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Impact of Reducing Nitrogen Fertilizer with Biochar on Flavor Substance and Nitrogen Balance in Different Swollen-Stem-Mustard Varieties

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Abstract: Excessive application of nitrogen fertilizer in the swollen-stem mustard cultivation leads to a series of environmental and quality issues. It was reported that reducing nitrogen fertilizer with biochar could increase crop yield and reduce environmental risks. However, the effect of nitrogen reduction combined with biochar application on the flavor substances was rarely reported. Thus, two genetic stem mustard varieties (Yx: Yong'an xiaoye and Fz: Fuza No. 2), and four N treatments (control: 0 N kg/ha with biochar; N150: 150 N kg/ha with biochar, N300: 300 N kg/ha with biochar, and N450: 450 N kg/ha) were chosen to study the effects of nitrogen reduction combined with biochar on the flavor substance content of mustard stem, and N balance. The results showed that the residual soil inorganic N in N300 was lower by 37% than that in N450 (156.5 kg/ha) in Fz mustard soil, and lower by 33% in N150 than in N450 (163.1 kg/ha) in Yx mustard. The highest biomass of stem mustard tumors of Fz (35.4×10^3 kg/ha) and Yx (35.7×10^3 kg/ha) was in N300. The content of umami amino acids, sweet amino acids, and bitter amino acids of Yx and Fz stem was the highest in N450, and N300, respectively. After comprehensive consideration, the Fz was recommended to be planted in the Three Gorges Reservoir Area with N300.

Keywords: swollen-stem mustard; nitrogen reduction; biochar; amino acids; nitrate

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1. Introduction

Swollen-stem mustard (*Brassica juncea* var. *tumida* Tsen et Lee) is an economically and nutritionally important vegetable crop of the Cruciferae family in China. The stem tumor of swollen-stem mustard can not only be eaten fresh but is also the main raw material for producing *Fuling* pickled mustard [1]. Swollen-stem mustard was widely cultivated in the Chongqing, Sichuan, Zhejiang, and Hubei provinces [2]. The cultivation of tuber mustard in Fuling District, Chongqing reached 50,000 ha, with an annual output of 1.6 million tons in 2020 accounting for 50% of the national output [3]. *Fuling* pickled mustard is a flavored food, famous for its "freshness, fragrance, and crispness". The flavor of *Fuling* pickled mustard is affected by seasonings added during the pickling process, and more importantly, by the quality of the swollen-stem mustard. Its umami flavor of pickled mustard is mainly due to its rich umami amino acids [4].

Vegetable production primarily relies on intensive horticultural systems. However, the shallow root system and limited nutrient absorption capacity of vegetables necessitate substantial inorganic fertilizer use, particularly nitrogen fertilizers [5]. Nitrogen plays a pivotal role in vegetable quality and yield formation [6–9]. Unfortunately, excessive chemical nitrogen application can compromise vegetable quality and lead to soil and environmental issues, including acidification and degradation of biological quality [10–13]. In *Fuling*, China, mustard crops receive up to 600 kg N/ha [2]. Excessive fertilization accompanied by water and soil erosion in swollen-stem mustard panting area has led to serious agricultural non-point source pollution and water eutrophication in the Three Gorges Reservoir area [14]. Research indicates that nitrogen reduction fertilization not only reduces soil nitrogen content and losses but also enhances crop nitrogen utilization efficiency, thereby increasing crop yield and quality [15,16]. Consequently, reducing nitrogen fertilizer input has become a point of consensus among scientists and even governments.

Excessive chemical nitrogen fertilization could reduce soil organic matter stocks because it enhances soil organic matter (SOM) mineralization [17]. However, substituting part of the chemical fertilizers with organic fertilizers can improve soil microenvironments. Organic fertilizers release their organic compounds slowly into the soil, promoting crop growth without compromising yield [18–21]. Biochar produced by anaerobic pyrolysis of biomass is widely used in soil improvement and crop yield improvement [22–26]. Studies show that using biochar to reduce chemical nitrogen effectively lowers environmental risks while increasing crop yield and quality [27–29]. For instance, research by Ning et al. [30] found that combining nitrogen reduction fertilization with rice straw biochar application enhances rice yield and nitrogen use efficiency. Similarly, Tian et al. demonstrated that reducing nitrogen to 144 kg N/ha combined with biochar application (10 t/ha) improves soil quality in purple soil regions of southwestern China and restores crop productivity. Additionally, biochar substitution for part of nitrogen fertilizers can enhance crop quality. Ahmed et al. found that compared to applying 250 kg/ha of nitrogen alone, applying 175 kg/ha of nitrogen with biochar significantly increases tomato soluble sugar content, sugar-to-acid ratio, and nitrogen productivity. Similarly, Bi et al. observed that combining nitrogen fertilizer with biochar reduces nitrate content in Chinese cabbage and improves fertilizer use efficiency while minimizing nutrient losses. Additionally, Cheng et al. found that nitrogen reduction combined with biochar application modulates maize root metabolism, promoting the secretion of amino acids and organic acids such as isoleucine, malic acid, and acetic acid [31]. However, the impact of nitrogen reduction combined with biochar on the flavor profile (amino acids) of swollen-stem mustard is rarely reported. Therefore, the two main swollen-stem mustard varieties in the Three Gorges Reservoir area were chosen to explore the effects of reducing nitrogen fertilizer with biochar on the flavor amino acid of the stem mustard tumor.

2. Materials and Methods

2.1. Site Description and Experimental Design

The experiment field is located in Wangjiagou's small watershed, Chongqing, China (N:107°29′43″; W: 29°53′27″). This region belongs to the subtropical monsoon climate, with average annual temperature and rainfall 22.1 °C and 1130 mm, respectively. The tested soil is purplish soil (Regosols in FAO soil taxonomy, Entisols in USA soil taxonomy) with the basic property listed in Table S1.

The cropping system was a maize (*Zea mays* subsp) and swollen-stem mustard crop rotation. The swollen-stem mustard was transplanted on 31 October 2019, and harvested on 22 February 2020. Each plot was 12 m^2 with 50 swollen-stem mustard. There was no irrigation during the growth period of swollen-stem mustard. The experiment used a split-plot design with two varieties of swollen-stem mustard and fertilizer treatments. The two varieties of swollen-stem mustard were Yong'an xiao ye (Yx) and Fuza No. 2 (Fz). The four fertilizer treatments were listed in Table 1, including the control (no nitrogen fertilizer with biochar), N150 (150 kg/ha nitrogen fertilizer with biochar), N300 (300 kg/ha nitrogen fertilizer with biochar), and N450 (450 kg/ha nitrogen fertilizer). The nitrogen, phosphorus, and potassium fertilizers were urea, calcium phosphate, and potassium sulfate, respectively. The biochar was brought from Huafeng Agricultural Bioengineering Co., Ltd., Yancheng, China, which was anaerobic pyrolysis with sawdust at 500 °C for 4 h. The C, H, N, O, and S contents of the biochar were 95.2%, 1.5%, 0.1%, 3.1%, and 0.03%. The whole calcium phosphate and biochar, 50% of potassium sulfate, and 20% of urea were applied before plowing the 0–20 cm floor soil and transplanting the swollen-stem mustard. The remaining 50% of potassium sulfate and 70% of urea were applied at the 12-leaf stage of the swollen-stem mustard. The remaining 10% of urea was applied in the 15-leaf stage of swollen-stem mustard. Each treatment has 3 replicates.

