horticulturae

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Nutrient Composition of Arugula Leafy Greens Following Application of Ascorbic Acid Foliar Sprays

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Abstract: Agronomic biofortification of vitamin C is a promising strategy to address vitamin C deficiencies in populations that lack access to diverse and nutritious diets. Different application methods can improve the vitamin C content in various crops; however, foliar application of ascorbic acid (AA) solutions has been under-explored. To determine if spray concentration, number of applications, and day of harvest would affect vitamin C in arugula leafy greens, foliar sprays consisting of 100 ppm and 200 ppm of AA and deionized (DI) water control were applied. Treatment application was initiated during the baby-leaf stage and subjected to a total of three sprays over the course of the experiment, followed by harvest at two days and four days after spraying (DAS). The harvested plants were measured for fresh and dry biomass and analyzed for vitamin C content, mineral composition, chlorophyll levels, and carotenoid content. The results of this study demonstrated a notably elevated total vitamin C concentration ($p = 0.0002$) and AA content ($p = 0.02$) in arugula leaves treated with a 200 ppm AA spray following the third application and harvested at 4 DAS. Additionally, the AA application improved the fresh and dry weight of leafy greens but did not exhibit any significant variances regarding the mineral composition of P, K, Ca, Mg, S, B, Zn, Mn, and Fe. Alternatively, AA foliar sprays reduced Cu content in leaves suggesting that AA reduced Cu accumulation in arugula leafy greens. In summary, the findings of this study establish that the foliar application of 200 ppm AA is an effective approach for increasing the vitamin C content in arugula leafy greens while improving the plant's biomass, mineral composition, and stress responses. These biofortified arugula leafy greens exhibit the potential to offer plant protection against environmental stresses and a more consistent supply of vitamin C to humans upon consumption.

Keywords: biofortification; horticulture; vitamin; biomass; baby leaf

Citation: Kathi, S.; Liu, H.; Laza, H.; Thompson, L.; Singh, S.; Li, W.; Simpson, C.R. Nutrient Composition of Arugula Leafy Greens Following Application of Ascorbic Acid Foliar Sprays. *Horticulturae* **2024**, *10*, 1126. https://doi.org/10.3390/horticulturae10111126

Academic Editors: Angelo Signore and Anoop Kumar Srivastava

Received: 11 September 2024
Revised: 11 October 2024
Accepted: 18 October 2024
Published: 23 October 2024

1. Introduction

Vitamin C, an antioxidant that protects the body against oxidative stress, is also crucial for the synthesis of collagen, a protein that helps maintain the health of connective tissues, bones, blood vessels, and skin [1]. In extreme cases, vitamin C deficiency causes scurvy, a multi-system condition that results in bleeding gums, wounds, and even death [2]. However, inadequate vitamin C intake is difficult to diagnose due to non-specific symptoms like depression, anxiety, fatigue, and apathy [3]. Vitamin C deficiencies are more common in developing countries, particularly in regions with limited access to fresh fruits and vegetables [4]. Individuals from low-income backgrounds are at a higher risk of vitamin C deficiency in both developed and developing countries [4]. Certain populations may be more susceptible to vitamin C deficiencies such as young children, older adults, and

individuals suffering from alcoholism, type I diabetes, gastro-intestinal diseases, eating disorders, and restrictive diets [5]. Comprehensive studies and surveys focusing specifically on vitamin C deficiency status are less widespread than other nutritional deficiencies. Nonetheless, it remains a concern in certain populations and regions.

Public health efforts, such as nutrition education programs, food fortification, and supplementation initiatives, can be implemented to combat micro-nutrient deficiencies [6]. However, the success and impact of these interventions varies. Another way to address micronutrient deficiencies like vitamin C is to biofortify produce [7]. Biofortification is a strategy that aims to increase the nutritional value of crops by enhancing their nutrient content [8]. Vitamin C has mostly been biofortified through breeding and genetic engineering methods [9]. Traditional breeding techniques have been used to select and cross different plant varieties that naturally possess higher vitamin C content [10]. This process involves several steps such as identifying plants with desirable traits, especially higher vitamin C levels, and breeding them over successive generations to develop new varieties with increased vitamin C content [9]. Genetic engineering approaches involve the identification of genes and enzymes involved in the vitamin C synthesis pathway and manipulating them to increase the production and accumulation of vitamin C in plants [11]. However, these methods, while important, are time and resource intensive. Alternatively, agronomic methods of biofortification are considered to be faster and less intensive [12].

Agronomic biofortification of vitamin C is a promising strategy to address vitamin C deficiencies in populations that lack access to diverse and nutritious diets. Vitamin C has been increased agronomically through fertilization with different nutrients [7,12]. Studies have shown that applying nutrients like nitrogen [12], silica [13,14], selenium [15,16], potassium [17], and so on increased vitamin C content in different horticultural crops like tomatoes, lettuce, strawberry, chard, kale, etc. Alternatively, ascorbic acid (AA) application has also been shown to increase vitamin C. For example, [18] found that leaf application of AA in soybeans increased AA content in soybean leaves; however, the increase could not be detected after 24 h. Similarly, bean leaves were also found to have increased vitamin C when AA was applied on leaves [19]. However, these previous studies increased vitamin C in plant parts that are not directly consumed (i.e., soybean leaves) and the increase was temporary, which led to desertion of AA application as a method of biofortifying vitamin C until recently.

