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Untargeted GC-TOFMS Analysis Reveals Metabolomic Changes in *Salvia miltiorrhiza* Bunge Leaf and Root in Response to Long-Term Drought Stress

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Abstract: *Salvia miltiorrhiza* Bunge (Danshen) is an important traditional Chinese medicine herb. This study aimed to investigate the drought-responsive metabolic profiling in *S. miltiorrhiza* using gas chromatography time-of-flight mass spectrometry (GC-TOFMS) analysis. Fifty day-old *S. miltiorrhiza* seedlings were treated with two (moderate drought, MD) and four weeks (high drought, HD) of withholding water. The *S. miltiorrhiza* leaf and root samples were prepared for the GC-TOFMS analysis. Differential metabolites with substantial changes in content in *S. miltiorrhiza* leaf and root were identified using multivariate and univariate statistics. A total of 178 and 157 annotated metabolites were detected in *S. miltiorrhiza* leaf and root, respectively. Multivariate analysis showed that significantly discriminant metabolites in *S. miltiorrhiza* leaf by drought were associated with "galactose metabolism" and "citrate cycle". In addition, the significantly discriminant metabolites in *S. miltiorrhiza* root were associated with "starch and sucrose metabolism". Univariate statistics showed that the content of succinic acid, D-glucose, and oxoglutaric acid in *S. miltiorrhiza* leaf was increased by drought (fold change, FC > 1.5). Allose, D-xylose, melibiose, mannose, sorbitol, quinic acid, sinigrin, and taurine in *S. miltiorrhiza* root were decreased by drought (FC < 0.67). There were different metabolic profiles between *S. miltiorrhiza* leaf and root. However, the influence of drought stress on the pharmacological value and accumulation of bioactive constituents in *S. miltiorrhiza* should be further investigated.

Keywords: *Salvia miltiorrhiza* Bunge; metabolic profiling; drought stress; bioactive constituents; gas chromatography time-of-flight mass spectrometry

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1. Introduction

Salvia miltiorrhiza Bunge (called Danshen in Chinese) is a plant of the genus Salvia of the Labiatae family. Its dried root and rhizome are important traditional Chinese medicine (TCM) materials. The bioactive constituents in the root of *S. miltiorrhiza*, including tanshinones, salvianolic acid B, and polysaccharides, exhibit potent bioactive activity such as free radical scavenging, antioxidant capacity, the prevention and treatment of vascular diseases, antianxiety, and anti-inflammatory effects [1–4]. Therefore, *S. miltiorrhiza* is widely used as a herbal medicine for the treatment of cardiovascular diseases, including coronary heart disease, atherosclerosis, myocardial infarction, and ischemia [4,5]. In addition, the market demand for *S. miltiorrhiza* remains high and its planting area is expanding with the increasing demand [6].

Drought has a devastating effect on crop production and quality [7,8]. Areas affected by water shortage account for ~52% of the land in China and the annual grain production

of global grain has been reduced by more than 1.00×10^{11} kg due to drought [9]. In addition, drought has become one of the most important environmental factors and a major constraint on grain production and quality in many parts of the world.

There is increasing evidence showing that drought significantly affected the accumulation of bioactive constituents and secondary metabolites in medicinal plants [6,10,11]. Mild drought promoted the synthesis and accumulation of tanshinone, cryptotanshinone, and salvianolic acid B in *S. miltiorrhiza* root [6,10]. However, the content of rosmarinic acid was decreased by drought stress in *S. miltiorrhiza* root [6]. Cao et al. showed that the "sweating" process changed the accumulation of metabolites, including lipids, lipid-like molecules, phenylpropanoids, and polyketides in *S. miltiorrhiza* [12]. In addition, there is evidence showing that bioactive constituents (including aurantio-obtusin, aloe-emodin, rhein, and chrysophanol) in the seeds of *Cassia obtusifolia* were increased by moderate drought (80–50% deficit irrigation) [12]. The determination of the metabolic profiling of the plant in response to drought stress is of significance for investigating TCM herbal quality [6,11,13]. However, the metabolic responses in *S. miltiorrhiza* in response to drought stress have not been reported systematically.

This study aimed to investigate the metabolic response in *S. miltiorrhiza* leaf and root to long-term drought stress using the gas chromatography time-of-flight mass spectrometry (GC-TOFMS) system. The effect of drought stress on metabolites in *S. miltiorrhiza* was identified, and different metabolites before and after drought stress were compared. This study might provide a theoretical basis for the cultivation and quality control of *S. miltiorrhiza*.

2. Materials and Methods

2.1. Plant Materials and Drought Treatment

S. miltiorrhiza seeds were obtained from the experimental base of Shandong Agricultural University, Tai'an, China. The seeds were planted into plastic basins (12 cm × 10 cm) filled with matrix soil (nutrient soil:vermiculite = 7:3, *v/v*) and were placed in a laboratory greenhouse with 300 mol·m^{-2}·s^{-1} of illumination intensity, a 16:8 h light: dark cycle, and a 65% relative humidity. Fifty day-old *S. miltiorrhiza* seedlings with consistent growth were selected and treated with two (moderate drought stress, MD) and four weeks (high drought stress, HD) of withholding water. Seedlings treated with regular watering were used as normal control. Each treatment was performed in triplicate with two plants of each replication (6 replicates × 3 groups). At the end of the experiments, the leaf and root samples were collected, snapped in liquid nitrogen, and stored at −80 °C.

