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The Combined Application of Urea and Fulvic Acid Regulates Apple Tree Carbon and Nitrogen Metabolism and Improves Anthocyanin Biosynthesis

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Abstract: Improving apple peel color has been an important objective in apple production. To better understand the effect and mechanism of the combined application of urea and FA (fulvic acid) regulation of anthocyanin biosynthesis, a field experiment was performed in 2022 and 2023, respectively, under five treatments of urea + FA (CK, urea only; FA50, urea + 50 kg ha^{-1} FA; FA100, urea + 100 kg ha^{-1} FA; FA150, urea + 150 kg ha^{-1} FA; FA200, urea + 200 kg ha^{-1} FA), using isotope (^{13}C and ^{15}N) marking to analyze the changes in carbon (C) and nitrogen (N) nutrient distribution as well as anthocyanin biosynthesis in fruits. We observed that, under FA application conditions, anthocyanin content in the peel was elevated in both years, with increases of 15.98~52.88% in 2022 and 15.93~52.94% in 2023. The best promotion effects were observed under FA150 treatment. Apart from the expression levels of anthocyanin biosynthesis-related genes and transcription factors in the apple peel, this positive effect on anthocyanin content induced by FA addition was also found to be associated with the optimization of C and N distribution in leaves and fruits. On the one hand, the application of FA not only enhanced leaf photosynthetic-related indexes (such as P_n, G_s, and Rubisco activity) and influenced (increased) S6PDH, SPS, and SS activities in leaves, but also elevated fruit sugar metabolism-related enzyme (SDH, SS-c, AI, and NI) activity and upregulated fruit stalk sugar transporter (*MdSOT1*, *MdSOT3*, *MdSUT1* and *MdSUT4*) gene expression, which ultimately promoted the synthesis and the leaf to fruit transport of photosynthates, thus promoting ^{13}C-photosynthate accumulation in fruits. On the other hand, FA application elevated leaves' N metabolism-related enzyme (GS and GOGAT) activity and optimized ^{15}N distribution in leaves and fruits. Moreover, we also observed that FA application altered the fate of N fertilizer in apple orchards, showed an elevation in apple tree ^{15}NUE and soil ^{15}N residuals and showed a decrease in soil ^{15}N loss. In summary, the appropriate application of FA150 (urea + 150 kg ha^{-1}) synergistically optimized C and N nutrient distribution, and promoted anthocyanin biosynthesis in apple trees.

Keywords: apple; anthocyanin; fulvic acid; ^{15}NUE; carbon (C) and nitrogen (N) metabolism

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1. Introduction

Apple, which has been cultivated wildly in China, plays an important role in promoting regional economic development [1–3]. As an important indicator of appearance quality, peel color directly affects the market value of apples, and red-skinned apple could easily attract consumers [4,5]. Anthocyanin, an important water-soluble pigment, plays a decisive role in red-skinned apple peel color, and also has a positive effect on human health (such as the prevention effects of cardiovascular disease) [6–10]. Therefore, exploring effective orchard management strategies to improve (red-skinned) apple peel color through the elevation of anthocyanin content has become an important research topic.

Fulvic acid (FA), a type of humic substance (HS), has great potential to maintain the sustainability of modern agricultural production, such as remediating heavy metal-polluted

soil as a chelating agent [11], mitigating the adverse effects of lettuce (*Lactuca sativa* L.) exposed to cadmium (Cd) stress condition through the way of improving photosynthesis performance and reducing total Cd content in lettuce [12], promoting plant nitrogen (N) utilization efficiency (NUE) in the way of promoting plant root growth [13], and prolonging the N fertilizer effectiveness via the enhancement of preservation and the decrease in N fertilizer loss [14]. As apple is an important economic crop with the ultimate purpose of fruit harvesting, apple peel color plays an important role in the realization of its economic value [15]. Although Jiang et al. [16] have reported that the application of FA could elevate the content of anthocyanin in the apple pericarp, the specific mechanism involved is still rarely reported.

It has been widely acknowledged that increasing C and N metabolism is highly important for increased productivity in modern agriculture [17,18], and FA application has great potential for enhancing plant C and N metabolism. For example, Yu et al. [1] noted that FA treatment could mitigate the inhibition of C and N metabolism induced by Cd stress, which could ultimately improve the Cd tolerance of plants. Gao et al. [19] noted that urea and FA combined-treated maize had a higher crop yield than that of urea applied alone and this promoting effect was found to be associated with the FA-induced positive influence on C and N metabolism. A similar tendency was observed by Yu et al. [13], who found that FA could regulate plant (apple seedlings) C and N assimilation, thus promoting apple plant growth and N utilization efficiency. Sugar is a key factor that influences anthocyanin synthesis and promoting sugar accumulation in fruits has been proved to be conducive to anthocyanin synthesis in peel [5,20]. Although authors such as Peng et al. [14] and Jiang et al. [16] both noted that FA application in apple orchards could elevate the content of sugar in fruits, the details regarding how FA application modulates apple anthocyanin biosynthesis through affecting fruit sugar accumulation via regulating apple C and N metabolism remains unclear. In this study, a two-year field experiment was performed, and the combined application of urea and five levels of FA were applied to "Fuji" apples to explore the effects of FA on the C and N nutrition of trees and anthocyanin content via the joint analysis of physiology and isotope (^{13}C and ^{15}N) marking. The results of the present study could provide a scientific basis for optimizing N management in apple orchards and will provide new insights for understanding the formation mechanism of apple fruit coloring under FA addition conditions.

2. Materials and Methods

2.1. Experimental Site Description

A field experiment was conducted in an apple orchard in Taishan District, Taian city, Shandong Province, China (117°59′55″ E, 35°38′58″ N) in 2022 and 2023, respectively. The mean temperatures from May to October were 19.5 °C, 23.0 °C, 24.9 °C, 27.3 °C, 22.1 °C, and 14.6 °C in 2022 and 18.2 °C, 22.3 °C, 24.2 °C, 26.9 °C, 21.6 °C, and 13.9 °C in 2023, respectively. The precipitation from May to October was 7.5, 39.8, 51.2, 126.6, 52.2, and 4.1 mm in 2022 and 8.5, 41.9, 56.3, 124.2, 51.3, and 3.5 mm in 2023, respectively. Five-year-old 'Yanfu3'/M26/*Malus hupehensis* Rehd. trees spaced at 2 m × 4 m were selected as the plant material. These apple trees had the same crop loads and developmental attributes. The physical and chemical properties of the soil are shown in the Table S1.