Supplementation with AA is becoming an effective method to biofortify vitamin C in several crops. For example, foliar application of AA increased synthesis of AA in barley leaves [20]. However, the plant part consumed in barley, i.e., seeds, did not show any increase. Alternatively, adding AA to nutrient solutions in hydroponics increased vitamin C and AA content in consumable parts of arugula [21] and broccoli [22] microgreens. This indicates that AA may need to be biofortified in consumable tissues, such as leaves in leafy greens, to realize nutritional benefits. In addition to nutritional potency, increasing AA content can also provide plants with several benefits such as protection against oxidative stress caused by factors like environmental pollutants, photoprotection, and pathogens [23]. This is achieved by increased availability of AA for recycling to neutralize harmful free radicals, thereby minimizing damage to plant cells and tissues [24]. Furthermore, increased levels of AA can enhance the plant's ability to withstand and recover from adverse environmental conditions by producing stress-related proteins and enzymes [24]. Therefore, vitamin C biofortification can benefit both plants and humans. Yet, there is not much in the recent literature on how AA application affects commonly consumable mature counterparts of microgreens such as leafy greens. Herein, we investigated the effect of AA foliar spray on AA and vitamin C content in arugula leafy greens, and also map out the persistence of increased vitamin C after each spray. Arugula was chosen to be the crop of investigation to see if AA application would have similar effects to that of our previous experiments on arugula at the microgreen stage [21,25]. The current study helps evaluate the difference in plant response to AA supplementation at different growth stages, i.e., arugula leafy greens and a different application method, i.e., foliar sprays to that of previous studies. Further-

more, we evaluated how the AA foliar application affected biomass, phytochemicals, and mineral nutrient content in arugula leafy greens to provide a more encompassing view of the overall nutritional quality of biofortified greens.

2. Materials and Methods

2.1. Experiment Set-Up

Two experiments were conducted in a greenhouse at Texas Tech University Greenhouse and Horticultural Gardens (33.58407° N, 101.88691° W) with average temperatures of 25.0 °C during trial 1 and 26.3 °C during trial 2 and photosynthetically active radiation of 540 µmoL. m^{-2} s^{-1} and 570 µmoL m^{-2} s^{-1}, respectively. The commercially bought arugula seeds (*Eruca sativa* 'Astro'; Johnny's Seeds, Fairfield, ME, USA) were sown in oasis cubes at a one seed/cube ratio and watered daily to facilitate germination. Around seven to ten days after germination, the seedlings were transferred [26] to deep water culture hydroponic units with deionized (DI) water and recommended fertilizer rate for hydroponics systems. The fertilizers used were liquid fertilizers (FloraGro and FloraMicro; General Hydroponics, Santa Rosa, CA, USA) diluted in water at the rate of 13.2 mL/10 L to provide N, P, K, and complete micronutrients. The spent nutrient solution was discarded every two weeks and replaced with a new nutrient solution to minimize algae and limit nutrient and pH fluctuations; plants were given the recommended dose of fertilizer until harvest. The pH was maintained between 5.5 and 6.5 by using pH down (General Hydroponics, Santa Rosa, CA, USA). The treatments included foliar sprays with DI water (control) and two different concentrations of ascorbic acid, i.e., 100 ppm AA (L-(+)-ascorbic acid, Alfa Aesar, Haverhill, MA, USA) and 200 ppm AA. Treatments were replicated 5 times in a completely randomized design and included a total of 6 plants per replication with a total of 30 plants per treatment. The foliar spray treatments began at the baby-leaf stage (i.e., leaf size of 10–15 cm, ~30 days after sowing) and were applied to all the leaves at a spray volume of 2 mL per plant in the form of fine droplets before 10 A.M. once every 5 days. The foliar treatments were sprayed a total of three times during the course of the study (Table 1). Hence, the leafy greens harvested after the first, second, and third sprays received AA foliar applications for one, two, and three times, respectively.

Table 1. Foliar spray and harvest schedule during trials 1 and 2. * DAS—days after spraying.

Trial	Spray	Harvest 1 (2 DAS *)	Harvest 2 (4 DAS)
1	First spray (4/22/22)	4/24/22	4/26/22
	Second spray (4/27/22)	4/29/22	5/1/22
	Third spray (5/2/22)	5/4/22	5/6/22
2	First spray (6/18/22)	6/20/22	6/22/22
	Second spray (6/23/22)	6/25/22	6/27/22
	Third spray (6/28/22)	6/30/22	7/2/22

2.2. Leafy Greens Harvest

Five randomly selected plants per treatment were harvested using sterile scissors at every harvest event. Plants were harvested following each foliar application at two and four days after spraying (DAS). The foliar application and harvest dates for trial 1 and 2 are shown below in Table 1. Following harvest, the leafy greens were washed with 0.1% HCl and DI water, blot dried, packed into plastic bags, and stored at −80 °C until they were freeze dried.