Table 1. The application of fertilizer and biochar in each treatment *.

Treatments		Fertilizer Amounts			Biochar
		N	P_2O_5	K_2O	
		kg/ha	kg/ha	kg/ha	t/ha
Yx	Control	0	90	150	15
	N150	150	90	150	15
	N300	300	90	150	15
	N450	450	90	150	0
Fz	Control	0	90	150	15
	N150	150	90	150	15
	N300	300	90	150	15
	N450	450	90	150	0

* The control presents no nitrogen fertilizer with biochar; N150 prensents150 kg/ha nitrogen fertilizer with biochar; N300 presents 300 kg/ha nitrogen fertilizer with biochar; and N450 presents 450 kg/ha nitrogen fertilizer. The Yx and Fz present the Yong'an xiaoye, and Fuza No. 2 swollen-stem mustard, respectively.

2.2. Sampling and Determination

The 10 strains of swollen-stem mustard in each treatment were randomly collected during the harvest period (22 February 2020). The plant samples were divided into stem mustard tumors and leaves to determine the quality and yield of swollen-stem mustard. Parts of the fresh stem mustard tumors and leaves were smashed in an ice box to determine the nitrate (NO_3^-), soluble sugar, vitamin C(Vc), and 17 amino acids. The remaining parts of the stem mustard tumors and leaves were oven-dried to determine the plant water content and total N content.

The plant water content was determined with the drying method (water content = (fresh mass − dry mass)/fresh mass). The total N was determined using a semi-micro Kjeldahl after digesting with H_2SO_4 and H_2O_2 [32]. The nitrate concentration of the mustard stem was determined by ultraviolet spectrophotometry [32]. The soluble sugar of the mustard stem was determined using an anthrone method [33]. The Vc concentration of the mustard stem was determined by the 2,6-dichlorophenol method [34]. The 17 amino acids, including aspartate (Asp), glutamate (Glu), threonine (Thr), serine (Ser), glycine (Gly), alanine (Ala), valine (Val), histidine (His), lysine (Lys), arginine (Arg), methionine (Met), isoleucine (Iso), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), proline (Pro), and cysteine (Cys) were detected by S-433D Amino acid automatic analyzer (SYKAM company, Munich, Germany). Briefly, 200 mg of fresh stem mustard tumor was added into the hydrolysis tube with 4 mL 6 mol/L of HCl. The hydrolysis tube was sealed after 15 min of nitrogen blowing. The sealed hydrolysis tube was hydrolyzed in an oven at 110 °C for 22–24 h. The sample was diluted to 100 mL after the tube cooled down. A total of 2 mL of the sample was accurately taken from the 100 mL solution and placed into a rotary evaporator. The sample was dried out at temperature 60 °C. A total of 2 mL 0.02 mol/L of HCl was added into the rotary evaporator. The sample in the rotary evaporator was determined using S-433D Amino acid automatic analyzer after filtration with 0.22 µm filter column [35].

The soil samples at 0–20 cm, 20–40 cm, and 40–60 cm were collected from the 5 points in each plot and mixed as 0–20 cm, 20–40 cm and 40–60 soil samples, respectively. Each soil sample was air-dried and passed through a 60-mesh sieve for the analyses of NH_4^+, and NO_3^-. A small portion of air-dried < 60-mesh samples was further ground and passed through a 100-mesh sieve for the analyses of total nitrogen (TN), total phosphorus (TP), soil organic matter (SOM), and pH. All air-dried samples were stored at room temperature (15° to 25 °C) in sealed polypropylene bags.

Soil pH was measured using a pH electrode (soil: water = 1:2.5). Content of SOM was determined using rapid dichromate oxidation followed by titrating with ferrous sulfate [5]. Total N was determined using a semi-micro Kjeldahl after digesting with H_2SO_4 and H_2O_2 [32].

The N content in amino acid, N content in amino acid, soil inorganic nitrogen residue, net mineralization of soil nitrogen, and apparent loss of soil nitrogen (kg/ha) were calculated with the following formula:

$$N_a(\mathrm{mg/kg}) = \frac{A \times\ M_n \times 14}{A_w \times (1-K)}$$

where N_a is the nitrogen content in amino acid. A is the amino acid concentration. M_n is the N molecule number in amino acid. A_w is the amino acid's molecular weight. K is the water coefficient of the mustard stem.

$$N_r(\mathrm{kg/ha}) = \text{soil depth}(\mathrm{cm}) \times \text{bulk density}\left(\mathrm{g/cm^3}\right) \times \text{soil inorganic } N \text{ content}(\mathrm{mg/kg})$$

where N_r is the soil inorganic nitrogen residue.

$$\text{Net } N_{min}(\mathrm{kg/ha}) = N_{pc} + N_c - N_{ic}$$

where N_{min} is the mineralization of soil nitrogen. N_{pc} is the nitrogen uptake of control-treated swollen-stem mustard (kg/ha). N_c is the soil inorganic nitrogen residue in control (kg/ha). N_{ic} is the initial inorganic nitrogen residue in control.

$$N_{loss} = \left(N_{fer} + N_{init} + Net\ N_{min}\right) - \left(N_{\ pc} + N_r\right)$$

where N_{loss} is the apparent loss of soil nitrogen (kg/ha). N_{fer} is the nitrogen application amount (kg/ha). N_{init} is the soil's initial inorganic nitrogen residue (kg/ha). N_{min} is the mineralization of soil nitrogen (kg/ha). N_{pc} is the nitrogen uptake of control-treated swollen-stem mustard (kg/ha). N_r is the soil's inorganic nitrogen residue.

2.3. Data Analysis

The effects of nitrogen reduction on biomass or yield and nitrogen concentration of mustard stem and leaves, as well as nitrate-nitrogen concentration, soluble sugar, and Vc, were analyzed with one-way ANOVA in SPSS 22.0. The figures were drawn using Origin 2018.

3. Results

3.1. Soil Nitrogen Content in Yx and Fz

In Fz mustard soil, the NH_4^+ content at the 0–20 cm, 20–40 cm, and 40–60 cm soil layers ranged from 2.60 mg/kg to 9.80 mg/kg, while the NO_3^- content fell within the range of 3.20 mg/kg to 21.00 mg/kg. As soil depth increased, NH_4^+ content decreased in each treatment. Notably, the highest NH_4^+ content occurred in the 0–20 cm layer of N450-treated soil, significantly surpassing that in the control and N150-treated soil. With the exception of N450, NO_3^- content decreased with increasing soil depth. In the 20–40 cm layer, N450 exhibited the highest NO_3^- concentration, whereas N300 or N150 had less than half the NO_3^- concentration of N450 in the 0–20 cm and 20–40 cm layers, respectively. These findings suggest that nitrogen reduction, combined with biochar application, can significantly reduce N residence in the soil after stem mustard harvest.

