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Abstract

Nitrogen (N) supply usually limits crop production and optimizing N-use efficiency (NUE) to minimize fertilizer loss is important. NUE is a complex trait that can be dissected into crop N uptake from the soil (NUpE) and N utilization (NUtE). We compared NUE in 14 genotypes of three-week old tomatoes grown in sand or hydroponic culture supplied with nitrate (NO_3^-). Culture method influenced measured NUE for some cultivars, but Regina Ostuni (RO) and UC82 were consistently identified as high and low NUE genotypes. To identify why these genotypes had contrasting NUE some traits were compared growing under 0.1 and 5 mM NO_3^- supply. UC82 showed greater root $^{15}NO_3^-$ influx at low and high supply, and stronger SlNRT2.1/NAR2.1 transporter expression under low supply when compared with RO. Conversely, RO showed a higher total root length and thickness compared to UC82. Compared with UC82, RO showed higher shoot SlNRT2.3 expression and NO_3^- storage at high supply, but similar NO_3^- reductase activity. After N-starvation, root cell electrical potentials of RO were significantly more negative than UC82, but nitrate elicited similar responses in both root types. Overall, for UC82 and RO, NUtE may play a greater role than NUpE for improved NUE.

Keywords: Nitrate influx; Nitrate reductase; Nitrate transporter genes; Nitrogen Uptake Efficiency – NupE; Nitrogen Utilization Efficiency – NutE; Nitrate uptake; Tomato (*Solanum lycopersicon* L.)

Introduction

Nitrogen (N) availability is one of the most important factors limiting plant growth and productivity in both natural and agricultural environments (Marschner, 1995). Plant roots acquire N from the soil mainly as ammonium (NH_4^+), nitrate (NO_3^-) or amino acids (Miller and Cramer, 2005). In temperate climates, NO_3^- is the dominant N supply form as in most agricultural soil microbial conversion of organic N and NH_4^+ to NO_3^- rapidly occurs (Forde and Clarkson, 1999). Nitrate is also an important signal for plant growth and development, regulating N metabolism and assimilatory pathways (Stitt, 1999). Nitrate uptake by roots is an active process with transport systems that operate over different concentration ranges allowing plants to maximize acquisition

depending on soil NO_3^- availability (Forde and Clarkson, 1999). A high affinity transport system (HATS) operates at low NO_3^- concentrations (0-0.2 mM) and has two defined parts. The constitutive transport system (cHATS) is always expressed and characterized by a greater NO_3^- affinity, and the inducible transport system (iHATS) generated by an increased NO_3^- supply and with a greater uptake capacity (Forde and Clarkson, 1999; Glass et al., 2002; Glass, 2009). The low affinity transport system (LATS) is also constitutively expressed and mediates NO_3^- uptake at high external concentrations (>1 mM) and displays linear kinetics (Glass, 2009). After NO_3^- is taken up by roots, it can be reduced to nitrite (NO_2^-) and then NH_4^+ and amino acids by N-regulated enzymes or translocated to the shoot where it is assimilated (Miller and Cramer, 2005; Glass, 2009). Within the *Arabidopsis* genome the NO_3^- influx transporters are encoded by two gene families, NPF or NRT1 (Léran et al., 2014) and NRT2 (Williams and Miller, 2001). Some of the NRT2 transporters require a small partner protein called NAR2.1 (or NRT3.1) for their function (Tong et al., 2005; Orsel et al., 2006). The activity of some root uptake transporters is regulated by internal and external N supply and is coordinated with N metabolism (Glass et al., 2002). Several transporters have a particular role in long distance xylem and phloem NO_3^- transport within the plant (Wang and Tsay, 2011; Xia et al., 2015). Nitrate uptake by roots requires energy to overcome the negative electrical potential across plasma membrane of root epidermal and cortical cells (Miller et al., 2001) which is provided by activity of the plasma membrane H^+-ATPase (PM H^+-ATPase), a key enzyme in plant nutrition (Palmgren, 2001).

The importance of optimizing N management practices together with genetic improvements to decrease excess fertilizer applications is well known (Good and Beatty, 2011) and much of the N fertilizer routinely applied to crops is leached, causing environmental damage (Good et al., 2004; Sebilo et al., 2013). The physiological and molecular steps involved in NO_3^- uptake and assimilation can be used to identify traits that are important for N Use Efficiency (NUE). This may be because the first step for this type of cultivar comparison requires a consistent definition of NUE. Plant NUE can be defined as the biomass produced per unit of applied N (Moll et al., 1982) or the dry mass production for N unit taken up from the soil (Hirose, 1971). Whatever the crop, root, leaf, fruit or seed the method to measure NUE usually depends on calculating the plant biomass production per unit of applied N (Good et al., 2004; Xu et al., 2011). Clearly, NUE is a complex trait that must be encoded by many different genes and their environmental interactions, but it can be dissected into two components. Firstly, the ability of the plant to take up N from the soil termed "nutrient uptake efficiency" and secondly the ability of the plant to transfer N to plant organs and yield, known as "nutrient utilization efficiency" (Xu et al., 2011). Several studies on model and crop species have highlighted the genetic variability and the complex regulatory mechanisms

controlling NUE under growth limiting and nonlimiting N supply (Krapp et al., 2011). Given the importance of the topic it is surprising that relatively few papers have compared measures of NUE for the same germplasm growing in different environments.

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural crops. Long storage types of tomato are of great interest for their adaptation to abiotic stress conditions and they are often cultivated in Mediterranean regions where both drought and N-limited conditions are frequent. Improving tomato NUE is particularly important as large amounts of N fertilizer are required to obtain the best yield. It follows then that the identification of high and low NUE tomato genotypes, and the subsequent identification of their contrasting physiological and molecular traits, can be used to provide tools for developing marker-assisted breeding strategies. Although both NO_3^- and NH_4^+ are important N sources for tomato, we have focused on NO_3^- as this form is more readily leached from the soil. A model simulating diurnal net uptake rate patterns have been set up in tomato assuming a homeostatic mechanism, i.e. negative feedback regulation by plant NO_3^- content on uptake rates (Càrdenas-Navarro et al.,1998). Several tomato NO_3^- transporter genes, belonging to the NPF and NTR2 families, have been characterized in roots and chiefly in root hairs (Lauter et al., 1996; Ono et al., 2000; Wang et al., 2001). Like Arabidopsis, the tomato genes NTR2.1 and NTR2.2 appear very similar in their coding regions (95% identity) and their expression was predominantly in roots (Ono et al., 2000), with transcription maximum achieved 4 h after a 200 mM NO_3^- treatment (Ono et al., 2000). The recently completed sequencing of the tomato genome (Tomato Genome Consortium, 2012) now provides access to the sequence for more key candidate genes previously identified in model species like Arabidopsis as being important in NO_3^- uptake and assimilation and therefore likely to have a role in NUE.

In the present study, biomass production was used to calculate NUE for a collection of Italian tomato cultivars and one from California supplied with NO_3^- grown in hydroponics and sand to identify contrasting NUE genotypes. Two lines representing consistently high and low NUE ranges were selected for a more detailed analysis comparing their morphological, physiological and molecular traits growing in hydroponic culture. Nitrate transporter activity measurements using NO_3^--elicited changes in root cell membrane potential and ^{15}N influx, tissue NO_3^- reductase (NR) activity, and root morphology were evaluated. Finally, the expression of some assimilatory (NR) and NO_3^- transporter (SlNPF6.3, SlNRT2.1, SlNRT2.3 and SlNAR2.1) genes was compared. This analysis identified some differences between the two contrasting phenotypes and these traits may be used as potential markers for tomato breeding to select for improved NUE.

Materials and methods

Plant materials

Thirteen recognized tomato landraces from distinct geographic regions of Southern Italy were chosen for this study. Ten landraces, namely Pizzutello di Paceco, Pizzutello di Nubia, Linosa, Buttighieddu, Piriddu, Sinacori, POP 2, Inverno, Stella, Patataro came from Sicily (University of Palermo, Italy), one, Regina Ostuni from Apulia (University of Bari, Italy) and two, Vesuviano and San Marzano, from Campania (CRA, Monsampolo del Tronto, Italy). In addition, the North American cultivar UC82, kindly supplied as seed by the Tomato Genetics Resource Center - Department of Plant Sciences, University of California Davis, was included.

