

Physiological, anatomical, and transcriptomic analyses reveal the potential mechanism of resistance of *Akebia trifoliata* to acid rain stress and mitigation effects of curcumin

Xingmei Tao

Kunming University

Kai Wang

Kunming University

Xiaoxu Bi

Kunming University

Yongfu Zhang

123017360@qq.com

Kunming University

Research Article

Keywords: *Akebia trifoliata*, curcumin, acid tolerance, physiological and biochemical, transcriptome, metabolic pathway

Posted Date: January 2nd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-5734927/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at *Biologia plantarum* on May 29th, 2025.
See the published version at <https://doi.org/10.32615/bp.2025.003>.

Abstract

Acid rain is a global ecological issue severely threatening crop growth. Curcumin (CUR), a natural antioxidant, can enhance the tolerance of plants to abiotic stresses via physiological and molecular modes. As both medicine and food, *Akebia trifoliata* exhibits high economic value. The resistance mechanism of *A. trifoliata* to acid rain and mitigation effects of CUR remain unclear. Therefore, in this study, we investigated the plant growth, physiological characteristics of leaves, anatomical structure, and gene expression of *A. trifoliata* under acid rain stress before and after treatment with exogenous CUR. The results indicated that under acid rain stress, the contents of chlorophyll a, chlorophyll b, total chlorophyll, and starch and thicknesses of upper and lower epidermis of leaves decreased by 58.16%, 77.88%, 64.77%, 63.85%, 58.93%, and 35.57%, respectively. Moreover, MDA, soluble sugar, soluble protein, and proline contents and production rate of oxygen free radicals increased by 82.55%, 43.20%, 44.55%, 64.40% and 345.77%, respectively. This suggested that acid rain stress affected the growth and development of *A. trifoliata*. *A. trifoliata* resisted acid rain stress by increasing SOD and CAT activities; thickness of leaf, palisade tissue, and spongy tissue; and ratio of palisade/spongy tissue. However, exogenous CUR could effectively facilitate plant growth, maintain integrity of anatomical structure of leaf, and relieve the damages to *A. trifoliata* caused by acid rain stress, and 50 $\mu\text{mol/L}$ (CUR50) was the most optimal concentration. Transcriptomic analysis revealed that CUR0 vs Control, CUR50 vs Control, and CUR50 vs CUR0 had 2978, 1760, and 323 DEGs, respectively. KEGG pathway enrichment analysis revealed that these DEGs were involved in eight pathways, among which protein processing in endoplasmic reticulum, plant hormone signal transduction, phenylpropanoid biosynthesis, and starch and sucrose metabolism were the key metabolic pathways via which CUR mitigated the effects of acid rain stress. This study revealed the potential mechanism of response of *A. trifoliata* to acid rain stress and mitigation effects of exogenous CUR via physiological, anatomical, and transcriptomic analyses, thereby providing theoretical references for phytoremediation in the acid rain zone.

1. Introduction

Acid rain is a global ecological issue that has attracted great attention (Xiao et al.,2020). China is the third largest acid rain area in the world after Europe and North America. In China, the area polluted by acid rain has reached 40% of the national land area, with the most prominent region being the south of the Yangtze River and east of Yunnan-Guizhou. This has seriously affected agricultural crop growth and biomass production in China (Ren et al.,2021; Yao et al.,2022). In actual production, crops exposed to acid rain stress exhibit spot necrosis, anatomical structural changes, chlorophyll content reduction, and other symptoms of leaf damage (Polishchuk et al.,2016). Moreover, acid rain leads to starch degradation and increased cell membrane permeability. Plants can regulate the contents of various osmoregulatory substances including soluble sugar, soluble protein, and proline to maintain the cellular osmotic balance, thus reducing stress-induced damage to cell membrane (Zhang et al.,2023). Under acid rain stress, absorption of excessive H^+ by plants can easily disrupt the metabolic balance of ROS, leading to rapid accumulation of free radicals and ultimately causing oxidative stress (Ren et al., 2021). To cope with

acid-rain-induced oxidative stress, plants can regulate the enzymatic antioxidant system including SOD, POD, and CAT to scavenge excessive ROS (Kováčik et al., 2011).

Curcumin (CUR) is a low-molecular-weight polyphenol compound isolated from the Zingiberaceae plants. It can ameliorate various disorders and diseases in human body and has many benefits for human health (Seibel et al., 2021). CUR is a natural antioxidant and has two phenolic sites. Therefore, it can immediately scavenge free radicals, thereby slowing down ROS-induced damage (Zaki et al., 2023). The exogenous application of CUR has been reported to attenuate the damage caused by abiotic stress in *Vitis vinifera* and *Spinacia oleracea*, via specifically activating stress response mechanisms (Li et al., 2024; Zhang et al., 2024).

Akebia trifoliata is an evergreen woody vine of the Lardizabalaceae family, with abundant germplasm resources in Yunnan, Guizhou, and Hunan provinces in China (Luo et al., 2008). The whole plant of *A. trifoliata* can be used as medicine. It can be used for detoxification, diuresis, stabilizing the fetus, relieving cough, regulating menstruation, and treating cancer (Jia et al., 2022). It has been planted on a large scale in many parts of China (Cai et al., 2022). It produces high-quality, third-generation fruits rich in amino acids, vitamin C, and other nutrients with sweet taste and unique flavor. *A. trifoliata* can develop specific survival strategies by changing its morphology and physiological and biochemical characteristics to resist or adapt to severely water-scarce ecological environment (Yu et al., 2022). Therefore, *A. trifoliata* has broad applications, including ecological restoration of plants, and high economical value as a medicine and food.

Currently, studies on *A. trifoliata* mainly focus on the chemical composition, pharmacological activity, nutritional value, resource distribution, DNA fingerprinting, and development of new fruits beneficial for human health (Wu et al., 2018). The tolerance of *A. trifoliata* to acid rain stress and its response to exogenous addition of CUR are unclear. The lack of molecular biology studies and relative scarcity of genetic information of *A. trifoliata* have severely limited its subsequent development and utilization. In this study, we aimed to explore the response of *A. trifoliata* to acid rain stress and role of exogenous CUR in mitigating the deleterious effects of acid rain in *A. trifoliata*. *A. trifoliata* cuttings were used for pot experiments, and acid rain stress was simulated after watering CUR on *A. trifoliata* roots. Alterations in the morphology, anatomical structure, contents of photosynthetic pigments and osmoregulatory substances, and activity of antioxidant enzymes were assessed. The non-parametric transcriptome sequencing analysis and bioinformatic analysis of the sequencing results were performed to explore the mechanism of *A. trifoliata* in response to acid rain stress and the mechanism of action of exogenous CUR. This study provided a theoretical basis for phytoremediation in the acid rain zone.

