

## Article

# Effects of Foliar Ca and Mg Nutrients on the Respiration of ‘Feizixiao’ Litchi Pulp and Identification of Differential Expression Genes Associated with Respiration

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**Abstract:** The ‘Feizixiao’ litchi cultivar, predominantly grown in Hainan Province, faces the issue of “sugar receding” during fruit ripening. The application of mixed foliar nutrients containing calcium and magnesium (Ca+Mg) during the fruit pericarp’s full coloring stage was investigated to overcome this issue. Experimental trials unveiled significant alterations in litchi pulp physiochemical properties, including the main nutrient and flavor quality, the total respiration rates of the main respiratory pathways, and the activities of some important enzymes associated with Embden–Meyerhof–Parnas (EMP), the tricarboxylic acid cycle (TCA) and the pentose phosphate pathway (PPP). The Ca+Mg treatment showed higher sugar levels than the control (CK) during ripening. Notably, the application of Ca+Mg in litchi pulp inhibited respiration rates through the EMP, TCA, and PPP pathways, resulting in a strong effect. RNA sequencing analysis revealed the impact of Ca+Mg treatment on respiratory pathways, revealing differentially expressed genes (DEGs) such as pyruvate PK1, PK2 (pyruvate kinase), and PDC (pyruvate dehydrogenase complex), validated through qRT-PCR with a significant correlation to RNA-seq results. In general, Ca+Mg treatment during litchi fruit ripening overcame “sugar receding” by inhibiting the expression of respiration key metabolic pathway genes. These findings provide insights for enhancing cultivation management strategies.

**Keywords:** litchi; sugar receding; Ca+Mg; respiratory pathways



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## 1. Introduction

Litchi (*Litchi chinensis* Sonn., Sapindaceae) is a perennial fruit tree native to China and is predominantly grown in regions such as Hainan, Yunnan, Guangxi, Guangdong, Taiwan and Sichuan [1]. It is well-known for its succulent flesh and nutritional richness and is named “king of Lingnan fruit.” China is the global leader in litchi production, with over 0.54 million hectares of cultivated land and a yield of approximately 2.3 million tons in 2018 [2]. The composition of litchi pulp includes an assortment of nutrients, including carbohydrates, proteins, lipids, vitamins, and minerals. The distinctive flavor of litchi primarily relies on the sugar and acid content and their concentration within the pulp [3]. This sweet and bitter taste profile has made litchi popular among consumers [4]. Sugars are essential components in the growth and development of plants, serving as vital building blocks, sources of energy, and mediators of signal transduction [5]. Respiration is essential for higher plant metabolism [6,7], and studies have shown that plant respiratory routes and intensity fluctuate in response to various environmental circumstances and treatments [8].

As a result, inhibiting respiratory metabolism has emerged as a critical target for protecting fruit quality and postharvest senescence [9]. Plant respiratory metabolic routes include the EMP, PPP, TCA, and cytochrome pathway (CCP) [10]. These metabolic pathways in the respiratory route directly affect energy metabolism [11]. Moreover, in the EMP, key enzymes include pyruvate kinase (PK), hexokinase (HK), and phosphofructokinase (PFK), whereas in the PPP pathway, crucial enzymes are 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD) [12–15]. In biochemical reactions, rate-limiting enzymes, such as phosphoglucose isomerase (PGI) and succinic dehydrogenase (SDH), play a pivotal role in TCA respiratory pathways and energy metabolism [16,17]. Coenzymes like NAD (H) and NADP (H) are crucial for various metabolic activities, including respiration and metabolism. Enzymes such as 6-phosphate dehydrogenase (6-PGDH) and glucose-6-phosphate dehydrogenase (G-6-PDH) are essential for pathways like the PPP pathway [18]. Cytochrome oxidase (CCO) acts as a terminal oxidation enzyme in respiration, generating energy and water [19]. Balancing these components significantly affect fruit quality, postharvest senescence, and metabolic pathways [20].

Calcium (Ca) and magnesium (Mg) are vital mineral nutrients crucial for plant growth, development, and fruit quality. Ca is renowned for decreasing fruit respiration, leading to desirable outcomes such as reduced fruit softening [21]. Studies on sweet cherry fruit have shown that higher Ca levels correspond to lower respiration rates and increased peel firmness [22]. Additionally, Ca ions participate in various physiological processes essential for plant health, including cell wall integrity, tissue repair, and pH regulation [23]. Similarly, Mg is critical in root development, nutrient absorption, and carbohydrate export while alleviating oxidative stress [24]. Ca and Mg are integral components of plant anatomy, influencing structures like vascular bundles and cell walls [25]. Their application is essential for maintaining plant integrity, as deficiencies can lead to significant structural damage [26]. Studies have demonstrated that Ca and Mg can delay ripening and hinder senescence during fruit growth and development [27,28]. Previous research has indicated that spraying a mixed solution of calcium and magnesium can alleviate the reduction of sugar sensation in the pulp of ‘Feizixiao’ litchi, suggesting a potential role in modulating fruit metabolism [29]. Furthermore, low-calcium treatment has significantly inhibited fruit respiration in mangoes, improving their storage quality [30]. The previous research results of our research group showed that calcium and magnesium are the main elements affecting the sugar and acid content in Feizixiao litchi [31]. The ‘Feizixiao’ litchi cultivar, prominent in Hainan Province and gaining global recognition, faces the issue of “sugar receding” during ripening, affecting its quality and market value [32].

