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Metabolic Regulation Profiling of Carbon and Nitrogen in Tea Plants [*Camellia sinensis* (L.) O. Kuntze] in Response to Shading

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ABSTRACT: Manipulating light transmission by shading is the most effective method of improving the nutritional value and sensory qualities of tea. In this study, the metabolic profiling of two tea cultivars ("Yulv" and "Maotouzhong") in response to different shading periods during the summer season was performed using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS) and gas chromatography-mass spectrometry (GC-MS). The metabolic pathway analyses showed that the glycolytic pathway and the tricarboxylic acid cycle (TCA cycle) in the leaves and shoots of "Maotouzhong" were significantly inhibited by long-term shading. The nitrogen metabolism in the leaves of the two cultivars was promoted by short-term shading, while it was inhibited by long-term shading. However, the nitrogen metabolism in the shoots of the two cultivars was always inhibited by shading, whether for short or long-term periods. In addition, the intensity of the flavonoid metabolism in both tea cultivars could be reduced by shading. These results revealed that shading could regulate the carbon and nitrogen metabolism and short-term shading could improve the tea quality to some extent.

KEYWORDS: *Camellia sinensis (L.) O. Kuntze, shading, metabolite profiling, metabolic pathway, nitrogen metabolism, carbon metabolism*

1. INTRODUCTION

Tea, prepared from the young leaves of the tea plant [*Camellia sinensis* (L.) O. Kuntz], is the most popular nonalcoholic beverage in the world. Tea has a number of health-promoting properties, a pleasant flavor, and cultural significance.[1−3] The quality of tea is primarily dependent on the sensory and health-promoting properties of its polyphenolic flavonoids, theanine, and alkaloids.[4,5] It is well known that the chemical composition and sensory quality of tea are influenced by various environmental factors and management practices, including the genetic background of the plant, growing region and altitude, climatic conditions, horticultural practices, harvest season, and insect or pathogen attacks.[4,6,7] Among these factors, the harvest season strongly affects the quality and value of tea. The chemical compositions of tea harvested during the spring, summer, and autumn seasons are dramatically different. For example, summer tea contains higher levels of catechins and lower levels of amino acids due to the strong light intensity and high temperature in the summer, which leads to changes in its sensory qualities.[6,8] Studies have shown that the high levels of catechins and caffeine in tea are responsible for its bitter and astringent taste, while amino acids, especially theanine, are associated with its sweetness and umami taste.[9−14]

Sunlight is an essential ecological factor that regulates photosynthesis and influences the growth, morphogenesis, and survival of plants.[15−17] The amount of sunlight is controlled by shading treatments, which is a traditional and effective practice of modifying the major natural product (catechins, theanine, and caffeine) accumulation in tea leaves and enhancing tea taste or quality.[18] During shade management, the levels of natural products such as tea polyphenols and amino acids are governed interactively according to the available carbon and nitrogen source in the plants. A recent study reported that shading tea plants reduced the soluble protein and chloroplasts in the leaves, thereby suggesting that the proteolysis of chloroplast proteins is responsible for the accumulation of free amino acids in tea leaves under shading.[19] Similarly, Ji et al.[20] found that the levels of alanine, asparagine, aspartate, isoleucine, threonine, leucine, and valine were conspicuously elevated in fresh tea leaves when the shading periods were increased, and they also reported that the increased biosynthesis of free amino acids largely reduced the glucose and chloroplast levels under dark conditions. Sano et al.[21] found that the shading cultivation method could decrease the epicatechin and epigallocatechin contents but increase the theanine and caffeine contents in new tea leaves. Recently, Fan et al.[22] reported that sunlight influenced the metabolism of the light-harvesting pigment and photosynthetic system in "Huangjinya". Zhang et al.[23] found that the class II homeodomain-leucine zipper (HD-ZIP) protein (AtHB2) negatively regulated the expression of anthocyanidin synthase (CsANS) in tea plants in response to light signals. These studies demonstrated that the light intensity and temperature under shading cultivations are affecting the metabolite compositions of tea, especially polyphenols, caffeine, and amino acids.

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Metabolomic analysis is a powerful and extensively used technology for the comprehensive profiling and comparison of metabolites in plant metabolism,[24,25] and it is also widely used on tea plants.[26] Metabolomic approaches, including ^{1}H NMR, gas chromatography-mass spectrometry (GC-MS), and ultra-performance liquid chromatography combined with time of flight mass spectrometry (UPLC-TOF-MS/MS), have been widely used to explore the biosynthesis and accumulation of quality-related metabolites in tea plants.[20,27] However, the changes in metabolites relating to the carbon and nitrogen metabolism in the shoots and leaves of different tea cultivars over different shading periods are unclear. Therefore, in this study, the metabolite profiling of the two different tea varieties cultivated in the summer (August) under different shading periods (0, 4, and 16 days) was analyzed by UPLC-TOF-MS/MS and GC-MS. The aim of this study is not only to provide a framework for a better understanding of carbon- and nitrogen-based metabolic regulation in the leaves and shoots of tea cultivars under different shading conditions but also to offer a better technology for improving the tea quality in the summer.

2. MATERIALS AND METHODS

2.1. Plant Materials and Shading Treatments. Three-year-old tea plant cultivars [*C. sinensis* (L.) O. Kuntze] of "Yulv" (YL) and "Maotouzhong" (MTZ) were planted at the tea plantation of the Tai'an Academy of Agricultural Sciences of China (117.08°E, 36.20°N). Based on the carbon/nitrogen and tea polyphenol/amino acid ratios analyzed during the pre-experiments, YL and MTZ were selected and used in this study. YL had higher carbon/nitrogen and tea polyphenol/amino acid ratios, while MTZ had lower ratios. A total of 30 healthy tea plants were used for each treatment, and the shading experiment consisted of three treatments, with tea plants grown under natural conditions (unshading, as the control) and tea plants grown under shading conditions (plants covered with black polyethylene net curtains) for 4 and 16 d. The shading treatments were performed on August 6, 2015, and August 18, 2015, and the shoots (apical bud with two leaves) and leaves (4th and 5th leaves) were collected on August 22, 2015. The samples were named as follows: YL shoots after 16 days of shading (16YS), YL leaves after 16 days of shading (16YL), YL shoots after 4 days of shading (4YS), YL leaves after 4 days of shading (4YL), YL shoots when unshaded (0YS), YL leaves when unshaded (0YL), MTZ shoots after 16 days of shading (16MS), MTZ leaves after 16 days of shading (16ML), MTZ shoots after 4 days of shading (4MS), MTZ leaves after 4 days of shading (4ML), MTZ shoots when unshaded (0MS), and MTZ leaves when unshaded (0ML). The samples in each group consisted of six biological replicates that were washed using distilled deionized water and divided into three parts. The first part was quickly frozen in liquid nitrogen and stored at −80 °C for metabolic analysis (3 g of the fresh weight). The second part was used to analyze the chlorophyll and carotenoids (fresh samples). The third part was dried at 80 °C for 6 h and used for physiological determinations (5 g of the dry weight).

2.2. Determination of Physiological Indexes. The dried samples were analyzed for the tea polyphenols and free amino acids in the shoots and the organic carbon and total nitrogen in the leaves. The measurements were performed in accordance with the State Standard of China for tea content determination and recorded as GB/T 8313-2008, GB/T 8314-2013, HJ 615-2011, and HJ 717-2014. Determinations of the chlorophyll *A*, chlorophyll *B*, and carotenoid contents in the freshly collected leaves and shoots were determined according to the previous report.[28] These contents were determined within one week after sampling. The photosynthetic indexes, including the light intensity, leaf temperature, photosynthetic/respiratory rate, transpiration rate, stoma conduction, and concentration of CO_2, in the dried tea leaves were determined with a TPS-2 portable photosynthesis system (PP SYSTEMS) on August 22, 2015.

2.3. GC-MS for Metabolomics. *2.3.1. Extraction Procedure.* Leaf sample extractions were prepared as previously reported, with little modification.[29−31] A total of 100 mg of tea leaves was extracted with 1.4 mL of chilled methanol (−20 °C). Then, 1.4 mL of ddH_2O (4 °C) and 750 μL of chloroform (−20 °C) were added for purification. After being dried with nitrogen, 60 μL of methoxypyridine solution (15 mg/mL) was added to vortex mixing for 30 s, and the reaction was conducted overnight for 16 h at room temperature. Next, 60 μL of BSTFA [*N*,*O*-bis (trimethylsilyl) trifluoroacetamide] with 1% trimethylchlorosilane was added and reacted for 60 min at room temperature. The mixture was prepared for GC-MS analysis, and ribitol was used as the internal standard (IS).

2.3.2. GC-MS Analysis. A total of 1 μL of the derivatized sample was injected into an Agilent 7890A/5975C GC-MS system HP-5 MS capillary column (Agilent) for profiling analysis. The injection temperature, interface temperature, and ion source temperature were 280, 150, and 250 °C, respectively. The initial temperature of the program was 70 °C and maintained for 2 min and then increased to 300 °C by 10 °C/min and maintained for 5 min. The carrier gas was helium and was constant at 1 mL/min. Mass spectra were recorded using a full-scan monitoring mode with a range of 35−780 *m*/*z*.

