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LC−MS and GC−MS Metabolomics Analyses Revealed That Different Exogenous Substances Improved the Quality of Blueberry Fruits under Soil Cadmium Toxicity

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ABSTRACT: Exogenous substances (ESs) can regulate plant growth and respond to environmental stress, but the effects of different ESs on blueberry fruit quality under soil cadmium (Cd) toxicity and related metabolic mechanisms are still unclear. In this study, four ES treatments [salicylic acid (SA), spermidine (Spd), 2,4-epibrassinolide (EBR), and melatonin (MT)] significantly increased blueberry fruit size, single-fruit weight, sweetness, and anthocyanin content under soil Cd toxicity and effectively reduced fruit Cd content to safe consumption levels by promoting mineral uptake (Ca, Mg, Mn, Cu and Zn). Furthermore, a total of 445, 360, 429, and 554 differentially abundant metabolites (DAMs) (LC−MS) and 63, 48, 79, and 73 DAMs (GC−MS) were identified from four comparison groups (SA/CK, Spd/CK, EBR/CK and MT/CK), respectively. The analyses revealed that ESs improved blueberry fruit quality and tolerance to Cd toxicity mainly by regulating the changes in metabolites related to ABC transporters, the TCA cycle, flavonoid biosynthesis, and phenylpropanoid biosynthesis.

KEYWORDS: *exogenous substance, Cd toxicity, metabolomics, blueberry, fruit quality*

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1. INTRODUCTION

Soil heavy metal pollution caused by industrial and agricultural activities and other environmental factors has a major impact worldwide.[1] Cadmium (Cd) is one of the most important heavy metal contaminating elements in agricultural soils, and approximately 25% of arable land in China is contaminated with heavy metals to varying degrees, with Cd contamination accounting for the highest percentage.[2] Due to the high mobility of Cd in the soil, it is easily absorbed by plants and transported to different tissues.[3] Excessive Cd accumulation can damage the structure and function of cell membranes, leading to plant nutrient deficiencies, reduced photosynthesis, and disorders of other physiological processes, such as oxidative stress, which can inhibit plant growth and even death.[4] In addition, in agricultural production, Cd can reduce crop yields and enter the human body through the food chain, thus posing a serious threat to food safety and human health.

Blueberries, known as "extreme superfruits", are popular among consumers because they are rich in anthocyanins, vitamin C (VC), polyphenols, and other bioactive substances that are beneficial to human health.[5] Currently, blueberry has become an important fruit crop for large-scale commercial cultivation worldwide, and China is already the largest blueberry producer in Asia.[6] Blueberry, as a calcifuge plant, is suitable for acidic soil (pH 4.5−5.5) rich in organic matter. However, Cd has increased solubility and mobility in acidic soil and is thus more easily absorbed by blueberry, and the application of agricultural fertilizers (phosphate fertilizers, etc.) further increases the risk of Cd toxicity to cultivated blueberry plants;[7] soil Cd toxicity has been reported to inhibit blueberry growth and increase Cd accumulation in fruits.[8] Therefore, there is an urgent need to find an effective strategy to mitigate and reduce the effects of soil Cd on blueberry toxicity for food safety.

In agriculture, the application of exogenous substances (ESs) is currently recognized as an efficient and economical strategy to mitigate heavy metal toxicity in crops.[9] Salicylic acid (SA), as a multifunctional endogenous phytohormone, can alleviate Cd toxicity in plants by enhancing antioxidant enzyme activity, increasing photosynthesis intensity, and maintaining cellular ion homeostasis.[10] Spermidine (Spd) is a hormone-like aliphatic nitrogenous compound widely distributed in plants that is closely related to plant tolerance, and exogenous Spd has been shown to enhance plant tolerance to Cd by upregulating antioxidant and osmotic metabolism mechanisms.[11] 2,4-Epibrassinolide (EBR), a synthetic and highly active analog of brassinosteroids, can influence the uptake of Cd in plants by stabilizing the electrical properties of cell membranes and enzyme activities and thus alleviating the toxic effects of Cd.[12] Melatonin (MT), a tryptophan derivative, can reduce Cd toxicity by promoting chlorophyll synthesis, improving the antioxidant capacity regulated by ascorbate peroxidase (APX), flavonoids and alkaloids, and activating ABC transporter proteins.[13] Overall, the current research on ESs to alleviate Cd toxicity in plants is mainly focused on the effects of single ESs on growth, physiological, and biochemical

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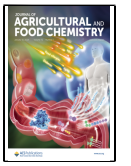

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aspects, while the effects of different ESs on fruit quality and the synthesis of related metabolites under soil Cd toxicity are still poorly understood.

Metabolomics is an effective technique for comprehensively analyzing and comparing metabolites in organisms and helps elucidate the connections between plant physiological metabolism and related metabolites.[14] Currently, liquid chromatography−mass spectrometry (LC−MS) and gas chromatography−mass spectrometry (GC−MS) are widely used in the study of phytochemical accumulation and secondary metabolism.[15] Furthermore, secondary metabolites in plants play an important role in the response to abiotic stresses. To date, many metabolomics studies of plants with Cd toxicity have been reported. Ye et al.[16] used LC−MS to demonstrate that rice roots and stems can respond to Cd toxicity by inducing the MT biosynthetic pathway, while an increase in Cd content in rice grain activates 13-(S)-hydroperoxy-9(Z) and 11(E),15(Z)-octadecatrienoic acid and upregulates D-mannitol and L-cysteine.[17] *Brassica napus* (CB671) can improve Cd tolerance through cell wall polysaccharides, lignin accumulation and the synthesis of antioxidant substances such as anthocyanins.[18] Amino acids (DL-tryptophan, L-aspartic acid, L-proline, L serine, and L-histidine), organic acids (eudesmic acid and chorismate) and fatty acids (oleic acid, linoleic acid, and stearic acid) in the roots of *Salvia miltiorrhiza* play an important role in the resistance to Cd toxicity.[19] Lapie et al.[20] applied LC−MS and GC−MS and found that with increasing Cd toxicity, the total carbon, sugar, and amino acids in maize root exudates and mucilage decreased. However, metabolomic studies on ESs to alleviate Cd toxicity in plants have been reported less frequently and are mainly based on LC−MS. Li et al.[21] found through LC−MS that exogenous MT could alleviate Cd toxicity in cotton seedlings by promoting the synthesis of alkaloids and flavonoids and inhibiting or reducing the synthesis of lipids, amino acids, and their derivatives. Overall, the effectiveness of different ESs in alleviating Cd toxicity in blueberries and their fruit metabolomics studies has not been reported.

To fill this knowledge gap, we evaluated the effects of four ESs (SA, Spd, EBR, and MT) on the ripening and appearance of three-year-old "Brightwell" blueberry fruits under soil Cd toxicity (15 mg/kg) in a pot experiment. The study objectives were to (i) evaluate the residual content of Cd and key quality indicators in fruits among different treatments, (ii) analyze the differentially abundant metabolites (DAMs) and the related metabolic pathways between different treatments by LC−MS and GC−MS under Cd toxicity, (iii) determine the optimal treatment ES type, and (iv) clarify the metabolic network of Cd accumulation, quality formation, and key DAMs in blueberry fruits among different treatments. This study will provide a scientific reference for the safe cultivation of blueberries and lay a theoretical foundation for the mechanism of ESs alleviating Cd toxicity in blueberries.