Understanding the respiratory pathways in ‘Feizixiao’ litchi pulp is crucial as they influence the senescence and ripening of fruit, particularly regarding the sensation of “sugar receding”. Initially, our investigation focused on understanding the biological mechanisms of aerobic respiration in the pulp, focusing on evaluating the effects of mixed foliar nutrient treatments incorporating calcium and magnesium [33]. This research aims to analyze the mechanism by which Ca+Mg treatment overcomes “sugar receding” during respiration. In this study, we aim to investigate the impact of foliar nutrients, specifically calcium and magnesium, on the key respiratory pathways of the ‘Feizixiao’ litchi pulp, and to identify genes differentially expressed to respiration. By analyzing metabolic responses and gene expression patterns, we aim to uncover the underlying mechanisms through which these nutrients modulate fruit metabolism and quality. By shedding light on physiological and molecular aspects, this research advances our understanding of litchi fruit physiology, with potential implications for improving cultivation practices and commercial success.

## 2. Materials and Methods

On 16 April 2022, in the litchi orchard of Jinpai Farm situated in Lingao County of Hainan Province, ten ‘Feizixiao’ litchi adult trees were selected carefully. These trees exhibited moderate growth and a uniform tree shape. They were free from mechanical damage, pests, and diseases, while boasting a high yield potential. Sited within the tropical

monsoon zone, the orchard experiences a consistent temperature range of 23–24 °C, with an average annual sunshine duration of 2175 h and an annual precipitation ranging from 1100 to 1800 mm. The synchronization of rain and heat characterizes the climate. The soil composition predominantly consists of brick-red soil. The primary phenological periods observed include February to March, signifying the flowering phase, followed by physiological fruit drop in early April, the fruit expansion phase commencing in late April, and the fruit ripening period initiating in mid-May.

### *2.1. Experimental Setup and Treatment*

The ten experimental trees were split into two groups: one underwent treatment with a foliar nutrient solution consisting of a 0.3%  $\text{CaCl}_2$  and 0.3%  $\text{MgCl}_2$  mixed aqueous solution (Ca+Mg), while the other served as the control and received clean water (CK). The experimental setup included single-plant plots, with each treatment replicated five times [34,35].

### *2.2. Procedures of Sample Collection*

On the 35th day after anthesis (DAA), field treatments commenced when the seed was fully coated in the peridium and the fruit base turned red, indicating the end of physiological fruit drop and the beginning of rapid fruit expansion. From that point forward, treatments were measured thrice weekly, between 9:00 a.m. and 10:00 a.m. Sampling began before the initial field treatment, with sample fruits collected from approximately the midpoint of the outer canopy of each experimental tree. Five medium-sized fruits from this region were selected as reference fruits for dynamic sampling, each marked with a hang tag. Sampling continued until the pericarp reached full redness, approximately 70 DAA. During each sampling session, we collected 30 sample fruits per tree, selected based on the size and color of the reference fruits from the midpoint of the outer canopy. These samples were rapidly frozen with liquid nitrogen in the field, then transferred to the laboratory and stored at  $-80$  °C temperature for further analysis.

### *2.3. Determination of Total Sugar, Total Acids, and Sugar–Acid Ratio*

To measure the total sugar content, we employed anthrone colorimetry. We began by grinding 0.1 g of the sample (whole fruit) then subjecting it to a boiling water bath (10 min), centrifugation, and another round of boiling water bath (10 min) treatment. After cooling, anthrone reacted with different sugar forms, including hexose, hexosyl, pentosyl, and hexuronic acid within polysaccharides. This reaction resulted in a blue-green solution with its maximum absorption at 620 nm. We determined the specific sugar content by comparing it to a standard curve [36]. To determine the total acid content, we utilized a sugar–acid analyzer (RX 5000, ATAGO, Tokyo, Japan), using citric acid as the standard. Additionally, we calculated the sugar–acid ratio in the fruit's flesh by dividing the ratio between the total sugar content and the total acid content within the same fruit.

### *2.4. Measurement of Total Respiration Rate and Rates of TCA, PPP, and EMP*

Pulp respiration rates through the TCA cycle, EMP, and PPP were measured using the Pictrip  $\text{O}_2/\text{CO}_2$  headspace analyzer (CheckMate 3, Dansensor, Denmark), expressed in  $\text{mL Cozkg}^{-1}$  (Wangetal, 10 mmol/sodium fluoride as EMP pathway inhibitor, 50 mmol  $\text{L}^{-1}$  malic acid as TCA cycle inhibitor, 10 mmol  $\text{L}^{-1}$  sodium phosphate as PPP pathway inhibitor) [37]. The measurement time was around 10:00 in the morning at a constant ambient temperature of 28 °C; the initial step involved measuring the total respiratory rate of the sample. Following this, the residual respiratory rate of each pathway was assessed after vacuum infiltration of the inhibitor. Each pathway's observed respiratory rate was calculated by subtracting its residual respiratory rate from the previously measured total respiratory rate [38].