Silver sand experiment

Seeds of each type of tomato were washed with 5% (v/v) NaClO for 15 min to surface sterilize the seed and then were germinated in a Petri dish (diameter 90 mm) on filter paper with 0.1 mM CaSO4. After 7 d of germination, seedlings of uniform size were selected and transferred to pots (diameter 7 cm, 110 cm3 volume), one plant per pot, filled with silver sand and the surface exposed to light was covered using black plastic film to prevent algal growth. Seedlings were daily watered with 5mL modified Hoagland nutrient solution, containing 2.5 mM K_2SO_4, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 1 mL L^{-1} Hoagland micronutrients and 2 mL L^{-1} FeEDTA. Nitrate was added as $Ca(NO_3)^2$ to the solution to give the following NO_3^- concentrations: 0, 0.1, 0.3, 0.5, 0.75, 1, 2.5, 5 and 10 mM. Furthermore, $CaSO_4$ in a range 0-5 mM concentration was added to the nutrient solution to adjust the Ca^{2+} concentration to the same value in all the treatments. The pH of the nutrient solution was adjusted to 5.8 with KOH. Tomato seedlings were placed in a growth chamber maintained at 23°C, 70% RH and 16 h photoperiod with a light intensity of 340 mmol m^{-2} s^{-1} for a further 2 weeks. Five tomato seedlings (21-days old), for each NO_3^- concentration and genotype, were collected and divided into leaves, stem and roots. Finally, the plant material was placed in an oven at 70°C for two days to determine leaf (LDW, g), stem (StDW, g) and root dry weight (RDW, g). Shoot dry weight (SDW, g) was calculated by adding LDW to the StDW.

Hydroponic experiments

Four tomato genotypes (UC82, Regina Ostuni, Linosa and Piriddu) were also grown in an aerated hydroponic system. After germination (as described above), seedlings of uniform size were placed into plastic pots (10 x 10 cm), with three plants per unit supported by netting above 700 mL of aerated nutrient solution, as described above containing 0, 0.1, 0.3, 0.5, 0.75, 1, 2.5, 5 and 10 mM NO_3^-. Care was taken to ensure there was the same amount of vigorous aeration in the pots. The nutrient solution was renewed every two days and the pH was adjusted to 5.8 with KOH. The plants

were placed in a growth chamber in the same experimental conditions as described above for 2 weeks. Shoot dry weight (SDW, g) was measured at the end of the experiment as reported above.

Tissue nitrogen concentration

The N concentration of the root and shoot of Regina Ostuni (RO) and UC82 tomato genotypes were measured by the micro-Kjeldahl technique (Lowther, 1980). After germination and growth for two weeks with 0.1 or 5mM NO_3^- supply (as described above), seedlings of both genotypes were harvested. Roots and shoots were washed with deionized water, then dried for several days at 70°C to determine shoot (SDW, g) and root dry (RDW, g) weights. Dry root and shoot samples were then placed into the Kjeldahl digestion flask containing 1 g of catalyst (Selenic mixture, Merck, Germany) and 15 or 5 mL of concentrated H2SO4, respectively. After 2 h of digestion by electrical heat and fume removal (Labonco, Kansas City, MO, USA), the samples were cooled at room temperature.

Thirty mL of distilled water containing 4-5 drops of phenolphthalein were added to each flask. Total N was trapped by distillation in a 4% (w/v) boric acid solution. Total N was determined by titration with 0.01 and 0.1 M HCl for root and shoot, respectively, with a mixed indicator endpoint (0.1 g $100mL^{-1}$ bromocresol green and 0.1 g 100 mL^{-1} methyl red in 95% ethanol). Data was expressed as milligrams of N g^{-1} of dry matter.

Nitrogen use efficiency definitions

Shoot dry weight (SDW) data obtained from both silver sand and hydroponic experiments were plotted as a function of different NO_3^- concentrations. These biomass curves were described by nonlinear regression analysis using the equation:

$$SDW(g) = \alpha [NO_3^-]/ \beta + [NO_3^-]$$

where α indicates the maximum shoot dry mass and β represents the NO_3^- concentration at half maximum shoot dry mass, also indicating the rate at which a was reached (see Fig. 1). This method of analysis linking yield or biomass to the concentration of nutrient supply was previously used for legumes and phosphate (Gourley et al., 1994). The screening method was used for NO_3^- supply to woody citrus rootstocks growing in perlite (Sorgonà et al., 2005, 2006). Germplasm performance can be analyzed by directly comparing α and β values in a way that is analogous to the well-known Michaelis-Menten enzyme kinetics Vmax and Km.

Additionally, NUE definitions for high and low NO_3^- supplies for the RO and UC82 genotypes, grown in hydroponic culture were calculated as follows:

(1) Total N Accumulation (TNA) calculated as the N concentration x total plant dry weight (mg N) (Lawlor, 2002);

(2) Nitrogen Efficiency Ratio (NER) calculated as the total plant dry weight divided by TNA (g TDW mg^{-1} N) (Gabelman and Gerloff, 1983);
(3) Nitrogen Utilization Efficiency (NUtE) calculated as the total plant dry weight divided by N concentration (g^2 TDW mg^{-1} N) (Siddiqi and Glass, 1981);
(4) Nitrogen Uptake Efficiency (NUpE) calculated as TNA divided by root dry weight (mg N g^{-1} RDW) (Elliot and Lauchli,1985).

Morphological root analysis

Root morphology of the UC82 and RO genotypes was compared as described previously (Romano et al., 2013). Eight seedlings of each genotype grown in hydroponic culture and supplied with full nutrient solution containing either 0.1 or 5 mM NO_3^- for 2 weeks as reported above divided into roots and shoots. The fresh weights of root (RFW, g) and shoot (SFW, g) were measured. The roots were immersed in 0.1% (w/v) toluidine blue for 5 min then rinsed in deionized water before scanning at 600 dpi resolution (WinRhizo STD 1600, Instrument Regent Inc., Canada) to determine the total root length (RL, cm), surface area (AS, cm^2) and root volume (RV, cm^3). Shoot and root dry weights (SDW and RDW, g, respectively) were determined as described above. 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.

Determination of root NO_3^- influx and shoot accumulation using $^{15}NO_3^-$

Plasma membrane influx of $^{15}NO_3^-$ was measured in UC82 and RO genotypes, grown in hydroponic culture supplied with either 0.1 or 5 mM NO_3^- for 2 weeks. Five tomato seedlings were transferred to 0.1 mM $CaSO_4$ for 1 min, and then to a modified Hoagland solution supplied either 0.1 mM or 5 mM ^{15} NO_3^- (atom% 15N: 98%) for 5 min, and finally to a 0.1 mM CaSO4 washing solution for 1 min (Orsel et al., 2006). Thereafter, roots were separated from shoots and frozen in liquid N. After homogenization using a pestle and mortar, the powder was stored at -80°C. An aliquot of the powder was weighed (1 mg) for each sample and analyzed using isotope ratio mass spectrometer system (model Integra CN; PDZ Europa, Crewe, UK). Influx of ^{15} NO_3^- was calculated from the total N and ^{15}N of the roots and shoots.

Tissue NO_3^- concentrations

Tissue NO_3^- concentrations were determined on boiling water extracts using a Nitrate/Nitrite colorimetric assay kit (Cayman Chemical Company USA, product number 780001). Seedlings of UC82 and RO were grown in hydroponic culture and supplied with 0.1 or 5 mM NO_3^- for 2 weeks as described above. Roots and shoots of each genotype were divided and homogenized in liquid N as described for the $^{15}NO_3^-$ influx experiments. A diluted boiled water extract was assayed by

measuring absorbance of the azo- compound (at 540 nm) using a spectrophotometer (model Varioskan flash Thermo Scientific) in a 100 mL sample. A NO_3^- standard curve was used to determine the NO_3^- concentration (mmol nitrate g^{-1} dry root or shoot) which was calculated following the manufacturer's instructions.

NR assays

NR activity was analyzed in shoots of UC82 and RO using a previously described method (Kuo et al., 1982). Seedlings were grown in hydroponics with either 0.1 or 5 mM NO_3^- supply as described above and whole leaf tissue samples were collected at the same time of the light/dark cycle (8 h into light period). Shoot material (0.6 g) was rapidly weighed and ground to a fine powder with a pestle and mortar under liquid nitrogen. Extraction buffer (1 mL), containing 50 mM Tris (pH 8.5), 10 mM DTT, 20 mM Leupeptin, 10 mM FAD and 10 mM Na_4EDTA or 10 mM $MgCl_2$, was added and the homogenate was then centrifuged at 13,000 rpm for 10 min at 4 C and the supernatant was used for NR assay.

The NR activity was measured in 250 mL of 0.1 mM potassium phosphate buffer, pH 7.5, containing 100 mM KNO_3 and 10 mM NADH. The reaction was started with the addition of 100 mL of supernatant to the buffer and the tubes were incubated for 20 min at 25 C in the dark. The calibration curve was prepared using buffer, without NADH, with final concentrations of KNO_2 in the range from 0 to 15 mM. In the blank KNO_2 was omitted. The reaction was stopped by adding 50 mL of a 1:1 1 M zinc acetate and 0.3 mM PMS solution. The tubes were left for 20 min to oxidise excess NADH. The NO_2^- was estimated by adding 250 mL of 3 M HCl containing 1% (w/w) sulphanilamide and 0.02% (w/w) *N*-1-(naphthyl) ethylenediamine (NED). After 15 min the OD was read at 540 nm. Each treatment was replicated four times and assayed by adding 10 mM EDTA to assay total activity (phosphorylated plus non-phosphorylated NR) or 10 mM $MgCl_2$ to measure actual activity (non-phosphorylated NR). NR activity state was defined as the ratio of the actual to total NR activity given as a percentage (MacKintosh et al., 1995).