2. MATERIALS AND METHODS

2.1. Plant Material and Experimental Design. In this experiment, potted "Brightwell" (*Vaccinium ashei* Reade, rabbiteye blueberry) plants grown in the artificial greenhouse of Lishui Baima Blueberry Test Base in Nanjing, Jiangsu Province (31°36′5.66″N, 119°11′49.39″E) were used as the test material. Three-year-old blueberries were planted in black pots (35 × 35 × 35 cm, with trays), and the culture medium was mixed substrate with $V_{\text{coarse peat soil}}$:$V_{\text{fine peat soil}}$:$V_{\text{pine bark}}$:$V_{\text{perlite}}$ = 3:3:1:1 (pH 5.2), and each pot contained 10 kg of culture substrate. In February 2022, blueberry plants with generally consistent growth and no pests were selected, and $CdCl_2 \cdot 5/2H_2O$ aqueous solution was used to treat potted blueberries with Cd toxicity (the concentration of Cd^{2+} in toxic soil was 15 mg/kg according to our previous studies, which showed that when the soil Cd content exceeded 15 mg/kg, the blueberry fruit Cd content was approximately 1.00 mg/kg, which already exceeded the safe consumption standard of 0.05 mg/kg). The treated potted blueberries were placed in a greenhouse with sufficient light and an open culture tank for culturing. Each pot was irrigated with 2.0 L water every 3 days, and the leachate under the pot was collected and reinjected. In April 2022, 20 potted blueberry plants under Cd toxicity with normal growth and similar flower buds were selected for the ES spraying mitigation test. The implementation plan was as follows: 200 μM SA, 200 μM MT, 500 μM Spd, and 20 μM EBR were used as the experimental groups, and distilled water was applied as the control group (CK). A whole tree spraying experiment was carried out at the flower bud stage, flowering stage, and fruit setting stage of potted blueberries under Cd toxicity. Each plant was sprayed with 120 mL of treatment solution 6 times, with four replicates of each treatment, and one potted plant was counted as one repeat. According to their maturity, the ripe blueberry fruits of each treatment were collected on June 28, July 5, July 11, July 18, and July 25 and August 5, 2022, stored in an incubator (including an ice bag), and then brought back to the laboratory to immediately determine the fruit shape and related physical and chemical indicators. In addition, appropriate quantities of ripening blueberry fruits at different periods after mixing under different treatments were frozen in liquid nitrogen and stored at −80 °C for subsequent metabolic analysis.

2.2. Determination of the Shape and Quality of Blueberry Fruits. The fruit shape index was calculated after measuring the transverse and vertical diameters of each treatment using a digital Vernier caliper, and the single-fruit weight was measured with an electronic balance. The anthocyanin content of the fruits of each treatment was determined using the pH difference method.[22] Soluble solids of fruits were measured using a portable hand-held Brix meter (Atago, WYT-A, Tokyo, Japan). The acid−base potentiometric titration method was used to determine the titratable acid content of fruits.[23]

2.3. Determination of Cd and Related Trace Elements in Fruits. Inductively coupled plasma−optical emission spectrometry (ICP−OES, Avio220, PE, USA) (K, Ca, Mg, Fe, Mn, and Zn) and inductively coupled plasma−mass spectrometry (ICP−MS, Nexion300X, PE, USA) (Cu and Cd) were used to determine the trace element contents in fruits from each treatment. In this case, the specific operation was referred to the National Food Safety Standards: Determination of Multielements in Food (GB 5009.268-2016)."

2.4. LC−MS Analysis. *2.4.1. Pretreatment.* A 60 mg fresh fruit sample was weighed into a 1.5 mL centrifuge tube, 600 μL of methanol−water was added (V:V = 7:3 with L-2-chlorophenylalanine, 4 μg/mL), and two small steel balls were added. After being placed in a freezer at −40 °C for 2 min, the contents of the centrifuge tubes were ground (60 Hz, 2 min), extracted by sonication in an ice−water bath for 30 min, and left to stand overnight at −40 °C. Then, the solution was centrifuged at low temperature for 10 min (13,000 × *g*, 4 °C), and 150 μL of the supernatant was pipetted using a crystal syringe, filtered through a 0.22 μm microfiltration chip, and transferred to the LC injection vial. Finally, the prepared samples were stored at −80 °C for LC−MS analysis. In addition, the quality control samples (QC) were prepared by mixing extracts from each sample equally.

2.4.2. LC−MS Analytical Conditions. This experiment was analyzed using a liquid chromatography−mass spectrometry system consisting of an ACQUITY UPLC I-Class (Waters Corporation, Milford, USA) plus an ultrahigh-performance liquid tandem QE high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). An ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.8 μm) column was used for the analysis in positive and negative ion modes. Water containing 0.1% formic acid and acetonitrile was used as

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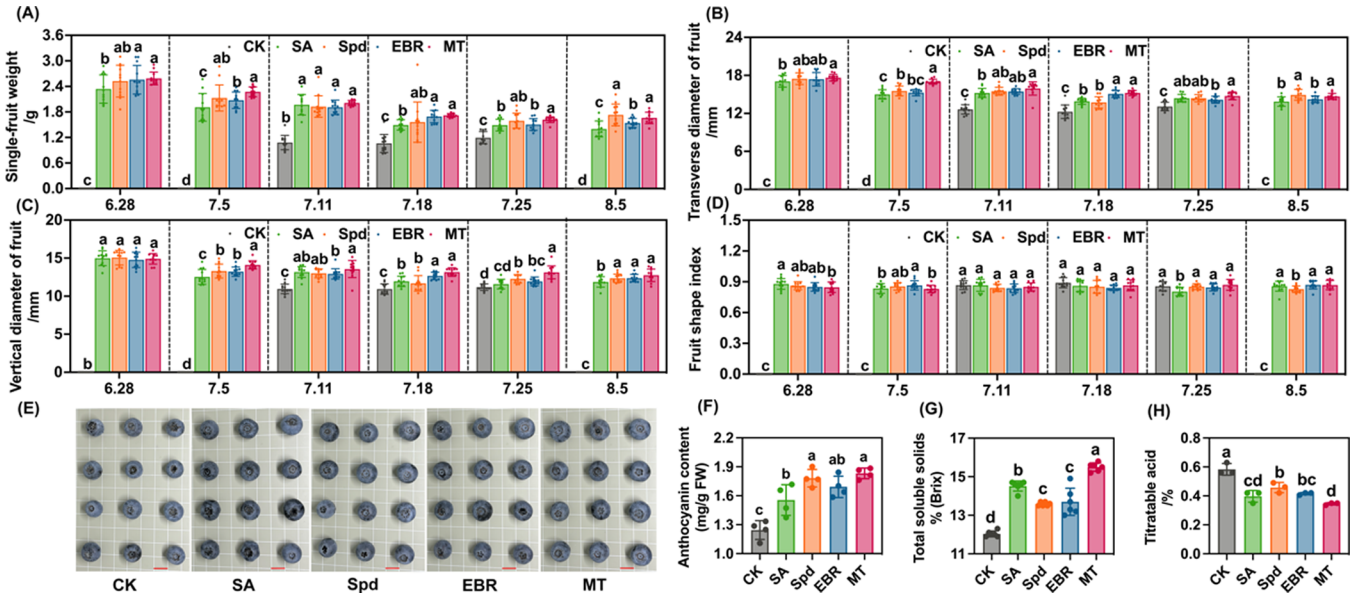

Figure 1. Effect of spraying different ESs on single-fruit weight (A), transverse diameter (B), vertical diameter (C), fruit shape index (D), phenotype (E, ripe fruit on July 25, 2022), anthocyanin (F), total soluble solids (G) and titratable acid (H) contents of blueberry fruits under Cd toxicity. The vertical bar represents the mean ± SD. The columns of different letters indicate that there were significant differences ($p < 0.05$) in blueberry fruits between different treatments by Duncan's multiple range test. Mature fruits were not harvested with Cd toxicity on June 28, July 5, and August 5, 2022, in the CK treatment. The red scale in Figure 1E is 1.0 cm.

mobile phases A and B, respectively, and the elution gradients are shown in Table S1. The column temperature was 45 °C, the flow rate was 0.35 mL/min, and the injection volume was 2 μL. Subsequently, the mass spectra of the samples were acquired using ESI as the ion source and positive and negative ion scan modes, and the relevant mass spectral parameters are shown in Table S2.