2.2. Experimental Design

In this study, five treatments were applied in 2022 and 2023, namely CK, FA50, FA100, FA150, and FA200, 0, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Each treatment contained three replicates, and the number of trees in each replicate was six. The trees of each replicate were evenly divided into two groups, a marking (^{13}C and ^{15}N) group and an unlabeled group, which were used for isotope (^{13}C and ^{15}N) analysis and other index measurements, respectively. In this study, the amounts of urea, calcium superphosphate, and K_2SO_4 applied to each tree were 435 g, 357 g, and 218 g, respectively. Except for the one-time input of 357 g calcium superphosphate applied to

each tree at flowering, urea and K_2SO_4 were added as a split application and the details of the fertilization are as follows, urea, 174 g at flowering, 174 g at 40 days after flowering, and 87 g at 100 d after flowering; K_2SO_4, 109 g at flowering and 109 g at 120 d after flowering. The FA treatments and ^{15}N labeling were applied at 40 days after flowering. Each tree of the unlabeled group was supplied with normal urea (174 g). For the marking group, 10 g of ^{15}N-urea (10.14%, abundance) and 164 normal urea were applied at 40 days after flowering. FA was evenly added to in the circular ditch after being dissolved in water. Moreover, all the treatments were subjected to the same field management practices.

^{13}C labeling on the trees of the marking group was conducted at 177 days after flowering. The entire ^{13}C labeling process is divided into three parts: the manufacturing of labeling chambers, the process of formal ^{13}C labeling and the collection of plant material at the end of ^{13}C isotope labeling. In brief, Mylar plastic bags (0.1 mm thick) and brackets, a beaker with $Ba^{13}CO_3$ (10 g, ^{13}C independence is 98%) and reduced iron powder, and the apple trees of the marking group comprised a marking chamber—a marking chamber contains one apple tree. After the marking chamber was assembled, the ^{13}C marking process was initiated. Labeling work started at 8:00 am through injecting (1 mol/L) hydrochloric acid into the beaker contained 10 g $Ba^{13}CO_3$ every 0.5 h. The (^{13}C isotope labeling) process lasted for 4 h. All apple trees of each treatment were destructively harvested at 180 days after flowering. Apart from the collection of plant (apple trees) samples, soil samples were also harvested on the same day. The 0~60 cm soil samples were collected and used to calculate soil ^{15}N residue according to the details reported by Wang et al. [21].

2.3. Measurement of ^{13}C-and ^{15}N-Related Indices

After the collection and the preparation of plant materials (fruits, leaves, roots, central stems and branches) according to Xu et al. [22], a MAT-251-Stable Isotope Ratio Mass Spectrometer was used to measure ^{15}N and ^{13}C abundance. The calculation equation of ^{15}N- and ^{13}C-related indices was as follows:

Calculation of ^{15}N

$$\text{Ndff}\ (\%) = \frac{\text{abundanceof15Ninplant} - \text{naturalabundanceof15N}}{\text{abundanceof15Ninfertilizer} - \text{naturalabundanceof15N}} \times 100\% \quad (1)$$

$$^{15}\text{N absorbed by each organ} = \text{Ndff}\ (\%) \times \text{total N content (mg)} \quad (2)$$

Calculation of ^{13}C

$$\text{Abundanceof}\ ^{13}\text{CC} : \text{Fi}(\%) = \frac{(\delta 13C + 1000) \times \text{RPBD}}{(\delta 13C + 1000) \times \text{RPB} + 1000} \times 100\% \quad (3)$$

RPBD is a constant value that represents the standard ratio of carbon isotopes. The value of RPBD is set to 0.0112372.

$$\text{C content of each organ: Ci} = \text{organ dry matter (g)} \times \text{organ total carbon content (\%)} \quad (4)$$

$$\text{Contentof}^{13}\text{C ineachorgan}: \ 13\text{Ci(mg)} = \frac{\text{Ci} \times (\text{Fi} - \text{Fnl})}{100} \times 100\% \quad (5)$$

The value of Fnl represents the 13C natural abundance of each organ

$$^{13}\text{C partitioningrate}: \ 13\text{C}\ (\%) = \frac{13\text{Ci}}{13\text{C netabsorption}} \times 100\% \quad (6)$$

2.4. Measurement of Photosynthetic Parameters

Before the collection of plant (apple trees) samples, the net photosynthetic rate (P_n) and stomatal conductance (G_s) of leaves were measured according to the operating instructions of the Li-6400 photosynthetic instrument (LICOR Inc., Lincoln, NE, USA). The light-saturation point was set to 1200 μmol (photon) m^{-2} s^{-1}, the ambient temperature of

the apple leaves was kept constant at 30 °C, the CO_2 concentration was 400 μmol (CO_2) mol^{-1}, relative humidity was 60%–65%, and air flow was 500 mmol s^{-1}. Leaves' Rubisco activity was also measured in this study by the method described by Hu et al. [23].

2.5. Measurement of Enzyme Activity

In this study, we measured the nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (GOGAT), sorbitol 6-phosphate dehydrogenase (S6PDH), sucrose phosphate synthase (SPS), and sucrose synthase (SS) activities in leaves and the sorbitol dehydrogenase (SDH), sorbitol oxidase (SOX), sucrose synthase decomposition direction activity (SS-c), acid invertase (Al), and neutral invertase (NI) activities in fruits, respectively. The measurements of NR, GS and GOGAT activities were conducted according to Hu et al. [24]. Subsequently, the activity measurements of S6PDH according to Berüter [25], and SPS and SS according to Huber and Israel [26] were performed. Moreover, the fruit SDH activity was determined as described by Rufly and Huber [27]. The SOX activity was determined as described by Keller and Ludlow [28]. The SS-c activities was determined, as described by Huber [29]. AI and NI activities were measured according to Merlo and Passera [30].