A similar trend was observed in Yx mustard soil. The NH_4^+ concentration in the 0–60 cm layer ranged from 3.15 mg/kg to 11.42 mg/kg, while the NO_3^- content in the same layer ranged from 3.25 mg/kg to 16.82 mg/kg. Notably, NH_4^+ and NO_3^- content in the 0–20 cm layer of Yx soil were significantly higher than those in Fz soil (Figure 1, $p < 0.05$).

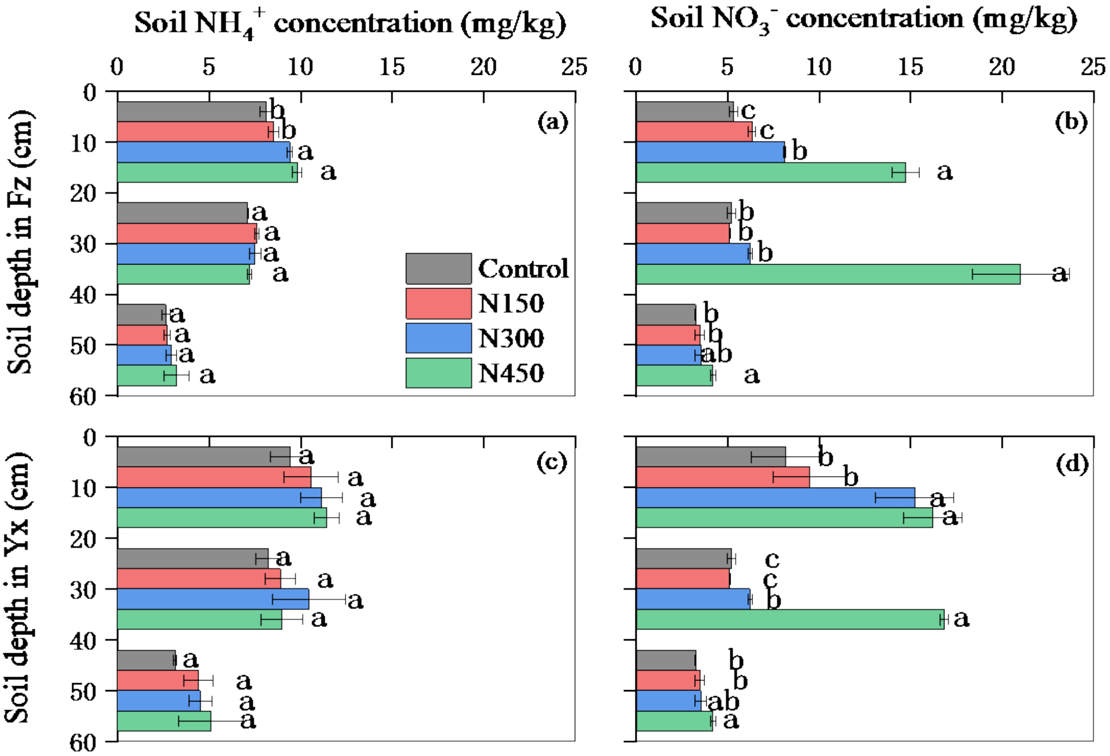

Figure 1. The soil NH_4^+ and NO_3^- concentration in different soil layers under Fz and Yx stem mustard. The soil NH_4^+ concentration in different soil layers under Fz stem mustard (**a**), the soil NO_3^- concentration in different soil layers under Fz stem mustard (**b**), the soil NH_4^+ concentration in different soil layers under Yx stem mustard (**c**), the soil NO_3^- concentration in different soil layers under Yx stem mustard (**d**). Control: no nitrogen fertilizer with biochar; N150:150 kg/ha nitrogen fertilizer with biochar; N300:300 kg/ha nitrogen fertilizer with biochar; and N450:450 kg/ha nitrogen fertilizer. Fz and Yx presented the stem mustard varieties of Fuza No. 2 and Yong'an Xiaoye, respectively. The different lower-case letters in the same swollen-stem mustard indicate significant differences between NH_4^+ treatments in 0–20 cm, 20–40 cm, or 40–60 cm soil layers at $p < 0.05$.

3.2. Biomass of Stem Mustard and Nitrogen Content in Yx and Fz

The stem yield and leaves biomass of Fz and Yx mustard in N150, N300, and N450 were significantly higher than those in the control (Figure 2a,c). This suggested that biochar alone, without N fertilizer, cannot adequately support stem mustard yield formation. Notably, both Fz and Yx mustard tumor yields were highest in the N300 treatment. This suggested that appropriately reducing the applied N fertilizer, combined with biochar application, significantly enhanced mustard tumor yield. Interestingly, the yield of Fz stem mustard tumors in the control, N150, or N300 treatments did not significantly differ from that in Yx stem mustard tumors (Figure 2a). However, the yield of Fz stem mustard tumors in the N450 treatment was significantly lower than that in Yx, suggesting that Fz is more suitable for nitrogen reduction combined with biochar.

Similarly, leaf biomass was highest in N300-treated Yx stem mustard leaves and N150-treated Fz stem mustard leaves. Remarkably, the stem mustard tumor-to-leaf biomass ratio for both Fz and Yx stem mustard was also highest in the N300 treatment among the three nitrogen fertilizers. This highlighted that appropriate nitrogen reduction, combined with biochar application, could promote biomass accumulation in stem mustard tumors.