2.3.3. Metabolite Identification and Data Analysis. The identified compounds were annotated by the National Institute of Standards and Technology (NIST) commercial database and Wiley Registry metabolome database. Ultimately, all of the peak areas were normalized by the internal standard (ribitol) method.

After Student's *t* test, metabolites with variable importance in the projection (VIP) > 1 (P < 0.05) were considered as differential metabolites. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were performed using SIMCA-P 13.0 (Umetrics AB, Umea, Sweden). Unit variance (UV) was used to scale all variables. In addition, a hierarchical clustering analysis (HCA) was also performed using R software (version 3.0.3). Subsequently, an independent permutation test was used to prevent the excessive fitting of the PLS-DA model.

2.4. UPLC-TOF-MS/MS for Metabolomics. *2.4.1. Extraction Procedure.* A leaf sample extraction was prepared as previously reported, with little modification.[30,31] The frozen sample was homogenized to a fine powder with liquid nitrogen. A total of 100 mg of the subsample was extracted with 1000 μL of chilled methanol (−20 °C). Then, 500 μL of chloroform and 1000 μL of ddH_2O were added. The mixture was vortexed for 1 min and centrifuged for 15 min at 4000 rpm. Finally, the supernatant was passed through a 0.2 μm filter membrane and used for LC-MS.

2.4.2. LC-MS Analysis. A total of 4 μL of the sample was injected into an Acquity Ultra Performance LC system (Waters). The autosampler temperature was kept at 4 °C. Aqueous formic acid (0.1%) and formic acid (0.1%) in acetonitrile were used as the mobile phase. The mass spectrometer was equipped using an electrospray ion (ESI) source with a capillary voltage of 4000 V, a source temperature of 120 °C, and a desolvation temperature of 300 °C. The mass spectra were recorded using full-scan monitoring mode with a mass scan range of 50−500 *m*/*z*.

2.4.3. Data Preprocessing. First, unprocessed MS files obtained from soft ProteinWizard were converted into the mzXML format. Then, the metabolic feature detection, chromatographic matching, and the alignment of all of the metabolite peaks in the LC/MS data were handled by the XCMS software. Finally, all of the peak areas were normalized and used for statistical analysis.

2.4.4. Statistical and Multivariate Analyses. Multivariate statistical analyses including PCA, PLS-DA, and HCA were performed using SIMCA-P 13.0. Variables with a VIP > 1 and P < 0.05 were consider as significantly different.

The MS/MS spectra were the key to identify the metabolites by comparison to authentic samples and data, and the databases used for the annotations were the Human Metabolome Database (HMDB), Metlin, and LipidMaps.

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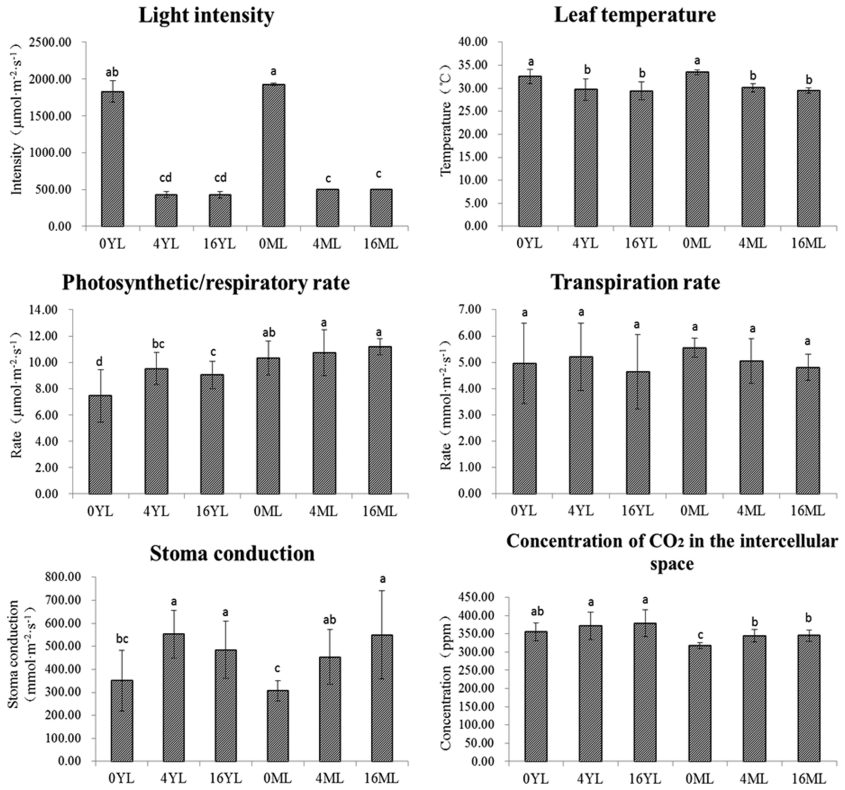

Figure 1. Analysis of the photosynthesis indexes in the leaves of the two tea cultivars under different shading periods. In each histogram, from left to right, the YL leaves when unshaded (0YL), the YL leaves after 4 days of shading (4YL), the YL leaves after 16 days of shading (16YL), the MTZ leaves when unshaded (0ML), the MTZ leaves after 4 days of shading (4ML), and the MTZ leaves after 16 days of shading (16ML) are shown.

Table 1. Contents of Chlorophyll and Carotenoid in the Shoots and Leaves of the Two Tea Cultivars under Different Shading Periods.[a]

	shading periods (day)	chlorophyll *A* (mg/g)	chlorophyll *B* (mg/g)	chlorophyll *A* + chlorophyll *B* (mg/g)	chlorophyll *A*/ chlorophyll B	carotenoid (mg/g)
YL (leaves)	0	1.36 ± 0.07ab	0.27 ± 0.02a	1.63 ± 0.05ab	5.04 ± 0.57ab	0.47 ± 0.02ab
	4	1.38 ± 0.03ab	0.24 ± 0.03a	1.62 ± 0.03ab	5.69 ± 0.76ab	0.44 ± 0.01abc
	16	1.68 ± 0.20a	0.27 ± 0.06a	1.95 ± 0.15a	6.21 ± 0.82a	0.53 ± 0.04a
MTZ (leaves)	0	1.10 ± 0.13b	0.22 ± 0.02a	1.32 ± 0.09b	4.92 ± 0.16ab	0.38 ± 0.04bc
	4	1.29 ± 0.30b	0.29 ± 0.06a	1.58 ± 0.22ab	4.44 ± 0.76b	0.37 ± 0.09c
	16	1.21 ± 0.22b	0.22 ± 0.07a	1.43 ± 0.16b	5.41 ± 1.86ab	0.37 ± 0.05c
YL (shoots)	0	0.85 ± 0.10b	0.11 ± 0.02b	0.96 ± 0.07b	7.76 ± 2.21a	0.38 ± 0.06a
	4	1.02 ± 0.06a	0.21 ± 0.01a	1.23 ± 0.04a	4.80 ± 0.25bc	0.39 ± 0.01a
	16	1.10 ± 0.07a	0.19 ± 0.08a	1.29 ± 0.08a	5.71 ± 0.37ab	0.43 ± 0.02a
MTZ (shoots)	0	0.57 ± 0.07d	0.11 ± 0.02b	0.68 ± 0.05c	5.01 ± 0.50abc	0.27 ± 0.01bc
	4	0.70 ± 0.06c	0.17 ± 0.01ab	0.87 ± 0.04b	4.00 ± 0.57bc	0.31 ± 0.04b
	16	0.61 ± 0.01cd	0.19 ± 0.06a	0.80 ± 0.04bc	3.13 ± 1.03c	0.24 ± 0.02c

[a]Correlations were determined by the least significant difference (LSD) analysis.

3. RESULTS

3.1. Analysis of Photosynthesis Indexes in the Leaves of the Two Tea Cultivars. To evaluate the phenotype changes in the two cultivars under shading treatments, we created photosynthesis indexes using the TPS-2 portable photosynthesis system (Figure 1). The results showed that the relative light intensity was significantly reduced in the leaves of the YL (4YL and 16YL) and MTZ (4ML and 16ML) cultivars, and the shading reached approximately 75%. The leaf temperature was gradually decreased in the leaves from YL and MTZ from 0 to 16 days of shading. The photosynthetic/respiratory rate of MTZ was significantly higher than that of YL. The highest photosynthetic/respiratory rate for YL and MTZ appeared in the leaves from the 4YL and 16ML treatments, respectively. The lowest transpiration rate was detected in the leaves under 16YL and 16ML. The stomatal conduction in the leaves of both tea cultivars was increased under shading, and the highest stoma conduction for YL and MTZ appeared in the leaves from the 4YL and 16ML treatments, respectively. The CO_2 concentration in the intercellular space of YL was significantly higher than that of MTZ. Thus, the results confirmed that the shading treatment greatly reduced the light intensity and leaf temperature in the leaves, thereby regulating photosynthesis and respiration in both tea cultivars.