### 2.5. Detection of Succinate Dehydrogenase (SDH) and Pyruvate Dehydrogenase Complex (PDC)

The SDH and PDC were prepared using the method [39]. The pulp of 2 g litchi pulp was extracted with  $0.005 \text{ mol L}^{-1}$  EDTA,  $0.5 \text{ mol L}^{-1}$  sucrose,  $1 \text{ mg mL}^{-1}$  bovine serum protein, and  $0.05 \text{ mol L}^{-1}$  PBS buffer, then ground after an ice bath. The enzyme solution was mixed with the reaction solution at 1:1 before the enzyme activity was determined. The samples were maintained at  $30^\circ\text{C}$  for 10 min, after which their absorbance was recorded at 600 nm. The enzyme activities in the samples were then determined by referring to a standard curve.

### 2.6. Detection of Glucose Phosphate Isomerase (GPI), Pyruvate Kinase (PK)

The method for extracting GPI and PK was measured [40]. It involved taking 2 g of litchi pulp, adding Tris-HCl buffer in an ice bath, grinding, and centrifuging. The culture medium was gathered, and its absorbance at a wavelength of 520 nm was determined using an enzyme-labeling instrument. Following this, the enzyme activity of the sample was determined using a standard curve established with fructose as the reference material.

### 2.7. Detection of Malate Dehydrogenase (NAD-MDH) Activity

The activity of NAD-MDH was determined. We weighed 2 g of litchi pulp, added Tris-HCl in a slow infusion, ground the mixture in an ice bath, and centrifuged the supernatant. The supernatant was added to the reaction solution composed of  $0.2 \text{ mol L}^{-1}$  Tris-HCl, 0.1% polyethylene glycol octyl phenyl ether, and  $5 \text{ mol L}^{-1}$  ascorbic acid. The volume of the reaction solution was 5 mL. The enzyme activity and absorbency values were measured at 340 nm using an enzyme marker, and the calibration curve calculated the activity of each enzyme [41,42].

### 2.8. Detection of Cytochrome Oxidase (CCO)

The CCO activity was measured according to the protocol [43]. Initially, 3 g of litchi pulp was ground in an ice bath with 7.5 mL of pH 7.4 phosphate buffer ( $0.05 \text{ mol L}^{-1}$ ). Following grinding, the mixture underwent centrifugation at  $3000 \times g$  for 10 min at  $4^\circ\text{C}$ , followed by filtration. A mixture was prepared by combining 0.3 mL of the filtered supernatant, 0.04% cytochrome C, and 3 mL of preheated double-distilled water at  $37^\circ\text{C}$  for 2 min. Subsequently, 0.3 mL of 0.4% dimethyl-p-phenylenediamine (DMPD) was added, and the solution was incubated at  $37^\circ\text{C}$  for 3 min until it turned red. The pH was adjusted to the range of 5.6–6.0 using  $0.1 \text{ mol L}^{-1}$  HCl. Finally, a 12.6 mL mixture comprising tetrachloroethylene and absolute ethyl alcohol in a 1:3 ratio was added, and the absorbance was measured at 510 nm. The CCO activity was expressed as  $\text{U kg}^{-1} \text{ FW}$ .

### 2.9. Analysis of Differential Gene Screening and Pathway Selection

According to the existing transcriptome [6], differential genes were screened based on  $\text{FDR} < 0.05$  and  $|\log_2(\text{FC})| \geq 1$ . The three respiratory pathways EMP, TCA, and PPP were investigated based on our phenotype and the existing literature.

### 2.10. Validation of qRT-PCR

Three differentially expressed genes were chosen, and real-time PCR primers synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China) and designed with Prime3 were employed. Fruit pulp RNA was extracted using the Plant RNA Extraction Agent Set from Beijing Kulebo Technology Co., Ltd. (Beijing, China). The extracted pulp RNA was reverse-transcribed into cDNA using a cDNA synthesis kit from Vazyme Biotechnology Co., Ltd. (Nanjing, China) with a T100FM Thermal Cycler PCR instrument from BIO-RAD (Hercules, CA, USA), following the kit instructions. Real-time PCR verification was conducted with a Taq Pro Universal SYBR qPCR Master Mix (Vazyme Code: Q712-02) from Vazyme Biotechnology Co., Ltd. (Nanjing, China) and a qTOWER3 instrument (Jena, Germany). Litchi actin was utilized as the internal reference gene. Detailed information regarding the primers used in the study is provided (Table 1).