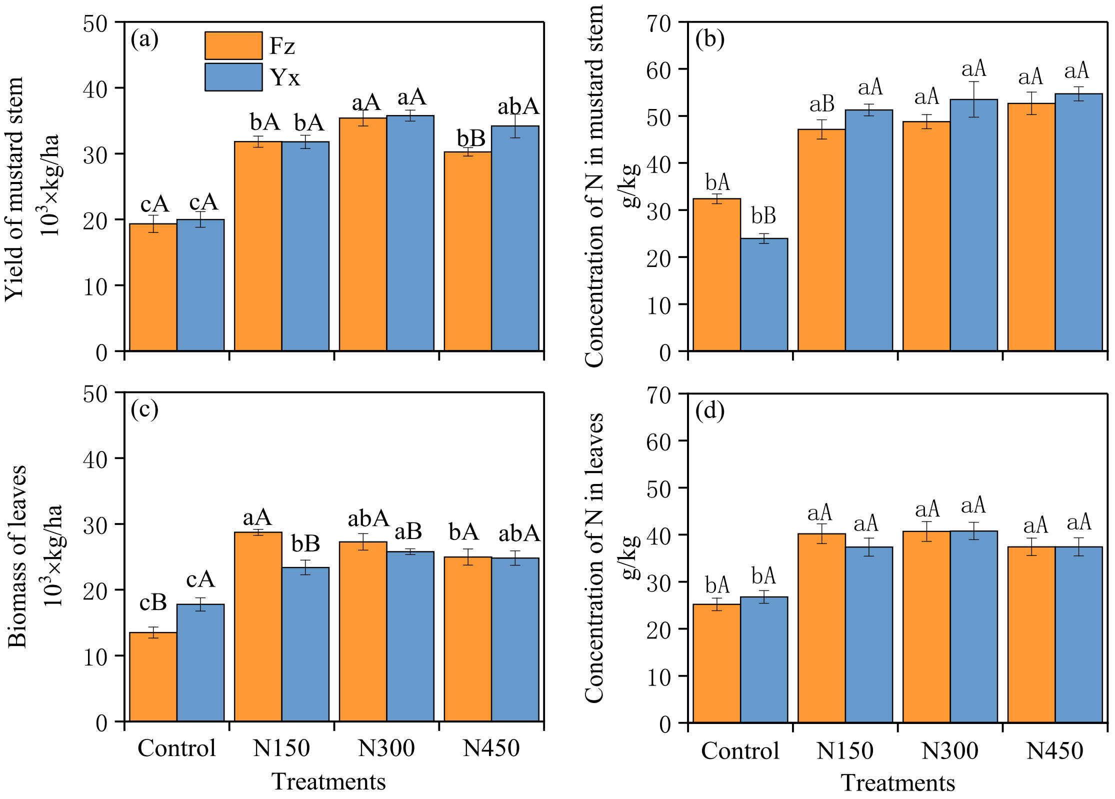

Figure 2. The yield of mustard stem (**a**), biomass of leaves (**c**), and N content of mustard stem (**b**) and leaves (**d**). The description of treatments and mustard varieties see the footnote of Figure 1. The different lower-case letters indicate a significant difference between N treatments at $p < 0.05$, and the capital letters indicate a significant difference between Fz and Yx mustard at $p < 0.05$.

Regarding nitrogen content, the stem mustard tumors in Fz and Yx fell within the range of 47.24–52.68 g/kg and 51.28–54.71 g/kg, respectively, while stem mustard leaves within 37.43–40.70 g/kg (Fz), and 37.36–40.79 g/kg (Yx), respectively (Figure 2b,d). The N content of Fz or Yx stem mustard tumors in N150 and N300 was lower than that in N450, even though the difference was not significant at $p < 0.05$. However, the N content of Fz or Yx stem mustard tumors in N150, N300, and N450 was significantly higher than that in the control ($p < 0.05$). Interestingly, the N content in the control-treated Yx tumors was lower than that in the control-treated Fz tumors. This discrepancy may be due to the higher yield of mustard stems in Yx, leading to N bio-dilution in Yx mustard stems. Furthermore, N content in Yx mustard treated with N150, N300, and N450 was notably higher than that in corresponding N fertilizer-treated Fz, suggesting Yx has a stronger N uptake ability than Fz.

3.3. Vc, Soluble Sugar, and Nitrate in Yx and Fz

The vitamin C (Vc) content in the stem mustard tumor of Yx and Fz fell within the range of 11.21–16.54 mg/kg, and 11.43–16.45 mg/kg, respectively (Table 2). These results were consistent with the other studies [36–38]. Notably, the Vc content of stem mustard tumors did not significantly differ among N150, N300, and N450 treatments in either Fz or Yx, but was significantly lower than that in the control.

A similar trend was also observed in the soluble sugar content of Yx and Fz tumors in each treatment. The soluble sugar content in the tumors of Yx and Fz was in the range of 15.73–26.00 mg/kg, and 15.94–28.27 mg/kg, respectively. The reduction effects of N combined with biochar on soluble sugar content were consistent between the two stem mustard varieties. The lowest soluble sugar content of the two varieties was in N450 (15.94 mg/kg in Fz and 15.73 mg/kg in Yx) and the highest was in the control (28.27 mg/kg in Fz, and 26 mg/kg in Yx). The sugar content in Fz was higher than that in Yx, but the difference was not significant at $p < 0.05$. Generally, the soluble sugar content would decrease when the N fertilizer application rates exceeded a certain threshold. Excessive N content depletes organic acids in the plant, leading to reduced sugar content due to increased amino acid or protein synthesis, which consumes organic acids [39].

Table 2. Effects of different variety and nitrogen dosage on Vc, nitrate, and soluble sugar content of mustard stem.

Treatments	**Vc (mg/kg Fm)**		**Nitrate (mg/kg Fm)**		**Soluble Sugar (mg/kg Fm)**	
	Fz	**Yx**	**Fz**	**Yx**	**Fz**	**Yx**
Control	16.45 ± 0.15 a	16.54 ± 0.14 a	820.4 ± 71.1 c	767.5 ± 65.12 c	28.27 ± 2.16 a	26 ± 1.14 a
N150	12.32 ± 0.11 b	11.7 ± 0.07 b	2041.1 ± 219.4 b	2237 ± 115.1 b	17.46 ± 0.61 b	17.19 ± 0.17 b
N300	11.4 ± 0.09 b	11.01 ± 0.08 b	2147.2 ± 334.7 b	2449.0 ± 108.24 a	16.81 ± 0.19 c	16.17 ± 0.68 c
N450	11.43 ± 0.21 b	11.21 ± 0.12 b	2842.4 ± 114.2 a	2520.6 ± 184.21 a	15.94 ± 0.21 c	15.73 ± 0.92 c

Fm presents fresh mass of mustard stem. Different lower-case letters in the same column indicate a significant difference among N treatments at $p < 0.05$. For the description of treatment and mustard varieties, see the footnote of Figure 1.

In contrast to vitamin C (Vc) and soluble sugar, nitrate content increased with higher applied N doses. The nitrate content of the Fz and Yx tumors was significantly higher than that of the control. Specifically, nitrate concentration was 191.5% to 228.4% higher in Fz and 148.8% to 246.5% higher in Yx compared to the control. Interestingly, when the nitrogen fertilizer of Fz mustard was reduced from N450 (2842.4 mg/kg) to N300 (2147.2 mg/kg), the nitrate content was significantly reduced ($p < 0.05$), while the nitrate content of Yx was reduced significantly until the nitrogen fertilizer was reduced to N150.

