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Full Length Research Paper

Effects of NH_4^+-N/ NO_3^--N ratios on growth, nitrate uptake and organic acid levels of spinach (*Spinacia oleracea* L.)

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The objective of this investigation was to study the effects of NH_4^+-N/ NO_3^--N ratios on the growth of spinach, the nitrate and organic acids contents of spinach fresh shoot. A completely randomized design was established as 5 treatments, that is, NH_4^+-N/ NO_3^--N ratio of 100/075/25, 50/50, 25/75 and 0/100, the total N concentration was 12 mmol l^{-1}. Each treatment had 3 replications, results showed that the spinach biomass significantly (P = 0.05) decreased as the NH_4^+-N/ NO_3^--N ratio was above 50:50 in nutrient solution. Spinach supplied with NO_3^--N as its sole nitrogen source had the highest biomass, but there was no notable difference (P = 0.05) in dry matter weight between 25: 75 and 0:100 of NH_4^+-N/ NO_3^--N ratio. A strongly (r = 0.9826) positively linear relationship was found between the nitrate content of spinach fresh shoots and the proportion of NO_3^--N in total nitrogen. When the NH_4^+-N/ NO_3^--N ratios was decreased from 100:0 to 0:100, the pyruvate, citrate, succinate, fumarate contents were all increased gradually and the malate content was highest at NH_4^+-N/ NO_3^--N ratio of 25:75. The water soluble oxalate content of the spinach fresh shoot at either NH_4^+-N or NO_3^--N as sole nitrogen resource, was higher than other combinations of NO_3^--N and NH_4^+-N and, for example, the soluble oxalate content was significantly (p = 0.05) decreased by 8% when 25% of NO_3^--N was replaced by NH_4^+-N. In conclusion, some replacement of NO_3^--N by NH_4^+-N could improve the spinach quality while the dry biomass was not significantly affected.

Key words: Spinach, NH_4^+-N/ NO_3^- ratio, organic acid, nitrate content, oxalate content.

INTRODUCTION

Nitrogen (N) is a unique nutrient because, unlike the other essential nutrient elements, plants can use it in either the cation form, ammonium (NH_4^+), or the anion form, nitrate (NO_3^-) (Miller and Donahue, 1990). Most of the NH_4^+ has to be incorporated into organic compounds in the roots. The assimilation of NH_4^+ in roots produces about 1 proton per molecule which has to be excreted into the external medium (Buchanan et al., 2002). Ammonium used as the sole source of N appears to have a negative effect on growth and morphogenesis (Cousson and Tran Thanh Van, 1993; Raab and Terry, 1994, 1995; Walch et al., 2000; Carl and Richard, 2002). The negative effect of NH_4^+ has been attributed to various factors such as changes in medium pH and toxic effects of free NH_4^+. NO_3^- is a major source of N for plants. Compared with NH_4^+, NO_3^- has the advantage of being a storage form in plants with no necessity to be assimilated in the roots. In addition, NO_3^- nutrition induces an increase rather than the decrease in rhizosphere pH and there is no risk of toxicity at alkaline pH (Marschner, 1995; Dev and Herbert, 2002).

As a result, maximium growth rates and plant yields could be obtained by combined supply of both NH_4^+ and NO_3^-. When both NH_4^+ and NO_3^- are supplied, pH stat may be achieved by similar rates of H^+ production (NH_4^+ assimilation) and H^+ consumption (NO_3^- assimilation) and thus has a very low energy requirement (Raven, 1985;

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Allen et al., 1988). This may, at least, partly explain that optimal growth for most plant species is usually obtained with mixed supply of NH_4^+ and NO_3^-. However, when the NH_4^+-N/ NO_3^--N ratio was above 50:50, a yield decrease occurred in most aerobic crops with the exception of rice which in most situations grew better when the NH_4^+-N/ NO_3^--N ratio was above 50:50 (Smiciklas and Below, 1992; Yang et al., 2003).

