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Growth, Quality, and Nitrogen Assimilation in Response to High Ammonium or Nitrate Supply in Cabbage (*Brassica campestris* L.) and Lettuce (*Lactuca sativa* L.)

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Abstract: Plants grow better when they are supplied with a combination of ammonium (NH_4^+) and nitrate (NO_3^-) than when either one is supplied as the sole N (nitrogen) source. However, the effects of N forms on N metabolism and major N assimilation enzymes in different plants, especially vegetables, are largely neglected. This study was conducted on two plants with distinct NH_4^+ tolerances to compare the responses of two popular leafy vegetables, Korean cabbage (*Brassica campestris* L.) 'Ssamchu' and lettuce (*Lactuca sativa* L.) 'Caesar green', to the N source. To this end, plant growth and quality, photosynthesis, carbohydrate, N contents (in the forms of NO_3^-, NO_2^-, NH_4^+, total protein), and key N assimilation-related enzyme (NR, NIR, GS, GDH) activities were investigated. When plants were subjected to one of three NH_4^+:NO_3^- regimes, 0:100, 50:50, or 100:0, lettuce was relatively more tolerant while cabbage was extremely sensitive to high NH_4^+. Both plants benefited more from being grown with 50:50 NH_4^+:NO_3^-, as evidenced by the best growth performance, ameliorated photosynthesis, and enriched carbohydrate (C) stock content. In addition, as compared to cabbage, the GS and GDH activities were reinforced in lettuce in response to an increasing external NH_4^+ level, resulting in low NH_4^+ accumulation. Our findings suggested that boosting or maintaining high GS and GDH activities is an important strategy for the ammonium tolerance in vegetables.

Keywords: nitrogen metabolism; ammonium toxicity; photosynthetic capacity; carbohydrate; nitrate reductase (NR); nitrite reductase (NIR); glutamine synthetase (GS); glutamate dehydrogenase (GDH)

1. Introduction

Nitrogen (N) is a primary and essential nutrient that affects the plant growth and agricultural production. Many higher plant species acquire their N in the form of nitrate (NO_3^-) and ammonium (NH_4^+) from the soil solution [1]. Both can always be absorbed and used via roots, but they vary greatly in the biochemical, energetic, and molecular features for assimilation. However, excessive NO_3^- is carried away through leaching, which pollutes the environment; furthermore, edible crops, especially leafy vegetables, have been found to accumulate an intermediate product nitrite (NO_2^-) during the nitrogen assimilation, which has toxic effects on both the plant growth and human health [2]. Fortunately, it has been well established that NH_4^+ uptake is a relatively energy-efficient process compared to NO_3^- uptake, leading to the fact that plenty of plant species prefer NH_4^+ as the N source [3,4].

Paradoxically, plants are often unable to grow optimally with high NH_4^+ concentrations or with NH_4^+ as the exclusive N source; intensive applications of ammonium-based fertilizers cause not only environmental problems, but strong toxicity for plant cells, such that excessive NH_4^+-induced toxicity has been considered as a factor for reduced plant species' richness [5,6]. Ammonium toxicity alters various physiological characteristics and biochemical attributes in plants. Detrimental symptoms, including reduced plant growth, yield, and root:shoot ratio as well as other side effects, such as leaf chlorosis and stunted root growth, are often observed [7]. Typically, certain integrated influences, such as the inhibition of cation uptake, increase of the oxidative stress, and interference of the photosynthetic activity, as well as limitation of carbohydrates, are dramatically caused by ammonium toxicity [8].

Researchers have observed that adding NH_4^+ to a NO_3^- solution or enhanced ammonium nutrition (EAN) and enhanced nitrate nutrition (ENN) can remarkably improve the nitrogen use efficiency (NUE) and promote plant growth. It is still important and necessary to gather new knowledge on how EAN and ENN induce biochemical and physiological changes and affect the N uptake and metabolism in marketable crops.

A large number of publications have reported the effects of N forms and different NH_4^+: NO_3^- ratios on photosynthesis. NH_4^+ was found to be able to uncouple the electron transport, which is linked to an important photosynthetic trait, Fv/Fm, which reflects the maximum quantum efficiency of photosystem II (PSII) and was adopted for early stress detection in plants [9,10]. Additionally, a declined photosynthetic rate was usually recorded when plants were supplied with NH_4^+ as the sole N source, because the conversion from NH_4^+ to amino acids required energy at the cost of carbon skeleton consumption [11]. As a consequence, the stock contents of soluble sugar or starch in plants dropped, which imposed a negative influence on the plant growth and development. Similarly, plants also withdrew the carbohydrates from vegetative tissues for NO_3^- assimilation; thus, the decrease of NO_3^- from a threshold level is generally accompanied by an increase of carbohydrates [12]. The photosynthetic capacity was found to be associated with the stomatal conductance, which is responsible for CO_2 fixation, diffusion, and assimilation [13].

The classical view of N use pathways in most plant species is conservative and has been well documented, involving uptake, assimilation, and translocation [14]. Two routes of ammonium assimilation have been identified: Usually, plants were unable to directly use NO_3^-, which is firstly reduced to nitrite (NO_2^-) by nitrate reductase (NR), then converted to NH_4^+ by nitrite reductase (NIR) and, finally, the NH_4^+ was incorporated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT) [15]. The NH_4^+ appeared to be alternatively catalyzed by glutamate dehydrogenase (GDH) [16]. Clearly, abundant NO_3^- also can result in the accumulations of NH_4^+ and affect not only the NR and NIR activities, but also the GS, GOGAT, and GDH activities. Although GS and GDH both play an important role in the N detoxification mechanism, the priorities of them differ among species. For instance, Cruz suggested that an increased GS activity level in *Solanaceae* was an important strategy in determining the ammonium tolerance, whereas GDH was evidenced as mainly responsible for ammonium detoxification in *Orchidaceae* [17,18]. Such information for vegetables, such as cabbage and lettuce, remain scarce. In addition, the analysis regarding relationships among GS, GDH, and other key enzymes (NR, NIR) and chemicals (free contents of NO_3^-, NO_2^- as well as NH_4^+) during EAN or ENN are not yet well known.

Therefore, the experiment undertaken herein assessed not only the responses of two different vegetables during EAN or ENN, but also the relationships between the key enzymes and chemicals involved in the N assimilation pathways. The growth attributes, photosynthetic capacity, soluble protein contents, and total carbohydrate (soluble sugar and starch) contents were investigated. Thereafter, the activities of key enzymes and contents of major chemicals involved in the N metabolism pathway were monitored in order to provide a potential rationale between the NH_4^+ tolerance and N assimilating enzymes.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The experiments were conducted in a fiberglass greenhouse, with 13 h of light (26 ± 2 °C) and 11 h of darkness (20 ± 2 °C) at Gyeongsang National University (35°90′ N, 128°06′ E, Jinju, Gyeongnam, Korea) from March to April and from September to October 2021. Seeds of two vegetables, Korean cabbage 'Ssamchu' and lettuce 'Caesar Green', were sown into 200 square-cell plug trays filled with BVB medium (Bas Van Buuren Substrate, EN-12580, De Lier, The Netherlands) and germinated under an intermittent mist for 5 days. They were subsequently transferred to a metal bench and allowed to grow for 5 additional days.