2.5. GC−MS Analysis. *2.5.1. Pretreatment.* A 60 mg fresh fruit sample was weighed into a 1.5 mL centrifuge tube, and two small steel balls and 600 μL of methanol−water solution (V:V = 1:1, containing L-2-chlorophenylalanine, 4 μg/mL) were added to the tube, which was then prechilled at −40 °C for 2 min, ground for 2 min at 60 Hz, and purged in an ice−water bath for 30 min with ultrasound. Next, 150 μL of chloroform was added, and the tube was vortexed for 2 min, extracted through ultrasonication in ice−water baths for 30 min, and rested at −40 °C for 30 min. Then, the sample was centrifuged at low temperature for 10 min (13,000 × g, 4 °C), and 150 μL of the supernatant was loaded into glass-derived vials, concentrated, and evaporated. Next, 80 μL of pyridinium methoxamine hydrochloride solution (15 mg/mL) was added for the oxime reaction (shaken at 37 °C for 60 min); the sample was removed, 50 μL of BSTFA derivatization reagent and 20 μL of hexane were added, 10 μL of 10 internal standards (C8/C9/C10/C12/C14/C16/C18/C22/C24, all in chloroform configuration) was added, and the reaction was carried out at 70 °C for 60 min. Finally, the samples were removed and left at room temperature for 30 min for GC−MS analysis. In addition, the QC sample preparation method was the same as that for LC−MS.

2.5.2. GC−MS Analytical Conditions. The test samples were analyzed on an Agilent 7890B GC system coupled to an Agilent 5977B MSD system (Agilent Technologies Inc., CA, USA). A DB-5MS silica gel fusion capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA, USA) was used for derivative separation, and the carrier gas was high-purity helium (≥99.999%) at a flow rate of 1.0 mL/min and an inlet temperature of 260 °C. The sample volume was 1 μL, with no split injection and a solvent delay of 5 min. The ramp-up procedure and mass spectra were acquired in full scan mode with the parameters shown in Tables S3 and S4, respectively.

2.6. Statistical Analysis. The raw LC−MS data were processed using Progenesis QI V2.3 (Nonlinear, Kinetics, Newcastle, UK) software, and the raw GC−MS data obtained in the. d format were converted to .abf format by the software Analysis Base File Converter. Then, the data were imported into MS-DIAL software for analytical processing. The processed LC−MS data and GC−MS data were subjected to principal component analysis (PCA) by importing matrices in R, and orthogonal partial least-squares discriminant analysis (OPLS-DA) and partial least-squares discriminant analysis (PLS-DA) were used to distinguish different metabolites between groups. Finally, the projected importance of variables (VIP) values obtained from the OPLS-DA model was used to rank the overall contribution of each variable to group discrimination. A two-tailed Student's *t*-test was further used to verify that the metabolites of between-group differences were significant. Differentially expressed metabolites with VIP values greater than 1.0 and *p* values less than 0.05 were selected.[24]

In addition, one-way analysis of variance (ANOVA) was performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA) on the blueberry fruit phenotype (single-fruit weight, transverse diameter, vertical diameter, and fruit shape index) and quality (anthocyanin, total soluble solids, and titratable acid) measurements for each treatment, and Duncan's multiple comparisons were used to test the significance of differences. GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA) was used to plot and visualize statistical data.

3. RESULTS

3.1. Effect of Different ES Sprays on Blueberry Fruit Growth and Quality under Soil Cd Toxicity. Cd toxicity was detrimental to the growth and development of blueberry fruits while spraying with ESs promoted fruit maturation and prolonged fruit production (Figure 1, no ripe fruit were harvested from the CK treatments under Cd toxicity on June 28, July 5, and August 5). Overall, the application of ESs can significantly increase the fruit size and single-fruit weight (by 58.91, 71.81, 69.01, and 77.85%, for the SA, Spd, EBR, and MT treatments, respectively) of blueberries under Cd toxicity ($p < 0.05$). However, the fruit shape indices of blueberries treated with different ESs were not significantly different from those of the CK (Figure 1A−D), and Figure 1E shows the

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J. Agric. Food Chem. 2024, 72, 904−915

mature fruit of blueberries harvested on July 25, 2022. In addition, spraying ESs can significantly increase the anthocyanin content of blueberries under Cd toxicity ($p < 0.05$), and the anthocyanin content of Spd- and MT-treated fruit increased by 43.19 and 47.21% compared with CK (Figure 1F); also, spraying ESs increased the sweetness of blueberries under Cd toxicity. Among them, the total soluble solids (TSS) of blueberry fruits under the SA, Spd, EBR, and MT treatments were 1.21, 1.13, 1.14 and 1.29 times that of the CK, respectively, while the titratable acid (TA) content was reduced by 32.00, 21.60, 28.80, and 40.80% compared to CK (Figure 1G,H). The above analysis showed that spraying ESs had a positive effect on improving the growth of blueberry fruits under Cd toxicity.

3.2. Effects of Different ES Sprays on Cd and Mineral Content in Blueberry Fruits under Soil Cd Toxicity. Under soil Cd toxicity, ES treatments affected the uptake and transport of mineral elements in blueberry fruits and significantly reduced Cd accumulation ($p < 0.05$) (Table 1), with the lowest Cd content in fruits treated with Spd and MT (approximately 0.02 mg/kg, which meets the requirement of Cd content no higher than 0.05 mg/kg in fruit). Compared with the CK, the Mn content in blueberry fruits increased by 44.84, 76.27, 37.78, and 90.12%, the Cu content increased by 101.69, 72.06, 105.30, and 61.11%, and the Zn content increased by 306.94, 216.85, 174.20, and 193.89%, respectively, while the macronutrients K, Ca and Mg showed different significant variabilities in the blueberry fruits of different treatments. In addition, the Fe content of blueberry fruits under different ES treatments did not significantly differ from that of the CK ($p > 0.05$). The above analysis indicated that ES treatments could promote the uptake and transport of mineral elements to inhibit the accumulation of Cd in blueberry fruits under soil Cd toxicity.

3.3. Multivariate Statistical Analysis and Metabolite Classification by LC−MS and GC−MS. Unsupervised PCA was used to observe the overall distribution among the samples and the stability of the whole analysis process, and the elliptical area indicates the 95% confidence interval. The samples were relatively distinguished between different groups in both LC−MS and GC−MS analyses, where CK significantly differed from each treatment. Overall, the degree of variability of GC−MS analysis among the different treatment samples was higher than that of LC−MS analysis; moreover, the variability of the three biological replicate samples was smaller within the group (Figure S1A,D). PLS-DA enables the prediction of sample categories, and its addition to the grouping variables also compensates for the deficiencies of PCA. The parameters R^2X ($c\mu m$) were 0.862 and 0.529, and the explanatory ratios R^2Y ($c\mu m$) were 0.981 and 0.724 in LC−MS and GC−MS analysis, respectively. The above analysis indicated that the PLS-DA model could better explain and predict the differences between the two groups of samples (Figure S1B,E). In addition, loading plots can be used to determine the strength of the effects of different comparison groups of metabolites; the farther the loadings are from 0, the stronger the effect of the variable on the component; the closer the loadings are to 0, the weaker the effect of the variable on the component (Figure S1C,F).