2.2. Sample Preparation

Samples (6 replicates × 3 groups × 2 types) were ground into powder and sieved with 50 mesh (300 μm). Eighty milligrams of powdery sample were transferred to 1.5 mL Eppendorf tubes supplemented with extract solvents: methanol, acetonitrile, and water (2:2:1 *v/v/v*). The samples were then ground (60 Hz for 2 min), sonicated three times (8 min), and rested at −20 °C for 1 h. After centrifugation (14,000× *g* for 10 min at 4 °C), the supernatants were collected, filtered through a 0.22 μm microfilter, vacuum dried, and stored at −80 °C. Then, all samples (n = 36) were reconstituted with 100% methanol, vacuum dried, oximated with methoxyamine hydrochloride in pyridine (50 μL, 20 mg/mL, for 90 min at 30 °C), and silylated with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (50 μL, for 30 min at 37 °C). Quality-control samples were prepared by mixing equivalent amounts of all samples.

2.3. GC-TOFMS Analysis

The metabolic profiling in *S. miltiorrhiza* leaf and root was detected using the GC-TOFMS system with an Agilent 4890 gas chromatograph (Palo Alto, CA, USA). A DB-5MS capillary column (30 m × 250 μm i.d., 0.25-μm film thickness; J & W Scientific, Folsom, CA, USA) was used for sample separation (1 μL). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The oven temperature was 75 °C and increased to 300 °C

(15 °C/min). Electron ionization energy was 70 eV and the full scan was 50–1000 m/z. The source and injection temperatures were set at 230 °C and 270 °C, respectively.

2.4. Data Preprocessing and Annotation

The XploreMET software (Metabo-Profile, Shanghai, China) and Chromma TOF software (V. 4.51.6.0, Leco, CA, USA) were used to analyze and process the range/scale data generated by GC-TOFMS. Metabolites were annotated using the JiaLib^{TM} comprehensive proprietary mammalian metabolite database. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze the pathways that were associated with metabolites in *S. miltiorrhiza*. Finally, Z-Score heatmap clustering of the differential metabolites was performed.

2.5. Multivariate and Univariate Statistical Analysis

Multivariate statistical analyses, including principal component analysis (PCA) and partial least squares discrimination analysis (PLS-DA), were carried out to compare the different metabolites between different experimental groups using the normalized data matrix. The significantly discriminant metabolites in *S. miltiorrhiza* leaf and root by drought stress were identified using the PLS-DA model with the criteria of variable importance in projection (VIP) ≥ 1 and correlation coefficient (Corr.Coeff) p value < 0.05. The identification of differential metabolites with substantial changes in contents in response to drought between groups was performed using the threshold of fold change (FC) ≥ 1.5 (increase) or $\leq$0.67 (decrease) and $p < 0.05$. The differential metabolites common to *S. miltiorrhiza* leaf and root were identified using the Venn diagram. The univariate statistical method Kruskal–Wallis H test was used to analyze the different levels of metabolites across three groups. A statistically significant difference was defined when p value < 0.05.

3. Results

3.1. Metabolite Profiles of S. miltiorrhiza Leaf and Root Identified by GC-TOFMS Technology

GC-TOFMS-based untargeted analysis produced a total of 447 and 349 metabolites in *S. miltiorrhiza* leaf and root, respectively, of which 178 (40%) and 157 (45%) metabolites were annotated (Supplementary Tables S1 and S2). The predominant metabolites in *S. miltiorrhiza* leaf and root were grouped into three metabolism categories: organic acids (22% and 27%), amino acids (24% and 22%), and carbohydrates (25% and 24%; Figure 1A,B). The results of the PCA analysis showed that there was a clear separation of *S. miltiorrhiza* leaf samples across groups (Figure S1A) and a high heterogeneity of *S. miltiorrhiza* root samples within groups (Figure S1B). However, the PLS-DA analysis showed that both *S. miltiorrhiza* leaf and root samples were separated clearly (Figure 1C,D). Accordingly, we used all the samples (n = 36) to analyze the metabolic profiles in *S. miltiorrhiza* leaf and root.

3.2. Identification of Differential Metabolites in S. miltiorrhiza Leaf and Root

Based on the PLS-DA model analysis and the criteria of VIP ≥ 1 and p value < 0.05, we identified that MD and HD treatment induced 63 and 71 significantly discriminant metabolites in *S. miltiorrhiza* leaf compared with control, respectively (Supplementary Table S3). In addition, MD and HD treatment induced 39 and 44 significantly discriminant metabolites in *S. miltiorrhiza* root compared with control, respectively (Supplementary Table S3).

Most of the above metabolites with substantial increase (FC ≥ 1.5 and $p < 0.05$) or decrease (FC ≤ 0.67 and $p < 0.05$), including 62 and 45 metabolites in *S. miltiorrhiza* leaf, were induced by HD and MD treatments (25 common metabolites, six downregulated and 19 upregulated; Figure 2A) as well as 34 and 44 metabolites in *S. miltiorrhiza* root induced by HD and MD treatments, respectively (14 common metabolites, 12 downregulated and two upregulated; Figure 2B). The common metabolites are listed in Table 1.