2.3. Plant Measurements

Measurements of the plant shoot fresh weight (wt.) were taken after every harvest (i.e., 2 and 4 DAS). The plants were then freeze dried (HarvestRight, North Salt Lake City, UT, USA), and dry weight was collected. The leaves were then ground in the presence of liquid

nitrogen and further analyzed for various minerals such as chlorophyll a, b, carotenoids, and vitamin C content.

2.4. Plant Analysis

Chlorophyll and carotenoid content analysis was performed according to [27] with few modifications. First, 100 mg of ground leaves sample was added to a 5 mL tube to which 1 mL of 100% methanol was added. The samples were vortexed and filtered and the 200 µL extracts and methanol as a blank was added to the microplate. Using a spectrophotometer (SpectroMax iD3, San Jose, CA, USA), the absorbance was read at 665.2, 652.4, and 470 nm for chlorophyll a, chlorophyll b, and carotenoids, respectively.

Vitamin C extraction was achieved as mentioned by [28] and analysis as per [29] with modifications as seen in [21,22,25]. Briefly, 100 mg of ground leafy greens samples were treated with 6% tri-chloroacetic acid, vortexed, and set on ice. Then, the samples were centrifuged at 25,000× *g* for 15 min at 4 °C. For total AA or total vitamin C assay, 20 µL of extractant was added to microplates and treated with dithiothreitol, N-ethyl maleimide, and color reagent. Subsequently, for the AA assay, equal amounts of phosphate buffer was used instead of dithiothreitol and then N-ethyl maleimide as per the analysis by [29]. The microplate was read at 550 nm using a spectrophotometer (SpectroMax iD3, San Jose, CA, USA) and the absorbance was recorded. The AA and vitamin C content were first calculated in mg 100 g^{-1} DW using the following formula: AA or vitamin C content = $(A - b)/a \times V_{TCA} \times 100/TS$, where A is absorbance, a and b are calibration curve parameters for y = ax + b, TS is mass of test sample, and V_{TCA} is volume of TCA used as mentioned in [28].

Since arugula is consumed fresh, the final results were presented in mg/100 g FW. The values obtained in mg 100 g^{-1} DM were converted to mg 100 g^{-1} FM using the following formula: AA (mg/100 g FW) = AA (mg/100 g DW) ×% DM/100, where %DM is percent dry matter.

The content of different minerals such as nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), boron (B), zinc (Zn), manganese (Mn), iron (Fe), and copper (Cu) in arugula leafy greens were analyzed by Waters Agricultural Laboratories, Inc. (Camilla, GA, USA) using ICP (iCAP 7600, Thermo Fisher, Waltham, MA, USA).

2.5. Experimental Design and Statistical Analysis

The experimental design was a completely randomized design with a total of 30 single-plant replications. The treatment design was a complete factorial design with AA rate (three rates: 0, 100, and 200 ppm), spray frequency (three types: first, second, and third harvest), and day of harvest (two options: 2 DAS and 4 DAS) as the three experimental factors, providing 18 treatment combinations. The significance of the main effect or interaction was determined by analysis of variance (ANOVA) using JMP Pro 16.0.0 software (SAS Inc., Cary, NC, USA). The significance level was set at $p \leq 0.05$. To determine variations, Tukey's HSD was employed to separate the means. If two means shared the same letter, it indicated that there were no significant differences between the factors.

3. Results

3.1. Arugula Leafy Greens Biomass

With additional sprays, there was a significant increase in fresh weight of leafy greens ($p < 0.0001$); leafy greens harvested after the third spray had the greatest fresh weight. Day of harvest had significant impact on the fresh weight with 4 DAS harvested leafy greens having greater fresh weight than 2 DAS harvested leafy greens. However, overall fresh weight was not influenced by the main effect of AA rate ($p = 0.156$) or its interactions with spray frequency or day of harvest. However, fresh weight of leafy greens harvest 4 DAS after the third spray were affected by AA rate, with 200 ppm treatment with greatest fresh weight (Figure 1C).