A total of 17,315 substance peaks (9253 cations; 8062 anions) and 5791 metabolites (3885 cations and 1906 anions) were identified from 15 blueberry fruit samples by LC−MS analysis; 322 metabolites were identified by GC−MS analysis. Furthermore, to show the classification of metabolites more

Table 1. Cd Accumulation and Mineral Content in Blueberry Fruit under Different ES Treatments[a]

treatment	Cd (mg/kg)	Fe (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	K (mg/kg)	Ca (mg/kg)	Mg (mg/kg)
CK	0.112 ± 0.021a	49.721 ± 3.319 ns	3.475 ± 0.645c	1.433 ± 0.286c	3.320 ± 1.048c	6951.246 ± 213.068a	263.963 ± 13.545c	283.135 ± 23.650b
SA	0.039 ± 0.001bc	55.399 ± 10.412 ns	5.033 ± 0.278b	2.890 ± 0.134a	13.512 ± 0.573a	6502.997 ± 169.384b	710.143 ± 40.492a	299.334 ± 3.291ab
Spd	0.022 ± 0.004d	55.324 ± 14.275 ns	6.125 ± 0.253a	2.466 ± 0.110b	10.521 ± 2.551b	6931.244 ± 153.512a	467.117 ± 148.615b	296.336 ± 0.915ab
EBR	0.043 ± 0.004b	43.970 ± 11.608 ns	4.788 ± 0.217b	2.942 ± 0.088a	9.105 ± 1.086b	6392.408 ± 152.769b	360.092 ± 78.734bc	277.493 ± 11.466b
MT	0.025 ± 0.002 cd	40.068 ± 12.145 ns	6.606 ± 0.210a	2.309 ± 0.127b	9.758 ± 0.446b	6906.337 ± 2.505a	272.540 ± 24.731c	317.659 ± 1.633a

[a]Results are shown as the mean ± SD ($n = 3$), different lowercase letters indicate significant differences in the same index across treatments according to Duncan's multiple range test at the $P < 0.05$ level.

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J. Agric. Food Chem. 2024, 72, 904−915

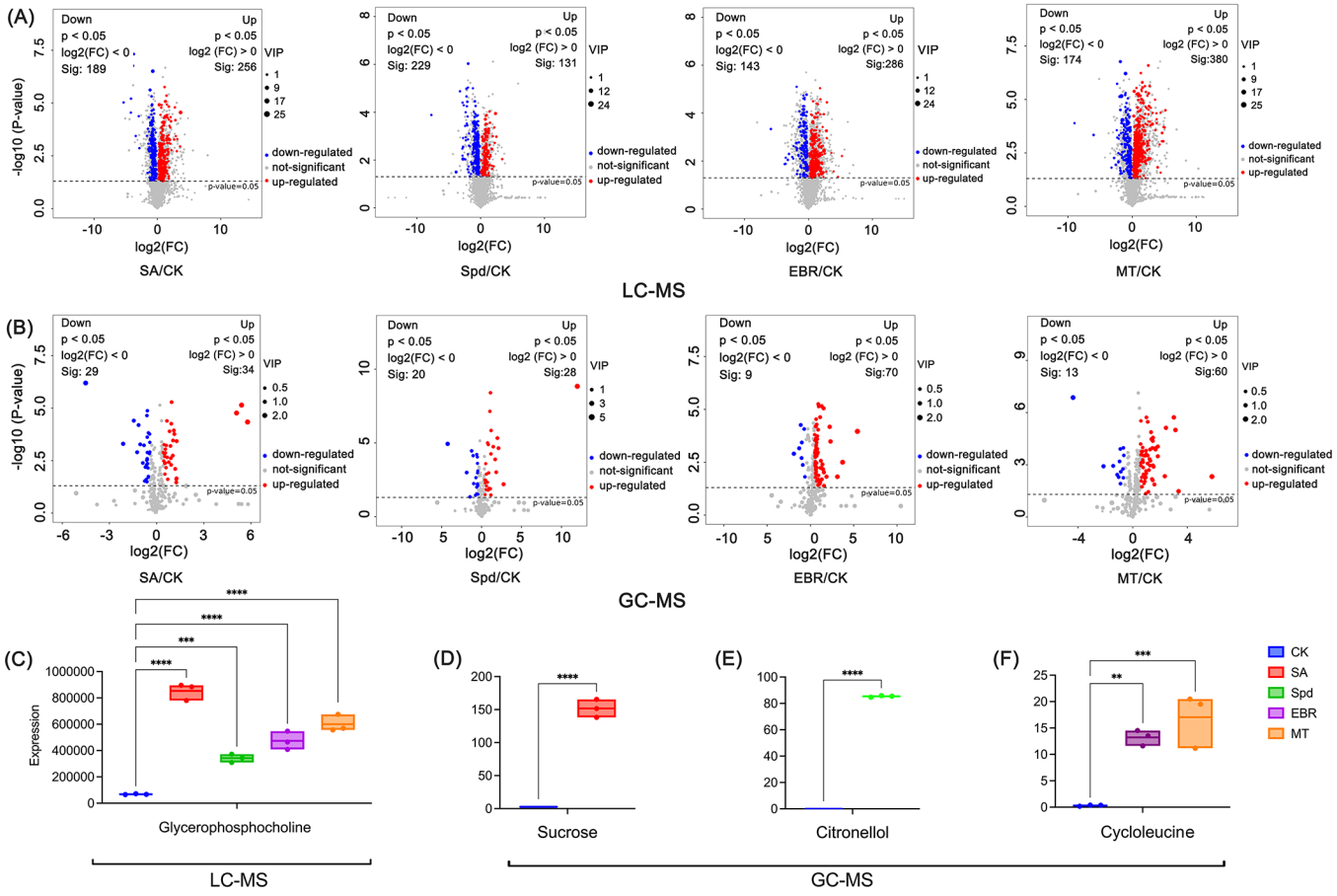

Figure 2. Statistical analysis of DAMs of blueberry fruits in different comparison groups under LC−MS and GC−MS analysis. Volcano plot of DAMs under LC−MS (A) and GC−MS (B); Box violin plot of maximum expression DAMs in SA/CK, Spd/CK, EBR/CK and MT/CK under LC−MS (C), SA/CK under GC−MS (D), Spd/CK under GC−MS (E), and EBR/CK and MT/CK under GC−MS (F). Each point in the volcano plot represents a metabolite, the horizontal coordinate is the $\log_2$ (FC) value of the two comparison groups, the vertical coordinate is the $-\log_{10}$ (p-value) value, red points are metabolites with $p < 0.05$ and FC > 1 (upregulation), blue points are metabolites with $p < 0.05$ and FC < 1 (downregulation), and gray points indicate metabolites with no significant difference. ** represents a significant difference at $p < 0.01$, and **** represents a significant difference at $p < 0.0001$ by Tukey's test in the box violin plot.

intuitively under the LC−MS and GC−MS analysis, the metabolites were statistically analyzed from the three classification levels of superclass, class, and subclass. Lipids and lipid-like molecules (fatty acids and conjugates, fatty acyl glycosides, etc.), organic oxygen compounds (carbohydrates and carbohydrate conjugates, carbonyl compounds, etc.) and phenylpropanoids and polyketides (flavonoid glycosides, flavonoids, etc.) were the main metabolites in the LC−MS analysis among the three classification levels (Figure S1G−I), while the metabolites that accounted for a large proportion in the GC−MS analysis included organic acids and their derivatives (amino acids, peptides, and analogues, beta-hydroxy acids and derivatives and dicarboxylic acids and derivatives, etc.) in addition to lipids and lipid-like molecules and organic oxygen compounds similar to the results of the LC−MS analysis (Figure S1J−L).

3.4. LC−MS and GC−MS Analysis of DAMs. The variable weight values (VIP > 1) and t-test p values ($p < 0.05$) of the first principal component of the OPLS-DA model were used as screening criteria for the DAMs of blueberry fruits in the LC−MS and GC−MS analyses, respectively. More DAMs were identified by LC−MS than by GC−MS; among them, 445, 360, 429, and 554 DAMs were identified in the LC−MS analysis in the SA/CK, Spd/CK, EBR/CK, and MT/CK comparison groups (Figure 2A), respectively; GC−MS analysis identified a total of 63, 48, 79, and 73 DAMs in the SA/CK, Spd/CK, EBR/CK and MT/CK comparison groups (Figure 2B), respectively. Detailed metabolite information is provided in Tables S5 and S6. Notably, the increase in glycerophosphocholine expression in the LC−MS analysis was the highest in all four comparison groups, 12.54-fold (SA/CK), 5.02-fold (Spd/CK), 6.96-fold (EBR/CK) and 8.82-fold (MT/CK) of the CK (Figure 2C). Furthermore, sucrose (SA/CK) and citronellol (Spd/CK) showed the greatest increase in expression under GC−MS analysis (Figure 2D,E), while cycloleucine showed the greatest increase in both the EBR/CK and MT/CK comparison groups (Figure 2F). Overall, the DAMs of blueberry fruits were more upregulated in the EBR and MT treatments than in the CK, suggesting that they may have a more pronounced effect on the metabolic processes of blueberry fruit growth under soil Cd toxicity.