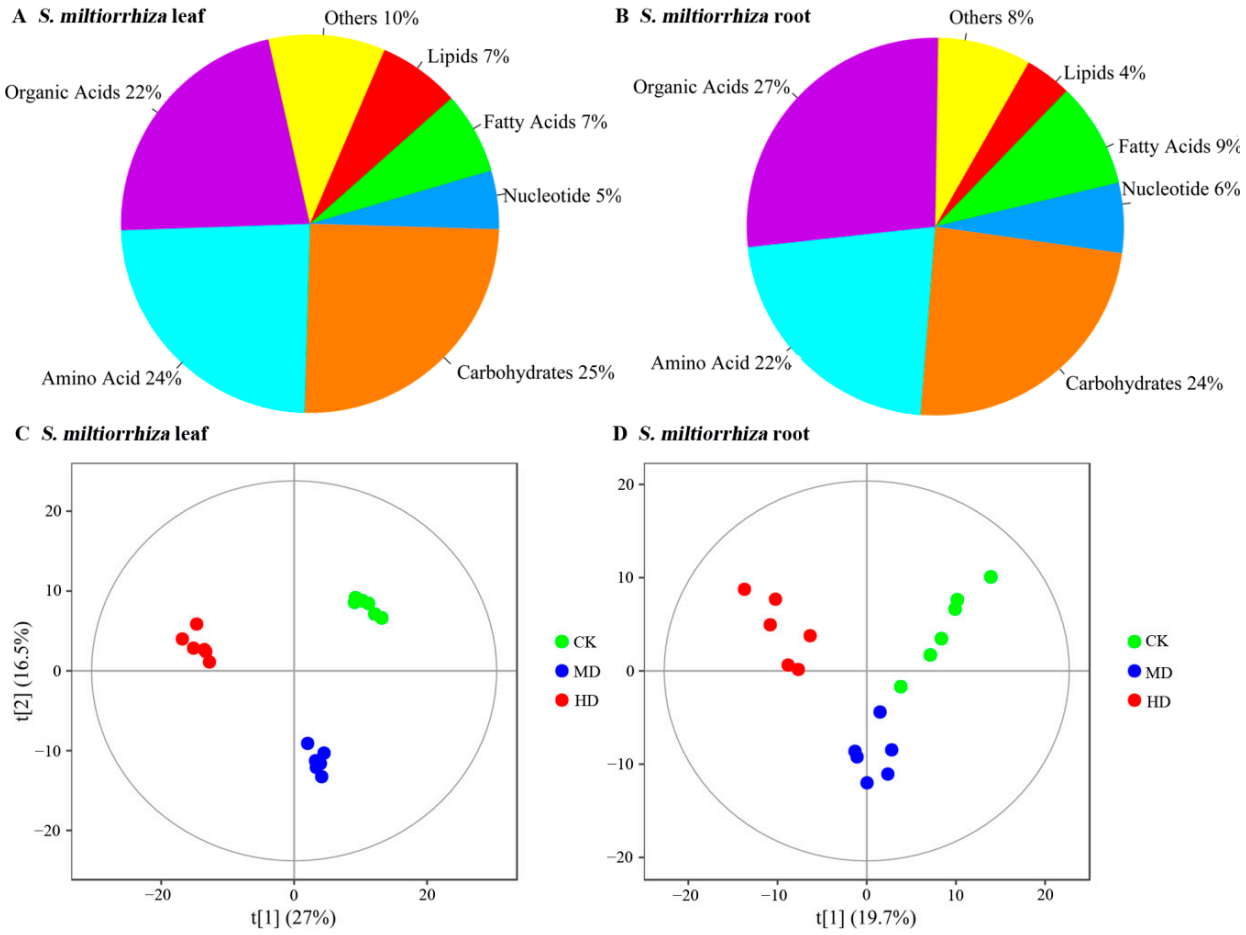

Figure 1. Overview of the metabolites in *Salvia miltiorrhiza* Bunge leaf and root. (**A**,**B**): The pie chart indicating the distribution of annotated metabolites. (**C**,**D**): The results of the partial least squares discrimination analysis (PLS-DA) of analyzed samples. MD: moderate drought. HD: high (severe) drought. CK: control.

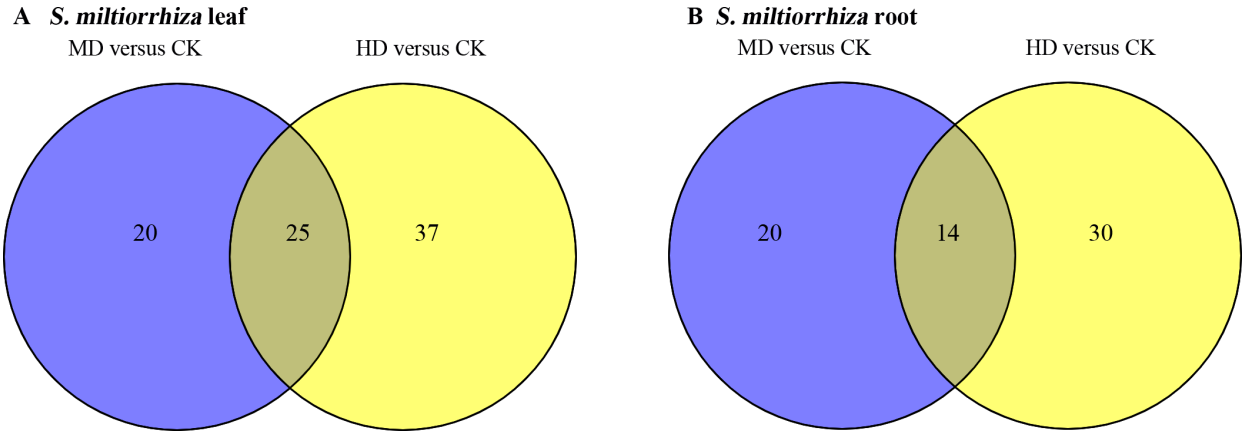

Figure 2. The Venn diagram of differential metabolites in *Salvia miltiorrhiza* Bunge. (**A**,**B**): the Venn diagram indicating differential metabolites in *S. miltiorrhiza* leaf and root, respectively. Differential metabolites were identified using the thresholds of fold change (FC) ≥ 1.5 or ≤ 0.67 ($|\log2FC| \geq 0.585$) and $p < 0.05$.

Table 1. Significantly differential metabolites in *Salvia miltiorrhiza* Bunge leaf and root induced by drought stress by univariate statistics.