3.4. Amino Acid Content in Two Varieties of Mustard Stem

3.4.1. Amino Acid Composition and Essential Amino Acid Content

Amino acids play a crucial role as nutritional components in food. Their composition directly impacts nutritional value and is closely related to human taste perception [40]. In our study, we detected 17 amino acids, of which 7—lysine, phenylalanine, methionine, threonine, isoleucine, leucine, and valine—are essential amino acids (Figure 3a,b). Glutamic acid content was the highest, accounting for 24.56% to 38.56% in Fz and 20.66% to 38.34% in Yx among the total 17 amino acids. Aspartic acid followed closely, comprising 8.21% to 10.31% in Fz and 7.61% to 9.97% in Yx. Conversely, methionine had the lowest proportion, representing 1.40% to 1.58% in Fz and 1.25% to 2.21% in Yx (Figure 3a,b). The remaining amino acids constituted 2% to 6% of the total amino acid content. Nitrogen had varying effects on different amino acids, with glutamic acid and proline being the most impacted by the applied nitrogen dose (Figure 3). Specifically, nitrogen application increased the proportion of glutamic acid and decreased the proportion of proline compared to the control. Aspartate and methionine were most abundant when no nitrogen was applied, but their share in total amino acids declined with increasing nitrogen doses.

In summary, seven essential amino acids (as shown in Table 3) constitute 24.1% to 30.8% of the total amino acids. The total content of these essential amino acids in Fz and Yx, under N fertilizer treatments, exceeded that in the control. Among the essential amino acids, lysine had the highest content, followed by phenylalanine, threonine, valine, and leucine, while methionine content was the lowest. Interestingly, in Fz, the threonine, valine, and methionine content were significantly higher than those in Yx under control, N150, and N300 treatments, but significantly lower in N450. This suggests that nitrogen reduction combined with biochar has a more pronounced effect on essential amino acid content in Yx compared to Fz.

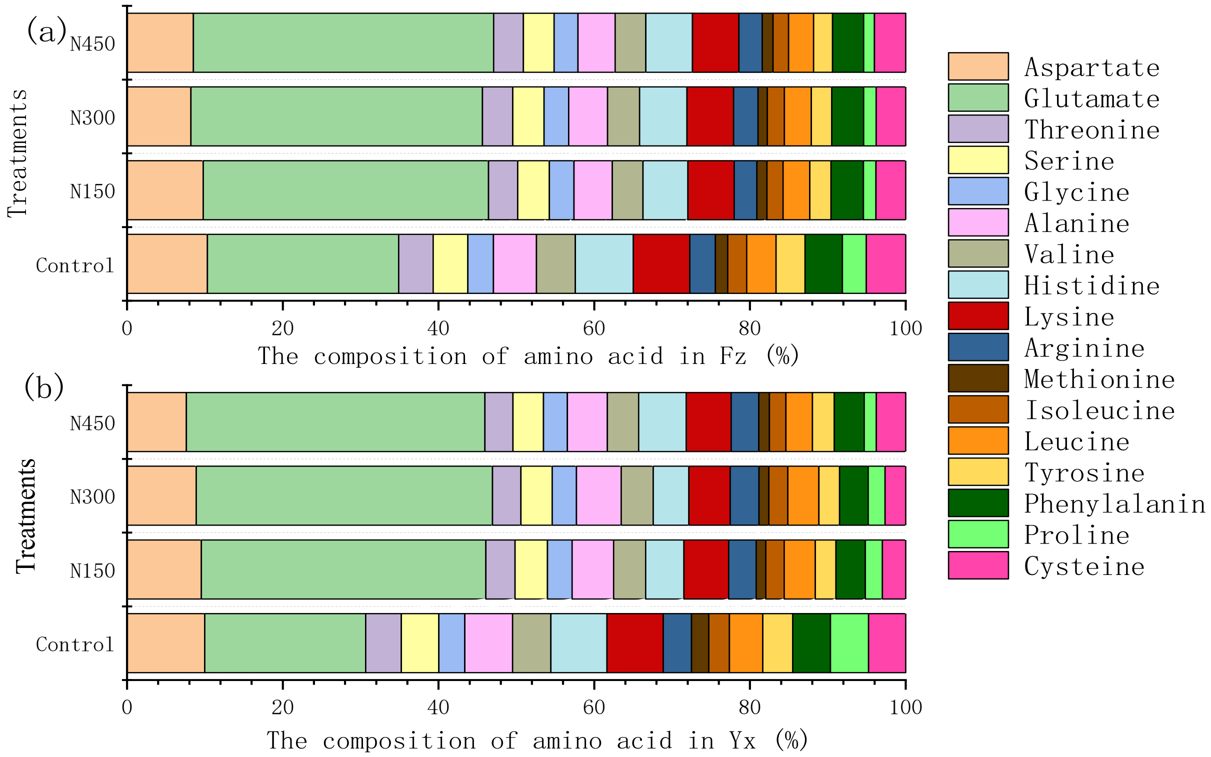

Figure 3. Effects of nitrogen reduction application on the composition of 17 amino acids in the stem of Fz (**a**) and Yx (**b**) mustard. For the description of treatment and mustard varieties, see the footnote of Figure 1.

Table 3. Effects of different varieties and nitrogen dosage on the essential amino acid content of mustard stem.

Essential Amino Acids	**Fz (mg/kg Fm)**				**Yx (mg/kg Fm)**			
	Control	**N150**	**N300**	**N450**	**Control**	**N150**	**N300**	**N450**
Threonine	0.498 ± 0.000 cA	0.516 ± 0.002 bA	0.534 ± 0.002 aA	0.502 ± 0.000 cB	0.412 ± 0.003 dB	0.469 ± 0.003 cB	0.485 ± 0.002 bB	0.542 ± 0.001 aA
Valine	0.559 ± 0.003 aA	0.542 ± 0.003 bA	0.563 ± 0.002 aA	0.516 ± 0.002 cB	0.441 ± 0.001 dB	0.515 ± 0.001 cB	0.548 ± 0.002 bB	0.599 ± 0.002 aA
Lysine	0.813 ± 0.002 aA	0.818 ± 0.001 aA	0.819 ± 0.002 aA	0.782 ± 0.002 bB	0.649 ± 0.003 dB	0.719 ± 0.002 bB	0.706 ± 0.001 cB	0.859 ± 0.001 aB
Methionine	0.177 ± 0.001 aB	0.175 ± 0.002 aA	0.164 ± 0.002 bB	0.183 ± 0.002 aB	0.198 ± 0.003 aA	0.156 ± 0.021 bA	0.172 ± 0.002 bA	0.203 ± 0.003 aA
Isoleucine	0.272 ± 0.002 cA	0.286 ± 0.002 bB	0.298 ± 0.001 aB	0.259 ± 0.002 dB	0.241 ± 0.002 dB	0.296 ± 0.003 cA	0.322 ± 0.002 aA	0.315 ± 0.003 bA
Leucine	0.422 ± 0.002 bA	0.474 ± 0.001 aB	0.476 ± 0.001 aB	0.423 ± 0.003 bB	0.386 ± 0.001 dB	0.499 ± 0.003 cA	0.533 ± 0.001 aA	0.506 ± 0.001 bA
Phenylalanine	0.536 ± 0.001 cA	0.573 ± 0.002 aA	0.56 ± 0.002 bA	0.522 ± 0.002 dB	0.435 ± 0.003 dB	0.474 ± 0.001 cB	0.489 ± 0.002 bB	0.572 ± 0.002 aA

Fm indicates fresh mass. The different lower-case letters in the same rows indicate the significant difference in each fertilizer amount at $p < 0.05$ in Fz or Yx; The different capital letters in the same rows indicate the significant difference between Fz and Yx in each fertilizer $p < 0.05$. The description of treatment and mustard varieties see the footnote of Figure 1.

