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Effects of NO_3^- Availability on Total Productivity, Root Morphology, Photosynthesis and Nitrogen Metabolism of Lettuce (*Lactuca sativa* L.) Recombinant Inbred Lines

J. He*, S.-F. Goh and L. Qin

Natural Sciences and Science Education Academic Group, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, 637616, Singapore

Abstract: We have identified certain heat-resistant (HR) and heat-sensitive (HS) lettuce (*Lactuca sativa*) recombinant inbred lines (RILs) from 113 lines under hot ambient temperature by studying the root morphology, shoot and root productivity. Except for temperature, one of the other major determinants of root morphology is nitrate (NO_3^-) availability. In this study, total productivity, root morphology, photosynthesis and nitrogen (N) metabolism of two RILs, 168 HS and 200 HR were studied under full N (100% NO_3^-), +N (125% NO_3^-) and –N (50% NO_3^-). The shoot and root productivity of both RILs under +N and –N treatments declined compared to those of full N plants. Reductions in root length, root surface area and total number of root tips were observed in 168 HS plants under both +N and –N treatments. For 200 HR plants, they all had similar values of root parameters regardless of N treatments. There were no significant differences in the light saturated CO_2 assimilation (A_{sat}) and stomatal conductance ($g_{s\ sat}$) between two RIL plants For each lettuce RIL, no differences in total chlorophyll (Chl) content and Chl a/b ratio were observed among the different N treatment. For both lettuce RILs, shoot NO_3^- concentration was highest in +N followed by full N plants and –N plants had the lowest values. There were no differences in root NO_3^- concentration between +N and full N plants but root NO_3^- concentration was significantly lower in –N plants than in +N and full N plants. For shoot total reduced N, +N plants had significantly higher concentration in both RILs compared to those of full N and –N plants. All plants had similar root total reduced N concentrations except for 168 HS under –N condition, which had significantly lower total reduced N concentrations. Differences in shoot maximal nitrate reductase (NR) activity among the different N treated plants were similar to those of total reduced N concentration. The relationships among NO_3^- availability, root morphology, productivity, photosynthesis and N metabolism were discussed.

Keywords: NO_3^- availability, NO_3^- reductase, photosynthesis, productivity, root morphology.

INTRODUCTION

Nitrogen (N) is one of the most important minerals required for plant growth. The major N source in soil is nitrate ($NO3^-$). Plant growth is most frequently limited by the availability of $NO3^-$ [1]. It was reported that growth of lettuce increases when the external N supply increases [2]. Many studies have shown that there is a regulatory and sensing mechanism between the carbon (C) production and N metabolism as they are sinks for ATP and $NADPH_2$ generated during photosynthesis [3].

NO_3^- is both a major N source for nutrition of plants and a signal to modulate plant development, suggesting that plant cells must have a sensor for NO_3^- availability [4]. Studies have shown that NO_3^- availability in the soil affects not only shoot and root production but also root morphology [5, 6]. For instance, the initiation and elongation of *Arabidopsis* lateral root development is stimulated by local availability of NO_3^- [7]. With lower NO_3^- available to *Arabidopsis thaliana*, lateral root (LR) initiation was suppressed. However, plants supplied with high concentration of NO_3^- showed that LR was initiated and elongated [8], which was due to increased influx of photosynthetic products at the site of NO_3^- uptake which is usually the roots [9]. It was also reported that birch (*Betula pendula*) plants allocate relatively less nutrients to the roots when NO_3^- is highly available in the soil [10]. These results indicate that the N uptake and assimilation is greatly promoted by increasing C supply. Therefore, N availability to the plants may directly or indirectly impact the N uptake and its assimilation.

NO_3^- is not used directly to synthesize amino acids in the plants. It is assimilated through a series of reduction catalysed by both nitrate reductase (NR) and nitrite reductase (NIR) into NH_4^+ which is then converted into amino acids and other organic N compounds [11-13]. In most grass species, the reduction of NO_3^- by NR is observed predominantly in shoots [14]. Synthesis of NR has been studied and proven to be promoted by light, NO_3^- and photosynthesis [13, 15]. It has been reported that tomato roots which were supplied with high NO_3^- had maximum NR activity and NO_3^- is believed to play a part in NR post-translational regulation [16].