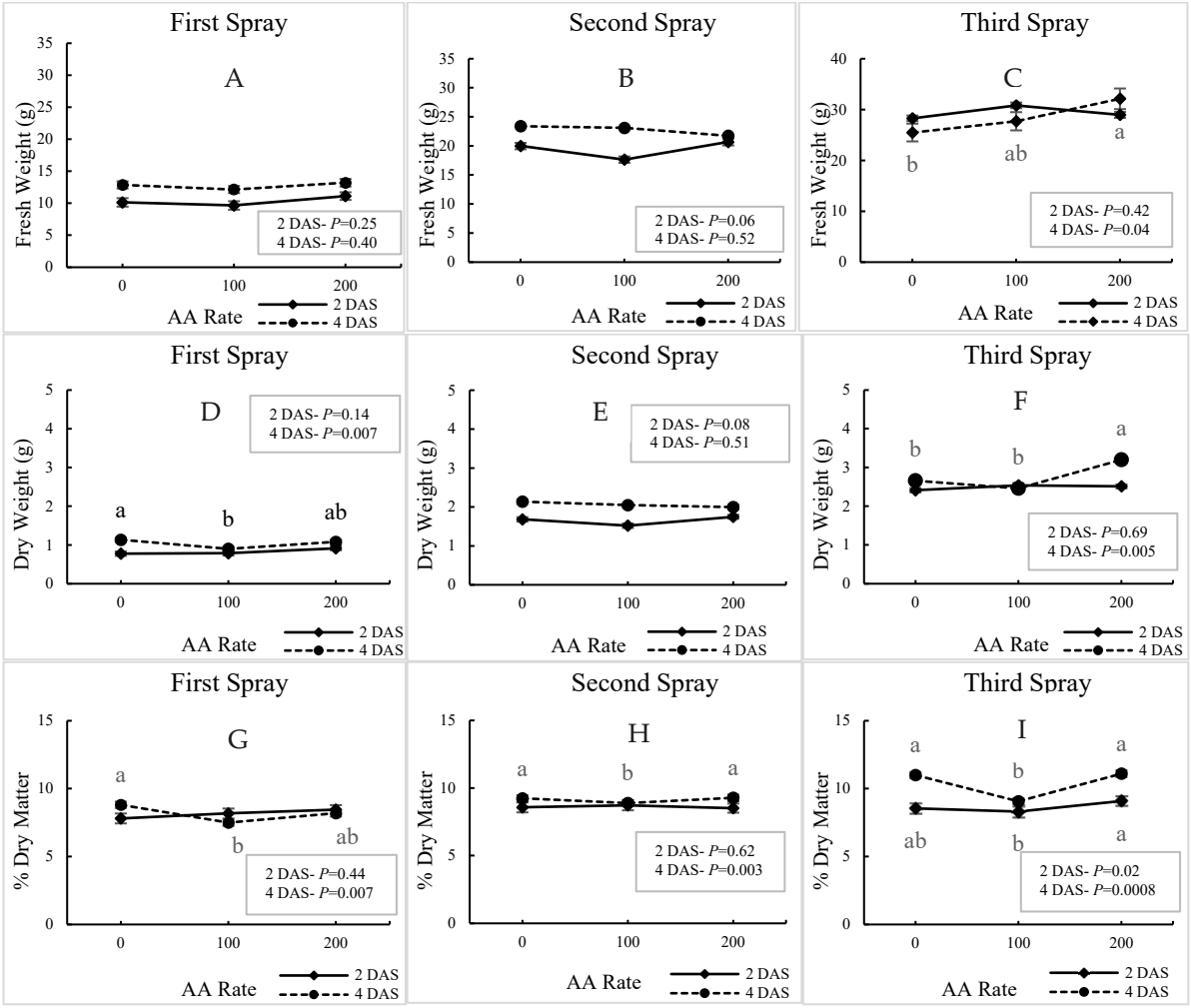

Figure 1. Impacts of the main effect of AA rate, spray frequency, and day of harvest on fresh weight (g) (**A**–**C**), dry weight (g) (**D**–**F**), and % dry matter (**G**–**I**) of leafy greens. Different lowercase letters indicate differences indicated by Tukey's HSD at $p \leq 0.05$ level.

Alternatively, dry weight of leafy greens was impacted by the main effects if AA rate ($p = 0.0034$), spray frequency ($p < 0.0001$), and day of harvest ($p < 0.0001$) but not by any of their interactions. Leafy greens that received three 200 ppm foliar spray had the greatest dry weight followed by control and 100 ppm treatments (Figure 1F). Overall, leafy greens harvested after the third spray had the greatest dry weight. Additionally, leafy greens harvested 4 DAS had greater dry weights than leafy greens harvested 2 DAS.

The % dry matter of leafy greens was significantly impacted by the main effects and their interactions. The 200 ppm and control treatments had greater % dry matter than the 100 ppm treatment ($p < 0.0001$) after the second and third sprays (Figure 1H,I). Spray frequency and day of harvest followed similar trends to that of fresh and dry weights with harvest after the third spray ($p < 0.0001$) and 4 DAS ($p < 0.0001$) having greatest % dry matter. When it comes to interactions between the main effects, leafy greens that received 200 ppm and 0 ppm of AA harvested after the third spray ($p= 0.002$), 200 ppm and 0 ppm harvested at 4 DAS ($p < 0.0001$), and the third spray and harvest at 4 DAS ($p < 0.0001$) had the great % dry matter.

3.2. Chlorophyll and Carotenoid Content

Chlorophyll a, chlorophyll b, total chlorophylls, and carotenoids were significantly influenced by spray frequency (Tables 2 and 3). Treatments that received the second and third sprays had greater chlorophyll a ($p = 0.002$), chlorophyll b ($p < 0.0001$), and total

chlorophylls ($p < 0.0001$) than treatments that received only the first spray. Conversely, treatments that received only the first application of AA foliar spray had greater carotenoids than treatments that received second and third sprays ($p < 0.0001$). However, chlorophylls and carotenoids were not impacted by AA rate and day of harvest or their interactions, except for chlorophyll b which was affected by the AA rate (Table 2) and the interaction between spray frequency and day of harvest with treatments that received AA having lowest chlorophyll b and treatments that received a second spray and harvested at 2 DAS having the greatest chlorophyll b concentration ($p = 0.007$).