2. Materials and methods

2.1 Materials and treatments

A. trifoliata cuttings used in this experiment were obtained from the germplasm resource nursery of Dongfang Farm Management Bureau in Mile City, Yunnan Province. CUR was purchased from Shanghai

Macklin Biochemical Technology Co., Ltd. Five treatments were set up: Control (no CUR treatment and acid rain stress) and CUR0, CUR25, CUR50, and CUR100 treated with 0, 25, 50, and 100 $\mu\text{mol/L}$ CUR, respectively, and subjected to acid rain stress. Each treatment included four healthy plants with consistent growth and height of approximately 110 cm. They were planted in individual white pots (length 100 cm, width 30 cm, and depth 25 cm). According to the main rainfall components of acid rain in Southwest China, sulfuric acid and nitric acid (in the molar ratio of $\text{SO}_4^{2-}:\text{NO}_3^- = 5:1$) were used to simulate acid rain (Zhou et al., 2017). A pre-test was conducted before the formal experiment. The pH of the simulated acid rain was set at 2.0. At 7 am, *A. trifoliata* roots were first watered with different concentrations of CUR solution (500 mL) or an equal amount of distilled water for Control and CUR0. Watering was repeated every 2 days. At 6 pm on the same day of watering, the plants were sprayed with simulated acid rain until all leaves had liquid droplets. Control was sprayed with an equal amount of distilled water, and the treatment was stopped after 28 days. For further analysis, the 10th – 15th leaves counted upward from the bottom of the plant were collected at 6 am 3 days later. After collection, leaf segments of 0.6 cm^2 area in the middle were cut along both sides of the main veins and quickly fixed in FAA (90 mL of 70% ethanol + 5 mL of formaldehyde + 5 mL of glacial acetic acid) to maintain the original state of the samples for anatomical structural observation. The other part was immediately used to measure the activities of SOD, POD, and CAT. The remaining samples were wrapped in tinfoil, snap-frozen in liquid nitrogen, and stored at -80°C for the determination of remaining physiological indexes and non-parametric transcriptome sequencing.

2.2 Measurement of growth indicators

The plant height was measured using a tape measure. The stem diameter was measured using a vernier caliper at 2 cm above the ground. The plant was pulled out from the substrate, washed with water, and dried at room temperature. The root was separated from aboveground part using scissors. Fresh weights of the aboveground part and root were weighed, and the root length was measured using a tape measure. The whole aboveground part was dried in an oven at 105°C for 0.5 h and further dried at 85°C until constant weight was obtained, which was noted as the dry weight.

2.3 Measurement of chlorophyll content

A. trifoliata leaves (0.1 g) were cut and placed in 5 mL of 95% ethanol. The extraction was performed in dark until the leaves whitened. The absorbance of the extract was measured at 665 and 649 nm to calculate the contents of chlorophyll a and chlorophyll b, respectively (Zou., 2003).

2.4 Measurement of MDA content and production rate of oxygen free radicals

Leaf sample (1 g) was ground with trichloroacetic acid and centrifuged. To 2 mL of supernatant, thiobarbituric acid solution was added, placed in boiling water bath, cooled, and centrifuged. The

absorbance of the supernatant was measured at 532, 600, and 450 nm. The MDA content was calculated using the method described by Kumar and Knowles (1993).

Leaf sample (1 g) was ground with potassium phosphate buffer and centrifuged. To 0.5 mL of supernatant, hydroxylamine hydrochloride was added and incubated in a water bath. Further, sulfonamide and α -naphthylamine were added for color reaction. After centrifugation, the absorbance was measured at 530 nm. The production rate of oxygen free radicals was calculated according to the method described by Li and Gong (2005).

2.5 Measurement of physiological indexes related to stress tolerance

SOD, POD, and CAT activities were determined using NBT method, guaiacol method, and UV-absorption method, respectively. The contents of soluble sugar, starch, soluble protein, and proline were determined using anthrone colorimetric method, perchloric acid hydrolysis method, Coomassie blue staining method, and indanone colorimetric method, respectively. These indexes were determined as described by Gao (2006).

2.6 Measurement of anatomical parameters of leaves

The fixed leaf samples were dehydrated, turned transparent, soaked in wax, embedded, and further sliced using a KD-2258 rotary slicer (thickness = 8 μ m). The slices were glued, baked, dewaxed, stained with saffron-green, sealed with neutral gum, and observed using an Olympus CX41 optical microscope. Image J was used for data measurement and image acquisition. The thickness of upper epidermis, lower epidermis, leaves, palisade tissue, and spongy tissue was measured. The mean value of each parameter from each slice was considered to represent the final measurement value, and the ratio of palisade/spongy tissue was calculated (Hu et al., 2022).

2.7 Transcriptome sequencing and bioinformatic analysis

2.7.1 Total RNA extraction, library establishment, and sequencing

The total RNA was extracted from 1 g of *A. trifoliata* leaves using the MJZol total RNA extraction kit (Shanghai Major Biomedical Technology Co., Ltd., China). RNA integrity and DNA/protein contamination were analyzed using agarose gel electrophoresis. RNA purity (OD260/OD280 and OD260/OD230 ratios) was measured using ultra-micro spectrophotometer, and RIN value was determined using a biological analyzer. After passing the test, the library establishment and non-parametric transcriptome sequencing were commissioned to Shanghai Major Biomedical Technology Co., Ltd.

2.7.2 Data quality control and de novo assembly

The cDNA library of *A. trifoliata* leaf tissue was subjected to high-throughput sequencing analysis using Illumina Novaseq 6000 sequencing platform. The raw reads obtained from sequencing were subjected

to quality control, which was done by removing low-quality reads, junction contamination, reads with high number of unknown base N, low-quality reads, and reads with all A bases. Therefore, high-quality clean reads were obtained that could be used for subsequent analyses. De novo assembly of the clean reads was performed using Trinity. The assembled transcripts were clustered and subjected to de-redundancy using Tgicl to obtain the unigenes, which were used as the reference sequences for subsequent analyses (Qian et al., 2022). Additionally, the assembly quality of all unigenes was evaluated, including GC content, Q20, and Q30.

2.7.3 Analysis of gene expression

The assembled reference sequences were mapped back to the transcriptome using Bowtie2 (Langmead and Salzberg., 2012). The RSEM and TPM formulae were used to calculate the gene and transcript expression levels in *A. trifoliata* leaf tissues (Li and Dewey., 2011), respectively, obtaining the standard expression levels of FPKM for each unigene. Further, the differential expression analysis was conducted using DESeq2. The DEGs were screened as per the criteria of $FDR < 0.05$ and gene expression fold change $|\log_2FC| \geq 1$ to compare the differences between different treatment groups. KEGG pathway enrichment analysis was performed using the DEGs. Metabolic pathways with corrected $P_{\text{adjust}} < 0.05$ were considered significant pathways.

2.7.4 Data processing and analysis

All experimental data were recorded and organized using Microsoft Excel 2019 and further statistically analyzed using SPSS 27.0. The results were expressed as mean $\pm$ SD. One-way ANOVA and Duncan's multiple comparisons were used to analyze the significance of differences in various indicators between different treatments. $P < 0.05$ was considered significant. The principal component analysis (PCA) of physiological indexes was performed by dimensionality reduction method. Plots were drawn using Origin 2021 and Graphpad Prism9.