3.4.2. Flavor Amino Acids

In recent years, the focus on vegetable quality has intensified, directly impacting nutritional value, processing, utilization, and human health. While vegetable production aims for higher yields, achieving excellent quality is equally essential. Pickled mustard, renowned for its umami, tender, and crisp flavor, exemplifies this balance. Beyond the seasonings added during pickling, its flavor significantly relies on amino acid content and composition. Its umami flavor, closely tied to the amino acid composition of swollen-stem mustard, particularly hinges on glutamic acid and aspartic acid (Figure 4a). These amino acids, known as umami amino acids, contribute significantly to pickled mustard's savory taste. Sweet amino acids (glycine, alanine, threonine, and serine) play a crucial role in regulating taste, surpassing the sweetness of granulated sugar (Figure 4b). Conversely, bitter amino acids (valine, histidine, lysine, and arginine) provide a contrasting flavor profile. Our data revealed that the total content of umami amino acids far exceeds that of bitter or sweet amino acids, constituting 45.6% to 47.1% of the total amino acids. This abundance is a vital source of pickled mustard's umami flavor.

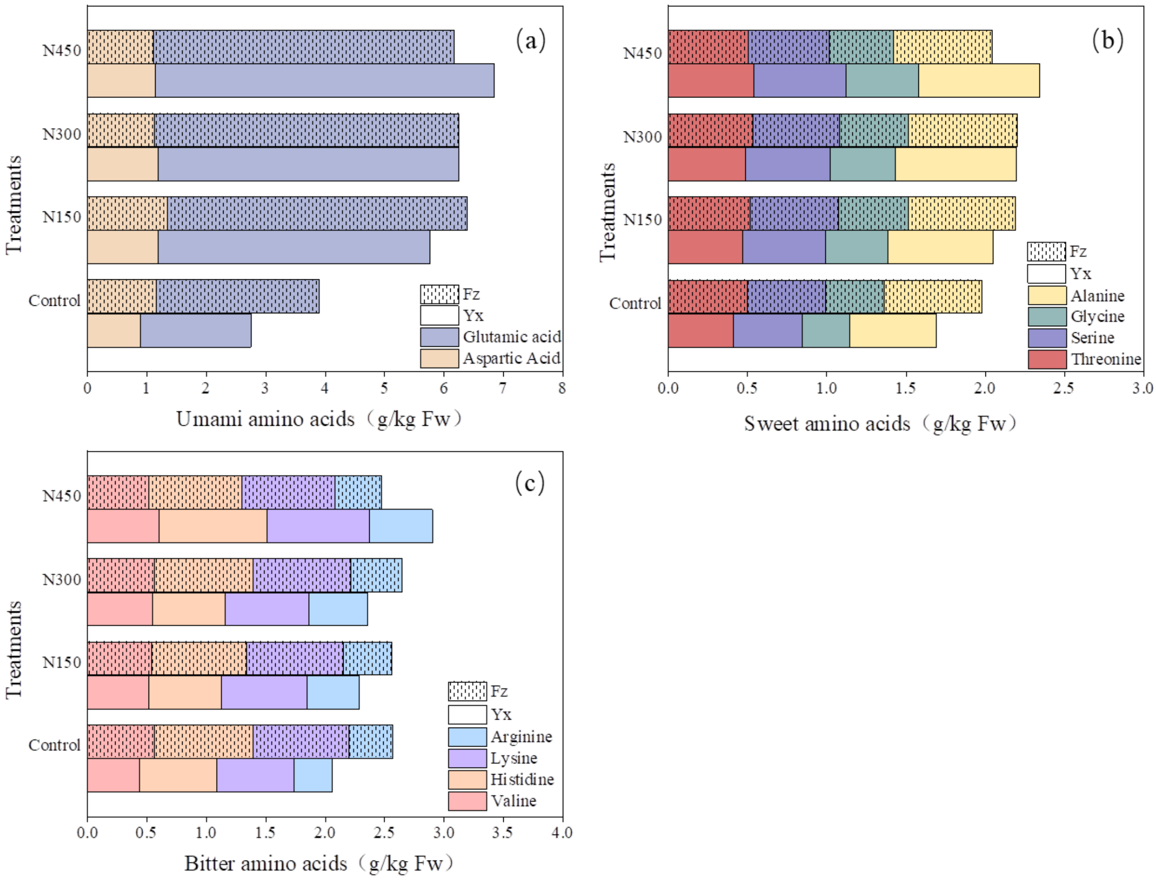

Figure 4. Effects of nitrogen reduction application on umami flavor acids (**a**), sweet amino acids (**b**), and bitter amino acids (**c**) content. For the description of treatment and mustard varieties, see the footnote of Figure 1.

The glutamic acid content was in the range of 4.57–5.71 g/kg, which was the most abundant amino acid among 10 flavor amino acids (Figure 4a). Glutamic acid was also the most crucial amino acid influenced by N fertilizer. In Yx, the glutamic acid content in N150, N300, and N450 was 132% higher than that in the control, while in Fz, it was 64% higher. Interestingly, the highest glutamic acid content in Yx occurred in N450 (5.71 g/kg), and in Fz, it was in N300 (5.13 g/kg). The highest aspartic acid contents in both Yx and Fz were observed in N150. This suggested the N application played a vital role in enhancing the umami taste of pickled mustard.

Compared to the control, N application increased the content of sweet amino acids (Figure 4b) but did not alter the ratio of these amino acids relative to the total of 17 amino acids (approximately 16%, as shown in Figure 3) in both Yx and Fz mustard tumors. Unlike sweet amino acids, the bitter amino acid content did not significantly differ among the control, N150, or N300. However, the ratios of bitter amino acids, accounting for the total of 17 amino acids in N150 and N300 (17.7–19.5%), were significantly lower than those in the control (23.0% in Fz and 23.5% in Yx).