**Table 1.** Primer table.

| Primer Names        | Right Primer Sequences (5' to 3') | Left Primer Sequences (5' to 3') | Amplification Length |
|---------------------|-----------------------------------|----------------------------------|----------------------|
| Cluster-6206.72872  | TGGAAAACGGTGGTGTGGAA              | AACAGCTTCTCCACCATCGG             | 100–200 bp           |
| Cluster-6206.110990 | TTGTGGCTGAGTGCCTAGG               | TCCACCACTTCCCAACTGG              | 100–200 bp           |
| Cluster-6206.117009 | TCCCCAACAAATGAACCAGGG             | CACTCGAATTCAAGCGTCGC             | 100–200 bp           |
| β-Actin             | AGTTTGGTTGATGTGGGAGAC             | TGGCTGAACCCGAGATGAT              | 100–200 bp           |

### 2.11. Data Analysis

Statistical analysis of the data was conducted using SAS 9.0 software. To expose the characteristics of the change tendencies as the time passed by, variance analysis was performed using an ANOVA, and a multiple-comparisons analysis was carried out using Duncan's method. The t-test method was used to expose the significance of the difference between the treatment and CK. A heatmap of differential gene expression was generated using TBtools-II: (Toolbox for Biologists) v2.070.

## 3. Results

### 3.1. Changes in Sugar and Acid Contents in Pulp

In Figure 1a–c, the total sugar content in treated litchi fruit pulp consistently increased during the experiment. By 63 days after anthesis (DAA), the Ca+Mg treatment exhibited notably higher sugar levels than the control (CK), consistent with observations at 70 days. This suggests that the Ca+Mg treatment may enhance soluble sugar accumulation in the pulp, effectively preventing “sugar receding” in the later stage of fruit maturity. Consequently, the “sugar receding” issue was effectively alleviated by the Ca+Mg treatment, with no occurrence observed. Regarding total acid content, a sharp decline was observed in both the Ca+Mg treatment and CK. These findings indicate a reduction in total acid content in pulp subjected to the Ca+Mg treatment prior to harvest, with a significant difference between the treatment and control during later stages of fruit development. Consequently, the flavor quality of the ‘Feizixiao’ litchi pulp primarily pivots on soluble sugar content at maturity. The sugar–acid ratio exhibited a steady increase in both the Ca+Mg treatment and CK, with the Ca+Mg treatment significantly higher than CK after 56 days after anthesis.

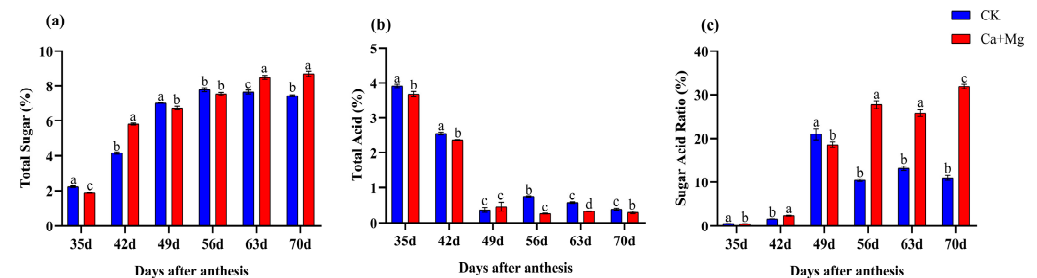


**Figure 1.** The effect of spraying calcium and magnesium fertilizer on litchi pulp as indicated by days after anthesis on the X-axis and respective sugar and acid contents on the Y-axis. (a) Total sugar, (b) total acid, and (c) sugar–acid ratio. Different letters represent the different kind of analysis in each figure and each portion.

### 3.2. Different Changes in Respiration Rate in Pulp

In Figure 2a–d, until 63 DAA, the total respiration rate of litchi fruit under the Ca+Mg treatment was significantly lower than that under CK, followed by a decrease observed at 70 days. In summary, CK partially enhances the total respiration rate, while the Ca+Mg treatment inhibits it. There were significant differences in the respiration rate of the EMP pathway in litchi pulp. The Ca+Mg treatment remained significantly lower than CK until 56 DAA. Overall, applying calcium and magnesium fertilizers resulted in varying degrees of inhibition of the respiration rate through the EMP pathway, with the Ca+Mg treatment exhibiting the most substantial inhibitory effect. The respiration rate through the TCA

pathway in litchi pulp displayed diverse trends. By 56 days after anthesis, the Ca+Mg treatment was significantly lower than CK, indicating that the Ca+Mg treatment began inhibiting the TCA pathway. The respiration rate of the PPP pathway in litchi pulp varied between the Ca+Mg treatment and CK. Specifically, the Ca+Mg treatment was significantly lower than CK by 56 DAA. Consequently, the Ca+Mg treatment demonstrated inhibition of the PPP pathway in the later stages.

