

Article

Response of Abscission Zone of Blue Honeysuckle (*Lonicera caerulea* L.) Fruit to GA₃ and 2,4-D Spray Application

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Abstract: The nutritional value of blue honeysuckle (*Lonicera caerulea* L.) fruit is abundant; however, its production faces challenges due to a short harvesting period and fruit drop issues. In this study, the effects and potential mechanisms of two different plant growth regulators, GA₃ (Gibberellins acid) and 2,4-D (2,4-Dichlorophenoxyacetic acid), on blue honeysuckle fruit abscission and abscission layer formation were investigated. The main cultivated variety of blue honeysuckle, ‘Berel’, was used as the experimental material. GA₃ and 2,4-D were sprayed on the plants from the veraison. The anatomical structure of the fruit abscission zone (FAZ) was observed after treatment. Key enzymes involved in cell wall degradation, pectinase, cellulase, polygalacturonase, and pectin methylesterase, were analyzed for their activities. Furthermore, the gene expression levels of cell wall detachment-related genes *CX1*, *CX2*, *PL20*, *PE*, and key genes for gibberellin and ethylene synthesis *GA2OX1*, *GA1*, *ACO*, and *ACO3* were examined. The results indicated that the application of GA₃ and 2,4-D could delay the formation of the abscission layer. In the FAZ treated with GA₃ and 2,4-D at 5 DAT, the activity of key enzymes involved in cell wall degradation decreased, the expression of genes related to cell wall degradation enzymes and key genes for ethylene synthesis was inhibited, and the drop of fruit reduced. In conclusion, exogenous application of GA₃ and 2,4-D suppresses the abscission of ‘Berel’ blue honeysuckle fruit, likely through the inhibition of cell wall degradation and abscission layer formation.

Keywords: *Lonicera caerulea*; GA₃; 2,4-D; abscission; abscission zone



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1. Introduction

Lonicera caerulea L., commonly known as blue honeysuckle, is a shrub plant belonging to the family Caprifoliaceae, genus *Lonicera* Linn. Its wild resources are mainly distributed in regions such as Xinjiang and the Greater and Lesser Khingan Mountains and Changbai Mountains in Northeast China. It is also found in Russia, Japan, and other regions [1]. The fruits of *Lonicera caerulea* can be processed and consumed fresh [2]. They are nutritionally rich, containing various nutrients such as flavonoids, anthocyanins, and polyphenols [3]. The fruits also possess antioxidative, blood glucose-lowering, and hepatoprotective effect [4], making them highly valuable in terms of nutrition and economics. With its unique flavor, blue honeysuckle has broad market prospects as a medicinal and edible berry [5]. However, in the development of the blue honeysuckle industry, factors such as the short harvesting period, fruit detachment during harvesting, and inconsistent ripening periods of

different varieties pose challenges, greatly affecting its economic and social benefits. Therefore, studying how to prolong the harvesting period of blue honeysuckle fruits and control their ripening and abscission is of great significance for the promotion and development of the blue honeysuckle industry.

Fruit abscission in tree organs can be summarized as the process of abscission zone formation, activation of abscission signals, enzymatic hydrolysis in the middle lamella of the abscission zone triggering abscission, and subsequent differentiation to form a protective layer [6]. The process of enzymatic hydrolysis in the middle lamella of the abscission zone is closely related to cell wall-degrading enzymes. Cellulose is the main component of the cell wall, and cellulases (Cx) and β -glucosidases (β -glu) play a role in cellulose degradation [7]. Polygalacturonases (PG) and pectin methylesterases (PME) play important roles in pectin degradation in the primary cell wall and middle lamella. These pectinases cause cell separation by disrupting cell adhesion [8], ultimately leading to fruit abscission.

The key to extending the harvesting period of blue honeysuckle lies in delaying the abscission of ripe fruits. Currently, the commonly used method to inhibit excessive fruit abscission in fruit trees is through exogenous application of plant growth regulators. GA₃ have been widely applied to delay fruit abscission in fruit tree crops. GA₃ plays an important role in the growth and development of fruit trees and is widely used for increasing yield and fruit retention [9]. Studies have shown that exogenous application of GA₃ significantly delays the abscission of citrus and sweet cherry fruits [10,11]. The correlation between endogenous auxin levels and fruit abscission has been demonstrated in research [12]. 2,4-D, as auxin-type plant growth regulators, are also used to delay fruit abscission. Exogenous application of 2,4-D effectively controls preharvest fruit drop in citrus, pomegranate, and other fruit trees [13,14]. Apart from exogenous application of GA₃ and 2,4-D, spraying naphthaleneacetic acid (NAA) has also been used to control fruit drop in most fruit trees. Exogenous application of 20 ppm NAA reduced fruit drop in sweet cherries [10].

Regarding the delayed fruit abscission in blue honeysuckle through exogenous application of GA₃ and 2,4-D, previous studies have determined the optimal concentrations for delaying fruit abscission [15]. However, the underlying mechanism of how GA₃ and 2,4-D delay fruit abscission in blue honeysuckle remains unclear. In this study, using the 'Berel' cultivar of blue honeysuckle, we aim to investigate the possible mechanisms of delayed fruit abscission through exogenous application of GA₃ and 2,4-D, with a focus on exploring the roles of GA₃ and 2,4-D in influencing abscission zone formation and gene expression. This research provides theoretical references for delaying fruit abscission and achieving efficient production in blue honeysuckle.

2. Materials and Methods

2.1. Plant Materials and Experimental Treatments

The experiment was conducted in the germplasm resource nursery of Northeast Agricultural University in Harbin, Heilongjiang Province, China, from May to August 2022. The main cultivar used for the experiment was 'Berel' blue honeysuckle. We selected 10 plants of each treatment and control for this study. Selected experimental plants had relatively consistent growth vigor and were planted in an east–west direction. The orchard was managed following regular irrigation and fertilization practices.

Starting from the flower drop stage, 10 different plants of 'Berel' were selected for fruit marking and observation. The concentrations of GA₃ and 2,4-D used for the experiment were determined based on previous hormone treatment results for 'Berel' blue honeysuckle. The GA₃ treatment concentration was set at 75 mg·L^{−1}, and the 2,4-D treatment concentration was set at 100 mg·L^{−1}. Spraying was carried out on sunny mornings, covering the entire plant, and water was sprayed as control. The first spraying was performed from the veraison (11 June 2022, 47 days after the peak flowering period), and the spraying was carried out continuously for three days. Each treatment had 10 biological replicates.

Fruit abscission zone samples were collected starting from the day before treatment as day 0. Fruits with relatively uniform maturity and no mechanical damage were harvested every 5 days. After harvest, the abscission zone tissue at the fruit pedicel junction was immediately separated. Sampling was performed five times. The materials were collected at 0, 5, 10, 15, and 20 days after treatment (DAT). Forty fruits were randomly selected from each treatment, with fruit stems approximately 0.5 cm long. The sampled tissues were used for anatomical observations of the fruit abscission zone and were also divided into two parts. One part was frozen in liquid nitrogen and stored at -80°C for measuring the activity of abscission-related enzymes and analyzing gene expression.