2.2. Ammonium–Nitrate Ratio Treatments

Subsequently, similar-size seedlings with two to three true leaves were screened and subjected to the different treatment solutions. A multipurpose nutrient solution (MNS), formulated according to our lab's pioneer publication [19], was modified in order to supply 13.0 me·L^{-1} N with three different A–N ratios (0/100, 50/50, 100/0) (Table 1). NH_4^+ or NO_3^- was used as the sole N source at concentrations of 13.0 me·L^{-1}. All plants were irrigated only with the treatment solutions. For each species, the experimental design was completely randomized, with three biological replicates per treatment, consisting of 60 plants each.

Table 1. Ion composition (me·L^{-1}) at a constant N concentration (13.0 me·L^{-1}), with three A–N ratios used as the treatment solutions.

NH_4^+: NO_3^- Ratio (%)	Cation (me·L^{-1})				Anion (me·L^{-1})				Total
	Ca^{2+}	Mg^{2+}	K^+	NH_4^+	NO_3^-	SO_4^{2-}	Cl^-	$H_2PO_4^-$	
Standard (MNS)	6.0	2.0	5.0	2.0	11.0	2.0	0.0	2.0	30.0
0:100	6.9	2.3	5.8	0.0	13.0	1.0	0.0	1.0	30.0
50:50	5.9	2.0	4.5	6.5	6.5	6.5	0.0	2.0	37.8
100:0	4.9	1.7	3.2	13.0	0.0	15.9	4.9	2.0	45.6

Additions of identical micronutrients (µM) (20 B, 0.5 Cu, 10 Fe, 10 Mn, 0.5 Mo, 4 Zn) to each solution.

2.3. Measurements of Growth Parameters

Three Weeks later, the growth attributes of juvenile plants were investigated during the harvest, which included shoot-related parameters (shoot length, leaf length and width, and chlorosis), whole plant weight, and root morphology. Specifically, leaf chlorosis was captured with a professional camera. The root morphological traits were determined using a WinRhizo Pro 2007a image analysis system (Regent Instruments, Sainte-Foy, QC, Canada) equipped with a scanner (Expression 1000XL, Epson America Inc., Long Beach, CA, USA).

2.4. Analyses of the Photosynthetic Capacity

The photosynthetic capacity was assessed and characterized herein by certain critical parameters. Chlorophyll a and b contents were spectrophotometrically estimated by using a protocol found in Arnon's study [20]. The maximum PSII intrinsic light energy conversion efficiency by means of the Fv/Fm and stomatal conductance were assessed using a FluorPen FP 100 (Photon Systems Instruments, Drásov, Czech Republic) and a Decagon Leaf Porometer (SC-1 mode, Decagon Device, Pullman, WA, USA), respectively.

2.5. Destructive Sampling and Quantification of the Total Souble Protein Content

At the end of the experiment, the plants were cut to separate the roots and shoots. Leaves with different sizes and colors were then individually collected, immediately frozen in liquid nitrogen, and stored at −70 °C for subsequent experiments.

The leaves in different treatments were individually labeled and ground into fine powder in a pre-cooled mortar. A total of 100-mg samples were homogenized in 50 mM of phosphate buffered saline (PBS) at pH = 7.0, which contained 1 mM of EDTA, 1 mM of polyvinylpyrrolidone, and 0.05% triton-X. The supernatant was obtained after centrifugation (13,000 rpm, 4 °C, 20 min). The total soluble protein estimations were conducted against the aqueous phase using Bradford's reagent [21].

2.6. Determinations of the Soluble Sugar and Starch Contents

The soluble sugar and starch contents were determined using an anthrone–sulfuric acid colorimetry with slightly modifications [22]. In brief, a 0.5 g finely ground leaf powder was mixed with 25 mL distilled water and placed in a 96 $\pm$ 2 °C water bath for 30 min. The mixture was then centrifuged (6500 rpm, 25 °C, 10 min) to obtain the supernatant that would be used afterwards for the soluble sugar content assays. The residue was collected to determine the starch content.

2.7. Measurements of NH_4^+, NO_3^-, and NO_2^- Concentrations

A colorimetric method based on the Berthelot reaction was used for the quantification of NH_4^+ in plant leaves [23]. A rapid and sensitive procedure via salicylic acid nitration [24] was employed to determine the NO_3^- concentration in plants. An approach developed based on the Griess reaction [25] was adopted to analyze the NO_2^- concentrations in plant samples. The detailed procedure can be found in Huang's publication [26].

2.8. Monitoring the Activities of Key Enzymes in N Metabolism Pathway

The activities of nitrate reductase (NR) and nitrite reductase (NIR) in plants were assayed in vitro in accordance with Hogberg et al. [27] and Ogawa et al. [28], respectively. NR activity is expressed as the amount of nitrite formed per gram of dry weight per hour, while NIR activity was calculated based on the reduction of NO_2^- in the assay, expressed as μmol NO_2^- reduced$\cdot h^{-1} \cdot g^{-1}$ dry weight.

Glutamine synthetase (GS) and NADH-dependent glutamate dehydrogenase (GDH) activities were spectrophotometrically estimated with slightly modified approaches proposed by Oaks et al. [29] and Kanamori et al. [30], respectively. Approximately 0.5 g of fine-ground frozen powder were extracted in a 3 mL extraction buffer (0.05 M Tris-HCl, pH 8.) consisting of 2 mM Mg^{2+}, 2 mM DTT, and 0.4 M sucrose. The crude extract was collected for the GS and GDH activity assay after centrifugation at 12,000$\times$ *g*, 4 °C for 20 min.

For the GS activity assay, a 0.7 mL crude enzyme extract was subjected to a 30-min incubation at 37 °C in a 2.3 mL assay solution (0.1 M Tris-HCl, pH7.4) containing 80 mM Mg^{2+}, 20 mM sodium glutamate and cysteine, 2 mM EGTA, 80 mM hydroxylamine hydrochloride, and 40 mM ATP (prepared daily). The reaction would then be terminated by adding 1 mL ferric chloride reagent (0.2 M TCA, 0.37 M $FeCl_3$ and 0.6 M HCl). Afterwards, the mixture was vigorously shaken and centrifuged (5000 g, Rt, 10 min), and the supernatant was spectrophotometrically measured at 540 nm. The GS activity was defined as the formation of 1 nmol γ-glutamyl hydroxamate per mg protein per minute.