Class	Name	HMDBID	KeggID	HD versus CK		MD versus CK	
				p Value	FC	*p* Value	FC
***S. miltiorrhiza* leaf**							
Amino acids	Ratio of L-Glutamic acid to Oxoglutaric acid	HMDB00148 HMDB00208	C00025 C00026	5.50e-03	0.06	2.40e-02	0.35
	L-Lysine	HMDB00182	C00047	3.50e-03	2.58	4.50e-04	1.85
	L-Leucine	HMDB00687	C00123	5.00e-06	0.11	2.00e-04	0.40
	L-Histidine	HMDB00177	C00135	6.10e-03	4.47	6.50e-06	2.98
	L-Alloisoleucine	HMDB00557	NA	1.60e-02	21.98	2.50e-05	41.37
Carbohydrates	Sucrose	HMDB00258	C00089	2.70e-02	2.48	1.20e-04	2.46
	Sorbitol	HMDB00247	C00794	4.90e-02	0.11	4.20e-02	0.07
	Mannitol	HMDB00765	C00392	3.30e-02	1.94	1.60e-02	1.53
	Lactulose	HMDB00740	C07064	2.10e-02	3.90	2.30e-05	3.05
	D-Xylose	HMDB00098	C00181	5.80e-03	1.95	8.00e-03	1.78
	D-Glucose	HMDB00122	C00031	2.40e-02	1.81	1.40e-02	1.71
	beta-Lactose	HMDB41627	C01970	5.30e-03	2.13	1.30e-04	6.38
Fatty acids	Docosahexaenoic acid	HMDB02183	C06429	2.70e-03	0.16	2.80e-02	0.49
Indoles	Indoleacetic acid	HMDB00197	C00954	4.90e-02	0.39	3.60e-02	2.48
Lipids	MG182	HMDB11568	NA	1.70e-02	3.01	3.20e-03	1.94
Nucleotide	Ratio of Uridine to Cytidine	HMDB00296 HMDB00089	C00299 C00475	1.30e-05	0.50	3.00e-06	0.24
	Ratio of Uracil to Uridine	HMDB00300 HMDB00296	C00106 C00299	2.70e-02	1.76	1.20e-02	2.52
Organic acids	trans-Ferulic acid	HMDB00954	C01494	5.50e-05	1.65	5.80e-04	1.51
	Succinic acid	HMDB00254	C00042	3.20e-07	3.71	6.60e-03	1.84
	Maleic acid	HMDB00176	C01384	5.00e-02	3.82	6.80e-05	1.78
	L-Malic acid	HMDB00156	C00149	5.60e-03	3.14	1.00e-02	2.90
	Fumaric acid	HMDB00134	C00122	3.70e-02	1.37	2.30e-05	1.59
	4-Hydroxycinnamic acid	HMDB02035	C00811	2.90e-02	2.02	4.50e-06	1.69
	3-Aminosalicylic acid	HMDB01972	NA	3.50e-04	0.08	2.80e-04	0.04
Vitamin	Pantothenic acid	HMDB00210	C00864	3.10e-02	2.46	3.90e-04	1.77
***S. miltiorrhiza* root**							
Amino acid	Urea	HMDB00294	C00086	1.50e-04	0.16	1.60e-04	0.20
	L-Alloisoleucine	HMDB00557	NA	1.00e-03	20.07	1.60e-03	59.69
Carbohydrates	Sorbitol	HMDB00247	C00794	1.30e-03	0.23	2.90e-02	0.50
	Ribonolactone	HMDB01900	C02674	6.00e-04	0.59	1.60e-04	0.65
	Ratio of L-Arabinose to L-Arabitol	HMDB00646 HMDB01851	C00259 C00532	1.70e-02	0.11	4.70e-02	0.30
	Melibiose	HMDB00048	C05402	1.60e-02	0.42	6.50e-03	0.29
	Isomaltose	HMDB02923	C00252	1.90e-02	5.43	1.50e-02	5.26
	Glucose 6-phosphate	HMDB01401	C00092	1.10e-02	0.47	2.80e-02	0.63
	Glucose 1-phosphate	HMDB01586	C00103	5.70e-03	0.53	7.20e-04	0.60
	D-Mannose	HMDB00169	C00159	6.70e-03	0.53	1.00e-02	0.61
Fatty acids	Myristoleic acid	HMDB02000	C08322	4.10e-02	0.56	2.30e-02	0.54
Lipids	MG182	HMDB11568	NA	5.80e-03	0.55	1.00e-03	0.30
Nucleotide	Uridine	HMDB00296	C00299	2.80e-02	0.52	4.50e-02	0.55
Organic acids	Taurine	HMDB00251	C00245	2.60e-02	0.42	4.40e-03	0.19

VIP: variable importance in projection; Corr.Coeff: correlation coefficient; FC: fold change; KEGG: Kyoto Encyclopedia of Genes and Genomes. NA: not applicable.

3.3. KEGG Pathway Annotation

The KEGG pathways related to the metabolites with substantial increase and decrease in *S. miltiorrhiza* leaf and root are shown in Figure 3A,B. The metabolites in *S. miltiorrhiza* leaf were related to "valine, leucine, and isoleucine" (hsa00290, including downregulated L-Leucine), "galactose metabolism" (hsa00052, including downregulated sorbitol and upregulated sucrose and D-glucose), and "citrate cycle (TCA cycle)" (hsa00020, including upregulated succinic acid and fumaric acid; Figure 3A), and the differential metabolites

induced by HD treatment in *S. miltiorrhiza* leaf were related to "butanoate metabolism" (hsa00650, including upregulated succinic acid), "D-glutamine and D-glutamate metabolism" (hsa00471, including upregulated oxoglutaric acid), and "arginine biosynthesis" (hsa00220, including upregulated fumaric acid and oxoglutaric acid).

