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Preharvest GA_3 Treatment Enhances Postharvest Storability of 'Brightwell' Blueberry by Bolstering Antioxidant Defenses and Modulating Glycerolipid Metabolism

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Abstract

This study evaluated the effects of the preharvest application of 0.2 g/L gibberellin A3 (GA_3) or 0.02 g/L forchlorfenuron (CPPU) at full bloom on postharvest storability and defense responses in 'Brightwell' blueberry. After ripening, berries were inoculated in vitro with a defined mixture of postharvest fungal pathogens. Fruit quality attributes and physio-logical indices were monitored during storage, and LC-MS metabolomics was performed to characterize treatment-associated metabolic alterations.GA_3-treated fruit exhibited higher antioxidant capacity and a lower decay incidence than CPPU-treated and control fruit. Metabolomic profiling showed that GA_3 was associated with the accumulation of specific polyphenols, coinciding with enhanced resistance to postharvest pathogens. In parallel, GA_3 treatment modulated glycerolipid metabolism and mitigated membrane lipid peroxidation, as indicated by reduced malondialdehyde levels, while enhancing enzymatic (superoxide dismutase and ascorbate peroxidase) and non-enzymatic (polyphenol) antioxidant defenses. Overall, these results suggest that preharvest GA_3 application can improve blueberry storability by coordinating redox homeostasis and lipid-related metabolic remodeling.

Keywords: CPPU; fungal pathogens; metabolomics; polyphenols; membrane lipid peroxidation

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1. Introduction

Blueberry (*Vaccinium* spp.), belonging to the genus *Vaccinium* in the Ericaceae family, is native to North America and has been introduced to many regions worldwide [1]. Blueberry fruits are blue to violet in color with a sweet–tart flavor and are valued for their high nutritional quality. They contain sugars, amino acids, vitamins, minerals, and abundant phenolic compounds that are beneficial to human health [2] and are widely recognized as a nutrient-dense fruit rich in health-promoting phytochemicals [3,4]. With the continuing expansion of blueberry cultivation, market demand for fruit quality has increased. Producing large berries with high nutritional value and good storability has become a key goal in blueberry production. However, blueberries are highly perishable, and postharvest decay and quality loss during distribution remain major constraints on marketability and shelf life [5]. Accordingly, regulating flowering and fruit ripening to advance or delay market supply is of considerable economic importance to the blueberry industry [6,7].

Gibberellin A3 (GA_3) is an important plant growth regulator that plays a pivotal role in fruit-tree growth and development and is widely used in commercial production. GA_3 has been reported to break seed dormancy [8], increase fruit set [9,10], induce parthenocarpy, stimulate cell elongation in stems and leaves [11–13], promote early ripening, and adjust the growth cycle [14]. In some fruit crops, GA_3 application has also been associated with delayed senescence or improved storage-related traits under specific regimes and storage conditions [15].

Forchlorfenuron (CPPU) is a synthetic cytokinin (CK) of the phenylurea class. Previous studies have shown that CPPU can promote cell division, increase fruit set, and delay ripening, thereby improving fruit quality [16]. In blueberries, CPPU has been reported to increase fruit size and soluble sugar content [17,18]. Notably, the responses to GA_3 or CPPU are often cultivar- and dose-dependent, and their postharvest consequences may vary with application regimes and storage conditions [19].

In China, the agricultural use of GA_3 and CPPU is governed by pesticide registration label requirements, including permitted crops, application rate, pre-harvest interval (PHI), and maximum number of applications. The application regime adopted in this study was designed to comply with the relevant national standard and label instructions.

The combined application of GA_3 and CPPU has been extensively explored in several fruit crops and is often associated with improved yield and quality. For instance, in 'Shine Muscat' grapes, combined GA_3 and CPPU treatment increased berry weight, berry dimensions, firmness, and soluble solids while decreasing titratable acidity [20]. However, whether these treatments influence postharvest storability remains unclear, and their effects on blueberry storability have not been established. Importantly, existing blueberry studies on GA_3 and CPPU have largely emphasized fruit set and berry enlargement, whereas evidence regarding postharvest decay resistance and associated metabolic adjustments—especially under pathogen pressure—remains limited [21]. Therefore, clarifying how preharvest GA_3 and CPPU management relates to postharvest performance is both scientifically and practically important, particularly for cultivars with strong inherent storability.

Gray mold caused by *Botrytis cinerea* Pers. is a major postharvest disease limiting blueberry storability. The pathogen can persist in soil and infected residues and spread via air currents, rain splash, and insects [22,23]. Disease development is strongly influenced by humidity and handling/storage conditions, and infection and colonization during storage can accelerate decay and quality deterioration, ultimately shortening shelf life [24–26]. Current strategies to improve blueberry storability have mainly focused on pre- or postharvest treatments with chemical elicitors such as melatonin (MT) and methyl jasmonate (MeJA) [22,26], postharvest coating technologies [25,27], and optimization of storage conditions [28].

In contrast, plant growth regulators (PGRs) such as gibberellic acid (GA_3) and forchlorfenuron (CPPU) have been widely studied for their roles in regulating fruit development (e.g., berry size and yield) in blueberries and other fruit crops, whereas their potential impacts on postharvest storability and defense responses remain less consistently documented and are highly dependent on cultivar, application regime, and storage context. Therefore, the effects of CPPU on blueberry storability, particularly when evaluated together with GA_3, have received relatively limited attention. To our knowledge, integrative studies linking preharvest PGR application with postharvest pathogen challenge and metabolome-wide responses in blueberry fruit are still scarce [29].

To address this gap, the present study combined preharvest GA_3 and CPPU treatments with in vitro inoculation of *Botrytis cinerea* and LC–MS-based metabolomics during storage to characterize postharvest storability and associated metabolic responses in blueberry fruit. This work highlights cultivar-specific postharvest responses to preharvest PGR management and identifies candidate pathways and metabolites associated with quality retention

and decay suppression. This integrative framework enables a more comprehensive evaluation of how preharvest PGR management may relate to postharvest performance and provides evidence to support the rational use of growth regulators to improve blueberry quality and storability through agronomic practices.

2. Materials and Methods

2.1. Experimental Materials

The experiment was conducted using 4-year-old 'Brightwell' rabbiteye blueberry (*Vaccinium virgatum* Aiton) plants grown in an open field at Nanjing Botanical Garden Mem. Sun Yat-Sen (118°49′46″ E, 32°03′29″ N). Three biological replicates (n = 3) were established per treatment, each with three plants per replicate, to ensure reliable results and biological variability. GA_3 (200 mg/L) was sprayed onto the fruit clusters at 5 and 10 days post-anthesis. CPPU (20 mg/L) was applied by spraying at 15 days post-anthesis. Four treatments were established: CK0 (distilled water), T1 (GA_3 applied twice), T2 (CPPU applied once), and T3 (GA_3 applied twice plus CPPU applied once), with three replicate plants per treatment. Blueberries were harvested at maturity (uniformly blue over 100% of the surface, including the stem-end, with no red/pink coloration), then visually screened for freshness, uniform appearance, and absence of pests and diseases, and were immediately transported to the laboratory for subsequent storage experiments.

2.2. Reagents and Instruments

AFS-830 Atomic Fluorescence Spectrometer (Haiguang Instruments, Chinese mainland); VISTA-MPX Inductively Coupled Plasma Emission Spectrometer (Varian, USA); Q Exactive^{TM} HF-X mass spectrometer (Thermo Fisher, USA); Vanquish UHPLC chromatograph (Thermo Fisher, USA); D3024R low-temperature centrifuge (Scilogex, USA).

2.3. Experimental Methods

Botrytis cinerea was isolated from the surface of naturally infected (moldy) blueberry fruit following the method described by Wang [26]. The isolate was authenticated as B. cinerea based on colony morphology and microscopic characteristics. Conidia were harvested and suspended in sterile water, and the conidial concentration was adjusted to 1.0×10^6 conidia/mL for inoculation. Fresh fruits were rinsed with tap water to remove surface dust and then stored in preservation boxes for subsequent use.