The NADH-GDH activity was determined with a reaction mixture consisting of 0.1 mL of 30 mM $CaCl_2$, 0.1 mL of 6 mM NADH (prepared daily, stored over ice when used), and 0.3 mL of distilled water, adjusted to a final volume of 3 mL with Tris-HCl (15.4 mM, pH8.0) containing 23.1 mM α-Ketoglutarate and 231 mM NH_4Cl. The reaction was triggered by the addition of 0.1 mL crude enzyme extract. The change of absorbance after 3 min in a 30 °C water bath was spectrophotometrically measured at 340 nm. The GDH activity was expressed as the consumption of nmol NADH per mg protein per minute.

2.9. Statistical Analysis and Data Processing

SAS statistical software (SAS 8.2 Inst., Cary, NC, USA) was used to perform the statistical analyses. Data from analysis of one-way ANOVA followed by the Duncan's multiple range test were considered significant at a probability (*p*) equal to 0.05. The acquired data were plotted using GraphPad Prism 8.0 software. All of the measurements were conducted with no less than three biological replicates.

3. Results

3.1. Plant Growth as Affected by the NH_4^+:NO_3^- Ratio

The growth parameters were significantly affected by different NH_4^+:NO_3^- ratios after 4 weeks of cultivation, regardless of the species. As is apparent in Figure 1A,B, large differences were observed among the plants in response to the different treatments. Plants treated with 50:50 NH_4^+:NO_3^- always showed the most optimal growth, regardless of the species. The fresh weight of cabbage treated with 50:50 NH_4^+:NO_3^- was 12.8% and 1008.49% higher, respectively, compared with that treated with 0:100 and 100:0 NH_4^+:NO_3^-. Similarly, the fresh weight of lettuce treated with 50:50 NH_4^+:NO_3^- was 89.22% and 197.66% higher than that treated with 0:100 and 100:0 NH_4^+:NO_3^-, respectively (Figure 1D).

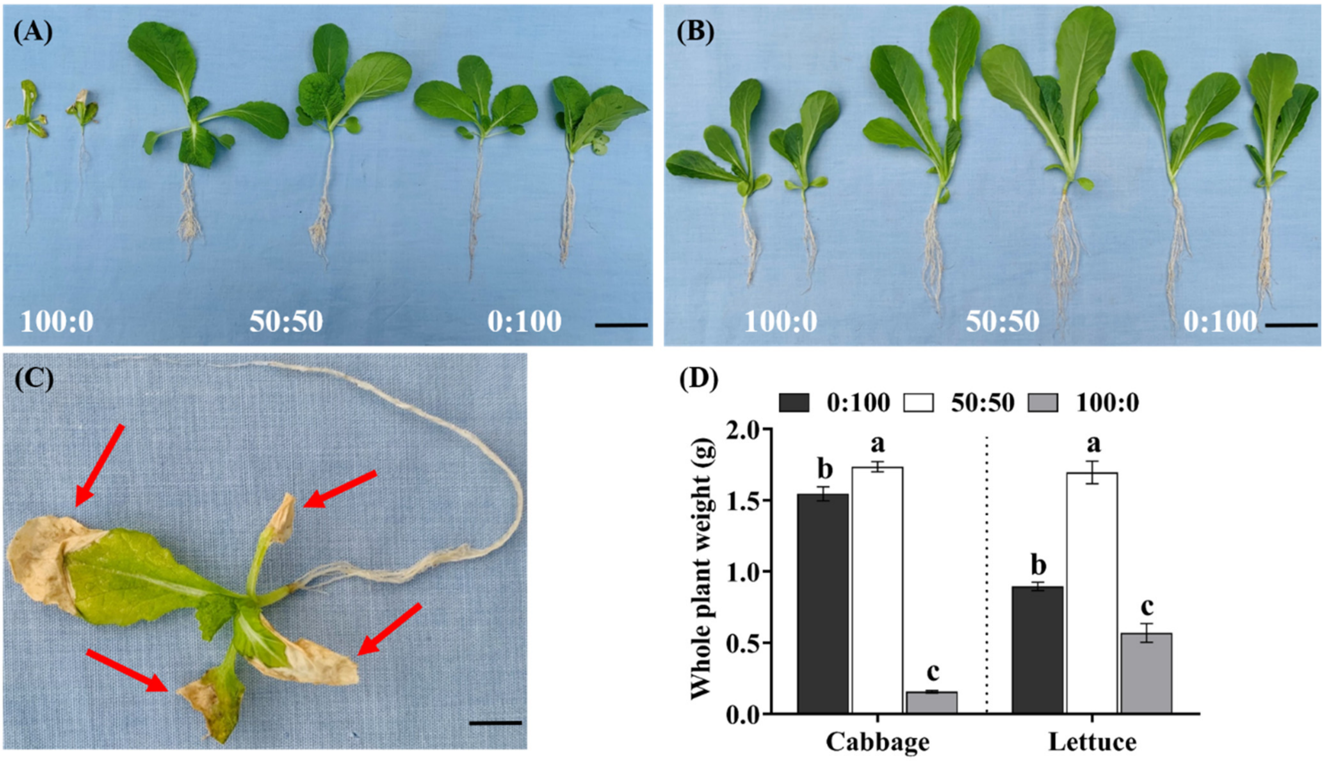

Figure 1. Plant growth of (**A**) cabbage and (**B**) lettuce as affected by different NH_4^+: NO_3^- ratios after weeks of treatments; 100:0, 50:50, 0:100 NH_4^+:NO_3^- are presented from left to right in the picture by two plant replicates with similar growth. (**C**) Enlarged image of ammonium toxicity symptoms developed in cabbage treated with 100:0 NH_4^+:NO_3^-. (**D**) Whole plant fresh weight (g) as affected by different NH_4^+: NO_3^- ratios in cabbage and lettuce; Values are expressed as means ± SE of n = 6 independent biological replicates. Error bars represent standard deviations of the means. Significant differences among treatments were indicated by different lowercase letters according to the one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

This enhancement in growth in response to the 50:50 NH_4^+:NO_3^- treatment was further evidenced by the growth data as listed in Table 2: the shoot length, root length, leaf length, and width. Among these four traits, the shoot length was the most prominently affected, where that of cabbage treated with 50:50 NH_4^+:NO_3^- was 28.53% and 143.25% greater than that of cabbage treated with 0:100 and 100:0 NH_4^+:NO_3^-, respectively (Table 2 'Cabbage part'). In parallel, lettuce treated with 50:50 NH_4^+:NO_3^- had an average shoot length that was 30.73% and 62.25% greater relative to that treated with 0:100 and 100:0 NH_4^+:NO_3^-, respectively (Table 2 'Lettuce part').