3.5. Correlation Analysis of DAMs Identified by LC−MS and GC−MS. To measure the degree of correlation between significant DAMs and further understand the interrelationship between metabolites during the changes in the biological state, the top 20 DAMs ranked by VIP in the LC−MS and GC−MS analyses were selected, and correlation heatmaps were drawn using Pearson correlation coefficients.

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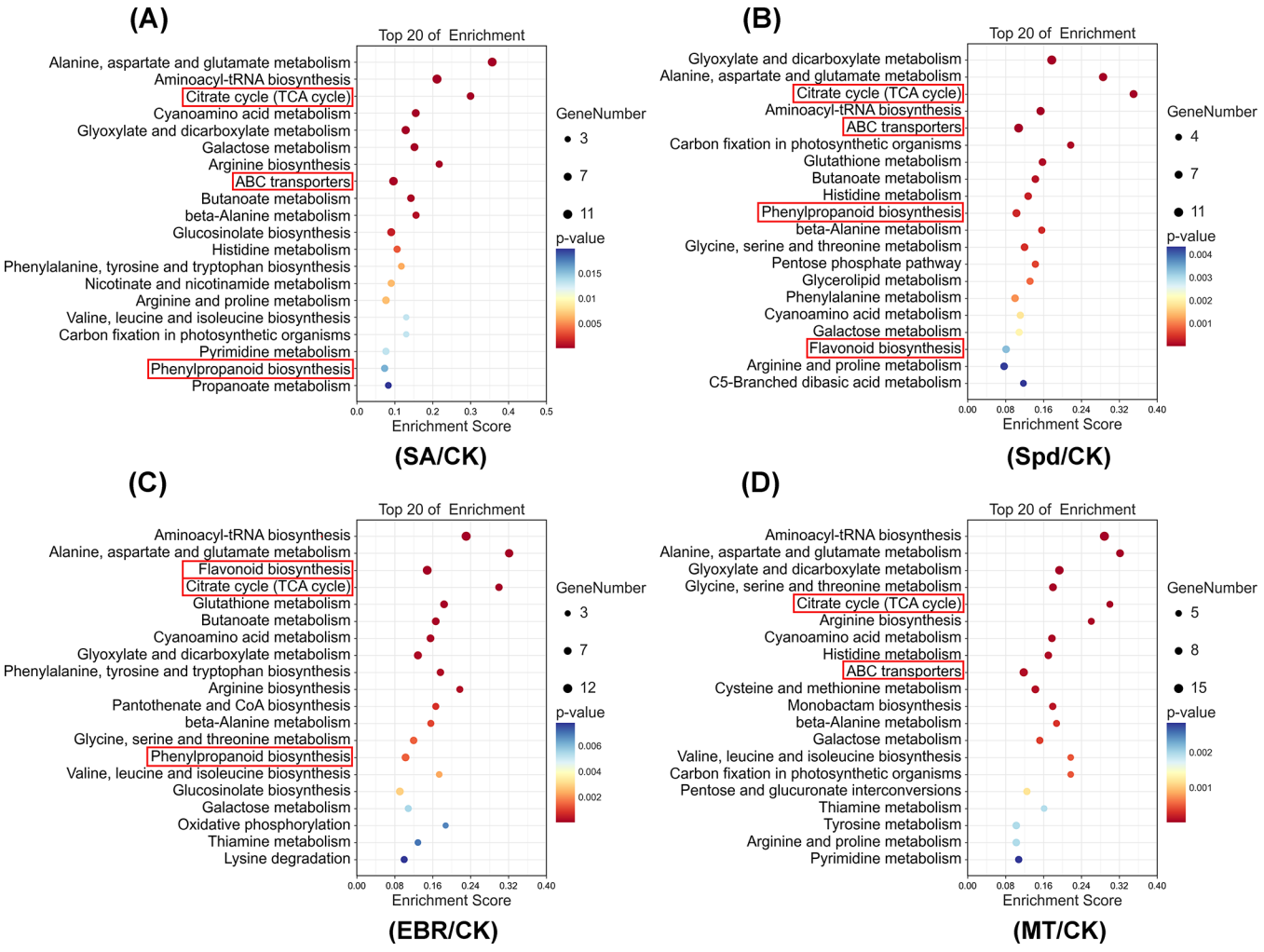

Figure 3. Enrichment bubble plots of metabolic pathways (top 20) for different comparative groups of blueberry fruit DAMs in LC−MS combined with GC−MS analysis. SA/CK (A), Spd/CK (B), EBR/CK (C) and MT/CK (D). The vertical coordinate is the name of the metabolic pathway, and the horizontal coordinate is the Rich factor (Rich factor = number of significant DAMs/total number of metabolites in the pathway); the larger the Rich factor, the greater the enrichment; the color from yellow to blue indicates that the *p* value decreases sequentially; the larger the dot, the greater the number of metabolites enriched to the pathway.

The main types of DAMs in the four comparison groups were organic oxygen compounds, organic acids and derivatives, lipids and lipid-like molecules, and phenylpropanoids and polyketides. These DAMs showed significantly more positive than negative correlations with each other in the SA/CK, EBR/CK, and MT/CK comparison groups. In contrast, this relationship was reversed in the Spd/CK comparison group (Figure S2A−D). In addition, common DAMs with positive and negative correlations showed similar expression abundances and associations in the different comparison groups.

3.6. KEGG Enrichment Analysis of DAMs Identified by LC−MS and GC−MS. Pathway enrichment analysis of DAMs helps elucidate the mechanisms of changes in metabolic pathways in different treated blueberry fruit samples. Through combined LC−MS and GC−MS analyses, we found that aminoacyl-tRNA biosynthesis and alanine, aspartate, and glutamate metabolism were the top two enriched metabolic pathways in the four comparison groups. In addition, the ABC transporter, the citrate cycle (TCA cycle), phenylalanine metabolism, and flavonoid biosynthesis pathways associated with Cd toxicity in plants showed different levels of enrichment in different comparisons, and the number of DAMs annotated in the pathways was also higher (Figure 3A−D). Taken together, the results of this analysis showed that different ES treatments could regulate the quality of blueberry fruits under Cd toxicity by affecting the relevant metabolic pathways.

