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RESEARCH ARTICLE

Nitric Oxide Treatment Improves the Antioxidant Capacity While Inhibiting Anthocyanin Biosynthesis of Peach Fruit ('Xiahui-8') During Ripening

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Abstract: Nitric Oxide (NO) is an important bioactive signal molecule that plays an important role in diverse physiological processes. This study aimed to explore the effects of Nitric Oxide (NO) treatments on postharvest peach fruit. Specifically, we examined changes in quality attributes, antioxidant ability, and anthocyanin profiles during storage, given the important role of NO as a bioactive signal molecule in physiological processes. The peaches (cv. Xiahui-8) were subjected to a 3-hour fumigation with 10 μL L^{-1} Nitric Oxide (NO) gas, followed by storage at room temperature (25 ± 2 °C) for 8 days. This treatment enhanced the fruit's antioxidant capacity by suppressing free radical (H_2O_2, $O_2^{\cdot-}$) and MDA generation and activating the activities of antioxidant enzymes (SOD, CAT) as well as their gene expression. Conversely, this treatment reduced red coloration intensity of peaches by repressing anthocyanin synthesis and the increase in colorimeter a* value. The content of pelargonidin 3-O-rutinoside appears to play a critical role in red pigment formation in peach fruit. This observation is supported by LC/MS and qPCR analyses, which revealed that anthocyanin levels and the transcript abundance of their structural genes were concomitantly reduced in NO-treated fruit. These findings collectively reveal a novel regulatory mechanism through which NO application influences anthocyanin synthesis in peach.

Keywords: Nitric Oxide, Anthocyanin, Ethylene, LC/MS, Gene Expression

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Introduction

Peach (Prunus persica L. Batsch) is cultivated worldwide as a popular fruit, prized for its appealing flavor, nutritional benefits, and attractive appearance. However, it undergoes immediate programmed senescence after harvest, triggering a series of biochemical and physiological changes which collectively lead to quality deterioration. Owing to their fragile skin and succulent flesh, peaches are susceptible to decay at high storage temperatures, resulting in significant commercial losses. Therefore, increased awareness of the commercial and nutritional values of peaches, extended to other fruits, has called for more in-depth research focused on postharvest biotechnological solutions.

Among the conserved developmental signals associated with post-harvest treatments, the participation of NO signal has attracted intense interest from researchers in recent years. Nitric oxide (NO), a small and highly diffusible free radical, is

involved in numerous physiological processes [1]. NO treatment has emerged as an effective method to extend the storage life of fruits and vegetables by directly inhibiting pathogenic infections and enhancing host resistance [2]. However, the effect of NO treatment on attributes such as fruit coloration has rarely been mentioned in previous studies.

The coloration of peach fruit, a key appearance quality attribute, is primarily governed by anthocyanins. Notably, these compounds are excellent natural pigments with significant physiological functions, which play major roles not only in color formation, reproduction, but also increase resistance to various abiotic and biotic stresses. Anthocyanins are increasingly recognized for their health-promoting properties, consequently making their metabolism a key target for both research and breeding programs [3]. The anthocyanin biosynthetic pathway is well-established, with its key enzymes thoroughly characterized (Fig. 1). In addition to encoding structural genes, anthocyanins are also regulated by various environmental or nonenvironmental factors, and series signals have been reported to promote anthocyanin synthesis. It was found that low temperatures (4–10 °C) upregulated the expression of structural genes involved in anthocyanin accumulation in blood oranges [4]. 1-MCP and UV-C were reported to be effective procedures that employed to induce anthocyanin accumulation in postharvest peaches [5, 6]. However, there have also been reports indicating that post-harvest treatments with auxin and methyl jasmonate can inhibit the synthesis of anthocyanins in red raspberries [7]. Furthermore, ethylene has also been reported to inhibit anthocyanin synthesis in tomato fruits [8]. This implies that the regulatory mechanisms of different postharvest treatments on anthocyanins are not consistent. To date, no report has explicitly addressed how postharvest Nitric Oxide (NO) treatment affects the synthesis of anthocyanins in peaches. Therefore, the aim of this study is to investigate the preservative effects of postharvest Nitric Oxide (NO) treatment on peaches, while simultaneously investigating its impact on the anthocyanin metabolism. To elucidate the mechanisms underlying the NO treatment effect, we evaluated the expression of key structural genes in conjunction with the levels of anthocyanin-derived compounds across the storage timeline. This research will provide a comprehensive evaluation of the effects of NO treatment on both the preservation and sensory qualities of peach fruit.