3. Results and analysis

3.1 Morphological characteristics of *A. trifoliata* under acid rain stress and CUR treatment

The morphological characteristics of *A. trifoliata* under acid rain stress and CUR treatment were observed on the 28th day of treatment. Compared with Control, the plants in CUR0 exhibited a large number of white–brown spots, chlorosis, and yellowing of leaves; plant wilting; severe reduction in the number of fibrous roots; slow root development. Under CUR treatment, the symptoms related to acid rain stress were alleviated to various degrees (Figs. 1A and B). Compared with CUR0, CUR25 and CUR50 treatments significantly reduced the necrotic spots of *A. trifoliata*, and the root morphology was closer to that of Control. Compared with CUR0, the plant height, stem diameter, fresh and dry weights of aboveground parts, and root length and fresh weight of plants in CUR25 and CUR50 increased by 26.48% and 33.45% ($P < 0.05$, Fig. 1C), 47.40% and 50.65% ($P < 0.05$, Fig. 1D), 25.50% and 28.90% ($P < 0.05$,

Fig. 1E), 41.92% and 58.46% ($P < 0.05$, Fig. 1F), 72.30% and 63.45% ($P < 0.05$, Fig. 1G), and 191.09% and 228.80% ($P < 0.05$, Fig. 1H), respectively. However, in plants in CUR100, the restoration of symptoms related to acid rain stress was significantly reduced, and leaf spot necrosis was significantly increased compared to CUR25 and CUR50. Moreover, the root system was severely distorted (Fig. 1B).

3.2 Physiological and biochemical indexes of *A. trifoliata* leaves under acid rain stress and CUR treatment

Compared with Control, the contents of chlorophyll a, chlorophyll b, total chlorophyll, and starch decreased by 58.16%, 77.88%, 64.77%, and 63.85%, respectively, and contents of soluble sugar, soluble protein, proline, and MDA; production rate of oxygen free radicals; and activities of SOD and CAT increased by 43.20%, 44.55%, 64.40%, 82.55%, 345.77%, 44.20%, and 34.78%, respectively ($P < 0.05$, Figs. 2D–F), in the leaves in CUR0.

Compared with CUR0, the contents of chlorophyll a, chlorophyll b, and total chlorophyll and activities of SOD, POD, and CAT increased by 51.02%, 154.75%, 72.84%, 93.69%, 145.61%, and 93.25%, respectively; the contents of soluble sugar, soluble protein, proline, starch, and MDA and production rate of oxygen free radicals decreased by 30.19%, 55.36%, 88.98%, 57.36%, 18.13%, and 54.18%, respectively ($P < 0.05$, Figs. 2A–L) in CUR25.

Compared with CUR0, the contents of chlorophyll a, chlorophyll b, total chlorophyll, soluble sugar, soluble protein, proline, and starch and activities of SOD, POD, and CAT increased by 71.03%, 175.31%, 92.97%, 93.33%, 108.62%, 198.39%, 142.55%, 130.74%, 276.22%, and 155.02%, respectively ($P < 0.05$, Figs. 2D–G); MDA content and production rate of oxygen free radicals decreased by 40.68% and 60.14%, respectively ($P < 0.05$, Figs. 2A–L) in CUR50.

However, the contents of chlorophyll a, total chlorophyll, soluble sugar, soluble protein, proline, and MDA were not significantly different in the leaves in CUR100 and CUR0 ($P < 0.05$, Figs. 2A, C–F, and H). The contents of chlorophyll b and starch were significantly lower and production rate of oxygen free radicals was significantly higher in CUR100 than in CUR25 and CUR50 ($P < 0.05$, Figs. 2B, G, and I). SOD activity was 24.04% and 36.24% lower and POD activity was 47.22% and 65.54% lower in CUR100 than in CUR25 and CUR50, respectively ($P < 0.05$, Fig. 2J and K).

Overall, acid rain stress accelerated degradation of chlorophyll and starch and caused severe osmotic and oxidative stresses. Under acid rain stress, increased contents of soluble sugar, soluble protein, proline, and other osmoregulatory substances and activities of antioxidant enzymes (e.g., SOD, POD, and CAT) were more conducive to coping with acid rain stress. CUR could effectively slow down the chlorophyll degradation rate, increase contents of osmoregulatory substances and antioxidant enzyme activity to maintain the osmotic balance and reduce the degree of cell membrane lipid peroxidation, and alleviate the damage to *A. trifoliata* due to acid rain stress.

3.3 Comprehensive evaluation of the physiological indexes using principal component analysis

To analyze the differences and correlations between physiological indexes and different concentrations of CUR, PCA was performed. PC1 and PC2 explained 51.00% and 44.30% of the total variance, respectively (Fig. 3). No significant difference was observed between CUR100 and CUR0 as well as between CUR25 and CUR50 ($P > 0.05$, Fig. 3). However, some of the physiological indexes of CUR25 and CUR100 were not significantly different from each other ($P > 0.05$, Fig. 3), whereas CUR50 and CUR100 were significantly separated from each other ($P < 0.05$, Fig. 3). Additionally, the Control group was significantly separated from the CUR groups ($P < 0.05$, Fig. 3). PCA indicated significant positive correlation between activities of SOD, POD, and CAT and soluble sugar content. A significant positive correlation was observed among the contents of starch, chlorophyll a, chlorophyll b, and total chlorophyll content. Production rate of oxygen free radicals and MDA content were significantly positively correlated ($P < 0.05$, Fig. 3). The results revealed that the effect of CUR50 was the most significant.

3.4 Anatomical structural changes in *A. trifoliata* leaves under acid rain stress and CUR treatment

To more comprehensively analyze the changes in *A. trifoliata* leaves under acid rain stress and the mitigation effect of CUR, anatomical structural changes were observed in *A. trifoliata* leaves in Control, CUR0, and CUR50. In the leaves in Control, the epidermis consisted of one layer of tightly arranged, regular shaped flat cells without intercellular spaces, and a distinction was observed between upper and lower epidermis (Fig. 4B). The mesophyll consisted of palisade and spongy tissues. The leaf was a typical bifacial leaf, with lateral veins. Palisade tissues consisted of 1–2 layers of long columnar or ellipsoid cells, which were tightly arranged and perpendicular to the cells of the upper epidermis. Spongy tissue was located under the palisade tissues, immediately adjacent to the lower epidermis; the cells were irregularly shaped, connected with each other to form a mesh, loosely arranged, and similar to sponges in appearance.

Interestingly, the leaf structures of Control, CUR0, and CUR50 significantly differed. Compared with Control, the lateral veins and epidermis of leaves in CUR0 were severely damaged and structurally incomplete; most of the palisade tissues were composed of two layers of long columnar cells arranged tightly, with tightly packed cells and small intercellular spaces (Fig. 4D). In CUR50, the degree of damage to the epidermis and lateral veins of leaves was significantly reduced; however, the palisade tissues were basically composed of two layers of columnar cells in a compact arrangement (Fig. 4F).