Table 2. Growth attributes of two vegetables treated with three NH_4^+:NO_3^- ratios.

Species	N Ratio (NH_4^+:NO_3^-)	Shoot Length (cm)	Root Length (cm)	Leaf Length (cm)	Leaf Width (cm)
Cabbage	0:100	7.6b [y]	10.0b	6.9b	3.3b
	50:50	9.7a	13.0a	8.7a	4.3a
	100:0	4.0c	8.1b	3.4c	0.9c
Lettuce	0:100	9.5b [y]	10.0b	7.0b	3.3b
	50:50	12.4a	13.0a	8.8a	4.3a
	100:0	7.6c	7.5c	6.7c	2.3b

[y] Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

Furthermore, it is notable that cabbage treated with 100:0 NH_4^+:NO_3^- developed ammonium toxicity symptoms, as characterized by chlorosis and leaf necrosis, inhibited growth, and stunted roots (Figure 1C).

3.2. Root Morphology as Affected by the NH_4^+:NO_3^- Ratio

Root morphology parameters, including the root volume and root surface area, were assessed in this study after sampling the shoots via destructive harvest. It is noteworthy that distinct differences were recorded in response to the different NH_4^+:NO_3^- ratios, irrespective of the species (Figure 2A). Apparently, plants treated with 50:50 or 0:100 NH_4^+:NO_3^- developed a larger root system, whereas high ammonium concentration (100:0 NH_4^+:NO_3^-) significantly restricted the growth of the adventitious roots and brought severe damages. As expected, values of the root volume and root surface area were in line with the scanned images. Cabbage treated with 50:50 NH_4^+:NO_3^- had a root volume that was, respectively, 59.11% and 670.78% greater than the root volume of cabbage treated with 0:100 and 100:0 NH_4^+:NO_3^-. Concomitantly, the root surface area of cabbage treated with 50:50 NH_4^+:NO_3^- was 10.05% and 207.06% higher compared to the root surface area of cabbage treated with 0:100 and 100:0 NH_4^+:NO_3^-, respectively (Figure 2B,C, 'Cabbage part'). However, lettuce failed to make such major differences among treatments against either the root volume or root surface area in response to the different NH_4^+:NO_3^- ratios (Figure 2B,C, 'Lettuce part'), probably because the roots possessed strong adaptability towards high concentrations of NH_4^+ or NO_3^-.

3.3. Effects of the NH_4^+:NO_3^- Ratio on the Photosynthetic Capacity

The photosynthetic capacity was determined in terms of the critical photosynthesis-related parameters, including the contents of leaf pigments (chlorophyll and carotenoids), Fv/Fm value, and the stomatal conductance. In Figure 3, it is clearly seen that plants supplied with a mixture of NH_4^+ and NO_3^- possessed not only higher contents of leaf pigments but also an increased Fv/Fm value together with greater stomatal conductance in both vegetables. For both vegetables, minor differences were observed in the leaf pigment contents between those treated with 50:50 NH_4^+:NO_3^- and those treated with 0:100 NH_4^+:NO_3^-, while those treated with 100:0 NH_4^+:NO_3^- had significantly lower leaf pigment contents (Figure 3A,B).

The Fv/Fm value in response to treatments of 0:100 or 100:0 NH_4^+:NO_3^- with varying degrees of decrease occurred as compared to 50:50 NH_4^+:NO_3^-. In cabbage, plants treated with 0:100 and 100:0 NH_4^+:NO_3^- had 3.85% and 18.8% lower Fv/Fm values than plants treated with 50:50 NH_4^+:NO_3^- did, respectively. In lettuce, plants treated with 0:100 and 100:0 NH_4^+:NO_3^- had 6.86% and 10.57% lower Fv/Fm values than those treated with 50:50 NH_4^+:NO_3^- (Figure 3C).

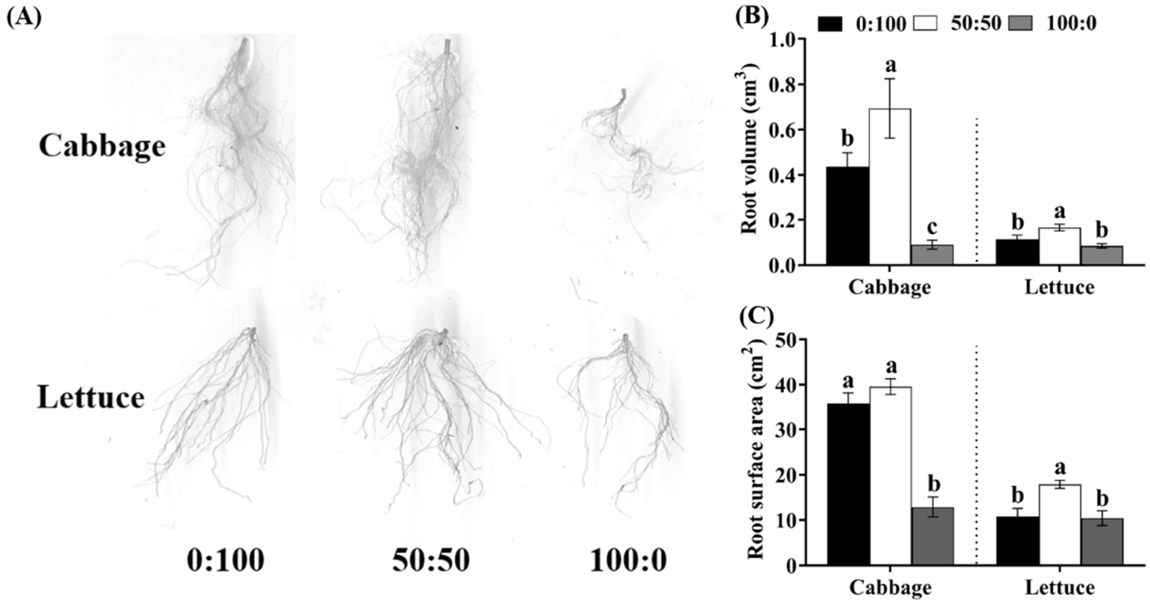

Figure 2. Root morphology of two vegetables in response to different NH_4^+: NO_3^- ratios after weeks of treatment; (**A**) Combined images of scanned root segments. (**B**) Root volume (cm^3) of two vegetables supplied with different NH_4^+: NO_3^- ratios. (**C**) Total surface area (cm^2) of two vegetables supplied with different NH_4^+: NO_3^- ratios; Values are expressed as means ± SE of n = 6 independent biological replicates. Error bars represent standard deviations of the means. Significant differences among treatments are indicated by different lowercase letters, according to the one-way ANOVA followed by the Duncan's multiple range test (p < 0.05).

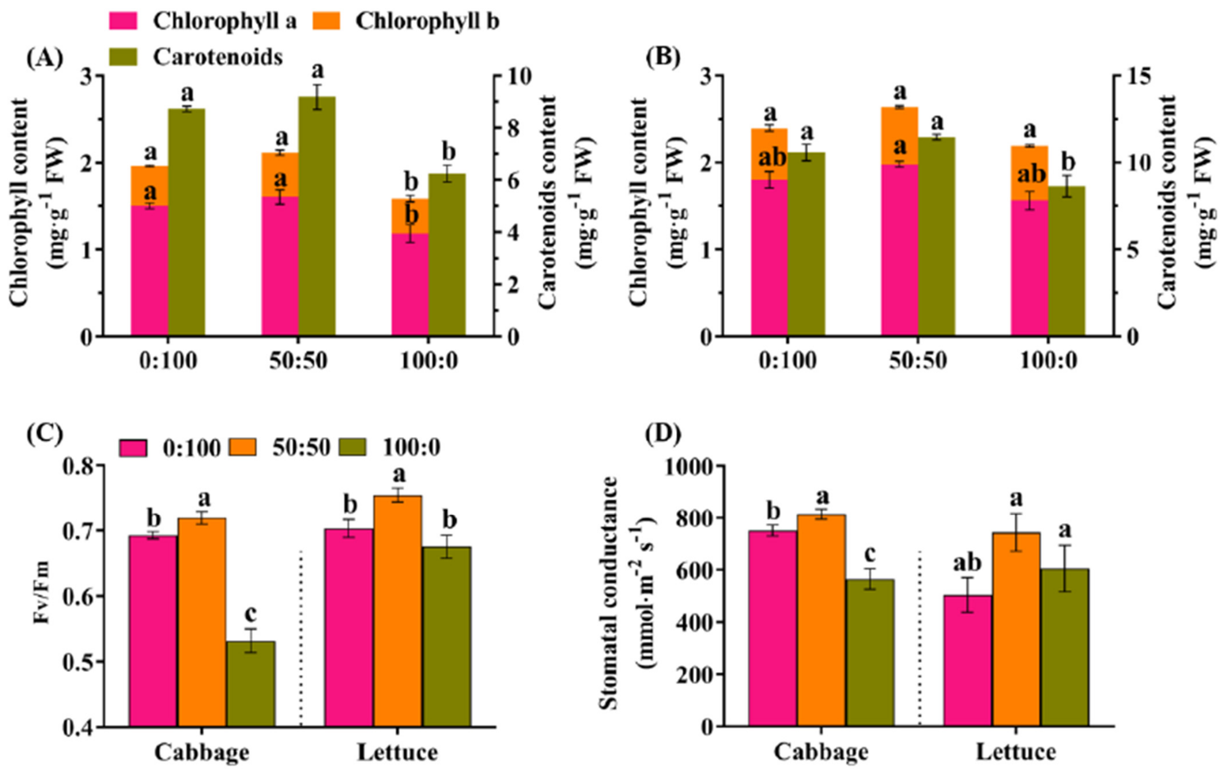

Figure 3. Photosynthetic performance of two vegetables in response to different NH_4^+:NO_3^- ratios. Pigments' contents (chlorophyll a, b and carotenoids) of (**A**) cabbage plants and (**B**) lettuce plants. (**C**) Fv/Fm value and (**D**) stomatal conductance of cabbage and lettuce. All data are expressed as means ± SE of n = 6 independent biological replicates. Error bars represent standard deviations of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Duncan's multiple range test (p < 0.05).

The stomatal conductance is regarded to be associated with the net photosynthesis and was accordingly analyzed in this study for the two vegetables in response to different NH_4^+:NO_3^- ratios. An Increase of the NH_4^+ concentration from 0% to 50% progressively reinforced the stomatal conductance, which was in accordance with the changes of other photosynthetic parameters mentioned above. Cabbage treated with 50:50 NH_4^+:NO_3^- showed a stomatal conductance of over 800 mmol·m^{-2}·s^{-1}. Those treated with 0:100 and 100:0 NH_4^+:NO_3^- had 751.55 mmol·m^{-2}·s^{-1} and 565.68 mmol·m^{-2}·s^{-1} of stomatal conductance, respectively (Figure 3D 'Cabbage part'). Curiously, lettuce treated with 100:0 NH_4^+:NO_3^- had only a slightly reduced stomatal conductance compared to plants treated with 50:50 NH_4^+:NO_3^-, which was still higher than the stomatal conductance of lettuce treated with 0:100 NH_4^+:NO_3^- (Figure 3D 'Lettuce part').

3.4. Carbohydrate Content as Affected by the NH_4^+:NO_3^- Ratio

In order to understand how the carbohydrate © status in plants is affected by EAN or ENN, we investigated the soluble sugar and starch contents in plants after harvest.

Plants obtained the greatest contents of C when grown with 50:50 NH_4^+:NO_3^-, regardless of the C form and species. As shown in Figure 4, lettuce had higher soluble sugar and starch contents relative to cabbage when comparisons were made based on the same treatments. We noticed that, in lettuce, soluble sugar and starch contents differed little between those treated with 100:0 NH_4^+:NO_3^- and those treated with 0:100 NH_4^+:NO_3^-, whereas in cabbage, the soluble sugar and starch contents markedly differed between those treated with 100:0 and 0:100 NH_4^+:NO_3^-.

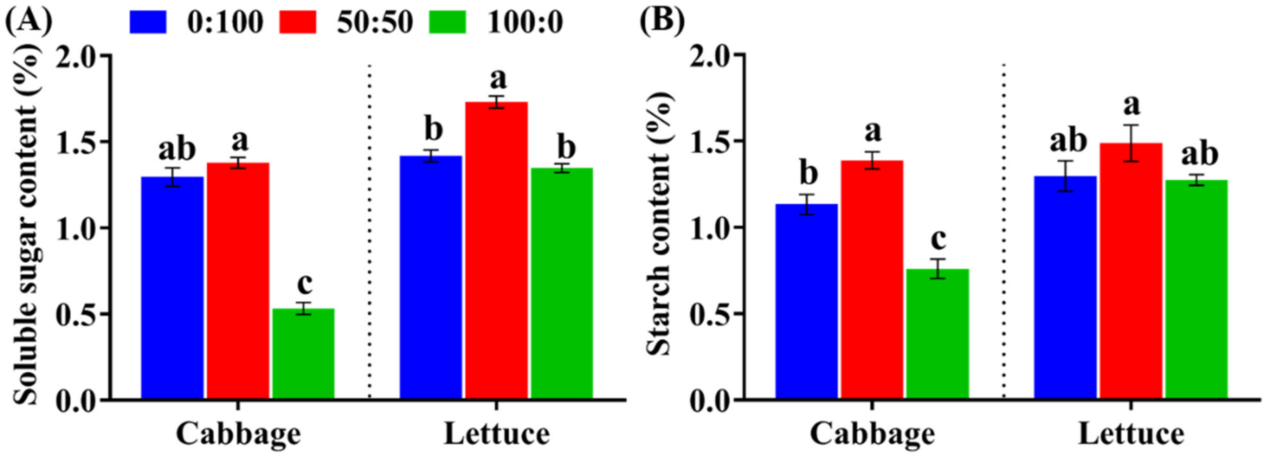

Figure 4. Carbohydrate levels in cabbage and lettuce in response to different NH_4^+:NO_3^- ratios. (**A**) Soluble sugar contents and (**B**) starch contents in two vegetables. All data are expressed as the means ± SE (n = 6 separate plants). Error bars represent standard deviations of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

Specifically, 100:0 NH_4^+:NO_3^- dramatically lowered the soluble sugar and starch contents in cabbage: Cabbage grown with 50:50 NH_4^+:NO_3^- had 1.38% soluble sugar content while those grown with 100:0 NH_4^+:NO_3^- merely had 0.53% soluble sugar content (Figure 4A 'Cabbage part'). In lettuce, the soluble sugar content was 1.35%, 1.73%, and 1.42% when grown with 100:0, 50:50, and 0:100 NH_4^+:NO_3^-, respectively (Figure 4A 'Lettuce part'). Similarly, the starch content in cabbage grown with 50:50 NH_4^+:NO_3^- exhibited a 1.83-fold increase compared with 100:0 NH_4^+:NO_3^-; on the other hand, the starch content in lettuce grown with 50:50 NH_4^+:NO_3^- was only 16.7% higher than that in lettuce grown with 100:0 NH_4^+:NO_3^- (Figure 4B).

3.5. Analysis of the NO_3^-, NO_2^-, NH_4^{+}, and Total Souble Protein Contents

The contents of various N forms, NO_3^-, NO_2^-, and NH_4^+, and total soluble protein in cabbage and lettuce were quantified and are given in Figure 5. Apart from the NO_2^- content in cabbage, it is noteworthy that other N contents were significantly influenced by the EAN or ENN. On average, on the basis of constant N input, the summed N contents were similar, regardless of the treatments and species.

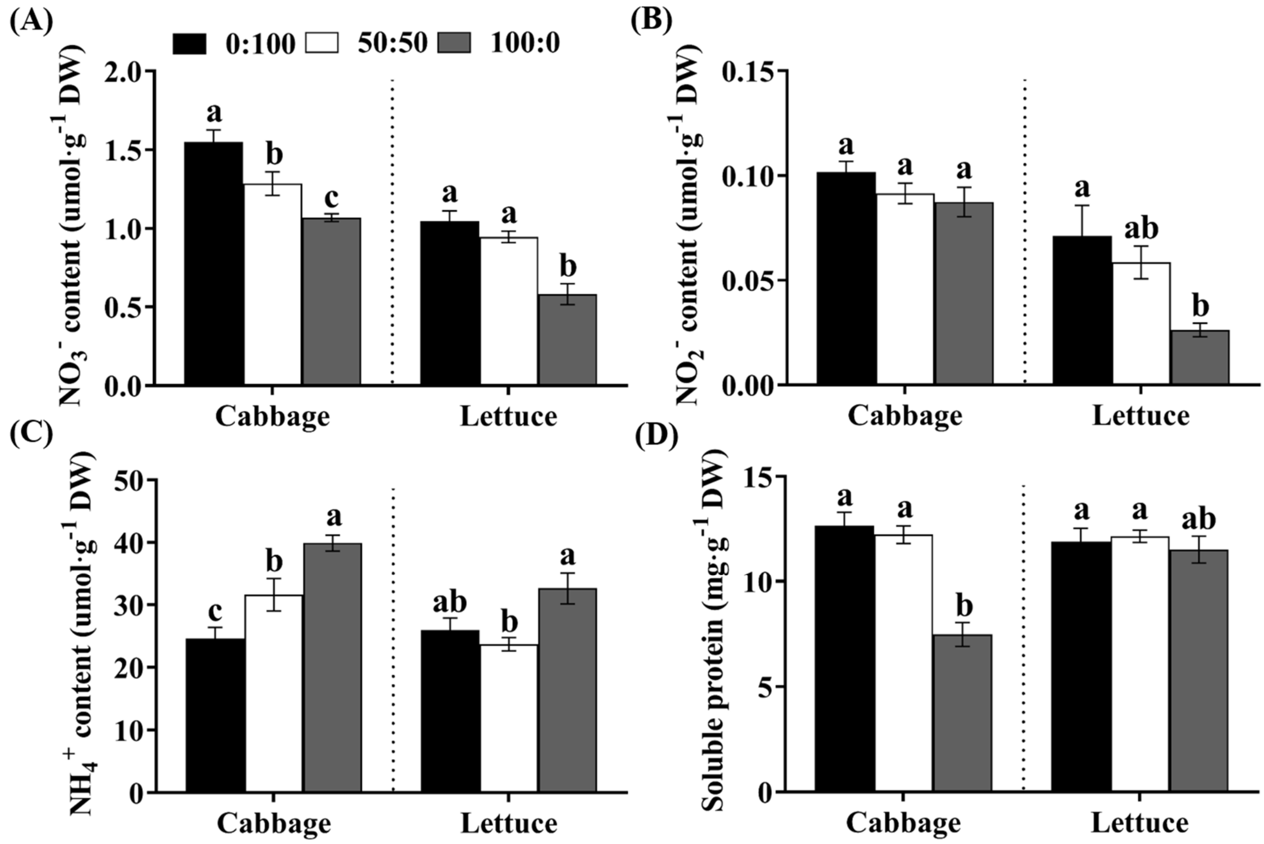

Figure 5. N contents as affected by different NH_4^+: NO_3^- ratios in cabbage and lettuce in the form of (**A**) nitrate (NO_3^-), (**B**) nitrite (NO_2^-), (**C**) ammonium (NH_4^+), and (**D**) soluble protein. Values are expressed as the means ± SE (n = 6 independent replicates). Error bars represent standard deviations of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

A substantially positive correlation was observed between a high NH_4^+ or NO_3^- supply and free NH_4^+ or NO_3^- content in plants, regardless of the species (Figure 5A,C). For cabbage, no significant or slight differences were observed in response to the different NH_4^+:NO_3^- ratios in the intermediate product NO_2^- content (Figure 5B, 'Cabbage part'); interestingly, lettuce grown at reducing NO_3^- concentrations displayed a parallel but significant decrease in the NO_2^- content (Figure 5B, 'Lettuce part').

Proteins are the final product in the N assimilation pathways and originate from the incorporation of NH_4^+. The total protein content was significantly affected not only by the different treatments of the A–N ratio, as described above, but also according to the species. As presented in Figure 5D, in general, the soluble protein content was positively correlated with the NO_3^- input. Furthermore, a dramatic reduction in the soluble protein content was observed in cabbage grown with 100:0 NH_4^+:NO_3^-, whereas that in lettuce treated with NH_4^+:NO_3^- was only slightly affected.

3.6. Activities of Key Enzymes in the N Assimilation Pathway

To better understand the changes of key enzymes in the N assimilation pathway, activities of those enzymes were examined when the plants were supplied with different

NH_4^+:NO_3^- ratios. Key enzymes in the N assimilation pathways were clearly and widely affected by the different NH_4^+:NO_3^- for both species (Figure 6).

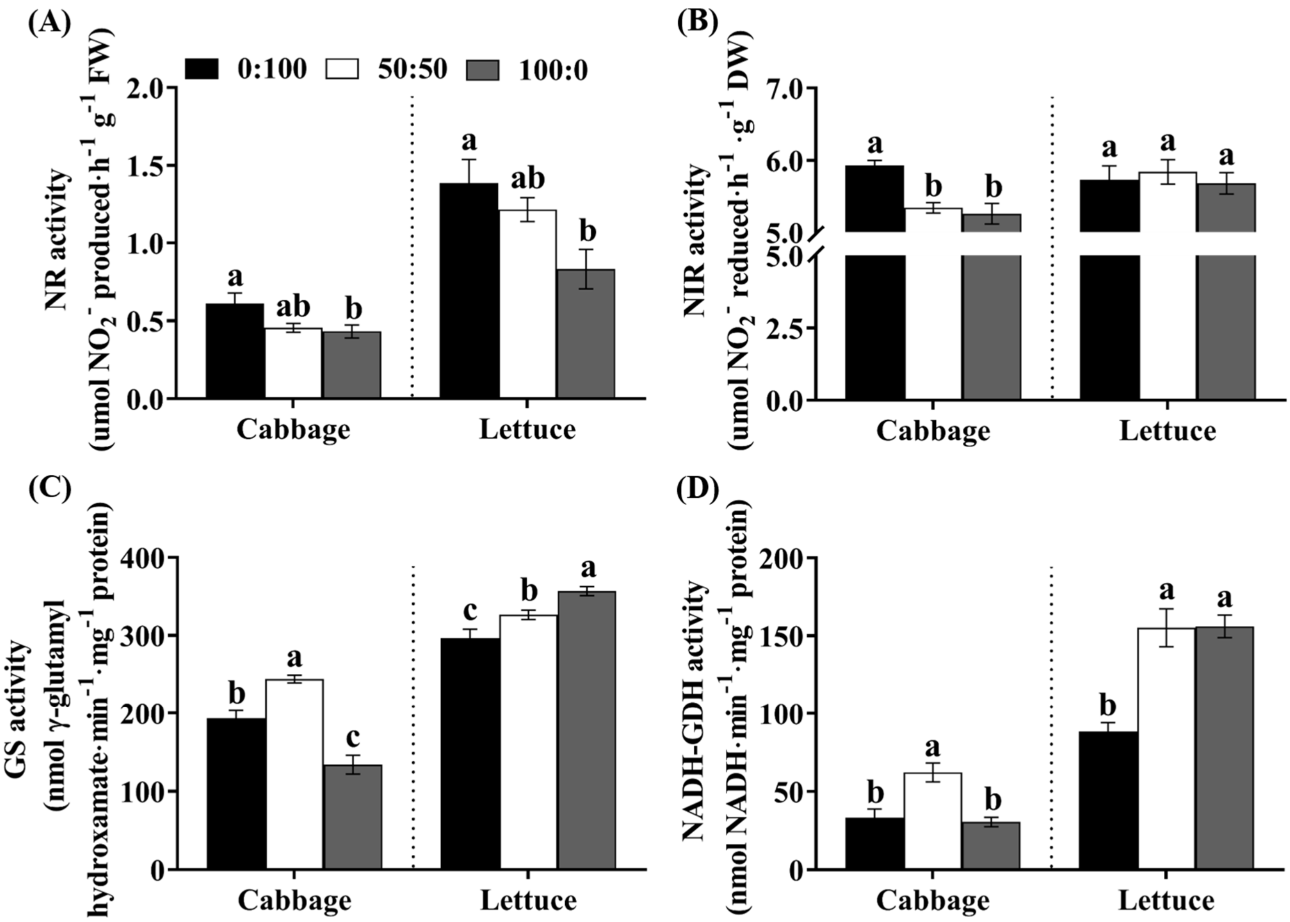

Figure 6. Activities of key enzymes in the N metabolism pathway as affected by different NH_4^+: NO_3^- ratios in cabbage and lettuce. (**A**) Nitrate reductase activity (NR); (**B**) Nitrite reductase activity (NIR); (**C**) Glutamine synthetase activity (GS); and (**D**) Glutamate dehydrogenase activity (GDH). Values are expressed as the means ± SE (n = 6 independent replicates). Error bars represent standard deviations of the means. Different lowercase letters indicate significant differences according to the one-way ANOVA followed by the Duncan's multiple range test ($p < 0.05$).

A gradual diminishment was observed in the NR activity with an increased concentration of NH_4^+, regardless of the species. Concomitantly, a parallel reduction of NO_3^- concentration was also observed, as presented above (Figures 5A and 6A). Similar tendencies are shown in Figure 6B, where the NIR activity was found to show a consistent tendency with the NR activity and NO_2^- contents in cabbage when the NH_4^+ concentration increased. However, interestingly, only minor changes were observed in the NIR activity in lettuce with regard to the different NH_4^+:NO_3^- ratios (Figure 6B 'Lettuce part').

More importantly, large differences were monitored in the activities of NH_4^+ assimilation enzymes (GS and GDH) in response to the different NH_4^+:NO_3^- ratios for both vegetables (Figure 6C,D). Additionally, the GS and GDH activity trends differed between the two vegetables. For instance, when the NH_4^+:NO_3^- ratio changed from 50:50 to 100:0, the GS and GDH activities in cabbage decreased sharply, by 45.06% and 50.94%, respectively, whereas in lettuce they both increased slightly, by 9.36% and 0.56%, respectively. Generally, the GS and GDH activities were higher in lettuce than they were in cabbage, regardless of the NH_4^+:NO_3^- ratio considered.

4. Discussion

In our trials, several morphological and physiological parameters were remarkably influenced by either high NH_4^+ or NO_3^- concentrations. A high NH_4^+ or NO_3^- significantly restricted the growth performance of cabbage and lettuce, as displayed by a reduced plant weight, decreased shoot length, and declined root-related traits including the root volume and root surface area (Figures 1 and 2; Table 2). Both cabbage and lettuce benefited more from being grown with the 50:50 NH_4^+:NO_3^- solution. However, this ratio is not a universal point that serves all the species. Even in this study, certain plants grown with 0:100 NH_4^+:NO_3^- grew better than those with 50:50 NH_4^+:NO_3^-, displaying greater soluble protein contents (Figure 5D). The data presented in this study were in agreement with a great deal of previous research [31–33]. More importantly, Korean cabbage 'Ssamchu' appeared to be an extremely NH_4^+-sensitive species, whereas lettuce was not, which was characterized by certain key responses, such as chlorosis, leaf necrosis, and stunted root development (Figure 1C).

Many studies have demonstrated that a mixture of nitrate and ammonium deliver outstanding benefits for plant growth and development; however, most of them focused on given plants and which NH_4^+:NO_3^- ratio was optimal [34–37]. Additionally, the variations of the key enzymes and intermediate chemicals involved in the N metabolism pathway in different NH_4^+-sensitive plants have yet to be thoroughly studied. Therefore, we designed, concentrated, and performed this work to investigate how EAN or ENN influences the plant growth and development, as well as the N metabolism, by using two plants with different NH_4^+ sensitivities.

Chlorophyll content in leaves has been widely adopted as a key indicator for the determination of photosynthesis behavior [38]. A low Fv/Fm value was believed to be related to dynamic photoinhibition, which may be caused by a high NH_4^+ stress [39]. Additionally, a higher stomatal conductance was found to be stimulated by the CO_2 absorbing rate, which promoted the photosynthetic capacity, as supported by certain plants [40–42]. Furthermore, photosynthesis has been well suggested to be associated with the N form, where it was often reported that high NH_4^+ decreased the photosynthetic capacity [43,44]. This was confirmed again in our study, where we observed reduced pigment contents and lower Fv/Fm and stomatal conductance rate in plants treated with 100:0 NH_4^+:NO_3^-, especially in cabbage (Figure 3).

In addition, plants exposed to high NH_4^+ levels were susceptible to a lower stock of carbohydrate contents, probably due to the fact that the assimilation of excessive NH_4^+ came at a cost of more carbon skeleton for the energy supply [45]. In our experiments, plants grown with 100:0 NH_4^+:NO_3^- experienced a sharp decline in the carbohydrate contents, which was more pronounced in cabbage than in lettuce (Figure 4). Lettuce was observed to be less sensitive to the NH_4^+ level, as those grown with 0:100 and 100:0 NH_4^+:NO_3^- displayed no significant differences in the carbohydrate content.

In order to figure out the differences in the N metabolism pathway between NH_4^+-sensitive species (cabbage) and tolerant species (lettuce) in response to different nitrogen sources, we not only measured the internal N concentrations (NO_3^-, NO_2^-, NH_4^+, total protein content) but also quantified the activities of key enzymes that catalyzed N reduction and assimilation (NR, NIR, GS, GDH). There were some great differences in the parameters mentioned above in the two species studied.

It is well known that the form of the external N source influences the internal N concentration in plants [46,47]. This differs among species, especially plants with distinct NH_4^+ sensitivities. An increasing supply of NH_4^+ or NO_3^- resulted in a relative increase in the content of each, regardless of the species. However, higher contents of NH_4^+ and NO_2^- was detected in cabbage grown with 50:50 and 100:0 NH_4^+:NO_3^- compared to lettuce (Figure 5C), which was in accordance with certain pioneer publications [48,49]. This indicated that NH_4^+-sensitive species are more prone to accumulate free NH_4^+ and NO_2^- in their tissues. Besides, a generally increased soluble protein content was produced in plants grown with 0:100 NH_4^+:NO_3^- (Figure 5D), which is likely due to a higher overall metabolic activity [50].

Nitrate reductase (NR) is regarded as the initial enzyme involved in the N metabolism, which catalyzes the reduction of nitrate to nitrite. Nitrite is reduced further into ammonium by nitrite reductase (NIR) [51]. A positive correlation between the NR activity and free NO_3^- content in plants was observed in this study (Figures 5A and 6A), which was in agreement with previous reports [17,32,52,53]. In parallel with NR, a similar regulatory pattern was monitored and displayed by NIR (Figure 6B). The summed higher activities regarding NR and NIR were exhibited in lettuce, which led to the boost of activities of downstream GS and GDH (Figure 6C,D). Most importantly, GS and GDH activities analyzed herein could be used to elucidate a primarily varying NH_4^+ tolerance within vegetables; for instance, lettuce was determined to be relatively tolerant to high NH_4^+. It had distinctly higher activities of GS and GDH in comparison to cabbage, which was extremely sensitive to NH_4^+ (Figure 6C,D). In response to increasing NH_4^+ concentrations, lettuce employed and reinforced GS and GDH activities for a rapid NH_4^+ assimilation, and it is thought that GS played an important role in NH_4^+ detoxification [7,17,32,54]. However, neither GS nor GDH in cabbage displayed parallelly enhanced activities as the NH_4^+ concentration increased, which may explain why ammonium toxicity symptoms developed only in cabbage. Still, GS activity in lettuce possessed a more rapid increase than GDH activity when the external NH_4^+ supply elevated from 50% to 100%, which suggested that GS was more important than GDH, at least in leaves, in determining the tolerance of vegetables exposed to a high NH_4^+ concentration. The results described in this section were also in line with results from studies performed on other plant species, such as rice [55], tomato [17,32], and pea [17,56].

5. Conclusions

Accordingly, this study provided evidence that Korean cabbage 'Ssamchu' was extremely sensitive while lettuce was relatively tolerant to high concentrations of ammonium. Concomitantly, in comparison to sole NH_4^+ or NO_3^- supply, a combination of the two forms of N appeared to be more beneficial to both vegetables, as characterized by the best growth performance, ameliorated photosynthesis, and enriched carbohydrate (C) stock content. Additionally, a positive correlation was found between the free NO_3^- and NO_2^- contents and the NR and NIR activities. The NH_4^+-sensitive species was more prone to accumulate free NH_4^+ as the external level of NH_4^+ increased, which could be attributed to the diminishment of GS and GDH activities. These results suggested that GS together with GDH appeared to underpin the ammonium tolerance of vegetables.

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