3.2. Determination of Chlorophyll and Carotenoids in the Leaves and Shoots of the Two Tea Cultivars. As shown

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Table 2. Contents of Tea Polyphenols, Amino Acids, and C and N in the Shoots and Leaves of the Two Cultivars under Shading Periods[a]

		shoot			leaf		
cultivars	shading periods (day)	tea polyphenols (mg/g)	amino acids (mg/g)	tea polyphenols/ amino acids	C contents (mg/g)	N contents (mg/g)	C/N
YL	0	301.7 ± 0.24a	32.8 ± 0.04a	9.20 ± 0.19d	312.6 ± 1.37c	30.7 ± 0.06ab	10.18 ± 0.47b
	4	297.8 ± 0.04b	28.5 ± 0.05b	10.45 ± 0.16c	352.2 ± 1.66ab	28.7 ± 0.34b	12.26 ± 2.06a
	16	296.0 ± 0.07b	20.7 ± 0.03e	14.29 ± 0.24a	334.0 ± 0.51bc	28.6 ± 0.06b	11.69 ± 0.36ab
MTZ	0	261.6 ± 0.27c	25.2 ± 0.02c	10.40 ± 0.05c	364.1 ± 1.68a	33.4 ± 0.14a	10.91 ± 0.49ab
	4	235.3 ± 0.32d	22.2 ± 0.02d	10.61 ± 0.22c	351.1 ± 1.67ab	31.2 ± 0.21ab	11.27 ± 0.89ab
	16	222.3 ± 0.08e	16.8 ± 0.04f	13.24 ± 0.30b	338.0 ± 0.79b	28.6 ± 0.19b	11.81 ± 0.52ab

[a]Correlations were determined by LSD analysis.

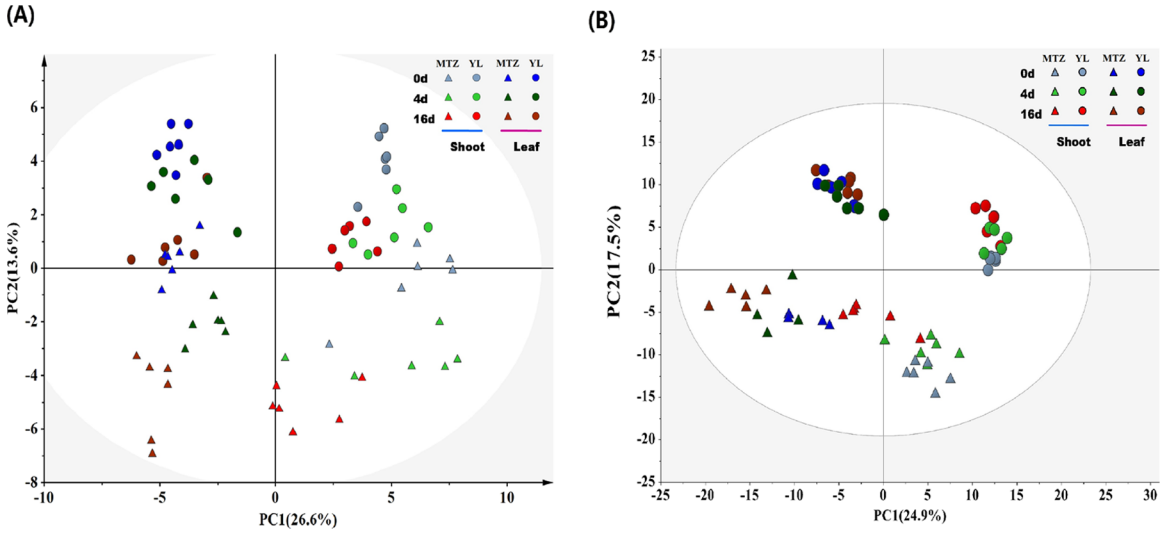

Figure 2. General clustering patterns between the leaves and shoots of the two cultivars with the shading period. (A) PCA score plot for 12 samples subjected to GC-MS-based metabolite analysis; quality parameters: $R^2X = 0.772$, $Q^2 = 0.569$. (B) PCA score plot for 12 samples subjected to UPLC-TOF-MS/MS-based metabolite analysis (positive ions); quality parameters: $R^2X = 0.95$, $Q^2 = 0.743$.

in Table 1, the chlorophyll *A* contents in the YL leaves and shoots were higher than those of MTZ. The chlorophyll *B* contents in the YL and MTZ shoots clearly increased with the shading conditions, whereas the chlorophyll *B* content of the leaves showed no obvious differences. Moreover, the total chlorophyll *A* and *B* contents were increased with the shading periods. The highest total chlorophyll *A* and B contents appeared in the leaves and shoots of YL after 16 days of shading, while the highest total chlorophyll *A* and B contents appeared in the MTZ leaves and shoots after 4 days of shading. Furthermore, the leaves under shading had higher chlorophyll *A* to chlorophyll *B* ratios (except for the 4ML), but the shoots under shading had lower chlorophyll *A* to chlorophyll *B* ratios (especially for the 4YS). The carotenoid analysis demonstrated that the carotenoid content was gradually increased in the YL and MTZ shoots with the shading periods (except for the 16MS). The highest carotenoid content was observed in the YL leaves under shading (16 days, 0.53 ± 0.04 mg/g), whereas there were no obvious differences in the carotenoid contents of the MTZ leaves. Thus, the shading treatment might influence the differential accumulation of pigments, including the chlorophyll and carotenoids in the leaves and shoots of the two cultivars.

3.3. Comparison of Tea Polyphenols, Amino Acids, and Carbon and Nitrogen Contents between the Two Tea Cultivars. As shown in Table 2, the tea polyphenol and amino acid contents in the YL and MTZ shoots were gradually decreased, while the ratios of tea polyphenols to amino acids gradually increased with the different shading periods. The highest ratios of tea polyphenols to amino acids in the YL and MTZ shoots under shading (16 days) were 14.29 ± 0.24 and 13.24 ± 0.30, respectively. The shading treatments reduced the nitrogen contents in both YL and MTZ, and the higher nitrogen levels appeared in 0YL and 0ML at 30.7 ± 0.06 and 33.4 ± 0.14 mg/g, respectively. The analysis of carbon contents between the YL and MTZ leaves showed that the carbon content was higher in 4YL, at 352.2 ± 1.66 mg/g, while the higher carbon level appeared in 0ML, at 364.1 ± 1.68 mg/g. However, the carbon-to-nitrogen ratio in the leaves of the two varieties under the shading treatments (4 and 16 days) was higher than it was in the control (0 days). Altogether, our results demonstrated that the tea polyphenol and amino acid contents in the YL shoots were higher than those of MTZ, whereas the carbon and nitrogen contents in the YL leaves were lower than those of MTZ after the shading treatment.

3.4. Multivariate Analysis of the Extracts from the Leaves and Shoots of the Two Tea Cultivars. To visualize the general clustering pattern and demonstrate the additional differences between the leaves and shoots of the two cultivars over different time periods, we performed a principal component analysis (PCA) and a partial least-squares discrimination analysis (PLS-DA) (Figure 2A,B). The results showed that 72 samples from the two tea cultivars were divided