2.2. Fruit Drop Evaluation

Before the treatment, three branches at different angles per tree were tagged for the subsequent evaluation of fruit drop. Fruits on the tagged shoots were counted at 0, 5, 10, 15, 20, 25, 30, and 35 DAT. Fruit drop rate was calculated using the following formula:

$$\text{Fruit drop rate(\%)} = \frac{\text{Number of total dropped fruits per bunch}}{\text{Number of total fruits per bunch}} \times 100\%$$

2.3. Preparation of Abscission Zone Paraffin Sections

For each sample point, six fruit abscission zone samples of each treatment and control group were randomly collected and used for paraffin section analysis. The abscission zone was dissected using a scalpel, including the upper and lower 0.4 cm regions of the fruit pedicel to fruit flesh. The dissected samples were immediately fixed in FAA fixative solution (70% ethanol:formaldehyde:acetic acid with a volume ratio of 90:5:5) for 24 h with following standard procedures [16]. Dehydrated through a series concentration of ethanol (70%, 85%, 95%, 100%, and 100%, each for 2 h, respectively). Transparent in xylene for 4 h (replace with new xylene every 1 h), and embedded in paraffin. The longitudinal sections were 8 μm thick cut with a rotary microtome (Leica HistoCore AUTOCUT, Wetzlar, Germany). In addition, the sections were fixed on slides and stained with safranin O and fast green for 10 min. Lastly, the well stained sections were sealed with resins and coverslips. The sections were observed under an inverted microscope (Leica DM IL LED, Wetzlar, Germany) at $\times 5$ and $\times 40$ magnitudes. Representative sections with typical structures were selected for photography.

2.4. Measurement of Abscission-Related Enzyme Activity in the Abscission Zone

For enzyme activity analysis, fruit abscission zone samples from 'Berel' blue honeysuckle treated at each sampling point (0, 5, 10, 15, and 20 DAT) were collected. Samples were randomly sampled for each treatment and control group. The tissues from the upper and lower 0.4 cm regions of the fruit pedicel were cut and immediately frozen in liquid nitrogen. The frozen samples were stored at -80°C . For enzyme activity measurement, the samples were placed in a mortar and ground with liquid nitrogen. Approximately 0.5 g of the powdered sample was weighed and added to 4.5 mL of $\text{pH} = 7.2\text{--}7.4$ $0.01 \text{ mol}\cdot\text{L}^{-1}$ PBS. The homogenate in the EP tube was centrifuged at 4°C and 4000 rpm for 20 min. The supernatant was collected and stored in a refrigerator at 4°C for further use. Enzyme activity was measured using the Cx assay kit, plant β -glu assay kit, plant PG assay kit, and plant PME assay kit from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). Each enzyme activity index was measured in triplicate for each sample.

2.5. Gene Expression Analysis

For gene expression analysis, fruit abscission zone samples from 'Berel' blue honeysuckle treated at each sampling point (0, 5, 10, 15, and 20 DAT) were collected. Twenty fruits abscission zone with approximately 0.5 cm long fruit stems were randomly sampled for each treatment and control group. The tissues from the upper and lower 0.4 cm regions of the fruit abscission zone were cut and immediately frozen in liquid nitrogen. The frozen

tissues were ground to a powder using a mortar and pestle. Total RNA was extracted using the plant RNA extraction kit (Omega Bio-Tek, Norcross, GA, USA), genomic DNA was removed, and cDNA was synthesized using the ReverTra Ace™ qPCR RT Master Mix With gDNA Remover kit (TOYOBO, Osaka, Japan). Based on the transcriptome database of Berel blue honeysuckle fruits abscission zone [17], eight genes related to fruit abscission were selected. Sequences were obtained from the transcriptome database and ORF were found through NCBI (<https://www.ncbi.nlm.nih.gov/orffinder/> (accessed on 19 December 2022)). Premier 5 was used to design the primer pairs for RT-qPCR. The qRT-PCR was performed in a 20 µL reaction system on qTOWER³G cycler (Analytik Jena AG, Jena, Germany): 2 µL cDNA, 0.8 µL forward primer (10 µM), 0.8 µL reverse primer (10 µM), 10 µL SYBR Green I Master (2×), and 6.4 µL ddH₂O. The reaction program consisted of 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 15 s, followed by a final termination step at 4 °C. Basic data processing was performed with qPCRsoft 4.1 software (Analytik Jena AG, Jena, Germany). The relative expression levels of the genes were calculated using the 2^{−ΔΔCT} method with β-Actin as the reference gene. Three RNA extractions and three RT-qPCR reactions were performed for each sample to account for biological and technical replicates. The primers for the target genes were list in Table 1.

Table 1. Specific primers used for gene expression analysis by Real-time quantitative PCR.

Gene Name	Forward Primer Sequence (5′–3′)	Reverse Primer Sequence (5′–3′)
β-Actin	ACCTGCTGACGAGTGCCGATAC	TCACCCCTTGAAACATCAGGAGACCA
CX1	TCGTTCGTCCTTTCTTCC	TAGCCATCAGCAGTCCAGTC
CX2	CTCCTATTCCCATACACTCC	CATCGTCGTATCCACTAACA
PE	AAAGTTTGATTGCTGGAGG	GGGTGGTGTGGCTTATT
PL20	ATAGCACCGAAAGGAGAAA	TATGGAACAGTCCGCAAGG
GA2OX1	AACGTGTATGGGCTTCTCA	ACCCTATCGCTATGTATGT
GA1	GCAAAGGTAGTTCCCGTCTA	TCATCCTCCACAATCTCG
ACO	GTACGAAGAGGGTGGTGT	CTGGATTGGGAGGATTAG
ACO3	GAGCCATCATAAGGAGGT	TCTCGCCCAATACACTAA

2.6. Data Analysis

Graphs were generated using Excel 2016 software, and correlation analysis was performed using SPSS 26 software. Three biological replicates were conducted for each treatment, and the mean ± standard error was calculated. One-way analysis of variance (ANOVA) was used to assess the differences between the control and the GA₃ and 2,4-D treatments in the fruit abscission zone at the same time points after treatment. Different lowercase letters indicate significant differences (*p* < 0.05) among each treatment (control, GA₃, and 2,4-D) within each evaluation time point.