Table 2. Effect of AA rate and spray frequency on chlorophylls and carotenoids content in arugula leafy greens. Different lowercase letters within a column denote significant differences between treatments.

AA Rate	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Carotenoids (µg/mL)	Total Chlorophylls (µg/mL)
0	25.818154	22.30 a	7.4080934	48.113557
100	25.005665	20.50 b	8.1440095	45.508819
200	25.725553	20.42 b	8.2260166	46.149551
p value	0.57	0.032	0.20	0.17
Spray Frequency	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Carotenoids (µg/mL)	Total chlorophylls (µg/mL)
First	23.84 b	17.32 b	9.34 a	41.16 b
Second	25.90 a	22.55 a	7.49 b	48.45 a
Third	26.81 a	23.35 a	6.95 b	50.16 a
p value	0.002	<0.0001	<0.0001	<0.0001

Table 3. Analysis of variance for both trials average fresh weight, dry weight, % dry matter, chlorophyll (Chl) a, chlorophyll b, carotenoids, total chlorophylls, total vitamin C (T-AsA), and ascorbic acid (AA) content of arugula leafy greens grown at three different rates of AA (0, 100, and 200 ppm), three different spray frequencies (first, second, and third) and two different days of harvest (2 and 4 days after spray).

	Parameter								
Treatment	**Fresh Weight**	**Dry Weight**	**%Dry Matter**	**Chl a**	**Chl b**	**Carotenoids**	**Total Chls**	**T-AsA**	**AA**
AA rRate	NS	***	***	NS	*	NS	NS	***	***
Spray frequency	***	***	***	***	***	***	***	***	***
Day of harvest	***	NS	***	NS	NS	NS	NS	***	***
AA rate * spray frequency	NS	***	***	NS	NS	NS	NS	***	***
AA rate * day of harvest	NS	NS	***	NS	NS	NS	NS	NS	NS
Spray frequency * day of harvest	***	NS	***	NS	**	NS	NS	NS	***

NS—non-significant. *, **, and *** are significant at $p \leq 0.05$, 0.01, and 0.001, respectively.

3.3. Mineral Composition

The AA rate had considerable effect on leafy greens average N content ($p = 0.027$). Specifically, 100 ppm treated leafy greens had greater N content than control (Table 4 and Figure 2A). Leafy greens had greater N content after the first and second spray than third spray (Table 5). The average P, K, Ca, Mg, S, B, Zn, Mn, and Fe content of arugula leafy greens were not influenced by AA rate, spray frequency, day of harvest, or their interactions (Table 5). The average Cu content in leafy greens was only impacted by AA rate ($p = 0.044$) and the treatment that received no AA (control) had the greatest amount of Cu (11.0 ppm) and the 200 ppm AA treatment had the lowest Cu in the leafy greens (9.31 ppm) (Figure 2B).

Higher AA rates resulted in lower Cu concentrations in leafy greens. However, Cu content was not affected by spray frequency nor day of harvest and no interactions were found.

Table 4. Effect of AA rate on mineral nutrient composition of arugula leafy greens. Significant differences between treatments ($p \leq 0.05$) are denoted by lowercase letters within a column.

AA Rate	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	B (ppm)	Zn (ppm)	Mn (ppm)	Fe (ppm)	Cu (ppm)
0	6.59 b	0.68	6.43	2.75	0.20	1.35	65.85	49.73	299.94	136.91	11.0 a
100	6.95 a	0.64	5.82	2.54	0.19	1.21	59.94	45.69	301.84	124.53	9.72 ab
200	6.67 ab	0.65	5.69	2.47	0.18	1.19	58.59	45.97	283.25	118.63	9.31 b
p Value	0.027	0.74	0.44	0.48	0.41	0.20	0.43	0.51	0.62	0.17	0.049