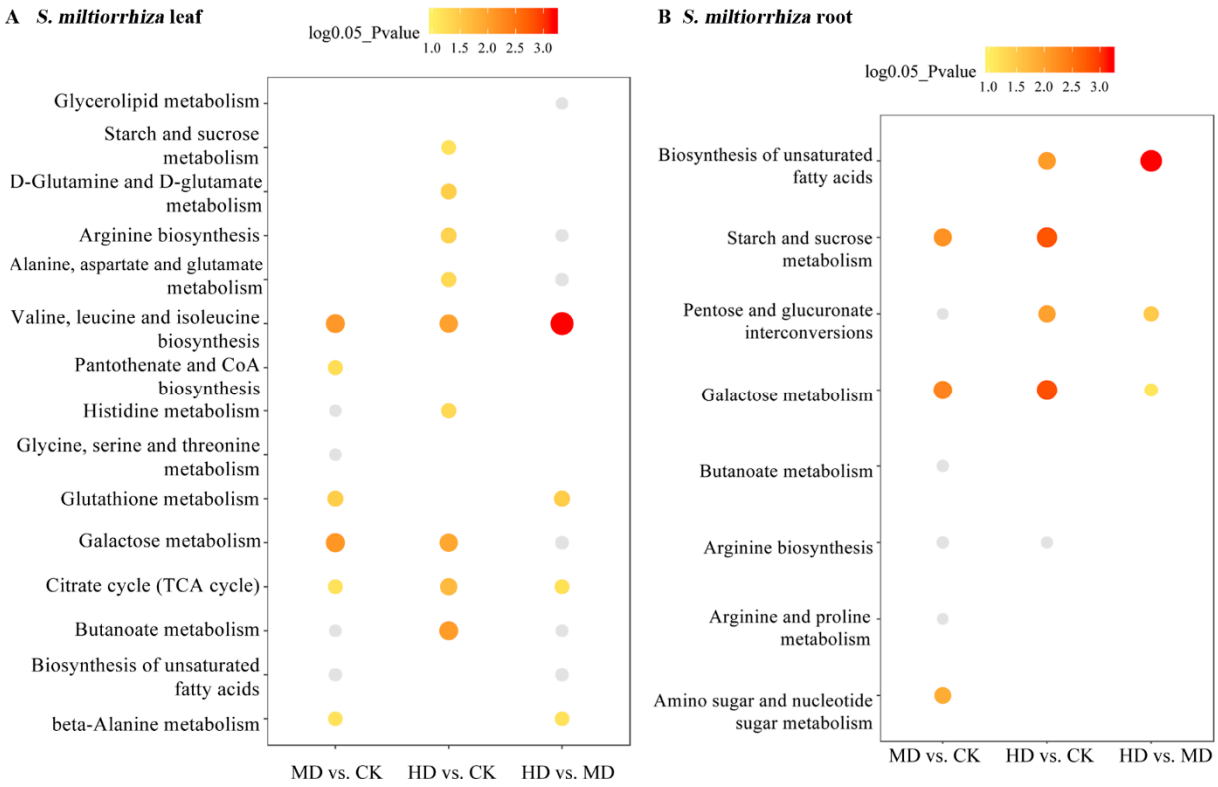

Figure 3. The metabolic pathway enrichment analysis of differential metabolites in *Salvia miltiorrhiza* Bunge. (**A**,**B**): the pathways related to differential metabolites in *S. miltiorrhiza* leaf and root, respectively. Significantly differential metabolites were identified using the thresholds of fold change (FC) ≥ 1.5 or ≤ 0.67 ($|\log2FC| \geq 0.585$) and $p < 0.05$.

In addition, we demonstrated that metabolites with substantial changes in *S. miltiorrhiza* root were related to "starch and sucrose metabolism" (hsa00500, including upregulated D-maltose and isomaltose) and hsa00052 (including downregulated glucose 1-phosphate, melibiose, and sorbitol; Figure 3B), and the HD-induced metabolites with substantial changes in *S. miltiorrhiza* root were associated with "pentose and glucuronate interconversions" (hsa00040, including downregulated glucose 1-phosphate). The sample clustering of metabolites enriched with the above pathways in *S. miltiorrhiza* leaf and root is shown in Figure S2A,B.

3.4. Metabolic Profiles of Metabolites Related to the Biosynthesis of Terpenoids in S. miltiorrhiza

The major pathways and the levels of key metabolites related to the biosynthesis of terpenoids are shown in Figure 4. The glycolysis and TCA cycle metabolites are the primary substances of terpenoid biosynthesis: the mevalonic acid (MVA) and 2-methyl-D-erythritol 4-phosphate (methylerythritol phosphate, MEP) pathways (Figure 4A). We showed that drought stress-induced differential metabolites were related to glycolysis and TCA cycle in *S. miltiorrhiza* leaf (Figure 4B). L-Pipecolic acid, phosphoenolpyruvic acid, isomaltose, and tartaric acid were the metabolites with the highest contents in *S. miltiorrhiza* leaf (peak area > 40,000, data not shown). The MD and HD stress increased the contents of succinic acid (1.84 and 3.71 FC), fumaric acid (1.59 and 1.37 FC), D-glucose (1.71 and 1.81 FC), oxoglutaric acid (MD: 10.83 FC), and L-malic acid (2.90 and 3.14 FC) compared with control (Figure 4B), while the HD stress decreased the accumulation of glucose 6-phosphate

(0.43 FC), glucose 6-phosphate/D-glucose ratio (0.26 FC), and fumaric acid/L-malic acid ratio (0.39 FC; $p < 0.05$ by Kruskal-Wallis H test).