2.6. Analysis of RNA Extraction and Gene Expression

In this study, the transcription levels of three *MdSOT*s and three *MdSUT*s fruit stalks as well as anthocyanin biosynthesis-related genes in peels were analyzed. Following a previously reported procedure described by Tian et al. [31], RNA extraction, reverse transcription, and qRT-PCR were performed. The $2^{-\Delta\Delta CT}$ method and the *MdActin* gene (the internal reference gene) were used in this study to determine the relative expression level of genes. Three biological replicates per treatment and three technical replicates per sample were used in the assay. The primers are listed in Supplementary Table S2.

2.7. Statistical Analysis

After the collection of data using Microsoft Excel, the analysis of data was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and a post hoc test (Duncan's) were adopted in this study for data analysis. The differences were considered statistically significant at a probability level of $p < 0.05$.

3. Results

3.1. Plant C Metabolism

3.1.1. Photosynthetic Characteristics

As shown in Figure 1, compared with those in the CK treatment, the P_n and G_s in the FA treatment group (FA50~FA200) were significantly greater, with increases of 14.85~53.47% (P_n) and 11.57~39.67% (G_s) in 2022 and 13.68~70.53% (P_n) and 11.28~33.83% (G_s) in 2023, respectively, and the highest values of P_n and G_s were obtained in the FA150 treatment in both years. Moreover, FA application also significantly enhanced the activity of Rubisco in leaves. Similar to the change in P_n and G_s, the activity of Rubisco in the FA150 treatment was greater than that in other treatment groups.

3.1.2. Enzyme Activities

In this study, the activities of sorbitol 6-phosphate dehydrogenase (S6PDH), sucrose phosphate synthase (SPS) and sucrose synthase (SS) in leaves were measured. As shown in Figure 2, with increasing FA addition, the abovementioned enzyme (except for SS) activities tended to increase and then decrease, and the highest values were observed under the FA150 treatment, which were increased by 53.82% (S6PDH), 54.49% (SPS) in 2022, 57.10% (S6PDH), and 51.26% (SPS) in 2023, respectively, compared to those under the CK treatment.

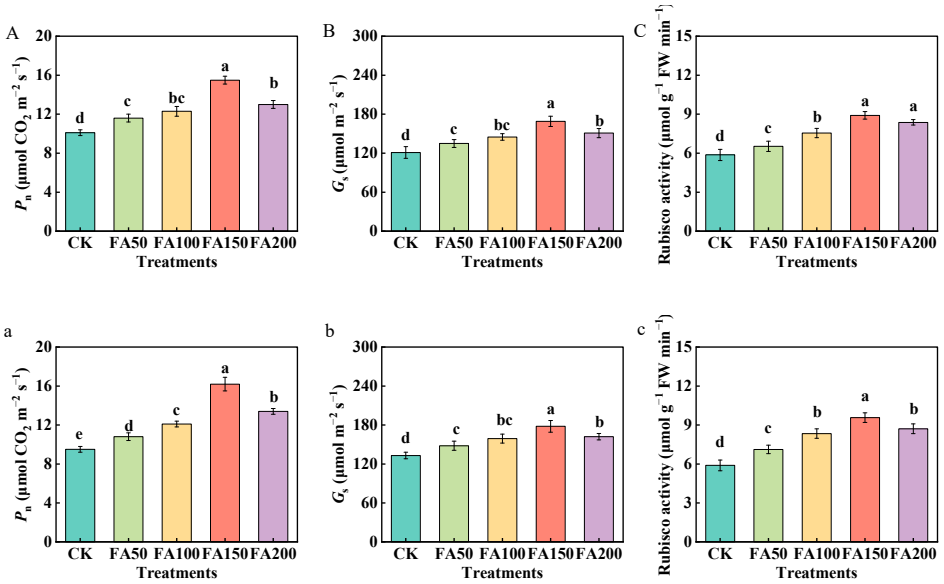

Figure 1. P_n (**A** and **a**), G_s (**B** and **b**), and Rubisco activity (**C** and **c**) in leaves in 2022 (**A**–**C**) and 2023 (**a**–**c**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

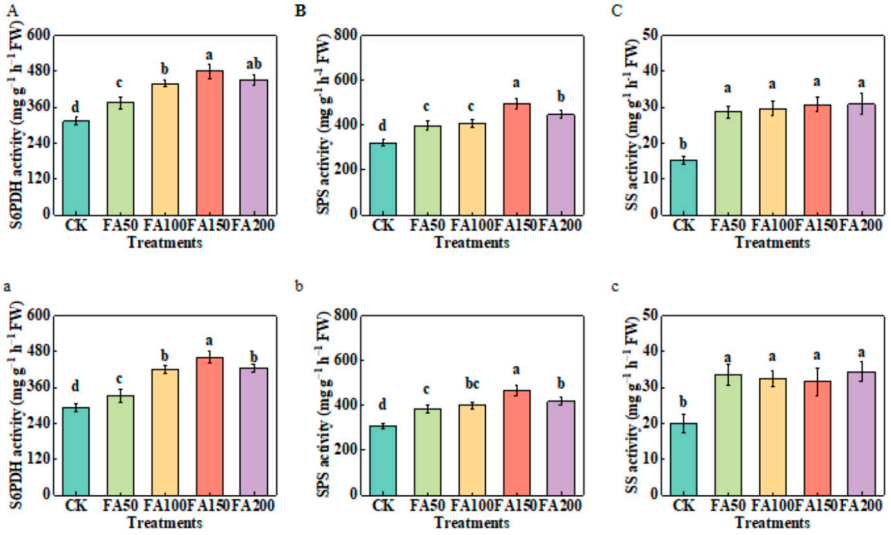

Figure 2. S6PDH (**A** and **a**), SPS (**B** and **b**) and SS (**C** and **c**) activity in leaves in 2022 (**A**–**C**) and 2023 (**a**–**c**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

Moreover, we also measured a series of sugar-metabolizing enzyme activities in fruit, such as sorbitol dehydrogenase (SDH), sorbitol oxidase (SOX), sucrose synthase decomposition direction activity (SS-c), acid invertase (AI) and neutral invertase (NI) (Figure 3). Under FA (FA50~FA200) treatments, the activity of the abovementioned enzymes in fruits were increased (except for SOX) and a dose modulation effect existed in the trend of SDH activity exhibited by the FA treatment, a tendency towards increasing and then decreasing. The highest SDH activity was observed under the FA150 treatment.