Inoculation method: A 0.3 cm deep hole was created using a 1 mL sterile pipette tip, followed by inoculation with 10 μL of spore suspension via a pipette. Each treatment included 10 fruits per replicate, with 3 biological replicates. Fruit were divided into non-inoculated groups (CK0, T1, T2, and T3) and in vitro inoculated groups (CKB, T1B, T2B, and T3B). All fruits were packaged in 0.03 mm polyethylene bags to prevent moisture loss and stored at 25 °C with a relative humidity of 90%. Although typical commercial blueberry storage occurs at 0–4 °C to maintain freshness and prevent decay, we selected 25 °C in this experiment to accelerate the observation of the treatment effects on postharvest storability. Storage characteristics and fruit quality parameters were evaluated on 0 d, 2 d, and 4 d of storage.

2.3.1. Determination of Blueberry Fruit Quality Parameters

a. Rot rate: Rot incidence was determined by visual counting. At each sampling time point, 20 fruits were randomly selected from each treatment group, and the number of rotten fruits was recorded. A fruit was classified as rotten when it showed visible mold growth, bacterial lesions, or at least one rotted spot on the surface. Rot rate

(%) was calculated as: Rot rate (%) = (number of rotten fruits/total number of fruits assessed) × 100%.

b. Phenotypic index determination (longitudinal diameter, transverse diameter, firmness, color): fruit were randomly selected to measure fresh weight, transverse diameter, longitudinal diameter, and firmness. Fresh weight was measured via an electronic analytical balance (METTLER TOLEDO, MS104TS/02, Germany). The transverse and longitudinal diameters were measured via a Vernier caliper (DL91150), and the fruit shape index was calculated as the ratio of the longitudinal diameter to the transverse diameter. Fruit firmness at the maximum diameter was measured via a Takeura Cat No. 9300 (KM-5 Japan) fruit hardness tester with a 1 mm diameter conical probe and a 5 mm downward pressure distance; the maximum breaking force was recorded in N. Ten fruits were measured per index, and the results are expressed as the means ± standard deviations.

c. Determination of sugar and acid contents: The soluble sugar content was determined via the anthrone colorimetric method. The titratable acid content was measured according to GB/T 12456-2008 [30] "Determination of Total Acid in Food". Briefly, 3 g of fruit homogenate was transferred to a 50 mL centrifuge tube, mixed with 30 mL of deionized water, ultrasonicated at 35 °C and 60 Hz for 20 min, and centrifuged at 5000× *g* for 5 min. The supernatant was used for soluble sugar determination: after appropriate dilution, anthrone and concentrated sulfuric acid were added, and the mixture was incubated in a boiling water bath for 10 min; the absorbance was measured at 620 nm. For titratable acid determination, 15 mL of the supernatant was transferred to a 30 mL beaker, and the initial pH was measured. The solution was titrated with 0.1 mol/L NaOH standard solution to pH 8.0, and the volume of NaOH consumed was recorded. The titratable acid content was calculated as follows:

$$\text{Titratable acid content} = [V \times c \times (V_1 - V_0) \times f]/(V_s \times m) \times 100\%$$

where V = the total volume of sample extract (mL), V_s = the volume of supernatant used for titration (mL), c = the concentration of NaOH (mol/L), V_1 = the volume of NaOH consumed for sample titration (mL), V_0 = the volume of NaOH consumed for distilled water titration (mL), m = the sample mass (g), and f = the conversion coefficient (kg/mol).

d. Determination of antioxidant indices:

① Total anthocyanin content: Total anthocyanin content was measured via the pH differential method. Fifty grams of blueberry fruit was pulped, and 3 g of pulp was mixed with 30 mL of 50% ethanol, ultrasonicated at 35 °C and 60 Hz for 20 min, and centrifuged at 5000× *g* for 5 min. A 0.3 mL aliquot of the supernatant was mixed with 2.7 mL of pH 1.0 HCl-KCl buffer and incubated in the dark at 25 °C for 20 min, after which the absorbance was measured at 510 nm. The anthocyanin content was calculated via the following standard curve equation: $y = 0.0455x - 0.0881$ ($R^2 = 0.9996$), where y = absorbance at 510 nm and x = anthocyanin content.

② Total polyphenol content: Determined via the Folin–Ciocalteu method. Three grams of pulp were mixed with 30 mL of 50% ethanol, ultrasonicated at 35 °C and 60 Hz for 20 min, and centrifuged at 5000× *g* for 5 min. A 200 μL aliquot of the supernatant was diluted with 300 μL of 50% ethanol, mixed with 2.5 mL of 0.1 mol/L Folin–Ciocalteu reagent, followed by the addition of 2 mL of a 7.5% saturated Na_2CO_3 solution, and incubated in the dark for 1 h. The absorbance was measured at 750 nm, and the polyphenol content was calculated via the standard curve equation $y = 0.0634x - 0.00179$ ($R^2 = 0.9992$), where y = the absorbance at 750 nm and x = the polyphenol content.

③ Total protein (TP) content: Total protein (TP) content was determined via a commercial kit (Nanjing Jiancheng Institute of Bioengineering, Chinese mainland) based on the Coomassie Brilliant Blue method. A 0.1 g aliquot of pulp was homogenized with 0.9 mL of 0.1 mol/L phosphate-buffered saline (PBS, pH 7.0–7.4) on ice to prepare a 10% tissue homogenate, which was subsequently centrifuged at 2500× *g* for 10 min. The supernatant was diluted appropriately and mixed with Coomassie Brilliant Blue chromogenic solution, and the absorbance was measured at 595 nm using distilled water as a blank and protein standard solution for calibration.

④ Determination of superoxide dismutase (SOD), nitric oxide (NO), ascorbic acid peroxidase (APX), malondialdehyde (MDA), chitin, and hydrogen peroxide (H_2O_2): Contents were determined via commercial kits (Nanjing Jiancheng Institute of Bioengineering). A 0.2 g aliquot of pulp was homogenized with 1.8 mL of pH 7.4 PBS on ice to prepare a 10% tissue homogenate, which was subsequently centrifuged at 3500× *g* for 10 min. The supernatant was used for analyses: reagents were added according to the kit instructions, and the absorbance was measured at 536 nm (SOD), 550 nm (NO), 290 nm (APX), 530 nm (MDA), 585 nm (chitin), and 405 nm (H_2O_2). For APX, the absorbance was measured at 10 s and 130 s after mixing.

2.3.2. LC–MS Metabolomic Analysis Conditions

Analyses were performed via an ACQUITY UPLC I-Class plus (USA)/Thermo QE liquid chromatography–mass spectrometry (LC–MS) system (USA) with a Hyperil Gold C18 column (USA). The column temperature was 40 °C, and the flow rate was 3.33×10^{-3} mL/s. Mobile phase A consisted of 0.1% formic acid (positive mode) or 5 mM ammonium acetate (pH 9.0, negative mode); mobile phase B was methanol. The elution gradient was as follows: 0–2 min, 2% B (isocratic); 1.5–3 min, linear gradient from 2% B to 85% B; 3–10 min, linear gradient to 100% B; 10.0–10.1 min, linear gradient back to 2% B; and equilibration for 14 min. The mass spectrometry conditions were as follows: spray voltage = 3.5 kV; sheath gas flow rate = 35 psi; auxiliary gas flow rate = 166.67 mL/s; capillary temperature = 320 °C; auxiliary gas heater temperature = 350 °C; polarity = positive and negative modes; and MS/MS secondary scanning = data-dependent scans.

2.4. Data Analysis

Duncan's multiple range test was used for significance analysis ($p < 0.05$). One-way analysis of variance (ANOVA) was performed via the "agricolae" package in R (version 4.0.1, New Zealand). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted via SIMCA 14.1 software (MKS Data Analytics Solutions, formerly Umetrics Company, Sweden). Bar plots were generated via the "ggplot2" package, and heatmaps were created via the "ComplexHeatmap" package in R (version 4.0.1, New Zealand).