Membrane potential measurements

All the electrophysiological measurements were performed on intact primary roots in cells 1-2 cm from the tip. The UC82 and RO tomato seedlings (7 days old) were placed in a Plexiglass chamber and perfused with a basic buffer solution containing 5 mM MES, 0.5 mM $CaCl_2$ and 0.05 mM KCl, adjusted to pH 6.0 with NaOH (Fan et al., 2007), before performing the electrode impalement of primary roots as described previously (Miller et al., 2001). The seed-lings were grown in a hydroponic nutrient solution with no added N (see above). Membrane electrical potentials were measured with glass single-barreled microelectrodes back-filled with 200 mM KCl

using a 70 mm long Microfil needle (World Precision Instruments Inc., Stevenage, UK). The reference salt bridge was filled with 200 mM KCl in 2% (w/v) agar and was placed in the perfusion chamber close to the root. The cell impalements were made into mature epidermal cells, 1e2 cm from the root tip. The potential difference (mV) between the inside of the cell and external bathing solution was then recorded on a pc as described previously (Fan et al., 2007). During each cell measurement the perfusion solution was changed to solution containing the same MES, K^+ and Ca^{2+} concentrations and 0.1 mM NO_3^- as $Ca(NO_3)_2$ to assay for NO_3^- transport activity (Miller et al., 2001). The pH was maintained to 6.0 and in order to keep the same Ca^{2+} concentration as the basic buffer solution, $CaCl_2$ was added in the treatment buffer solution to reach 0.4 mM.

Gene expression analysis

Total RNA was isolated both from roots and shoots of UC82 and RO seedlings grown in hydroponic culture and exposed to 0.1 and 5 mM NO_3 for 2 weeks. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Milano, Italy) according to the manufacturer protocol and its quality and quantification was assayed by NanoDrop 2000 (Thermo Scientific).

The first-strand cDNA was synthesized from 2 mg of the total RNA (Tetro cDNA synthesis kit), using oligo-dT primers as sug- gested by the Bioline manufacturer. The real-time PCR (qPCR) was performed on DNA Engine Opticon2 (Bio Rad) using SYBR Green master mix kit (Sigma-Aldrich) according to the manufacturer's instructions. The qPCR was carried out starting from 2 min at 95 C (initial denaturation), then for 40 cycles consisting of 30 s at 94 C, 30 s at 60 C and 1 min at 72 C. Three replicate experiments for each NO_3 concentration and genotypes were carried out. Specific primers for *SlNPF6.3* (formerly *SlNRT1.1* accession number: X92853), *SlNTR2.1* (accession number: NM001279334), *SlNTR2.3* (NM001247198), *SlNAR2.1* (XM004236225) and *SlNR* (NADH dependent: accession number XM004250307.1), tomato ubiquitin and actin genes (accession numbers: TC193502 and TC194780, respectively), the latter two were used as internal standards, were designed to amplify specific fragments (Table S1). The sequences used for designing the primers showed the following percentage of identities to the equivalent genes from other plants; *SlNRT2.1* (NM001279334) showed 87%, 86% and 84% identity to *NRT2.1* from potato, *Arabidopsis* and tobacco, respectively (Ono et al., 2000); *SlNRT2.3* (NM001247198) showed 97% identity to potato and to- bacco *NRT2.3* and *SlNAR2.1* (XM004236225) showed 90% identity to potato *NAR2.1*.

The qPCR results were analyzed by the $2^{-\Delta Ct}$ comparative method as previously described (BioRad Real-time PCR Application guide; Livak and Schmittgen, 2001). Based on the logarithmic fluorescence graph, the fitting threshold was chosen by calculating Ct using the 7000 System SDS

software (RQ Study Application, Applied Biosystems). This method can detect relative changes in gene expression, where ΔCt is the difference in threshold cycles for target (Ct sample) and reference (Ct ubiquitin) genes. The Ct of each sample was normalized to ubiquitin to account for variability in the original concentration and quality of the total RNA, and the con- version efficiency of the reverse transcription reaction.

Statistical analysis

All the tomato experiments were set up in a completely randomized design with at least five replications for each. In hydroponic and silver sand experiments, the parameters of the non-linear equations were estimated by the "Least Squares" method of non-linear regression (Table Curve 2D Version 4.0 Software, Jandel Scientific Ekrath, Germany) using the Levenburg-Marquardt algorithm. All data were checked for normality (Kolmogorov-Smirnov test) and tested for homogeneity of variance (Leven median test). The data were than analyzed by one-way ANOVA comparing among genotypes, and means were separated by Tukey's Honest Significant Difference (HSD) test (p 0.05). Statistical analysis in NUE, ^{15}N influx, N concentration and content, NR activity, root morphology and gene expression, was performed by Student's unpaired *t*-test comparing the two genotypes within the NO_3 concentration using the Systat software (Systat Software Inc., Chicago, USA).

Results

Nitrogen use efficiency

Shoot dry weight analysis of genotypes grown in silver sand supplied with a range of NO_3^- concentrations enabled a series of response curves to be plotted (see Fig. 1). From these response curves the a and b parameters, the half maximum shoot dry mass (g) and the NO_3^- concentration (mM) at which a was reached were calculated using non-linear regression analysis (Gourley et al., 1994). The parameters α and β showed statistically significant differences ($p < 0.01$) among the fourteen tomato genotypes (Table 1). The α values ranged from 0.012 to 0.057 g with RO and UC82 both showing significantly higher values than all other genotypes. Lower, but not significantly different α values were shown by Linosa, Piriddu, POP2, Buttighieddu, Sinacore, Inverno, Patataro, Vesuviano, Stella and Paceco. The Nubia genotype showed the lowest a value. The β values ranged from 0.12 to 0.14 (Stella and Linosa) to 0.87 and 2.41 mM (Piriddu and UC82), the latter two were significantly different from one another. UC82 required a significantly higher NO_3^- concentration to achieve half maximal biomass when compared with all other genotypes.

We selected four of these cultivars (RO, PI, LI and UC82) that gave a spread of α and β values to compare their responses when grown in hydroponics with the same range of NO_3^- supplies (Table 2). The results revealed that although α values in UC82 and Regina Ostuni were much lower in hydroponics when compared with sand, the same relative pattern was found. For example, comparison of the half maximal NO_3 concentrations again showed that UC82 exhibited a significantly larger β value compared to all other cultivars (Table 2). Among the Italian cultivars, although a different relative arrangement emerged RO showed a similar consistent pattern. Therefore, the genotypes RO and UC82 that exhibited contrasting responses in both growth systems (sand and hydroponics) were chosen for more detailed phenotyping.
To further test their contrasting responses to NO_3^- supply the RO and UC82 genotypes were grown in hydroponics at two NO_3^- concentrations (0.1 and 5 mM) and several different commonly used NUE definitions were used to compare the two types of plants (Fig. 2). At 0.1 mM NO_3^- supply all the NUE definitions showed significant differences between the genotypes. The cultivar RO showed greater TNA, NER and NUtE when compared to UC82 (Fig. 2A-D). While UC82 exhibited a higher NUpE compared to RO (Fig. 2D) at low NO3 supply. At 5 mM NO_3^-, RO showed significantly higher TNA and NUtE values compared to UC82, while no significant differences for NUpE and NER values were observed (Fig. 2A-D). To better understand the reasons for these differences further phenotypic analysis was undertaken using assays that compare uptake and utilization of nitrate in these two cultivars.

Membrane potential measurements

Root cell membrane potentials of RO and UC82 of N-starved plants were recorded before and during NO_3^- treatments. After microelectrode impalement a stable cell membrane potential (E_m) was recorded for at least 5 min before the root was treated with a nutrient solution containing NO_3^- (steady state, Table 3). These initial E_m values were significantly different between RO and UC82 genotypes (Table 3); RO root cells showed more negative electrical potentials than UC82 (142 mV *vs*. 122 mV). Supplying a nutrient solution containing 0.1 mM NO_3^- caused a transient depolarization (less negative electrical potential) in both tomato genotypes occurring in a few minutes and was followed by a slower hyper- polarization to a more negative electrical potential in both genotypes (UC82 and RO, 137 and 144 mV, respectively). Continued longer exposure to NO_3^- (30 min) caused a mean hyperpolarization of around 146 mV for RO and 155 mV for UC82, this difference was significant between cultivars (Table 3).

Determination of root NO_3^- influx and accumulation using ^{15}N-NO_3^-

Nitrate influx rates were compared for roots of RO and UC82 genotypes growing in nutrient solutions containing either 0.1 or 5 mM NO_3^- concentrations. The results showed significant differences in HATS, but not LATS activity between two genotypes (Fig. 3). At 0.1 mM NO_3^-, UC82 showed significantly higher root influx rates when compared to RO (see Fig. 3A). We also compared the $^{15}NO_3^-$ transfer rates to the shoots between genotypes after exposure to low (0.9 vs 0.4 mmol h 1 g 1SDW for UC82 and RO) and high NO_3^- (33.7 vs. 41.5 mmol h 1 g 1 SDW for UC82 and RO).