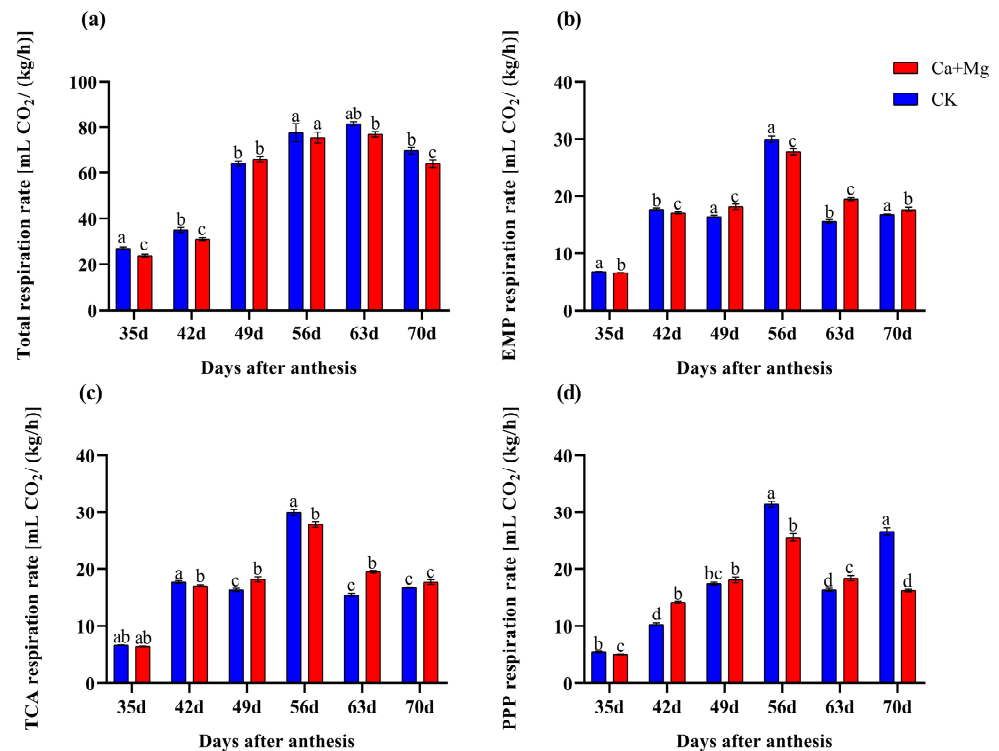


**Figure 2.** The effect of spraying calcium and magnesium fertilizer on litchi pulp as indicated by days after anthesis (DAA) on the x-axis and respective enzyme activities on the y-axis. (a) Total respiration rate: (b) EMP, (c) TCA, and (d) PPP. Different letters represent the different kind of analysis in each figure and each portion.

### 3.3. Changes in Enzyme Activities in Pulp

In Figure 3a–f, SDH activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. By 49 DAA, the Ca+Mg treatment showed a significantly lower level than CK. The decreased activity of Ca+Mg treatment than CK at 70 days may contribute to the inhibition of the TCA pathway. PDC activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. At 56 DAA, the Ca+Mg was lower than CK and stable until 70 days. Consequently, the Ca+Mg treatment demonstrated inhibition of the PDC pathway at 70 days. GPI activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. The Ca+Mg treatment showed higher levels than CK in the early stages, but after 56 days, the Ca+Mg treatment significantly decreased and became the lowest compared to CK. In brief, Ca+Mg treatments boosted GPI activity early on but inhibited it later, suggesting that Ca+Mg treatment inhibited the EMP pathway through GPI activity at full maturity.

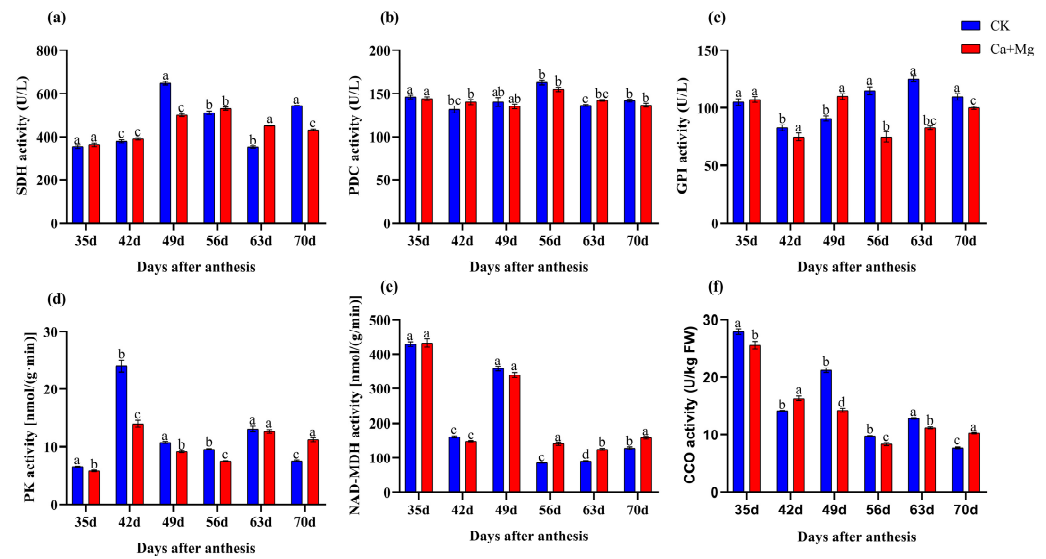


**Figure 3.** The effect of spraying calcium and magnesium fertilizer on litchi pulp as indicated by days after anthesis (DAA) the X-axis and respective enzyme activities on the Y-axis. (a) SDH activity, (b) PDC activity, (c) GPI activity, (d) PK activity, (e) NAD-MDH activity, and (f) CCO activity. Different letters represent the different kind of analysis in each figure and each portion.