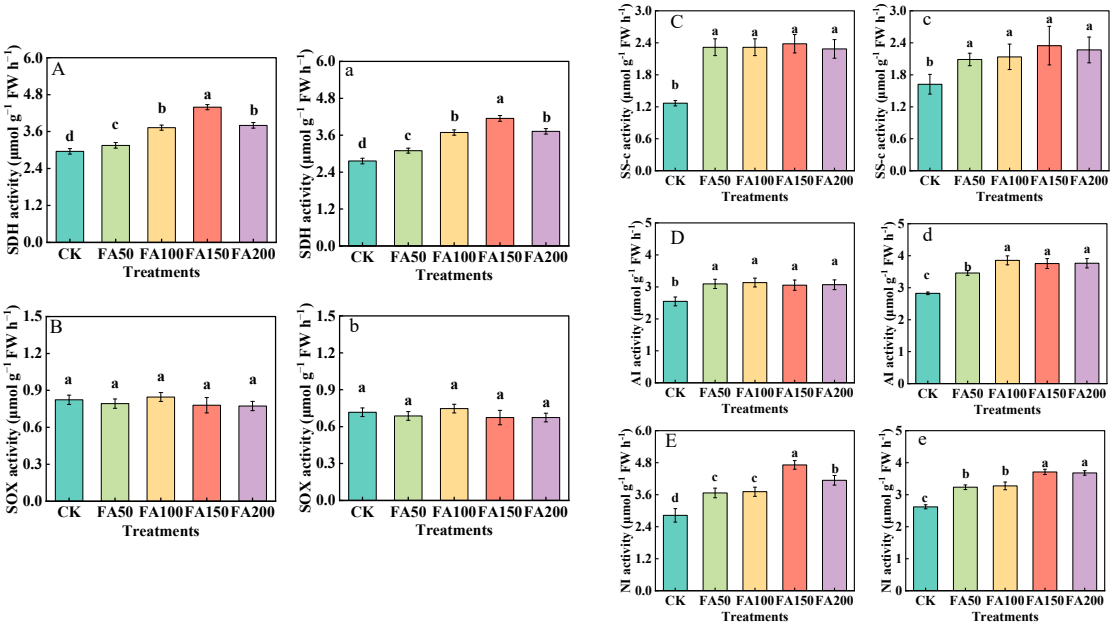

Figure 3. The activities of SDH (**A** and **a**), SOX (**B** and **b**), SS-c (**C** and **c**), AI (**D** and **d**) and NI (**E** and **e**) in fruits in 2022 (**A**–**E**) and 2023 (**a**–**e**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

3.1.3. ^{13}C Distribution Rate, Fruit ^{13}C Accumulation, and Sugar Transporter-Related Gene Expression

According to the results shown in Figure 4, apple trees under FA application conditions displayed a relatively higher fruit ^{13}C distribution rate and a relatively lower leaf ^{13}C distribution rate than those of the CK treatment. FA150 treatment resulted in the highest fruit ^{13}C distribution rate and the lowest leaf ^{13}C distribution rate. The values were 44.87% (2022) and 46.37% (2023) in fruits and 13.33% (2022) and 15.93% (2023) in leaves. Subsequently, we analyzed the expression of sugar transporter-related genes in fruit stalks. Under FA application conditions, the expression of *MdSOT1*, *MdSOT3*, *MdSUT1* and *MdSUT4* was upregulated. Further analysis demonstrated that the highest expression levels of the abovementioned genes (except for *MdSUT4*) were observed in the FA150 treatment in both years (Figure 4B,b).

3.2. N Absorption and Metabolism

3.2.1. Plant N Metabolism-Related Enzyme Activities

With increasing FA amounts, the activities of GS and GOGAT initially increased and then decreased, and the greatest increase was observed under the FA150 treatment. However, no significant difference in the change in NR activity was detected among the treatments (Figure 5).

3.2.2. Plant ^{15}N Utilization and ^{15}N Distribution

In this study, the ^{15}N utilization efficiency (^{15}NUE) and ^{15}N distribution rate were detected and are presented in Figure 6A,a. In both the 2022 and 2023 treatments, compared with those in the CK treatment, the ^{15}NUE in the FA treatment tended to initially increase and then decrease with increasing FA amounts, and the highest ^{15}NUE values were observed in the FA150 treatment. The values were 19.37% (2022) and 21.89% (2023), which were 69.58% and 74.70% greater than those in the CK treatment, respectively. Moreover, with increasing FA amounts, the leaf ^{15}N distribution rate first increased and then decreased. However, the opposite trend was observed for the fruit ^{15}N distribution rate. Compared with the CK treatment, the FA treatment reduced the fruit ^{15}N distribution to

varying degrees, and the lowest value was detected under the FA150 treatment, which was 32.97% and 35.80% lower than that of the CK treatment in both years (Figure 6B,b).