Nitrate concentration and NR assay

Nitrate accumulations in both shoots and roots were significantly different between genotypes at both 0.1 and 5 mM NO_3^- supplies (Fig. 4). At the lower NO_3^- supply, UC82 showed a larger NO_3^- concentration in the root when compared to RO (77 vs. 133 mmol g 1 RDW). At high NO_3^- supply the opposite pattern was observed with a significantly higher NO_3^- concentration in RO compared to UC82 (764 vs 485 mmol g 1 RDW Fig. 4A). Furthermore, RO showed a significantly higher shoot NO_3^- concentration than UC82, at low (39 vs 23 mmol g 1 SDW) and high (350 vs 200 mmol g 1 SDW) NO_3^- supply (Fig. 4B).

Leaf NR activity of RO and UC82 genotypes was measured at both 0.1 and 5 mM NO_3^- supplies, but only at low supply were there significant differences between the two genotypes (Fig. 5). The NR activity was larger in RO compared to UC82, for both total and non- phosphorylated enzyme activity (Fig. 5BC). The activity state of NR [calculated as (Mg-assay/EDTA-assay) x 100] was significantly different at 43 and 32% for RO and UC82, respectively. At the higher NO_3^- supply the NR activity state was not significantly different, at about 42% in both genotypes (Fig. 5A).

Root morphology

The root morphology of the RO and UC82 genotypes was compared for plants growing in hydroponic culture supplied with 0.1 or 5 mM NO_3^-. Root dry weight (RDW), root length ratio (RLR), root mass ratio (RMR) and root tissue density (RD) were not significantly different between RO and UC82 at both NO_3^- concentrations (Fig. 6ACDF). However, total root length (TRL) and root fineness (RF) were significantly higher in RO compared to UC at both NO_3 supplies (Fig. 6B-E).

Gene expression analysis

The expression pattern of SlNPF6.3 (formerly, NRT1.1), two NRT2 family member genes (SlNRT2.1 and SlNTR2.3), together with partner SlNAR2.1 in tomato roots exposed to 0.1 or 5

mM NO_3^- was investigated by RT-PCR. The expression of SlNPF6.3 was very low and no significant change was detected under our experimental conditions in both genotypes (data not shown). Expression of the two-component high affinity uptake system, SlNRT2.1/NAR2.1 was significantly higher in UC82 when compared with RO at low NO_3^- supply (Fig. 7A-B). At 5 mM NO_3^- supply, SlNRT2.1 expression did not show any significant difference between genotypes, but SlNAR2.1 expression was significantly higher (59%) in RO. At low NO_3^- supply, SlNRT2.3 expression was significantly greater (82%) in RO than UC82 (Fig. 7C). When grown in 5 mM there much lower expression and this was similar for both genotypes.

The level of NR transcript was investigated by RT-PCR in tomato roots and shoots grown in 0.1 or 5 mM NO_3^-. NR expression was not significantly different in roots of both genotypes at low NO_3^- supply, while it was significantly higher (72%) in UC82 compared to RO at 5 mM NO_3^- (Fig. 7D). At low NO_3^- supply, significant differences were observed in shoots between genotypes, with higher NR expression in RO compared to UC (Fig. 7E). The shoot NR expression was not significantly different between genotypes growing in 5 mM NO_3^- (Fig. 7E).

Discussion

Screening N-use efficiency

A major goal for sustainable agriculture is to increase yield using less N fertilizer input and thereby improve NUE by crops (Good et al., 2004). Measuring NUE can be technically demanding and a simplified method to compare many cultivars is very attractive to crop breeders. There are many different ways to measure NUE and we began by comparing tomato germplasm using a definition based on shoot biomass relative to the NO_3^- supply concentration. When this method was previously used for legumes and phosphate, it was argued that equivalent α and different β values (Table 1) could be used to select P efficient/inefficient genotypes (Gourley et al., 1994). In other words, in an efficient genotype the same shoot biomass (α) is achieved growing at a lower phosphate concentration or β value. For example, comparing RO and UC82 in sand culture shows similar shoot biomasses (Table 1), but the Italian cultivar can achieve this biomass at a much lower NO_3^- concentration. Among all the other Italian genotypes screened none can achieve this amount of shoot biomass suggesting they may have limited 'genetic potential' by this NUE criterion (Gourley et al., 1994). Of course, it is risky to extrapolate directly from shoot biomass to crop yield of tomatoes as although nitrogen supply is required for the production of the plant canopy it may not result in greater fruit yield. Some of the Italian cultivars produced vigorous plants at remarkably low NO_3^- concentrations when comparing β values with UC82, for example Stella and Linosa. Although this NUE method is not widely adopted there are positive

aspects to the screen. It has the advantage of identifying germplasm that is able to grow well at a low concentration and may therefore require lower inputs thereby minimizing the amount of NO_3^- lost by leaching through the soil profile.

We further tested this method of measuring NUE by growing four tomato genotypes in hydroponics with the same series of NO_3^- concentrations and environmental conditions. We found the α values were the same for all four cultivars, but again UC82 achieved this shoot biomass at a significantly higher NO_3^- concentration relative to all three Italian cultivars screened (Table 2). The β values for all the genotypes tested were much lower in hydroponics when compared with sand culture and this difference must depend on the different growth medium, silver sand vs. hydroponics. This might suggest there is a difference in the delivery of NO_3^- to roots and the effective concentration at the root surface; however, this does not fit with the observation that in agar and sand, the NO_3^- activities at the root surface were higher than those supplied in hydroponic nutrient solution (Chapman et al., 2011). Another explanation might be that NO_3^- supply was depleted in the sand culture, but the daily watering with nutrient solution makes this unlikely. It has been argued that NUE screening of plants in inert substrates might be a more suitable system to improve root growth and nutrient transport function when compared to aerated hydroponic culture (Gabelman and Gerloff, 1983). However, our data suggest that screening in sand may not be ideal for NUE screening. Furthermore, in contrast to RO and UC82, the tomato genotypes Linosa and Piriddu had larger α values in hydroponics when compared with sand showing they produced more biomass in this system. Taken together these results suggest another factor, such as root oxygen supply may be important for some genotypes in the sand system. Despite the limitations of various screening methods, we were able to consistently show contrasting differences between the UC82 and RO genotypes in sand and hydroponics. As a further check we compared the NUE performance of these two cultivars in hydroponics at high and low NO_3^- using some more widely used definitions based on plant N accumulation. We tested RO and UC82 using micro-Kjeldahl N biomass methods, and the results confirmed that when comparing the pair, RO has improved NUE showing better TNA, NER and NUtE values when compared to UC82 (Fig. 2). Interestingly at low NO_3^- supply, UC82 showed a significantly higher NUpE when compared with RO (Fig. 2D) yet it showed poorer NUE when compared with RO (Tables 1 and 2 and Fig. 2AB). This may suggest NUpE is not a good indicator for NUE for tomato at low NO_3^- supply. To identify important tomato NUE traits we focused on comparing the molecular and physiological properties of these two genotypes. As a function of multiple interacting genetic and environmental factors, NUE is a complex trait which can be dissected into two key plant physiological components, NUpE and

NUtE (Good et al., 2004; Xu et al., 2011). The NUpE defines the capacity of roots to acquire N from the soil and it is associated with the activity of the NO_3^- uptake system and improved root architecture for acquisition. The NUtE components of NUE may be more dependent on N assimilation and storage in roots and shoots. We have dissected apart some of the components of NUE and compared them in UC82 and RO.

Electrophysiology

The size of the cell membrane electrical potential difference indicates the energy status of the plasma membrane and is important for driving transport and growth; furthermore, the size of NO_3^--elicited changes in the cell potential can be used to measure NO_3^- transport activity (Miller et al., 2001; Lupini et al., 2016). In N-starved plants a significantly more negative root membrane potential was measured in RO relative to UC82 (Table 3; column 1), suggesting more energy is available for plasma membrane trans- port. However, on addition of NO_3^- to the perfusion solution to assay for transporter activity there was no significant difference between tomato genotypes (Table 3, column 2). After a longer exposure (30-60 min) to 0.1 mM NO_3^- in the perfusion solution, the mem- brane potential of UC82 became more negative and no longer significantly different from RO. These results identify an important difference between the tomato genotypes, the N-starved cell membrane potential of RO was maintained at a significantly more negative voltage when compared with UC82, suggesting this tomato is better able to withstand low N supply and deprivation. When both genotypes are N-replete the root cell membrane potentials were not significantly different (data not shown).