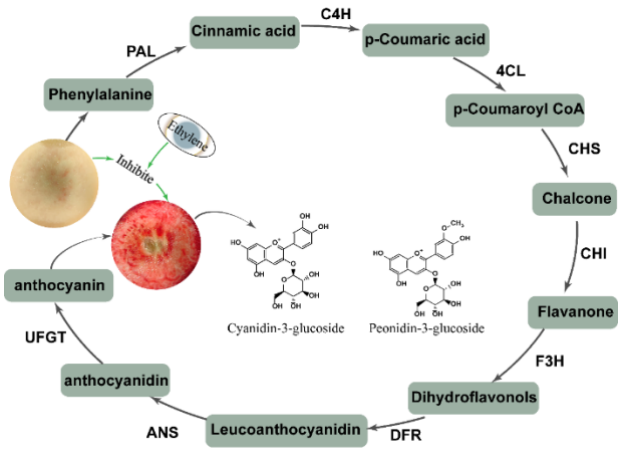

Fig.1: Anthocyanin biosynthesis pathway in fruit and the potential role of ethylene in this pathway

Materials and Methods

Fruit Materials and Treatment

Nitric Oxide (NO), purchased from Nanjing Special Gas Co., Ltd. (Nanjing, China), was delivered to the laboratory in a specific gas cylinder at a pre-prepared concentration of 10 μL L^{-1} for subsequent applications. The 'Xiahui 8' peach, a honey peach cultivar noted for its desirable taste, exhibits a distinct red coloration development as it ripens postharvest. This key phenotypic trait rendered it a suitable model system and was therefore selected for the present investigation. 'Xiahui-8' peaches were picked from local orchards in Nanjing, Jiangsu Province. A total of 300 peaches, uniform in size and shape and free of disease or mechanical damage, were selected and promptly transported to the laboratory. Upon arrival, these freshly harvested fruits (designated as D0) were randomly divided into two groups, each of which underwent distinct treatments. In the Nitric Oxide-Treated (NT) group, approximately 150 peaches were put into an airtight plastic bin and fumigated with 10 μL L^{-1} NO gas for 3 h based on published literatures, then peaches were taken out and stored afterward at room temperature

(25 ± 1°C) [9, 10]. Considering that the shelf life of peaches at room temperature is generally around 7 days, samples were taken every two days after harvest (D2, D4, D6, D8) to monitor the changes in quality indices during fruit ripening. Fruit put in an airtight container without fumigation was taken as control. For subsequent analysis, the collected samples were peeled, diced, snap-frozen in liquid nitrogen, and preserved at −80°C.

Ethylene Production, Firmness and Electrolyte Leakage Measurement

Ethylene production was analyzed according to Cai et al. [11]. Firmness was evaluated with a GY-3 handheld penetrometer (Tuopu, China) using the method of Wu et al. [12]. Electrolyte leakage was quantified following Huan et al. [13].

$O_2^{\cdot-}$, H_2O_2 and MDA Content Determination

MDA content was measured following Wu et al. [14]. O_2 and H_2O_2 levels were assessed using commercial assay kits (Jiancheng, Nanjing) for each analyte, respectively.

SOD and CAT Activity

For enzymatic measurement of SOD and CAT, peach samples were ground into powder with liquid nitrogen, and subsequently measured regarding to published report [15].