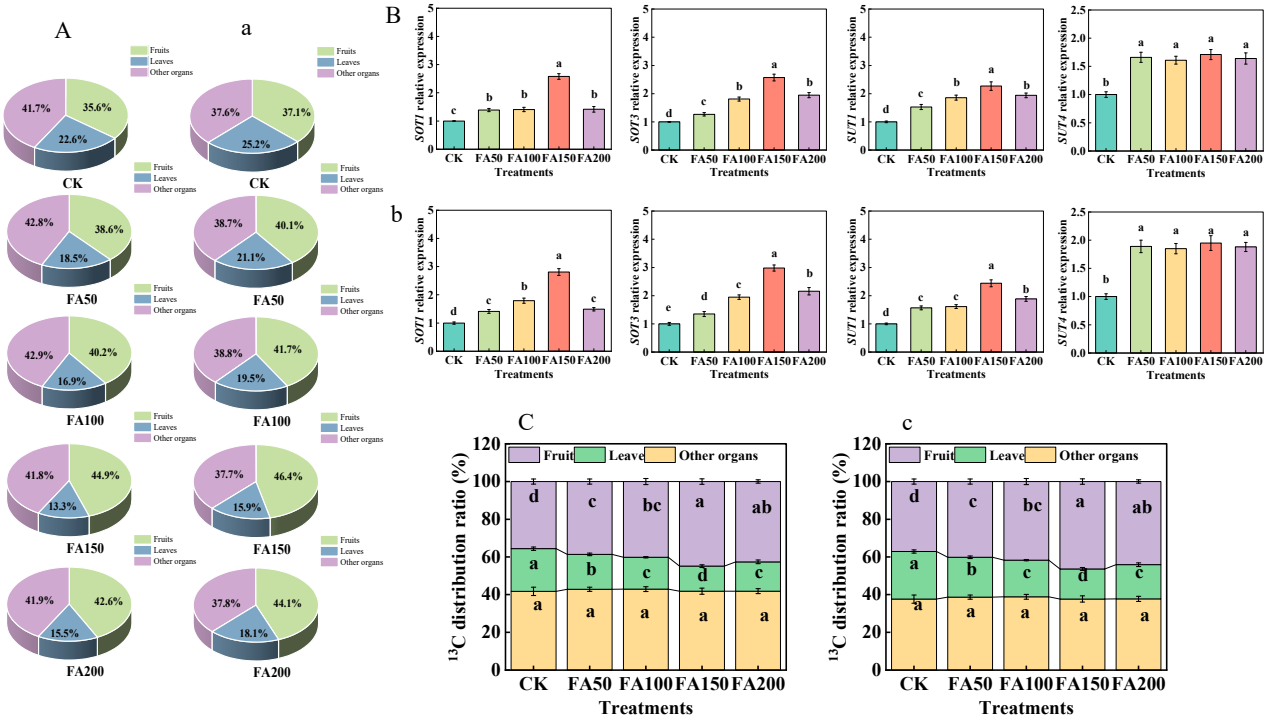

Figure 4. Organs' ^{13}C distribution rate (**A** and **a**; **C** and **c**) as well as fruit stalks' *MdSOT*s and *MdSUT*s gene expression (**B** and **b**) under different treatments in 2022 (**A**–**C**) and 2023 (**a**–**c**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

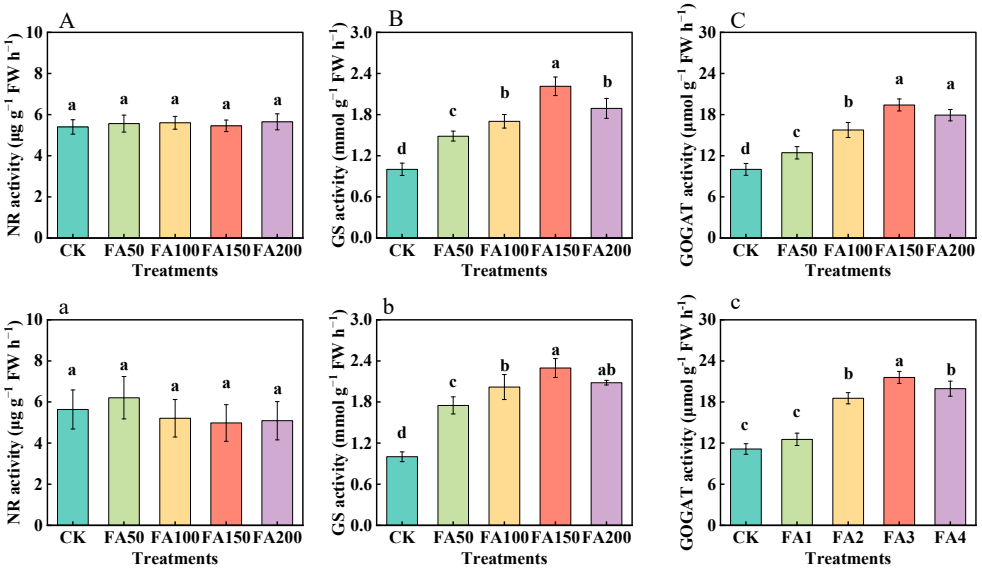

Figure 5. The activities of NR (**A** and **a**), GS (**B** and **b**) and GOGAT (**C** and **c**) in leaves under different treatments in 2022 (**A**–**C**) and 2023 (**a**–**c**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