*Address correspondence to this author at the Natural Sciences and Science Education Academic Group, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, 637616, Singapore;
Tel: 65-67903817; Fax: 65-68969432;
E-mail: jie.he@nie.edu.sg

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There is increasing use of fertilisers N in the cultivation of crops to ensure yield and concern about potential health risks of taking in excess NO_3^- concentration in lettuce. Hence, optimising or even reducing the supply of N such as NO_3^- to the vegetable crops becomes critical. In addition, there is more awareness about the impact of climate change to food safety and food security. It will be cost effective if we are able to identify crops that are more heat resistant yet able to produce high yield given lesser NO_3^- supplied to the growth mediate. In our previous study, some heat resistant (HR) and heat sensitive (HS) RILs of *Latuca sativa* have been identified from 113 lines [17, 18]. In this study, two lettuce RILs, namely 168 HS and 200 HR, were supplied with different concentrations of NO_3^-. The effects of NO_3^- availability on productivity, root morphology, photosynthetic CO_2 assimilation and N metabolism were investigated.

MATERIALS AND METHODS

Plant Materials and Culture Methods

Lettuce RILs (F_9 generation) were obtained from repeated crosses between HS *L. sativa* (cv. 'Salinas') and HR *L. serriola* (accession UC96US23). Previously identified HR (Line 200) and HS (Line 168) RILs were used for this study. After germination for three days, the plants were then grown in the greenhouse for 4 days for acclimatization before transplanting onto the hydroponic trays. All plants were exposed to ambient temperature fluctuating from 26 – 36°C under 100% of full sunlight with average maximal photon flux density (PPFD) of 1000 to 1200 µmol m^{-2} s^{-1} and 75% of relative humidity. Plants were divided into three groups and each of them was supplied with one of the three different NO_3^- concentrations: full N (100% NO_3^-), +N (125% NO_3^-) and –N (50% NO_3^-). Full N plants were supplied with full strength Netherlands Standard Composition. Nutrient solution conductivity and pH were maintained at 2 ± 0.2 mS and 6.5 ± 0.5, respectively. For +N treatment (125% NO_3^-), nutrients solution was added with additional 65% HNO_3. For –N treatment (50% NO_3^-), supply for the KNO_3 and $Ca(NO_3)_2$ were reduced by half as compared to the control nutrient solution and 50 ml of KCl and $CaCl_2.2H_2O$ were added respectively into the nutrient solution.

Measurement of Fresh Weight (FW) and Dry Weight (DW) of Shoot and Root

All plants were harvested after 6 weeks of different NO_3^- treatments. After plant removal from the troughs (09:00h to 10:00h), the sponge cube was removed from individual root system carefully. Each plant was divided into shoot and root. The shoots and roots were weighed separately before wrapping in aluminum foil. The DW of shoots and roots were recorded after they were dried at 80 °C for 4 days.

Analysis of Root Morphology

Root morphology was analysed with WIN MAC RHIZO V 3.9 programme after different NO_3^- treatments for two weeks. Total root length, surface area, average root diameter and number of root tips were determined by the programme.

Measurements of Light-Saturated Photosynthetic CO_2 Assimilation (A_{sat}) and Stomatal Conductance ($g_{s\ sat}$)

Five weeks after different NO_3^- treatments, A_{sat} and $g_{s\ sat}$ of attached fully expanded leaves (the 6th leaves from the base) were recorded simultaneously between 09:00 h to 11:00 h in the greenhouse with an open infrared gas analysis system with a 6-cm^2 chamber (LI-6400, Biosciences, U.S.). Readings were taken with an LED light source in the wavelength range 660 to 675 nm under a PPFD of 1200 µmol m^{-2} s^{-1}. Average relative humidity and ambient [CO_2] in the chamber were 70% and 400 ± 5 µmol mol^{-1}, respectively. Leaf chamber temperature was set according to greenhouse conditions (30 – 32 °C). It usually took about 3–5 min for both A_{sat} and $g_{s\ sat}$ to be stable. For each treatment, five readings were obtained from five different leaves of five different plants (n = 5).

Determination of Chl

Fresh tissues of 0.5 g from each treatment were soaked in 5 ml of N, N-dimethylformamide (N, N-DMF, Sigma Chemical Co.) in the dark for 48 h at 4 °C. The absorptions of Chl were measured at 647 nm and 664 nm, respectively using a spectrophotometer (Du 650, Beckman, USA). Total Chl content was calculated according to Welburn [19].

