Effect of Nitrogen Sources on Lettuce Root Growth and Development in Physiological and Molecular Levels

ผลของแหล่งไนโตรเจนต่อการเจริญเติบโตของรากผักกาดหอม ในระดับสรีรวิทยาและระดับโมเลกุล

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ABSTRACT

Lettuce (*Lactuca sativa*) is an important hydroponic-grown crop that requires extensive care of nutrient supplement especially nitrogen to promote optimal growth. The purpose of this study was to investigate the effect of different ratio of nitrogen sources (ammonium and nitrate) on lettuce root growth and development at physiological and molecular levels. The results showed that green oak lettuce prefers nitrogen source in form of nitrate by observing lateral root density. Nitrate accumulation analysis also showed nitrate content profile changed in shoot and roots according to ammonium to nitrate ratio. To characterize nitrate uptake at molecular level, nitrate transporter gene (*NRT1*) expression, was analyzed using semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The result showed that the expression of *NRT1* was found when lettuce was grown with high concentration of nitrate (1/2 (NH_4^+/NO_3^-)) but the expression tended to increase when nitrogen source was absent which indicated that *NRT1* in lettuce might be upregulated to increase nitrate uptake when nitrogen from rhizosphere is depleted continuously. The knowledge gained from this study will help us understand more about nitrogen source preference in lettuce and might suggest beneficial ratio of NH_3^+/NH_4^+ to promote optimal root system in hydroponically-grown green oak industry.

บทคัดย่อ

ผักกาดหอม (Lettuce) เป็นพืชเศรษฐกิจสำคัญที่ได้รับความนิยมปลูกแบบไฮโดรโปนิกส์อย่างแพร่หลายซึ่งต้องการแร่ธาตุอาหารโดยเฉพาะไนโตรเจนเพื่อเพิ่มการเจริญเติบโต การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของแหล่งไนโตรเจนต่อการเจริญเติบโตของรากผักกาดหอมในระดับสรีรวิทยาและโมเลกุล ผลการศึกษาพบว่าผักกาดหอมมีแนวโน้มการเกิดรากเพิ่มขึ้นในกลุ่มที่มีความเข้มข้นของไนเตรตสูง และพบการสะสมไนเตรตของรากและใบที่แตกต่างกันเมื่อความเข้มข้นของอัตราส่วนระหว่างแอมโมเนียมและไนเตรตมีการเปลี่ยนแปลงไป นอกจากนี้ผลการศึกษาระดับโมเลกุล พบว่ามีการแสดงออกของยีนขนส่งไนเตรต (*NRT1*) ในกลุ่มที่มีความเข้มข้นของไนเตรตสูง แต่การแสดงออกของยีนขนส่งไนเตรตมีแนวโน้มเพิ่มขึ้นเมื่อไม่มีแหล่งไนโตรเจน ทั้งนี้อาจเป็นไปได้ว่ามีการเพิ่มการแสดงออกของยีนขนส่งไนเตรตในผักกาดหอมเพื่อเพิ่มความสามารถในการดูดซึมไนเตรตในสภาวะที่รากขาดไนโตรเจนอย่างต่อเนื่อง โดยประโยชน์จากการศึกษาในครั้งนี้เพื่อการนำไปประยุกต์ใช้ในเรื่องของสัดส่วนของธาตุอาหารจากแหล่งไนโตรเจนเพื่อทำให้เกิดผลผลิตที่สูงและสามารถลดต้นทุนการผลิตได้

Keywords: Root system, Lateral root development, Nitrate transporter

คำสำคัญ: ระบบราก การเจริญของรากแขนง ตัวขนส่งไนเตรต

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Introduction

Green oak lettuce (*Lactuca sativa*) is one of the most widely produced vegetable crops. This annual leaf vegetable is rich in antioxidants making it a vegetable good for the health (Zhong et al., 2018). Since 2012, lettuce was developed to fresh-market and expanded to the industry. In 2015, it was considered to be the United State's most valuable crops after reaching $1.9 billion in sales. Nowadays, the rising cost of shipping produce tends to increase demand for lettuce production in other regions of the country such as Northwestern, and Southeastern of the United States. In agriculture, nitrogen is an essential macronutrient for plant growth and development (Okamoto et al., 2003). It is generally used to increase crop productivity, and the nitrogen level available to plants is one of the most limiting factors in crop productivity. Plants use many forms of available nitrogen sources including nitrate, nitrite and ammonium. Plants normally absorb nitrogen in form of nitrate and ammonium. Ammonium is taken up by ammonium transporters, and nitrate is absorbed by plants via nitrate transporters (Razgallah et al., 2017). Nitrate accumulation in plants is influenced by light intensity, photoperiod, temperature and fertilizer applied.