3.7. Analysis of DAMs in the ABC Transporter, Flavonoid Biosynthesis, Phenylalanine Metabolism, and TCA Cycle Pathways. A total of 41 DAMs were identified from four metabolic pathways in the four comparison groups, including 16 in the ABC transporter pathway, 10 flavonoids, 9 phenylalanines, and 6 organic acids (Figure 4). Among them, in the flavonoid biosynthesis pathway, naringin was downregulated in the four comparison groups; (+)-catechin was downregulated in SA/CK but upregulated in Spd/CK, EBR/CK, and MT/CK. Myricetin was upregulated in SA/CK, EBR/CK, and MT/CK, and (+)-catechin was most significantly upregulated in EBR/CK (3.72-fold) (Figure 4A). In the phenylalanine metabolism pathway, spermidine and coniferin were significantly upregulated in the four comparison groups; interestingly, L-tyrosine was downregulated in SA/CK, Spd/CK, and EBR/CK but upregulated in MT/CK. In addition, L-phenylalanine was upregulated in SA/CK, EBR/CK, and MT/CK but down-

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J. Agric. Food Chem. 2024, 72, 904−915

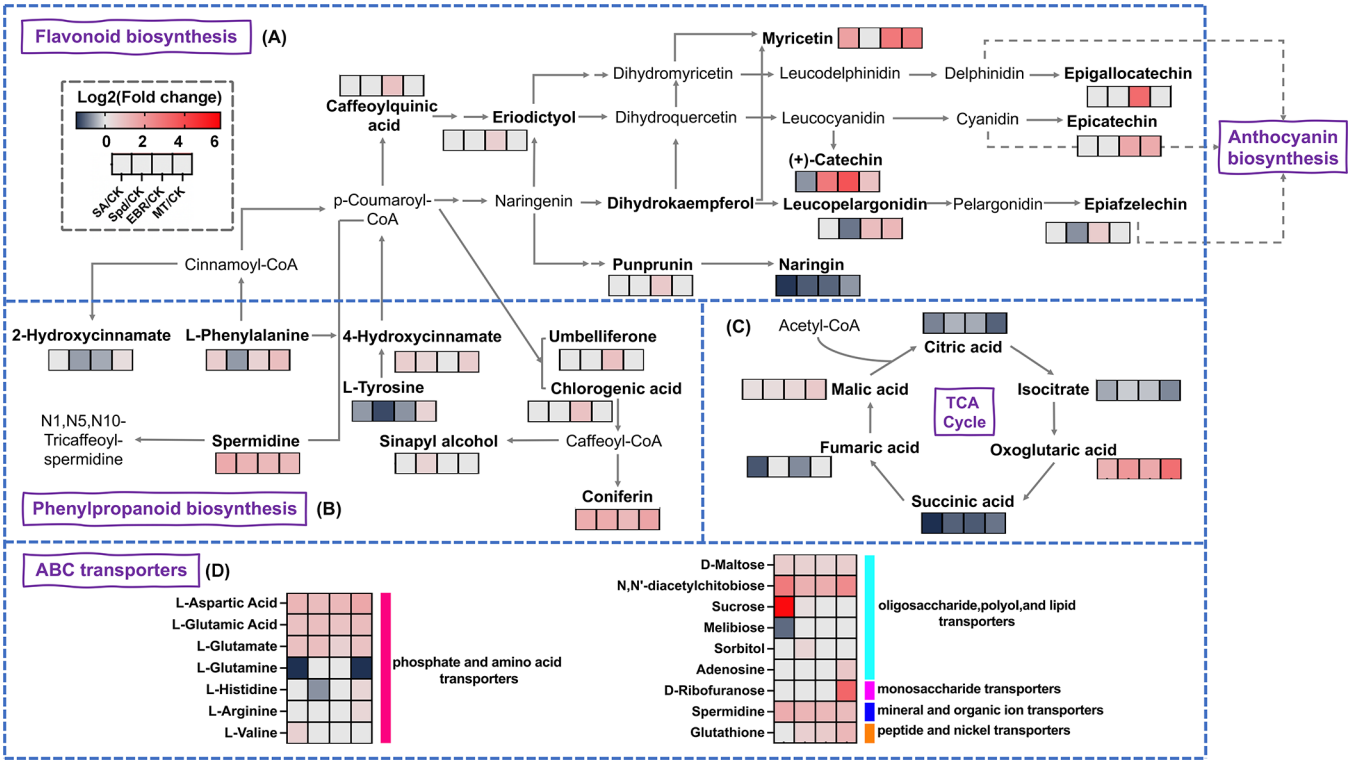

Figure 4. Heatmap of DAMs in the four metabolic pathways in different comparative groups of blueberry fruits under LC−MS combined with GC−MS analysis. Flavonoid biosynthesis pathway (A), phenylalanine metabolism pathway (B), citrate cycle (TCA cycle) pathway (C) and ABC transporter pathway (D). Heatmaps were produced using $\log_2$(fold change) values, with red indicating upregulation and blue indicating downregulation.

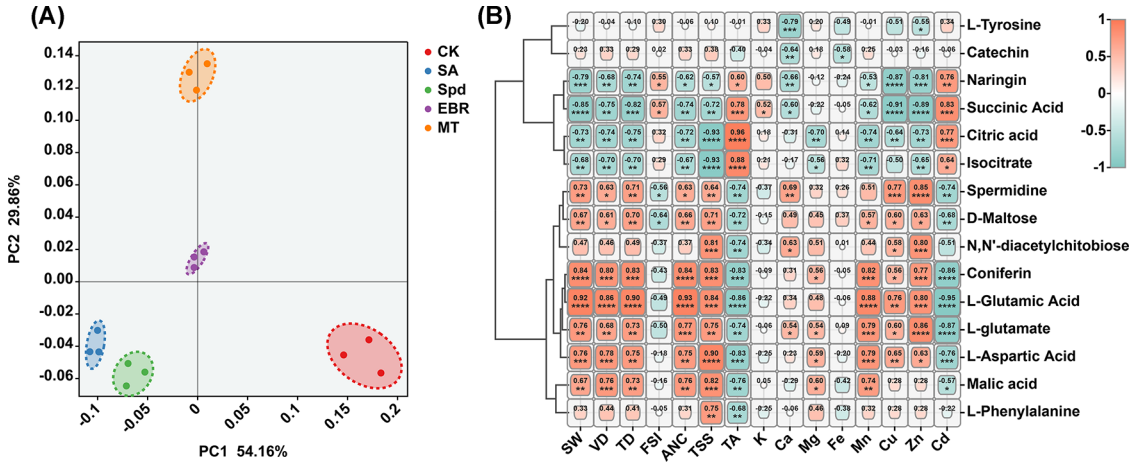

Figure 5. PCA of different treatments and Spearman's rank correlation analysis of fruit physiological indices and DAMs. According to Spearman's rank correlation coefficient, (∗) $p < 0.05$, (∗∗) $p < 0.01$, (∗∗∗) $p < 0.001$, (∗∗∗∗) $p < 0.0001$. SW, single-fruit weight; VD, vertical diameter; TD, transverse diameter; FSI, fruit shape index; ANC, anthocyanin content; TSS, total soluble solids; TA, titratable acid; K, potassium; Ca, calcium; Mg, magnesium; Fe, iron; Mn, manganese; Cu, copper; Zn, zinc; Cd, cadmium.

regulated in Spd/CK (Figure 4B). Succinic acid, isocitrate, and citric acid in the TCA cycle pathway were significantly downregulated in the four comparison groups, while malic acid and oxoglutaric acid were upregulated; furthermore, fumaric acid was only downregulated in the SA/CK and EBR/CK comparison groups (Figure 4C). In the ABC transporter pathway, the levels of DAMs related to phosphate and amino acid transporters (L-aspartic acid, L-glutamic acid, and L-glutamate), oligosaccharide, polyol, and lipid transporters (D-maltose and N, N′-diacetylchitobiose) and mineral and organic ion transporters (spermidine) were significantly increased in all four comparison groups. Sucrose was the most upregulated in the SA/CK comparison group, with levels 5.79 times that of CK, while L-glutamine was significantly downregulated in the SA/CK and MT/CK comparison groups (Figure 4D).