Determination of NO_3^-

Dried plant tissue of 0.03 g was ground with deionised water and then incubated at 37 °C for 2 h. Prior to analysis, sample turbidity was removed by filtration through a 0.45 m pore diameter membrane filter. The NO_3^- was determined according to Allen [20] using a Flow Injection Analyser (Model QuikChem 8000, Lachat Instruments Inc, Milwaukee, WI, USA). In this measurement, NO_3^- was first reduced to NO_2^- by passage of the sample through a copperized cadmium column. The NO_2^- was then determined by diazotizing with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride. The resulting water soluble dye was read at 520 nm.

Determination of Total Reduced N

For each treatment, dry samples of 0.05 g from each treatment were placed into a digestion tube with a Kjeldahl tablet and 5 ml of concentrated sulfuric acid. After the digestion was completed, the N content was determined by a Kjeltec auto 1030 analyser after the digestion was completed.

Determination of Maximal Nitrate Reductase (NR) Activity

Leaf or root samples (harvested during light period between 09:30 to 10:30 h) were immediately frozen in liquid nitrogen after weighed and stored at – 80°C until use. Tissues were extracted using Tris-HCl buffer (pH 8.5) developed by [21]. A frozen sample of 0.5 – 1 g was powdered in liquid nitrogen and ground with 3 ml of extraction buffer, with a mortar and pestle in the presence of 0.2 g/g fw insoluble PVP. The extraction buffer includes 0.25 M Tris-HCl (pH 8.5), 3 mM dithiothreitol (DTT), 10 µM flavin adenine dinucleotide (FAD), 1 µM sodium molybdate, 1 mM ethylenediamine-tetra-aceticacid (EDTA). The extracts were cen-

trifuged at 15,000 g for 10 min at 4 °C. NR activity was measured immediately in the supernatant.

In vitro NADH: NR activity assay was derived from Kaiser and Huber with modification. The maximum activity of NR was determined by assaying NR with Mg^{2+} (10 mM). In all cases, the total reaction medium was 700 µl which contained 50 mM Hepes-KOH (pH 7.5), 1 mM DTT, 10 µM FAD, 10 mM KNO_3, 0.2 mM NADH, NR extraction, and 10 mM $MgCl_2$ or 15 mM EDTA. The reaction was started by adding of 200 µl NR extraction. Incubation was performed at 25°C for 20 min, and the reaction was then terminated by the addition of an equal volume (700µl) of sulfanilamide (1% (w/v) in 3 N HCl) and the naphthylethylenediamine dihydrochloride (0.02% w/v). After 30 min at room temperature, the absorbance at 540 nm (A_{540}) of all the samples was read. The blank was identical to the samples. NR activity was expressed as nmol nitrite min^{-1} mg^{-1} protein.

Statistical Analysis

Means across all treatments were discriminated using ANOVA and followed by Tukey's multiple comparison test. The difference among the means were considered significant at $p<0.05$. All statistical analyses were conducted using MINITAB software (MINITAB, Inc., Release 15, 2007).

RESULTS

FW and DW of Shoots and Roots

At harvest (6 weeks after transplanting), the FW and DW of both RILs under +N and –N treatments declined significantly compared to those of full N plants (Figs. **1A** to **1D**). Figs. (**1E** and **1F**) show that shoot/root FW ratio was higher in both RILs grown under +N compared to full N and –N treatments. The shoot/root DW ratio of 168 HS plants under full N was lower than those grown under +N and –N conditions. However for 200 HR plants, the highest shoot/root DW ratios were found in +N plants followed by full N plants and the lowest shoot/root DW ratio were obtained from –N plants (Fig. **1F**).

Root Morphology

Reductions in root length (Fig. **2A**), root surface area (Fig. **2B**) and number of root tips (Fig. **2C**) were observed in 168 HS plants under both +N and –N treatments. However, there were no significant differences in root diameters