4. Discussion

4.1. The Effects of Reducing Nitrogen Combined Biochar Application on Nitrogen Allocation in Amino Acid and Nitrate in Fz and Yx

The application of urea significantly enhanced nitrogen (N) accumulation in the Yx and Fz mustard tumors (Figure 2b). This N content in the stems was partly in the form of nitrate and partly as amino acids, with the remainder supporting mustard growth in other forms. Analyzing the proportion of amino acids and nitrate nitrogen in the mustard stems (Figure 5), we found that N in the control-treated stems was primarily used for amino acid formation (accounting for 42% in Fz and 46% in Yx) and other life processes, while N in the form of nitrate constituted less than 30%. Studies have shown that N application could significantly promote the amino acid content [41–43], which was supported by our study. The content of 17 amino acids and 7 essential amino acids in Fz or Yx tumors was higher than that in the control (Figure 3, and Table 3). Interestingly, the ratio of the seven essential amino acids to total amino acids decreased with increasing applied N rates (Table 3). This implies that N fertilizer application more significantly promotes non-essential amino acids compared to essential ones. Therefore, reducing nitrogen fertilizer application is of significant importance for improving the nutritional quality of pickled mustard. Additionally, the content and composition of flavor amino acids are also affected by the amount of nitrogen fertilizer used. The N application increased the content of umami, sweet and bitter amino acids (Figure 4a–c). However, in both Fz and Yx tumors, the ratios of umami amino acids to total of 17 amino acids in N150, and N300 were significantly higher than that in the control, while the ratios of bitter amino acid in N150 and N300 were significantly lower than those in the control (Figure 3). Studies indicate that umami and sweet amino acids can mitigate the bitterness of certain amino acids and synergistically enhance flavor and freshness [4]. Interestingly, the total content of bitter amino acids in Yx mustard stem in N450 was largely higher than that in N150 and N300. However, this phenomenon was not observed in Fz mustard stem. The umami, sweet, and bitter amino acids content of Yx stem was lower than those of Fz stem in the control, N150, or N300, but higher than those of Fz stem in N450. This suggested that reducing N combined with biochar had a greater impact on flavor amino acids in the stem of Yx mustard than in Fz.

However, the increasing proportion of N in nitrate with N dose was connected the decreasing of N in amino acids (Figure 5). This suggested the nitrogen absorbed by the stem was more formed into nitrates than into amino acids. Numerous studies have demonstrated a positive linear correlation between nitrate concentration and applied nitrogen rates in leafy and root vegetables [44–46]. Our data showed the highest nitrate content of Fz and Yx tumors were in N450 (Fz: 2842.4 mg/kg, and Yx: 2520.6 mg/kg, respectively) and the lowest in the control (Fz: 820.4 mg/kg, and Yx: 767.5 mg/kg, respectively). Interestingly, the nitrate content of Fz tumors in N150 and N300 was significantly lower than in N450, while that of Yx tumors in N150 was significantly lower than N300, and N450. Additionally, the proportion of N in amino acid in Yx (about 28%) did not change much among N150, N300, and N450. However, the proportion in Fz was increased from 25% in N450 to 34% in N150. This suggested reducing N was more conducive to the accumulation of amino acids and decrease in nitrate in Fz. Plants generally take up nitrate and ammonium ions from the soil. This is assimilated into amino acids in either roots or shoots, depending on the plant species [47–51]. Different from ammonium, plants can store high levels of nitrate, or they can translocate it from tissue to tissue without deleterious effects. The ammonium can dissipate transmembrane proton gradients, which are required for both photosynthetic and respiratory electron transport, causing toxicity to plants [52]. Thus, plants assimilate ammonium near the site of absorption or generation and rapidly store any excess in their vacuoles, thus avoiding toxic effects on membranes and the cytosol. The soil N in the control treatment was mainly in the form of NH_4^+ (Figure 1) which might be the reason for the highest proportion of N in amino acids. Increased nitrogen application led to an increase in soil nitrate (Figure 1) and nitrate content in mustard tumors. Therefore, the use of high doses of nitrogen has an adverse effect on the quality of pickled mustard. Reducing the nitrogen rate is an important way to control and improve the quality of pickled mustard.

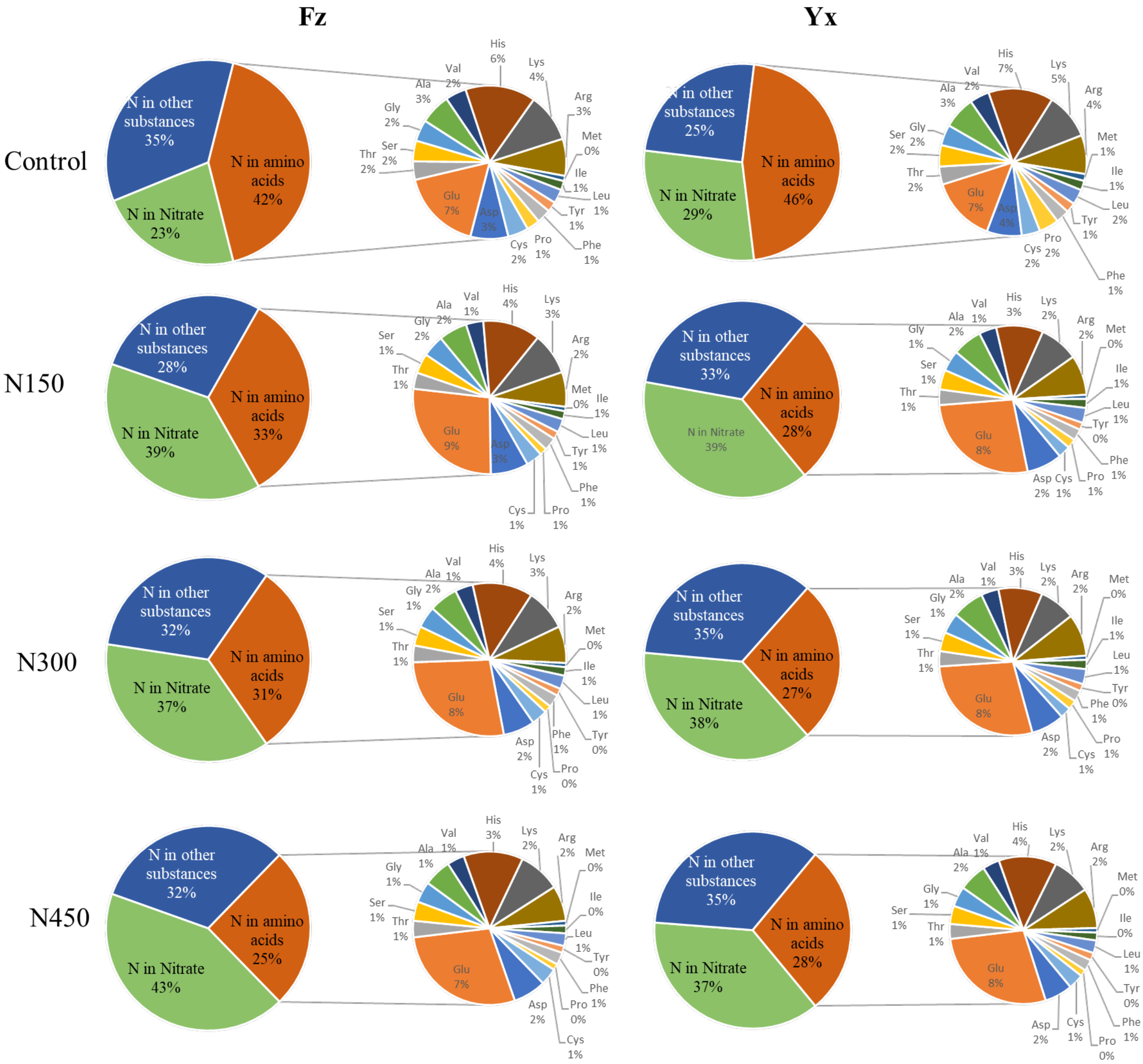

Figure 5. Effects of reducing nitrogen application on the proportion of amino acid and nitrate nitrogen in the stem of mustard. For the description of treatment and mustard varieties, see the footnote of Figure 1.