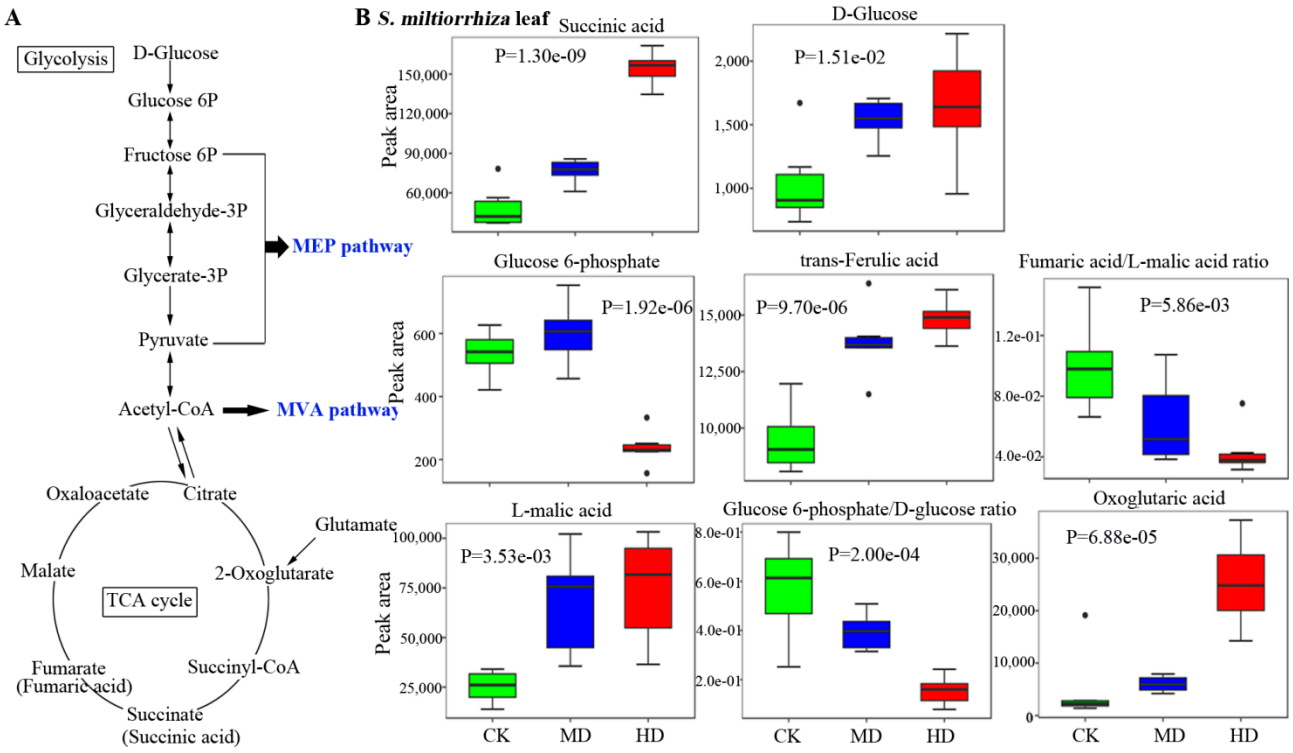

Figure 4. Metabolic profiles of terpenoid biosynthesis metabolites in *Salvia miltiorrhiza* Bunge. (**A**): the metabolic pathways (glycolysis and TCA cycle) producing primary substances of terpenoid biosynthesis. MVA, mevalonic acid. MEP, 2-methyl-D-erythritol 4-phosphate/methylerythritol phosphate. (**B**): the profiles of differential metabolites in *S. miltiorrhiza* leaf. CK, control. MD, moderate drought. HD, high (severe) drought. Univariate statistic method Kruskal–Wallis H test was used to analyze the different levels of metabolites. Data were expressed as median and range (interquartile range).

Also, we identified that metabolites with the highest contents in *S. miltiorrhiza* root were fructose 6-phosphate, galactinol, allose, and sinigrin (peak area > 10,000, data not shown). We also identified that three differential metabolites (glucose 6-phosphate, glucose 1-phosphate, and D-glucose) that were related to glycolysis and TCA cycle were decreased in *S. miltiorrhiza* root by drought stress, especially by HD treatment (Figure 5). Besides, MD and HD stresses gradually decreased the contents of allose (0.87 and 0.61 FC), D-xylose (0.55 and 0.16 FC), melibiose (0.29 and 0.42 FC), and D-mannose (0.61 and 0.53 FC) compared with control (Figure 5), while the content of isomaltose was increased by drought (MD and HD: 5.26 and 5.43 FC; $p < 0.05$ by Kruskal-Wallis H test). These results might show that the biosynthesis of terpenoids and the accumulation of carbohydrate metabolites in *S. miltiorrhiza* root were inhibited by drought stress.

3.5. Drought Decreases the Bioactive Constituents in S. miltiorrhiza Root

We also showed that the contents of bioactive constituents in *S. miltiorrhiza* root, including beta-sitosterol (0.26 and 0.47 FC), sorbitol (0.50 and 0.23 FC), myristoleic acid (0.54 and 0.56 FC), quinic acid (0.85 and 0.37 FC), sinigrin (0.67 and 0.47 FC), and taurine (0.19 and 0.42 FC), were decreased by drought stress compared with control (Figure 6). However, the content of L-alloisoleucine in *S. miltiorrhiza* root was increased by drought (MD and HD: 59.69 and 20.07 FC). These results showed that the pharmacological value of *S. miltiorrhiza* might be decreased by drought stress.

