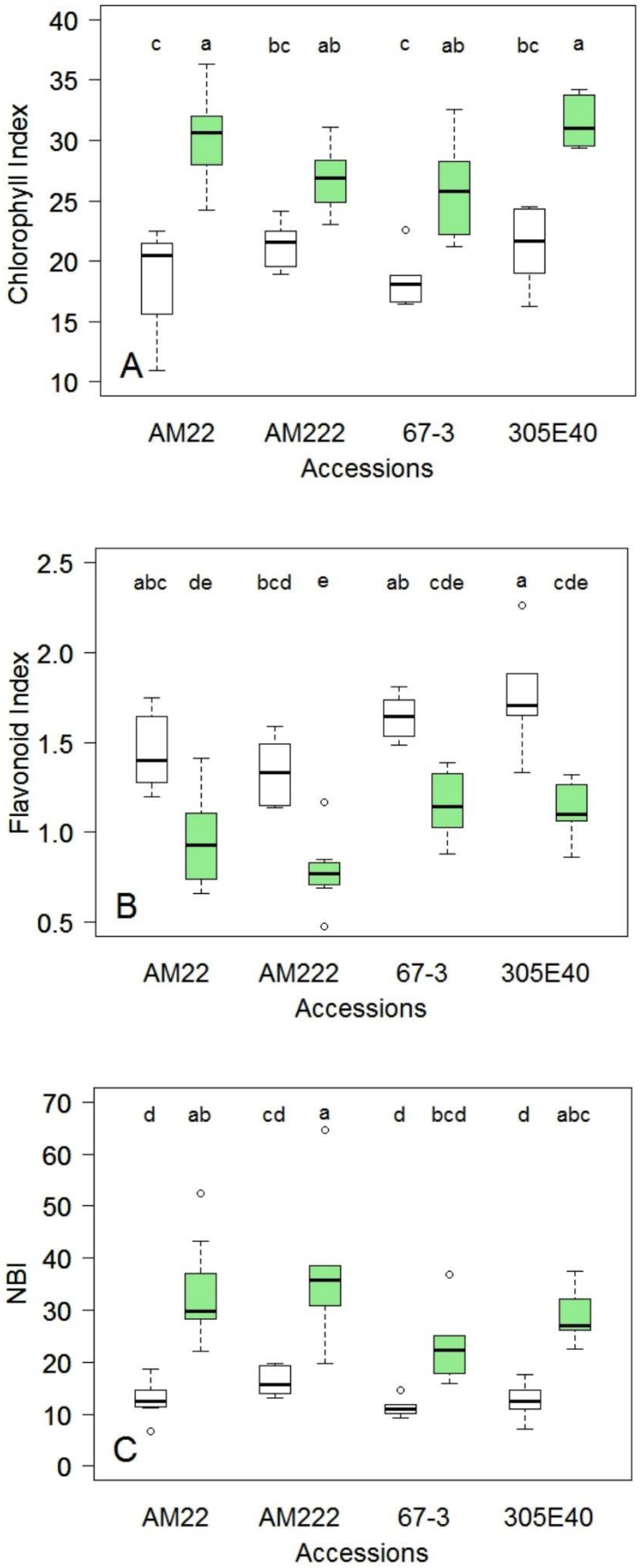
Chlorophyll Index
40
35
30
25
20
15
10
c
a
bc
ab
c
ab
bc
a
A
AM22
AM222
67-3
305E40
Accessions
Flavonoid Index
2.5
2.0
1.5
1.0
0.5
abc
de
bcd
e
ab
cde
a
cde
B
AM22
AM222
67-3
305E40
Accessions
NBI
70
60
50
40
30
20
10
0
d
ab
cd
a
d
bcd
d
abc
C
AM22
AM222
67-3
305E40
Accessions

Figure 11.