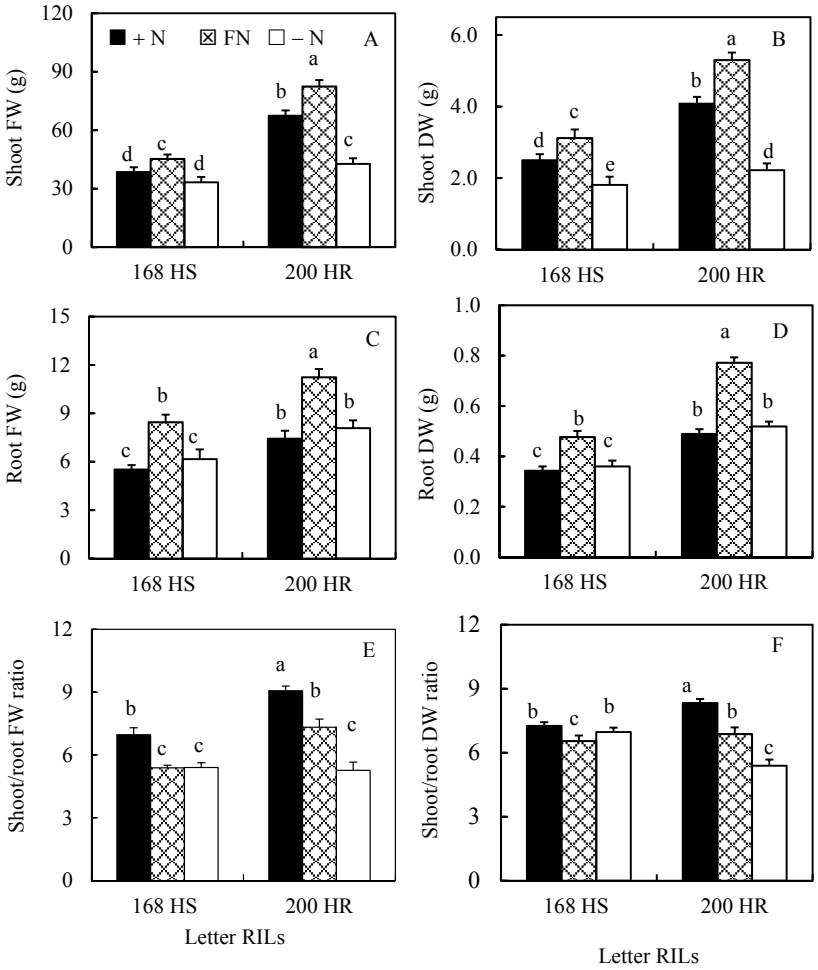

Fig. (1). Shoot FW and DW (**A, B**), root FW and DW (**C, D**), shoot/root ratio FW and DW (**E, F**) of 168 HS and 200 HR lettuce RILs grown under different N treatments for 6 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

among the different N treatments in 168 HS plants (Fig. **2D**). For 200 HR plants, all plants had similar values of all root parameters regardless of N treatments (Fig. **2**).

A_{sat}, $g_{s\ sat}$ and Chl Content

Fig. (**3**) shows A_{sat} and $g_{s\ sat}$ of both lettuce RILs that were grown under different N treatments for 5 weeks. A_{sat} and $g_{s\ sat}$ of different plants ranging from 11 – 12.5 μmol CO_2 m^{-2} s^{-1} and 1080 –1200 mmol H_2O m^{-2} s^{-1}, respectively. However, statistically, there were no significant differences for both A_{sat} (Fig. **3A**) and $g_{s\ sat}$ (Fig. **3B**) among the different N treatments in both lettuce RILs. Total Chl content was higher in 200 HR plants than in 168 HS plants. For instance, the total Chl contents for 200 HR and 168 HS plants were, respectively, about 2000 – 2060 μg g^{-1} FW and 1700 –1870 μg g^{-1} FW. However, for each RIL, all plants under different N treatments had similar total Chl content (Fig. **4A**) and Chl a/b ratios, ranging from 2.5 – 2.7 (Fig. **4B**).

NO_3^-, Total Reduced N Concentration and Maximal NR Activity

For both lettuce RILs, shoot NO_3^- concentration was highest in +N plants followed by full N plants and –N plants had the lowest values (Fig. **5A**). However, no differences were found in root NO_3^- concentrations between +N and full N plants but root NO_3^- concentration was significantly lower in –N plants compared to +N and full N plants (Fig. **5B**). For shoot total reduced N, +N plants had significantly higher concentration in both RILs than those of full N and –N plants (Fig. **5C**). All plants had similar root total reduced N concentrations except for 168 HS under –N condition, which had significantly lower total reduced N concentrations (Fig.