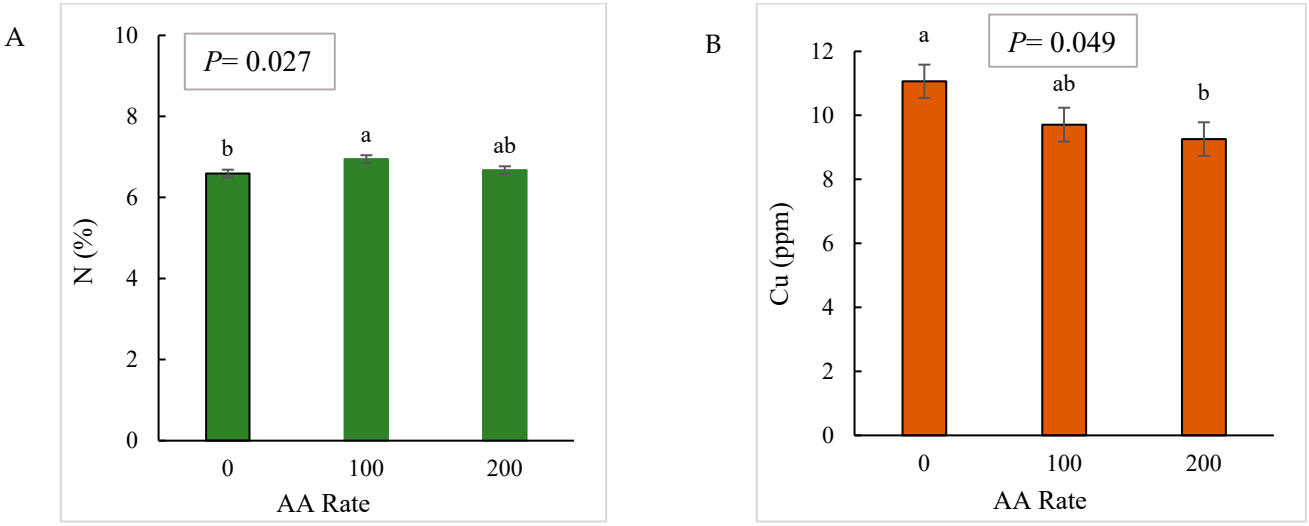

Figure 2. (**A**) Nitrogen (N) content of arugula leafy greens grown under three different ascorbic acid (AA) rates. (**B**) Copper (Cu) content of arugula leafy greens affected by three different ascorbic acid rates. Different lowercase letters denote significant differences between treatments at $p < 0.05$.

Table 5. Analysis of variance for N, P, K, Mg, Ca, S, B, Zn, Mn, Fe, and Cu content of arugula leafy greens grown at three different rates of AA (0, 100, and 200 ppm), three different spray frequencies (first, second, and third), and two different days of harvest (2 and 4 days after spray).

	Parameter										
Treatment	**N**	**P**	**K**	**Mg**	**Ca**	**S**	**B**	**Zn**	**Mn**	**Fe**	**Cu**
AA rate	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
Spray frequency	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Day of harvest	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AA rate * spray frequency	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AA rate * day of harvest	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Spray frequency * day of harvest	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS—non-significant. * and *** are significant at $p < 0.05$, and 0.001, respectively.

3.4. Ascorbic Acid and Total Vitamin C Content

Ascorbic acid content in leafy greens was significantly influenced by AA rate ($p = 0.003$), spray frequency ($p < 0.0001$), and day of harvest ($p < 0.0001$). Specifically, leafy greens that received 200 ppm foliar spray had the greatest amount of AA (26.6 mg/100 g FW) followed by 100 ppm (22.4 mg/100 g FW) and control (21.0 mg/100 g FW). Additionally, AA content in the leafy greens increased with an increased number of

sprays received by the plants. The leafy greens harvested after third spray (34.4 mg/mg/100 g FW) had significantly greater AA content than leafy greens harvested after second (23.2 mg/ 100 g FW) and first sprays (12.4 mg/100 g FW). Furthermore, arugula leafy greens harvested 4 DAS (26.9 mg/100 g FW) had greater amounts of AA than leafy greens harvested 2 DAS (19.8 mg/100 g FW). The AA content is also affected by the interactions between AA rate * spray frequency ($p < 0.0001$) and spray frequency * day of harvest ($p = 0.003$). Leafy greens that received 200 ppm AA foliar spray and harvested after third spray had the greatest amount of AA (Figure 3F). Further, treatments that received three AA sprays and harvested at 4 DAS had the most AA in leafy greens (Figure 3F).