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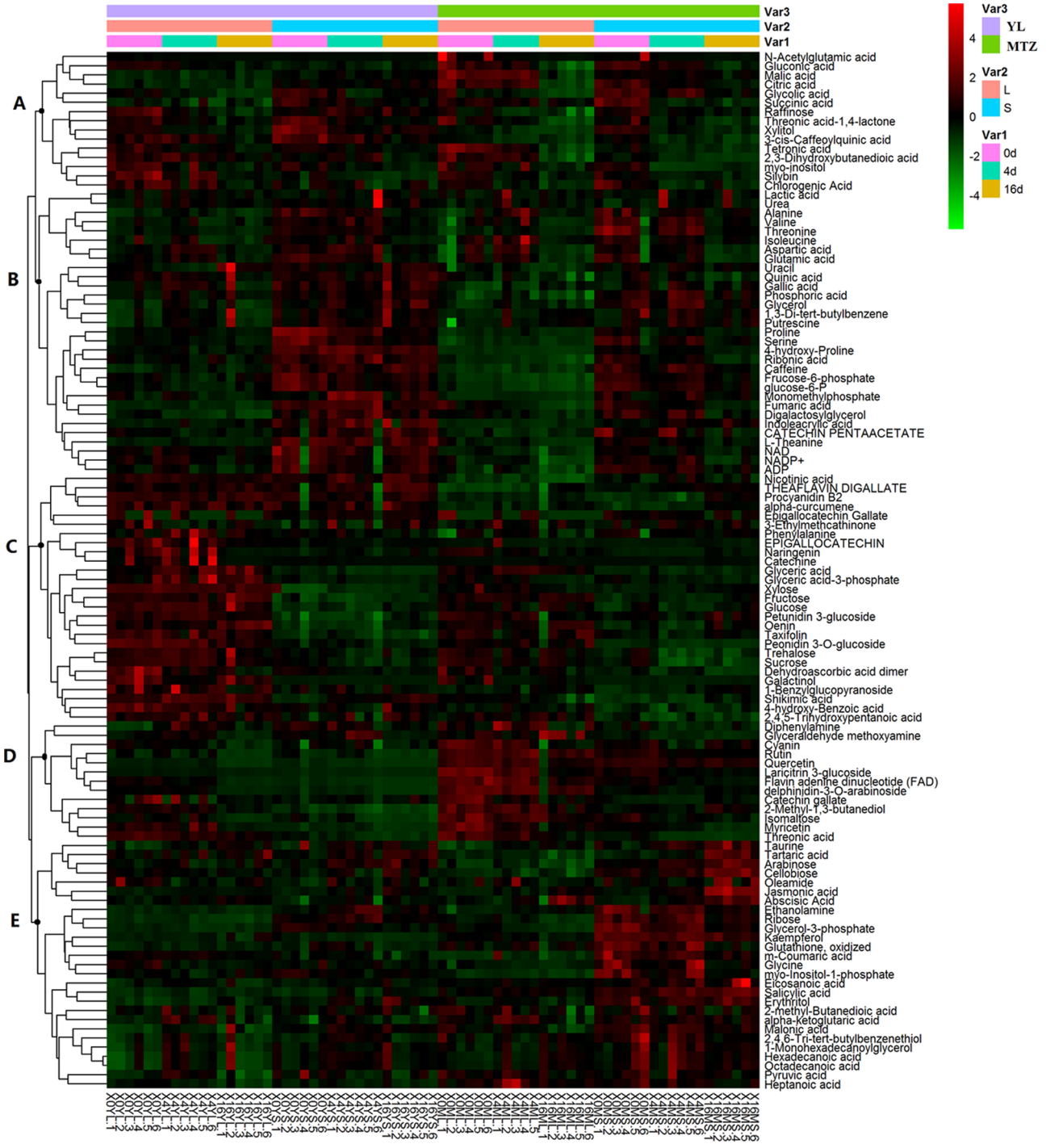

Figure 3. Hierarchical clustering of 113 metabolites identified from the leaves and shoots of the two tea cultivars with different shading periods. In this heat map, the columns and rows represent different samples and individual metabolites, respectively. From left to right, the YL leaves when unshaded (0YL), the YL leaves after 4 days of shading (4YL), the YL leaves after 16 days of shading (16YL), the YL shoots when unshaded (0YS), the YL shoots after 4 days of shading (4YS), the YL shoots after 16 days of shading (16YS), the MTZ leaves when unshaded (0ML), the MTZ leaves after 4 days of shading (4ML), the MTZ leaves after 16 days of shading (16ML), the MTZ shoots when unshaded (0MS), the MTZ shoots after 4 days of shading (4MS), and the MTZ shoots after 16 days of shading (16MS) are shown. Each sample has six biological replicates (1−6). The metabolite levels changed, and they could be organized into five clusters, named A, B, C, D, and E. Green indicates relatively low and red indicates relatively high intensity.

into 12 groups. The quality parameters from the PCA model for GC-MS and UPLC-TOF-MS/MS were $R^2X = 0.772$, $Q^2 = 0.569$ and $R^2X = 0.95$, $Q^2 = 0.743$, respectively. This PCA model showed a separation trend between the shoots and leaves of the two cultivars. As shown in Figure 2A,B, except the 16MS, the leaves from YL and MTZ were separated to the left side of the PC1 axis, while the leaves and shoots of MTZ were separated to the right side of the PC2 axis. The first principal component (PC1) of the GC-MS and UPLC-TOF-MS/MS accounted for 26.6 and 24.9% of the variation, respectively, and the samples obtained from the two cultivars were separated by the sampling time. The second component (PC2) of the GC-MS and UPLC-

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TOF-MS/MS accounted for 13.6 and 17.5% of the variance, respectively, and the samples across the two tea cultivars were separated by the sampling time.

To identify the different metabolites between the shoots and leaves of the two tea cultivars under shading, the data from the GC-MS and UPLC-TOF-MS/MS analyses were used to construct a model in a similar way to that of PCA but in combination with a discrimination analysis for all of the samples (Figures S1 and S2). The first principal component (PC1) of GC-MS and UPLC-TOF-MS/MS accounted for 26.6 and 24.9% of the variation, respectively. The second component (PC2) of the GC-MS and UPLC-TOF-MS/MS accounted for 13.5 and 17.5% of the variance, respectively.

3.5. Identification of Metabolites by GC-MS and UPLC-TOF-MS/MS. To identify the metabolic differences between the two tea cultivars under shading, we detected the metabolites in the leaves and shoots using GC-MS and UPLC-TOF-MS/MS. A total of 113 metabolites were identified with 78 metabolites by GC-MS and 35 metabolites by UPLC-TOF-MS/MS (Supporting Information Table S1). All of the metabolites identified by GC-MS and UPLC-TOF-MS/MS could be classified into several classes of chemicals, including 32.74% organic acids (37 metabolites), 17.70% flavonoids (20 metabolites), 11.50% amino acids (13 metabolites), 10.62% sugars (12 metabolites), 9.73% polyols (11 metabolites), 4.2% nucleotides (5 metabolites), 4.2% amines (5 metabolites), 2.65% fatty acids (3 metabolites), and 6.19% other components (7 metabolites). The abundance of these identified metabolites in the leaves and shoots of YL and MTZ varied significantly during at least one shading time point.

3.6. Hierarchical Clustering Analysis (HCA) of Metabolites. For the possible differences in the metabolisms among these two tea cultivars to be investigated, a total of 113 metabolites identified by GC-MS and UPLC-TOF-MS/MS were organized and visualized with the HCA using the Euclidean distance coefficient and the average linkage method.[32,33] As shown in Figure 3, the clustering presented a good separation of the metabolites between the leaves and shoots from YL and MTZ. Across the metabolite patterns, the compounds could be grouped into five clusters, which were named A (15 metabolites), B (31 metabolites), C (27 metabolites), D (13 metabolites), and E (27 metabolites). During the 16-day shading treatment, the metabolites in cluster A contained lower levels of compounds in the MTZ leaves and shoots than those of the YL; *N*-acetylglutamic acid, tetronic acid, citric acid, gluconic acid, and chlorogenic acid were included. Cluster B consisted of compounds such as fumaric acid, glucose-6-phosphate, $NADP^+$, and L-theanine at lower levels in the MTZ leaves and shoots than in the YL shoots and leaves under shading conditions. Cluster C contained compounds with higher levels in the YL and MTZ shoots under shading periods (4 and 6 days). It contained 27 metabolites, such as xylose, shikimic acid, α-curcumene, and 3-ethylmethcathinone. Cluster D contained compounds (rutin, quercetin, and delphinidin-3-*o*-arabinoside) that were present at lower levels in the leaves and shoots of YL than those of MTZ. Cluster E contained compounds such as tartaric acid, arabinose, abscisic acid, and taurine contents that were more abundant in the shoots of both YL and MTZ than in the leaves under the shading treatments.

3.7. Different Metabolites in Tea Leaves and Shoots with Different Shading Periods. To study the effects of shading on the metabolic profiling in the shoots and leaves of the two tea cultivars, we chose the different metabolites according to the fold changes (FCs) calculated as the $\log_2$ values (FC $\geq$ 0.5 or ≤ -0.5) with significant differences ($P < 0.05$) between the samples (Supporting Information Table S1). For the YL leaves, a total of 61 metabolites were differentially accumulated, including 39 metabolites of lower abundance and 22 metabolites of higher abundance. In particular, the contents of catechin, flavin adenine dinucleotide (FAD), and quercetin showed −2-fold decreases in 16YL compared to those in 0YL, whereas the catechin, naringenin, and quercetin contents showed −2 to −4-fold decreases in 16YL compared to those in 4YL. For the YL shoots, a total of 56 metabolites showed different accumulation levels, including 33 metabolites that showed lower abundance and 23 metabolites that showed higher abundance. The lactic acid and monomethyl phosphate in 4Y, as well as the oleamide and jasmonic acid in 16YS, showed more abundance with more than a 1-fold increase compared to those in 0YS.