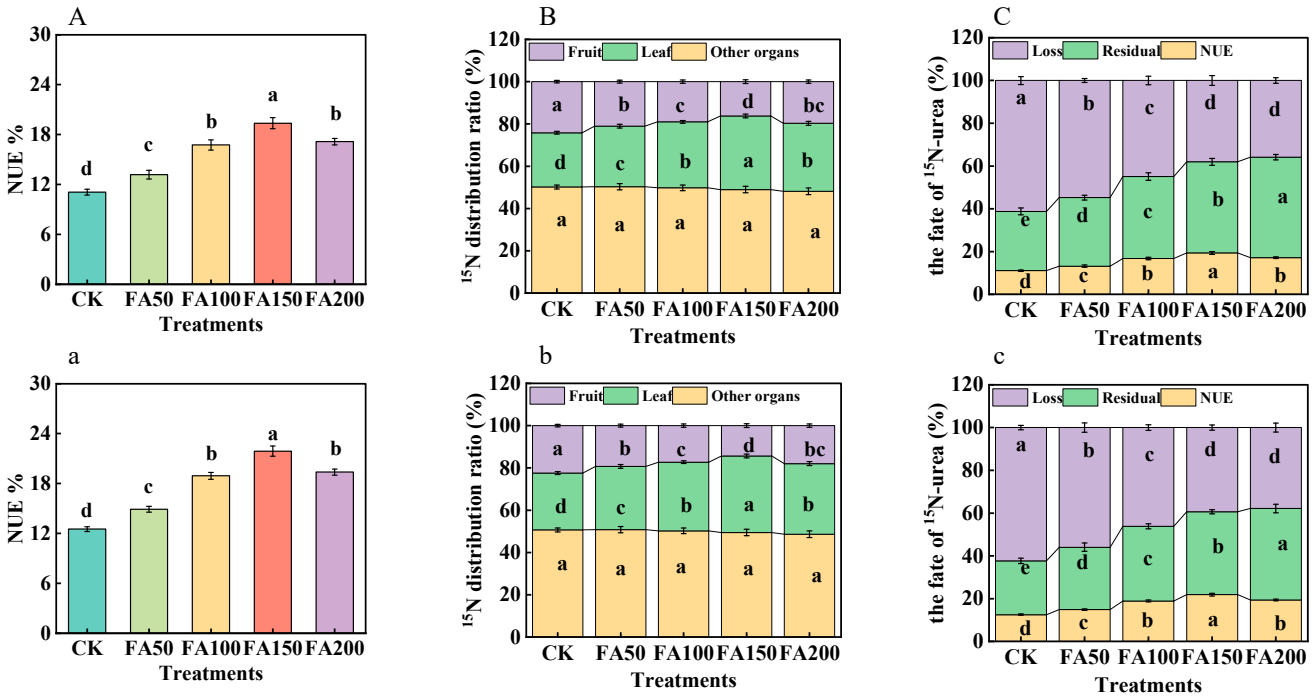

Figure 6. ^{15}NUE of apple trees (**A** and **a**), organs' ^{15}N distribution rate (**B** and **b**) and the fate of ^{15}N-urea (**C** and **c**) in 2022 (**A**, **B** and **C**) and 2023 (**a**–**c**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

3.2.3. The Fate of ^{15}N-Urea in Orchard

In this study, the fate of the ^{15}N-urea in apple orchards consists of three phases: utilization, residue, and loss. As shown in Figure 6C,c, the FA treatments elevated the soil ^{15}N residue to varying degrees. With increasing FA applied to the orchard, the soil ^{15}N residue tended to gradually increase, and the highest soil ^{15}N residue was observed under the FA200 treatment. In contrast, FA150 and FA200 treatment both decreased soil ^{15}N loss rate, compared with the CK treatment, with decreases of 37.93% (FA150), 41.49% (FA200) in 2022 and 36.84% (FA150), 39.25% (FA200) in 2023, respectively.

3.2.4. Anthocyanin Content and Anthocyanin Biosynthesis-Related Gene Expression in the Peel

Under FA application conditions (FA50~FA200), the content of anthocyanin in the peel was elevated in both years, compared with the CK treatment, with increases of 15.98~52.88% in 2022 and 15.93~52.94% in 2023. The best promotion effects were observed under FA150 treatment, compared with the CK treatment in both years. Subsequently, we observed that, under FA150 treatment, the *MdCHS* and *MdMYB1* gene expression levels were higher than that of other treatments in both years. Although FA addition treatments upregulated the *MdF3H* and *MdbZIP44* gene expression levels, the levels of FA (50~200 kg ha^{-1}) had no significant effect on the changes in the expression of the abovementioned genes (*MdF3H* and *MdbZIP44*). As for *MdCHI*, *MdDFR* and *MdUFGT* gene expression, there is no significant difference among all treatments (Figure 7).

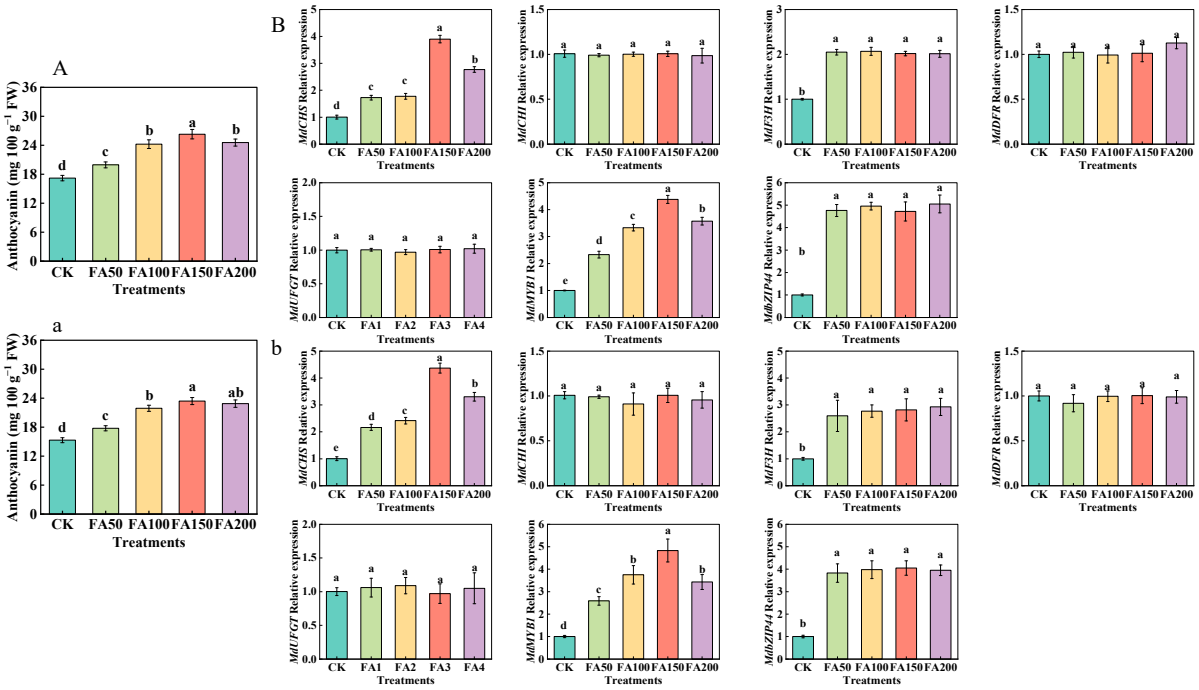

Figure 7. Anthocyanin content (**A**,**a**) and anthocyanin biosynthesis-related gene expression level in peels (**B**,**b**) in 2022 (**A**,**B**) and 2023 (**a**,**b**). CK: control, without FA addition; FA50, FA100, FA150, and FA200, 50, 100, 150, and 200 kg ha^{-1} of fulvic acid (FA) application, respectively. Vertical bars on the histograms indicate ± SD. Different letters indicate statistically significant differences ($p < 0.05$).