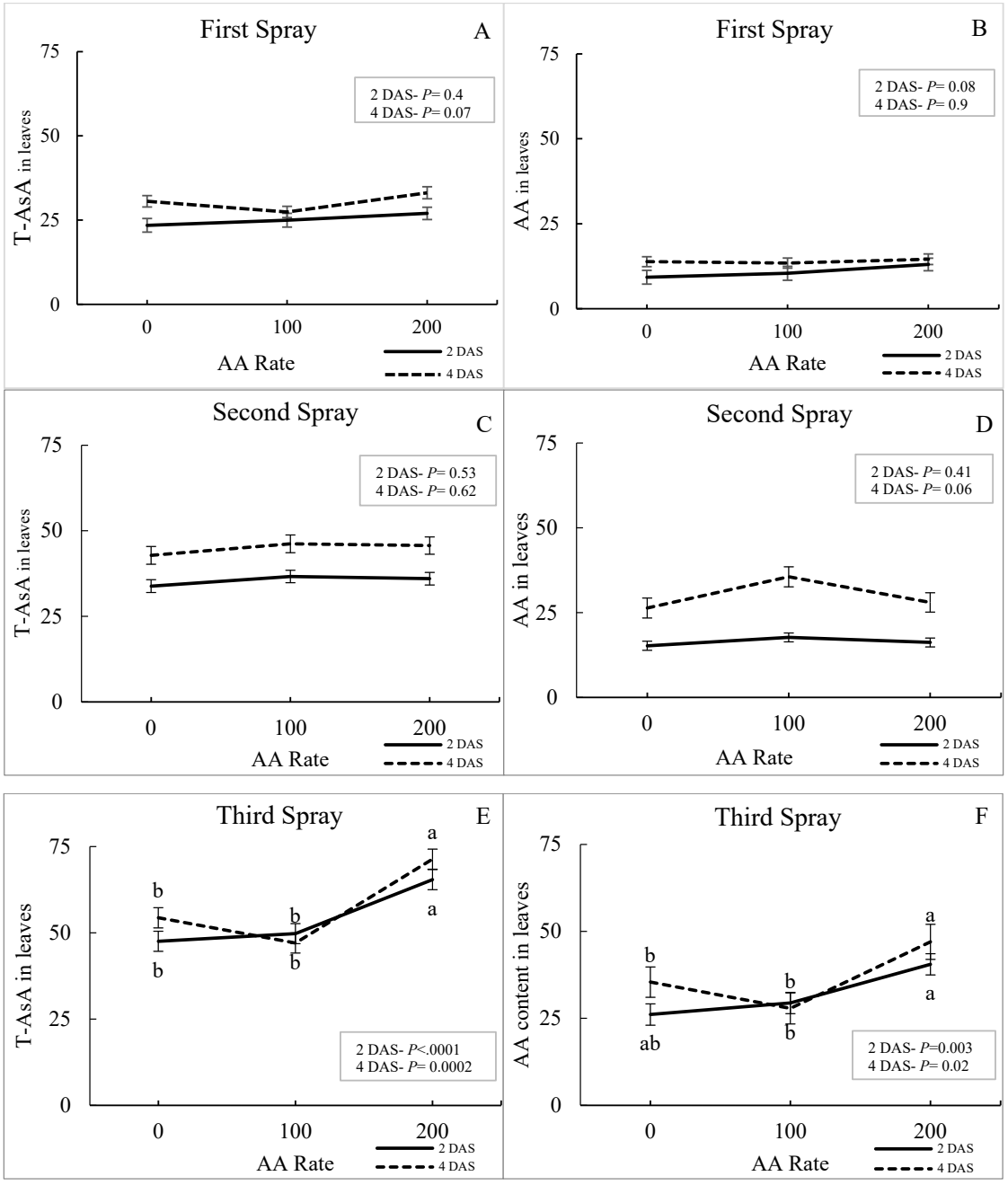

Figure 3. Concentrations of total vitamin C (T-AsA) and ascorbic acid (AA) in leafy greens harvested after first spray (**A**,**B**), second spray (**C**,**D**), and third spray (**E**,**F**) affected by the AA rate. Different letters indicate significant differences between treatments within each category indicated by Tukey's HSD at $p \leq 0.05$.

Similar to AA content in leafy greens, total vitamin C content was also impacted by AA rate, spray frequency, and day of harvest; leafy greens that received 200 ppm AA ($p < 0.0001$), three total sprays ($p < 0.0001$), and harvested 4 DAS ($p < 0.0001$) had the greatest amount of total vitamin C in leafy greens (Figure 3E). However, only the interactions between AA rate * spray frequency were significant ($p < 0.0001$). The treatments that received 200 ppm AA foliar spray three times had the greatest amount of total vitamin C in the leafy greens.

4. Discussion

Agronomic biofortification through AA supplementation has proven to be an effective method of biofortification in various crops like arugula [21] and broccoli [22] microgreens. Another way to increase vitamin C agronomically is through foliar application of AA. However, this method has only been explored in a few agronomic crops like soybean [18] and barley [20]. Herein, we investigated the effect of AA foliar spray on vitamin C content in arugula leafy greens and found that 200 ppm AA increased the total vitamin C and AA content in leafy greens. Exogenous application of AA resulted in the increased absorption and accumulation of AA in arugula leafy greens. The most substantial increase in total vitamin C (Figure 3E) and AA (Figure 3F) was seen after a total of three sprays of 200 ppm, which shows that at least three sprays before leafy greens harvest were necessary to increase vitamin C content in arugula leafy greens to a significant degree.

Furthermore, the 100 ppm foliar spray treatment did not increase total vitamin C (Figure 3A,C,E) or AA (Figure 3B,D,F) content in the leaves; thus, 200 ppm is the suggested dose of foliar spray to improve vitamin C content in arugula leafy greens. We also found that the increase in vitamin C was more noticeable two days and four days after the third spray, contrasting the study by [18], where the increase in vitamin C disappeared after 24 h. The noticeable increase in vitamin C up to four days after spray indicates that there is some longevity in foliar applications of AA, in contrast to those previous studies. The increase in vitamin C in the consumable leaves of arugula is significant, as previous studies by [18,20] increased AA in leaves of soybeans and barley, respectively, while the plant part that is consumed is the seed. It is worth noting that target edible tissues like seeds did not show increases in vitamin C in previous studies but showed increases in leaf tissues, indicating that foliar-applied AA is not mobile in plant tissues. As per the current study results, it is recommended to apply AA to leaves in leafy greens to see increased vitamin C content in edible plant parts. This is of increased importance because vitamin-C-biofortified arugula leafy greens could provide increased amounts of vitamin C to humans when consumed if applied to consumable tissues.