Many studies indicated that a reasonable NH_4^+-N/ NO_3^--N ratio could increase crop yield and reduce NO_3^- content of vegetable (Shen et al., 2003; Dong et al., 2004; Chen et al., 2005). Moreover, different ratios of NH_4^+-N/ NO_3^--N in a nutrient solution or in soil with controlled nitrification not only affected plant growth, but also the organic acid content to a large extent. An increase in a suitable proportion of NH_4^+-N in nutrient solution led to a significant decrease in malate, citrate and fumarate (Dong et al., 2004). NO_3^- acts as the signal to initiate co-ordinated changes in carbon and N metabolism and organic acid production (Scheible et al., 1997). During rapid vegetative growth, the rates of NO_3^- reduction, carboxylate and amino acid synthesis are fast. NO_3^- reduction implies the formation of excessive alkaline ions. These ions can not be efficiently expelled from the cell, therefore, the plant synthesizes organic acids (principally citrate and malate) in leaves which are transported to the sites where NO_3^- reduction is occurring to maintain pH homeostasis (Touraine et al., 1988; Imsande and Touraine, 1994; Jose et al., 2000). Thus, the effect of NH_4^+-N/ NO_3^--N ratio on oxalate levels of plants should be paid attention to. In a number of plant species, a higher oxalate content in plant was commonly found in NO_3^- medium than in NH_4^+ (Gilbert et al., 1951; Libert and Franceschi, 1987). Oxalate concentrations were the highest in the leaves and stems of purslane that were grown in nutrient solutions with no NH_4^+ and the lowest in leaves and stems that were grown with 75% NH_4^+ in the nutrient solution (Usha et al., 2004).

Spinach (*Spinacia oleracea* L.) is a cool season annual herb. It is a good source of vitamin C, vitamin A and minerals especially ferrum. With respect to its utilization, spinach is classed in the group of leafy vegetables, thus potentially disposed to accumulate excessive amounts of NO_3^- and oxalates which are the main indexes of the quality (Grevsen and Kaack, 1996; Jaworska, 2005). Apparently, N fertilizer with different NH_4^+-N/ NO_3^--N ratio will affect these 2 parameters. Thereby, understanding the relationship between NH_4^+-N/ NO_3^--N ratio and organic acid metabolism and practically controllable nitrification in soil could be the key point to improve the quality of spinach.

The objectives of this study were to study the effects of NH_4^+-N/NO_3^--N ratio on spinach growth, the NO_3^- and organic acid contents of spinach shoot to find the balanced point between the biomass yields and quality of spinach so that it can provide basic theory for the practice of cultivation of spinach with high quality.

MATERIALS AND METHODS

Plant material and growing conditions

A hydroponic experiment was conducted in a greenhouse of Anhui Science and Technology University, P. R. China in January 2004. Seeds of spinach (*S. oleracea* L., Yinchuandayuan) were surface-sterilized with 50 ml of water for 30 min. The disinfected seeds were washed thoroughly with deionized water and they were soaked in deionized water for 12 h. Subsequently, the seeds were germinated in an incubator at 25℃. After a week the germinated seeds were sown on a nursery bed with clean and moist vermiculite prepared. 30 days after sowing, the seedlings of uniform size and vigor with 4 leaves were transferred into a plastic tank containing 12 l of nutrient solution. 30 plants were grown in each tank (15 tanks in total) on 22nd February 2004.

A completely radomized design was adopted in the experiment with 5 treatments (combinations) of NO_3^--N/ NH_4^+ that is, percentages of 100/0, 75/25, 50/50, 25/75 and 0/100. Each treatment had 3 replications. Total N concentration was 12 mmol l^{-1} in all treatments. The full strength macronutrient solution containing 12 mmol l^{-1} N, 1 mmol l^{-1} P, 6 mmol l^{-1} K, 5 mmol l^{-1} Ca and 2 mmol l^{-1} Mg. Micronutrients were supplied as H_3BO_3(0.50 mg B L^{-1}), Fe-EDTA(2.8 mg Fe^{-1}), $MnCl_2 \cdot 4H_2O$ (0.50 mg Mn l^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.05 mg Zn l^{-1}), $CuSO_4 \cdot 5H_2O$ (0.02 mg Cu l^{-1}), and H_2MoO_4 (0.09 mg mol^{-1}). A nitrification inhibitor $C_2H_4N_4$ (7µmol l^{-1}), was added to all the nutrient solutions to prevent nitrification. Throughout the experiment, the nutrient solutions in the hydroponic system were aerated for 30 min twice a day with an electronic inflator and every day the pH of each nutrient solution was adjusted to 6.50 by adding 0.5 mol l^{-1} HCl or NaOH as needed. The nutrient solution was discarded and replaced by new nutrient solution in every 10 days.

All plants grew in a greenhouse under the same temperature (15/25℃) and natural irradiance conditions.