4.2. The Effects of Reducing Nitrogen Combined with Biochar Application on the Nitrogen Balance of Soil

Nitrogen in crops mainly originates from fertilization, soil residual inorganic nitrogen, and soil organic nitrogen mineralization [53]. Our data reveal that relying solely on soil organic nitrogen and organic nitrogen mineralization without nitrogen fertilization poses challenges in supporting pickled mustard yield and quality. Specifically, the control exhibited significantly lower stem mustard yield and quality compared to the fertilization treatment (Figure 2). Excessive fertilization also led to a decline in stem mustard yield and quality. While nitrogen (N) fertilization can enhance plant photosynthesis, leaf area production, and net assimilation rate [54] through the introduction of NH_4^+ and NO_3^-, excessive N does not consistently improve crop yield and may even cause reductions [55].

The decrease in grain or rhizome biomass with increasing N dose is attributed to overgrown plant leaves, which aligns with our findings [56,57]. Notably, the stem yield and amino acid content (including umami, sweet, and bitter amino acids) in Fz at N450 were lower than those at N300 and N150.

Furthermore, a nitrogen surplus significantly contributes to nitrate accumulation in soil profiles [58–60]. This was supported by the high soil NH_4^+-N and NO_3^--N residues (Figure 1). Specifically, in the 0–20 cm and 20–40 cm soil layers of Fz or Yx stem mustard, N was predominantly in the form of $NH_4 \pm N$ in the control and N150 treatments, while it shifted to NO_3–N in the N450 treatment. This suggested that N reduction combined with biochar would lead to the reduction of soil nitrate nitrogen content. The accumulation of nitrate nitrogen profiles contributes soil acidification and salinization [61,62], and negative environmental effects such as groundwater nitrate pollution [63]. Interestingly, the NH_4^+-N content in the 0–20 cm soil layer exceeded the NO_3^--N content in N300-treated Fz mustard, whereas the opposite was observed in Yx. Consequently, nitrate accumulation in Yx mustard stems was higher than that in Fz (Table 2).

The apparent equilibrium results of nitrogen in the soil-plant system (Table 4) revealed that the soil organic nitrogen mineralization in Yx (101.0 kg/ha) significantly exceeded that in Fz (72.7 kg/ha). This disparity suggests that soil organic nitrogen mineralization is notably influenced by vegetation types [22,64–66]. Our data further demonstrate that ammonium nitrogen and nitrate nitrogen contents in Yx control soil were significantly higher than those in Fz control soil (Figure 1), with crop uptake in Yx also surpassing that in Fz (Table 4). These findings imply that Yx mustard promotes soil organic nitrogen mineralization, albeit at the expense of sustained soil fertility.

Table 4. Nitrogen balance of soil layer under different nitrogen dose application.

Treatment		N Input (kg/ha)				N Output (kg/ha)		
		N Application Rate	N in Biochar	Initial Soil Inorganic N	Soil N Mineralization	Crop Uptake	Residual Soil Inorganic N	N Apparent Loss
Fz	CK	0	15	105.5	72.7	110.9 ± 12.5 ^{b}	82.3 ± 0.2 ^{b}	0
	N150	150	15	105.5	72.7	251.9 ± 5.4 ^{a}	87.8 ± 0.3 ^{b}	3.5 ± 5.7 ^{c}
	N300	300	15	105.5	72.7	277.4 ± 4.7 ^{a}	98.1 ± 0.2 ^{b}	117.7 ± 4.9 ^{b}
	N450	450		105.5	72.7	234.3 ± 8.3 ^{a}	156.5 ± 0.8 ^{a}	237.4 ± 9.1 ^{a}
Yx	CK	0	15	105.5	101	124.1 ± 9.1 ^{b}	97.4 ± 0.7 ^{a}	0
	N150	150	15	105.5	101	255.7 ± 1.2 ^{a}	109.1 ± 0.9 ^{a}	6.7 ± 2.1 ^{c}
	N300	300	15	105.5	101	279.6 ± 3.2 ^{a}	132.7 ± 1.1 ^{a}	109.2 ± 4.3 ^{b}
	N450	450		105.5	101	266.9 ± 2.4 ^{a}	163.1 ± 0.9 ^{a}	226.5 ± 3.3 ^{a}

Different lowercase letters stand for significance among all treatments at 0.05 level.

The apparent nitrogen loss in the Fz or Yx mustard field increased with the applied nitrogen rate beyond N150 (Figure S1). While residual nitrogen after mustard harvest in the surface soil layer can benefit subsequent rotation crops, excessive residue may migrate and accumulate in deeper soil layers, elevating the risk of nitrogen loss and environmental pollution [62]. Fertilization significantly impacts soil nitrogen residues; as nitrogen application levels rise, nitrogen residue and fertilizer residue rates increase in vegetable fields (Table 4), while nitrogen fertilizer utilization rates decline. This underscores the critical role of reducing urea nitrogen transformation in mitigating soil nitrogen residue and loss. Notably, compared to N450, N300 not only enhanced crop nitrogen uptake but also increased Fz mustard stem yield (Figure 2). This effect may be attributed to biochar in N300 stimulating rhizosphere soil microbial activity, thereby enhancing nitrogen uptake [67]. Overall, our results highlight that judicious nitrogen reduction combined with biochar can enhance nitrogen uptake, reduce inorganic nitrogen residue in soil, and mitigate nitrogen loss. However, it is important to acknowledge that our nitrogen balance calculations did not account for nitrogen loss forms such as nitrous oxide emissions and ammonia volatilization.

Additionally, the impact of exogenous nitrogen fertilizer supplementation (e.g., atmospheric deposition nitrogen) was not considered in this study, warranting further investigation.

5. Conclusions

In this study, appropriate nitrogen reduction combined with biochar can greatly reduce soil nitrogen residues, and simultaneously maintain the yield of mustard stem. Applied 300 kg/ha N combined with biochar resulted in the highest yield of Fz and Yx mustard stem. The content of umami amino acids, sweet amino acids, and bitter amino acids of Yx and Fz stem was the highest in N450, and N300, respectively. Yx mustard was more sensitive to nitrogen reduction combined with biochar in terms of content and was more consistent with amino acids than Fz mustard. For a more comprehensive consideration, Fz is recommended to be planted in the Three Gorges Reservoir Area with nitrogen application, with a nitrogen application rate lower than N300.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14061254/s1, Figure S1: Relationship between N fertilizer application rate and nitrogen apparent loss; Table S1: The elements content of the biochar.

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