The uptake of nitrate requires active transport system depending on nitrate concentration including two high-affinity transport systems (HATS) which can take up nitrate at very low concentration (<250 µM) and two low-affinity transport systems (LATS) which can take up nitrate at high concentration (>250 µM). Genes involved in the nitrate pathway have been identified in plants; NRT1 for LATS and NRT2 for HATS. The LATS (*AtNRT1.1* or *CHL1*) was the first considerable nitrate transporter gene in higher plants to be cloned from *Arabidopsis* (Loque et al., 2003). However, NRT1 in lettuce is still poorly studied.

Roots system (RS) is an important factor that determines how efficiently plants can absorb nutrients. It varies widely between species, and different species have evolved different RSs depending on environment in which plants can grow (Ristova et al., 2014). RS direction is useful in breeding of crops, because it affects plant' ability to absorb water and nutrients leading to impact on the final yield of crops (de Dorlodot et al., 2007). When focusing on the important factors of RS, lateral root growth and development is one of the most important components of the adaptability of root to respond to its environments.

In lettuce, there is very little information about genes of *NRT* family and genotype differences in characteristic of *NRT1*. The absorption of nitrate by root relies on a specific plasma membrane nitrate transporter NRT1.1 (CHL1/NPF6.3) (Noguero and Lacombe 2016). NRT1.1 is a key regulator for the nitrate regulation of RS, because it acts as a dual transporter/sensor function by triggering a specific nitrate signaling pathway in response to lateral root growth and development (Sun et al., 2017). However, there is no evidence yet that the mechanisms of NRT1.1 in response to nitrate affect on root system in lettuce.

In this study, we investigated effect of different ratio of nitrogen sources (ammonium and nitrate) on root growth and development and nitrate contents in order to evaluate response of RSA to different nitrogen source in physiological and molecular levels as well as preference of nitrogen forms in lettuce. Moreover, this study also identified genes involved in nitrate transport in lettuce, and evaluated the relationship between nitrate accumulation and expression pattern of nitrate transporter genes in lettuce. The knowledge gained from this study will help us understand how nitrate can control RS in green oak lettuce physiologically and molecularly as well as provide beneficial ratio of NH_3^+/NH_4^+ to give the preferential RSA for green oak growing industry.

Objective of the study

The aims of this study were to investigate the effect of different ratio of nitrogen sources (ammonium and nitrate) on root growth and development and nitrate contents in lettuce in order to evaluate nitrogen form preferences, and observed the expression of nitrate transporter gene (*NRT1*) in root by semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Materials and methods

Plant material and growth condition for hydroponic system

Hydroponic experiments were conducted in growth chamber at Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. The lettuce species used in this experiment was Green Oak lettuce (*Lactuca sativa*). The Green oak seeds were germinated on hydroponic planting material (sponge) in a plastic tray which was floated on the commercial solution (AB solution). The tray was kept moist at 25°C until germination. After approximately 28 days of germination, the plants were transferred to a hole in gully that linked to the storage tank contained 10 L of nutrient solution by using Nutrient Flim Technique system (NFT) with different concentrations of nitrate including control, no-N source, high NO_3^-, high NH_4^+ (NH_4^+/NO_3^- ratios of 1/1.5, 0/0, 1/2, 1.5/1, respectively). (Table 1).

The nutrient solution also contained monopotassium phosphate (KH_2PO_4), potassium nitrate (KNO_3), ammonium sulfate ($(NH4)_2SO_4$), calcium nitrate ($Ca(NO_3)_2•4H_2O$), calcium sulfate ($CaSO_4$), hydrate magnesium sulfate ($MgSO_4•7H_2O$), ammonium nitrate (NH_4NO_3), potassium sulfate (K_2SO_4), boric acid (H_3BO_3), manganese sulfate ($MnSO_4•H_2O$), zinc sulfate ($ZnSO_4•7H_2O$), copper sulfate ($CuSO_4•5H_2O$), sodium molybdate ($NaMoO_4•2H_2O$) and Fe (in Fe-sequestrene). Throughout the experiment, the pH of each nutrient solution was adjusted to 5.6. The solution was completely renewed every 7 days.