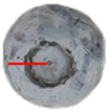
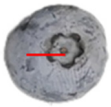
3. Results

3.1. Comparison of Fruit Phenotypic Indicators Before Storage

As shown in Table 1, fruit treated with T1 exhibited significant improvements in size, weight, and hardness, with an 8.58% increase in longitudinal diameter and a 7.81% increase in hardness. Compared to the CK0 treatment, the T2 treatment showed more pronounced effects, with a 44.5% increase in single-fruit weight, the largest increase among all four treatments; however, the fruit's hardness was slightly lower. In the T3 treatment, compared to CK0, the transverse diameter, longitudinal diameter, and single-fruit weight increased, along with a 16% improvement in fruit hardness. Additionally, the L* values of the three growth regulator-treated groups (T1, T2, and T3) significantly increased, reflecting a notable

change in fruit color. The hardness and color of blueberries are closely related to their surface waxy substances and anthocyanins [31]. GA_3 and CPPU, as growth regulators, may influence the synthesis of waxy substances and anthocyanins in blueberry fruit.

Table 1. Pre-storage phenotypic indicators of 'Brightwell' fruit.

Indicators	CK0	T1	T2	T3
Fruit diameter (mm)	15.87 ± 0.72 c	16.28 ± 1.11 bc	18.35 ± 0.63 a	16.77 ± 0.79 b
Fruit longitudinal diameter (mm)	13.76 ± 0.68 b	14.94 ± 1.43 a	14.86 ± 0.67 a	14.13 ± 0.59 ab
Fruit weight (g)	2.00 ± 0.23 c	2.28 ± 0.45 bc	2.89 ± 0.28 a	2.32 ± 0.19 b
Fruit firmness (N)	3.84 ± 0.47 ab	4.14 ± 0.36 a	3.66 ± 0.21 c	4.02 ± 0.38 a
Fruit color L*	28.69 ± 3.61 b	37.39 ± 7.6 a	34.81 ± 5.73 a	34.54 ± 6.73 a
Fruit photo				

Note: 1. Each row of different letters indicates significant differences ($p < 0.05$) between each variety for each metric according to Duncan's test. 2. L* value is the lightness parameter in the CIE L*a*b* color space, ranging from 0 to 100; a higher value indicates a lighter color, while a lower value indicates a darker color.

3.2. Storage Indicators: Contamination Rate, Total Acid Content, and Soluble Sugars

Results presented in Figure 1 reveal a higher mold contamination rate in the in vitro mold-inoculated group versus the non-inoculated control. Marked differences were also evident in the inhibitory efficacy of the tested growth regulators against contamination. Among them, the T1 treatment had the lowest contamination rate among all the treatment groups and for all the periods. Compared with that of the control group (CK0), the contamination rate of the group exposed to mold for 4 d decreased by 51.34% under the same treatment during the same period, demonstrating the strongest anti-mold contamination ability. The T2 treatment with CPPU alone had a relatively high contamination rate. When there was mold contamination for 4 d, the contamination rates both with and without mold contamination were higher than those of the control group under the same treatment during the same period, indicating that the inhibitory effect of CPPU alone on mold contamination was relatively weak. The T3 treatment with the combination of GA_3 and CPPU had a synergistic regulatory effect. When the samples were exposed to mold for 4 d, the contamination rate was 30.72% lower than that of T2 under the same treatment during the same period, making it the treatment group with the second lowest contamination rate. In terms of the other storage indicators, the overall changes in the total acid and soluble sugar contents of each treatment group were not significant. However, in general, the soluble sugar content tended to decrease during storage; under mold infection, the total acid content tended to first increase but then decrease.

3.3. Antioxidant Indicators

Figure 2 illustrates that different growth regulator treatments (T1, T2, and T3) significantly affected the physiological metabolism and antioxidant indicators of blueberries during postharvest storage. Among these treatments, the T1 treatment showed the best storage adaptability. Specifically, the anthocyanin content in the T1, T2, and T3 treatment groups was higher than in the control group (CK0), with the T3B group, which was exposed to mold for 4 days, showing the most significant increase (62.79% compared to CKB). The polyphenol content peaked in the middle of storage. In the T3 treatment at day 2, the polyphenol content reached 105.29 mg/g, a 61.84% increase compared to the CK0 treatment during the same period. The chitin content generally decreased, and in vitro mold infection accelerated its reduction. Among all treatments, the T1B group exhibited the smallest decrease in activity (only 16.60%), making it the most stable under infection conditions.

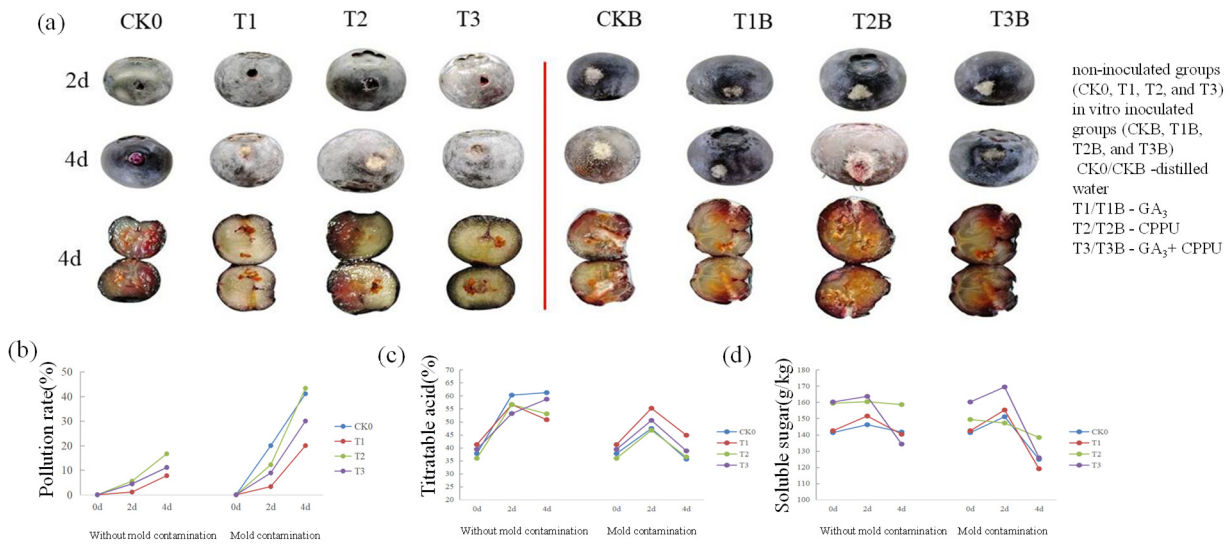


Figure 1. Postharvest Quality Dynamics of 'Brightwell' Blueberry (**a**), visual quality progression (2–4 d); (**b**), contamination incidence (%); (**c**), titratable acidity (%); and (**d**), soluble solids content (g/kg).

Indicators related to oxidative damage showed distinct differences: malondialdehyde (MDA) content increased with storage time, but the MDA content in the T1 treatment was consistently the lowest, indicating that oxidative damage to the cell membrane was the mildest. Similarly, the content of superoxide dismutase (SOD) remained stable, with no significant trends observed.

The protein and nitric oxide (NO) contents showed phased changes: protein content increased over time, except in the T2 treatment during the middle stage of storage, and fluctuated in the later stages. While the overall trend of NO content was not clear, the NO levels in the T1, T2, and T3 groups that were not infected at the end of storage were higher than those in the infected groups during the same period. The NO content in the T1 group was 39.56% higher than in the T1B group at 4 days.