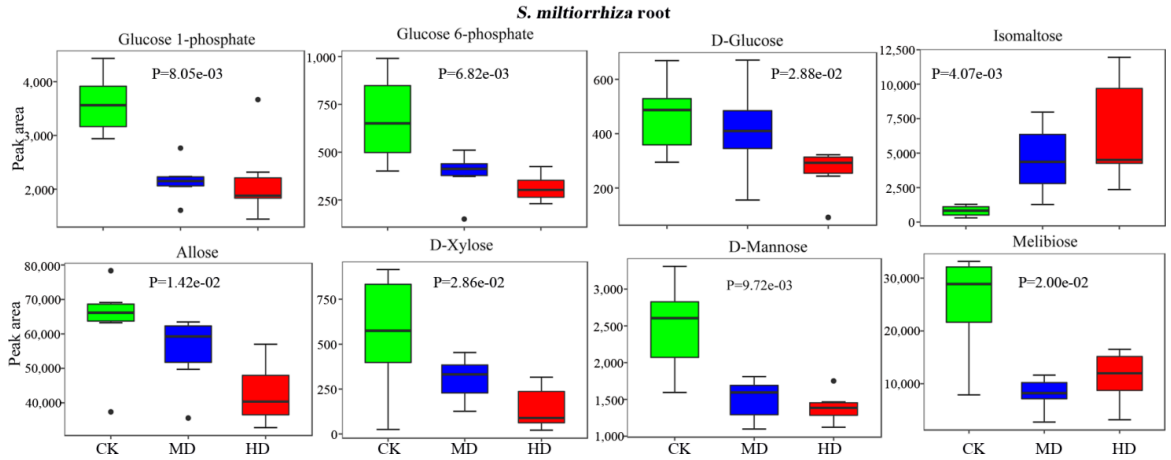

Figure 5. Metabolic profiles of glycolysis metabolites and carbohydrate metabolites in *Salvia miltiorrhiza* Bunge root. CK: control. MD: moderate drought. HD: high (severe) drought. Univariate statistic method Kruskal–Wallis H test was used to analyze the different levels of metabolites. Data were expressed as median and range (interquartile range).

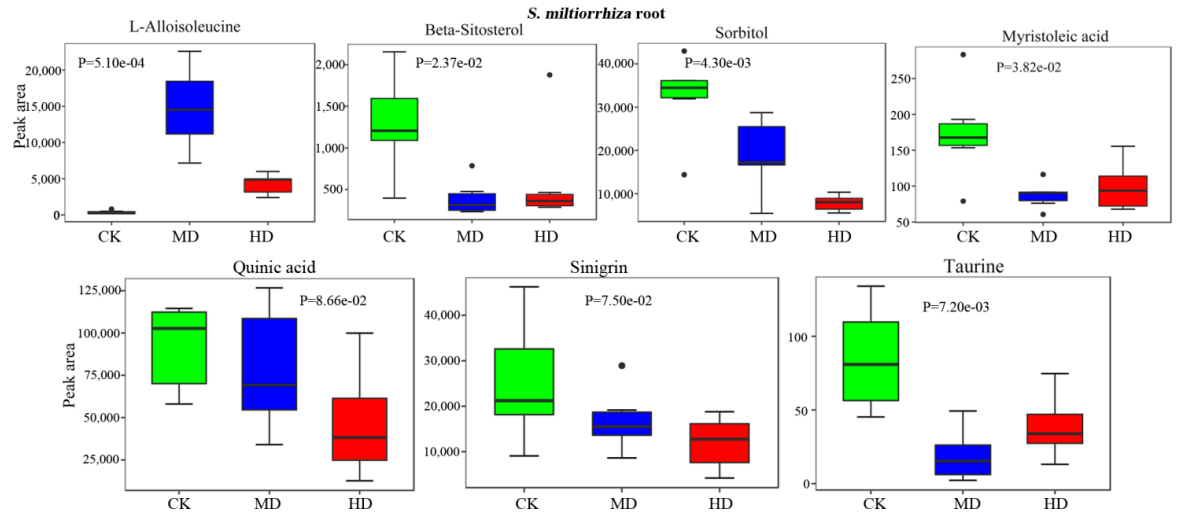

Figure 6. Metabolic profiles of several bioactive constituents in *Salvia miltiorrhiza* Bunge root. CK: control. MD: moderate drought. HD: high (severe) drought. Univariate statistic method Kruskal–Wallis H test was used to analyze the different levels of metabolites. Data were expressed as median and range (interquartile range).

4. Discussion

Both biotic and abiotic stresses influence the quality, production, and accumulation of secondary metabolites in the plant [8,13,14]. Our present study showed that most of the differential metabolites in *S. miltiorrhiza* leaf were increased by drought stress and most differential metabolites in *S. miltiorrhiza* root were decreased by drought stress. Moreover, we showed that most of the metabolites related to the biosynthesis of terpenoids, glycolysis, and TCA cycle in *S. miltiorrhiza* leaves were upregulated by drought stress. However, several bioactive constituents and most of the metabolites related to the biosynthesis of terpenoids, glycolysis, TCA cycle, and carbohydrate metabolism in *S. miltiorrhiza* root were decreased by drought stress. These results showed that the pharmacological value and the accumulation of bioactive constituents in *S. miltiorrhiza* leaf and root were influenced by drought.

Plant growth and development are frequently stressed by various environmental factors (including salinity, drought, temperature, and water), organisms (diseases, pests, and weeds), and deficiency of nutrients (including nitrogen, phosphorus, and potassium) [14–17]. The above factors influence transcriptomics, proteomics, and metabolomics

in plants [17–19]. Chai et al. [18] showed that most of the metabolites in *Vitis amurensis* plantlets identified by the GC-TOMFS system were grouped into three categories (carbohydrates, amino acids, and organic acids) and were positively correlated with decreasing temperatures. They showed that metabolites including galactinol, melibiose, xylose, glucose, maltose, lactose, psicose, raffinose, fructose, mannose, glycerol, 2-oxoglutarate, glycine, alanine, arginine, glutamine, aspartate, succinate, malate, and fumarate were gradually increased by cold treatment at 4 °C for 24 h and 72 h [18]. Zhu et al. [10] revealed that the accumulation of saikosaponin a/b in *Bupleurum chinense* DC. seedlings were gradually increased with the degree of drought stress. They demonstrated that the contents of saikosaponin a/b in *B. chinense* DC. seedlings were increased by mild drought stress (water deficit for 3 and 6 days) and the content of saikosaponin a was decreased by severe drought stress (water deficit for 9 days) compared with control. Chen et al. [20] demonstrated that the amounts of guanosine and succinic acid in *Pinellia ternata* were reduced by drought stress compared with control treatment. In addition, Jia et al. [11] identified that the contents of calycosin-7-O-β-D-glycoside and ononin in *Radix Astragali* roots were enhanced with the degree of drought stress. These results showed that drought stress has different influences on the biosynthesis and accumulation of metabolites.