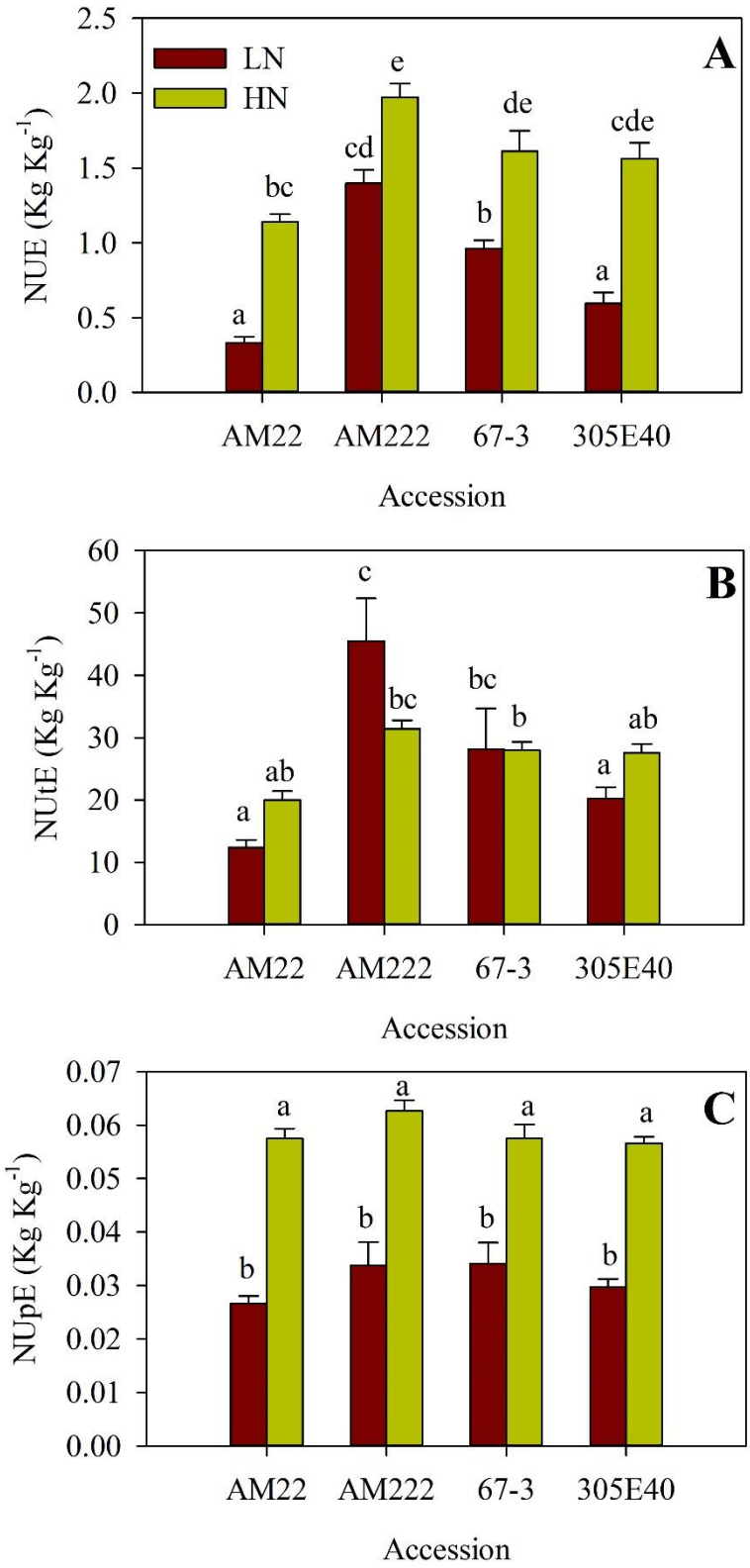
A
LN
HN
NUE (Kg Kg-1)
2.5
2.0
1.5
1.0
0.5
0.0
a
bc
cd
e
b
de
a
cde
AM22
AM222
67-3
305E40
Accession
B
NUtE (Kg Kg-1)
60
50
40
30
20
10
0
a
ab
c
bc
bc
b
a
ab
AM22
AM222
67-3
305E40
Accession
C
NUpE (Kg Kg-1)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
b
a
b
a
b
a
b
a
AM22
AM222
67-3
305E40
Accession

Figure legends

Figure 1. Variation in shoot and root dry weight of 19 eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days. CV= coefficient of variation.

Figure 2. Root morphology traits of 19 eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days. TRL, total root length; PRL, primary root length; LRL, lateral root length; NRL, number of lateral roots; RLR, root length ratio; RMR, root mass ratio; SRL, specific root length; RF, root fineness; RTD, root tissue density. CV = coefficient of variation.

Figure 3. Nitrate use efficiency (A, NUE), Uptake Efficiency (B, NUpE) and Utilization Efficiency (C, NUtE) of 19 eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days.

Figure 4. Global ANOVA for biomass, root morphology and NUE definition traits of the eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days. Different grid indicates effect of the genotype (accession), nitrate and their interaction as percentage of the observed variation.

Figure 5. Clustering of the eggplant accessions based on biomass, root morphology and NUE definition traits exposed to 0.5 and 10mM nitrate for 18 days.

Figure 6. Nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT) activities in shoot (A, C and E, respectively) and root (B, D and F, respectively) of the eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days. The values are mean± SE (n = 8). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Figure 7. Heatmap of N-related gene expression in root (A) and shoot of the eggplant accessions exposed to 0.5 and 10mM nitrate for 18 days.

Figure 8. Fruit (A), leaf (B) and stem dry weight (C) of the eggplant accessions grown in pots and exposed to low (LN) or high (HN) nitrate. The values are mean± SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Figure 9. Fruit (A), leaf (B) and stem N content (C) of the eggplant accessions grown in pots and exposed to low (LN) or high (HN) nitrate. The values are mean± SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Figure 10. Chlorophyll index (A), Flavonoid index (B) and Nitrogen Balance Index (C, NBI) of the eggplant accessions grown in pots and exposed to low (LN, white) or high (HN, green) nitrate. The values are mean± SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Figure 11. Nitrate Use efficiency (A, NUE), Utilization Efficiency (B, NUtE) and Uptake Efficiency (C, NUpE) of the eggplant accessions grown in pots and exposed to low (LN) or high (HN) nitrate. The values are mean± SE (n = 6). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Figure S1. Performance of three groups of *Solanum melongena* for all the traits under evaluation.