Regarding other antioxidant substances, hydrogen peroxide (H_2O_2) content in the T1 treatment was lower than in the other treatments. On day 0, it was only 62.17% of that in the CK0 group at the same time. This was directly related to the increase in ascorbic acid peroxidase (APX) content as storage progressed. The efficient expression of APX enhanced the H_2O_2 scavenging ability. Overall, the T1 treatment resulted in the lowest contamination rate and the best postharvest storage performance by optimizing the antioxidant system (low H_2O_2, high APX, and low MDA), maintaining chitin stability, and regulating NO metabolism.

3.4. Differentially Abundant Metabolites

The proportions of various substances detected through metabolomics, based on the ClassyFire classification, are shown in Figure 3a. Lipids and lipid analogs were the predominant metabolite category, accounting for 23.43% of the metabolites. This was followed by phenylpropanoids and polyketides (14.10%), organic acids and their derivatives (10.92%), organic heterocyclic compounds (8.26%), and organic oxides (7.66%). Principal component analysis (PCA) revealed that, in positive ion mode, PC1 (55.30%) and PC2 (10.80%) together explained 65.80% of the total variation. Notably, the metabolic differences induced by mold contamination were significant, whereas the differences between samples treated with GA_3 during the same period were relatively minor.

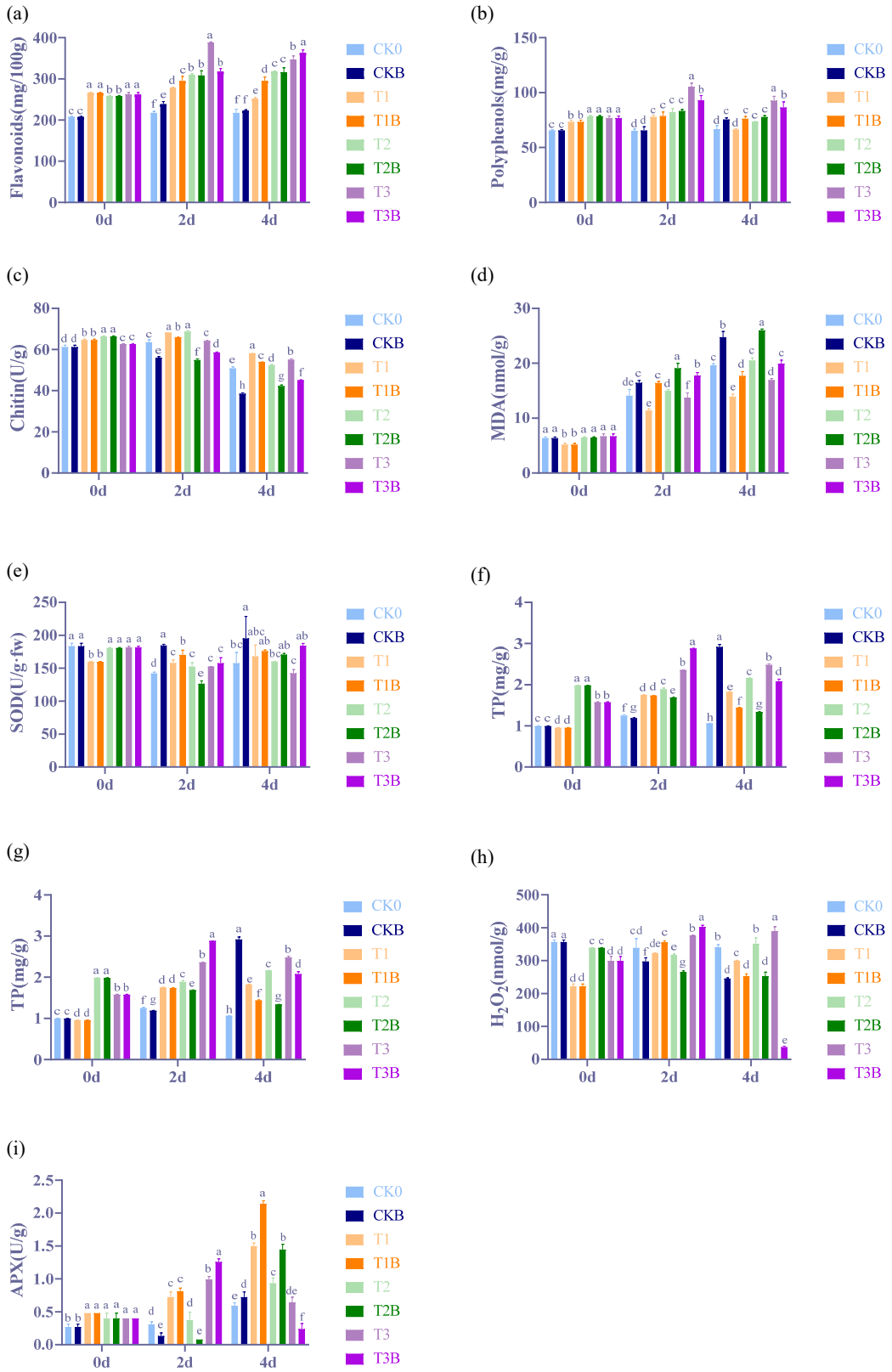


Figure 2. Antioxidant Dynamics in 'Brightwell' Blueberry during Storage (**a**), Flavonoids; (**b**), Polyphenols; (**c**), Chitin; (**d**), MDA (Malondialdehyde); (**e**), SOD (Superoxide Dismutase); (**f**), TP (Total protein); (**g**), NO; (**h**), H_2O_2; and (**i**), APX (Ascorbate Peroxidase). Note: 1. Different lowercase letters indicate significant differences ($p < 0.05$) among treatments at each time point by Duncan's test. 2. Error bars = SD.

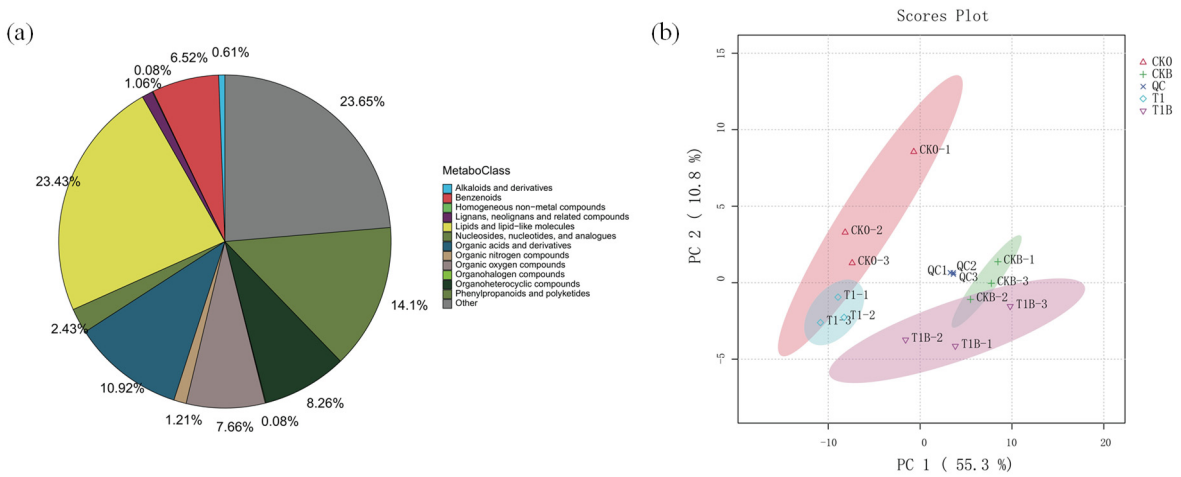


Figure 3. Comparative Metabolite Profiles (**a**), DAM distribution: Proportional overlap in T1 vs. CK0 and T1B vs. CKB; (**b**), PCA score plot: Group separation (T1/CK0 vs. T1B/CKB).