3.8. Relationship between Fruit Quality and DAMs in Blueberry Treated with Different ESs under Soil Cd Toxicity. To further explore the relationships between blueberry fruit quality and DAMs in related metabolic pathways, a Spearman's rank correlation analysis was

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J. Agric. Food Chem. 2024, 72, 904−915

performed by calculating Pearson's correlation coefficients (Figure 5). The results of PCA showed that the changes in blueberry fruit quality and metabolites under different ES treatments were significantly different from the CK, while the differences between the SA and Spd treatments were smaller and those in the MT treatments were the most significantly different (Figure 5A). The correlation heatmap showed that the blueberry fruit quality outcome was closely related to the changes in metabolites. Among the aspects of quality, SW, VD, TD and the contents of anthocyanins, TSS, Mn, Cu, Zn, Ca, and Mg were mostly significantly and positively correlated with spermidine, D-maltose, coniferin, L-glutamic acid, L-glutamate, L-aspartic acid, and malic acid but negatively correlated with naringin, succinic acid, citric acid, and isocitrate. Notably, the TA and Cd contents of blueberry fruits showed completely opposite and significant relationships with these metabolites. In addition, the relationships between other fruit physiological indicators and metabolites showed sporadic and significant correlations (Figure 5B). The above results suggest that ES treatments can affect the changes in blueberry fruit quality under soil Cd toxicity by regulating the expression of metabolites in the ABC transporter, phenylalanine metabolism, flavonoid biosynthesis, and the TCA cycle pathways.

4. DISCUSSION

Cd is a strongly toxic nonessential element in plants that can adversely affect plant growth and productivity by disrupting related physiological and biochemical metabolic processes.[28] The application of ESs has been widely used as an effective strategy to regulate plant growth and alleviate abiotic stress.[9] Our results showed that soil Cd toxicity had an adverse effect on the growth of blueberry fruits (Figure 1), and spraying exogenous SA, Spd, EBR, and MT could advance fruit ripening and significantly improve the appearance quality of blueberries. Fruit ripening involves a series of physiological, biochemical, and sensory changes, and exogenous EBR and MT can promote fruit ripening by activating the ethylene signaling pathway to synthesize ethylene.[26] Interestingly, studies have shown that SA and Spd are able to delay the ripening and senescence process of postharvest fruits,[27] in this study, exogenous SA promoted blueberry fruit ripening, which may be due to the fact that low concentrations of SA (<1 mM) increased endogenous ethylene biosynthesis.[28] Meanwhile, exogenous Spd promoted fruit coloration[29] and synergized with ABA, IAA, and ethylene pathways to promote fruit ripening,[30] which is consistent with our findings. In addition, ESs can promote cell division and elongation and play a positive role in fruit expansion.[31−33] In our study, exogenous SA, Spd, EBR, and MT treatments also significantly increased the single-fruit weight of blueberry fruits under Cd toxicity compared with CK ($p < 0.05$) (Figure 1A).

Mineral elements, especially the trace elements Fe (the main component of chlorophyll synthesis), Mn, Cu, and Zn, play an important role in plant growth and physiological metabolic activities, and are involved in processes related to photosynthesis, fruit quality, and yield formation;[34] moreover, Cu and Zn as components of most oxidative enzymes directly affect the function of plant oxidative systems, while Mn acts as a catalyst which is closely related to the plant enzyme activity.[35] However, the excessive accumulation of Cd in plants disrupts the uptake and translocation of mineral elements and ultimately inhibits plant growth. Studies have shown that the application of exogenous MT significantly promoted the uptake of Fe, Mn, Cu, and Zn and reduced the accumulation of Cd in tissues of *Platycladus orientalis* seedlings.[36] Moreover, exogenous Spd, EBR, and SA exerted similar effects in inhibiting the uptake and translocation of metal-toxic elements in plants. Similarly, our study also revealed that different ES treatments significantly increased the levels of Mn, Cu, Zn, and Ca in blueberry fruits subjected to soil Cd toxicity while decreasing the accumulation of Cd; meanwhile, the contents of K, Mg, and Fe showed different patterns of variation among treatments (Table 1). The fact that ESs can inhibit the uptake of Cd by plants and limit its translocation to fruits.[37] Meanwhile, plants do not have Cd^{2+} channel proteins, and ESs can reduce Cd uptake by facilitating the translocation of Mn, Cu, Zn, and Ca through cation channel/translocator proteins.[38] However, the molecular mechanism of action of ESs in inhibiting the accumulation of Cd in blueberry fruits still needs to be further investigated.

ESs can improve fruit quality by regulating changes in fruit metabolism and physiological characteristics, such as color, solid−acid ratio, bioactive substances, etc. Our results showed that spraying SA, Spd, EBR, and MT significantly increased anthocyanin and TSS content and reduced TA accumulation in blueberry fruits under Cd toxicity ($p < 0.05$), with higher anthocyanin content in the Spd and MT treatments and higher fruit sweetness in the SA and MT treatments (Figure 1F−H). Similarly, exogenous MT can increase anthocyanin accumulation in strawberry seedlings with Cd toxicity.[39] Meanwhile, fruit is the most metabolite-rich plant organ; fruit quality and flavor formation are closely related to metabolite accumulation and/or consumption, while environmental stresses and ESs can affect the metabolic pathways of the fruit, resulting in changes in metabolites. In this study, the most up-regulated metabolite expression in blueberry fruits treated with four ESs under LC−MS was glycerophosphocholine (Figure 2C), which, as a major phospholipid in eukaryotic cell membranes, plays an important role in the maintenance of the integrity, permeability, and fluidity of cell membranes.[40] Moreover, the metabolites with the highest up-regulated levels in exogenous SA, Spd, EBR, and MT-treated blueberry fruits detected by GC−MS were sucrose, citronellol, and cycloleucine (Figure 2D−F), all of which are closely associated with fruit sweetness or flavor.[41,42] In addition, we found that ESs affected the metabolites in ABC transporters, flavonoid, phenylpropanoid biosynthesis, and the TCA cycle in blueberry fruits in response to Cd toxicity (Figure 4). Related studies have also shown that MT can alleviate Cd toxicity in cotton seedlings by stimulating the synthesis of alkaloids and flavonoids and by activating ABC transporters.[21]

ABC transporters are a large family of transmembrane transport proteins widely present in living organisms, mainly relying on ATP hydrolysis for intra- and extracellular transmembrane transport of biomolecules, such as amino acids, metal ions, sugars, peptides, and lipids, etc.; moreover, they also play important roles in regulating plant growth and responding to abiotic stresses.[43] In this study, DAMs from four ES-treated blueberry fruits under Cd toxicity were enriched in the ABC transporters and alanine, aspartate, and glutamate metabolic pathways with significantly higher levels of L-aspartate, L-glutamate, and L-glutamate. Moreover, aminoacyl-tRNA biosynthesis was significantly enriched in all four comparisons, and only the MT treatment significantly increased the expression levels of L-isoleucine, L-arginine, L-histidine and L-tyrosine (Figures 3, 4D and Table S5). Amino

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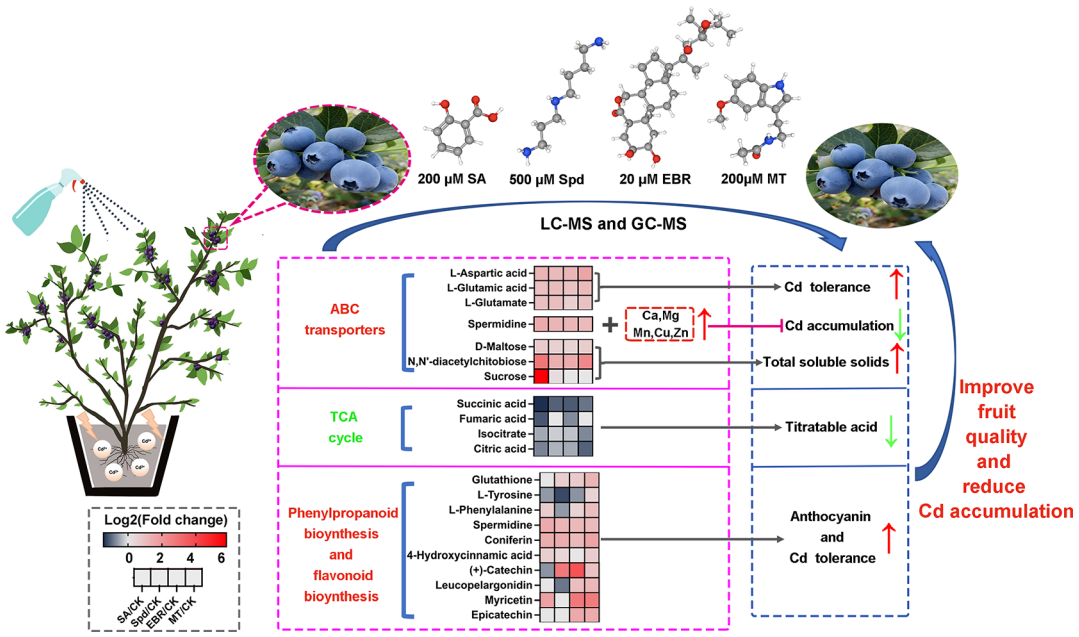