Total Phenolics, Total Anthocyanin and Color Determination

To evaluate color parameters, the peel and pulp of ten peaches per group were analyzed at two opposite equatorial sites using a Minolta CR-400 colorimeter (Konica Minolta, Japan) within the CIELAB (L, a, b*) color space. For quantification of total phenolic content, the method described by Kuljarachanan et al. was followed, using a gallic acid standard curve for calibration [16]. Regarding anthocyanin concentration, it was determined spectrophotometrically employing the differential pH method, as outlined by Wu et al. [17].

Phenolic Compounds Analysis Using LC/MS

Samples from the fourth and eighth day of storage were selected for LC/MS analysis due to the rapid changes in anthocyanins at D4 and the final stage of senescence at D8. Briefly, peach tissue was subjected to grinding with liquid nitrogen, extraction with 95% acidic (0.1 M HCl) methanol, centrifugation and evaporation. For LC/MS analysis, we employed the method detailed in our previous research [17].

Gene Expression Analysis

Gene sequences encoding anthocyanin biosynthesis enzymes were retrieved from the Peach Genome Database (www.rosaceae.org). Specific primers were designed using Primer 5.0 software. Translation elongation factor 2 (PpTEF2), known for its stable expression during ripening [18] (Tong et al., 2009), served as the reference gene. All primer sets exhibited amplification efficiencies comparable to PpTEF2, and their sequences are listed in Supplementary Materials. Total RNA extraction, cDNA synthesis, and qRT-PCR were conducted following the established protocol of Wu et al. [17].

Statistical Analysis

Data are means ± SD of at least three biological replicates; different letters indicate significant differences ($P < 0.05$, Student's t-test).

Results

Effects of NO Treatment on Ethylene Production and Firmness of Peach

As shown in Fig. 2A, ethylene biosynthesis increased throughout the storage time, and the values between two groups were not significant except in the middle storage period. NO treatment enhanced ethylene production from D4 to D6. Firmness showed an overall decreasing trend (Fig. 2B), and NO treatment maintained a higher level compared to the control.

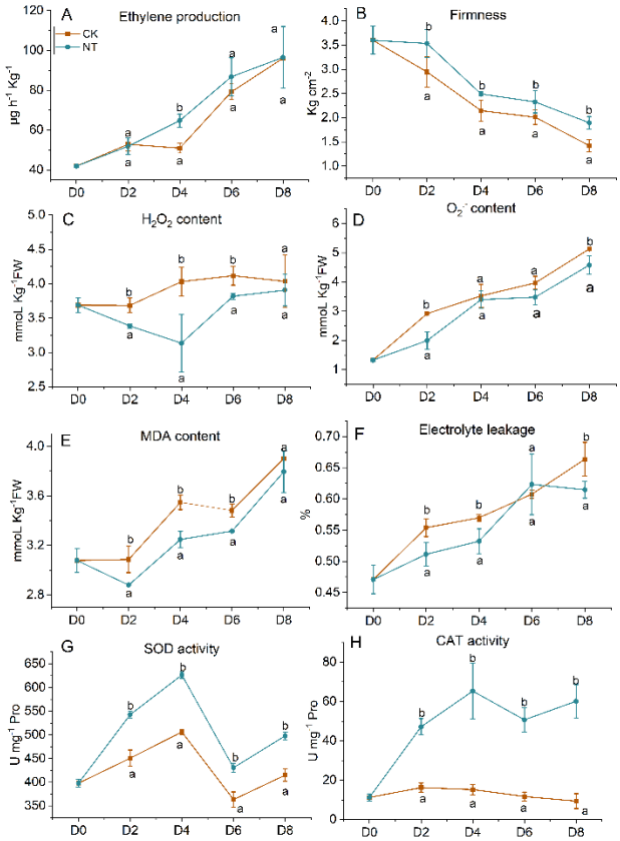

Fig. 2: Physiological and biochemical indices of peach fruit during ripening. Ethylene production (A), Firmness (B), H_2O_2 content (C), O_2.- content (D), MDA content (E), Electrolyte leakage (F), SOD activity (G) and CAT activity (E). Each value represents the mean for three replicates, with vertical bars indicating standard errors. The different lower-case letters indicate significant difference at p≤0.05 by Student's T-test

NO Treatment on Electrolyte Leakage, ROS Production and MDA Content

ROS values and MDA content varied similarly, showing an overall increase with the continuation of the storage period, and NO treatment inhibited ROS and MDA production (Fig. 2C-E). Electrolyte leakage increased continually during fruit ripening (Fig. 2F), which was lower in the NO-treated group than that in the control group, except for the 6th day of the storage period.

NO Treatment on Antioxidant Enzyme Activities

As shown in Fig. 2G-H, SOD and CAT activities exhibited comparable trends in both control and NO-treated peaches, peaking on day 4 before declining and then rising again. Throughout the storage period, NO treatment led to a sustained and significant increase in the activities of both enzymes.

NO Treatment on Color Change, Total Polyphenolics, and Total Anthocyanins

Phenotypically, the flesh color of peach fruit slowly turned red during storage, but NO application inhibited the reddening process as compared with that in control group (Fig. 3A). In terms of chromatic aberration, a* value was also progressively increased during the first stage of the storage period and stabilized from the sixth day onwards. a* value of peach fruit pericarp was not significant between two groups, but NO treatment significantly inhibited increasing trend of that in peach pulp (Fig. 3B-C).

As shown in Fig. 3D, the total phenolic content of the two groups showed an overall upward trend, except for a slight decrease on the third day of the storage period in NO group. Compared with that in control group, the concentration of the total phenolics of peach fruit in the NO group remained high levels from day 2 to day 6, whereas the phenolic content was

suppressed at the end of the storage. Likewise, the anthocyanin content between the CK and NO group showed a similar increasing trend, but NO treatment inhibited anthocyanin synthesis throughout the entire storage period (Fig. 3E).

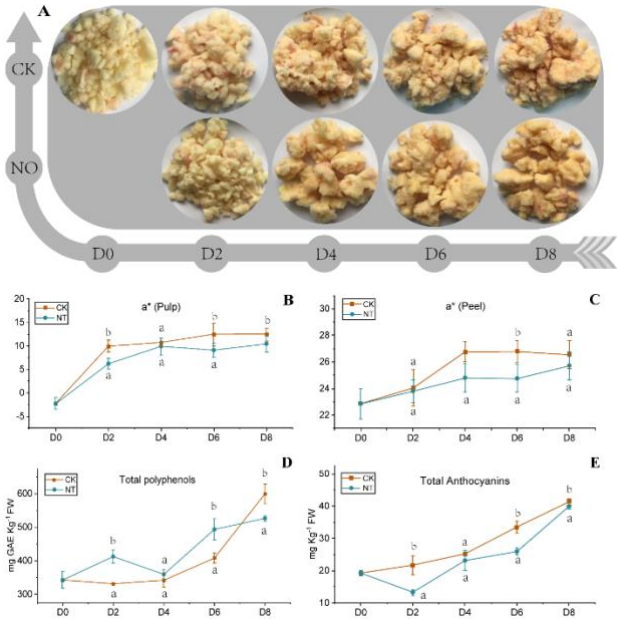

Fig. 3: Phenotype (A), a* value of pulp (B), a* value of peel (C), total polyphenols (D) and total anthocyanins (E), changes of peach fruit. Each value represents the mean for the replicates, with vertical bars indicating standard errors. The different lower-case letters indicate significant difference at p≤0.05 by Student's T-test

NO Treatment on Anthocyanin Derivatives in Peach Fruit

Following LC–MS/MS analysis, six anthocyanin compounds were identified by matching their specific fragments, MS data, and retention times, including pelargonidin 3-O-rutinoside, pigment A, malvidin 3-O-galactoside, cyanidin 3-O-xylosyl-rutinoside, isopeonidin 3-O-rutinoside, and peonidin 3-O-(6"-p-coumaroyl-glucoside). The total abundance of cyanidin-3-O-(6"-p-coumaroyl-glucoside), which showed the highest overall level, was defined as 1 across all time points. The contents of all other anthocyanins were then expressed as relative values normalized to this monomer (Fig. 4). NO treatment significantly reduced the anthocyanin compounds' synthesis of the fruits, especially pelargonidin 3-O-rutinoside and cyanidin 3-O-xylosyl-rutinoside, which were not detected in the NO-treated group. In addition, isopeonidin 3-O-rutinoside and peonidin 3-O-(6"-p-coumaroyl-glucoside) were significantly reduced in the NO-treated group.

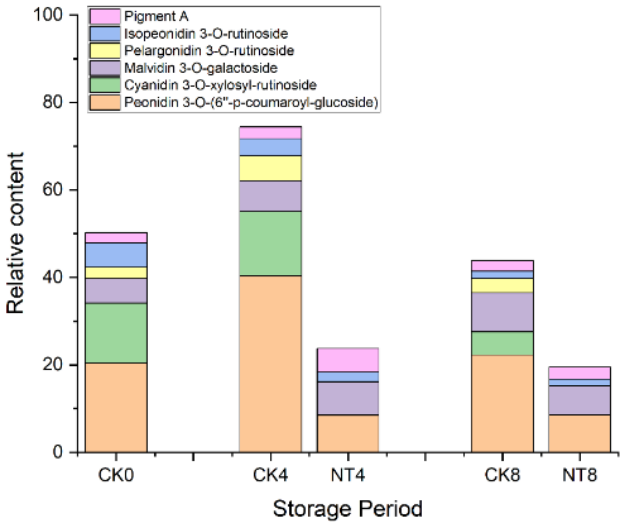

Fig. 4: Relative content of anthocyanin compounds in peach fruit

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Effects of NO Treatment on Gene Expression

The relative expression of PpSOD showed a general increasing trend, and NO treatment remarkably enhanced PpSOD expression on the fourth day of the storage period, while the effect of treatment at other times was not significant (Fig. 5A). PpCAT expression rose to a maximum value on the second day, declined on the fourth day, and then maintained at a stable value until the end of storage (Fig. 5B). At specific time points (days 2 and 4 of storage), the relative expression of PpCAT was up-regulated by NO treatment. The expression of PpPAL continued to decline after a slight increase on the second day of the storage period, and compared to the control, NO-fumigated peaches maintained a higher level of PpPAL expression on storage days 2-4, which then became lower from day 6 onward during ripening. (Fig. 5C). PpCHS and PpCHI showed a similar trend, with an overall decrease during the storage period (Fig. 5D-5E), and values in the NO-treated group exceeded those of the control on the second day. However, from the fourth day of the storage period onwards, NO treatment suppressed the relative expression of these two genes. PpF3H and PpF35H expression continued to decline after rising on the second day of storage time (Fig. 5F-5G), and NO treatment suppressed the expression of these two genes. The relative expression of PpANS and PpUFGT decreased throughout the storage period, and NO treatment significantly suppressed their expression in the later stages of storage (Fig. 5H-5I).

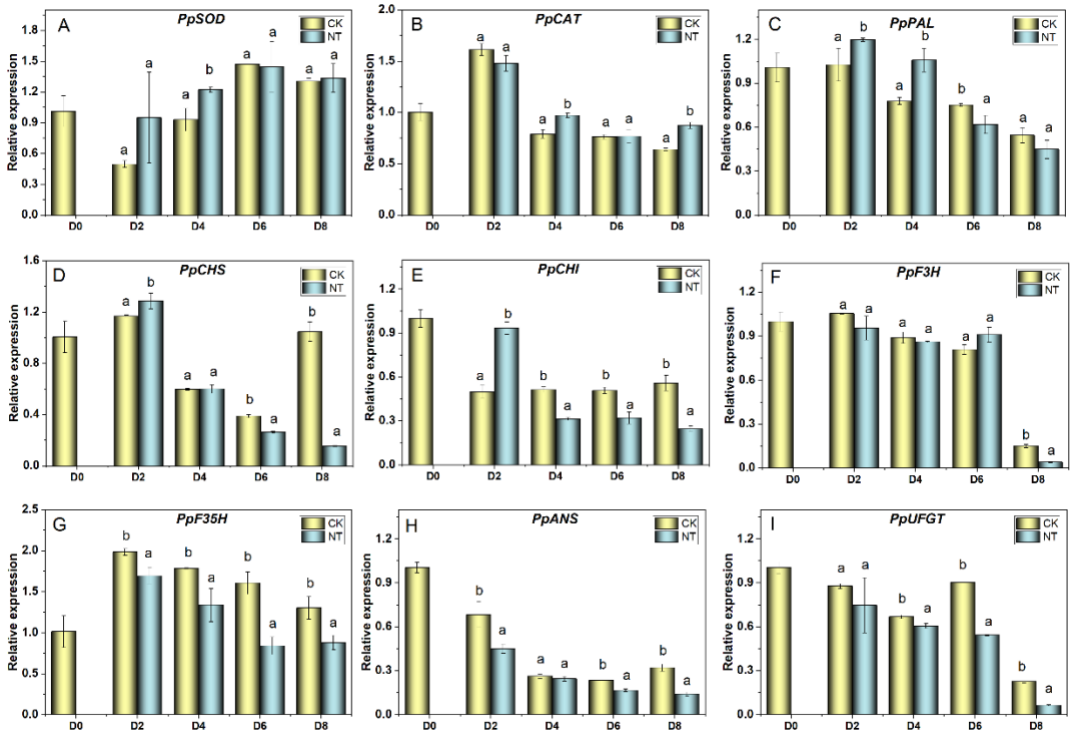

Fig. 5: Gene expression of peach fruit during ripening. Each value represents the mean for three replicates, with vertical bars indicating standard errors. The different lower-case letters indicate significant difference at p≤0.05 by Student's T-test

Discussion

Peach fruit is a typical climacteric fruit, which undergo a rapid and programed ripening accompanied by increased ethylene release and respiration, softening, reddening of color and decay incidence. NO treatment has been widely reported to delay fruit decay during storage and has been demonstrated in fruits such as pear and tomato [2, 19]. Nevertheless, our understanding of how NO treatment modulates color development, in particular during the postharvest phase of peach fruit, remains limited. Furthermore, the mechanisms promoting anthocyanin synthesis have been extensively investigated in prior studies, such as haomethyl jasmonate treatment on Radish Sprouts, UV-B treatment on tomato, low-temperature treatments in blood oranges, but studies about the negative effect of post-harvest treatment on anthocyanin synthesis are rare [20-22]. This study investigated the changes in stress response and anthocyanin metabolism elicited by NO treatment in postharvest

peach fruit (cv. Xiahui-8). Interestingly, we found that NO treatment increased the antioxidant capacity of the fruit while inhibiting anthocyanin accumulation within the fruit ripening period.

Fruit decay in climacteric species is often attributed to, and exacerbated by, a concurrent rise in ethylene synthesis and ROS generation [23]. In our research, it is worth noting that NO treatment did not inhibit ethylene synthesis (Fig. 2A). However, the NO-treated group exhibited higher firmness, consequently enhancing resistance to mechanical damage. This treatment also significantly suppressed ROS accumulation, reduced MDA content and electrolyte leakage. Furthermore, the notable boost in CAT and SOD activities revealed that NO treatment strengthened the fruit's intrinsic antioxidant defense system. These results align with previous reports documenting similar dynamics in ROS levels and antioxidant enzyme activities [24-25]. It should be noted that the tendency of high ethylene level and antioxidant capacity seem to contradict each other, as high antioxidant activity is often accompanied by limited ethylene emission in previous studies. However, a similar regulation pattern was also observed in proteomic analysis of ‘Xiahui-6’ peach fruit, in which NO treatment upregulated ethylene synthase while inducing antioxidant enzyme such as SOD, APX and GST [25]. The positive correlation between ethylene metabolism and antioxidant capacity has also been reported in tomatoes [26] (Steelbeart et al., 2019). Specifically, Shu et al. found that exogenous ethylene application enhanced the fruit's antioxidant capacity and chilling tolerance [27]. The results revealed a complex and dynamic relationship between NO, ethylene molecules, and plant metabolism.

Anthocyanins, which are key water-soluble pigments, determine the coloration of numerous fruits and flowers. In addition to their visual role, they provide beneficial qualities to plants owing to their antioxidant properties [28]. In the present study, the total anthocyanin content was found to increase continuously throughout the storage period (Fig. 3E), a trend that corresponded with the apparent reddening of the peach fruit. Nevertheless, NO treatment inhibited anthocyanin synthesis, and this changing pattern was further corroborated by changes of a* values of both peel and pulp in the NO-treated group (Fig. 3B-3C). NO application was preliminarily found to inhibit anthocyanin accumulation from peach phenological and total anthocyanin indices. Therefore, we decided to focus on deciphering the role of this inhibitory effect in depth at the metabolic and gene expression levels. Six anthocyanin substances were successfully identified by LC/MS assay (Fig.4), and cyanidin 3-O-xylosyl-rutinoside and peonidin 3-O-(6’’-p-coumaroyl-glucoside) were the main components of anthocyanin in terms of their relative expressions. This finding is consistent with the published literature, which stated that the distribution of cyanidin and peonidin and their derivatives occupies a major portion in the edible parts of plants [29]. Notably, two specific anthocyanin derivatives, pelargonidin 3-O-rutinoside and cyanidin 3-O-xylosyl-rutinoside, were strikingly undetectable in the NO-treated group, underscoring the potent inhibitory effect of NO on their synthesis (Fig. 4). Previous research has established that pelargonidin-based anthocyanins impart orange to red coloration in horticultural produce [30]. Therefore, we hypothesize that the decreased content of pelargonidin 3-O-rutinoside in this study is detrimental to the formation of red pigment in the NO-treated group, which is in accordance with the phenotype in peach fruit.

From the perspective of gene expression (Fig. 5), NO treatment inhibited PpPAL expression from day 6 to the end, which is consistent with the lower total polyphenol content observed on day 8 in the NO group (Fig. 3D). During early storage, key genes (PpPAL, PpCHS, and PpCHI) showed higher expression in NO-treated peaches than in the control (Fig. 5C-5E), and we hypothesized that the enhanced antioxidant capacity in NO-treated peaches may be attributed to the observed higher accumulation of polyphenols or flavonoids, thereby boosting the fruit’s overall antioxidant potential [31]. Notably, NO treatment inhibited the expression of genes in downstream pathways of anthocyanin synthesis, such as PpF35H, PpANS and PpUFGT, and this suppression effect was pronounced during the later storage period (Fig. 5G-5I). Overall, in terms of gene expression in anthocyanin synthesis pathway, NO treatment was reconfirmed to prevent anthocyanin accumulation.

To summarize, this study demonstrates that NO treatment inhibits anthocyanin biosynthesis in postharvest peaches, affecting gene expression, metabolite levels, and fruit coloration. We hypothesize that this inhibition may be mediated through ethylene metabolism. Ethylene appears to have a dual role in regulating anthocyanin accumulation: on one hand, it is typically known to promote accumulation during ripening [32]; on the other hand, emerging evidence reports an inhibitory function in some species, notably in pear [33], black rice [34], and peach [5]. For instance, in Fragaria chiloensis fruit, ethylene downregulated key genes and reduced pigment synthesis, aligning with our observations [35].

In the present study, NO treatment generally activated ethylene release during the ripening stage of ‘Xiahui 8’ peach fruit. Similar results were observed about positive effect of NO on ethylene release has been mentioned in published literatures. Kang et al. performed proteomic analysis of NO-treated peaches and revealed that NO fumigation significantly elevated 1-Aminocyclopropane-1-carboxylate oxidase (ACO) and S-adenosylmethionine synthetase (SAM) to 5.01-fold and 2.42-fold, respectively [23]. Critically, NO itself is a known inducer of ethylene biosynthesis, as evidenced by its upregulation

of *ACS2* gene expression and elevation of ethylene levels [36], this parallels findings from studies on the breaking of embryo dormancy in apple [37]. The variation in results across studies may be attributed to differences in the underlying genetic mechanisms that regulate coloration, as well as distinct genetic backgrounds. Considering the results of the current study, for 'Xiahui-8' peaches, NO treatment hampers anthocyanin accumulation by enhancing release of ethylene, which further prevented the reddening of the fruit coloration. This inhibitory effect of NO is not unique to peach. A similar delay in reddening and lower anthocyanin content was observed in NO-treated Chinese winter jujube [38]. Furthermore, in pistachios, NO treatment was found to inhibit flavonoid synthesis and reduce the activity of phenylalanine ammonia-lyase (PAL), which would adversely affect anthocyanin biosynthesis [39].

The metabolic pattern resulting from NO application, in terms of regulating anthocyanin synthesis via ethylene metabolism, was similar to that induced by 1-MCP. This is notable because 1-MCP functions by blocking ethylene perception, which typically promotes anthocyanin synthesis in peach, whereas NO appears to act differently within the ethylene pathway [5, 17]. Likewise, Zhang et al. treated peach fruit with ethylene and found that anthocyanin content was significantly reduced [5]. Overall, in this study, NO application inhibited anthocyanin synthesis by promoting ethylene emission in peach fruit. The detailed governing mechanism between NO and anthocyanin biosynthesis, with special reference to the metabolism of cyanidin and pelargonidin and their derivatives, requires further investigation. While this study has preliminarily revealed a potential relationship between ethylene and anthocyanin synthesis, whether anthocyanin biosynthesis is regulated by other plant signaling molecules, such as abscisic acid (ABA) or jasmonate, remains to be further explored.

Conclusion

In summary, this work demonstrated that NO treatment strengthened fruit antioxidant capacity by activating antioxidant enzyme, inhibiting free radical accumulation, which contributed to delay of fruit senescence. In postharvest peaches, however, anthocyanin accumulation was reduced by NO treatment, which was associated with the downregulation of key upstream structural genes. This further hampered the biosynthesis of specific anthocyanins, including cyanidin and pelargonidin derivatives, which may consequently have an adverse effect on fruit sensory quality. These results provide a new perspective on the pattern of anthocyanin synthesis affected by NO and ethylene signals in fruits. The ethylene-responsive transcription factors associated with anthocyanin accumulation, the genetic links among pathways of NO generation are needed to identify and characterize more. Future research will focus on comprehensively evaluating the impact of NO treatment on various attributes of peach fruits or seeking an optimal balance between extending storage life and promoting anthocyanin accumulation.

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Author's Contributions

Mrs Z. W. is responsible for the determination of antioxidant indices in peach fruit. Mrs Y.L. was involved in the determination of anthocyanin-related indices, including those in Figure 3 and Figure 5. Mrs.Y.D. is involved in data curation and statistical analysis. Mrs. C.Z. specializes in the design and beautification of graphs, and she is responsible for the data analysis, and the design and plotting of Figure 3. Mrs. Y.Z. is responsible for data curation. Dr. X.W. is responsible for the experimental design, and the organization of the experiments.

Ethics

The authors declare no competing interests.

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