PK activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. The Ca+Mg treatment showed a significantly lower level than CK at 42 DAA, and this difference remained stable until 70 days. This suggests that before 56 DAA, the CK enhanced PK activity while the Ca+Mg treatment boosted PK activity postharvest, indicating that PK is not the primary enzyme inhibiting the TCA pathway in the Ca+Mg treatment. NAD-MDH activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. At 35 and 49 DAA, the Ca+Mg treatment showed lower levels than CK. In summary, Ca+Mg inhibited NAD-MDH activity in the early stage, but it was promoted later, suggesting that NAD-MDH is not the key enzyme responsible for TCA inhibition in the Ca+Mg treatment. CCO activity in litchi pulp exhibited varying trends among Ca+Mg treatment and CK. At 35 and 49 DAA, the Ca+Mg treatment exhibited the lowest levels compared to CK, while at 70 DAA, the Ca+Mg treatment showed significantly higher levels than CK. This suggests that the Ca+Mg treatment promoted CCO activity at the harvesting stage. Consequently, CCO is not the primary enzyme inhibiting the TCA pathway in the Ca+Mg treatment.

### 3.4. Differential Gene Screening and Role of EMP, PPP, and TCA

In our study of sugar-related conversions in Ca+Mg-treated litchi fruit, we focused on key metabolic pathways, the EMP, PPP, and TCA cycle, as shown in Figure 4. We hypothesized that these pathways play critical roles in litchi respiration under Ca+Mg treatment. These pathways, such as (a) glycolysis (EMP), involve the breakdown of glucose into pyruvate, producing ATP and NADH. Under Ca+Mg treatment, specific proteins associated with glycolysis may have been upregulated, indicating increased activity in glucose metabolism. (b) The PPP pathway produces ribose-5-phosphate and NADPH, which are important for nucleotide synthesis and antioxidant defense. Proteins related to PPP may have been affected by Ca+Mg treatment, potentially influencing sugar metabolism and antioxidant responses. (c) TCA is central to cellular respiration, generating ATP and reducing equivalents (NADH and FADH<sub>2</sub>) from acetyl-CoA derived from various carbon sources. Proteins associated with the TCA cycle may have been modulated by Ca+Mg treatment, affecting energy production and metabolism.

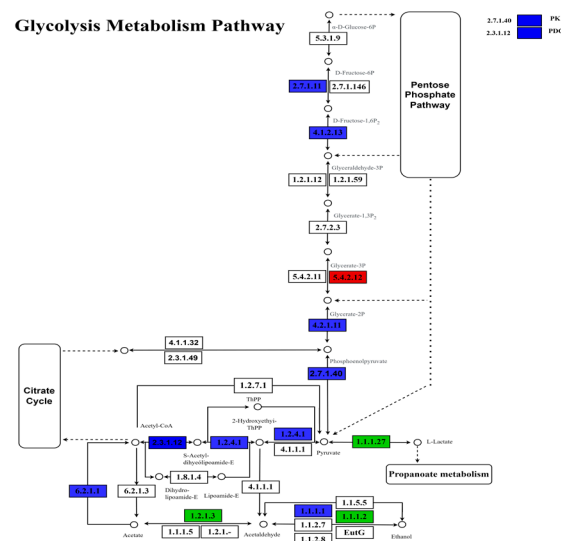


**Figure 4.** Respiratory pathways map (EMP, TCA, and PPP). The ECs (enzyme numbers) marked by red boxes are related to up-regulated genes, and green boxes are related to down-regulated genes, whereas the ECs marked with blue boxes are related to both up-regulated and down-regulated genes.

In our investigation into the influence of EMP, TCA, and PPP respiratory pathways on alleviating fruit “sugar withdrawal” with Ca+Mg treatment, we conducted a screening of 143 genes associated with these respiratory enzymes using transcriptome data. The Log2FC values of these genes were visually represented in a heatmap (Figure 5). Comparative analysis against the control group revealed significant alterations in genes related to respiratory enzymes. Notably, we emphasized genes encoding pyruvate kinase and pyruvate dehydrogenase E2 components due to their recognized importance in fruit respiration, as highlighted in the existing literature. We compared 35d vs. 63d, 35d vs. 70d and 63d vs. 70d. In the comparison between 35d and 63d DAA, 10 genes associated with pyruvate kinase displayed differential expression, with 8 genes upregulated and 2 downregulated. Similarly, in the comparison between 35d and 70d, six genes showed upregulation while four exhibited downregulation, and there was no expression level in 63d vs. 70d DAA. The upregulation and downregulation of the *PK* gene in our experiment indicate changes in its expression or activity level compared to control conditions. *PK* gene catalyzes the conversion of ADP,  $H^+$  ion, and phosphoenolpyruvate to ATP and pyruvate, the final step of glycolysis. Upregulation suggests increased activity, potentially enhancing ATP production and glycolytic flux. Downregulation indicates decreased activity, which could impact ATP generation and cellular energy metabolism. These changes in *PK* expression may have significant implications for cellular energy production and metabolism. The *PDC* E2 component (dihydrolipoyllysine-residue acetyltransferase) showed dual regulation, with two genes identified within the *PDC* family. One gene exhibited consistent upregulation across all comparisons: 35d vs. 63d, 35d vs. 70d, and 63d vs. 70d, whereas the other gene showed downregulation in the 35d vs. 63d and 63d vs. 70d comparisons but was upregulated in the 35d vs. 70d. The reaction catalyzed by the *PDC* E2 component (dihydrolipoyllysine-residue acetyltransferase) involves the conversion of phosphoenolpyruvate (PEP) to pyruvate, accompanied by the generation of ATP from ADP and an  $H^+$  ion. This process is a key step within the *PDC* gene and is pivotal for cellular metabolism, especially in transforming carbohydrates into energy.