Quantitative analysis was performed on leaf epidermis and mesophyll cells. Compared with Control, the thickness of the upper and lower epidermis in CUR0 decreased by 58.93% and 35.57%, respectively; thickness of leaf, palisade tissue, and spongy tissue and the ratio of palisade/spongy tissue increased by 13.75%, 50.75%, 34.58%, and 19.90%, respectively ($P < 0.05$, Figs. 4G–L). Compared with CUR0, the

thickness of upper epidermis and spongy tissue in CUR50 increased by 103.66% and 13.50%, respectively ($P < 0.05$, Figs. 4G and K); however, the lower epidermis thickness was not significantly different ($P > 0.05$, Figs. 4H and I), and palisade tissues thickness and ratio of palisade/spongy tissues decreased by 17.02% and 7.84%, respectively ($P < 0.05$, Fig. 4J and L). No significant difference was observed in the ratio of palisade/spongy tissues between CUR50 and Control ($P > 0.05$, Fig. 4L).

The structural changes in palisade and spongy tissues of *A. trifoliata* leaves may be a resistance or adaptation mechanism to acid rain stress, and the appropriate concentration of CUR could effectively alleviate the damage to epidermis and lateral veins of *A. trifoliata* leaves due to acid rain stress.

3.5 Transcriptome sequencing of CUR-treated *A. trifoliata* leaves

3.5.1 Assessment of transcriptome sequencing data

To investigate the mechanism by which *A. trifoliata* responded to acid rain stress and CUR enhanced its stress resistance at the gene level, non-parametric transcriptome sequencing was performed on *A. trifoliata* leaves of Control, CUR0, and CUR50 with three biological replicates. A total of 9 cDNA libraries were constructed, with a total of 63.10 Gb of clean data and more than 6.52 Gb of clean data per sample (Table 1). For the Control group (Control_1, Control_2, and Control_3), 47,644,476, 44,619,286, and 49,530,660 high-quality clean reads were obtained, respectively. For the CUR0 (CUR0_1, CUR0_2, and CUR0_3) and CUR50 (CUR50_1, CUR50_2, and CUR50_3) groups, 48,197,900, 49,175,852, 45,072,782, 46,214,632, 51,695,644, and 50,944,140 high-quality clean reads were obtained, respectively (Table 1). The average error rate of sequencing bases corresponding to clean data was less than 0.05%; the Q20 and Q30 of each sample exceeded 97.00% and 93.00%, respectively, and the GC content of total bases of clean data in each sample reached $> 44.00\%$ (Table 1). These results indicated that the high-throughput sequencing platform for transcriptome sequencing of *A. trifoliata* could provide high quality and quantity of data, which could be used for the next splicing process.

Table 1
Statistics of transcriptome sequencing data of *A. trifoliata* leaves

Sample	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
Control_1	48548786	47644476	6966674116	0.0256	97.78	93.73	44.52
Control_2	45677648	44619286	6525992624	0.0255	97.80	93.81	44.50
Control_3	50593486	49530660	7189291230	0.0254	97.84	93.92	44.52
CUR0_1	49343926	48197900	6991767400	0.0252	97.92	94.14	44.58
CUR0_2	50254668	49175852	7171140825	0.0253	97.86	93.97	44.71
CUR0_3	46186546	45072782	6531896696	0.0252	97.93	94.15	44.72
CUR50_1	47377258	46214632	6731354533	0.0256	97.77	93.75	44.61
CUR50_2	52812006	51695644	7544830614	0.0259	97.70	93.41	44.61
CUR50_3	52108326	50944140	7442409659	0.0253	97.87	94.01	44.97
Following the clean reads, which are high-quality sequencing data for all entries; the whole amount of sequencing data following quality control is known as clean bases. The average mistake rate of a sequencing base related to quality control data is the error rate (%). Q20, Q30 (%), denotes the sequencing quality in percentage terms of the total base, which is 99.9% and 99%, respectively; the percentage of the total bases that correspond to the quality control data for G and C bases is known as the GC content (%)							

Table 2
Assembly quality for transcript and Unigene of *A. trifoliata*

Type	Unigene	Transcript
Total number	82321	127383
Total base	76165113	138563235
Largest length (bp)	16296	16296
Smallest length (bp)	201	201
Average length (bp)	925.22	1087.77
N50 length (bp)	1623	1826
Total number, the number of sequence entries of the assembled unigene/transcript; total base, the number of bases of all unigene / transcripts assembled; largest length, the longest unigene / transcript length obtained through assembly; smallest length, the shortest unigene / transcript length obtained through assembly; average length, the average length of all unigene / transcripts assembled; N50 length, sort the assembled unigene / transcript in descending order of length, and accumulate the length of the transcript to half of the total length, corresponding to the length of the transcript		

The Trinity software was used for sequence assembly of sample data. In total, 127,383 transcripts were obtained, with an average length of 1087.77 bp and N50 length of 1826 bp. The obtained transcripts were further assembled to obtain 82,321 unigenes, with an average length of 925.22 bp and N50 length of 1623 bp. The filtered clean reads of each sample were compared with the reference sequences obtained from Trinity assembly, and the number of clean reads that could be matched to the assembled transcripts ranged from 17,686,746 to 20,571,698 with the matching efficiency ranging from 79.16–80.00% (Table 3). Overall, the transcriptome sequencing and assembly integrity were sufficiently good for subsequent analysis.

Table 3

Comparison and statistics of *A. trifoliata* transcriptome sequencing data and assembly results

Sample	Clean reads	Mapped reads	Mapped ratio
Control_1	23822238	18857616	79.16%
Control_2	22309643	17686746	79.28%
Control_3	24765330	19765848	79.81%
CUR0_1	24098950	19253469	79.89%
CUR0_2	24587926	19642332	79.89%
CUR0_3	22536391	18029666	80.00%
CUR50_1	23107316	18381064	79.55%
CUR50_2	25847822	20571698	79.59%
CUR50_3	25472070	20281272	79.62%
Clean reads (pair reads), the number of filtered sequencing data entries; mapped reads, the number of clean reads (pair reads) that can be compared to the assembled transcript; mapped ratio, the percentage of clean reads (pair reads) that can be located on the assembled transcript			

3.5.2 Gene expression analysis

Quantitative analysis of the expression levels of unigenes was performed using Bowtie2 and RESM. The log FPKM + 1 values of the nine samples ranged from approximately - 2 to 5; thus, FPKM was in the range of 0–10⁵ (Fig. 5A). Figure 5B shows the PCA analysis of the correlation between samples from three different treatment groups. In Control, CUR0, and CUR50, the three samples in the same group were more concentrated. This indicated that the correlation was high; repeated experiments were good, and no outliers were present. DEseq2 was used to compare the samples between different treatment groups pairwise to obtain the DEG set between different treatment groups. In CUR0 vs Control, CUR50 vs Control, and CUR50 vs CUR0, 2978 (1561 up- and 1417 downregulated DEGs), 1760 (818 up- and 942 downregulated DEGs), and 323 (124 up- and 199 downregulated DEGs) DEGs were obtained, respectively (Figs. 5C–F). This indicated that under acid rain stress, *A. trifoliata* may have transcribed more gene fragments, whose transcription levels significantly reduced after applying CUR. Moreover, CUR0 vs

Control and CUR50 vs Control shared 1209 DEGs; CUR50 vs Control and CUR50 vs CUR0 shared 84 DEGs; CUR0 vs Control and CUR50 vs CUR0 shared 161 DEGs (Fig. 5C); CUR0 vs Control, CUR50 vs Control, and CUR50 vs CUR0 shared 25 DEGs (Fig. 5C). These shared gene fragments may have the same mode of action under acid rain stress and during the addition of CUR.