Chlorophylls and carotenoids were not impacted by AA rate. However, there were increases in chlorophylls and carotenoid content with increased sprays. As seen in tea leaves [30], chlorophyll increase in arugula leafy greens can be associated with leaf stage and maturity. Furthermore, maturation in many vegetable crops also results in increased carotenogenesis, thereby resulting in higher carotenoid content [31]. When it came to the effects of foliar-applied AA on yield, an increased AA rate increased the fresh and dry biomass of arugula leafy greens. The 200 ppm AA-treated leafy greens after three sprays had greater fresh weight and dry weight. This suggests that the AA foliar spray improved yield and vitamin composition of arugula leafy greens. This agrees with the study by [22], where 0.25% AA in supplemental solution increased yields of broccoli microgreens due to uptake and accumulation of more solutes like AA from foliar application. However, it is worth noting that the concentrations of AA used in current study and [22] were different and may have resulted in varying impacts on biomass. In the study by [32], application of 2000 and 3000 ppm AA improved Manzanillo olive tree yields. This illustrates the importance of the current study, proving that different plants respond differently to AA, and the growth environments and treatment rates play a vital role in the influence of AA on plants biomass.

AA foliar application did not alter mineral nutrient levels and maintained similar concentrations to that of the control. This illustrated that exogenous application of AA did not affect the ability of cells to retain other essential nutrients like P, Ca, Mg, S, Zn, and Fe. Alternatively, in studies by [21,22], nutrient solutions containing AA and K improved AA and K content in arugula and broccoli microgreens, respectively. However, the current study differs from the studies by [21,22] by applying AA alone, proving that AA by itself can improve AA and vitamin C content in arugula leafy greens without adding any other essential nutrients. Spraying of non-essential minerals like silica [14] has improved Si content without any known effects on yield, unlike essential nutrient nutrients like N, P, K, etc. that improve yield and other nutrient content as explained by [12,33], respectively. This is likely due to non-essential nutrients' limited role in plant functions and nutrient absorption [34,35]. Similarly, AA is also not an essential nutrient for plants; hence, AA was able to increase vitamin C and AA content while not affecting yield parameters or most nutrient concentrations in arugula leafy greens. Alternatively, 100 ppm treated leafy greens had the greatest N content followed by 200 ppm treated leafy greens and control, which suggests that AA application at lower rates could improve N uptake and accumulation. Similar results were seen in wheat, where 200 mg L^{-1} AA application resulted in greater N content in straw [36]. Additionally, AA application decreased the Cu accumulation in the leaves of AA with 200 ppm AA treatment having lowest amount of Cu, followed by 100 ppm and control. The presence of excess Cu in plants is known to result in oxidative stress [37] due to formation of OH radicals due to non-enzymatic reaction catalyzed by transition metals like Cu and Fe between superoxide and hydrogen peroxide (Haber–Weiss reaction) [38]. Additionally, the role of the ascorbate glutathione cycle in protecting against oxidative stress induced by copper has been well researched [38,39]. A reduction of Cu content in AA-treated leafy greens suggests that AA inhibited Cu uptake and translocation, thereby reducing overall Cu uptake [40]. Hence, we can assume that AA foliar application can enhance tolerance to Cu toxicity in arugula leafy greens, thereby protecting the plants from physiological and biochemical stress responses.

Overall, this study provides evidence that AA foliar application can improve vitamin C and chlorophyll content in arugula leafy greens while maintaining yields and nutrient composition. Additionally, foliar application of AA can increase vitamin C content in arugula leafy greens up to four days after harvest. The increased AA can provide plants with added protection against environmental stressors and provide humans with more vitamin C upon consuming these biofortified arugula leafy greens. However, the current lack in the literature on agronomic biofortification of vitamin C using AA underscores the importance of assessing its effects across different crops and different growing conditions to understand the broader implications. Further research on the application of AA in more commonly consumed leafy greens like lettuce and spinach could further elaborate on the economic implication and wide-scale adoption of this method.

5. Conclusions

The current study demonstrated a novel approach to increase vitamin C content in arugula leafy greens using exogenous foliar application of AA. Furthermore, AA application also increased yields and helped reduce Cu accumulation in arugula leafy greens without affecting other mineral nutrients content. It is important to note that the benefits of increased AA may vary depending on the plant species, specific environmental conditions, and other factors. Therefore, future research should explore the implications of AA applications on various crops in different growing conditions. Finally, proper dosage and application methods should be considered to achieve optimal results.

Author Contributions: S.K.: conceptualization, methodology, investigation, writing—original draft, and formal analysis. H.L. (He Liu): investigation. H.L. (Haydee Laza), L.T., S.S. and W.L.: writing—review and editing. C.R.S.: conceptualization, supervision, resources, validation, formal analysis, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are available upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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