^{15}N influx and gene expression analysis

Significantly greater $^{15}NO_3^-$ influx in roots was measured in UC82 compared with RO at both 0.1 and 0.5 mM NO_3^- supply (Fig. 3A). This difference in influx rate in the HATS range can explain why UC82 showed a significantly higher NUpE when compared with RO (Fig. 2D) at 0.1 mM NO3 supply. However, in the LATS range no significant difference in NUpE was measured and confirmed by $^{15}NO_3^-$ influx rate at 5 mM too (Fig. 3A). For Arabidopsis and Brassica napus it has been shown that HATS makes a major contribution to NO_3^- acquisition over the LATS concentration range (Orsel et al., 2002). Although the tomato influx and NUpE experiments were only measured at one high concentration (5 mM) in the earlier sand and hydroponic screen the β values were always larger concentrations for UC82 when compared with RO.

Another reason for differences in NO_3^- uptake of UC82 could be due to changes in gene expression. As discussed above genes encoding many of the key transporters for uptake are now

well- known. As was reported in Arabidopsis (Orsel et al., 2006), also in tomato SlNTR2.1 expression is strongly correlated with HATS NO_3^- influx and with its functional partner SlNAR2.1 (Fig. 7AB). The expression levels of both SlNTR2.1/NAR2.1 were significantly higher in UC82 compared to RO at low NO_3^-, mirroring the ^{15}N influx data which indicated UC82 as more efficient at acquiring NO3 at low concentrations. Although, induction of SlNTR2.1 in tomato after NO_3^- treatment was already described (Lauter et al., 1996; Ono et al., 2000; Wang et al., 2001), the co-expression of SlNAR2.1 and SlNTR2.1 was first reported here (Fig. 7AB). At 5 mM NO_3^- supply the co-expression pattern of SlNAR2.1 and SlNTR2.1 between genotypes is different providing more evidence of other functions of this protein (Orsel et al., 2006; Yan et al., 2011; Feng et al., 2011).

The expression of NTR2.3 has been demonstrated to interact with NAR2.1 to provide NO_3^- uptake in rice (Feng et al., 2011). Based on the rice model (Tang et al., 2012), SlNTR2.3 expression was measured in both root and shoot tomato genotypes, at low and high NO_3^- concentrations. The results showed high expression levels of SlNTR2.3 in the root of RO compared to UC82, while it was very low or undetectable in shoots of both tomato genotypes (data not shown). This relatively high expression in the root at 0.1 mM NO_3^- supply does not support a major role for the transporter in root uptake as the $^{15}NO_3^-$ influx rate was found to be higher in UC82 (Fig. 3B). In rice NTR2.3 was found to have a role in long distance transport too (Tang et al., 2012) and this idea is discussed below.

Root morphology

It is well known that a plants ability to respond to N fluctuation is associated with root morphological changes (Ryser and Lambers, 1995). Root length is generally considered the below-ground functional trait which best describes the capacity of the root system to explore the soil (Ryser, 1998) and it is strictly associated with the competitive ability for NO_3^- acquisition (Hodge et al., 1999). The important role of root system in this process has been already re- ported in several species (Sullivan et al., 2000). RO showed a higher total root length (TRL) than UC82, at both NO_3^- concentrations. Since root length ratio (RLR, root length per unit of plant dry biomass) is a trait more important than TRL in plant potential for resource acquisition under stress conditions (Ryser, 1998), it has been also analyzed. The RLR contains the allocation component, root mass ratio (RMR), and two structural components: root fineness (RF, root length per unit root volume) and tissue density (RTD, root dry mass per unit root volume) (Ryser and Lambers, 1995; Ryser, 1998). Plants may produce longer roots either by increasing biomass allocation or by adjusting root fineness (thickness) and/or reducing root tissue density. At both NO_3^- concentrations, RO showed

higher RF but not significantly different RLR, RMR and TD parameters when compared to UC82. These findings suggested that RO has a thinner and more efficient root system compared to UC82. Thinner roots increasing the root-soil interface providing a larger surface for uptake (Larcher, 1995), radial conductivity by lesser resistance to the radial flow (Huang and Eissenstat, 2000), root hydraulic conductance per leaf unit surface area (Pemana et al., 2006). These root morphology traits may be more relevant to plants in soil but are less likely to be important for sand and hydroponically grown plants.

Nitrate concentration and NR activity

At low NO_3^- concentration, RO showed a higher NO_3^- concentration in shoot but not in the root, whereas UC82 showed a significantly higher NO_3^- concentration. Accumulation of more NO_3^- may be due to low NR activity since a negative correlation between NR and tissue NO_3^- concentration has been reported (Sienkiewicz-Porzucek et al., 2008). The activity state of tomato leaf NR was shown to be very dependent on water status, decreasing by 60% in 4 h as leaf water content fell (Brewitz et al., 1996), but for these measurements in hydroponics this cannot be relevant. Root NR gene expression at low NO_3^- supply was not significantly different between the tomato genotypes. Conversely, RO showed a significantly higher activity in shoot at low NO_3^- supply together with a higher NR gene expression compared to UC82. Increased NO_3^- accumulation is accompanied by larger stores in the vacuole when the N supply concentration is larger and this is the basis for tissue testing as an indicator of crop N status (Miller et al., 2007). Furthermore, as suggested above, the higher NO_3^- accumulation in RO root could be supported by lower NR gene expression observed compared to UC82. On the other hand, NR gene expression in shoot at higher NO_3^- concentration did not highlight a significant difference between genotypes, as expected, while NR activity was higher in RO compared to UC82. The NR expression data (Fig. 7DE) fit the pattern of NR activity measured in the same tissue in similar plants with higher values in RO growing in low NO_3^- supply (Fig. 5). Regarding long distance transport within the tomato plants, we have few measurements of this process. The $^{15}NO_3^-$ transfer to shoots occurred significantly faster in UC82 when compared with RO growing in low NO_3^- supply, but this difference was not maintained at 5 mM supply (Fig. 5B). Yet the expression of SlNRT2.3 was much higher in RO when compared with UC82 (Fig. 7C) and this related transporter in rice has an important role in long distance transport (Tang et al., 2012). In rice genome, OsNTR2.3 mRNA was spliced into two gene products, OsNTR2.3a and OsNTR2.3b, respectively (Feng et al., 2011; Yan et al., 2011) and OsNTR2.3a operated in long distance NO3 transport from roots to shoots (Tang et al., 2012). Further studies will need to demonstrate if there is alternate splicing in SlNRT2.3.

In conclusion, the application of the Gourley criteria identified some significant differences among 14 tomato cultivars grown in sand culture and supplied with various NO_3^- concentrations. Curiously for some cultivars their NUE ranking depended on the culture method. This variation may result from phenotypic changes in response to the altered culture method or it may reflect limitations in fitting the Gourley parameters. Either way, it illustrates the potential pitfalls of defining and measuring NUE. The cultivars RO and UC82 consistently showed contrasting phenotypes in this screen, and this difference between the genotypes was independent of growth in sand or hydroponics. These two cultivars also showed contrasting NUE phenotypes when tested using other more conventional criteria that are based on biomass, N acquisition and accumulation. These two cultivars were then studied in more detail and the results identified various root/shoot traits that differ be- tween the two cultivars when growth occurs at low or high NO3 concentrations. The results for RO growing at low NO_3^- supply, suggest total TRL and RF may be important root morphological traits for NUE. Also, at low NO_3^- supply, the higher shoot NO_3^- concentration/content, the higher activity and gene expression of NR in shoot, together with a high root SlNTR2.3 expression seem to be parameters which may be associated with the higher NUE of the RO tomato genotype. The highly significant difference in the expression of SlNTR2.3 between the two cultivars may make it a good marker for the identifying improved NUE tomato genotypes. When compared with UC82, RO showed high SlNRT2.3 expression and shoot NO_3^- content in shoot at high NO_3^- supply, but similar NRA suggesting an improved capacity to store leaf NO_3^- that may also be a potential phenotypic marker for improved NUE genotypes. For UC82 relative to RO, with efficient NO_3^- acquisition at high NO_3^- supply, high influx rate and higher NTR2.1/NAR2.1 expression defined the genotype. Overall, the results indicate that in our experimental conditions for UC82 and RO, NUtE component may play a greater role than NUpE in conferring NUE in tomato. This data enforces the idea that both genotype and environment are important contributors to the NUE phenotype.

Contribution

AL, AJM, FS and MRA conceived and designed the experiments. AL analyzed statistically all experimental data. AL and CL performed the experiments. FA and CL performed the enzyme assays. FM, CL and MPP performed molecular experiments. AL, AJM, FS and MRA wrote the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at

http://dx.doi.org/10.1016/j.plaphy.2016.04.021.

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Table 1
Kinetic parameters for fourteen tomato genotypes comparing the shoot dry-weight (g plant^{-1}) and NO_3^- supply response curves in silver sand culture (R^2 or coefficient of determination was $P < 0.001$ for all genotypes).

