INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–084/2018/20–10–2243–2250
DOI: 10.17957/IJAB/15.0773
http://www.fspublishers.org

Full Length Article

High Quality Yield in Lettuce in Response to Low Nitrate Content can be Achieved by Reduced Nitrogen Application

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Abstract

A nitrogen starvation trial was performed using nitrogen-free Yamazaki Solution. A solution culture trial using 100, 95, 90, 85, and 80% full nitrogen strength Yamazaki Solution and a pot trial at nitrogen rates of 0.60 (N1), 0.55 (N2), 0.50 (N3), 0.45 (N4), and 0.40 (N5) g N kg^{-1} soil were performed to investigate the balance between low nitrate content and quality fresh yield of lettuce (*Lactuca sativa* L.). The results showed that vacuolar nitrate activity was much higher than cytoplasmic nitrate activity. As exposure increased from day 1 to day 5 in the nitrogen starvation trial, vacuolar nitrate activity decreased rapidly, in contrast to cytoplasmic nitrate activity, which remained stable and the ratios of root biomass and shoot biomass increased. With a decline in the nitrogen supply, the biomass and nitrate content decreased significantly. Therefore, a balance between low nitrate content and quality fresh yield can be achieved by reducing the normal level of nitrogen fertilizer by 10%.

Keywords: Biomasses; Fresh yield; Lettuce (*Lactuca sativa* L.); Nitrate accumulation; Nitrate activity

Introduction

Consumers are becoming increasingly concerned about the high nitrate content in vegetables, because it presents a potential hazardous risk to their health when it is reduced to nitrite (Pavlou *et al.*, 2007; Özdestan and Üren, 2010). Hence, numerous studies have investigated the nitrate content in plants (Jaworska, 2005; Özdestan and Üren, 2010). Vegetables play an important role in human nutrition because they are a good source of vitamins, minerals, and biologically active compounds. It has been estimated that plants contribute about 72–94% of the dietary nitrate intake in some societies (Dich *et al.*, 1996). Thus, reducing the nitrate content of plants is a key step in the maintenance of low nitrate content in the human population. Managing the nitrate content of plants can be achieved through low nitrate production systems (Andersen and Nielsen, 1992). In this system, many factors can affect nitrate content in plants, such as plant species, cultivar, level and type of fertilization, nitrate reductase activity, nitrification step, organic acids, light conditions, temperature, humidity, growing season, photosynthesis, soil structure, soil pH, and even the application of plant protection agents (Olday *et al.*, 1976; Biemond *et al.*, 1996; Siomos and Dogras, 1999; Zhang and Forde, 2000; Kraus *et al.*, 2004; Chen *et al.*, 2005; Yuan *et al.*, 2005; Li *et al.*, 2018). N fertilization has been identified as a major factor that influences the nitrate content in vegetable crops (Cantliffe, 1973).

Karaman *et al.* (2000) found that the nitrate content of plants increased with increasing regional nitrogen use. Reducing the strength of N fertilizer could significantly reduce the nitrate content of plants. Unfortunately, plant yield decreases as the strength of the applied N fertilizer decreases. Usually, farmers supply N in excess because it is critical for plant growth (Anjana and Iqbal, 2007; Martin, 2012). At the same time, the area of cultivated land per capita in China is lower than the average in the rest of the world. Furthermore, the population is increasing and cultivated land is being transformed into construction land at an ever-increasing rate. This means that China requires an increased level of agricultural production on a reduced area of arable land. Reducing the intensity of nitrogen application is a prerequisite for increasing plant yield. Obtaining a balance between low nitrate content and quality fresh biomass of plants is a challenge for the Chinese population.

Nitrate is found in all organs (Dechorgnat *et al.*, 2011). However, during nitrate metabolism, most of the nitrate taken up by plants is stored within the vacuole (Zhen

To cite this paper: Wang, L., H. Yuan, B. Wang, X. Sun, X. Lu and W. Yu, 2018. High quality yield in lettuce in response to low nitrate content can be achieved by reduced nitrogen application. *Int. J. Agric. Biol*., 20: 2243–2250

et al., 1991; Miller and Smith, 1992; Anjana and Iqbal, 2007) where it functions as an osmotic ion or provides a stock that can be subsequently mobilized when the nitrogen supply is insufficient to meet demand (McIntyre, 1997; Van *et al.*, 1998; Dechorgnat *et al.*, 2011). However, compared with the vacuole, the cytoplasm is a more important compartment for nitrate metabolism. Experiments with NO_3^--specific microelectrodes have indicated that the level of cytoplasmic nitrate is somewhat constant, while that of vacuolar nitrate is higher in maize and barley (Zhen *et al.*, 1991). These microelectrode results suggest that the activity of cytoplasmic nitrate is somehow maintained constant, perhaps by the competing processes of influx, efflux, xylem and vacuolar loading, and nitrate reduction. Trials have also shown that plants are able to mobilize nitrate from the vacuole to the cytoplasm in order to maintain N metabolism, at least during early N starvation (Van *et al.*, 1998). Meanwhile, the capacity of plants to take up nitrate from the root medium usually increases during the first 2 days of N starvation, and then decreases thereafter (Siddiqi *et al.*, 1989). Evidence indicates that plants are able to cope with a short period of N deficiency without serious consequences on growth (Richard-Molard *et al.*, 2008; Wang and Shen, 2012). A previous greenhouse study from our research group found that the nitrogen content in plant tissues decreases markedly during the early period of nitrate starvation (Wang and Shen, 2012). Remobilization and reassimilation of nitrate in plants can not only reduce nitrate content but can also increase the efficiency of N use by plants. Consequently, the amount of applied N fertilizer will be decreased.

Our research group has performed some greenhouse experiments on nitrogen nutrition, especially regarding nitrate content, in species such as Chinese cabbage (*Brassica chinensis* L.) (Chen *et al.*, 2005), spinach (*Spinacia oleracea* L.) (Wang *et al.*, 2008), and lettuce (*Lactuca sativa* L.) (Wang and Shen, 2011; Wang and Shen, 2012). Overall, a better understanding of the decreasing nitrate content of plants and the quality fresh yield is needed. The aims of this study were as follows: (1) to depict the characteristics of nitrate mobilization and remobilization in vacuoles; (2) to balance the low nitrate content with the quality fresh yield of lettuce.

Materials and Methods

Plant Material and Experimental Conditions

Seeds of lettuce cultivar Sx1, are extensively cultivated in southeast China, were surface-sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min, rinsed with deionized water, and then germinated in sterilized moist quartz sand in a growth chamber with a 12-h day/night temperature of 25/15°C with light exposure of approximately 210 μmol m^{-2} s^{-1}. After 20 days, seedlings of equal height were transferred to plastic containers containing nutrient solution.

The plastic tanks used for hydroponics could hold 22.5 L, with PVC covers containing 24 holes, with one seedling planted per hole. Each tank was filled with 20 L nutrient solution, and each seedling was wrapped around and supported by a strip of sponge to reduce evaporation and equipped with plant holes. Each treatment consisted of four growth tanks, and each growth tank supported 24 plants, for a total of 96 plants per treatment. Growth tanks were randomly arranged in a climate-controlled greenhouse with a 12-h day/night temperature of 25/15°C with light exposure of approximately 210 μmol m^{-2} s^{-1}.

Nutrient solutions (Wang and Shen, 2012) were replaced weekly and the pH of each solution was adjusted to 6.3–6.5 using $Ca(OH)_2$ or H_2SO_4 as required every day. To obtain a balanced nutrient composition among the treatments, the K:Ca:Mg ratio of all the nutrient solutions was kept constant on an equivalent basis (Table 1). Dicyandiamide (DCD) (7 μmol L^{-1}) was added to all hydroponic culture solutions to prevent nitrification in the solutions. One-quarter of the original strength solutions was used during the first week, which was the period of pre-culture after transplanting, and then the plants were transferred into different nutrient solutions for the subsequent experiment.

The pH was adjusted every day as required to 6.3–6.5 using $Ca(OH)_2$ or H_2SO_4. All the reagents were analytical reagents (AR).

Nutrient Solution Culture Experiment

Plants were transferred into full-strength nutrient solution. Other culture conditions were the same as those described above. This trial was conducted at Nanjing Agricultural University, Nanjing, Jiangsu Province, P. R. China.

N Starvation Experiment

As soon as the NO_3^- activity in the root vacuoles and cytoplasm was maintained at a steady state for 2 days in half-strength Yamazaki Solution, the plants were transferred to N-free nutrient solution for an N starvation experiment. During the experimental period, vacuolar and cytoplasmic nitrate activities in the root and shoot were determined and the nutrient solutions were changed every day. This trial was also conducted at Nanjing Agricultural University, Nanjing, Jiangsu Province, P. R. China.

Pot Experiment

An outdoor pot experiment was established during the growing seasons in Dushuhu campus of Soochow University, Suzhou, Jiangsu Province, P.R. China. Plants were transplanted in 25 × 30-cm (diameter × depth) plastic pots filled with 5 kg field soil. Soil was taken from a fallow field of the Horticulture Experimental Station of

Department of Horticulture, passed through a 4-mm sieve to remove plant litter, and then mixed uniformly. Some physicochemical characteristics of the soil used were as follows: organic matter content 15.29 g kg^{-1}, total N 0.97 g kg^{-1}, NO_3^--N 45.47 mg kg^{-1}, NH_4^+-N 18.21 mg kg^{-1}, Olsen P 24.34 mg kg^{-1}, available K 8.29 mg kg^{-1}, and pH 6.5. In each pot, 0.10 g P and 0.15 g K were incorporated at the same stage of transplanting by split application and were well mixed with the soil. Before transplanting, soil was irrigated with deionized water to 20% (w/w) water content. Twelve plants per pot were transplanted at six points, the same distance apart. A week after transplanting, six uniform plants remained per pot. The pots were placed in the glasshouse, scarified, and weeded weekly. Soil water content was controlled with deionized water to 20% (w/w) using the commonly used weight method. Each treatment was planted in four replicate pots.

Five nitrogen treatments, 0.60 (N1), 0.55 (N2), 0.50 (N3), 0.45 (N4), and 0.40 (N5) g N kg $soil^{-1}$ in the form of $CO(NH_2)_2$ were applied in two parts. One half was applied before transplanting, and the other half was applied 4 weeks after transplanting. N1 is the conventional level of nitrogen fertilizer used in southeastern China.

Sample Collection and Measurements

Plants were harvested at the 9–10 leaf stage from the experiment using nutrient solution culture, which could be obtained from the N starvation experiment. The plants were then cut and separated into roots and shoots from rootstock. Roots were washed using deionized water and dried with a towel paper. The shoot was harvested from the pot experiment. The fresh biomass of shoots and roots was weighed and the samples were immediately dried in a forced-air oven at 108°C for the first 30 min and at 60°C until a constant weight. The dried biomass of shoots and roots was recorded and the samples were then ground to a fine powder in a Wiley mill. NO_3^- was determined using the standard micro-Kjeldahl procedure in four replications, each in two parallel samples. Consequently, the nitrate level in the filtrate was measured using a continuous-flow-autoanalyzer (Autoanalyzer 3, Bran + Luebbe GmbH, Norderstedt, Germany) (Schortemeyer *et al.*, 1997).

Double-barreled nitrate-selective microelectrodes, as well as the microelectrode system used to determine vacuolar and cytoplasmic NO_3^- activities in roots and shoots, were generated as described by Miller and Zhen (Miller and Zhen, 1991).

Statistical Analysis

Statistical analyses were performed using the statistical software program SPSS (Version 11.0; SPSS, Inc., Chicago, Illinois, USA). Treatment means were compared using Fisher's protected least significant difference ($LSD_{0.05}$) test.

Results

Biomass and Nitrate Activity of the Vacuole and Cytoplasm in Nutrient Solution with Nitrogen

The biomass of shoots and roots as observed in Yamazaki solution at the 9–10 leaf stage was 1.17 and 0.14 g $plant^{-1}$ dry weight (dw), respectively. The ratio of shoot dw to root dw was 8.36. The nitrate content of shoots and roots was 3531.78 and 3970.26 mg N kg^{-1}*FW, respectively, and the nitrate content of shoots was 88.96% that of the roots. In contrast, the nitrate levels in the shoots and roots were 141.98 and 7.42 mg N $plant^{-1}$, respectively, with that of the former being much higher than that of the latter.

Nitrate activity in the vacuole and cytoplasm of shoots was 97.05 and 4.01 mmol L^{-1}, respectively (Table 2). Vacuolar and cytoplasmic nitrate activity in roots was 119.63 and 4.14 mmol L^{-1}, respectively. And it was significantly higher in the vacuole than in the cytoplasm. Based on the volume of the vacuole and the cytoplasm, and the nitrate activity in both the shoot and the root, most of nitrate was thus stored in the vacuole.

Biomass and Nitrate Activity of the Vacuole and Cytoplasm in N-Free Nutrient Solution

With an increasing number of exposure days to N-free nutrient solution, nitrate activity in the cytoplasm of roots and shoots was almost unchanged in the maintenance of normal plant growth, whereas the nitrate activity in the vacuole of shoots and roots decreased rapidly (Fig. 1). Nitrate activity in root vacuoles after 1, 2, 3, 4 and 5 days' exposure to N starvation solution was 64.1, 49.0, 34.0, 23.0, and 15.7% before transferring, respectively. Nitrate activity in shoot vacuoles after 1, 2, 3, 4, and 5 days' exposure to N starvation solution was 61.9, 41.2, 27.0, 18.1, and 15.1% before transferring, respectively. The rate of decline of nitrate activity in the shoot vacuoles was faster than that in the root, and the correlation coefficient reached 0.989. This implied that the nitrate activity in the root vacuole could be used to predict that in the shoot.

Data represent arithmetic means ± standard deviation (S.D.). Each value is the mean of four replicates. Differences were tested using one-way ANOVA followed by Duncan's test. Within the same organelles, means followed by a different letter are significantly different at $p < 0.05$.

With an increase in exposure days to N-free nutrient solution, the biomass of roots and shoots increased generally (Fig. 2). Compared with the biomass of roots and shoots before transferring, the values on the 5th day after transferring had increased by 0.5057 and 1.4794 g FW $plant^{-1}$, respectively. In contrast, the rate of increase in the roots and shoots decreased as the number of exposure days increased. At the same time, the ratio of root biomass to shoot biomass increased gradually (Fig. 3). This implied

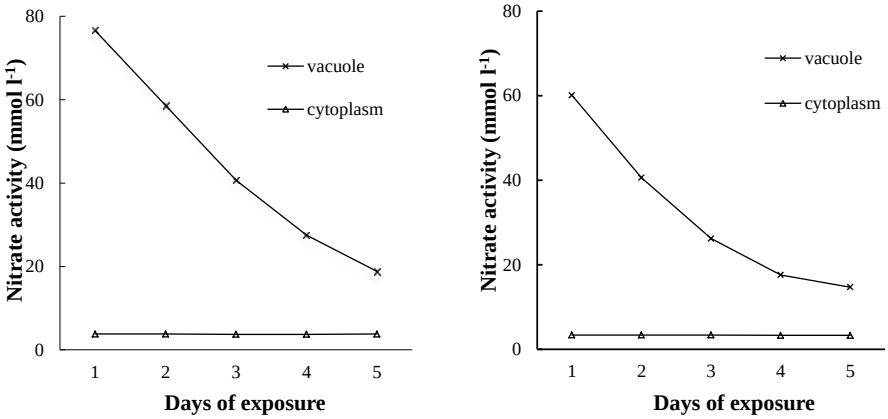

Fig. 1: Effects of nitrogen starvation on vacuolar nitrate activities of the root (left) and shoot (right). Each value represents the mean of 6–10 replications. The days of exposure show that plants were transferred for that number of days in N-free Yamazaki Solution. Before being transferred into this solution, nitrate activity in the vacuole of roots and shoots was 119.6 and 97.1 m mol l^{-1}, respectively

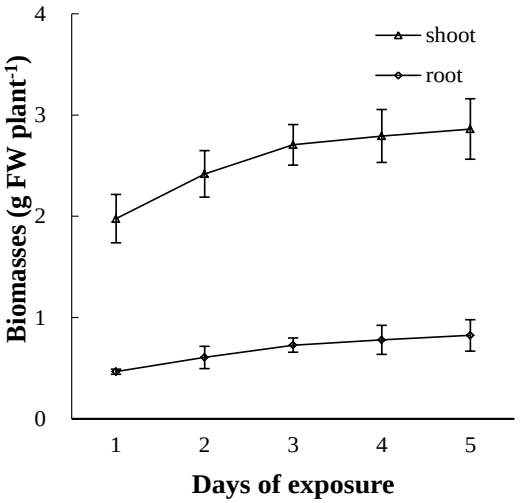

Fig. 2: Effects of nitrogen starvation on fresh biomass of roots and shoots. Each value represents the mean of four replicates. The days of exposure shows that plants were transferred for that number of days in N-free Yamazaki Solution. Before being transferred into this solution, the biomass of roots and shoots were 0.3183 and 1.3831 g FW $plant^{-1}$, respectively. Vertical bars represent the standard deviation of the mean (n = 4)

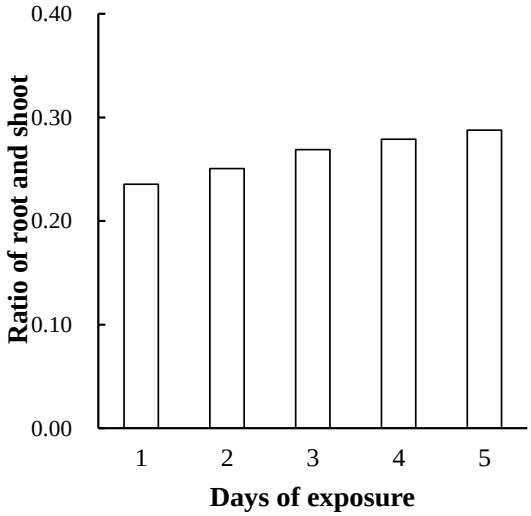

Fig. 3: Effects of nitrogen starvation on the ratio of root fresh biomass and shoot fresh biomass. Each value represents the mean of four replicates. The days of exposure shows that plants were transferred for that number of days in N-free Yamazaki Solution

that low nitrate content in plants could be induced by reducing the level of nitrate activity in the vacuole appropriate with steady nitrate activity in cytoplasm.

Biomass and Nitrate Content in Nutrient Solutions at Different N Levels

Shoot and root biomass decreased continually with the decrease in nitrogen concentrations in the nutrient solution (Fig. 4). There was no significant difference in shoot biomass between the 100 and 95% treatments. As the nitrogen concentration further reduced from 95 to 90, 85, and 80%, the difference between the shoot biomass of all treatments was significant. Whereas no significant difference in root biomass was observed between the 100, 95, 90, and 85% treatments, the different between the 80% treatment and the others was significant for root biomass.

With decreasing concentrations of nitrogen in the nutrient solution, the nitrate content in the roots and shoots decreased (Fig. 5). The differences among the 100, 95, 90, 85, and 80% treatments were significant for the nitrate content in the roots and shoots. This shows that reducing

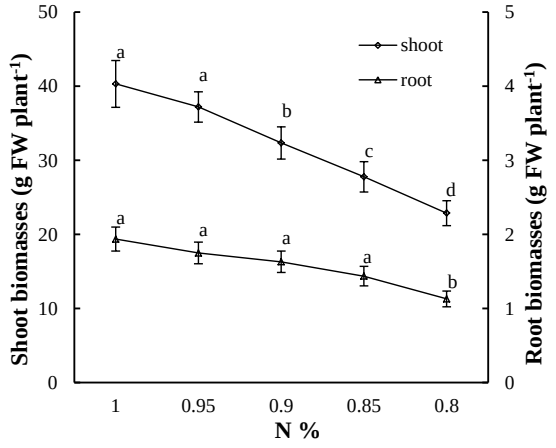

Fig. 4: Effects of different N concentrations of Yamazaki Solutions on the biomass of shoots and roots. The N content was converted to a percentage and compared with the N concentration of Yamazaki Solution. Each value represents the mean of four replicates. Vertical bars represent the standard deviation of the mean (n = 4). Differences in biomasses were tested using one-way ANOVA followed by Duncan's test

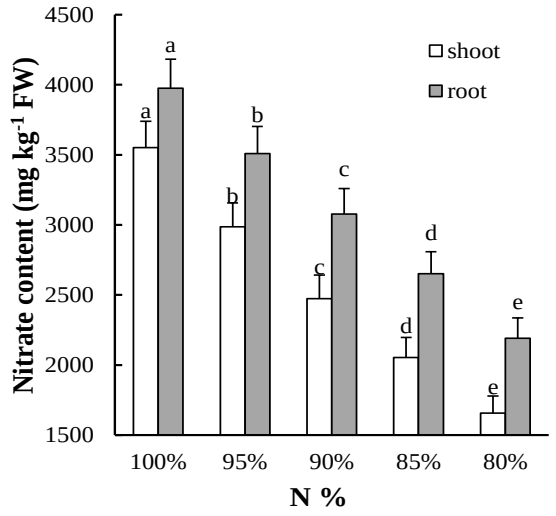

Fig. 5: Effects of different N concentrations of Yamazaki Solutions on shoot and root nitrate content. The N content was converted to a percentage and compared with the N concentration of Yamazaki Solution. Each value represents the mean of four replicates. Vertical bars represent the standard deviation of the mean (n = 4). Differences in NO_3^- concentrations were tested using one-way ANOVA followed by Duncan's test

the nitrogen supply can significantly reduce the nitrate content in plants. The nitrate content in roots was significantly higher than that in shoots exposed to the same nutrition solution.

As shown in Fig. 6, decreasing application of N led to decreased shoot fresh weights. However, there was no significant effect on shoot fresh weights between the treatments of N apply rate in soil with N1 and N3, N4, and N5 g N kg^{-1} was significant, different treatments between of N apply rate in soil with N2 and had a significant influence on shoot fresh weights. With decreasing rates of N application to the soil, the shoot nitrate content decreased, and the differences among all treatments were significant for shoot nitrate content (Fig. 7).

Table 1: Composition of nutrient solution (Yamazaki Solution)

Macronutrients	mg l^{-1}	Micronutrient	mg l^{-1}
KNO_3	404	H_3BO_3	2.86
$Ca(NO_3)_2 \cdot 4H_2O$	236	$MnSO_4 \cdot 4H_2O$	2.13
$MgSO_4$	123	$ZnSO_4 \cdot 7H_2O$	0.22
$NH_4H_2PO_4$	57	$CuSO_4 \cdot 5H_2O$	0.08
FeNaEDTA	20	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02

Table 2: Cytoplasmic and vacuolar nitrate activity in shoots and roots at the 9–10 leaf stage (mmol L^{-1})

Organ	Vacuole	Cytoplasm
Shoot	97.05 ± 10.15b	4.01 ± 0.34a
Root	119.63 ± 12.34a	4.14 ± 0.27a

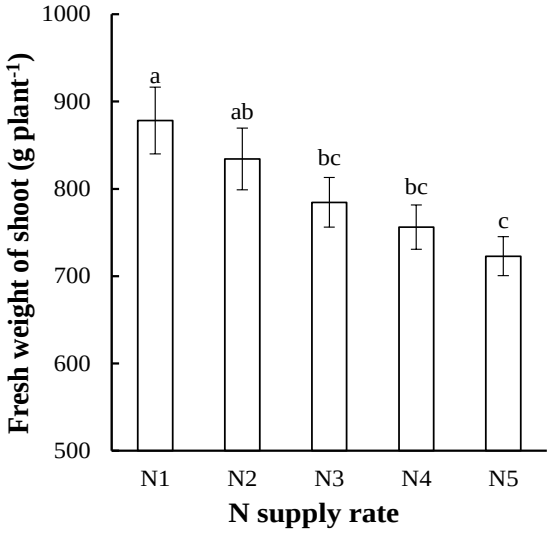

Fig. 6: Effects of different N supply rates on the biomass of shoots. N supply rates were 0.60 (N1), 0.55 (N2), 0.50 (N3), 0.45 (N4), and 0.40 (N5) g N kg $soil^{-1}$. Each value represents the mean of four replicates. Vertical bars represent the standard deviation of the mean (n = 4). Differences in biomasses were tested using one-way ANOVA followed by Duncan's test. Means followed by different letters are significantly different at $p < 0.05$

Discussion

Because nitrate accumulation in plant fresh yield is

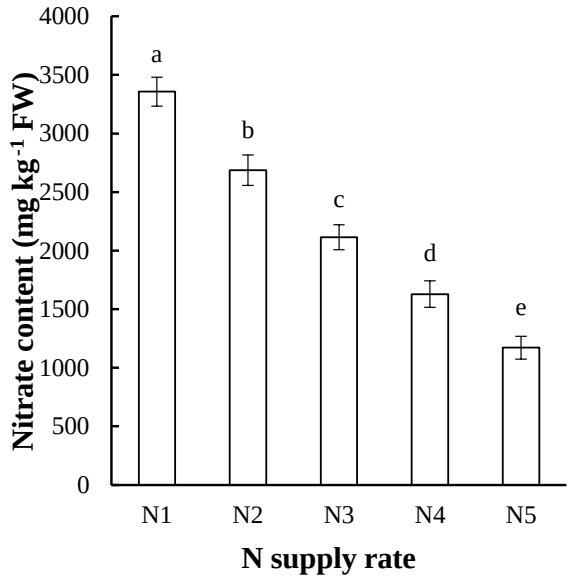

Fig. 7: Effects of different N supply rates on shoot content. N supply rates were 0.60 (N1), 0.55 (N2), 0.50 (N3), 0.45 (N4), and 0.40 (N5) g N kg soil^{-1}. Each value represents the mean of four replicates. Vertical bars represent the standard deviation of the mean (n = 4). Differences in biomass were tested according using one-way ANOVA followed by Duncan's test. Means followed by a different letter are significantly different at $p < 0.05$

undesirable for a number of reasons, numerous studies have investigated the factors contributing to the accumulation of nitrate in plants (Wang and Shen, 2011). It is well established that N fertilization is a major factor influencing nitrate content in vegetable crops (Cantliffe, 1973). Plants accumulate more nitrate as the N fertilization level increases (Chen *et al.*, 2004), whereas a decrease in nitrogen availability can significantly decrease the nitrate content in plants (Wang and Shen, 2011). However, reducing the amount of nitrogen fertilizer supply results in a reduction in plant fresh yield. This is unacceptable in developing countries. Therefore, based on our understanding of the characteristics of vacuolar and cytoplasmic nitrate ion influx and efflux, the optimal amount of N fertilizer is important in order to balance the low nitrate content with the quality fresh yield of plants.

Fig. 4 and 5 showed that plants exposed to nutrient solutions containing from 100 to 95, 85 and 80% of N, generally exhibited the decreases of fresh yields and the nitrate content. Compared with plant development when exposed to the 100 and 95% treatments, the difference in fresh yield did not reach significance; in contrast, the difference in nitrate reached significance. This was probably due to a significant decline in nitrate activity in vacuoles, with stable nitrate activity in the cytoplasm leading to a significant reduction in the nitrate content in plants; however, the decline of vacuole nitrate activity did not cause a significant decline in fresh yield. We further reduced the nitrogen concentration in the nutrient solution from 100 to 90%, and the differences in fresh yield and nitrate content between these treatments were significant. We also observed a similar trend in the pot experiment (Fig. 6,7). This implied that the decline in vacuolar nitrate activity caused a significant decline in fresh yield.

Most of the nitrate taken up by plants is stored within the vacuole (Zhen *et al.*, 1991; Miller and Smith, 1992; Anjana and Iqbal, 2007), where it functions as an osmotic ion or as a stock that can be mobilized when the nitrogen supply is insufficient to meet the nitrogen demand (McIntyre, 1997; Van *et al.*, 1998; Dechorgnat *et al.*, 2011). Experiments with NO_3^--specific microelectrodes indicated that nitrate activity in the cytoplasm is more stable, with lower values of 3.1 mol m^{-3} in maize (Zhen *et al.*, 1991) and 4.9 mol m^{-3} in barley (Miller and Smith, 1992), than in the vacuole, where levels of 26 mol m^{-3} in maize (Zhen *et al.*, 1991) and 39 mol m^{-3} in barley (Miller and Smith, 1992) have been reported. The results of our previous study in lettuce showed that nitrate is mainly stored in the vacuole (Wang and Shen, 2011). Since the vacuolar nitrate activity is much higher than that of cytoplasm, and the vacuolar volume is also of far greater than that of the cytoplasm, the amount of nitrate in the vacuole dominates the nitrate content of plants (Van *et al.*, 1998).

In the present study, the cytoplasmic nitrate activity in shoots decreased sharply from the 1st day (60.1 mmol l^{-1}), 2nd day (40.6 mmol l^{-1}), 3rd day (26.2 mmol l^{-1}), 4th day (17.6 mmol l^{-1}), to the 5th day (14.7 mmol l^{-1}). In contrast, the vacuolar nitrate activity remained stable at approximately 3.3 mmol l^{-1} in the shoot; a similar trend was observed in the root (Fig. 1). Additionally, the fresh biomass of shoots and roots generally increased with the increasing number of exposure days in N-free nutrient solution (Fig. 2). This was confirmed by the fact that nitrate effluent occurred continuously from the vacuole to the cytoplasm in order to maintain stable nitrate concentration. This result is consistent with the idea that vacuolar nitrate serves as a pool, providing a reservoir of stored nitrate that can be used to maintain cytoplasmic nitrate activities for nitrate metabolism.

We also observed that with a further increase in exposure days, less and less nitrate could be transported from the vacuole to the cytoplasm; meanwhile, cytoplasmic nitrate activity began to decrease (data not shown). The fact that the vacuolar nitrate activity decreased to a certain extent and then remained stable suggested there is a "minimum value" of vacuolar nitrate activity. Therefore, this requires further study.

Methods used to measure intracellular nitrate concentrations include the compartmental tracer efflux method (Miller and Smith, 1996), cellular nitrate reductase assays (Siddiqi and Glass, 2002), single-cell sampling and microelectrodes (Zhen *et al.*, 1991), nitrate selective-microelectrodes (Britto and Kronzucker, 2003), and ^{133}Cs

nuclear magnetic resonance (NMR) (Radcliffe *et al.*, 2005). The nitrate content measured depends on the technique used, the cell type, the sample point, and the growing conditions (Zhen *et al.*, 1991; Miller and Smith, 2008; Dechorgnat *et al.*, 2011).

In this experiment, an interesting phenomenon was observed; changing the insertion depth within a single vacuole changed the nitrate activity when the vacuolar nitrate activity was determined using nitrate-selective microelectrodes. Different positions in the vacuole were found to have different nitrate activities. This implied that vacuole nitrate is unevenly distributed, and it might be affected by different protein groups within the tonoplast membrane and accompanying NO_3^- ions. In the present study, we took the arithmetic mean of 6–10 samples as the vacuolar nitrate activity (Fig. 1). Thus, accurately measuring the activity of nitrate in vacuole merits further investigation.

Conclusion

Considering results obtained here, we could achieve the balance` between low nitrate content and quality fresh yield by cut 10% of normal level of N fertilizer.

Acknowledgements

We acknowledge the financial supports of the National Key Research and Development Program of China (2017YFD0800100), National Natural Science Foundation, and Jiangsu Agricultural Science and Technology Innovation Fund (CX (17) 2016) , and the Suzhou Science and Technology Project No. SNG 201609 and No. SYN201501.

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(Received 26 April 2018; Accepted 02 June 2018)