Harvest and sampling

The seedlings were harvested, washed with distilled water, separated into roots and shoots for the determination of fresh weight. 5 plants among these were used for measurement of dry weight at 65℃ in an oven for 48 h after they were kept in a 108℃ for 30 min to kill the enzymatic activity of the plant samples (Zhang et al., 2005; Nakata, 2003; Noonan and Savage, 1999).

Sample preparation

Other 20 plants from the same tank were separated into shoots and roots. The shoots and roots from different plants were cut into small pieces and mixed thoroughly, respectively. And then the mixed segments of shoots and roots were divided into several sub-samples with weight of 2 g each for measurement of NO_3^-, organic acid content and other items. All samples were quickly frozen in liquid N after that the samples were stored at -40°C until assay.

Nitrate determination

2 g fresh shoot samples were added to 20 ml deionized water and ground into a paste in an agate mortar (Niu, 1992; Chen et al., 2005). The extraction was decolorized by activated carbon and filtered to prevent chlorophyll pigment interference. The filtrate was measured for NO_3^- using a continuous-flow autoanalyzer (Autoanalyzer 3, Bran + Luebbe GmbH, Germany).

Table 1. Effect of NH_4^+-N/NO_3^--N ratios on spinach biomass (g $plant^{-1}$).

NH_4^+-N/NO_3^--N ratios	Fresh weight			Dry weight		
	Shoot	Root	Total	Shoot	Root	Total
100:0	1.05 ± 0.12a [a)]	0.25 ± 0.06a	1.30 ± 0.15a	0.22 ± 0.03a	0.05 ± 0.03a	0.27 ± 0.04a
75:25	1.83 ± 0.12b	0.61 ± 0.11ab	2.44 ± 0.22b	0.35 ± 0.09a	0.11± 0.05a	0.46 ± 0.13a
50:50	2.42 ± 0.23c	0.77 ± 0.26b	3.19 ± 0.12c	0.59 ± 0.08b	0.13 ± 0.04a	0.72±0.12b
25:75	5.18 ± 0.17d	1.52 ± 0.34c	6.70 ± 0.23d	0.99 ± 0.12c	0.26 ± 0.03b	1.25 ± 0.12c
0:100	6.20 ± 0.26e	1.60 ± 0.25c	7.80 ± 0.51e	1.02 ± 0.04c	0.27 ± 0.10b	1.29 ± 0.14c

The data represent mean values ± deviations for the there replications. Values followed by the same letter are not significantly different ($P < 0.05$) using Duncan's multiple range test.

Organic acid determination

2.0 g frozen spinach shoot samples were ground in a mortar with 2 ml super-pure water and homogenized. The homogenized samples were thoroughly transferred to a 7 ml centrifugal tube with 3 ml super-pure water and centrifuged at 16 000 rpm min^{-1} for 20 min. The supernatant was collected for analysis of organic acids (malate, citrate, fumarate, pyruvate and succinate) by HPLC (high-performance liquid chromatography). Standard organic acid samples in this experiment were analytically pure grade and the chromatographic conditions were as previous studies (Dong et al., 2005; Zhang and Huang, 2007). Chromatographic column: waters symmetry RP_{18} (250 mm × 3.9 mm, 5 μm), mobile phase: acetonitrile, 0.1% phosphoric acid solution (4:96), injection volume: 10 μl, flow rate: 0.8ml min^{-1}, column temperature: 30℃ detection wavelength: 326 nm.

Soluble oxalate content was determined as follows (Wang and Du, 1989). 2.0 g frozen spinach shoot samples were ground in a mortar and pestle to a fine homogenate to which 20 ml of distilled water was added. The homogenate was then filtered and washed for three times with 50 ml of distilled water. The entire filtrate was transferred to a 200 ml volumetric flask. 5 ml of the filtrate was transferred into a centrifuge tube which contained 2 ml of 2 mol l^{-1} $CaCl_2$ and 0.5 ml 2 mol l^{-1} NaOH, the flocky yellow green precipitate was removed by centrifuging at 4,000 rpm min^{-1} for 5 min, the supernatant was taken out and diluted with 5 ml distilled water. Then, this emulsion was irradiated with uviol lamp for 5 h at 365 nm to yield powder white precipitate.

The powder white precipitate was separated and dissolved in 5 ml 1.00 mol L^{-1} perchloric acid. This solution was thoroughly transferred into a 25 ml color comparison tube which contained 6 ml 10.0 mmol l^{-1} $KMnO_4$ and diluted to 25 ml by deionized water. After 5 min, the absorbance of the solution was measured at 520 nm (756 spectrophotometer).