The Venn diagram analysis in Figure 4a,b revealed that there were 312 differentially abundant metabolites between CK0 and CKB, and 279 between T1 and T1B, with 113 overlapping differentially abundant metabolites between the two comparisons. Additionally, there were 101 differentially abundant metabolites between T1 and CK0, 94 between T1B and CKB, and 17 overlapping differentially abundant metabolites between these two comparisons. The results of partial least squares discriminant analysis (PLS-DA) further confirmed that significant differences in metabolite composition existed between CK0 and CKB, as well as between CKB and T1B.

Figure 4c presents the volcano plot of differentially abundant metabolites, visually displaying the distribution of metabolite differences among each group: red indicates significant upregulation, blue indicates significant downregulation, gray indicates metabolites that did not reach the fold change threshold, and yellowish-brown indicates metabolites that met the fold change threshold but not the *p*-value threshold. Among the 94 differentially abundant metabolites between T1B and CKB, 30 were upregulated and 64 were downregulated. Among the 77 differentially abundant metabolites between T1 and CK0, 29 were upregulated and 72 were downregulated.

3.5. Correlation Analysis of Differentially Abundant Metabolites

The chord diagram of differentially abundant metabolites in Figure 5 visually presents the classification and attribution of differentially abundant metabolites as well as the correlation between their content changes. In this diagram, each point on the outermost circle represents a distinct metabolite, and the color of the point corresponds to the classification of the metabolite to which it belongs. The lines connecting the points indicate a high correlation among metabolites (with the absolute value of the correlation coefficient being $\geq$0.99), among which red lines represent a positive correlation, blue lines represent a negative correlation, and the thickness of the lines is positively correlated with the degree of correlation. Specifically, the differentially abundant metabolites between T1 and CK0 were classified into five main categories: benzoic acids, lipids and lipid molecules, organic acids and derivatives, phenylpropanoids and polyketides, and others. The classification of differentially abundant metabolites between T1B and CKB was more diverse. On the basis of the above five categories, two new categories, namely, nucleosides and nucleotides, and organic heterocyclic compounds, have been added, reflecting greater diversity in the classification of differentially abundant metabolites in vitro mold infection conditions (Group B).

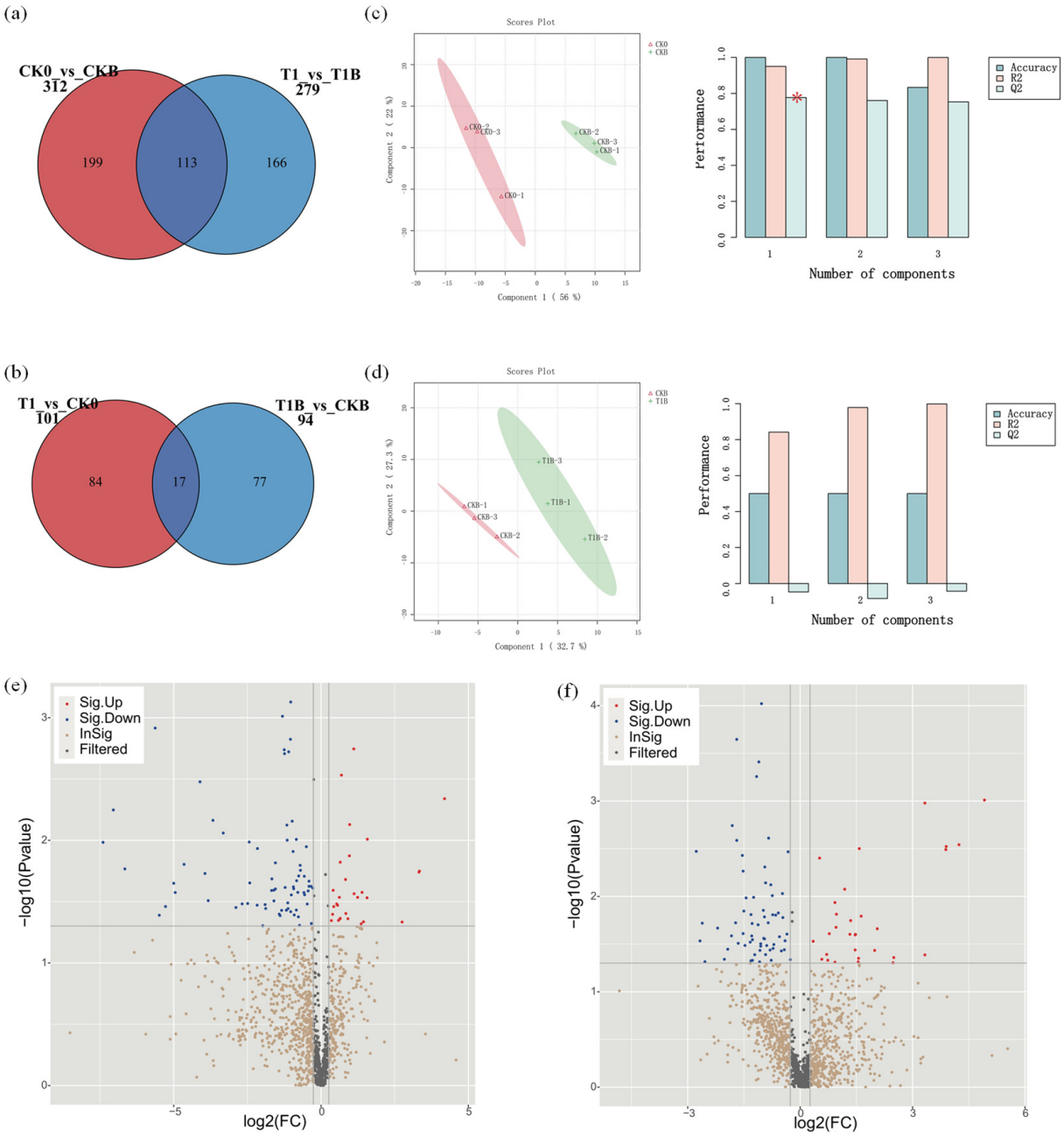


Figure 4. Differential Metabolite Analysis (**a**), Venn diagram: Shared DAMs in CK0 vs. CKB and T1 vs. T1B; (**b**), Venn diagram: Shared DAMs in T1 vs. CK0 and T1B vs. CKB; (**c**), PLS-DA score plot: CK0 vs. CKB separation; (**d**), PLS-DA score plot: CKB vs. T1B separation; (**e**), Volcano plot: DAMs in T1 vs. CK0; and (**f**), Volcano plot: DAMs in T1B vs. CKB. Note: * Indicates significant difference in (**c**).

3.6. Enrichment Analysis of Metabolic Pathways for Differentially Abundant Metabolites

As shown in Figure 6, the differentially abundant metabolites between CK0 and CKB were enriched in 30 metabolic pathways, with the most significantly altered pathways being neuroactive ligand-receptor interaction, niacin and nicotinamide metabolism, β-alanine metabolism, and proximal tubular bicarbonate reclamation. Compared to the differentially abundant metabolite pathways in T1B, the number of significant pathway differences under infection conditions was notably lower. The newly enriched metabolic pathways included alanine, aspartate, and glutamate metabolism; glutathione metabolism; and the synaptic vesicle cycle. Among these, nicotinamide in the niacin and nicotinamide metabolism pathway is an amide compound of vitamin B3 and a precursor of NAD+ and

NADP+ in multiple metabolic pathways. These two coenzymes play crucial roles in energy metabolism, redox reactions, and DNA repair [32]. Therefore, changes in this pathway may suggest that exogenous GA_3 influences the accumulation of vitamins in blueberry fruit as well as redox balance.