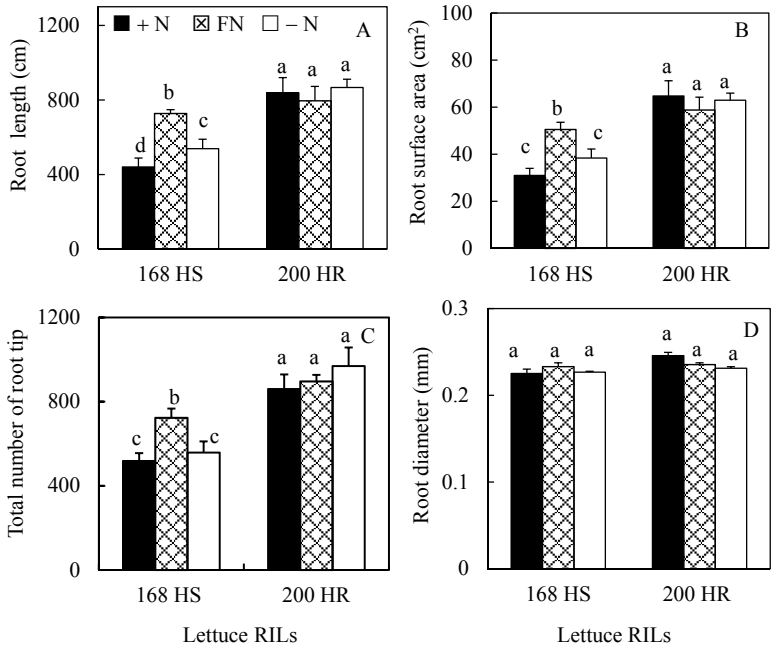

Fig. (2). Root length (**A**), root surface area (**B**), total number of root tips (**C**) and root diameter of 168 HS and 200 HR lettuce RILs grown under different N treatments for 6 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

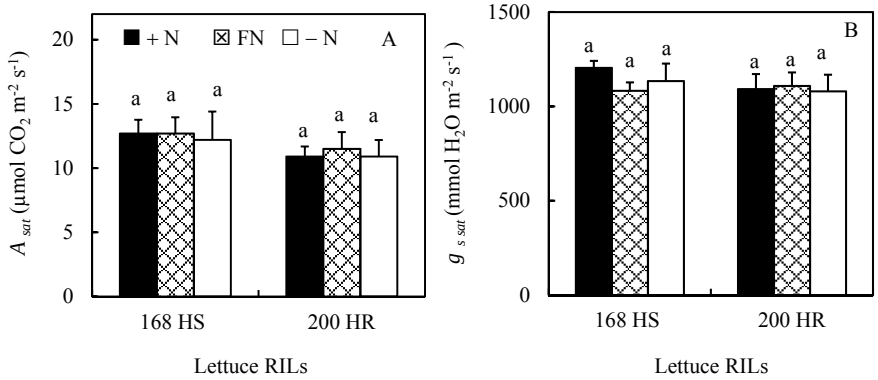

Fig. (3). A_{sat} (**A**) and $g_{s\ sat}$ (**B**) of 168 HS and 200 HR lettuce RILs grown under different N treatments for 5 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

5D). Changes in shoot maximal NR activity in both RILs and in root maximal NR activity in 200 HR plants were very similar to those of total reduced N (Fig. **6A**). However, 168 HS plants had significantly higher root maximal NR activity under +N conditions compared to those under full N and –N conditions (Fig. **6B**).

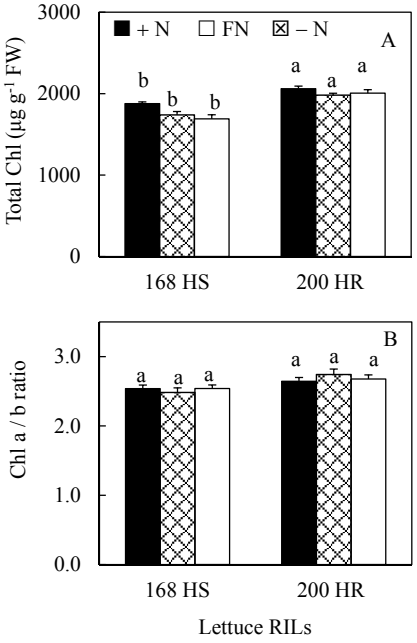

Fig. (4). Total Chl content (**A**) and Chl a/b ratio (**B**) of 168 HS and 200 HR lettuce RILs grown under different N treatments for 6 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