All data were analyzed by Duncan's test from the SPSS software.

RESULTS AND DISCUSSION

Spinach biomass yields

Different NH_4^+-N/NO_3^--N ratios significantly affected the growth of spinach (Table 1). With the NH_4^+-N/ NO_3^--N ratios being decreased from 100: 0 to 0: 100, the shoot and root of both the fresh and dry weight of plant increased. There were notable difference ($P = 0.05$) in fresh weight among the 5 treatments. Compared with the treatment of 0:100 of NH_4^+-N/ NO_3^--N ratio, the shoot fresh weight of the relative values of other 4 treatments were 16.9, 29.5, 39.0 and 83.5%, respectively.

Spinach supplied with NO_3^--N as its sole N source had the highest fresh and dry weight, which were 6 and 5 fold higher respectively of the treatment supplied with sole NH_4^+-N treatment. Previous studied also showed that the highest yield of plant was produced at 0: 100 of NH_4^+-N/ NO_3^--N ratio (Ge, 2002; Wang et al., 2005). Apparently, at higher and exclusive supply, NH_4^+-N tends to inhibit plant growth (Walch et al., 2001) or to generate toxicity in aerobic crops (Britto and Kronzucker, 2002). In addition, plant growth, particularly root growth, is poor in NH_4^+-N -fed plants when NH_4^+-N concentration is high (Marschner, 1995)

However, although the fresh and dry weight of plant treated with 25:75 of NH_4^+-N/ NO_3^--N ratio were 85.9 and 96.9% of 0:100 NH_4^+-N/NO_3^--N ratio respectively, statistical analysis showed that there was no significant difference in dry weight between 25:75 and 0:100 of NH_4^+-N/ NO_3^--N ratio. This implies that increasing the proportion of NO_3^--N in total N had no advantage of biomass accumulation when the proportion was above 75%. Unlike other aerobic crops, spinach crop did not have any response of increased biomass yield when NO_3^--N was replaced by NH_4^+-N. This is the same as the results reported by Wang et al. (2005) who found that the biomass yield of spinach was the highest in the treatment of 100% of NO_3^--N. But Zhang et al. (1990) reported that spinach had the highest yield when the ratio of NH_4^+-N and NO_3^--N is 50:50 and 25:75. Spinach is a typical crop that prefers NO_3^--N to NH_4^+-N.

Many studies have shown that most of the aerobic plants also seem to grow better in a mixed supply of NH_4^+ and NO_3^- (Dong et al., 2004; Chen et al., 2005). If the NH_4^+-N form is used, it does not surely have reduction process inside the plant before being incorporated into the amino form ($-NH_2$) as NO_3^--N does. This would save plant energy. In addition, increasing the NH_4^+-N/ NO_3^--N ratio could reduce the NO_3^- content of crops (Chen et al., 2005). As Wang and Li (2003) pointed out, mixed N as NH_4NO_3 could reduce the accumulation of NO_3^- in cabbage and spinach compared with sole NO_3^--N as $NaNO_3$ with no significant difference in yields. Therefore, it was

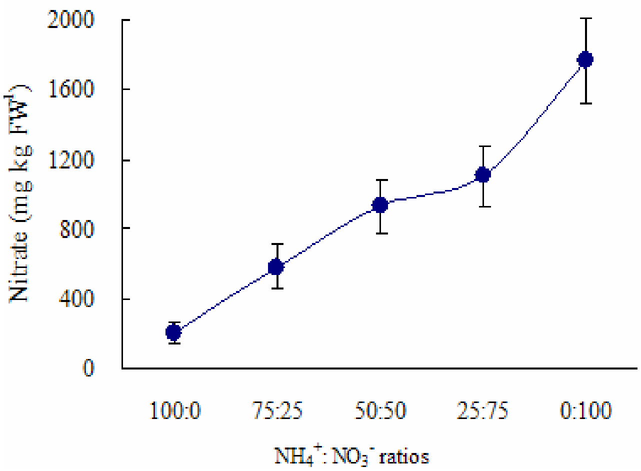

Figure 1. Nitrate concentrations in spinach shoots with five NH_4^+-N/NO_3^--N ratios in nutrient solutions. Data represent means of six replications, and values of nitrate concentrations marked by the same letter are not significantly different (P = 0.05).

worthwhile replacing some NO_3^--N in the solution with NH_4^+-N to improve plant quality with no significantly negative effect of spinach biomass yields.