Genotype	α	β	R^2
Inverno	0.024^{ad}	0.23^{ab}	0.74
Paceco	0.030^{cd}	0.62^{ab}	0.68
Stella	0.016^{ac}	0.12^{a}	0.52
UC82	**0.057^{e}**	**2.41^{c}**	**0.60**
Buttighieddu	0.028^{bd}	0.48^{ab}	0.87
Linosa	**0.022^{ad}**	**0.14^{a}**	**0.65**
Nubia	0.012^{a}	0.30^{ab}	0.81
Piriddu	**0.028^{d}**	**0.87^{b}**	**0.66**
R. Ostuni	**0.052^{e}**	**0.74^{ab}**	**0.44**
S. Marzano	0.017^{ab}	0.61^{ab}	0.68
Sinacori	0.018^{ad}	0.54^{ab}	0.26
Vesuviano	0.015^{ab}	0.41^{ab}	0.75
Patataro	0.022^{ab}	0.58^{ab}	0.78
POP2	0.025^{ad}	0.86^{ab}	0.75

The value are presented as mean ($n = 12$). Different letters within a column indicate means that differ significantly (Tukey's HSD test at $P \leq 0.05$). Genotypes marked in bold indicate pairs (R. Ostuni-UC82 and Linosa-Piriddu) with different nitrate efficiency according to Gourley's method (see Materials and Methods).

Table 2
Kinetic parameters for four tomato genotypes of the shoot dry-weight (g plant^{-1}) and NO_3^- supply response curves in hydroponic culture (R^2 or coefficient of determination was $P < 0.001$ for all genotypes).

Genotype	α	β	R^2
Linosa	0.038a	0.057b	0.69
Piriddu	0.046a	0.053b	0.81
UC82	0.024a	0.130a	0.72
Regina Ostuni	0.030a	0.042b	0.78

Each value is a mean ($n = 5$). Different letters within a column indicate means that differ significantly (Tukey's HSD test at $P \leq 0.05$).

Table 3
Comparing root cell membrane potentials of two tomato genotypes (UC82 and Regina Ostuni) measured before and after treatment with NO_3^-.

Genotype	SS	Dep	15 min	30 min
UC82	-122.7^a (± 1.6)	3.33^a (± 2)	-137^a (± 1)	-155^a (± 1.3)
Regina Ostuni	-142^b (± 4)	4.67^a (± 2.3)	-143.6^a (± 5.4)	-146.3^a (± 3.1)

SS, membrane potential at steady state or before NO_3^- contact; Dep, membrane depolarization after about 5 min of NO_3^- contact; 15 min, membrane potential after 15 min of NO_3^- contact; 30 min, membrane potential after further 15 min of NO_3^- contact. The values are mean ($\pm$SE) with $n = 15$ for each cultivar. Different letters within a column represent a significant difference (Student's unpaired *t*-test at $P \leq 0.05$).

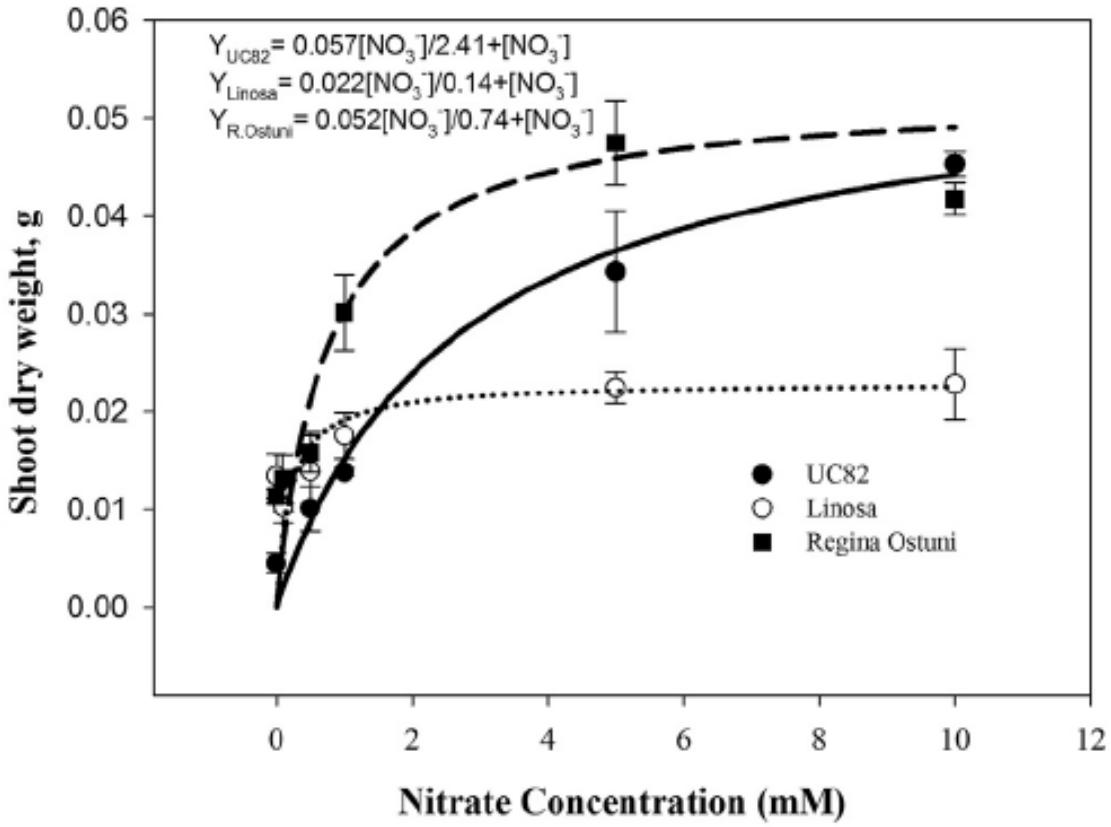

Fig. 1. Examples of the relationship between shoot dry weight and NO_3^- supply concentration for UC82, Linosa, Regina Ostuni tomato cultivars grown in sand culture used to calculate the kinetic parameters α and β shown in Tables 1 and 2. R^2 or coefficient of determination was $P < 0.001$ for all genotypes. The values are presented as mean $\pm$ SE ($n = 5–8$).

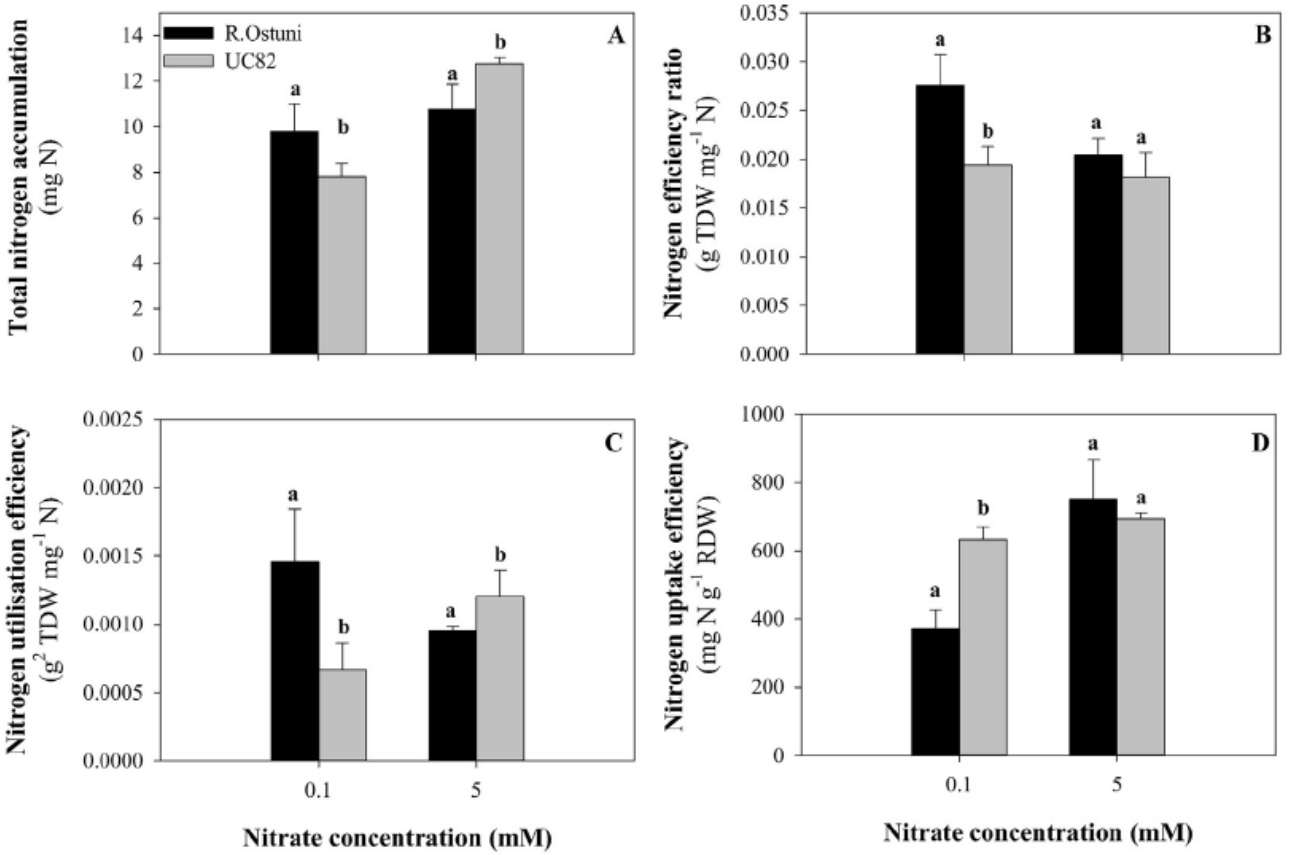

Fig. 2. (A) Total N accumulation (TNA), (B) N efficiency ratio (NER), (C) N utilization efficiency (NUtE) and (D) N uptake efficiency (NUpE) of Regina Ostuni (RO) and UC82 seedlings grown over 21 days at 0.1 or 5 mM NO_3^-. The values are presented as mean ± SE (n = 5–8). Different letters within nitrate concentration indicate means that differ significantly (Student's unpaired *t*-test at $P \leq 0.05$).