DISCUSSION

In present study, the results demonstrated that by limiting N supply in the nutrient solution, the FW and DW of the shoots and roots declined significantly for both 168 HS and 200 HR plants (Figs. **1A** to **1D**) as less NO_3^- is made available to the lettuce plants (Figs. **5A**, **5B**). N deficiency most frequently limits plant growth [1, 2]. However, subjecting to N deficient but constant low N concentrations in the root medium, plants normally reduce shoot growth rate without affecting photosynthesis [22-24]. This was also observed in the present study (Fig. **3**), that was, no significant differences in photosynthetic gas exchanges (Fig. **3**) and total Chl content (Fig. **4**) among the different N treatments were observed. It was reported that when plants supplied with limited N, newly fixed carbon is mainly channeled toward the below part of the plants so that root elongation can be stimulated [25]. This results in a decrease in the shoot/root biomass ratio [24, 26, 27]. The results of both lettuce RILs in the present study (Fig. **1E**) supported these earlier findings. Walker *et al.* [2] reported that lettuce growth increase when the external N supply increases. In the present study, it was surprise to see that increase the supply of NO_3^- (+N plants) also resulted in the decreases of shoot and root productivity in both lettuce RILs (Figs. **1A** to **1D**) although there was no change in photosynthetic gas exchange (Fig. **3**) compared to those of full N plants.

Both intrinsic factors such as the supply of photoassimilate and extrinsic factors including NO_3^- availability affect growth and development of a root system [5-10, 28-30]. In the present study, it was shown that by changing N (both +N and –N treatments) to the 168 HS plants changed the root morphology in terms of reduction in their root length and root surface area and the number of root tips (Fig. **2**). Zhang *et al.* [8] reported that low NO_3^- availability suppressed the

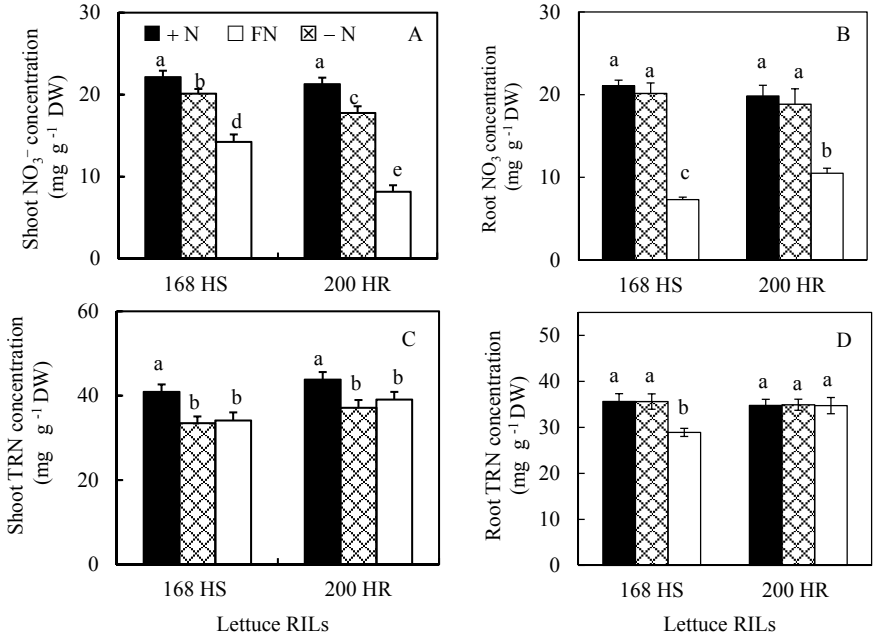

Fig. (5). Shoot and root NO_3^- (**A, B**) and total reduced N (**C, D**) concentrations of 168 HS and 200 HR lettuce RILs grown under different N treatments for 5 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

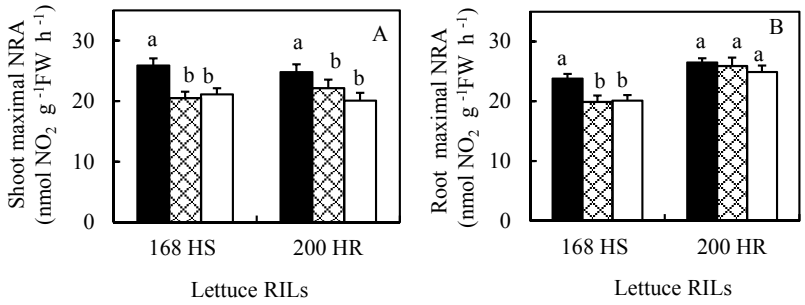

Fig. (6). Shoot (**A**) and root (**B**) maximal NR activity of 168 HS and 200 HR lettuce RILs grown under different N treatments for 5 weeks. Each bar is a mean of 5 measurements. Vertical bars represent standard error. Means with different letters above the bars are statistically different ($p < 0.05$) by Tukey's multiple comparison test.