Nitrate content in spinach plants

Nitrate accumulation in spinach shoots was dramatically increased with the increase of NO_3^--N percentage (Figure 1). From NH_4^+-N/ NO_3^--N ratio of 0:100 to the ratio of 25:75, NO_3^- content of fresh spinach shoots was decreased by 37.5%. This was accordance with Chen et al. (2005) who found that from NH_4^+-N/ NO_3^--N ratios of 0:100 to the ratio of 25:75 and 50:50, NO_3^- contents in 3 Chinese cabbage cultivars decreased, on average, by 37 and 52%, respectively. A small amount of NO_3^--N replacement by NH_4^+-N could significantly decrease the NO_3^--N content in spinach and this implies that we can find the balanced point between the maximum spinach biomass yield and the minimum NO_3^- content in the plants. More gradients of NH_4^+ proportion to total N is worthwhile doing in soil condition since normal nitrifica-tion of soil usually makes soil NH_4^+ content too low and any practice to slow down the nitrification to maintain a certain amount of is NH_4^+ of great significance.

Regression analysis indicated that there was a strong positive linear relationship between the NO_3^- content of spinach fresh shoot and the proportion of NO_3^--N in total N (r = 0.9826).

Organic acid contents in spinach plants

With the NH_4^+-N/ NO_3^--N ratios decreasing from 100:0 to 0:100, the pyruvate, citrate, succinate, fumarate contents all increased gradually (Figure 2). Malate content was highest at NH_4^+-N/ NO_3^--N ratio of 25:75 and a small, but not significant (P = 0.05) decrease at the ratio of 0:100. However, the change of the oxalate (water soluble) content was different from other organic acids, the oxalate content was highest at NH_4^+-N/ NO_3^--N ratio of 100:0 and lowest at NH_4^+-N/ NO_3^--N ratio of 25:75.

Effects of NH_4^+-N/ NO_3^--N ratio on organic acid levels of spinach was in accordance with Dong et al. (2004) who reported that increasing the ratio of NH_4^+-N/ NO_3^--N (25, 50 and 75%) in the nutrient solution led to a significant decrease (P = 0.05) in malate, citrate and fumarate.

The relationship between NO_3^- assimilation and organic acid metabolism has been elegantly explored in mutant plants with low NO_3^- reductase activity (Nia 30 mutants), which accumulate large amounts of NO_3^-. When wild type tobacco plants are grown on low NO_3^-, a large decrease in malate and citrate levels has been documented. These organic acids increased 3/10 fold in plants growing on high NO_3^- (12 mM). These results showed that NO_3^- initiated a coordinated increase in the expression of several genes involved in organic acid synthesis (Jose et al., 2000). Hence, N assimilation interacts very closely with organic acid metabolism (Imsande and Touraine, 1994; Dong et al., 2004). Malate, which acts as a counterion related to NO_3^- uptake, is known to be one of the most important organic acids to ongoing NO_3^- assimilation and also in maintaining pH-stat (Martinoia and Rentsch, 1994). Citrate also acts as a counterion for NO_3^- uptake (Scheible et a1., 1997).

Regression analyses were performed on the percentage of NO_3^--N in total N in nutrient solution to the contents of malate and citrate in spinach fresh shoot. The results revealed that the percentage of NO_3^--N in total N (x%) were highly correlated with the contents of malate (Y_{Mal}, mg kg^{-1} FW) and citrate (Y_{Cit}, mg kg^{-1} FW) in spinach. The linear regression equations were as follows:

$Y_{Mal} = 185.6+8.525x \quad R^2 = 0.9207$
$Y_{Cit} = 246.1+3.804x \quad R^2 = 0.9685$

Another most important organic acid -oxalate was also marked affected by the N form. The present experimental results showed the water soluble oxalate content of the spinach fresh shoot at either NH_4^+-N or NO_3^--N as sole N resource, was higher than other treatments. 25% replacement of NO_3^--N by NH_4^+-N could significantly (p = 0.05) decrease the soluble oxalate content by 8%. In a number of plant species, NO_3^- as the source of nitrogen as opposed to ammonium lead to a higher oxalate content of the plant (Libert and Franceschi, 1987). Meeuse and Campbell (1959) identified NO_3^- as a potent inhibitor of oxalic acid oxidase in beta vulgaris. If photorespiratory glyoxylate was a precursor of oxalate, the supply of N could influence whether glyoxylate would accumulate and thus be further oxidized or be transaminated to glycine (Kpodar et al., 1978). Moreover, the source of N appears to affect the aggregation state of glycolate oxidase, which