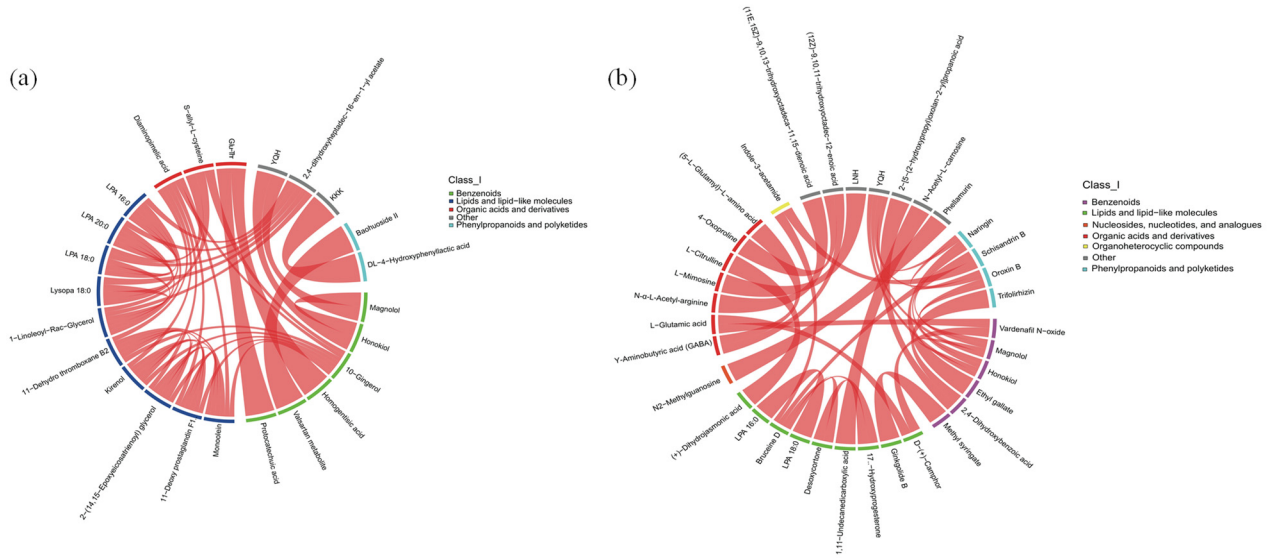


Figure 5. Metabolic Pathway Associations of DAMs (**a**), Chord diagram: Enriched pathways in T1 vs. CK0 and (**b**), Chord diagram: Enriched pathways in T1B vs. CKB.

Additionally, most of the significant metabolic pathways are involved in amino acid metabolism, which plays a central role in protein synthesis, energy metabolism, and nitrogen balance [33]. These metabolic changes may reflect the redistribution and utilization of nitrogen sources by blueberries in response to infection. Furthermore, the newly identified differential metabolic pathway of glutathione metabolism between T1 and T1B indicates that glutathione, an important antioxidant in plants, may have increased in response to oxidative stress, such as that caused by mold infection [34]. Neuroactive ligand-receptor interactions, proximal tubular bicarbonate reclamation, and the newly identified differential metabolic pathway of the synaptic vesicle cycle between T1 and T1B may also contribute to regulating signal transduction and response mechanisms in plants [35].

3.7. Thermal Map Analysis of Differentially Abundant Metabolites

Through the analysis of the clustering heatmap in Figure S1, the evolutionary trends of metabolites during storage can be identified. As shown in the figures, the content of lipids and lipid molecules (such as LPA) in the differentially abundant metabolites of T1 and T1B was lower than in CK0 and CKB. After in vitro infection by gray mold, the contents of polyphenols (such as lichenic acid and chlorogenic acid) in these groups were higher than in the CK0 and CKB groups. Amino acid derivatives, such as citrulline, aspartic acid-glutamic acid (asp-glu), glutamine, and lysine, were present in relatively high amounts in CK0 and CKB.

Lipids and lipid molecules accounted for the greatest proportion of differentially abundant metabolites. The content of differentially abundant metabolites (such as LPA) in the T1 and T1B groups was lower than in CK0 and CKB. GA_3 treatment predominantly reduced the accumulation of fatty acids and phospholipids. LPA may be rapidly utilized as a precursor in the synthesis of other functional lipids (such as membrane phospholipids), thereby enhancing cell membrane stability and improving the stress resistance of the fruit [36].

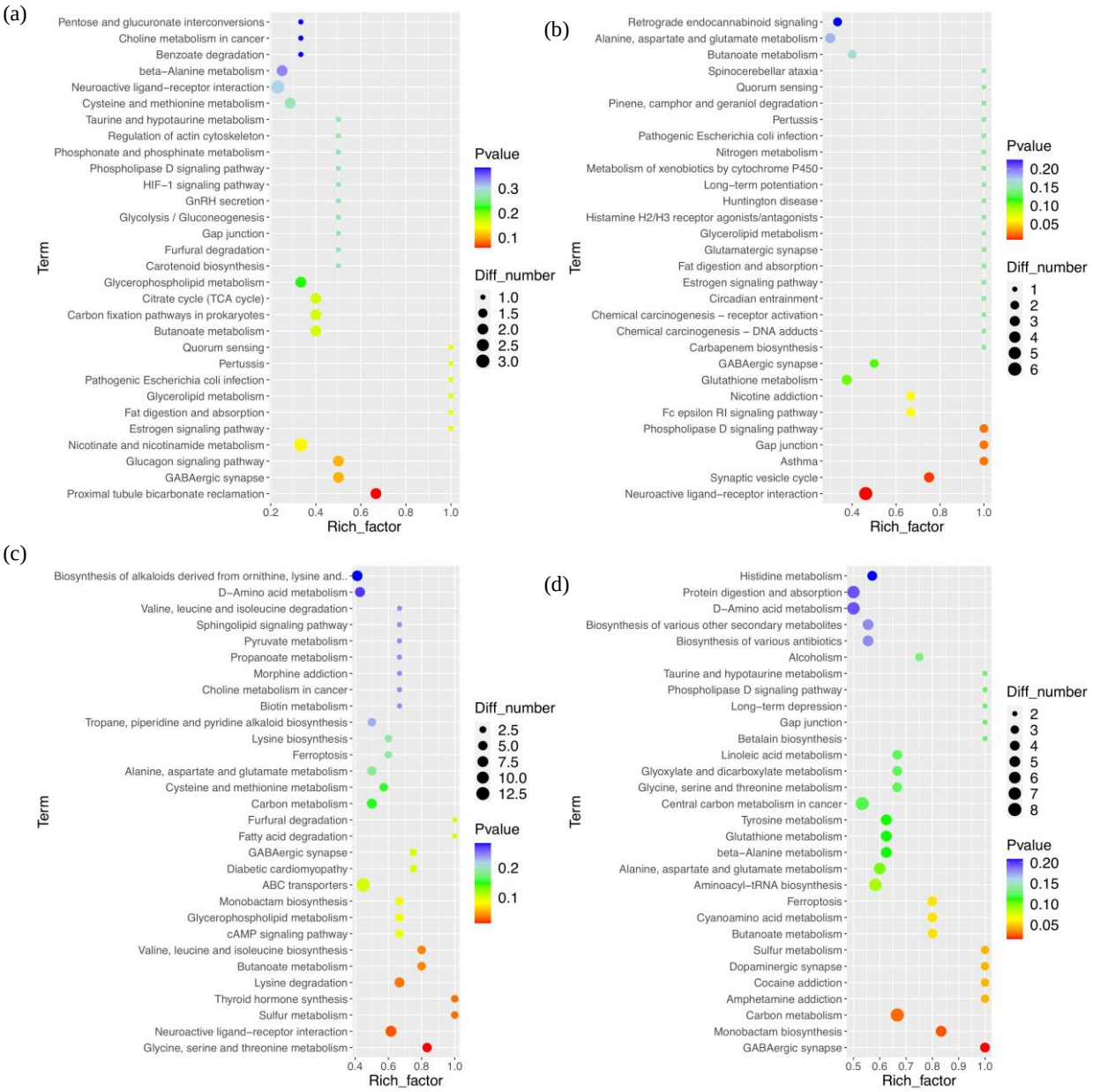


Figure 6. Metabolic Pathway Enrichment Analysis (**a**), T1 vs. CK0; (**b**), T1B vs. CKB; (**c**), CK0 vs. CKB; (**d**), T1 vs. T1B. Note: Enrichment factor (x-axis) = (DAMs in pathway)/(annotated metabolites in pathway). Circle size ∝ DAM count per pathway. Color gradient: blue ($p > 0.05$) → red ($p < 0.001$).