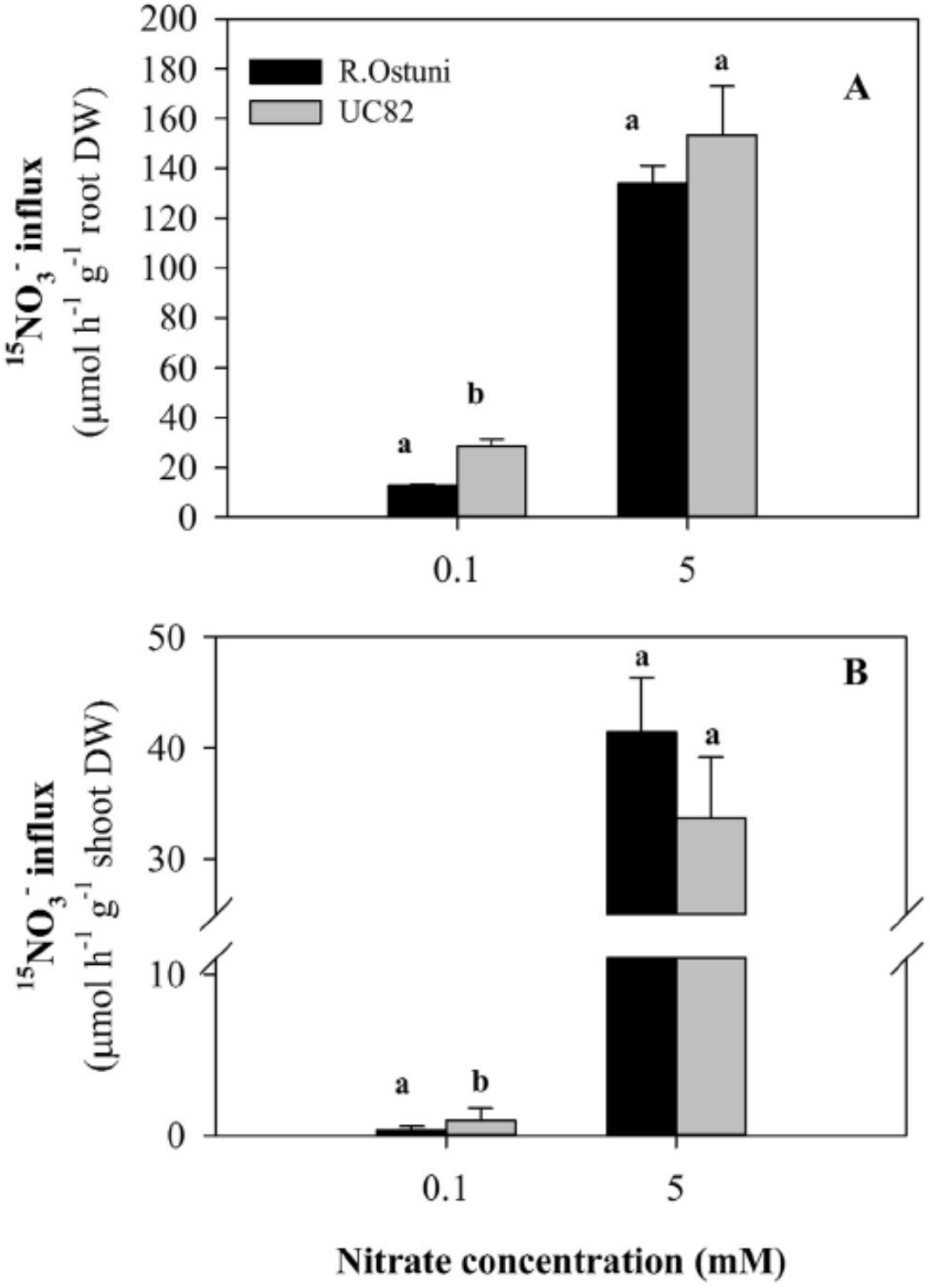

Fig. 3. Nitrate influx rate in roots (A) and shoots (B) of UC82 and Regina Ostuni (RO) genotypes measured using ^{15}N-enriched sources. The values are presented as mean ± SE (n = 5). Different letters indicate means that differ significantly between genotypes at each NO_3^- concentration (Student's unpaired *t*-test at $P \leq 0.05$).

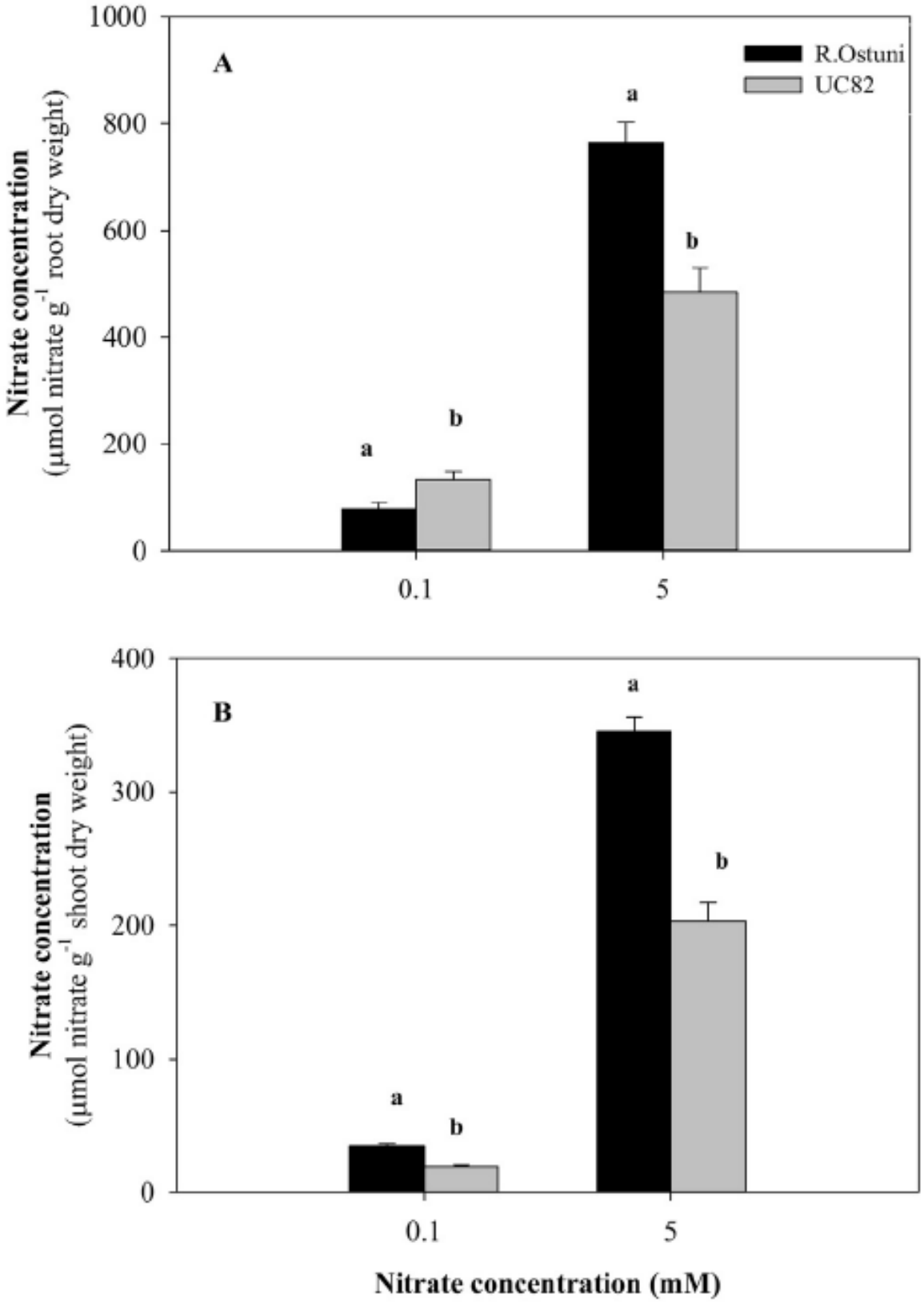

Fig. 4. Nitrate concentration (µmol nitrate g^{-1} dry weight) in root (A) and shoot (B) of Regina Ostuni (RO) and UC82 genotypes. The values are presented as mean ± SE ($n = 5$). Different letters within NO_3^- concentration indicate means that differ significantly (Student's unpaired *t*-test at $P \leq 0.05$).