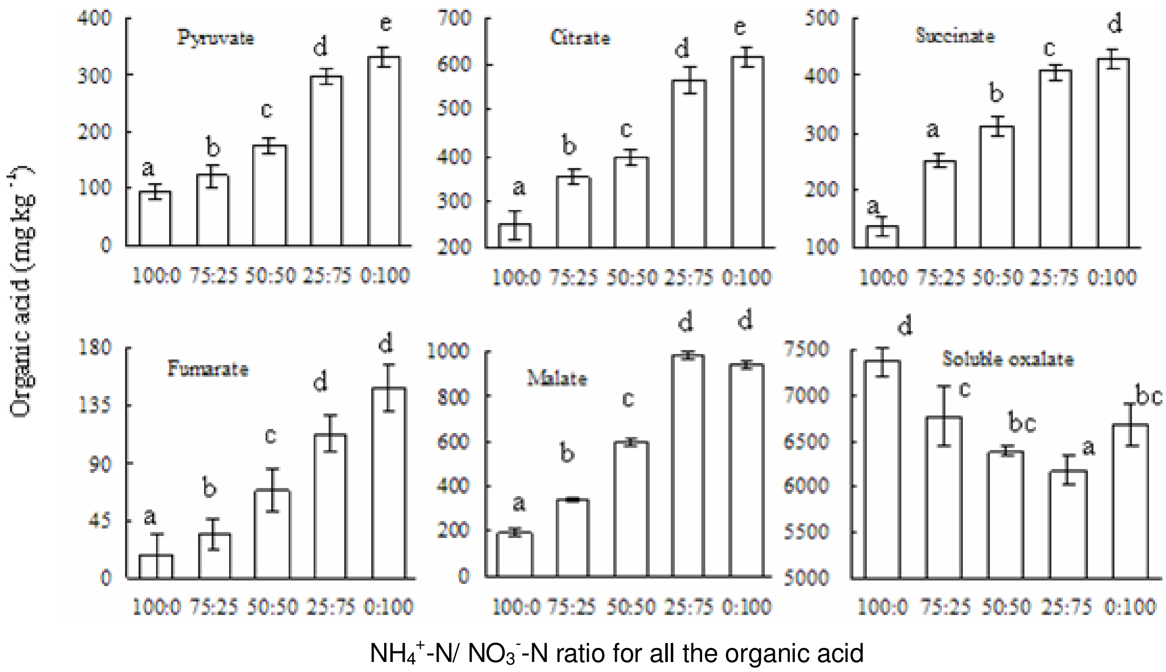

Figure 2. Organic acid contents in spinach shoots with 5 NH_4^+-N/ NO_3^--N ratios in nutrient solutions. Data represent means of three replications, and values of organic acid contents marked by the same letter are not significantly different (P = 0.05).

may have physiological significance (Emes and Erismann, 1982). So far, although the important aspects of oxalate biosynthesis, accumulation, and catabolism are not well understood , one definite concept from our present results is that a suitable proportion replacement of NO_3^--N by NH_4^+-N may effectively reduce the oxalate content of spinach, which proposed a novel way to improve the quality of spinach.

Conclusions

Different NH_4^+-N/ NO_3^--N ratios in a nutrient solution not only affected plant growth, but also affected the NO_3^- and organic acid content to a great extent. Spinach supplied with NO_3^--N as its sole N source had the highest biomass yield, but the biomass yield of spinach supplied with NH_4^+-N/ NO_3^--N ratio of 25:75 was not significantly reduced.

The NO_3^- content of spinach fresh shoot was a strongly (r = 0.9826) positively related to the proportion of NO_3^--N in total N. The highest contents of pyruvate, citrate, succinate, fumarate in spinach fresh shoots were found at the NH_4^+-N/ NO_3^--N ratio of 0:100 while the highest content of malate was at the ratio of 25: 75. Water soluble oxalate content of the spinach fresh shoots was decreased by 8% as 25% replacement of NO_3^--N by NH_4^+-N.

Therefore, a combination of N nutrition at a ratio of 25:75 in hydroponic cultivation of spinach would optimize the biomass yield and quality of spinach. Any practice of maintaining a certain amount of NH_4^+-N in soil condition is greatly useful to producing an optimized yield with high quality of spinach.

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