Figure 6. Potential metabolic mechanism of spray treatment with different ESs to improve the quality of blueberry fruits under soil Cd toxicity. The blunted arrows (—|) indicate inhibition; the red arrows indicate an increase, and the green arrows indicate a decrease.

acids, as essential compounds for plant growth, play an important role in physiological metabolism, such as plant nutrition and resistance to Cd toxicity. Studies have shown that accumulation of L-aspartic acid alleviates the toxicity of Cd to *Salvia miltiorrhiza*,[19] and exogenous L-glutamic acid can reduce oxidative damage and inhibit Cd accumulation in *Lens culinaris* Medik.[44] Meanwhile, ABC transporters can also maintain cellular osmotic homeostasis by regulating the uptake and transport of secondary metabolites.[43] Our study showed that the upregulation of carbohydrates (*N*,*N*′-diacetylchitobiose, D-maltose, sucrose, melibiose, sorbitol, adenosine, and D-ribofuranose, etc.) in ABC transporters was positively correlated with the TSS in blueberry fruits under different ES treatments (Figures 1G and 5B), and sugars are also good osmoregulators and membrane protectors in plants in response to environmental stresses.[45] Glutathione plays an active role in antioxidant and detoxification in plants,[46] and the glutathione levels in blueberry fruits were significantly increased under Spd, EBR, and MT treatments (Figure 4D), suggesting that ESs can enhance the tolerance of blueberry fruits to soil Cd toxicity. In addition, the TCA cycle serves as a hub for the metabolic linkage and interconversion of carbohydrates and amino acids, and its metabolic production of organic acids such as citric acid directly determines the acidity of the fruit.[47] In this study, ES treatment downregulated the levels of metabolites succinic acid, fumaric acid, isocitrate, and citric acid in blueberry fruits under soil Cd toxicity, which may be an important factor in the decreased TA in blueberry fruits (Figures 1H and 5B). Similarly, ESs can respond to abiotic stresses by regulating fruit organic acid accumulation or consumption.[48,49] Notably, malic acid was upregulated in all four comparisons (Figure 4C), which may be the fact that blueberry fruit ripening requires it to provide energy for the associated carbon metabolism and sugar xenobiotic pathways.[50]

Phenylpropanoid biosynthesis is the main pathway for the synthesis of secondary plant substances, and its metabolites, such as flavonoids, lignans, phenolics, and anthocyanins, are closely related to fruit aroma, flavor, pigmentation, and nutritional quality.[51] Meanwhile, as a classical metabolic pathway for inducing plant resistance, it can play an antioxidant role in response to abiotic stress and improve plant tolerance to heavy metal stress.[52] In this study, ES treatments increased the expression of L-phenylalanine (the initial substrate for phenylpropanoid biosynthesis) and 4-hydroxycinnamate in blueberry fruits under Cd toxicity (Figure 4B), which may be the result of ESs promoting the production of phenolic metabolites to alleviate the oxidative damage of Cd toxicity to blueberry fruits.[53] Coniferin, a downstream product of cinnamic acid, plays an important role in pericarp lignification and response to abiotic stress.[54] Our results showed that ES treatments significantly elevated coniferin levels in blueberry fruits under Cd toxicity. Besides, ESs also significantly up-regulated the expression of Spd (Figure 4B), which, as an important amino acid metabolite involved in the phenylpropanoid biosynthesis, is closely related to antioxidant and anti-inflammatory bioactivities of fruits.[55] In addition, Spd plays an important role in plant defense against heavy metal toxicity, and related studies have shown that Spd can alleviate Cd toxicity by inhibiting Cd accumulation and transport in rice,[11] which is one of the reasons why ES treatments reduced the Cd content in blueberry fruit (Figures 4B and 5B).

Flavonoids play an important role as defense metabolites and antioxidants in plant responses to abiotic stresses. Currently, the plant flavonoid biosynthesis pathway has been studied more systematically through Phenylpropanoid biosynthesis.[56] Flavonoid compounds in fruits are mainly present in the free state or in the form of glycosides and contribute to fruit coloration and nutritional quality.[57,58] Catechin and epicatechin are widely distributed in plants, and in this study, ESs promoted the expression of the metabolites epigallocatechin, epicatechin, and (+)-catechin in blueberry fruits under Cd toxicity (Figure 4A), which act as natural defense substances and play an important role in enhancing fruit resistance and quality. Recent studies have shown that catechins were positively correlated with the TSS of peach[59]

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J. Agric. Food Chem. 2024, 72, 904−915

and anthocyanin synthesis in mangosteen.[60] Eriodictyol is distributed as a flavonoid in both fruits and vegetables, where it masks the bitter flavor of the fruit.[61] Our results suggest that ESs also promote the expression of eriodictyol in blueberry fruit with Cd toxicity. Notably, the expression level of myricetin, a metabolite with various biological activities such as antioxidant and antifungal,[62] was also significantly increased in ES-treated blueberry fruits. In addition, ES treatments significantly reduced naringin levels in blueberry fruits, which was related to the fact that ESs promoted the ripening of blueberry fruits with Cd toxicity.[63]

In conclusion, exogenous SA (200 μM), Spd (500 μM), EBR (20 μM), and MT (200 μM) sprays can effectively improve blueberry fruit quality under soil Cd toxicity, as well as increase the uptake of mineral elements (Ca, Mg, Mn, Cu, and Zn) to suppress Cd accumulation in the fruit. Overall, spraying exogenous 200 μM MT was the most effective. In addition, the upregulation of D-maltose, *N*,*N*′-diacetylchitobiose, and sucrose in the ABC transporter pathway and the downregulation of succinic acid, fumaric acid, isocitrate, and citric acid in the TCA cycle is a potential factor for the ESs-induced changes in TSS and TA of blueberry fruits under Cd toxicity, while the upregulation of L-aspartic acid, L-glutamic acid, and L-glutamate improved the tolerance of blueberry fruits to Cd toxicity. Furthermore, the upregulation of metabolites such as coniferin, L-phenylalanine, 4-hydroxycinnamic acid, myricetin, and spermidine in the flavonoid and phenylpropanoid biosynthesis pathways were closely associated with increased anthocyanin content and resistance in blueberry fruits with soil Cd toxicity (Figure 6). However, the potential molecular mechanisms for ESs to stimulate changes in specific metabolite contents in blueberry fruit growth and quality formation warrant further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c05879.

Chromatographic column elution gradient of LC−MS; mass spectrometric parameters of LC−MS; furnace program of GC−MS; mass spectrometric parameters of GC−MS; total DAMs detected and annotated in soil Cd-toxic blueberry fruits in four different comparison groups under LC−MS analysis; total DAMs detected and annotated in soil Cd-toxic blueberry fruits in four different comparison groups under GC−MS analysis; multivariate statistical analysis of blueberry fruit metabolites under LC−MS and GC−MS analysis; heatmap of the correlation of DAMs in different comparative groups of blueberry fruits under LC−MS combined with GC−MS analysis (Top 20) (PDF)

List Item LC−MS and GC−MS plots of SA/CK, Spd/CK, EBR/CK, and MT/CK plots (ZIP)

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Notes

The authors declare no competing financial interest.

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