initiation of lateral root. However, their results also showed that high NO_3^- concentration stimulate the initiation and elongation of lateral root due to high photosynthate partitioned to the roots [9]. In the present study, although all 168 HS plants had very similar photosynthetic rate higher shoot/root FW ratio grown under +N compared to full N and –N treatments imply that the supply of photosynthetic product from the shoot could in part responsible for the changes of root morphology. High NO_3^- availability in *Arabidopsis* plants could cause localized stimulatory effects on lateral root proliferation and a systemic inhibitory effect on the root elongation indicating that there are signalling pathways that regulate root development in response to the availability of NO_3^- [8]. However, our results are controversy with those of Zhang *et al.* [8]. In this study, hydroponic system is used to cultivate the lettuce plants. Roots were immersed in the nutrient solutions at all time. There is no necessity to increase root length as well as root surface area [8]. The resource optimization theory about plants which tend to allocate relatively lesser to the roots when nutrient availability increases [10] is reaffirmed. Zhang and Forde [31] reported that the LR of *Arabidopsis* showed two contrasting responses to high NO_3^-. Uniformly high NO_3^- reduces lateral root elongation throughout the root system. However, in plants grown on a low NO_3^- concentration, exposure of a section of the primary root to high NO_3^- induces a local stimulation of LR elongation [31]. In the present study, the +N plants were grown under uniformly high NO_3^- concentration. However, for 200 HR plants, all plants had similar values of all root parameters regardless of N treatments (Fig. **2**). The development of root for two different heat resistant RILs was affected not only by NO_3^- availability but also other external factors such temperature, especially the root-zone temperature [17]. Using other lettuce cultivars, our previous studies showed that high root-zone temperature inhibited root growth and development [32-34] due the alternation of photoassimilate partitioning [35].

NO_3^- plays a part in NR post-translational regulation [16]. On the other hand, NR activity is affected by NO_3^- and photosynthesis [13, 15]. There were significant increases of shoot NO_3^- and total reduced N concentrations in both RILs exposed to +N treatment (Figs. **5A**, **5C**). This could be due to a higher of conversion of NO_3^- into reduced N by NR [14]. These results corresponded well with a significant higher maximal NR activity in the shoots of RILs under +N conditions compared to those of full N and –N plants (Fig. **6**). Although shoot NO_3^-concentrations in both RILs supplied with lower NO_3^- (–N plants) were lower compared to that of full N plants, the –N plants had similar total reduced N concentrations (Figs. **5C**, **5D**) and NR activity (Fig. **6**) as full N plants of both RILs. NO_3^- uptake and assimilation is an energy demand process [3, 13, 15]. Chen *et al.* [36] reported that NR activity might be induced only when reaching a threshold of NO_3^- concentration for leafy vegetables. This may contribute to the high maximal NR activity in both shoot and roots of both RILs that had much higher shoot and root NO_3^- concentration grown under +N condition. Carbon metabolism seems to be essential both for provision of energy for NO_3^- uptake and for regulation of NO_3^- assimilation [37]. In the present study, High NO_3^- uptake and conversion in +N plants; and high NO_3^- assimilation with lower NO_3^- concentration could be at the costs of new fixed carbon and thus reduce the accumulation of biomass in both +N and –N plants (Fig. **1**).

CONCLUSION

Both excessive and limited NO_3^- supply reduced the productivity of shoots and roots. It is not economically practical to supply higher or lesser NO_3^- to the growth media in order to increase or to maintain the productivity of HR and HS lettuce RILs, respectively. There was no clear correlation between NO_3^- availability and root morphology. However, it was evident that the responses of two different heat resistant lettuce RILs to NO_3^- availability were different in terms of root length, root surface area and the number of roots tip. There are multiple factors such as NO_3^- availability, the supply of photoassimilate and temperature, which are responsible for lettuce root growth and development. Lower biomass accumulation in both +N and –N plants could be due to the high energy demand for both NO_3^- uptake and assimilation.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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