When blueberry fruits were infected by Botrytis cinerea, the accumulation of polyphenols (such as lichenic acid and chlorogenic acid) in the differentially abundant metabolites of the T1 group increased. Polyphenols are important secondary metabolites in plants, possessing various biological activities, including antioxidant, antibacterial, and anti-inflammatory properties [37]. Studies have shown that both lichenic acid and chlorogenic acid exhibit antibacterial activity: lichenic acid has potential as an antibacterial functional food additive, and chlorogenic acid can effectively prevent and control gray mold in peach fruit [37,38]. Under in vitro gray mold infection, the GA_3-induced increase in polyphenols in blueberries (T1 group) may represent a plant defense mechanism, aiding the plant in resisting pathogens or environmental stress.

The contents of amino acids and their derivatives (such as γ-aminobutyric acid (GABA), citrulline, aspartic acid-glutamic acid, glutamine, and lysine derivatives) in the T1 group were lower than in the CK0 group. This may be linked to the redistribution

of nitrogen metabolism in plants, which is consistent with the observation that most of the significant metabolic pathways among the enriched pathways are involved in amino acid metabolism.

3.8. Metabolic KEGG Analysis

Analysis of differentially abundant metabolites (DAMs) and enriched pathways (Figures 6 and S1) identified key conserved metabolic alterations during storage, including shared pathways such as triglyceride metabolism, GABAergic synapse, and alanine/aspartate/glutamate metabolism, as well as core DAMs like lysophosphatidic acid (LPA) and GABA.

LPA, traditionally considered an intermediate in glycerol metabolism, is now recognized as an important bioactive lipid mediator with multiple biological activities. It can trigger various cellular responses, including both mitotic [39,40] and antimitotic [41] effects on the cell cycle, as well as intracellular calcium mobilization [42]. LPA's ability to influence cell mitosis suggests that during postharvest storage of blueberry fruit, changes in LPA metabolism may delay fruit senescence by regulating the cell cycle. Inhibiting unnecessary cell division helps maintain fruit integrity and extends its shelf life. Additionally, lipid accumulation and cellular peroxidation are linked to aging [43]. A combination of metabolomics data and KEGG metabolic diagrams (Figure 7) revealed that LPA content in T1 and T1B was decreased, indicating that the T1 treatment slowed the increase in LPA, thereby inhibiting cellular peroxidation and delaying cellular aging. Moreover, under certain conditions, LPA and glycerides can be interconverted, further participating in the regulation of adipocyte decomposition. Glycerides, which are involved in the synthesis and decomposition of fatty acids, are closely linked to lipid metabolism, helping maintain the energy balance and material metabolism of cells.

GABA, an important plant signaling molecule [44], regulates plant growth, development, and metabolism [45], and plays a critical role in plant tolerance to abiotic stress [46–48]. When plants are exposed to environmental stresses, reactive oxygen species (ROS), including superoxide radicals (O_{2-}) and hydrogen peroxide (H_2O_2), accumulate [49]. These ROS are primarily produced through oxygen reduction, known as the Mehler reaction. The accumulation of ROS can cause lipid peroxidation, leading to the production of malondialdehyde (MDA), which damages cell structure and function [50]. To counteract the adverse effects of ROS, plants activate enzymatic and non-enzymatic antioxidant systems. As shown in Figure 1, T1 treatment reduced MDA and H_2O_2 levels in the fruit, lowering ROS levels, weakening cellular peroxidation stress signals, and inhibiting the activation of glutamate decarboxylase (GAD). This resulted in a reduction in GABA synthesis [51].

As an anaplerotic route connecting glutamate metabolism to the tricarboxylic acid (TCA) cycle, the GABA shunt can influence the replenishment of TCA intermediates by channeling carbon toward succinate formation and thereby affecting overall energy metabolism [52]. In our data, metabolites at the glutamate/GABA node showed a decreasing trend under T1-related treatments, accompanied by a reduction in α-ketoglutarate, whereas upstream organic acids (e.g., citrate and malate) and titratable acidity remained higher in T1B than in the control (CKB). These coordinated changes are consistent with a weakened contribution from the glutamate–GABA branch and a compensatory accumulation of upstream organic acids that may help sustain TCA-cycle intermediate pools and maintain metabolic homeostasis [53].

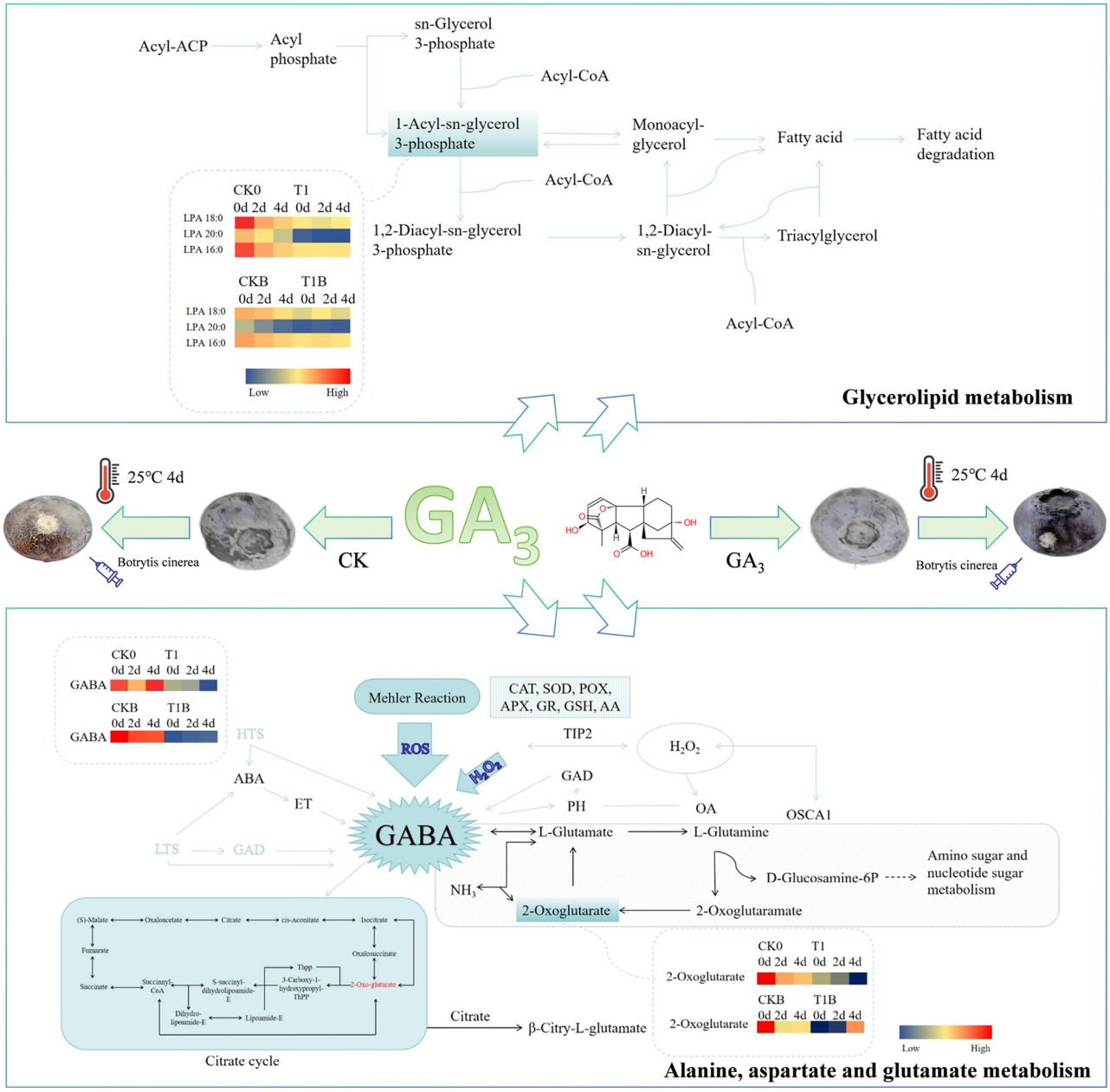


Figure 7. Schematic Diagram of Two Major Metabolic Pathways Involved in Blueberry Storage.