Table 1 Macronutrient and micronutrient quantities (g $100L^{-1}$)

Macronutrient quantities (g $100L^{-1}$)

Treatments	KH_2PO_4	KNO_3	K_2SO_4	$Ca(NO_3)_2•4H_2O$	$CaSO_4$	$MgSO_4•7H_2O$	NH_4NO_3	$NH_4(SO_4)_2$
control	14.17	56.68	48.80	28.80	68.66	42.51	127.8	-
No N-source	14.17	-	48.80	-	68.66	42.51	-	-
High NO_3^-	14.17	56.68	48.80	122.31	-	42.51	85.2	-
High NH_4^+	14.17	-	48.80	-	68.66	42.51	127.8	25.56

Micronutrient quantities (g $100L^{-1}$)

treatment	H_3BO_3	$MnSO_4•H_2O$	$ZnSO_4•7H_2O$	$CuSO_4•5H_2O$	Fe (in Fe-Sequestrene)	$NaMoO_4•2H_2O$
All treatments	1.760	0.880	0.018	0.009	7.000	0.000008

Nitrate content measurement by salicylic acid-UV/VIS spectrophotometry

Sample preparation

Lettuce was dried in hot air oven at 60°C for 5 days. Then, 0.2 g of lettuce was grounded with 1ml of distill water in a new 1.5 ml microcentrifuge tube and incubating at 48°C for 1 hour. The mixture was centrifuged at 15,000 rpm for 20 minutes. Supernatant was collected at 0.2 ml and added to 0.8 ml 5% (w/v) Saliccylic acid in concentrated H_2SO_4 and leave the solution in room temperature until cool down. The absorbance measurement was made at 410 nm.

Chemical preparation

KNO_3^- standard solution (0.5 mg NO_3^--N/ml) was prepared by dissolve 3.6090 g anhydrous KNO_3^- in distilled water with the final volume 1L, and then add 2 ml chloroform. The solution was kept at 4°C and can be used for 6 months. Five grams of salicylic acid was added in 100 ml of H_2SO_4. 2N of NaOH was also prepared into 1L.

Standard curve preparation

KNO_3^- standard solution (0.5 mg NO_3^--N/ml) was prepared at following concentration: 0 µg, 50 µg, 100 µg, 150 µg, 200 µg, 250 µg and 300 µg for nitrate analysis in shoot and 0 µg, 20 µg, 40 µg, 60 µg for nitrate analysis in root. Nineteen ml of 2 N NaOH were added to 0.2 ml of distill water with 0.8 ml 5% (w/v) Saliccylic acid in concentrated H_2SO_4 and leave the solution in room temperature until cool down. The absorbance measurement was made at 410 nm. Standard curve between Abs_{410} and KNO_3 standard solution concentration were plotted and R-square value was determined.

Molecular identification

RNA extraction and cDNA synthesis

To extract RNA and perform cDNA synthesis, 0.2 g of frozen root tissue was cut off into a fine powder in liquid nitrogen and total RNA was isolated following the total RNA mini kit (plant) protocol (Geneaid). For each sample of total RNA was treated with 25 µl of DNA-digested solution (Geneaid) for 30 minutes at 37°C to remove any DNA contamination. The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). All transcription reactions were added in a total volume 20 µl with 10 µl of RNA sample (1,000 ng of RNA + DEPC water), 2 µl of 10x RT buffer, 0.8 µl 25x dNTP mix, 2 µl of 10x RT random primers, 1 µl of MultiScribe™ Reverse Transcriptase and 4.2 µl of Nuclease-free water. Reactions were run by following the conditions of High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems): 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes and 16°C infinity.

Polymerase chain reaction

The reaction was in triplicate with 25 µl volumes containing 2.5 µl of 10x reaction buffer, 0.5 µl of 10 mM dNTP mix, 0.5 µl of each primer (0.2 µM of final concentration), 0.2 µl of Taq DNA polymerase (5 U/ µl) and 20.8 µl of sscDNA sample including 2 µl of diluted sscDNA template (200 ng of final concentration) and 18.8 µl of nuclease free water. Reactions were run in a T100™ thermal cycler using the following cycling program: 94 °C for 7

minutes, 34 cycles at 94°C for 30 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds. To extend all incomplete PCR products, 72 °C for 7 minutes at final extension step and 16 °C infinity for storage in the cycler. Gene-specific PCR primers (table 2) were designed following Razgallah et al (2017). To normalize amounts of template cDNA, the actin gene from *L. sativa* was used as an internal control. Gene expression analysis was calculated using Molecular Imager® Gel Doc™ XR + Imaging System.