4. Discussion

4.1. The Appropriate Application of FA Promoted Apple Tree NUE and Optimized N Partitioning in the Tree

As the main research objects of precise nutrient management in apple orchards, improving N utilization efficiency (NUE) and securing a stable supply of N are beneficial for the formation of fruit quality at fruit maturity. FA has been demonstrated to have many potential benefits in regulating plant NUE. Consistent with the previous studies, in the present study, we observed that FA-treated apple trees had higher ^{15}NUE values than CK-treated apple trees, indicating that FA application promoted the N uptake of apple trees. Further analysis showed that, with increasing FA application, the ^{15}NUE tended to initially increase and then decrease, indicating that a dose modulation effect existed between the ^{15}NUE and FA amounts in apple trees. Although it is frequently reported that FA improves plant NUE in terms of plant physiological and biochemical aspects [13,19], the increase in NUE could also be closely related to the increase in soil ^{15}N residues and the decrease in soil ^{15}N loss caused by exogenous organic amendments [32]. Therefore, the variations in N fertilizer preservation and loss could also explain why FA-treated apple trees had higher ^{15}NUE than non-treated trees in this study.

Plant uptake, soil residues and losses are the three main destinations of N fertilizer in the apple orchard system [14]. The main apple production areas in Shandong Province are generally subject to high temperatures and rainy weather in the late stage of apple fruit expansion. Moreover, the soil N nitrification process could be enhanced under high temperature and rainy weather conditions [33]. These effects could increase the risk of surface runoff and leaching losses of NO_3^--N. In addition to the abovementioned plant ^{15}NUE, we also measured the residue and loss of N fertilizer. In this study, the lowest soil ^{15}N residue and highest soil ^{15}N loss were both observed under CK conditions (without FA addition). Wang et al. [21] reported that the root distribution of dwarf apple is mainly concentrated in the 0~60 cm soil layer. According to the analysis of soil ^{15}N residues (^{15}N within the 0~60 cm soil layers), we found that FA treatments elevated soil ^{15}N residues,

which could be propitious to the improvement of apple NUE; additionally, we also observed that FA treatments decreased soil ^{15}N loss to varying degrees. The greater soil ^{15}N residue and lower soil ^{15}N loss in the FA treatments than in the CK treatment may be closely related to the delay in the decomposition of urea caused by FA application through the inhibition of urease activity [19,32]. Moreover, the formation of stable FA-ammonium salts through the reaction between FA and $NH4^+$ [34] could also be a vital reason as to why the FA treatments had a lower ^{15}N loss rate than that of the CK treatment. Due to the regulation of soil ^{15}N residues and loss caused by FA application, the goals of plant N fertilizer uptake improvement, soil N fertilizer preservation and soil N fertilizer loss reduction have been achieved, which not only optimizes orchard N management, but is also beneficial for improving apple fruit quality (to a certain degree).

N absorbed by the root system undergoes N assimilation in the plants, which is catalyzed by a series of N-metabolizing enzymes, such as NR, GS, and GOGAT [35]. In this study, we detected no significant difference in the NR activity of leaves among all of the treatments. Moreover, we observed that, compared with CK, all of the FA treatments elevated the activities of GS and GOGAT in leaves, indicating that the process of N assimilation was enhanced under FA addition conditions via the regulation of N metabolism-related enzyme activities in leaves. Tian et al. [31] reported that the promotion of N metabolism in leaves could enhance the requisition and allocation of N in leaves. In this study, we observed that the ^{15}N distribution rate in leaves increased under FA treatment, indicating that FA application could increase the competition for N in leaves, which could provide a foundation for the optimization of leaf photosynthetic performance under FA treatment. Previous studies have shown that the overaccumulation of N in fruit can not only reduce the transport of sugar from leaf to fruit, but also increase the fruit titratable acid content [21,36]. Wang et al. [37] also noted that the overaccumulation of N in fruit could inhibit the biosynthesis of anthocyanin, thus negatively influencing the fruit color. In this study, although plant (apple) NUE was elevated by FA application, the ^{15}N distribution rate of fruits were decreased under FA treatments, indicating that FA could optimize N distribution in fruits and avoid the overaccumulation of N in fruits to a certain degree. This effect could be an important reason as to why FA treatments had higher anthocyanin content in peel. Combined with the abovementioned variations, we concluded the following: (1) FA application increased apple tree ^{15}NUE, which could provide a material foundation (scientific and effective N supply) for the various apple plant physiological and biochemical processes, including leaf photosynthesis; and (2), FA optimized N distribution in leaves and fruits. In this study, FA improved the ^{15}N distribution rate in leaves, which could enhance the photosynthetic performance and photosynthetic synthesis ability of leaves, due to the closely related C-N metabolism. These effects could increase the anthocyanin content to a certain degree [37,38]. In contrast, less N allocation in fruits may also further promote leaf-to-fruit photosynthate transport, which could not only be beneficial for improving anthocyanin biosynthesis, but also be conducive to reducing the titratable acid content in fruits.

4.2. FA Promoted C Metabolism and the Transport of Photosynthates from Leaves to Fruits

Numerous studies have proven that facilitating sugar accumulation in fruits is not only beneficial for increasing the intrinsic quality, but also conducive to improving the appearance quality of fruits, due to the critical role of sugar in anthocyanin synthesis. The essence of fruit sugar accumulation is the process of the synthesis of photosynthates in leaves and the transport of photosynthates from leaf to fruit [3,5]. In this study, compared with those in the CK treatment, the P_n, Rubisco activity and G_s in the FA treatment groups were significantly greater, indicating that the photosynthetic performance of the leaves in the FA treatment group was optimized. Subsequently, we observed that FA application also elevated the activities of S6PDH, SPS, and SS in leaves. These effects (the variations in photosynthate synthesis-related indices) could be conducive to proving that FA application improves the synthesis of photosynthates (to a certain degree). The greater leaf ^{15}N

distribution rate and N-metabolizing enzyme activity caused by FA treatments might explain why the synthesis of photosynthates in leaves was promoted under FA application conditions (to a certain degree), due to the close relationship between C and N metabolism. Similar results were also obtained by Tian et al. [31] and Yu et al. [13]. In addition, we observed that, although the photosynthates synthesis-related indexes were improved, the ^{13}C distribution rate in leaves under FA treatments was lower than that of CK, according to the results obtained by 13C labeling. This effect could be closely related to the increase in the amount of photosynthates that are transferred from leaves to fruits.

In addition to the production of photosynthetic products (the synthesis of photosynthates) in leaves, the effective transfer of photosynthates to fruits is also a key factor that affects the biosynthesis of anthocyanin in peel. In this study, we found that, compared with that under CK, the ^{13}C distribution rate in fruits under FA treatment increased, indicating that the accumulation of photosynthates in fruits under FA application conditions increased to a certain degree. Subsequently, we analyzed the variations in *MdSOT*s and *MdSUT*s. The results showed that FA application upregulated the expression of *MdSOT1*, *MdSOT3*, *MdSUT1* and *MdSUT4* in fruit stalks, thus indicating that the transport efficiency of sugar (sorbitol and sucrose) was improved by FA. The reason for this effect may be related to the increase in nutrient (such as potassium) absorption under FA treatment conditions [39]. This result might explain why the FA treatments led to a lower ^{13}C distribution rate in leaves and a greater ^{13}C distribution rate in fruits than the CK treatment. Photosynthate transported to the fruit undergoes sugar metabolism catalyzed by a series of sugar-metabolizing enzymes [40]. The activity of sugar-metabolizing enzymes, which are important factors that affect sink activity, could reflect the ability of sink organs to compete with the photosynthates produced by source organs (to a certain degree) [15]. In this study, the activities of SDH, SOX, SS-c, AI, and NI in fruits were measured and were elevated by FA application (except for SOX), indicating that the processes of sucrose and sorbitol decomposition were facilitated. However, no significant difference was observed in the change in SOX activity among all of the treatments, which was in conjunction with the results obtained by Tian et al. [31]. The reason for these findings may be the special form of SOX and its minor activity at the fruit maturity stage [41]. Sha et al. [2] and Wang et al. [37] noted that the overaccumulation of N in fruit limits the transport of sugar to fruit, thus decreasing the accumulation of sugar in fruits. Additionally, Tian et al. [31] reported that a greater N distribution in fruits could decrease the enzyme activities involved in sugar metabolism, thus decreasing competition for photosynthates in fruits. Therefore, the lower ^{15}N distribution rate of fruits caused by FA application could also be conducive to explaining why FA treatment had a greater effect on the ^{13}C distribution rate and sugar content of fruits than no FA treatment. Overall, we concluded that FA application could enhance the "source" (leave) strength and the "sink" (fruit) strength, as well as *MdSOT* and *MdSUT* expression, thus ultimately improving sugar accumulation in fruits, which could provide the basis for anthocyanin biosynthesis in the peel.

In addition to the potential benefits of FA application from the perspective of C and N metabolism, the anthocyanin content is also closely related to the expression of two types of genes, structural genes and transcription factors (TFs) [42,43]. In this study, we observed that, compared with CK, FA treatment upregulated the expression of *MdCHS*, *MdF3H*, *MdMYB1*, and *MdBZIP44*, and the anthocyanin content also increased. The reason for these findings may be related to the changes in the N signal and sugar signal, and the specific mechanism must be further studied.

5. Conclusions

In summary, an appropriate FA supply is beneficial for the biosynthesis of anthocyanins in the peel (Figure 8). In this study, our results showed that an appropriate FA supply level (i) elevated apple tree NUE and decreased N fertilizer loss; (ii) optimized N distribution in leaves and fruits; (iii) promoted the synthesis of photosynthates in leaves and the transport of photosynthates from leaves to fruits; and (iv) upregulated anthocyanin

biosynthesis-related gene expression. Comprehensive analysis demonstrated that appropriate (150 kg ha^{-1}) FA application can coordinate the C-N metabolism of trees and optimize C and N partitioning, ultimately improving anthocyanin biosynthesis in the peel. The results of our study provide a scientific basis for improving NUE and apple appearance quality in orchards.

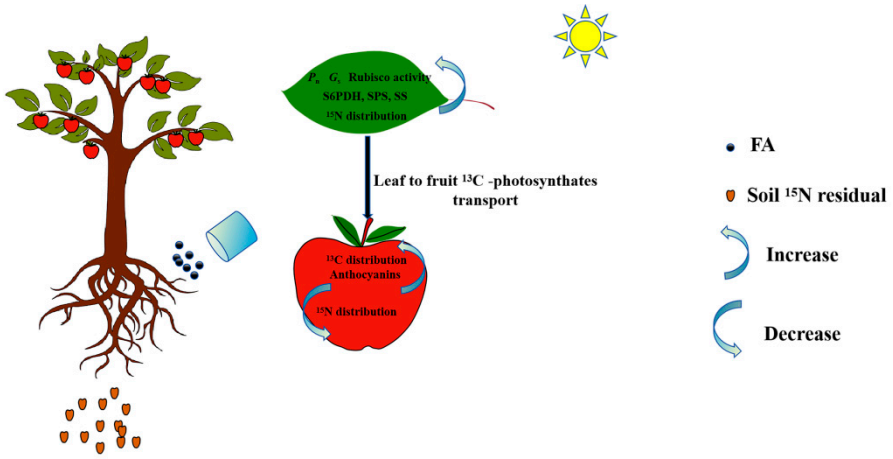

Figure 8. Schematic model displaying the role of FA-mediated improvement in the increase in anthocyanin content in red-skinned apple fruits.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14092062/s1. Table S1: Basic physicochemical properties of the experimental soil. Table S2: Primers used for qRT-PCR.

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