Our present study showed that drought stress (two and four weeks of withholding water) reduced the contents of carbohydrate metabolites in *S. miltiorrhiza* root, including glucose 1-phosphate, glucose 6-phosphate, D-glucose, allose, D-xylose, D-mannose, and melibiose. However, we also showed that the contents of oxoglutaric acid, trans-ferulic acid, D-glucose, and succinic acid in *S. miltiorrhiza* leaf were increased by drought stress. Moreover, the contents of bioactive constituents in *S. miltiorrhiza* root, including beta-sitosterol, sorbitol, myristoleic acid, quinic acid, sinigrin, and taurine, were gradually decreased by moderate and severe drought stresses. These results indicated different metabolic profiling between *S. miltiorrhiza* root and leaf in response to drought stress. Moreover, drought might be a major constraint on the accumulation of bioactive constituents and the pharmacological value of *S. miltiorrhiza* root.

Sugars mainly come from photosynthesis, gluconeogenesis, and polysaccharide degradation in plant cells. In this study, the contents of D-glucose, allose, D-xylose, D-mannose, melibiose, beta-sitosterol, sorbitol, myristoleic acid, quinic acid, sinigrin, and taurine in *S. miltiorrhiza* root increased significantly under drought stress. Quinic acid has been shown to exhibit potent bioactive activity, including anti-neuroinflammatory, antivirulence, radioprotection, and anti-oxidant capacity [21–23]. Sinigrin has anti-inflammatory, anti-asthmatic, and anti-atherosclerosis activities [24–26]. Lee et al. [25] showed that sinigrin reduced the lipopolysaccharide-induced production of inflammatory cytokine tumor necrosis factor (TNF)-α and interleukin (IL)-6 in RAW 264.7 macrophages and inhibited the nuclear factor-kappa B (NF-κB)/MAPK pathway. Quinic acid and sinigrin both inhibit TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) [21,26]. In addition, taurine shows a neuroprotection function in a mouse model with the manganism-induced locomotor deficit [27]. The above evidence shows that long-term drought stress reduced the accumulation of the above bioactive constituents and might decrease the pharmacological value of the *S. miltiorrhiza* root.

Salvianolic acid B and tanshinones (including tanshinone I/IIA and cryptotanshinone) are diterpenoids: secondary metabolites of *S. miltiorrhiza* [28]. Photosynthesis, glycolysis, and the TCA cycle provide basic stacking modules for the biosynthesis of terpenoids through the MVA and MEP pathways [28]. Tanshinones have antitumor properties [29–31]. Studies show that drought stress reduced the terpenoid emissions of cork oak [32] and the needle terpenoid contents of the coastal-provenance Douglas fir [33]. The decreased glycolysis and the TCA cycle in *S. miltiorrhiza* root might show that the biosynthesis of terpenoids is inhibited by drought stress. However, Liu et al. showed that the amounts of salvianolic acid B and tanshinones in *S. miltiorrhiza* root were decreased and increased by drought stress, respectively [6]. In addition, Xue et al. showed that drought stress (80–50% deficit irrigation) increased the contents of aurantio-obtusin, aloe-emodin, rhein,

and chrysophanol in *C. obtusifolia* seeds [12]. However, our study did not identify terpene metabolites in *S. miltiorrhiza* leaf and root using GC-TOFMS-based untargeted metabolic analysis. The studies from Liu et al. [6] and Xue et al. [12] showed that drought stress might be a factor conducive to the accumulation of tanshinones in *S. miltiorrhiza*. The results showed that drought stress is a controversial factor in the accumulation of bioactive constituents and the pharmacological value of *S. miltiorrhiza*.

5. Conclusions

In summary, this study demonstrated that the long-term drought stress increased the glycolysis and TCA cycle in *S. miltiorrhiza* leaf. In addition, drought stress decreased the glycolysis and TCA cycle metabolism and the biosynthesis of the bioactive constituents in *S. miltiorrhiza* root, including D-glucose, allose, D-xylose, D-mannose, melibiose, beta-sitosterol, sorbitol, myristoleic acid, quinic acid, sinigrin, and taurine. These results demonstrated that there were different metabolic profiles between the leaf and root of *S. miltiorrhiza*. However, the influence of drought stress on the pharmacological value and accumulation of bioactive constituents in *S. miltiorrhiza* should be further investigated by more experiments.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7070175/s1, Table S1. All metabolites in *Salvia miltiorrhiza* Bunge leaf. Table S2. All metabolites in *Salvia miltiorrhiza* Bunge root. Table S3. Significantly discriminant and differential metabolites in *Salvia miltiorrhiza* Bunge leaf and root induced by drought stress. Figure S1. Principal component analysis (PCA) of the *Salvia miltiorrhiza* Bunge leaf and root samples. MD, moderate drought. HD, high (severe) drought. CK, control.

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Abbreviations

FC	fold change;
GC-TOFMS	gas chromatography time-of-flight mass spectrometry;
HD	high drought;
KEGG	Kyoto Encyclopedia of Genes and Genomes;
MD	moderate drought;
MEP	2-methyl-D-erythritol 4-phosphate;
MVA	mevalonic acid;
NF-κB	nuclear factor-kappa B;
PCA	principal component analysis;
PLS-DA	partial least squares discrimination analysis;
TCA	citrate cycle;
TCM	traditional Chinese medicine;
VCAM-1	vascular cell adhesion molecule-1;
VIP	variable importance in projection;

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