Table 2 Primers of putative nitrate transporter genes used for qRT-PCR analysis Razgallah et al. (2017)

GenBank (db EST) Accession number	Forward primer 5'-3'	Reverse primer 5'-3'	Temperature (°C)
DY975195 (LsNRT1)	F-ACACCTTGCCCTTCATTCAC	R-CTGCCTTTGTAGTCCCAAG	58
AY260165 (Actin)	F-GTGATGGTAGGTATGGGCCA	R-TCCATGTCATCCCAGTTGCT	58

Influence of nitrogen form (ammonium and nitrate) on root development in lettuce

Root growth and development

To characterize the effect of different ratio of nitrogen sources (ammonium and nitrate) on root growth and development in order to evaluate nitrogen form preference, green oak was grown in the bottle with different NH_4^+/NO_3^- ratio in nutrient solutions. The results showed that lettuce could not grow under nutrient starvation which acted as negative control compared to other groups. On the other hand, the primary root length and lateral root elongation obviously increased in sufficient nutrient solution (Hoagland solution) which acted as a positive control compared to nutrient starvation treatment. The lateral root surprisingly increased when lettuce was grown in nitrate treatment alone (0/2 (NH_4^+/NO_3^-)). The inhibition of lateral root slightly appeared when presented ammonium (1/1 (NH_4^+/NO_3^-)) compared to nitrate treatment alone (0/2 (NH_4^+/NO_3^-)) and the inhibition of primary and lateral root length strongly appeared in ammonium treatment alone (2/0 (NH_4^+/NO_3^-)) (Figure.1).

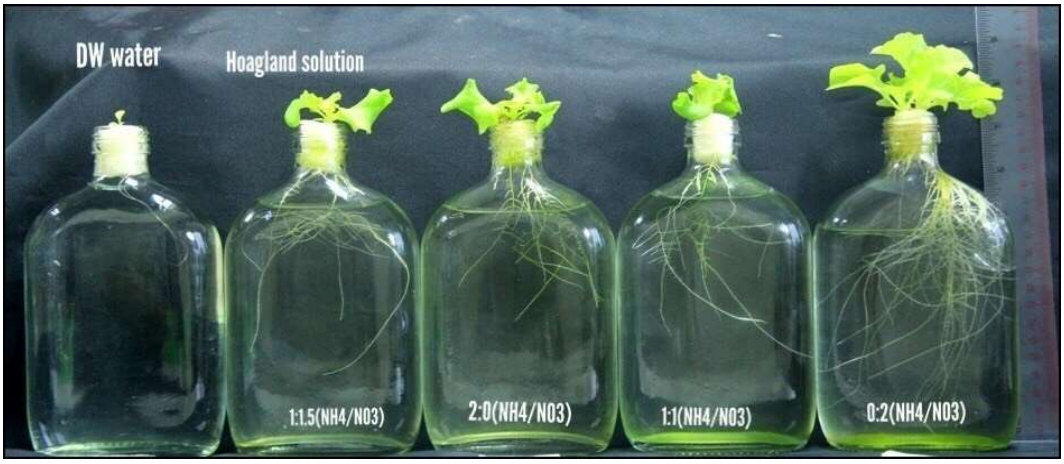

Figure 1 Growth of root system under different concentration of ammonium to nitrate ratio. DW represented distilled water using as a negative control and Hoagland solution using as a positive control

Influence of ammonium to nitrate ratio on root development in lettuce

Root growth and development

To understand more about how nitrate is important to root growth and development in lettuce, the experimental design was divided into 4 groups including control, no-N source, high NO_3^-, high NH_4^+ (NH_4^+/NO_3^- ratios of 1/1.5, 0/0, 1/2, 1.5/1, respectively). When lettuce was grown in high concentration of nitrate (1/2 (NH_4^+/NO_3^-)), the lateral root elongation tended to increase (figure 3) whereas the inhibition of root growth seemed to appear in high concentration of ammonium treatment (1.5/1 (NH_4^+/NO_3^-)) (figure 4). Furthermore, the length of primary root growth found in without nitrogen group (0/0(NH_4^+/NO_3^-)) (figure 5).

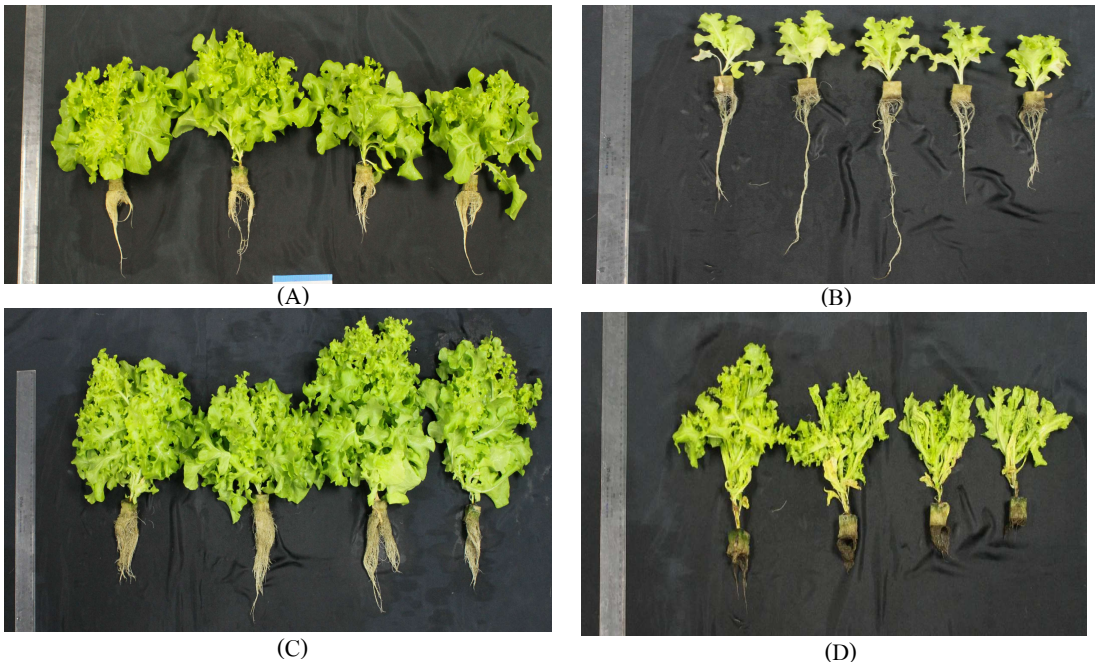

Figure 2 Growth of lettuce grown with treatment: (A) Control (1/2 (NH_4^+/NO_3^-), (B) No nitrogen (0/0 (NH_4^+/NO_3^-)), (C) high nitrate concentration (1/2 (NH_4^+/NO_3^-)) and (D) high ammonium concentration (1.5/1 (NH_4^+/NO_3^-)), respectively

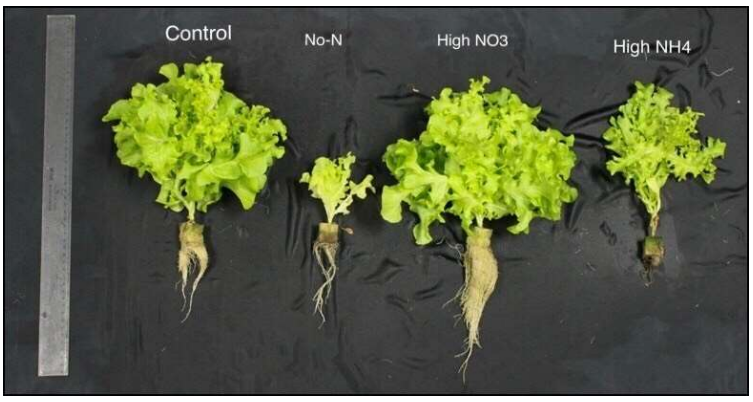

Figure 3 The comparison of physiological appearances between groups including control (1/1.5 (NH_4^+/NO_3^-)), no nitrogen (0/0 (NH_4^+/NO_3^-)), high nitrate concentration (1/2 (NH_4^+/NO_3^-)), high ammonium concentration (1.5/1(NH_4^+/NO_3^-))

Nitrate content

To analyze nitrate content in lettuce, the Salicylic acid was used by interacting with nitrate and measuring with UV/VIS spectrophotometry. Both of the shoot and root parts showed the highest accumulation of nitrate content in high nitrate group (1/2 (NH_4^+/NO_3^-)) compared to all groups of each part. As expected, the nitrate content in root part under high ammonium (1.5/1 (NH_4^+/NO_3^-)) showed the lowest of nitrate accumulation. When lettuce was grown without nitrogen (0/0(NH_4^+/NO_3^-)), the nitrate accumulation in root tended to increase compared to control (1/1.5 (NH_4^+/NO_3^-)) whereas tended to decrease in shoot part (Figure 4).

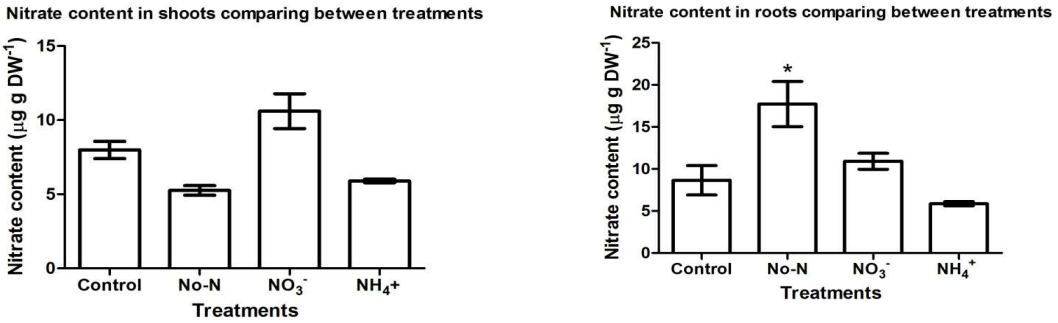

Figure 4 Nitrate content in shoot (left) and root (right) of lettuce. Bars represent the means ±standard errors of 3 replicates. Asterisk represent statistical significance observed between treatments compared to control group (Dunnett's test, one-way ANOVA; $P<0.05$)

Gene expression analysis

A unique feature of nitrate is that nitrate not only a substrate for transport, but also for induction of nitrate transport system at physiological and molecular levels. Therefore, characterization of nitrate response in lettuce was done by analyzing expression pattern of *NRT1* using qRT-PCR technique and shown in figure 8.

From the result, *NRT1* was slightly expressed in high nitrate treatment (1/2 (NH_4^+/NO_3^-)) significantly compared to control. Interestingly, the expression level tended to increase when absent nitrogen source (0/0 (NH_4^+/NO_3^-)).

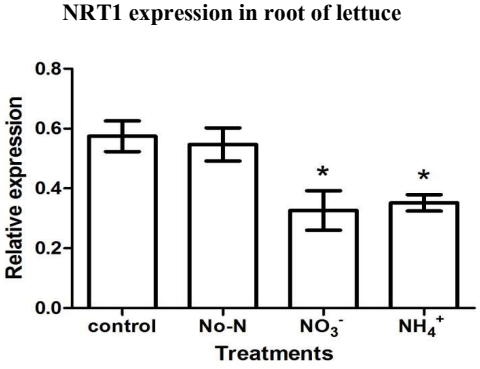

Figure 5 Expression of *NRT1* gene in root of lettuce under different ammonium to nitrate ratio. The treatments include control group (1/1.5 (NH_4^+/NO_3^-)), No-N (0/0 (NH_4^+/NO_3^-)), high nitrate concentration (1/2 (NH_4^+/NO_3^-)) and high ammonium concentration (1.5/1 (NH_4^+/NO_3^-)). Bars represent the means ±standard errors of 3 replicates. Asterisk represent statistical significance observed between treatments compared to control group (Dunnett's test, one-way ANOVA; $P<0.05$)

Discussion

In this study, we observed the effect of different NH_4^+/NO_3^- ratio using hydroponic which was hypothesized that different NH^+_4 / NO^-_3 ratio should affect lettuce root growth and development differently. We designed experiment by dividing into 4 groups with different concentrations of nitrate including control (distilled water), nitrate alone, ammonium alone and both nitrate and ammonium (NH_4^+/NO_3^- ratios of 0/0, 0/2, 2/0, 1/1, respectively). The results showed that the primary root length seemed to increase under nutrient starvation (control) and tended to promote root hair density which is one of mechanism for adaptation in plant under stress by increasing absorption area developed from epidermis cell in root. It could identify that nutrient is one of important factors for plant growth and development. When lettuce was grown in nitrate treatment (0/2 (NH_4^+/NO_3^-)), the primary root length tented to increase compared to ammonium treatment (0/2 (NH_4^+/NO_3^-)) and both ammonium and nitrate treatment (1/1 (NH_4^+/NO_3^-)). In addition, the inhibition of primary root found in ammonium treatment (2/0 (NH_4^+/NO_3^-)) which have been previously reported as important cues for ammonium toxicity in plants. Liu et al. (2013) demonstrated when the primary root growth grew with 1 mM ammonium; root growth was significantly inhibited compared to 1mM nitrate suggesting the reduction in primary root growth caused by ammonium due to decrease cell length. To further characterize root growth that response to nitrogen sources in lettuce in order to evaluate nitrogen form preference, we continued this experiment which was divided into 4 groups including control (1/1.5 (NH_4^+/NO_3^-)), no-nitrogen (0/0 (NH_4^+/NO_3^-)), high nitrate concentration (1/2 (NH_4^+/NO_3^-)) and high ammonium concentration (1.5/1 (NH_4^+/NO_3^-)). The results were going as expected: the elongation of primary and lateral root strongly appeared in high nitrate concentration (1/2 (NH_4^+/NO_3^-)) compared to all treatments because nitrate can modify root system architecture, especially by stimulating lateral root elongation. (Remans et al., 2006).

Gene expression analysis indicated that *NRT1* was correlated with nitrate and differently expressed in different concentration of nitrate. The expression of *NRT1* was the highest levels in no-nitrogen treatment and showed the lowest levels in high nitrate concentration compared to all treatments. This might be because nitrate response by upregulation of *NRT1* gene might just happen during early stage on nitrogen treatments. Whereas, the long term exposure to no-nitrogen treatment might cause continue upregulation of *NRT1* gene in order to seek for nitrate. In addition, Liu et al. (1999) showed a constitutive expression pattern of *NRT1*gene which was already present under nitrate starvation in *Arabidopsis* (0 hrs), and the expression levels did not change substantially during 72 hrs in order to exposure nitrate. However, there is little evidence about *NRT1* expression in lettuce and the role of NRT regulation in lettuce remains unclear.

Conclusion

1. Nitrogen is an important factor that control root growth and development as well as root structure.
2. N source in form of nitrate promote more growth of lettuce when compared with ammonium.
3. *NRT1* gene expression responded to nitrate and ammonium treatments as well as no-N treatment indicated dynamic response on NRT1 to Nitrogen status in lettuce.

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References

de Dorlodot S, Forster B, Pages L, Price A, Tuberosa R, Draye X. Root system architecture: opportunities and constraints for genetic improvement of crops. Trends Plant Sci 2007; 12(10): 474-481.

Loque D, Tillard P, Gojon A, Lepetit, M. Gene expression of the NO3- transporter NRT1.1 and the nitrate reductase NIA1 is repressed in Arabidopsis roots by NO2-, the product of NO3- reduction. Plant Physiol 2003; 132(*2*): 958-967.

Noguero M, Lacombe B. Transporters Involved in Root Nitrate Uptake and Sensing by Arabidopsis. Front Plant Sci 2016; Doi:10.3389/fpls.2016.01391.

Okamoto M, Vidmar J, Glass A. Regulation of *NRT1* and *NRT2* gene families of *Arabidopsis thaliana*: response to nitarte provision. Plant Cell Physiology 2003; 44(3): 304-317.

Razgallah N, Chikh-Rouhou H, Abid G, M'hamdi M. Identification of Differentially Expressed Putative Nitrate Transporter Genes in Lettuce. International Journal of Vegetable Science 2017; 23(5): 390-399.

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A. A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. Plant Physiol 2006; 140(3): 909-921.

Ristova D, Busch W. Natural variation of root traits: from development to nutrient uptake. Plant Physiol 2014; 166(2): 518-527.

Sun CH, Yu JQ, Hu DG. Nitrate: A Crucial Signal during Lateral Roots Development. Front Plant Sci 2017; Doi:10.3389/fpls.2017.00485

Zhong F, Wang, S, Lin J, Roan S, Lin B, Zhou X, et al. Characterization of nitrate asimilation in Lactuca sativa L. under different nitrogen sources. Plant Growth Regulation 2018; 86: 1-10.