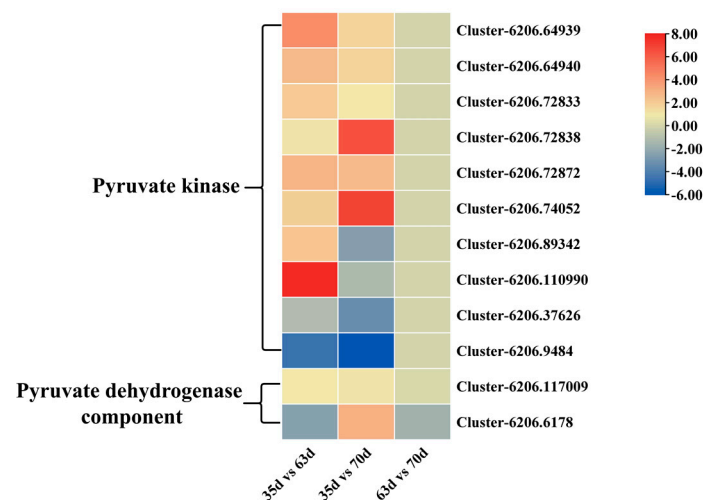


**Figure 5.** Differential gene expression heatmap of respiratory pathways indicating some important gene families with their respective ECs.

### 3.5. Quantitative RT-PCR

As shown in Figure 6a–c, three differentially expressed genes were selected, and their expression levels were detected using real-time PCR in samples collected at 35d, 63d, and 70d DAA. The qRT-PCR expression data for these genes exhibit similarity with the transcriptome sequencing results, thereby confirming the reliability of the RNA sequencing.

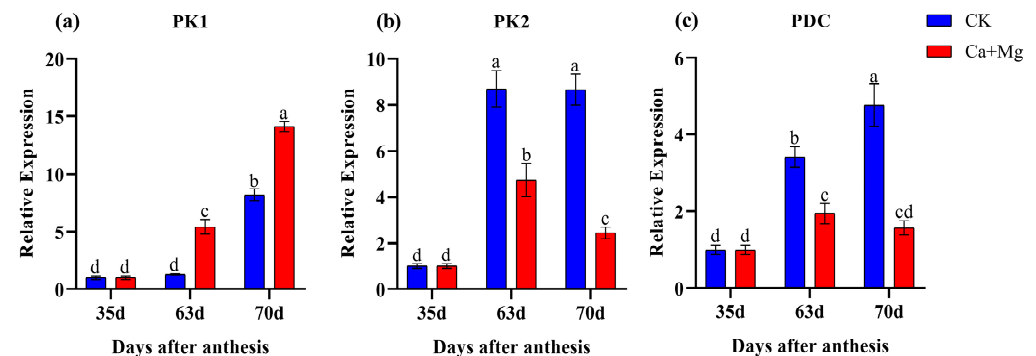


**Figure 6.** Differentially expressed genes upon qRT-PCR validation related to respiratory pathways: (a) *PK1*, (b) *PK2*, and (c) *PDC* refer to real-time PCR trends from samples taken at 35d, 63d, and 70d of Ca+Mg treatment and CK. Different letters represent the different kind of analysis in each figure and each portion.

## 4. Discussion

Minerals, whether sourced naturally or synthetically, are chemical substances that can be applied to seeds, plants, and soil. They can modify crucial physiological and structural processes within plants, thereby influencing growth, yield, and overall quality [44]. Studies have revealed that plants treated with organic or inorganic chemicals and natural bio-stimulants often exhibit increased activity levels of antioxidant enzymes [45,46]. During senescence, fruits experience significant changes in membrane composition and structure. Calcium is critical in maintaining cell wall structure and plasma membrane integrity, thereby reducing spoilage and physiological disorders in plant cells [47,48]. Magnesium plays a critical role in plant growth by directly influencing physiological and biochemical systems. It promotes root development, improves the absorption of water and nutrients, facilitates the export of carbohydrates, and mitigates the generation of reactive oxygen species (ROS) and photo-oxidative damage to cells under stressful conditions [49]. Mg is

an indispensable nutrient in various metabolic processes throughout plant growth and development [50].