In addition, a total of 88 metabolites (23 high and 65 low) and 66 metabolites (17 high and 49 low) showed different accumulation patterns in the MTZ leaves and shoots. Most of the identified metabolites, such as xylose, malonic acid, NAD, xylitol, ribonic acid, raffinose, and myo-inositol, showed lower abundance, while three metabolites, theaflavin digallate, putrescine, and abscisic acid, showed higher accumulations in 16ML than in 0ML. In a 16MS/0MS comparison, 10 metabolites, such as glucose-6-phosphate, glycolic acid, fructose-6-phosphate, and ethanolamine, showed lower abundance, and 4 metabolites, eicosanoic acid, arabinose, 1-benzylglucopyranoside, and taurine, were more abundant in 16MS compared to that in 0MS. Taken together, these results indicated that the differential accumulation of these metabolites could be caused by the shading treatments.

3.8. Different Metabolites in the Leaves and Shoots of Two Cultivars with Corresponding Shading Periods. To study the metabolite changes in the leaves and shoots of the two tea cultivars with the corresponding shading periods, we also chose the different metabolites according to their fold changes (Supporting Information Table S2). A total of 50 (21 low and 29 high), 48 (18 low and 30 high), and 58 (15 low and 43 high) metabolites showed differential accumulations in the 0YL/0ML, 4YL/4ML, and 16YL/16ML comparisons, respectively. In addition, a total of 44 (29 low and 15 high), 40 (17 low and 23 high), and 45 (20 low and 25 high) metabolites exhibited different accumulation patterns in the 0YS/0MS, 4YS/4MS, and 16YS/16MS comparisons, respectively. In particular, the levels of delphinidin-3-*o*-arabinoside, oxidized glutathione, quercetin, rutin, flavin adenine dinucleotide (FAD), salicylic acid, and laricitrin 3-glucoside showed significantly lower accumulation, while the levels of glucose-6-phosphate, nicotinic acid, theaflavin digallate, epigallocatechin, serine, 4-hydroxy-proline, proline, and fructose-6-phosphate showed significantly more abundance in the YL leaves compared to that in the MTZ leaves. In addition, 11 metabolites, such as delphinidin-3-*o*-arabinoside, petunidin 3-glucoside, flavin adenine dinucleotide (FAD), and kaempferol, showed lower abundance, and 9 metabolites, such as epigallocatechin, L-theanine, proline and peonidin 3-*o*-glucoside, showed higher abundance in YL shoots compared to that in MTZ shoots.

4. DISCUSSION

4.1. Shading Periods Dramatically Affected the Photosynthesis and Biochemical Compositions in Different Tea Cultivars. The light intensity and temperature are two major environmental factors that play significant roles in plant

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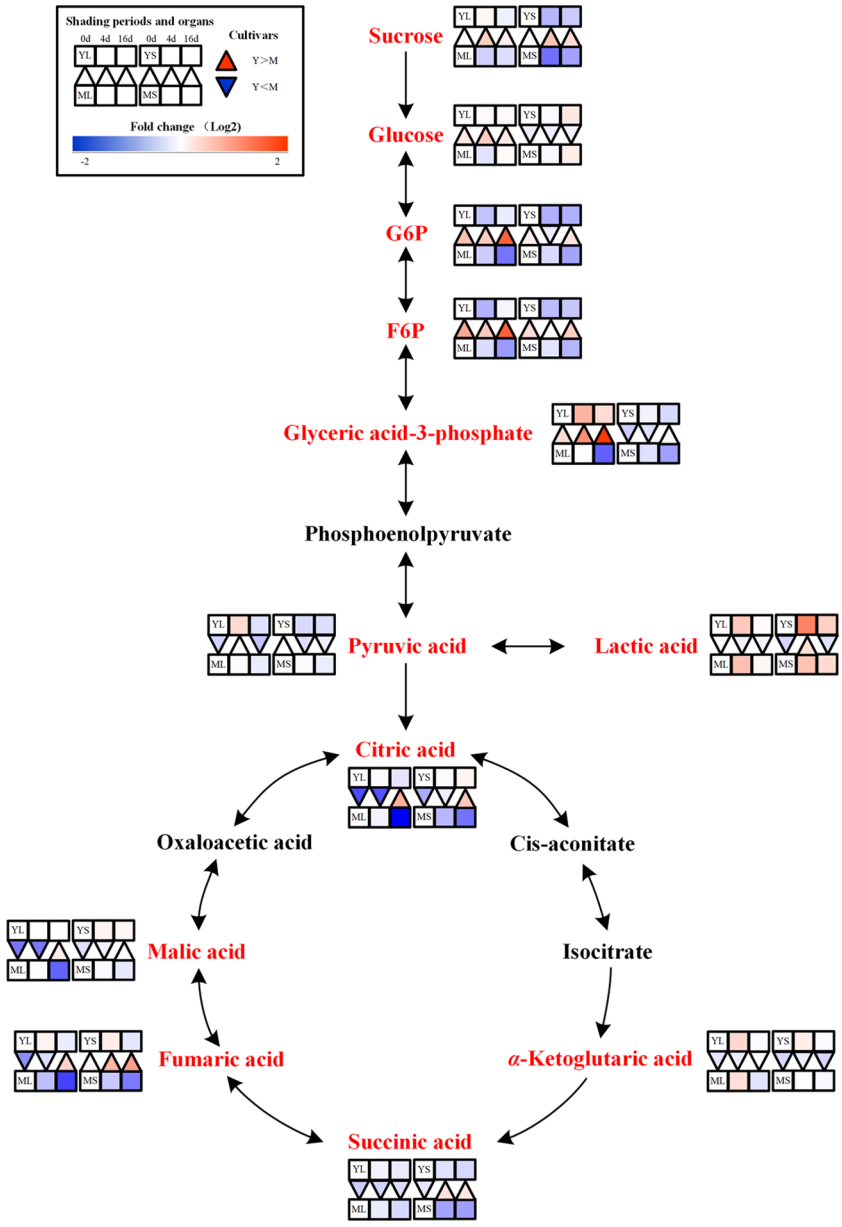

Figure 4. Metabolite differences during energy metabolism in the two tea cultivars under different shading periods. The identified metabolites are marked with a red color. In the legend, the squares at two lines express the different cultivars, YL and MTZ. The squares at six columns express the different shading periods. The squares in the left part represent the leaves, and the right part represents the shoots. Blue indicates relatively low and red indicates relatively high intensity. The red triangle indicates the relatively higher intensity of metabolites in YL (Y) than in MTZ (M), and the inverted blue triangle indicates the relatively lower intensity of metabolites in YL (Y) than in MTZ (M). The degree of change was described with the depth of color, and the depth of the colors is based on the $\log_2$-fold change value between the samples. G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; YL: leaf of "Yulv"; YS: shoot of "Yulv"; ML: leaf of "Maotouzhong"; MS: shoot of "Maotouzhong".

growth and development. In addition to plant growth and development, light manipulation is also important for regulating the nutritional and sensory qualities of tea. In this study, we measured the leaf temperature and the light intensity in the leaves. The results showed that the shading treatment greatly reduced the light level intensity and the leaf temperature in the shaded leaves for both YL and MTZ. Similarly, Pallas et al.[34] demonstrated that the leaf temperature was positively correlated with the light intensity. Photosynthesis and respiration play important roles in plant metabolism. We observed that the photosynthetic/respiratory rate was negatively correlated with the transpiration rate in the MTZ leaves, while the highest photosynthetic/respiratory and transpiration rates were observed in 4-day shaded YL leaves, indicating that the shading treatment might differentially regulate the accumulation of photosynthetic products for plant growth. Reductions in photosynthesis decreased the CO_2 diffusion into the leaves and the metabolic potential inhibition for photosynthesis.[35,36] However, our results showed that the CO_2 concentration was slightly increased in shaded YL and MTZ leaves than in unshaded leaves. The stomata control the flow of gases between plants and the atmosphere. In this study, the stomatal conduction was increased in shaded MTZ leaves, while a little variation occurred in shaded YL leaves. Altogether, the photosynthetic/respiratory rate, transpiration rate, and stomatal conduction results showed significant differences between the

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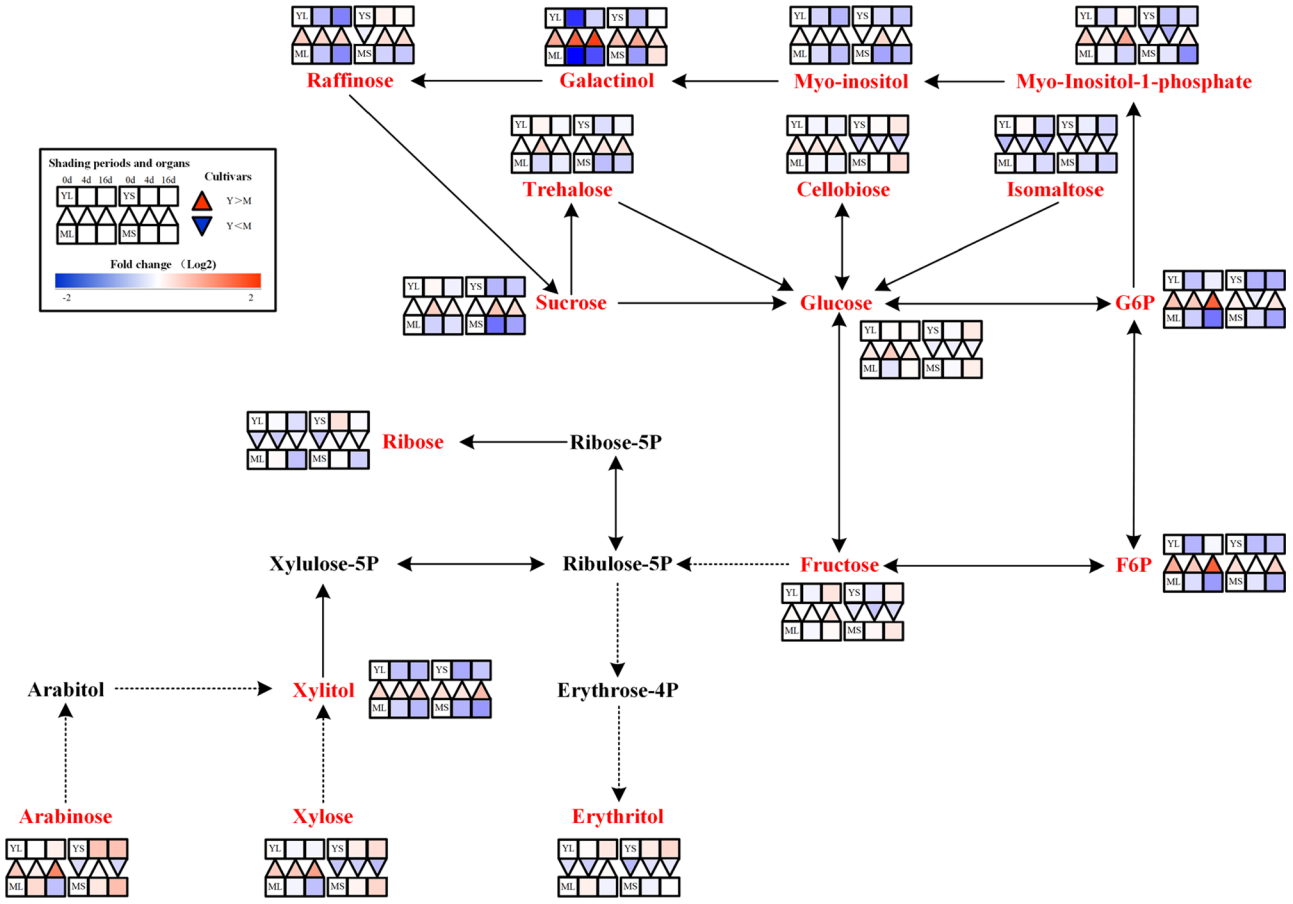

Figure 5. Metabolite differences during polyol metabolism in the two tea cultivars under different shading periods. The identified metabolites are marked with a red color. In the legend, the squares at two lines express the different cultivars, YL and MTZ. The squares at six columns express the different shading periods. The squares in the left part represent the leaves, and the right part represents the shoots. Blue indicates relatively low and red indicates relatively high intensity. The red triangle indicates the relatively higher intensity of metabolites in YL (Y) than in MTZ (M), and the inverted blue triangle indicates the relatively lower intensity of metabolites in YL (Y) than in MTZ (M). The degree of change was described with the depth of color, and the depth of the colors is based on the $\log_2$-fold change value between the samples. YL: leaf of "Yulv"; YS: shoot of "Yulv"; ML: leaf of "Maotouzhong"; and MS: shoot of "Maotouzhong".

two cultivars, suggesting that the shading treatment might play important roles in the leaves of the YL cultivar because it had low carbon and nitrogen contents.

The levels of chlorophyll and carotenoids, which are the major pigments that influence the leaf color, are involved in light harvesting and are indispensable for photo protection against excess light.[37] The reduced light intensity significantly increased the chlorophyll content in *Schefflera arboricola*.[38] In the tea plant, Liu et al.[18] also found that shading significantly enhanced the accumulation of chlorophyll. Li et al.[39] reported that the carotenoid content increased in the leaves of yellowish tea plants compared to that in purplish and green leaves. In this study, we found that the total chlorophyll *A* and chlorophyll *B* contents increased in the leaves and shoots of YL and MTZ under different shading periods. The carotenoid content was increased only in the YL leaves and shoots, indicating that the shading treatment might differentially regulate the accumulation of carotenoid pigments in the MTZ cultivar, which consisted of higher carbon and nitrogen levels.

Extensive study has indicated that high light intensity regulates the expression of the structural genes associated with the biosynthesis of flavonoids and the activity of several important enzymes, which lead to increase contents of secondary metabolites including anthocyanin, catechins, and flavanols.[40−42] Recently, Liu et al.[18] also found that shading tea plants significantly decreased the catechins, epicatechin, epigallocatechin, and epicatechin-3-gallate contents in tea buds. Moreover, Sano et al.[21] revealed that the shaded culture showed decreased epicatechin and epigallocatechin contents and increased theanine and caffeine contents. The quality of the tea is dependent on the chemical components in young shoots. Therefore, in this study, we measured the tea polyphenol and amino acid contents between the shoots of the two cultivars. The results revealed that the tea polyphenols and amino acids gradually decreased in both YL and MTZ when they were exposed to shading. Thus, the findings indicated that the control of the light intensity alters the composition of tea polyphenols and amino acids in different tea plant species.

4.2. Differences in the Metabolic Pathways of the Two Tea Cultivars under Different Shading Periods. Carbon-based compounds include soluble sugar and starch (photosynthetic products) that are used as a substrate to produce tea polyphenols (flavonoids) through the shikimic acid pathway.[43] Caffeine and amino acids are the primary nitrogenous compounds; caffeine is synthesized by the pentose phosphate pathway, and amino acids are produced by glycolysis, the tricarboxylic acid cycle, and the oxidative pentose phosphate pathway.[39] Thus, the carbon and nitrogen metabolisms are

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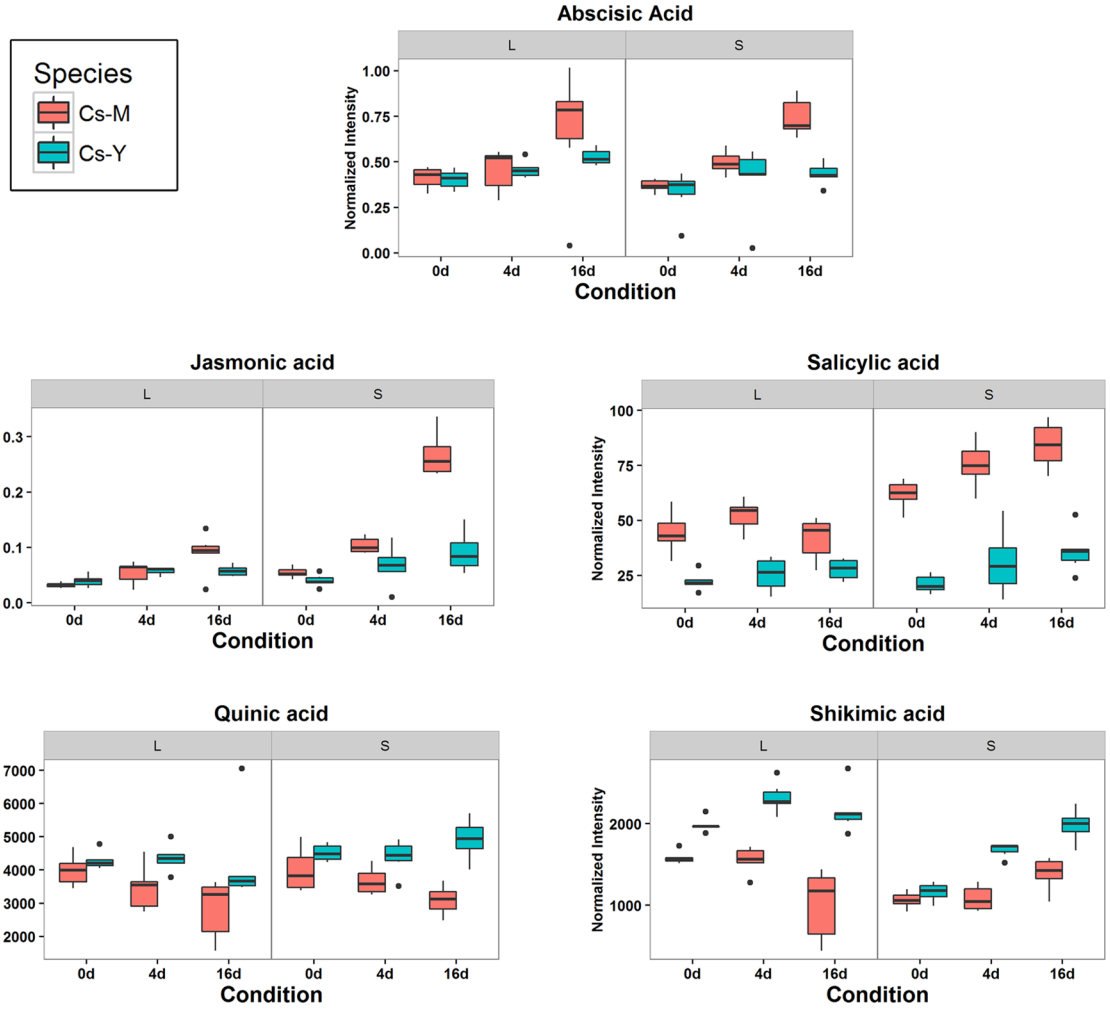

Figure 6. Differences during organic acid metabolism in the two tea varieties under different shading periods. The box plot shows the metabolite intensity, the red box represents MTZ, and the green box represents YL. The left parts represent the leaves (L), and the right parts represent the shoots (S).

closely integrated and regulated.[44] To analyze the different metabolites between the two tea cultivars, we proposed different metabolic pathways in reference to the KEGG database and other literature.[45−49]

4.2.1. Energy Metabolism. In plants, glycolysis and the tricarboxylic acid cycle (TCA cycle) provide not only energy and cofactors but also substrates for metabolite synthesis or signals for feedback. Interestingly, in this study, we found that there were obvious differences in the abundances of metabolites associated with energy metabolism (Figure 4). During glycolysis, sucrose, glucose, glucose-6-phosphate, fructose-6-phosphate, glyceric acid-3-phosphate, and pyruvic acid were identified. In the TCA cycle, citric acid, α-ketoglutaric acid, succinic acid, fumaric acid, and malic acid were identified. Sucrose and glucose are regarded as the primary substrates, and other metabolites are known as intermediate products in glycolysis. We found that the intermediate products in MTZ under shading treatments had lower intensities. However, there were no obvious rules for the metabolites in glycolysis and the TCA cycle in YL. The metabolites displayed changes similar to the intermediate products in glycolysis, except for α-ketoglutaric acid. α-Ketoglutaric acid is the precursor for glutamic acid synthesis, and it might be the reason for the special change in α-ketoglutaric acid in the TCA cycle of MTZ. Similar to our result, two studies have shown that in coffee fruits, shade led to a significant reduction in the sucrose content.[50,51] In contrast to our study, Geromel et al.[50] reported that shade led to an increase in the contents of reducing sugars (glucose and fructose) in coffee fruits. Under natural conditions, the tea leaves were shown to have higher contents of metabolites, such as glucose and fructose derived from the hydrolysis of sucrose, which generally act as osmoprotectants and are involved in the glycolytic pathway.[31] The important roles of these sugars are energy and signaling molecules to help plants to cope with stress.[52,53] Rodziewicz et al.[54] showed that the contents of osmoprotectants, such as sugar alcohols, were increased and played a part in adaptive mechanisms to cold stress. Our results indicated that energy metabolism would be reduced in tea plants under shade treatments. This finding might indicate that under shade conditions, the need for energy by tea plants is lower, which causes a reduction in the synthesis of glucose and might cause a feedback mechanism by shifting stored glucose to amino acid metabolism instead of normal carbon metabolism. This shift leads to the accumulation and synthesis of necessary amino acids, which are known to increase the freshness of green tea.

4.2.2. Polyol Metabolism. Carbohydrates are important structural components and ergastic substances for living organisms, and they can also provide a carbon backbone for

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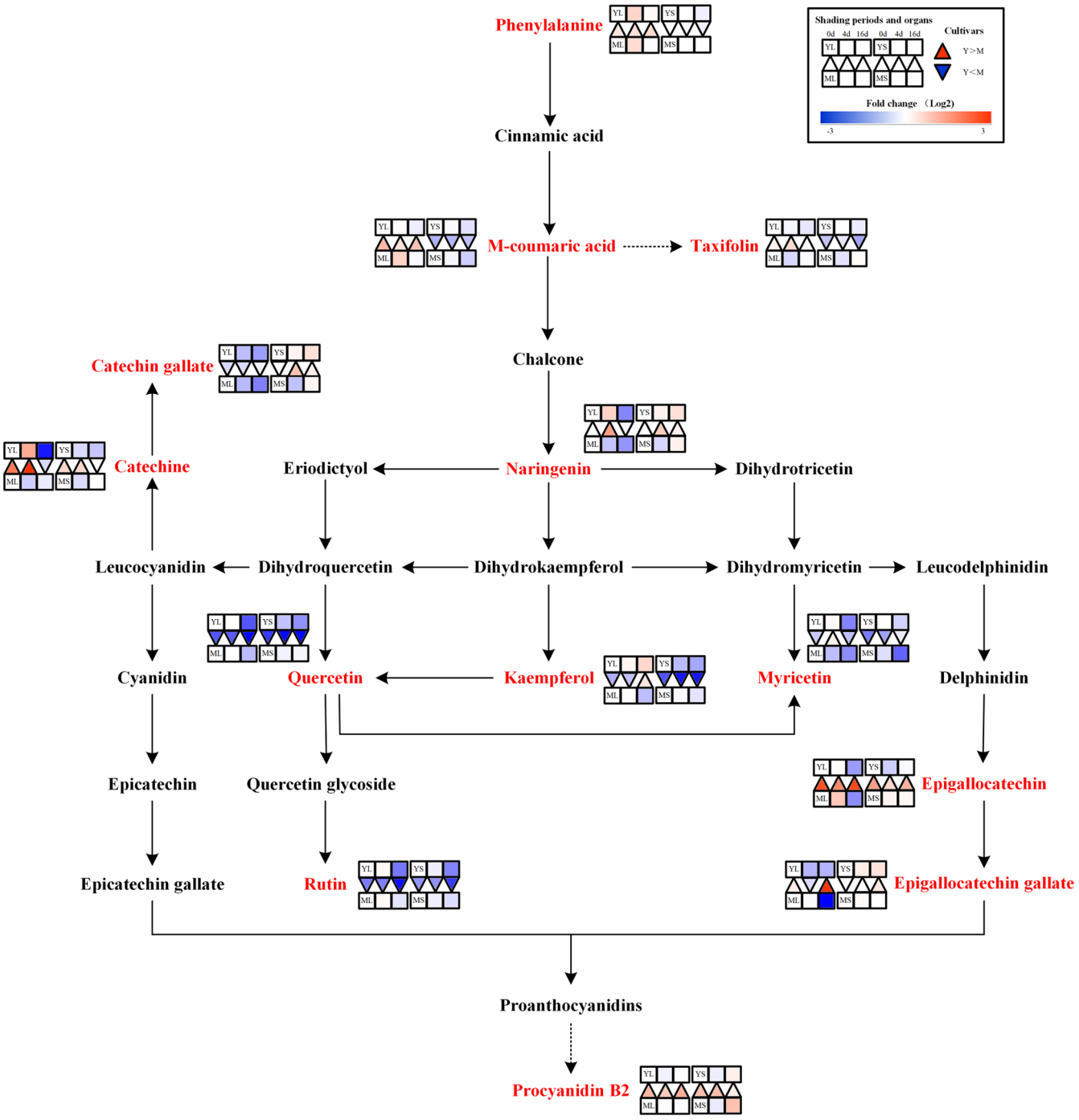

Figure 7. Metabolite differences during flavonoid metabolism in the two tea cultivars under different shading periods. The identified metabolites are marked with a red color. In the legend, the squares at two lines express the different cultivars, YL and MTZ. The squares at six columns express the different shading periods. The squares in the left part represent the leaves, and the right part represents the shoots. Blue indicates relatively low and red indicates relatively high intensity. The red triangle indicates the relatively higher intensity of metabolites in YL (Y) than in MTZ (M), and the inverted blue triangle indicates the relatively lower intensity of metabolites in YL (Y) than in MTZ (M). The degree of change was described with the depth of color, and the depth of the colors is based on the log2-fold change value between the samples. YL: leaf of "Yulv"; YS: shoot of "Yulv"; ML: leaf of "Maotouzhong"; and MS: shoot of "Maotouzhong".

the synthesis of amino acids and nucleotides. Polyol could be regarded as intercellular infiltration, and it could also transfer the carbon backbone and energy within an organism.[55,56] Sucrose, the primary product of photosynthesis, is also the primary form of stored, accumulated, and transported carbohydrates.[57] In addition, sucrose is also a signal molecule in controlling other metabolism pathways.[58] As shown in Figure 5, the shading treatments could reduce the accumulation of the sucrose in the leaves and shoots of MTZ, and the same occurs in the shoots of YL. However, the shorter shading treatment could accelerate the accumulation of the sucrose in the YL leaves. This result is consistent with the photosynthetic indexes. Ribose is an important ingredient in RNA. It has been reported that a high ribose content is related to active metabolism.[59] In Figure 5, the

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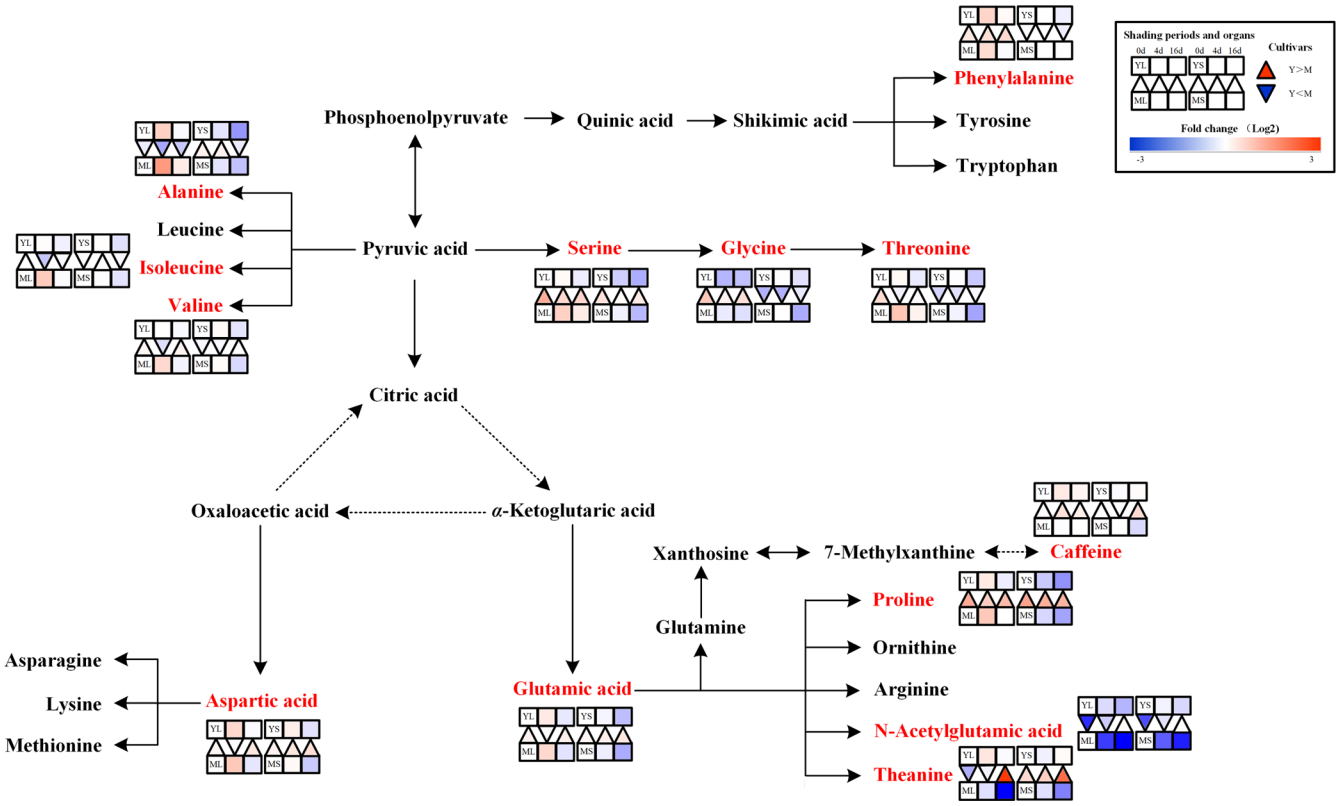

Figure 8. Metabolite differences during nitrogen metabolism in the two tea cultivars under different shading periods. The identified metabolites are marked with a red color. In the legend, the squares at two lines express the different cultivars, YL and MTZ. The squares at six columns express the different shading periods. The squares in the left part represent the leaves, and the right part represents the shoots. Blue indicates relatively low and red indicates relatively high intensity. The red triangle indicates the relatively higher intensity of metabolites in YL (Y) than in MTZ (M), and the inverted blue triangle indicates relatively lower intensity of metabolites in YL (Y) than in MTZ (M). The degree of change was described with the depth of color, and the depth of the colors is based on the log2-fold change value between the samples. YL: leaf of "Yulv"; YS: shoot of "Yulv"; ML: leaf of "Maotouzhong"; and MS: shoot of "Maotouzhong".

ribose intensities of YL and MTZ under 4-day shading treatments displayed no significant changes compared to those of the control, but the ribose intensities of the two tea varieties under the 16-day shading treatments displayed clear down-regulation. However, the YL shoots showed significant upregulation when the plants were shaded for 4 days. This result indicated that YL shoots under 4 days of shading treatment had a more active metabolism. In summary, it could be inferred that 4-day shading treatment seems to be advantageous for the growth of YL.

4.2.3. Organic Acid Metabolism. We identified 36 acids in this study and selected several organic acids for further analysis (Figure 6). Abscisic acid and jasmonic acid have similar structures and similar physiological functions. The shading treatments could induce intense abscisic and jasmonic acid upregulation, and a change in their concentrations in MTZ, especially under 16-day shading treatment, is obvious. Abscisic acid could enhance the stress resistance to low light intensity in plants,[60] so it could be inferred that the longer shading treatment might induce the resistance reaction of MTZ to the low light intensity. The shading treatments could also increase the intensity of salicylic acid expression in the tea shoots, especially in the MTZ shoots. In addition, quinic acid and shikimic acid are the keys to concatenation between primary metabolism and the benzene propane metabolic pathway.[61] The shikimic acid pathway, benzene propane metabolic pathway, and flavonoid synthesis pathway are the primary biosynthesis pathway of tea polyphenols.[62] Most plant phenolics were synthesized through the shikimic acid pathway. Through this pathway, soluble carbohydrates are used as basic components to produce phenolic components.[39] As a result, the changes in the quinic acid and shikimic acid intensities could cause changes in the secondary metabolism. In summary, organic acids have important roles in the growth and development of tea varieties under shading treatments.

4.2.4. Flavonoid Metabolism. It is understood that flavonoids are carbon-based secondary metabolites, and flavonoids have important roles in determining tea qualities.[12] As shown in Figure 7, the shading treatments reduced the concentrations of most of the flavonoids, and this result is similar to that of previous studies.[24,63,64] In addition, the chlorophyll *A* concentration increased along with the increase in epicatechin and epigallocatechin and the decline of catechin, suggesting that chlorophyll could play a crucial role in regulating individual catechins.[65] It is worth noting that the epigallocatechin changes in the flavonoid metabolism and the chlorophyll *A* in the previous section of our study follow this rule. In addition, the flavonoid metabolism is reportedly influenced by the TCA cycle and the biosynthesis of carbohydrates and amino acids.[61] Moreover, the biosynthesis of flavonoids competed with the biosynthesis of lignin.[66,67] Thus, the flavonoid metabolism is controlled in many ways. Moreover, the contents of most of the metabolites in the flavonoid metabolism pathway, including quercetin, myricetin, rutin, epigallocatechin gallate, and catechin

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gallate, were decreased during the shading and therefore we believe that the shading treatments could reduce the intensity of flavonoid metabolism in tea plants.

4.2.5. Nitrogen Metabolism. Nitrogen metabolism includes amino acid and caffeine metabolism. The biosynthesis of amino acids occurs primarily in glycolysis, the TCA cycle, and the oxidative pentose phosphate pathway. A common purine alkaloid, caffeine, is synthesized by the pentose phosphate pathway.[39] In this study, 11 amino acids and caffeine were identified (Figure 8). Nearly all of the amino acids and caffeine have a similar rule of change under shading treatments; the 4-day shading treatments increased the concentrations of amino acids and caffeine in the tea leaves, while the shading treatments reduced their concentrations in the tea shoots. These results indicated that the shading treatments reduced the intensity of the N metabolism in the tea shoots, while the shorter shading treatment was conducive to N metabolism in the tea leaves. This conclusion is different from that of previous studies.[63] In general, the N provision for sustaining growth is contributed by the translocation of soil N from new uptake by the root and the remobilization of the N reserves stored within the plants.[68,69] In addition, low transpiration may decrease the plant N uptake and lead to N deficiency.[70,71] It has been widely recognized that the C-to-N ratio is important, and if there is not enough carbon available, the metabolism of nitrogenous compounds might be inhibited.[72] Thus, this ratio might explain the different conclusions drawn from this study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b05858.

Analysis of differential metabolite accumulations in the leaves and shoots of the two cultivars between different shading periods (Table S1) (XLSX)

Analysis of differential metabolite accumulations in the leaves and shoots between the two cultivars with their corresponding shading time periods (Table S2) (XLSX)

PLS-DA loading plot for 12 samples subjected to GC-MS-based metabolite analysis between the leaves and shoots of the two cultivars with the shading periods (Figure S1); PLS-DA loading plot for 12 samples subjected to UPLC-TOF-MS/MS-based metabolite analysis between the leaves and shoots of the two cultivars with the shading periods (Figure S2) (PDF)

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Author Contributions

‖Y.L. and A.J. contributed equally to this study.

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Notes

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