3.5.3 KEGG pathway enrichment analysis of the DEGs

According to the screening criteria of $P_{\text{adjust}} < 0.05$, the up- and downregulated DEGs were enriched in five and two KEGG pathways, respectively, in CUR0 vs Control (Figs. 6A and B). Among them, according to P_{adjust} from low to high, the upregulated DEGs were enriched in protein processing in endoplasmic reticulum (map04141, 25 DEGs), phenylpropanoid biosynthesis (map00940, 17 DEGs), plant hormone signal transduction (map04075, 24 DEGs), starch and sucrose metabolism (map00500, 18 DEGs), and cyanoamino acid metabolism (map00460, 9 DEGs) (Fig. 6A). The downregulated DEGs were enriched in ribosome (map03010, 80 DEGs) and oxidative phosphorylation (map00190, 42 DEGs) (Fig. 6B).

In CUR50 vs Control, up- and downregulated DEGs were enriched in five and two KEGG pathways, respectively (Figs. 6C and D). Among them, according to P_{adjust} from low to high, the upregulated DEGs were enriched in phenylpropanoid biosynthesis (14 DEGs), tryptophan metabolism (map00380, 9 DEGs), plant hormone signal transduction (18 DEGs), protein processing in endoplasmic reticulum (18 DEGs), and starch and sucrose metabolism (11 DEGs) (Fig. 6C). The downregulated DEGs were enriched in ribosome (80 DEGs) and oxidative phosphorylation (36 DEGs) (Fig. 6D). No significantly enriched KEGG pathways were observed in CUR50 vs CUR0. Ribosome and OP were significantly downregulated KEGG pathways in CUR0 vs Control and CUR50 vs Control, and both pathways had multiple associated DEGs (Figs. 6B and D). This indicated that osmotic stress and oxidative damage induced by acid rain stress may have mainly impaired the rRNA biosynthesis of *A. trifoliata* and reduced the oxidative phosphorylation efficiency of mitochondria. Starch and sucrose metabolism, phenylpropanoid biosynthesis, protein processing in endoplasmic reticulum, and plant hormone signal transduction were significantly upregulated KEGG pathways in CUR0 vs Control and CUR50 vs Control, indicating that the enhanced expression of genes in these pathways may be related to the morphological and physiological responses of *A. trifoliata* to acid rain and mitigation effect of CUR (Figs. 6A and C).

3.5.4 Key DEGs

To further elucidate the mechanism by which *A. trifoliata* responded to acid rain stress and CUR enhanced its stress resistance at the gene level, we analyzed the FPKM expression patterns of DEGs in starch and sucrose metabolism, phenylpropanoid biosynthesis, protein processing in endoplasmic reticulum, and plant hormone signal transduction pathways (Fig. 7). In protein processing in endoplasmic reticulum, 18 DEGs were involved in encoding heat stress proteins, among which HSP20-encoding DEGs were the most abundant (15 in CUR0 vs Control and 12 in CUR50 vs Control; Fig. 7A). In plant hormone signal transduction, 15 DEGs were involved in auxin biosynthesis, of which SAUR-encoding DEGs were the most abundant (10 in CUR0 vs Control and 5 in CUR50 vs Control; Fig. 7B). In phenylpropanoid biosynthesis, the DEGs were extensively involved in lignin synthesis as follows. In CUR0

vs Control, one, six, and two DEGs were involved in encoding 4CL, E1.11.1.7, and COMT, respectively; in CUR50 vs Control, two, five, and three DEGs were involved in encoding 4CL, E1.11.1.7, and COMT, respectively (Fig. 7C). In starch and sucrose metabolism, 11 DEGs were involved in encoding glycoside hydrolases, including E3.2.1.21 and E3.2.1.4 (Fig. 7D). Among these DEGs, CUR0 vs Control included eight and three DEGs, whereas CUR50 vs Control included four and two DEGs (Fig. 7D).

Overall, the molecular mechanism by which *A. trifoliata* responds to acid rain stress and CUR enhances its stress resistance may involve multiple genes with the synergistic regulation of multiple biological processes. It is suggested that the expression patterns and related functional studies of DEGs involved in encoding HSP20, SAUR, 4CL, E1.11.1.7, COMT, E3.2.1.21, and E3.2.1.4 are important for further exploring this mechanism.

4. Discussion

Acid rain stress inhibits plant growth by inducing osmotic stress and oxidative damage. The most direct effect of acid rain stress on plants is on leaves, causing serious damage to the integrity of anatomical structure of leaves (Ren et al., 2021). In this study, *A. trifoliata* plant growth and development were significantly inhibited under acid rain stress, resulting in decreased biomass and a large number of white–brown necrotic spots on the leaves. The addition of exogenous CUR significantly improved the damage to *A. trifoliata* leaves. Both CUR25 and CUR50 could significantly reduce necrotic spots on the leaves and increase the biomass, whereas the necrotic spots were significantly higher in CUR100 than in CUR25 and CUR50. Additionally, the root system of *A. trifoliata* was severely distorted in CUR100. This suggested that the appropriate concentration of CUR could effectively alleviate the damage due to acid rain stress on *A. trifoliata*; however, the effect was weakened when CUR concentration was too high. This was consistent with the results obtained by Zhang et al. (2024) in spinach.

Chlorophyll is an important pigment for photosynthesis in plants. Its content is an important basis for assessing growth, development, and physiological condition of plants (Gharibiyan et al., 2023). In this study, acid rain stress alone significantly reduced the chlorophyll content of *A. trifoliata* leaves. This could be because of two reasons. First, acid rain corroded leaf surfaces, causing chlorophyll degradation. Second, the chloroplast was damaged when the plant was under stress, and the activity of chlorophyll-degrading enzyme increased, which accelerated chlorophyll degradation (dos Santos et al., 2023). In this study, the contents of chlorophyll a, chlorophyll b, and total chlorophyll in *A. trifoliata* leaves significantly increased under acid rain stress after CUR25 and CUR50 treatments. However, the chlorophyll content significantly reduced in CUR100, which was consistent with the degree of spot necrosis of plant leaves and changes in root morphology. The effect of increasing chlorophyll content was the best in CUR50, demonstrating that the appropriate concentration of CUR could effectively reduce the inhibitory effect of acid rain stress on the growth of *A. trifoliata*. Therefore, CUR can be used as an inhibitor of degradation of photosynthetic pigments to protect the structure and function of the photosynthetic system and improve the acid resistance of plants.

Osmoregulation plays an important role in the stress resistance of plants mainly via two aspects. First, it improves plants' water absorption capacity through osmotic pressure regulation. Second, it maintains the integrity of cell structure and function through osmotic protection (Lambers et al., 2006). Soluble sugar and soluble protein are important intracellular osmoregulatory substances, and increase in their contents is favorable for the tolerance of plants to acid rain stress (Du et al., 2023). Accumulation of proline, which is very sensitive to changes in the external environment, reflects the adversity resistance of plants (Maach et al., 2020). In this study, acid rain stress alone accelerated starch degradation and significantly increased the contents of soluble sugar, soluble protein, and proline in *A. trifoliata* leaves. This was conducive to cope with acid rain stress. The application of exogenous CUR further increased the contents of soluble sugar, soluble protein, and proline. Particularly, CUR50 exhibited the highest content of these substances. This indicated that CUR could promote the biosynthesis of osmoregulatory substances under acid rain stress, thereby maintaining the osmotic balance and integrity of cell structure and function.