#### *4.1. Impact of Ca+Mg on Sugar Content and Fruit Quality*

Ca+Mg, being a secondary macronutrient, is essential for the normal growth and development of tomato. Calcium plays a vital role in cell walls and membranes' structure, fruit growth, and development [51]. Sugar receding, characterized by a drastic decrease in the total soluble solids (TSS) in the pulp, leads to reduced sweetness and deterioration of longan fruits during on-tree preservation [52]. In our experiment, applying Ca+Mg fertilizers significantly overcame "sugar receding" and increased sugar content in litchi fruit pulp. When Ca+Mg treatments were combined, they resulted in even higher sugar levels compared to CK, which aligns with the findings [53]. This rise in sugar content is attributed to improved transport of carbohydrates to storage organs, facilitated by increased fruit calcium content. Additionally, Mg's role in photosynthetic efficiency and chlorophyll synthesis supports these findings, influencing carbohydrate synthesis and transport [54]. Both the CK and Ca+Mg treatments demonstrate a decline in total acid content after flowering, consistent with research stressing the significance of organic acid composition in fruit quality [55,56]. These results emphasize the pivotal role of mineral nutrition, particularly Ca+Mg, in shaping sugar accumulation and total acid content in litchi fruit, crucial for optimizing both its quality and nutritional value [57].

#### *4.2. Influence of Ca+Mg on Respiration and Metabolic Pathways*

The study found that the total respiration rate of litchi fruit was significantly lower in the Ca+Mg treatment than the CK until 63 DAA, followed by a decrease at 70 days. This indicates an inhibitory effect of Ca and Mg on respiration, suggesting a unique interaction between these minerals [58]. This inhibition aligns with previous findings suggesting that foliar spraying of calcium–magnesium mixed fertilizer can slow down the sugar-reducing phenomenon in litchi pulp. Regarding specific metabolic pathways, the respiration rate through the EMP pathway was significantly lower in the Ca+Mg treatment than in CK until 56 days after anthesis, with the Ca+Mg treatment showing the most substantial inhibitory effect [59]. Similarly, the TCA pathway and PPP showed significant differences, with the Ca+Mg treatment inhibiting both pathways, particularly in the later stages [60,61]. Overall, these findings highlight a complex interaction between calcium and magnesium treatments and fruit metabolism, potentially affecting the sensation of pulp sugar reduction and fruit quality.

#### *4.3. Effect of Ca+Mg Treatment on Enzymatic Activity and Metabolic Processes*

The observed variations in succinate dehydrogenase (SDH) activity in litchi pulp across treatments and CK are consistent with its crucial role in cellular respiration. Elevated SDH activity in the CK indicates heightened metabolic processes, while decreased activity in the Ca+Mg treatment suggests potential inhibition of the tricarboxylic acid (TCA) cycle and electron transport chain [62]. Similarly, the findings regarding PDC activity suggest a potential influence on metabolic pathways, with Ca+Mg treatment exhibiting inhibition of the PDC pathway [63]. Glucose-6-phosphate isomerase (GPI) activity initially rose but was later suppressed by Ca+Mg treatment, indicating inhibition of the EMP pathway upon full maturity. Additionally, fluctuations in pyruvate kinase (PK) activity suggested a potential collaborative effect of Ca+Mg fertilizers in enhancing PK activity post anthesis [64]. NAD-malate dehydrogenase (NAD-MDH) activity was initially inhibited but later stimulated by Ca+Mg treatment, suggesting a complex influence on TCA pathway dynamics, while CCO activity initially dipped but later significantly rose in the Ca+Mg treatment, indicating potential promotion of CCO activity during the maturity stage, as observed [65]. These findings underscore the interconnected and intricate nature of metabolic pathways and indicate that Ca+Mg treatments can affect enzymatic activities and metabolic fluxes in litchi fruit pulp. In summary, Ca+Mg plays a crucial role in maintaining fruit quality by

influencing membrane integrity, sugar accumulation, respiration rates, and enzymatic activities. Their combined application not only enhances fruit sugar content and reduces spoilage but also affects various metabolic pathways, thereby optimizing fruit quality and nutritional value. These findings emphasize the importance of adequate mineral nutrition for the growth and development of high-quality fruits.

## 5. Conclusions

In addressing the challenges related to “sugar receding” in ‘Feizixiao’ Litchi, the foliar application of Ca+Mg significantly influenced the biochemical composition and respiratory pathways of ‘Feizixiao’ litchi fruit pulp. The treatment effectively enhanced sugar accumulation, overcoming the “sugar receding” issue and improving flavor quality. However, it also inhibited the total respiration rate and key enzymes involved in glycolysis (EMP), PPP, and the TCA cycle, suggesting a modulation of energy metabolism. Transcriptomic analysis revealed differential regulation of genes encoding pivotal enzymes such as pyruvate kinase (PK) and pyruvate dehydrogenase complex (PDC), indicating complex regulatory mechanisms underlying the observed metabolic changes. Overall, the findings highlight the elaborate interchange between foliar nutrient application, metabolic pathways, and gene expression in litchi fruit physiology, providing valuable insights for optimizing fruit quality management strategies.

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