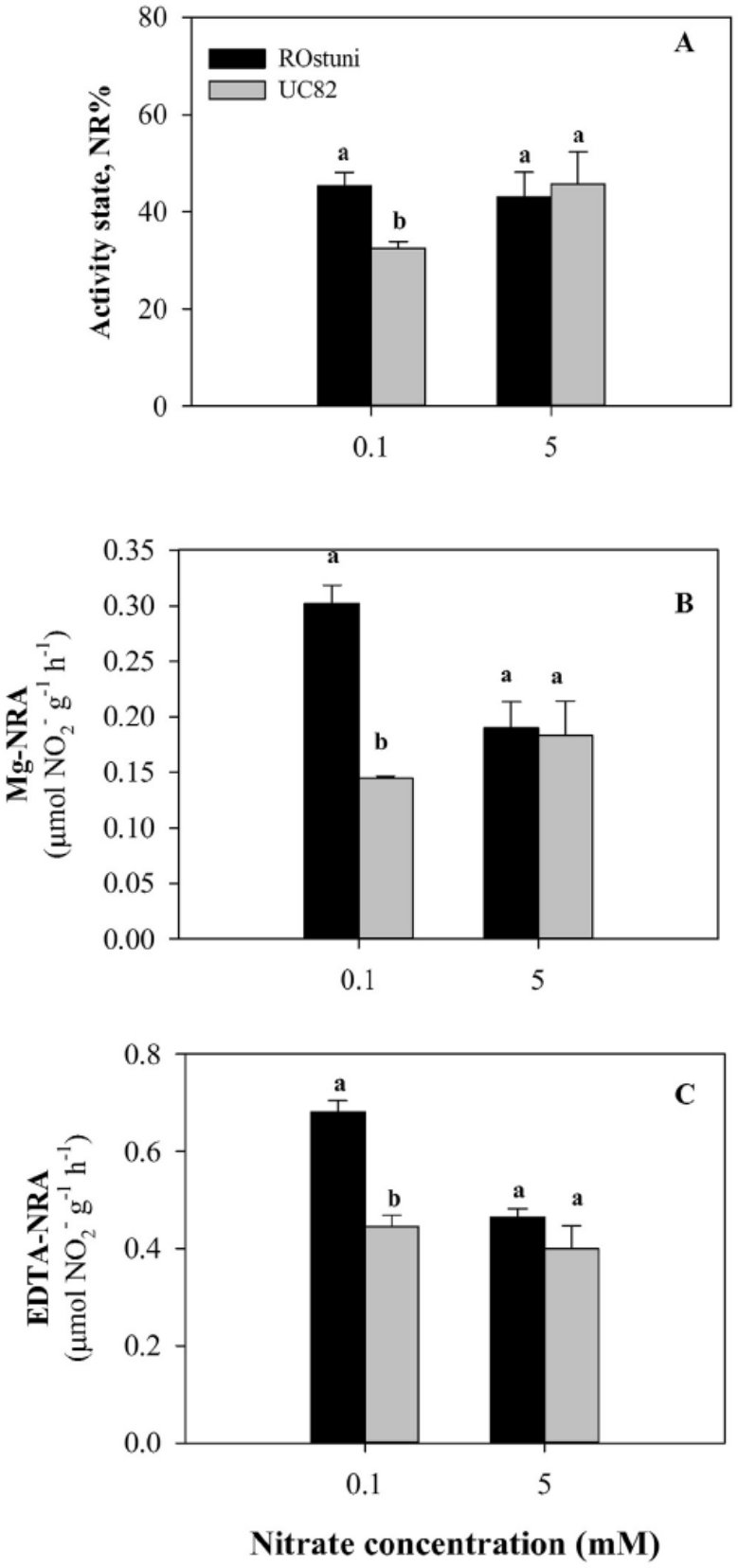

Fig. 5. Nitrate reductase activity on shoot of Regina Ostuni (RO) and UC82 seedlings grown at 0.1 and 5 mM NO_3^-. (A) Nitrate Reductase Activity (NRA) state [(Mg-assay/EDTA-assay) × 100]; (B) NRA assayed in presence of Mg^{2+} reflecting active non-phosphorylated enzyme; (C) NRA total activity reflecting phosphorylated plus non-phosphorylated enzyme activity. The values are presented as mean ± SE (n = 5). Different letters within NO_3^- concentration indicate means that differ significantly (Student's unpaired *t*-test at $P \leq 0.05$).

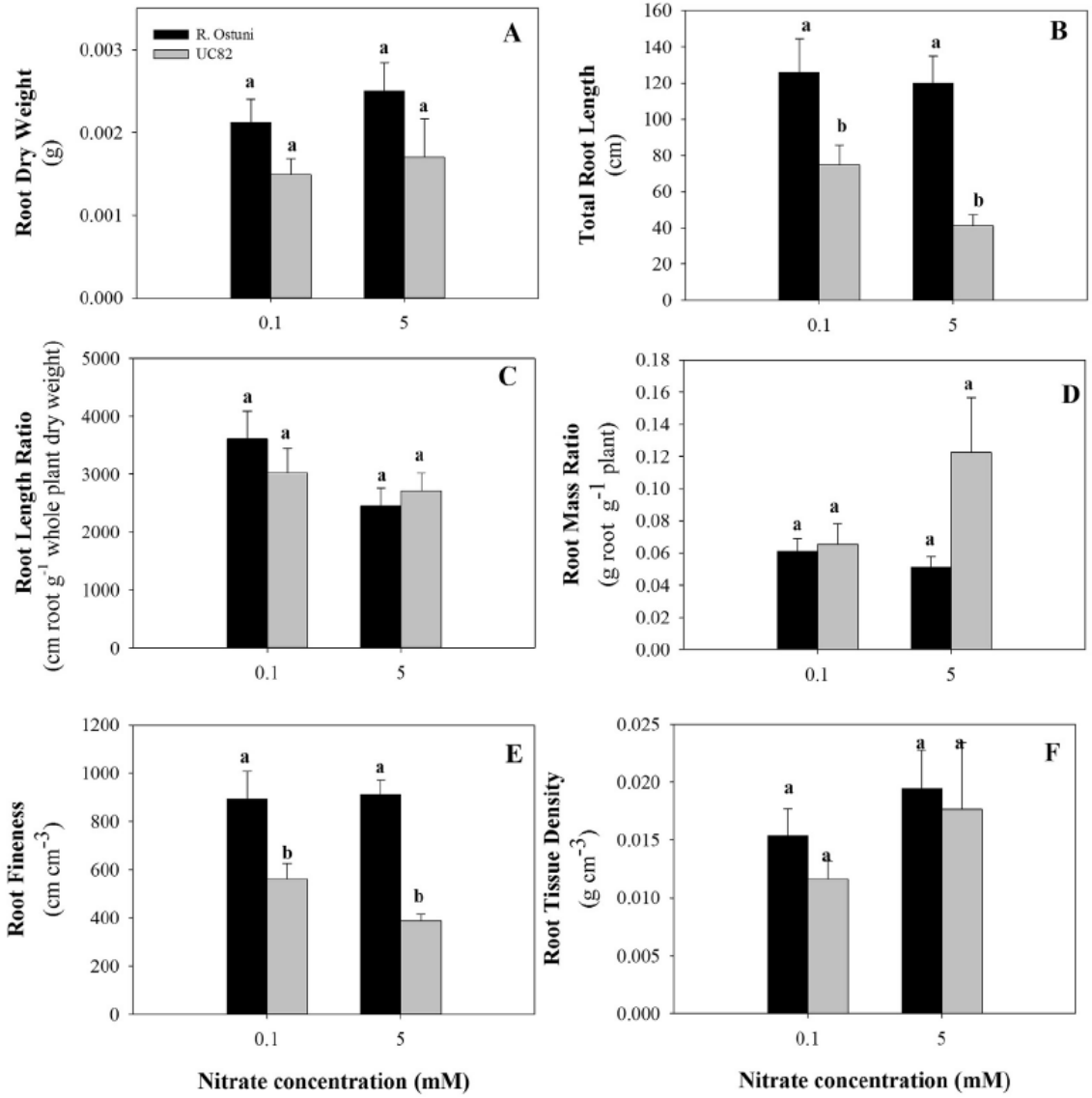

Fig. 6. Root morphology of Regina Ostuni (RO) and UC82 seedlings grown at 0.1 and 5 mM NO_3^-. A. Root dry weight, B. Total root Length, C. Root Length ratio, D. Root mass ratio, E. Root finesses, and F. Root tissue density. The values are presented as mean ± SE (n = 5). Different letters within NO_3^- concentration indicate means that differ significantly (Student's unpaired *t*-test at $P \leq 0.05$).