Under normal conditions, the ROS content in plants is relatively low, which plays an important role in maintaining the intracellular physiological balance. Under acid rain stress, plants produce more ROS, exacerbating the degree of membrane lipid peroxidation, leading to cell death, and thus inhibiting plant growth (Debnath et al., 2018). Oxygen free radicals and MDA are important indicators of membrane lipid peroxidation levels and degree of damage (Wang et al., 2023). In this study, the MDA content and production rate of oxygen free radicals were significantly higher in CUR0 than in Control. This suggested that acid rain stress led to more ROS production in *A. trifoliata* leaves, disrupted the physiological balance, and increased cell membrane permeability. CUR25 and CUR50 effectively reduced the MDA content and production rate of oxygen free radicals in *A. trifoliata* leaves, with CUR50 being the most effective. This may be related to the direct scavenging of free radicals by CUR (Zaki et al., 2023). Overall, exogenous CUR played important roles in reducing ROS, minimizing plasma membrane damage, and protecting and repairing cell membranes.

SOD, POD, and CAT are important components of the antioxidant defense system, are involved in the scavenging of oxygen free radicals, and play a role in the protection of the stability and integrity of cell membrane under stress conditions. In this study, under acid rain stress alone, the activities of SOD, POD, and CAT were elevated in *A. trifoliata* leaves. This suggested that injury to *A. trifoliata* leaves and stress response simultaneously occurred, increasing the activity of antioxidant enzymes and resisting the harmful effects of acid rain stress. Exogenous CUR could further effectively increase the activities of SOD, POD, and CAT, thus effectively reducing the damage caused by acid rain stress to *A. trifoliata* and playing a protective role. However, the effect was dependent on the concentration of CUR. The activities of the three antioxidant enzymes were the highest in CUR50. In summary, the comprehensive evaluation of changes in plant morphology; content of chlorophyll, osmoregulatory substances, and membrane lipid peroxidation products; activity of antioxidant enzymes; and physiological indexes indicated that CUR50 was an optimal treatment exhibiting important mitigation effect on the damage due to acid rain stress in *A. trifoliata*.

In the process of plant growth and development, leaf is one of the most sensitive organs to environmental changes. Under stress conditions, plants can adapt to the external environment through changing the anatomical structure of leaves (Li et al., 2022). In this study, the leaves of *A. trifoliata* (control) grown under normal conditions were relatively thin, and the leaf epidermis was completely wrapped around the periphery of the entire leaf blade and played a protective role. The palisade tissues consisted of 1–2 layers of long columnar or ellipsoidal cells, which were mainly involved in photosynthesis. However, under acid rain stress alone, the epidermis and lateral veins of *A. trifoliata* leaves were severely damaged, and the leaf thickness significantly increased. The palisade tissues mostly consisted of 2 layers of long columnar cells, which were more compact, and the ratio of palisade/spongy tissues significantly increased. Indeed, thickening of leaves, increase in the number of palisade tissues, and shortening of intercellular spaces could help to improve the water utilization rate of the plant and provided the adaptability to water scarcity; additionally, the increase in the leaf thickness could help to reduce excessive water transpiration (Dong and Zhang., 2001). Thus, acid rain stress may cause severe physiological water deficit in *A. trifoliata*; however, *A. trifoliata* may reduce water consumption by increasing leaf thickness and water transport efficiency by thickening palisade tissues and shortening intercellular spaces. In CUR50, the structural integrity of leaves under acid rain stress remained intact, whereas the growth of palisade tissues in terms of thickness gradually slowed down. This indicated that exogenous CUR could alleviate the damage due to acid rain stress to the tissue structure of *A. trifoliata* leaves.

Under abiotic stress, plants regulate the expression of relevant genes to regulate the physiological and biochemical mechanisms involving various metabolism pathways to alleviate the damage caused by external environmental changes. Ribosomes play an important role in plant metabolism by translating mRNA into functional proteins (Ramakrishnan., 2002). Additionally, external stress may lead to increased activity of uncoupling proteins in plant mitochondria and uncoupling of oxidative phosphorylation. This results in decreased oxidative phosphorylation efficiency of mitochondria, thereby affecting the normal energy metabolism of cells (Jacoby et al., 2011). In this study, ribosome and OP were the significantly downregulated KEGG pathways in CUR0 vs Control and CUR50 vs Control, with multiple relevant DEGs. Hence, it was deduced that osmotic stress and oxidative damage induced by acid rain stress may mainly impair the rRNA biosynthesis and reduce the oxidative phosphorylation efficiency of mitochondria in *A. trifoliata*.

The endoplasmic reticulum plays an important role in the abiotic stress resistance of plants (Cao et al., 2022). In this study, protein processing in endoplasmic reticulum was the most significant pathway in CUR0 vs Control, indicating that endoplasmic reticulum may be involved in response to acid rain stress. Most DEGs were involved in encoding heat stress proteins. HSP20 is a class of small molecular heat stress proteins in plants with molecular chaperone function and is widely involved in the regulation of plant stress response (Wu et al., 2022). In *Solanum lycopersicum*, the sustained expression of genes involved in encoding endoplasmic reticulum HSP20 led to increased salt resistance (Fu et al., 2016). HSP20 interacts with biological membranes, regulating membrane fluidity and maintaining membrane integrity (Tsvetkova et al., 2002). Based on our results, one of the reasons for the adaptation of *A.*

trifoliata to acid rain stress could be that HSP20 overexpression may maintain the structural integrity of the endoplasmic reticulum membrane by interacting with it, thus maintaining the normal function of the endoplasmic reticulum and ultimately alleviating ROS-induced damage. protein processing in endoplasmic reticulum-related DEGs were significantly reduced in CUR50 vs Control, which may be due to the action of CUR.

Plant hormones can induce stress-responsive genes through synergistic and antagonistic effects (Fujita., 2006). Optimized auxin level is required for the regulation of growth and development of plants. During auxin biosynthesis, SAUR, as the largest gene family among the early auxin-responsive genes in plants, plays an important role in the growth, development, and abiotic stress resistance of plants (Hagen and Guilfoyle., 2002). Overexpression of SAUR-encoding DEGs could improve the survival rate of *Triticum aestivum* under drought and salt stresses (Guo et al., 2018). In this study, most SAUR-encoding DEGs were significantly upregulated in CUR0 vs Control. This suggested that the enhanced expression of these DEGs may also be related to the increased acid resistance of *A. trifoliata* under acid rain stress. SAUR expression was reduced in CUR50 vs Control, which may be related to the mitigation effect of CUR.

Most defense-related secondary metabolites in plants undergo direct or indirect biosynthesis through the phenylpropane metabolic pathway. In this study, phenylpropanoid biosynthesis was the significantly affected secondary metabolic pathway in CUR0 vs Control. Similar results were reported in plants such as *Glycine Max* and *Celosia argentea* (Wang et al., 2024; Liang et al., 2024) under adversity, which are usually endowed with stress resistance in the adversity they are exposed to. This indicated that phenylpropane metabolic pathway may play an important defensive role in plants' resistance to abiotic stress. 4CL, E1.11.1.7, and COMT are three key enzymes of lignin biosynthesis in phenylpropane metabolic pathway. Lignin, as a complex phenol, plays an important defensive role in stress resistance by plants (Kováčik and Klejdus., 2008). 4CL is a key enzyme in the upstream stage of the lignin biosynthesis pathway, catalyzing the formation of p-coumaric acid coenzyme-A from cinnamic acid and controlling the movement of phenylpropanine toward different metabolic pathways (Sui et al., 2019). In CUR0 vs Control, one differentially expressed 4CL gene was identified in *A. trifoliata* leaves. In CUR50 vs Control, two differentially expressed 4CL genes were identified and were upregulated, which may be beneficial to enhance the flux of the whole metabolic pathway. In the lignin biosynthesis pathway, phenylalanine (the substrate) is deaminated, hydroxylated, methylated, and reduced to generate lignin monomers, which are further polymerized to generate lignin polymers. This is catalyzed by E1.11.1.7, laccase, and phenolase. COMT can catalyze the methylation of caffeic acid to generate the precursor material for lignin biosynthesis (Xin et al., 2022). Lignin accumulation is beneficial for increasing cell wall thickness (Dong et al., 2023). In this study, in CUR0 vs Control, six DEGs were involved in encoding E1.11.1.7 and two DEGs were involved in encoding COMT, and the significant thickening of palisade tissues was clearly observed. Hence, it was deduced that *A. trifoliata* may increase the cell wall thickness of palisade tissues by regulating lignin biosynthesis, thus preventing the entry of acidic substances into the cells. In CUR50 vs Control, Phenylpropanoid biosynthesis was the most significant secondary metabolic pathway, CUR50 could favor the activation of this pathway. Additionally, in

medicinal plants, Phenylpropanoid biosynthesis is involved in the accumulation of various compounds (Li et al., 2024). Therefore, this study provided a reference for further exploration of active components in *A. trifoliata*.

In the process of sugar metabolism, E3.2.1.21 plays an important role in the glycolytic hydrolysis of cellulose. E3.2.1.4 can hydrolyze cellulose to cellobiose, which is hydrolyzed to glucose by E3.2.1.21 to release energy and resist the internal stress (Zhang et al., 2023). In this study, in CUR0 vs Control, 11 DEGs involved in encoding E3.2.1.21 and E3.2.1.4 in the SSM pathway were upregulated. The starch content was significantly lower in CUR0 than in Control. It was hypothesized that the upregulation of these DEGs promoted the formation of glucose in *A. trifoliata* under acid rain stress, which may provide energy for *A. trifoliata* to resist acid rain stress. The starch content was significantly higher in CUR50 than in CUR0, and at the same time, the expression levels of these DEGs decreased. This indicated that exogenous CUR could mitigate the effects of acid rain stress in *A. trifoliata*.

5. Conclusion

Physiological, anatomical, and transcriptomic analyses revealed that acid rain stress significantly inhibited the normal growth of *A. trifoliata*. It led to chlorosis, large numbers of white–brown spots, chlorophyll degradation, and excessive accumulation of membrane lipid peroxidation products in the leaves. It destroyed the structural integrity of the upper and lower epidermis and lateral veins of the leaves and significantly downregulated the DEGs involved in ribosome and oxidative phosphorylation metabolism. Further, the tolerance mechanism of *A. trifoliata* to acid rain stress was investigated. Under acid rain stress, contents of osmoregulatory substances and activities of antioxidant enzymes increased; the leaf blade was significantly thickened; the number of palisade tissue layers significantly increased; the cell morphology was altered; the intercellular spaces became smaller, and the ratio of palisade/spongy tissues increased. Transcriptomic analysis revealed that various DEGs related to abiotic stress, including those from protein processing in endoplasmic reticulum, plant hormone signal transduction, phenylpropanoid biosynthesis, and starch and sucrose metabolism pathways, were upregulated. The DEGs encoding HSP20, SAUR, 4CL, E1.11.1.7, COMT, E3.2.1.21, and E3.2.1.4 were important factors involved in this mechanism. Meanwhile, appropriate concentration of exogenous CUR could effectively improve plant growth, slow down the degradation rate of chlorophyll, and increase the contents of osmoregulatory substances and antioxidant enzyme activity. Thus, it maintained the osmotic balance and integrity of the anatomical structure of the leaves, lowered the degree of cell membrane lipid peroxidation, and alleviated the damage due to acid rain stress in *A. trifoliata*. The most significant effect was observed at 50 $\mu\text{mol/L}$ of CUR. Additionally, genes involved in protein processing in endoplasmic reticulum, plant hormone signal transduction, phenylpropanoid biosynthesis, and starch and sucrose metabolism pathways were regulated by exogenous CUR, indicating that exogenous CUR could mitigate the effects of acid rain stress in *A. trifoliata* via these pathways. This study provided insights for further studying the growth, development, and potential mechanism of molecular responses of *A. trifoliata* under acid rain stress and demonstrated the mitigation effect of exogenous CUR under acid rain stress.

Declarations

Author Contribution

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Interest Statement:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Xiao, S.S., Wang, G.G., Tang, C.G., Fang, H.Y., Duan, J., Yu, X.F., 2020. Effects of one-year simulated nitrogen and acid deposition on soil respiration in a subtropical plantation in China . *Forests*. 11 (2), 235. <https://doi.org/10.3390/f11020235>.
2. Ren, X.Q., Zhang, J.E., Xiang, H.M., Shi, Z.J., Hei, Z.W., Li, M.K., 2021. Research advances and prospects for effects of acid rain on aboveground physiology of plants and related alleviation countermeasures. *Chin J Appl Environ Biol*. 27(6), 1716–1724. <https://doi.org/10.19675/j.cnki.1006-687x.2020.07054>.
3. Yao, L.R., Wang, X.J., Zhuo, C., Leng, M.Z., Ni, W.Z., 2022. Effects of simulated acid rain on dissolution characteristics and fraction of phosphorus in tea garden soil. *Acta Agriculturae Zhejiangensis*. 34 (12), 2700–2709. <https://doi.org/10.3969/j.issn.1004-1524.2022.12.13>.
4. Polishchuk, O.V., Vodka, M.V., Belyavskaya, N.A., Khomochkin, A.P., Zolotareva, E.K., 2016. The effect of acid rain on ultrastructure and functional parameters of photosynthetic apparatus in pea leaves. *Cell Tiss Biol*. 10(3), 250–257. <https://doi.org/10.1134/S1990519X16030093>.
5. Zhang, Z.H., Chai, X.T., Zhang, B., Lu, Y., Gao, Y.J., Tariq, A., Li, X.Y., Zeng, F.J., 2023. Potential role of root-associated bacterial communities in adjustments of desert plant physiology to osmotic stress. *Plant physiol bioch*. 204, 108124. <https://doi.org/10.1016/j.plaphy.2023.108124>.
6. Kováčik, J., Klejdus, B., Bačkor, M., Štork, F., Hedbavny, J., 2011. Physiological responses of root-less epiphytic plants to acid rain. *Ecotoxicology*. 20(2), 348–357. <https://doi.org/10.1007/s10646-010-0585-x>.
7. Seibel, R., Schneider, R.H., Gottlieb, M.G.V., 2021. Effects of spices (safron, rosemary, cinnamon, turmeric and ginger) in Alzheimer's disease. *Curr Alzheimer Res*. 18(4), 347–357. <https://doi.org/10.2174/1567205018666210716122034>.
8. Zaki, F.S., Khater, M.A., El-Awadi, M.E., Dawood, M.G., Elsayed, A.E., 2023. Curcumin-polyvinyl alcohol nano-composite enhances tolerance of *Helianthus annuus* L. against salinity stress. *Beni-Suef Univ J Basic Appl Sci*. 12(1), 60. <https://doi.org/10.1186/s43088-023-00397-2>.

9. Li, X., Zhang, Y., Ren, Z., Wang, K., Liu, Z., Xu, S., Qiao, Z., 2024. Curcumin leads to responses of grapes to aluminum stress by inducing whole genome hypo-methylation, Horticulture, Environment, and Biotechnology. 65, 375–389. <https://doi.org/10.1007/s13580-023-00565-4>.
10. Zhang, L.L., Zengin, G., Konakci, C.O., Yildiztugay, E., Arıkan, B., Ekim, R., Koyukan, B., Elbasan, F., Lucini, L., 2024. Exogenous curcumin mitigates As stress in spinach plants: A biochemical and metabolomics investigation. Plant physiol bioch. 211, 108713. <https://doi.org/10.1016/j.plaphy.2024.108713>.
11. Luo, K.M., Liu, X.W., Yang, T.Y., Ban, X.C., Wan, M.C., Shi, L.J., Zhang, C.J., Li, Z.J., Hao, Y.C., 2008. The growth and fruiting habit of *Akebia trifoliata* (Thunb.) Koidz under the cultivation condition. Guizhou Agricultural Sciences. (3): 123–124.
12. Jia, Y.X., Yang, Q.X., Zeng, L., Xu, Q.L., Liu, S.B., Xu, X.L., Tan, J.W., 2022. Triterpenes from stems of *Akebia trifoliata* and their antibacterial activities. J Trop Subtrop Bot. 30(1), 144–150. (in Chinese). <https://doi.org/10.11926/jtsb.4489>.
13. Cai, F.L., Zou, S.Y., Gao, P.X., Jia, T.J., Cheng, C.S., Fan, S.Q., Ding, J.M., Huang, H. W., 2022. Analysis on quality variations of *Akebia trifoliata* fruit at different harvesting times. J Plant Res Environ. 31(1), 83–85. <https://doi.org/10.3969/j.issn.1674-7895.2022.01.10>.
14. Yu, X.S., Wang, X.H., Li, L., Liu, Z., Yang, N.X., Zhang, M.S., 2022. Effects of rocky desertification habitat on growth and physiological characteristics of *Akebia trifoliata*. Acta Ecol Sin. 42(6), 2382–2393. <https://doi.org/10.5846/stxb202102250522>.
15. Wu, Z.H., Yu, L.F., Yan, L.B., Zhou, C., Cai, G.J., Zhang, J.L., 2018. Responses of *Akebia trifoliata* leaf anatomical structure and photosynthetic characteristic to drought stress. Journal of Southern Agriculture. 49 (6), 1156–1163. <https://doi.org/10.3969/j.issn.2095-1191.2018.06.16>.
16. Zhou, X.D., Xu, Z.F., Liu, W.J., Wu, Y., Zhao, T., Jiang, H., 2017. Progress in the studies of precipitation chemistry in acid rain areas of southwest China. Environ Sci. 38(10), 4438–4446. <https://doi.org/10.13227/j.hjhx.201702069>.
17. Zou, Q. 2003. Plant Physiology Experiment Guidance. Beijing: China Agriculture Press.
18. Kumar, G.N.M., Knowles, N.R., 1993. Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during aging and sprouting of potato (*Solanum tuberosum*) seed-tubers. Plant physiology. 102, 115–124. <https://doi.org/10.1104/pp.102.1.115>.
19. Li, Z.G., Gong, M., 2005. Improvement of measurement method for superoxide anion radical in plant. Plant Diversity. (2), 211–216.
20. Gao, J.F., 2006. Experimental Instruction of Plant Physiology. Beijing: Higher Education Press, 2006.
21. Hu, Y., Yang, L., Gao, C., Liao, D., Long, L., Qiu, J., Wei, H.L., Deng, Q.E., Zhou, Y.C., 2022. A comparative study on the leaf anatomical structure of *Camellia oleifera* in a low-hot valley area in Guizhou Province, China. PloS one. 17 (1), e0262509. <https://doi.org/10.1371/journal.pone.0262509>.
22. Qian, C.C., Zhao, L.Q., Yang, Y.T., Han, B.X., Wu, J.W., Ou, J.M., 2022. Analysis of the transcriptome and discovery of key enzyme genes of the triterpenoid saponin biosynthesis pathway in *Akebia*

- trifoliata* (Thunb.) Koidz. Plant Science Journal. 40(3), 378–389.
<https://doi.org/10.11913/PSJ.2095-0837.2022.30378>.
23. Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods. 9, 357–359. <https://doi.org/10.1038/nmeth.1923>.
 24. Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 12, 323. <https://doi.org/10.1186/1471-2105-12-323>.
 25. Gharibyan, P., Roozban, M.R., Rahemi, M., Vahdati, K., 2023. Exogenous salicylic acid improves growth and physiological status of two *Pistacia* species under salinity stress. Erwerbs-Obstbau, 65 (5), 1441–1452. <https://doi.org/10.1007/s10341-023-00841-8>.
 26. dos Santos, L.A., Batista, B.L., Lobato, A.K., 2023. 24-Epibrasinolide Delays Chlorophyll Degradation and Stimulates the Photosynthetic Machinery in Magnesium-Stressed Soybean Plants. J Plant Growth Regul. 42, 183–198. <https://doi.org/10.1007/s00344-021-10539-4>.
 27. Lambers, H., Shane, M.W., Cramer, M.D., Pearse, S.J., Veneklaas, E.J., 2006. Root structure and functioning for efficient acquisition of phosphorus: Matching morphological and physiological traits. Annals of Botany. 98(4), 693–713. <https://doi.org/10.1093/aob/mcl114>.
 28. Du, X., Chen, H.M., Qin, X., Xu, W.T., Luo, Q.J., 2023. Physiological and biochemical response of *Enteromorpha* and *Ulva* to simulated acid rain stress. Journal of Nuclear Agricultural Sciences. 37 (5), 1067–1075. <https://doi.org/10.11869/j.issn.1000-8551.2023.05.1067>.
 29. Maach, M., Baghour, M., Akodad, M., Gálvez, F.J., Sánchez, M.E., Aranda, M.N., Venema, K., Rodríguez-Rosales, M.P., 2020. