

Full Length Research Paper

Nitrate concentration and nitrate/ammonium ratio on lettuce grown in hydroponics in Southern Amazon

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Lettuce has a predisposition to nitrate accumulation and has led many researchers to develop research to reduce this accumulation. The objective of this study was to evaluate the effect of nitrate concentration (NO_3^-) and nitrate/ammonium ratio ($\text{NO}_3^-:\text{NH}_4^+$) on growth, production and accumulation of these nitrogen compounds in lettuce grown in a hydroponic system in southern Amazon. There were performed two experiments with Iceberg lettuce type, in a floating-hydroponic system under protected cultivation. In the first experiment, five nitrate concentrations (2.5, 5.0, 7.5, 10.0 and 15.0 mmol L^{-1}) were evaluated. In the second experiment, the best $\text{NO}_3^-:\text{NH}_4^+$ ratio was evaluated with five proportions 0:100, 25:75, 50:50, 75:25 and 100:0 in the nutrient solution. At 35 days after transplanting, the evaluations were performed. The nutrient solution containing nitrogen (N) as nitrate source in the concentration of 15.0 mmol L^{-1} contributed to higher lettuce productivity, being the concentration recommended for hydroponic grown system in southern Amazon. It is recommended to use about 23% of the N in nutrient solution in ammonium form, where higher N-ammonium concentrations are not indicated for Iceberg lettuce type because it reduces the productivity of this green leaf vegetable, causing toxicity symptoms.

Key words: Nitrogen, vegetable, nutrient solution, food security.

INTRODUCTION

The consumption of vegetables has increased in recent years; Nielsen (2016) showed that 60% of the population of North America has been trying to change eating habits by the consumption of healthy foods.

Lettuce is the foremost green leafy vegetable consumed in the world, standing out for being a good

source of vitamins and minerals (Stagnari et al., 2015). To supply the demand of the market for vegetables, farmers have invested in hydroponic systems, an advantageous option compared to conventional cultivation, which leads to higher quality products, higher productivity associated with less time for harvesting, as

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well as lower costs with hand labor, water and agricultural inputs. On the other hand, mishandled hydroponic crops may cause excessive nitrate accumulation in plants, especially in leaf vegetables such as lettuce, which has a predisposition to accumulate nitrate (Byrne et al., 2002).

Due to nitrogen (N) being the most required nutrient for the most plants, the fertilizers recommendation in higher doses has been increased, which raises concerns in two ways: first by the contamination of waters and springs, which is likely to increase with increasing population and second, by increasing the nitrate content (NO_3^-) in vegetables which is considered one of the main sources of human nitrate intake (Santamaria, 2006; Di Gioia et al., 2013; Pandorf et al., 2020). Some estimates indicate that the vegetables correspond to the group of food that mostly contributes to nitrate ingestion by humans, accounting for about 72 and 94% of the daily intake (Turazi et al., 2006; Takahashi et al., 2007).

The nitrate accumulation in plants can occur due to many factors, such as: genetic characteristics, light intensity, temperature, availability of ions in the substrate, molybdenum availability, cultivation season and time of harvest (Pôrto et al., 2008). However, researchers have been trying to reduce the accumulation of nitrate in lettuce using techniques, such as molybdenum supply in plants (Taiz et al., 2017) and the supply of the N in the nutrient solution in the ammonium form, allowing a reduction in the accumulation of NO_3^- in the vacuoles (Andriolo et al., 2006; Rocha et al., 2020).

The hydroponic producers have numerous nutritional formulations for lettuce cultivation in this system (Hoagland and Arnon, 1950; Castellane and Araújo, 1995; Furlani et al., 1999); however, the nutrient sources can vary depending on the economic bias, which can cause excessive use of nitric fertilizers, resulting in higher nitrate accumulation by plants.

The hydroponic cultivation system has been intensifying in Amazon region and due to the luminous intensity and temperature are factors that influence the accumulation of nitrate, with a preoccupation in knowing the nitrogen compounds level in lettuce, because of the specific climate conditions of this region. Allied to this, the usual commercially available nutrient solutions are all nitrics and vary a lot with respect to nitrate concentration, not being found in the literature research results about this region.

Given the above, this study aimed to evaluate the effect of nitrate concentration and the nitrate/ammonium ratio on growth, production, nitrate reductase enzyme activity and accumulation of nitrogen compounds in lettuce grown hydroponically in southern Amazon.

MATERIALS AND METHODS

Location and meteorological data

The research was conducted in a Deep Floating Technique at 30°C

hydroponic system (DFT) also known as Floating at the University of the State of Mato Grosso, located in Alta Floresta-MT city, Brazil, from May to August 2018. The city is geographically located at 09° 54' S latitude and longitude 55° 54' W with an altitude of 292 m, and a tropical Am type climate.

Site characterization and installation of the experiments

The experiments were conducted in greenhouse arch type, covered with transparent polyethylene film 150 μ thick, and the side closed with a 50% shade cloth. It was grown with lettuce 'Great Lakes 659', iceberg type. Seeds were sown in a 162-cell polystyrene tray containing commercial substrate. Two seeds were placed per cell, and after seedling emergence, thinning was performed, leaving one plant per cell. At 15 days after sowing, the seedlings were transplanted to the hydroponic system under proper treatments. There were utilized polystyrene pots of 7 L capacity, covered with perforated polystyrene plates to support the plants. The nutrient solution was maintained under constant aeration through forced injection of air with compressor model II Super power 5 W, and the electric conductivity was kept in the range from 1.5 to 2.5 mS cm^{-1} , by changing the value of the solution when the solution reached levels below 1.5 mS cm^{-1} . The pH of the nutrient solution was measured daily, aiming to keep it from 5.8 to 6.2 by using dilute solutions (0.1 M) of HCl or NaOH.

Treatments

The study was conducted in two stages. In the first experiment, five nitrogen concentrations were assessed in NO_3^- form: 2.5, 5.0, 7.5, 10.0 and 15.0 mmol L^{-1} corresponding to the 25, 50, 75, 100 and 150% of the N concentration in the standard nutrient solution. In the second experiment, the best N doses found in the first experiment was utilized and the best $\text{NO}_3^-/\text{NH}_4^+$ ratio was evaluated with five variations: 0:100, 25:75, 50:50, 75:25, and 100:0 corresponding to proportions of 0, 25, 50, 75 and 100% of nitrate related to ammonium. For both experiments, calcium concentrations among the treatments were adjusted using the calcium chloride source $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, being necessary to vary the calcium nitrate dose utilized in the experiments due to the treatments. The nutrient solution adopted for the study followed the recommendations of Hoagland and Arnon (1950) with changes in the concentration of nitrogen as nitrate in the first experiment and nitrate and ammonium ratio in the second one. The experiments were conducted in randomized block design with four replications (two plants per replication).

Evaluations

At 35 days after transplanting to hydroponic system and treatments application, the plants were harvested, and then vegetative growth was evaluated by determination of the following variables: number of leaves per plant (NL), stem diameter (SD), leaf length (LL), root volume (RV), leaf fresh mass (LFM), fresh root mass (FRM), leaf dry mass (LDM) and root dry mass (RDM). To determine the dry mass, the plants were placed into air forced circulation oven at 65°C until constant weight.

The qualitative variables represented by the activity of the enzyme nitrate reductase, total nitrogen content and ammonium nitrate content were evaluated in leaf tissues. The activity of nitrate reductase was estimated *in vivo* using the method described by Jaworski (1971) via the fresh leaf tissue. From each sample, 300 mg of leaf fragments were taken and placed in a dark pot with 2.5

ml of phosphate buffer solution (285 mmol dm⁻³ pH 7.25); 2.5 ml KNO₃ (300 mmol dm⁻³); 2.5 ml of n-butanol 1% (v/v); 1.0 ml Triton X-100 0.1% (v/v) and 1.5 ml of deionized water. After vacuum infiltration, vials containing the samples were incubated in waterbath for 90 min in the dark, and at the end of this process, the reaction was stopped by adding 1.0 ml of 1% sulfanilamide solution in the same vials. Then, a 0.5 ml aliquot was collected and transferred to the reaction vial for determination of NO₂⁻ formed. In this tube, 0.5 ml of 1% sulfanilamide (P.A = pure for analysis) and 0.5 ml of solution dihydrochloride N-(1-naphthyl)-ethylenediamine 0.02% (P.A) solutions were added, and after 20 min, the volume was completed with 2.5 ml of deionized water. The reading of the reactions was performed on a UV spectrophotometer at 540 nm and the nitrate reductase enzyme (NR) activity, based on the equation of a standard curve made with dilute solutions of NaNO₂⁻ (329 µg ml⁻¹) (P.A), expressed in micromoles of nitrite released per gram of fresh weight of leaf tissue per hour of incubation (µmol g⁻¹ h⁻¹ of NO₂⁻ FW).

The determination of total nitrogen has been done followed the methodology described by EMBRAPA (2009). Furthermore, 0.1 g of leaf dry mass, which was placed in a test-tube with the addition of 1.0 g of mixture of salts (copper sulfate and potassium sulfate) was used. Subsequently, the tubes were taken to the exhaust hood and 3 ml of 98% sulfuric acid (P.A) and 1 ml of 35% hydrogen peroxide (P.A) were added. After that, the material was digested with temperatures up to 350°C with manual adjustment through digester block, the temperature gradually rose until a greenish colored liquid was obtained. After the digestion, the tubes were completed with 50 ml of distilled water and the digested material passed to the semi-micro Kjeldahl distiller. A 125-ml beaker containing 15 ml of boric acid (20 g L⁻¹) was laced at the end of the distiller tube, and 20 ml of 40% NaOH (P.A) was added to the digested material. The color of the sulfuric acid solution turned from wine to green at the end of the distillation, and at the end of this procedure, the titration was carried out with HCl solution 0.01 mol L⁻¹ to obtain initial coloring wine.

The ammonium content was determined using the method described by Weatherburn (1967). To the extraction, was added 100 mg of dry leaf tissue in an oven at 30°C. In each sample, 5 ml of deionized water was added and the plant material was taken to water bath for 1 h at 100°C and then the extract was filtered through quantitative filter paper Unifil™. For the determination, 1 ml of the filtrated solution was removed, and transferred to a vial, in which were added 9 ml of distilled water, 5 ml of reagent (100 ml sodium hydroxide (0.3 M), 100 ml of sodium salicylate (1.06 M) (P.A), 100 ml of distilled water and 2 ml of dichloroisocyanurate solution (39.1 mM) (P.A). After 30 min rest, the reading was performed in a spectrophotometer at 630 nm. The calculations were made using a standard curve prepared with dilute solutions of NH₄Cl (1000 µg NH₄⁺-N ml⁻¹) (P.A).

The nitrate content was determined using the method described by Cataldo et al. (1975), from fresh leaf tissue. For the extraction, 0.1 g of chopped fresh leaves was used. The material was placed in a beaker with 20 ml of ethyl alcohol 80% and remained refrigerated for 12 h. Subsequently, the material was crushed in a mortar and filtered through four layers of gauze and clinical qualitative filter paper Whatman™. The filtrate was transferred to separatory funnel and added to an equal volume of chloroform. After gentle mixing, the material was kept in rest for 40 min for complete separation. The non-polar fraction was discarded, and the polar fraction collected. Its volume was completed to 25 ml with 80% ethanol and stored in a refrigerator. For the determination, a 0.3 ml aliquot received 1.2 ml of solution of salicylic acid (HC₇H₅O₃), 5% in 98% concentrated H₂SO₄ and 8.5 ml of NaOH 2 mol L⁻¹ (P.A), and then the absorbance reading was taken at 410 nm. The results were expressed in mg of N-NO₃⁻ in fresh plant mass, with the aid of a calibration curve prepared from dilute solutions of KNO₃, which received the same treatment of the samples.

The data were subjected to variance analysis and the study of

polynomial regression, using the significance level of 5% with the statistical program SISVAR® version 5.6.

RESULTS

In the first experiment, the increase of nitrogen concentration in the form of NO₃⁻ in the nutrient solution contributed to the linear increase in the number of leaves, stem diameter, leaf length and leaf dry mass of lettuce. For the leaf fresh mass, the nitrate concentration of 15.0 mmol L⁻¹ promoted higher yield through of fresh mass (Table 1).

The increase in nitrogen as nitrate also contributed to higher root development, and the root volume and fresh mass obtained linear rise with the increase of this nitrogen source in the solution (Table 1).

In the second experiment, as the ammonium concentration was increased in relation to the nitrate concentration in the nutrient solution containing proportions nitrate/ammonium, the number of leaves, stem diameter, root fresh and dry mass were linearly reduced (Table 2). For leaf length, fresh and dry leaf mass and root volume, the proportion of ammonium in the nutrient solution that resulted in the best increments was 27, 23, 6.7 and 3%, respectively.

The increase of nitrogen as nitrate doses in the nutrient solution (experiment I) linearly increased the nitrate reductase activity and the nitrate content in the plants. Also, the leaf ammonium content was quadratically reduced. For total nitrogen content, the concentration of 14 mmol L⁻¹ of nitrate resulted in higher nitrogen content in the dry mass (Table 3).

Evaluating the experiment about nitrate/ammonium ratio (experiment II), it is perceived that the increase in the ammoniacal nitrogen in the nutrient solution linearly reduced the enzymatic activity and leaf nitrate content (Table 3), which however, contributed to the linear increase of ammonium content in leaf dry mass. The concentration of 39% of ammonium was one that provided the highest total-N content in the dry mass of lettuce. This result demonstrates that the proportions greater than 39% ammonium may lead to severe toxicity, since the plant significantly reduced the ion absorption (Table 3).

It was observed that higher ammonium proportions lead to reduction in the production and visual quality of the lettuce crop as well. On the other hand, the nitrate at high concentrations did not cause toxicity to plants, contributing to production increase and higher visual quality in lettuce (Figure 1).

DISCUSSION

The rise in nitrate doses in the nutrient solution resulted in increases for root and leaf mass of lettuce. This is because, according to Salomez and Hofman (2009),

Table 1. Regression equations for the effect of nitrate doses in the nutrient solution on the number of leaves (NL), stem diameter (SD), leaf length (LL), root volume (RV), leaf fresh mass (LFM), leaf dry mass (LDM), root fresh mass (RFM) and root dry mass (RDM), of lettuce grown hydroponically in southern Amazon.

Variable	Equation	F	R ²	¹ PM
NL	$y=0.401x + 7.851$	23.59**	0.94	-
SD	$y=0.049x + 0.841$	11.62*	0.89	-
LL	$y=0.875x + 24.323$	9.85*	0.87	-
LFM	$y=-0.750x^2 + 22.325x + 18.617$	21.94*	0.97	15.0
LDM	$y=0.258x + 3.767$	62.02**	0.97	-
RV	$y=0.106x + 1.815$	16.37*	0.91	-
RFM	$y= 0.587x + 8.267$	10.64*	0.88	-
RDM	$y=-0.001x + 0.655$	0.03 ^{ns}	0.10	-

*,** and ns: significant at 5 and 1% probability and not significant, respectively, ¹Maximum point.

Table 2. Regression equations for the effects of nitrate/ammonium ratio in the nutrient solution on the number of leaves (NL), stem diameter (SD), leaf length (LL), root volume (RV), leaf fresh mass (LFM), leaf dry mass (LDM), root fresh mass (RFM) and root dry mass (RDM) of lettuce grown hydroponically in southern Amazon.

Variable	Equation	F	R ²	¹ PM
NL	$y=-0.03x + 11.800$	75.97**	0.98	-
SD	$y=-0.004x + 1.236$	698.07**	0.99	-
LL	$y=-0.003x^2 + 0.160x + 29.269$	88.94**	0.99	2.6
LFM	$y=-0.005x^2 - 0.235x + 103.154$	32.57*	0.98	2.3
LDM	$y=-0.0003x^2 + 0.004x + 4.702$	5.38*	0.91	0.7
RV	$y=-0.0001x^2 - 0.0006x + 2.107$	8.06*	0.94	0.3
RFM	$y=-0.071x + 10.506$	82.16**	0.98	-
RDM	$y=-0.002x + 0.486$	32.14**	0.95	-

*,** and s: significant at 5 and 1% probability and not significant, respectively, by the F test. Maximum ¹Point.

nitrogen favors vegetative growth and in low concentrations promotes consequent reduction of yield, poor quality and delay of plant maturity.

In a study on lettuce, Becker et al. (2015) observed that low concentrations of nitrogen influenced plant growth, causing a decrease in leaf number and fresh leaf mass. In our study, nitrogen in nitrate form, also promoted the growth of lettuce root system, as stated by Liu et al. (2008). Increase in the root volume can be attributed to the fact that the nitrate supply to the plant promotes the activation of genes involved in cell development, which promotes the increase in turgor and cellular absorption, with consequent growth.

The highest yield of lettuce due to high concentrations of nitrate in solution was also verified by Kováčik et al. (2014), Galieni et al. (2015), Qadir et al. (2017) and Sapkota and Liu (2019) who found that there was higher yield with high nitrate levels, and reduction in productivity of plants and slow growth at lower nitrate concentrations. In this context, it is notorious that the increase in nitrogen availability is a major factor in the accumulation of nitrate in plants, reinforcing the point proposed in the literature

by Chen et al. (2004), Gülser (2005), Turan and Sevimli (2005) and Pôrto et al. (2008).

The levels of nitrate in the present study (225 mg kg⁻¹ of fresh weight was the maximum found) and do not present risks to human health, as they are lower than the one established by the regulation n° 1258/2011 of the Committee of European Communities (2011), which sets the maximum permissible content of 5000 mg kg⁻¹ fresh weight for the culture of lettuce. As regards research on nitrate accumulation in curly lettuce in the hydroponic system, Urlić (2017) also found nitrate levels below that established by law, noting that the observed NO₃⁻ concentrations for the cultivar reached a maximum value of only 450 mg kg⁻¹ in fresh matter.

The availability of nitrate in the nutrient solution also influenced the activity of nitrate reductase enzyme in lettuce leaves. The results show that the higher nitrate concentrations in the solution promote higher enzymatic activity. This observation was expected because the nitrate reductase is an enzyme induced by the presence of nitrate. According to Kawachi (2002), the activity of this enzyme depends mainly on light and continuous

Table 3. Regression equations for the effect of nitrate concentration and ratio of nitrate/ammonium in the nutrient solution, over the activity of nitrate reductase enzyme, total nitrogen content and ammonium content of lettuce leaves for plants grown hydroponically in southern Amazon.

Variable	Equation	F	R ²	¹ PM
Nitrate content in the nutrient solution				
Enzyme (NR)	$y=0.112x - 0.178$	17.92*	0.92	-
Total-N	$y= -0.175x^2 + 4.897x + 7.286$	7.53*	0.93	14.0
Nitrate content	$y=11.087x + 58.259$	29.44**	0.95	-
Ammonium content	$y=0.176x^2 - 4.186x + 37.965$	1.19 ^s	0.73	12.0
Nitrate/ammonium ratio in the nutrient solution				
Enzyme (NR)	$y=-0.030x + 1.280$	43.60**	0.96	-
Total-N	$y=-0.002x^2 + 0.156x + 46.474$	126.85**	0.99	3.9
Nitrate content	$y=-1.043x + 202.420$	6.27*	0.82	-
Ammonium content	$y=0.185x + 34.530$	10.21*	0.87	-

*,** and s: significant at 5 and 1% probability and not significant, respectively, by the F test. ¹ Maximum or Minimum Point.