3. Results

3.1. Effects of 2,4-D and GA₃ Treatments on ‘Berel’ Fruit Drop Dynamics

The data (Figure 1G) clearly showed that natural fruit drop occurred throughout the period after veraison (11 June 2022). Compared to the control group, both exogenous sprays significantly affected the fruit drop rate. The ‘Berel’ fruit drop rate displayed prominent peaks at 5 and 25 DAT. At 5 DAT, the control group exhibited a drop rate of 39.17%, while the GA₃-treated and 2,4-D-treated groups recorded rates of 22.90% and 11.89%, respectively. Notably, both the GA₃ and 2,4-D treatments considerably decreased fruit abscission in comparison to the control group. At 25 DAT, the second peak manifested. The fruit drop rates were 51.19% for the control group, 44.83% for the GA₃-treated group, and 32.87% for the 2,4-D-treated group. Both GA₃ and 2,4-D treatments significantly reduced fruit abscission compared to the control group. At 35 DAT, a significant majority of ‘Berel’ fruits in the control group had naturally fallen, with a drop rate reaching 94.22% (Figure 1A), and the unfallen fruits showed signs of dehydration and wrinkling (Figure 1D). In contrast,

many fruits in the GA₃ and 2,4-D treatment groups remained on the tree and did not fall off (Figure 1B,C). Among them, the 2,4-D treatment group had the most unfallen fruits (Figure 1C), and each result branch had more unfallen fruits than the control group (Figure 1F). Similarly, the 2,4-D treatment group reported the lowest drop rate at 72.38%, while the GA₃ treatment group stood at 87.95% (Figure 1G). Both treatments showcased a notable reduction in fruit abscission. Moreover, from veraison to late ripening, both GA₃ and 2,4-D spray treatments consistently ensured lower fruit drop rates for blue honeysuckle compared to the control group, except that at 15 DAT, where no significant difference in fruit drop rate existed between the control and GA₃-treated groups (Figure 1A). In terms of delaying fruit shedding rate, 2,4-D treatment was better than GA₃.

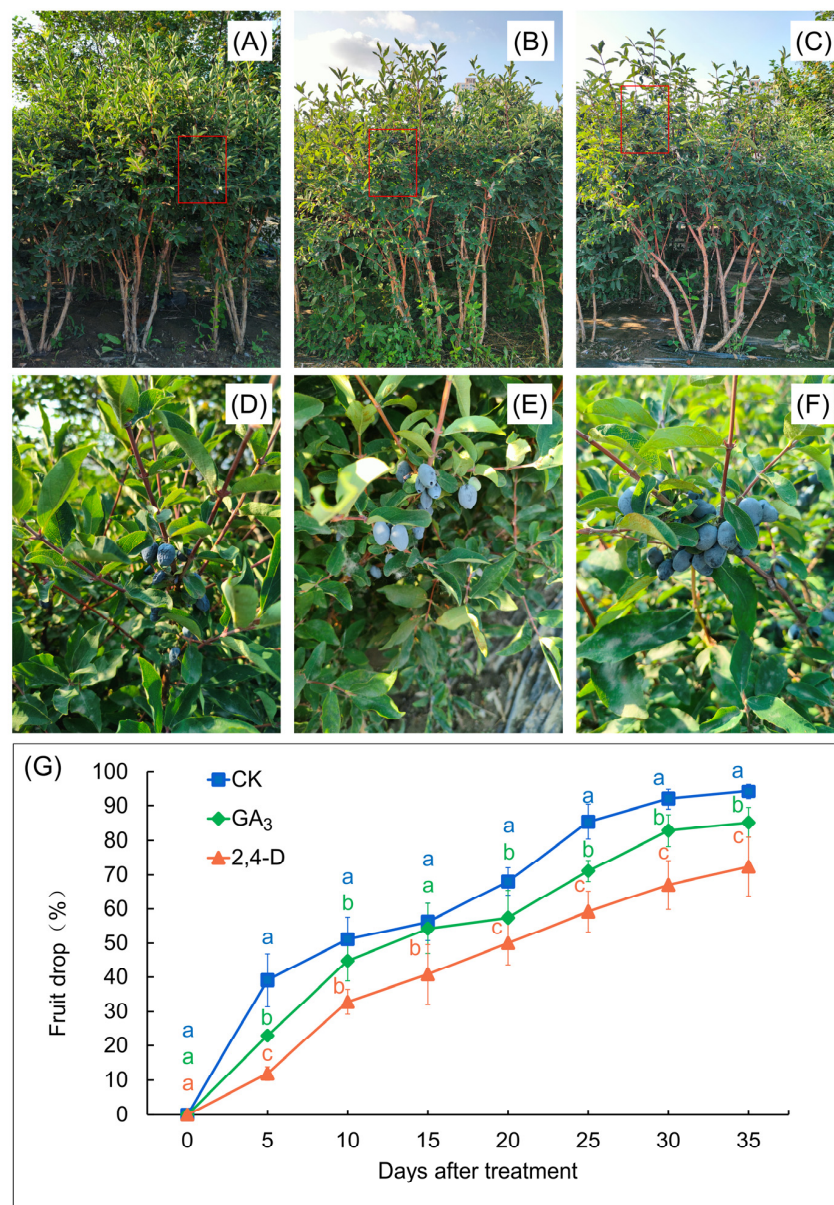


Figure 1. Effects of blue honeysuckle fruit abscission on ‘Berel’ after 35 days of different treatments and effect of different treatments on fruit drop rate of ‘Berel’ blue honeysuckle fruit (G). (A) Control. (B) GA₃ treatment. (C) 2,4-D treatment. (D–F) Magnification of the portions of (A–C) enclosed in a red frame, respectively. Data are mean ± SE. Different letters represent significant differences between different treatment at each time point ($p < 0.05$). Same letters represent not significant differences.

3.2. Effects of 2,4-D and GA₃ Treatments on Fruit Abscission Zone Formation

The FAZ cells in the three treatments of blue honeysuckle all underwent a sequence of changes. Initially, at 0 DAT, both treatment groups exhibited the FAZ cell structure that was tight and evenly distributed. At 5 DAT, the FAZ cells in the control group became loose and showed slight cellular deformation. The schizogenous process occurred when these changes in the control group. Intercellular cavities also appeared during schizogenous process in the control group. In contrast, the GA₃-treated group only showed slight cellular deformations in the FAZ with no apparent schizogenous process. The 2,4-D-treated FAZ cells remained densely packed and uniformly distributed. At 10 DAT, the intercellular gaps in the control group became larger, with more pronounced intercellular cavities than observed at 5 DAT. At 10 DAT, in the control group, cells in the FAZ ruptured, indicating that AZ cells began to degrade. The FAZ cells deformed further, revealing a deeper indentation between the fruit and its stalk, along with multiple small cracks at the connecting vascular bundles of the stalk-fruit. These changes weakened the connection between the fruit and its stalk, culminating in fruit abscission. For the GA₃-treated group, cells in the FAZ remain more relatively intact. Moreover, the indentation between the fruit and its stalk in the GA₃-treated group was less pronounced, likely due to the expansion of the adjacent cells. In the 2,4-D- treated group, cells remained tightly packed at 10 DAT. FAZs occurred adjacent to the phloem in the longitudinal profile of control group fruits. At 15 DAT, the control group showed ruptures in the vascular bundle, along with visible cracks at the junction of the fruit and its stalk (Figure 2B). Notably, in the 2,4-D treatment group the intercellular space was smaller than that of the control group at 15 DAT, apparently the crack of FAZ cell was smaller, which allowed FAZ cellular structure to maintain longer. At 20 DAT, certain FAZ cells in the control group had already separated, indicating the weakening and eventual breaking of cellular connections. In the GA₃-treated group, the FAZ intercellular gaps expanded at 15 DAT, yet these was no significant deformation. At 20 DAT, the abscission zone cells had loosened, but without any discernible ruptures or cracks. For the 2,4-D-treated group, the abscission zone cells began to deform at 20 DAT, although this deformation was not significant compared to that of the control group at the same time.

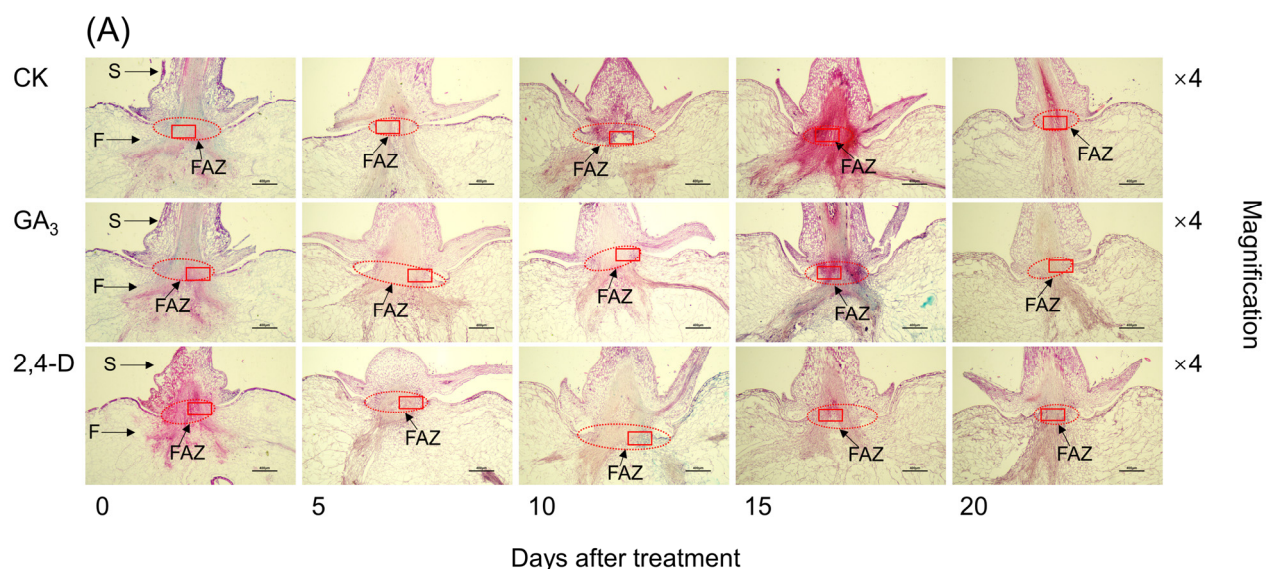


Figure 2. Cont.

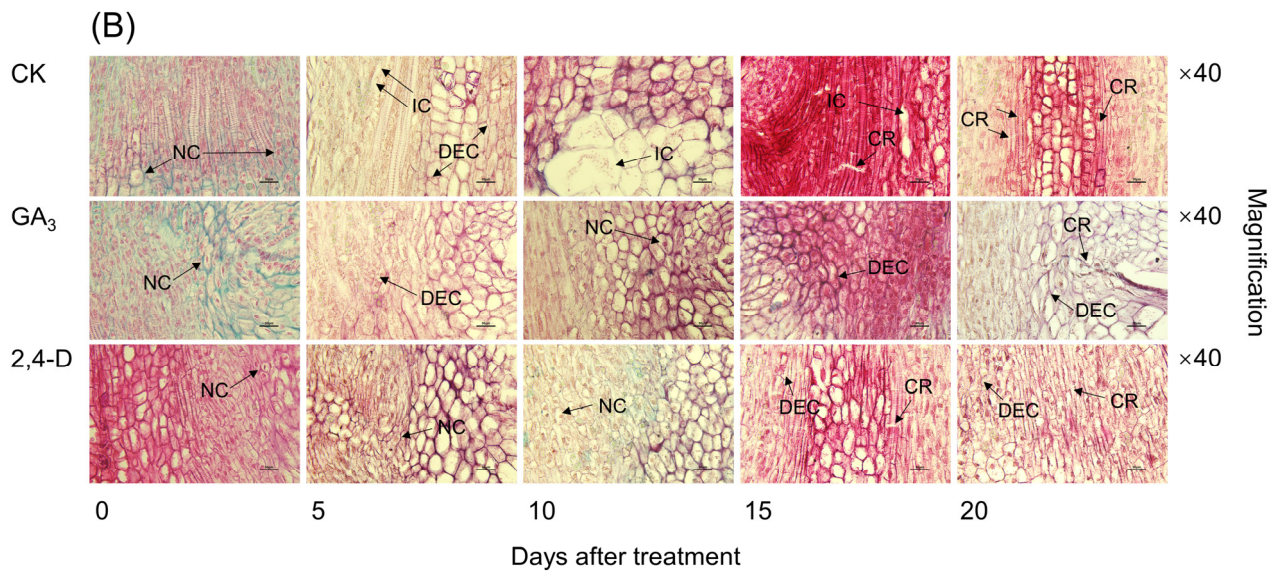


Figure 2. Effects of different treatments on the disparate anatomy of 'Berel' fruits abscission zone. (A) Anatomy of different treatments of 'Berel' fruits abscission zone and adjacent area in different periods of separation. (B) Magnification of the portions of the red square area in (A). S: stalk, F: fruit, FAZ: fruit abscission zone, IC: intercellular cavities, DEC: deformed cells, CR: crack, NC: normal cells. Scale bars: (A) 400 μm , (B) 30 μm .

Based on microscopic observations, the control group showed FAZ specific cells as early as 5 DAT, whereas the 2,4-D-treated group the FAZ cell structure was relatively intact. At 5 DAT, the GA₃-treated in the FAZ was with no apparent schizogenous process. At 10 DAT, in the control group more pronounced intercellular cavities was observed at 5 DAT. In the two treated group, cells in the FAZ remain more tightly packed. In the control group at 15 DAT and 20 DAT, cracks at the edge connecting the fruit and the stalk were observed. However, these noticeable cracks were absent in the treated groups, indicating that the formation of the abscission layer was notably delayed when treated with 75 $\text{mg}\cdot\text{L}^{-1}$ GA₃ and 100 $\text{mg}\cdot\text{L}^{-1}$ 2,4-D sprays.

3.3. Effects of 2,4-D and GA₃ Treatments on the Activity of Cellulolytic Enzymes in the Fruit Abscission Zone

3.3.1. Cx Activity

From Figure 3, it can be observed that in the fruit coloring stage, at 5 DAT, the Cx activity in the abscission zone of 'Berel' fruits treated with GA₃ and 2,4-D was significantly lower than that in the control group. At 10 DAT, the Cx activity in the treatment groups slightly increased compared to the control group. At 15 DAT, the GA₃ treatment group showed lower Cx activity compared to the control group, while the 2,4-D treatment group and the control group exhibited higher Cx activity. At 20 DAT, both the GA₃ and 2,4-D treatment groups had higher Cx activity than the control group, with the GA₃ treatment group showing slightly higher Cx activity than the control group, and the 2,4-D treatment group showing significantly higher Cx activity. This indicates that both treatments significantly reduced cellulase activity in the FAZ at 5 DAT, and the effect was more pronounced with GA₃ treatment at a concentration of 75 $\text{mg}\cdot\text{L}^{-1}$.

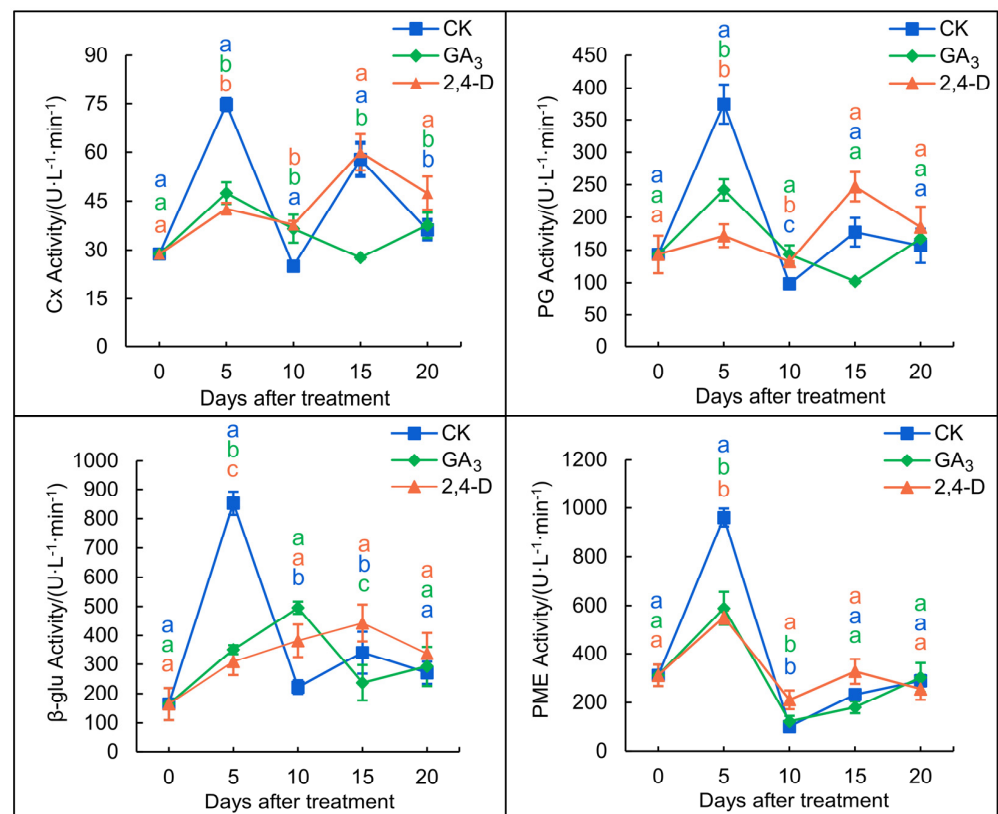


Figure 3. Effects of different treatments on the activities of Cx, β -glu, PG, and PME in 'Berel' fruit abscission zone. Data are mean $\pm$ SE. Different letters represent significant differences between different treatment at each time point ($p < 0.05$). Same letters represent not significant differences.

3.3.2. β -glu Activity

From Figure 3, it can be observed that at 10 DAT, the β -glu activity in the control group initially increased and then decreased. In contrast, both treatment groups showed a gradual increase in β -glu activity. At 5 DAT, the enzyme activity in the control group increased, while the β -glu activity in both treatment groups was significantly lower than that in the control group. At 10 DAT, the β -glu activity in the control group decreased to its lowest value, while the β -glu activity in both treatment groups slowly increased and was higher than that in the control group. The β -glu activity in the control group gradually increased and then decreased at 10 DAT, while the GA_3 treatment group showed a decrease in β -glu activity at 10 DAT, reaching its lowest value at 15 DAT, which was lower than the control group. At 20 DAT, there was a slight increase in β -glu activity in the GA_3 treatment group. In contrast, the 2,4-D treatment group showed a slow increase in β -glu activity at 10 DAT, reaching its highest value at 15 DAT, which was higher than the control group, and then gradually decreased until 20 days. At 20 DAT, the activity of β -glu in both treatment groups was slightly higher than that in the control group.

3.3.3. PG Activity

From Figure 3, it can be observed that at 10 DAT, the PG activity in both the control group and the two treatment groups initially increased and then decreased. At 5 DAT, the enzyme activity in the control group increased, while the PG activity in both treatment groups was significantly lower than that in the control group. At 10 DAT, the PG activity in the control group decreased to its lowest value, and the PG activity in the treatment groups also decreased, with both treatment groups showing higher PG activity than the control group. The PG activity in the control group gradually increased and then decreased at 10 DAT, while the GA_3 treatment group showed a decrease in PG activity at 10 DAT, reaching its lowest value at 15 DAT, which was lower than the control group. At 20 DAT,

there was a slight increase in PG activity in the GA₃ treatment group. In contrast, the 2,4-D treatment group showed a slow increase in PG activity at 10 DAT, reaching its highest value at 15 DAT, which was higher than the control group, and then gradually decreased until 20 DAT. At 20 DAT, the activity of PG in both treatment groups was slightly higher than that in the control group.

3.3.4. PME Activity

From Figure 3, it can be observed that the PME activity in the control group exhibited a rapid increase followed by a rapid decrease, and then a slow increase. The overall trend of PME activity in both treatment groups was similar to that in the control group, with lower activity than the control group at 5 and 20 DAT.

3.4. Effects of 2,4-D and GA₃ Treatments on the Expression Levels of Abscission-Related Genes in the Fruit Abscission Zone

3.4.1. Effects of 2,4-D and GA₃ Treatments on the Expression Levels of Cell Wall Degradation-Related Genes in the Fruit Abscission Zone

The expression levels of cell wall degradation-related genes (CX1, CX2, PE, PL20) were analyzed in the abscission zone of ‘Berel’ fruits treated with 100 mg·L⁻¹ 2,4-D, 75 mg·L⁻¹ GA₃, and the untreated control. The results showed that both treatments inhibited the expression of these four genes compared to the control group at 5 DAT (Figure 4). At 10 DAT, the relative expression levels of the two cellulose-related genes were higher in the 75 mg·L⁻¹ GA₃ treatment group compared to the control group, while they were lower in the 100 mg·L⁻¹ 2,4-D treatment group. At 15 DAT, the expression levels of cellulose-related genes significantly decreased in both treatment groups and were much lower than those in the control group. The relative expression level of CX2 in the 2,4-D-treated FAZ was extremely low and consistently significantly lower than that in the control group.

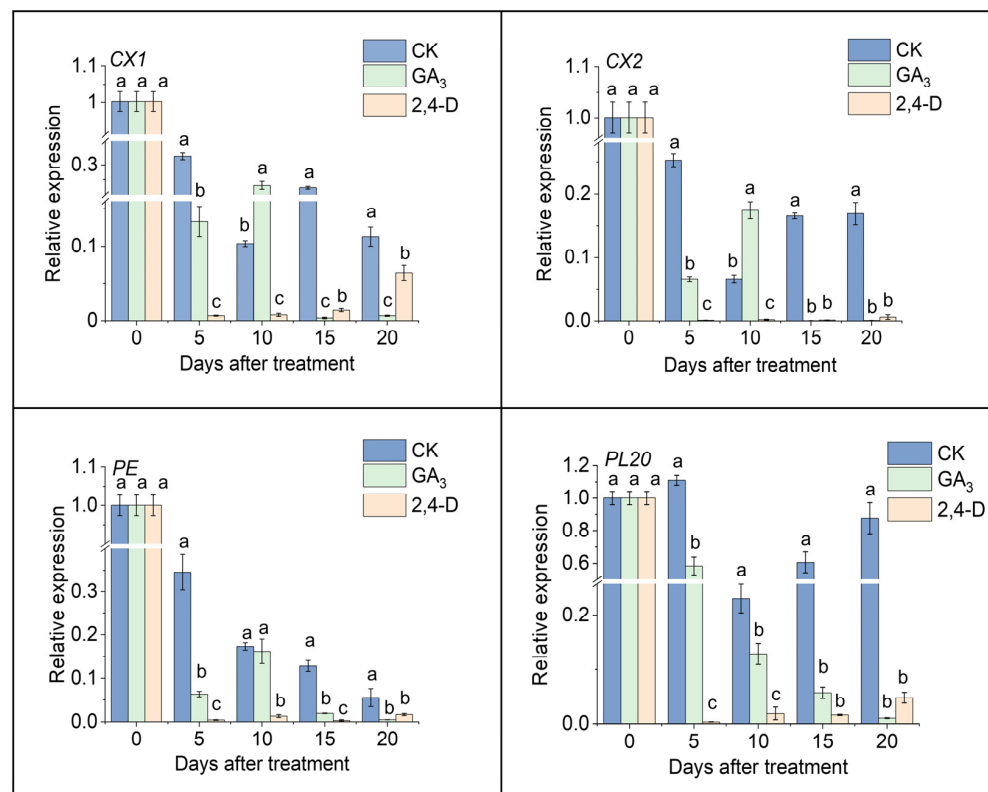


Figure 4. The effect of GA₃ and 2,4-D treatment on genes related to cell wall degradation expression during the veraison stages of ‘Berel’ fruit abscission zone. Data are mean ± SE. Different letters represent significant differences between different treatment at each time point ($p < 0.05$). Same letters represent not significant differences.

The relative expression level of *PE* in the control group showed a continuous decrease after treatment. In both treatments, the expression level of *PE* initially decreased and then increased, with both treatments showing lower expression levels than the control group at 5 DAT, and further decreased at 10 DAT. The relative expression level of *PE* in the 2,4-D-treated FAZ was extremely low and consistently significantly lower than that in the control group. The relative expression level of *PL20* in the control group initially increased, then decreased, and increased again at 10 DAT, reaching the highest expression level at 5 DAT. The expression level of *PL20* in the GA₃ treatment group consistently decreased, while in the 2,4-D treatment group, it initially decreased and then slightly increased. The expression levels in both treatment groups were lower than that in the control group. Therefore, both treatments reduced the expression levels of cell wall degradation-related genes in the FAZ.

3.4.2. Effects of 2,4-D and GA₃ Treatments on the Expression Levels of Ethylene and Gibberellins Key Synthesis-Related Genes in the Fruit Abscission Zone

Ethylene has been demonstrated to play a pivotal role in plant organ abscission by promoting the synthesis and secretion of hydrolytic enzymes in the cell wall and middle lamella [18], which has an important effect on fruit abscission. We examined the expression patterns of key gibberellin and ethylene synthesis-related genes (*GA2OX1*, *GA1*, *ACO*, *ACO3*) in the abscission zones of 'Berel' fruits subjected to treatments with 100 mg·L⁻¹ 2,4-D, 75 mg·L⁻¹, and an untreated control. The results showed that in the control group the relative expression levels of the two-gibberellin synthesis-related genes (*GA2OX1*, *GA1*) displayed a pattern of initial decrease, subsequent increase, and then a decrease again by 15 DAT (Figure 5). For fruits treated with GA₃, the expression of *GA2OX1* and *GA1* initially dropped, rose thereafter, and displayed a sustained decrease by 10 DAT. Notably, in the GA₃-treated FAZ, the *GA2OX1* expression consistently remained subdued compared to the control, while *GA1* expression exceeded the control group at 10 DAT. In contrast, the 2,4-D treatment led to a sharp drop in *GA2OX1* expression, reaching its nadir at 5 DAT, before rebounding. By 15 DAT, this expression in the 2,4-D treated FAZ was still below the control group, but by 20 DAT, it surpassed it. As for *GA1* in the 2,4-D treated FAZ, its expression first waned and then waxed. Notably, its lowest expression was observed at 10 DAT, persistently staying below the control levels after treatment. In contrast, the 2,4-D treatment led to a sharp drop in *GA2OX1* expression, reaching its nadir at 5 DAT, before rebounding. By 15 DAT, this expression in the 2,4-D treated FAZ was still below the control group, but by 20 DAT, it surpassed it. As for *GA1* in the 2,4-D treated FAZ, its expression first waned and then waxed. Notably, its lowest expression was observed at 10 DAT, persistently staying below the control levels after treatment.

In the control group, the relative expression levels of the two-ethylene synthesis-related genes (*ACO*, *ACO3*) initially decreased, then increased, and decreased again at 10 DAT. In the GA₃-treated FAZ, the relative expression level of *ACO* decreased and was lower than that in the control group at 5 DAT. At 10 DAT, the expression level of *ACO* increased and was higher than that in the control group, but it consistently decreased and remained lower than that in the control group at 10 DAT. In the 2,4-D-treated FAZ, the relative expression level of *ACO* initially decreased, then increased, and decreased again, while at 10 DAT, it remained consistently lower than that in the control group. The relative expression level of *ACO3*, an ethylene synthesis-related gene in the control group, initially decreased, then increased, and decreased again, reaching the lowest value at 20 DAT. In the GA₃-treated FAZ, the relative expression level of *ACO3* initially decreased, then increased, and decreased again, reaching the lowest value at 20 DAT. At 10 DAT, the expression level of *ACO3* in the GA₃ treated group was higher than that in the control group, but in other periods, it was lower than that in the control group. The expression level of *ACO3* in the 2,4-D-treated group initially decreased, then increased, decreased again, and increased. At 15 DAT, the expression level of *ACO3* was the lowest, while it was higher than that in the control group at 10 and 20 DAT, but significantly lower than that in the control group in other periods.

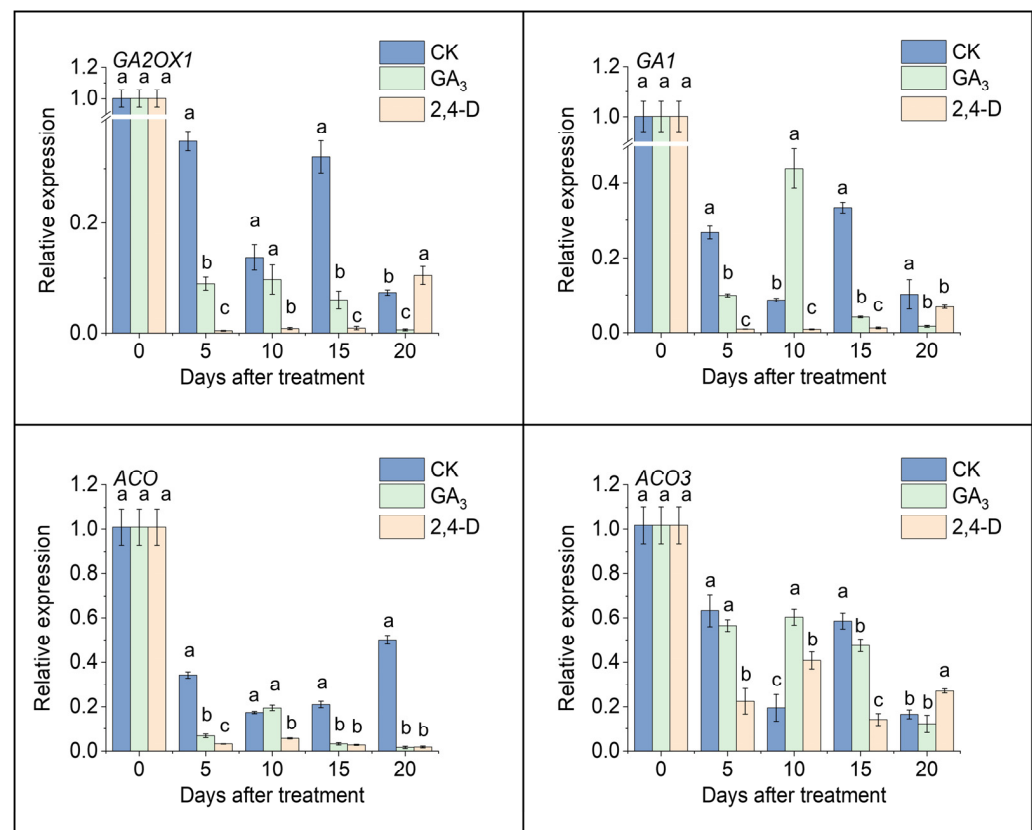


Figure 5. The effect of GA₃ and 2,4-D treatment on genes related to Gibberellin and ethylene synthesis expression during the veraison stages of ‘Berel’ fruit abscission zone. Data are mean $\pm$ SE. Different letters represent significant differences between different treatment at each time point ($p < 0.05$). Same letters represent not significant differences.

4. Discussion

The physiological phenomenon of organ abscission is widely observed in the plant kingdom. Abnormal fruit abscission that occurs in fruit tree organs can to some extent affect fruit quality and yield, thereby impacting the economic benefits of the fruit tree industry. Fruit organ abscission is influenced by various factors, including intrinsic physiological activities, external environments, plant endogenous hormones, and related enzymes. Therefore, the application of appropriate fruit retention techniques to prevent abnormal fruit abscission in fruit trees is of great significance for improving fruit quality and yield.

Research has shown that auxin is involved in the formation of the abscission layer and plays a role in delaying plant organ abscission [19]. 2,4-D is a growth regulator that belongs to the auxin class and has been used in citrus and other fruit trees to retain fruit and delay fruit abscission [20–22]. One of the ways in which GA inhibits fruit ripening and senescence is by suppressing the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis, thereby inhibiting ethylene production [23]. The application of GA₃ and 2,4-D significantly reduced fruit drop in date palm and Satsuma mandarin, respectively [24,25]. Based on these research findings, we conducted this experiment to verify whether GA₃ and 2,4-D can be used to delay the abscission of blue honeysuckle fruits.

The formation of the abscission zone in fruits is related to the stimulation of external signals. Abscission layer cells have specific cell structures with small and dense cytoplasm. When these cells perceive abscission signals, the connections between the cells start to break, leading to fruit abscission [26]. In this study, observation of paraffin sections of the FAZ revealed that in the control group, the cells in the FAZ gradually became compact, increased in number, and deformed over time. The connections between the cells decreased, and

obvious cracks appeared, ultimately resulting in fruit abscission. This finding is consistent with our results. Comparing the ripening stage fruits treated with GA₃ and 2,4-D, as time passed after treatment, the abscission zone cells also gradually became compact. However, compared to the abscission zone cells in the control group at the same period, the distribution of cells in the treated groups was more uniform, with fewer or no visible cracks or ruptures. Therefore, it is speculated that GA₃ and 2,4-D treatments delayed the formation of the abscission layer, further postponing the abscission of blue honeysuckle fruits. We found that exogenous GA₃ and 2,4-D treatment could inhibit the activity of cell wall degrading enzymes by inhibiting the expression of cell wall lysis related genes, weakened the dissolution of intercellular junctions, maintained the structure of cells, and further delayed fruit abscission. Research has shown that exogenous application of 2,4-D delays the formation of the abscission layer in citrus fruits [27], which is consistent with our experimental results.

The abscission of fruit tree organs is regulated by enzymes associated with the abscission process. The dissolution of the middle abscission layer or shared cell wall regulated by cell wall-degrading enzymes is a fundamental step in abscission. This study demonstrates that the activities of four cell wall-degrading enzymes, PME, PG, Cx, and β -glu, in the abscission zone of fruit were reduced after treatment with two plant growth regulators. Among them, the most significant reduction in enzyme activity compared to the control group was observed after 5 days of both treatments. There were differences in the enzyme activities between the two treatments after 5 days, but they were not significant. As the post-treatment time progressed, there was a slight decrease in enzyme activity in the GA₃ treatment group compared to the control group, although the difference was not significant. This indicates that GA₃ has the best effect in reducing the activity of the four cell-wall-related enzymes. At 10 DAT, there was no significant difference between the two treatment groups and the control group. This phenomenon may be due to the discontinuation of exogenous spraying after continuous treatment for three days, resulting in a gradual decrease in the impact on the activity of cell wall-degrading enzymes over time. The activities of PME, PG, Cx, and β -glu are related to cell wall degradation, and an increase in cellulase and pectinase activities accelerates cell wall degradation [26,28]. In a study on strawberry, exogenous application of auxin resulted in a decrease in cellulase activity, leading to the breaking of the half-cellulosic chains that support the fruit weight, resulting in fruit abscission [29]. We believe that a similar situation may occur in the treated 'Berel' FAZ. Therefore, at 5 DAT (Figure 3), the activities of PME, PG, Cx, and β -glu were reduced, thereby delaying the cellulose rupture and pectin dissolution between the cells in the abscission zone, further maintaining the original morphology of the abscission zone and delaying fruit abscission.

In this study, after treatment with GA₃ and 2,4-D, the expression of CX1, CX2, PE, and PL20 genes in the abscission zone of 'Berel' fruit at 5 DAT was significantly lower than that in the control group. We believe that this result may be due to the inhibition of cellulase, pectinase, and pectin lyase gene expression in the FAZ at 5 DAT. The reduced expression levels of cellulase and pectinase genes delay the degradation of cellulose and pectin, further delaying fruit abscission. Previous studies have shown that exogenous application of GA to carrot roots upregulates the expression levels of cellulose degradation-related genes due to the effect of GA on the formation of intermediates required for cell wall lignification [30]. In our results, the expression levels of CX1 and CX2 genes in the GA₃ treatment group were significantly higher than those in the control group at 10 DAT. This suggests a possible relationship between GA₃ and lignin synthesis in the abscission zone, which requires further investigation into the relationship between GA₃ signaling and lignin-related genes. Based on the analysis of gene expression levels of cellulase and pectinase in each treatment group, we propose that one mechanism by which GA₃ and 2,4-D delay fruit abscission in 'Berel' blue honeysuckle fruit is by inhibiting tissue or organ cell wall degradation, thereby delaying fruit abscission.

Ethylene plays an important role in the abscission process of plant organs, and studies have shown that ethylene levels increase during organ abscission [18,31,32]. In this study, the expression levels of *ACO*, a key gene involved in ethylene synthesis, in the abscission zone of fruit after treatment with GA₃ and 2,4-D were suppressed, indicating a possible inhibition of ethylene biosynthesis. Ethylene primarily alters cell wall metabolism in the abscission zone through degradation and new synthesis [33,34]. Research has shown that ethylene is associated with PME gene expression [35,36], so we believe that the decreased expression levels of key genes involved in ethylene synthesis result in reduced ethylene production, further reducing the activity of pectinase in the cell wall, delaying pectin degradation, and consequently delaying fruit abscission. However, the mechanisms by which GA₃ and 2,4-D inhibit ethylene synthesis and affect the expression of cell wall-degrading enzyme genes in blue honeysuckle fruit are not yet clear and require further experimental validation.

GA negatively regulates the formation of the abscission layer during fruit abscission in fruit trees to prevent fruit drop [37]. In our results, the expression of two genes related to the GA synthesis pathway was significantly inhibited in both treatment groups, possibly due to negative feedback regulation. In loquat, it has been shown that the combination of GID1-GA-DELLA is inhibited when exogenous GAs are supplied, reducing the impact of Gas [38]. In our conclusions, the expression levels of *GA2OX* at each time point after exogenous GA₃ spraying were significantly lower than those in the control group, which may be related to this phenomenon. In a study on grapes, the expression of *GA2OX* genes in flowers was upregulated after exogenous GA₃ application [39], which is contrary to our results and may be related to feedback regulation. However, at 20 DAT, the expression of *GA2OX* in the 2,4-D treatment group was higher than that in the control group. In pear, the application of GA₄₊₇ has a better effect on fruit setting and fruit drop rate than GA₃ [40]. *GA2OX* drives the synthesis of GA₄, so the increase in *GA2OX* expression in the 2,4-D treatment group may delay fruit abscission by promoting GA₄ synthesis. *GA1* is a key gene in gibberellin synthesis, and the expression of *GA1* was significantly higher than that in the control group at 10 DAT, indicating that exogenous gibberellin treatment may increase gibberellin synthesis in the abscission zone, further delaying fruit abscission.

5. Conclusions

In this study, the treatment of 'Berel' blue honeysuckle fruit with GA₃ and 2,4-D reduced the activity of cell wall-degrading enzymes (PME, PG, β -glu, and Cx) and simultaneously suppressed the expression levels of genes related to fruit abscission in the abscission zone. This delayed the formation of the abscission layer, postponed fruit abscission, and reduced the fruit drop rate, as proposed in Figure 6. The findings provide a theoretical basis for the application of exogenous GA₃ and 2,4-D treatments to improve the fruit retention of blue honeysuckle and propose a new approach for blue honeysuckle harvesting and delayed fruit drop. This study has important implications for promoting the development of the blue honeysuckle industry.

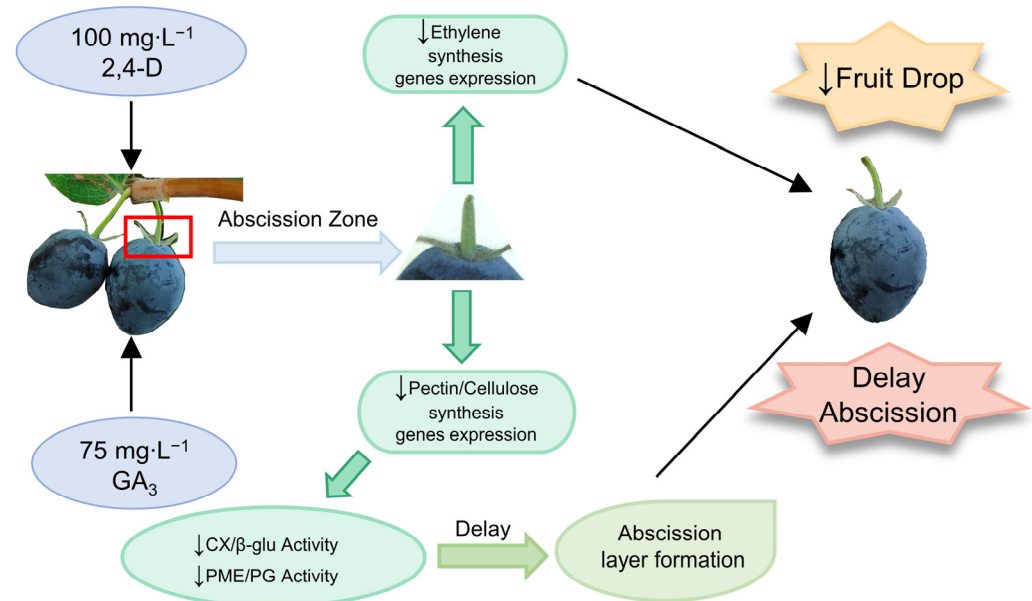


Figure 6. Proposed mode of action of GA₃ and 2,4-D treatment on ‘Bere’ blue honeysuckle fruits. Portion of the red square area represents fruit abscission zone.

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