4. Discussion

The application of GA_3 and CPPU promoted berry enlargement, reduced the incidence of contamination during storage, improved postharvest storability, and extended shelf life. Although soluble sugar and titratable acidity did not differ significantly among treatments, GA_3-treated fruit exhibited higher anthocyanin and total polyphenol contents. This response is consistent with previous reports showing that exogenous plant growth regulators, particularly gibberellins, can promote the accumulation of phenolic compounds and improve quality-related attributes in blueberries [54] and other horticultural crops, e.g., litchi [55], grapefruit [56], sweet cherry [57].

Moreover, MDA, a product of lipid peroxidation, reflects the extent of cellular damage [58]. SOD is a key antioxidant enzyme involved in scavenging reactive oxygen species (ROS) and is commonly used as an indicator of antioxidant capacity. Therefore, the combined assessment of MDA and SOD is useful for evaluating oxidative damage and antioxidant status in postharvest fruit tissues. During storage, the gradual increase

in MDA content suggests that lipid peroxidation and oxidative damage intensified over time, whereas the absence of a clear trend in SOD suggests that antioxidant capacity remained relatively stable under these conditions. In contrast, the T1 treatment showed an increasing tendency in SOD content over the storage period, suggesting that GA_3 treatment was associated with enhanced antioxidant-related status, which may be related to the lower contamination incidence observed during storage. Previous studies have shown that exogenous plant growth regulators can influence antioxidant indices (e.g., SOD, APX, CAT, and POD) [59,60] and lipid-peroxidation markers (e.g., MDA) [61,62] during postharvest storage, although the magnitude and direction of these effects often depend on cultivar, application regime, and storage environment. Collectively, our findings support the interpretation that GA_3 treatment is associated with improved oxidative-stress-related performance during storage, consistent with reports in other horticultural crops [63].

Nitric oxide (NO), an important signaling molecule in plant defense responses, has been reported to enhance resistance against pathogens by triggering hypersensitive-like responses and inducing the expression of defense-related genes [64–66]. NO can also interact with ROS during stress responses, modulating superoxide-related processes and limiting lipid peroxidation, thereby reducing oxidative damage [67].The increase in NO content in the T1 treatment group under storage conditions suggests a potential role of GA_3 in modulating defense mechanisms, which may enhance the resistance of blueberry fruit to gray mold infection [26].

Furthermore, APX content increased over storage time. APX (ascorbate peroxidase) is a key enzyme involved in H_2O_2 detoxification in plant cells by using ascorbate as an electron donor. H_2O_2 is a reactive oxygen species generated in multiple cellular compartments during metabolism and stress responses [68]. In this study, H_2O_2 content in T1 was lower than that in the other treatments, and the concurrent increase in APX content over storage time may contribute to H_2O_2 scavenging [69]. Together with the SOD results, these findings indicate that GA_3 treatment (T1) was associated with enhanced antioxidant-related indices during storage, which may contribute to improved storability and reduced contamination incidence [70].

Metabolomics, a core discipline within systems biology, enables comprehensive analysis of metabolic dynamics through qualitative and quantitative profiling of metabolites, typically using chromatography-mass spectrometry techniques [71]. In this study, among the differentially abundant metabolites identified, lipids and lipid analogs represented the largest proportion, followed by phenylpropanoids and polyketides, organic acids and derivatives, organic heterocyclic compounds, and organic oxides. The increase in lipids and lipid analogs in the T1 treatment group suggests a response to lipid peroxidation caused by environmental stress and fungal infection, supporting the idea that GA_3 treatment mitigates stress-induced lipid damage [70,72]. The lipids and lipid molecules with the highest proportions were primarily fatty acids and phospholipids, which respond to lipid peroxidation caused by environmental stress. The T1 treatment effectively reduced lipid peroxidation. This is consistent with findings from other studies on the role of phospholipids in mitigating oxidative damage in postharvest fruits [73].

Under *Botrytis cinerea* infection conditions, polyphenols in the T1 treatment group increased. Polyphenols act as effective antibacterial agents, helping plants resist pathogens or environmental stress. This suggests that GA_3 treatment may not only affect fruit development but also modulate the fruit's metabolic pathways, enhancing its ability to combat pathogens during storage [63]. Furthermore, the enriched pathways revealed that most of the significant metabolic pathways were associated with amino acid metabolism, which plays a core role in protein synthesis, energy metabolism, and nitrogen balance. Amino acid metabolism plays a key role in regulating stress responses, and the redistribution

of nitrogen sources could be a metabolic adaptation to stress [62,74]. These metabolic changes likely reflect the redistribution and utilization of nitrogen sources by blueberries in response to gray mold infection, consistent with the fact that amino acids are prominent in the metabolite difference heatmap.

Additionally, LPA and GABA, both differentially abundant metabolites, help delay fruit senescence, increase stress resistance, and regulate organic acid content in fruit. They achieve this by regulating the cell cycle, inhibiting lipid peroxidation, and maintaining TCA cycle homeostasis during postharvest storage, thus improving fruit quality. While the role of GABA in postharvest storage has been hypothesized, the exact mechanistic pathways through which it regulates senescence and stress resistance remain to be fully elucidated and should be treated as a speculative discussion [75–77]. The nontargeted metabolomics analysis method used in this study cannot precisely calculate the absolute content of each compound. However, it provides valuable theoretical insights into the metabolic changes during 'Brightwell' blueberry storage and offers a reference for future research on storage and quality control of blueberries treated with GA_3.

5. Conclusions

In conclusion, the application of GA_3 and CPPU treatments significantly improved the postharvest storability of 'Brightwell' blueberry fruits by enhancing their resistance to oxidative stress and reducing the contamination rate during storage. The results suggest that GA_3 treatment, in particular, increased the contents of anthocyanins, polyphenols, and key antioxidant enzymes, including SOD and APX, which are essential for combating reactive oxygen species (ROS) and lipid peroxidation. Furthermore, metabolomics analysis revealed that GA_3 treatment modulated several metabolic pathways, particularly those involved in GABA metabolism and polyphenol biosynthesis, which may contribute to enhanced stress resistance and improved fruit quality during storage. Although the findings highlight the potential of GA_3 and CPPU to improve blueberry quality and storability, further research is needed to confirm the precise mechanistic pathways and explore the effects under cold chain storage conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy16070686/s1, Figure S1: Clustered Abundance Patterns of DAMs.

Author Contributions: X.P. and X.W. (Xiaoming Wang) designed and performed the experiments. X.P., X.W. (Xingru Wei) and X.W. (Xiaoming Wang) analyzed the data. X.P. wrote the first manuscript version. X.W. (Xiaoming Wang), Q.Z., Y.W. and W.W. reviewed the manuscript, and all the authors accepted the final version of the manuscript. Conceptualization, X.P. and X.W. (Xiaoming Wang); methodology, X.P.; software, X.P.; validation, X.P., X.W. (Xingru Wei), H.L. and X.W. (Xiaoming Wang); formal analysis, X.P.; investigation, X.P.; resources, X.P.; data curation, X.P.; writing—original draft preparation, X.P.; writing—review and editing, X.W. (Xiaoming Wang), Q.Z., Y.W. and W.W.; project administration, X.P.; funding acquisition, X.W. (Xiaoming Wang). All authors have read and agreed to the published version of the manuscript.

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