Figure 1. Effects of nitrate concentration (25, 50, 75, 100 and 150% of the recommended dose) and proportion of nitrate/ammonium (0:100, 25:75, 50:50, 75:25 and 100/0) in the nutrient solution for hydroponics, on the development of lettuce Iceberg type.

supply of nitrate through the xylem.

The use of high nitrogen as nitrate concentrations in the nutrient solution contributed to the reduction of the ammonium content in leaf tissue. However, the reduction of nitrogen as nitrate in the solution and the substitution for N-ammonium, contributed to greater accumulation of ammonium ion by the plant. The factor that may be related to the increase of ammonium concentration in plants is the reduction in sugar content (carbon skeletons) of the plant from photosynthesis. According to Mifflin and Lea (1977) and Pate (1980), ammonium assimilation depends on the availability of sugars for

amino acids formation.

Based on the above and on the results of the present work, it is believed that the losses in growth and production variables caused by low levels of nitrogen as nitrate or high levels of N-ammonium are due to the limitation of the plant in performing photosynthesis, least amount of carbon skeletons, and providing greater accumulation of ammonium.

The use of ammoniacal sources in high doses in the nutrient solution negatively influenced the leaf and root development of lettuce. Studies like those of Borgognone et al. (2013) and Villarreal et al. (2015), respectively with

the species *Solanum lycopersicum* L., and *Eustoma grandiflorum* L., also indicated negative effect of the increase in ammonium concentration on the plant development. Our results are also similar with those obtained by Liu et al. (2017) who studied the effect of nitrate/ammonium on the growth of tomato seedlings, and found that plants respond significantly, since the higher plant height was obtained by $\text{NO}_3^- : \text{NH}_4^+$ ratio up to 75:25%. In studies with ammonium proportions in lettuce, Tian et al. (2003) and Weil et al. (2021) reported that 50% of nitrogen as NH_4^+ significantly decreased the development of the root system making plants susceptible to pathogens.

The reduction of lettuce growth with increases of ammonium content in the solution can be attributed to several factors, including the fact that the NH_4^+ ion when in excess is toxic to plant cells as it acts as a decoupling between the electron flow and oxidative phosphorylation or photophosphorylation. Besides acting as a decoupler between the electron flow, excess of ammonium (NH_4^+) in plant tissue dissipates proton gradient and this gradient is required for photosynthesis and respiration processes, besides being important in transpiration and accumulation of metabolites into the vacuole (Taiz et al., 2017). Another effect that ammonium causes in plants is the imbalance between the ions, due to the nutritional disturbances in the plants and the competitive effect of NH_4^+ in relation to the cations, since they are ions that compete for the same sites of absorption (Marschner, 2012).

However, the harmful effects of ammonium on lettuce were mitigated with proper proportions of nitrate/ammonium in the solution, reducing the accumulation of nitrate by the plant. These results confirm the proposition stated by Andriolo et al. (2006) who report that a part of the N supplied in the nitrate (NO_3^-) form may be replaced by ammonium (NH_4^+), reducing the accumulation of NO_3^- in the vacuoles. The nutrient solution containing nitrate and ammonium in appropriate concentrations may be a great ally to decrease toxic effects caused by ammonium. This is because the absorption of this anion is associated with alkalizing rhizosphere and stimulating cation uptake, relieving some harmful effects of the NH_4^+ (Britto and Kronzucker, 2005).

There is a concern that the specific climatic characteristics of the Southern Amazon region may favor the accumulation of nitrate in lettuce grown in a hydroponic system. However, the results observed in the present research are similar to the results of the aforementioned studies, ensuring, above all, that the lettuce produced and commercialized in this region has good quality, without containing high levels of nitrate.

Conclusion

The nutrient solution containing nitrate source in concentration of 15.0 mmol L^{-1} contributes to higher

productivity of lettuce, owing to a recommendation of this concentration in hydroponic cultivation in southern Amazon.

It is suggested that about 23% of nitrogen be used in nutrient solution in the form of ammonium, being that upper N-ammonium concentrations are not suitable for lettuce Iceberg type because they can reduce the productivity of this vegetable, causing toxicity symptoms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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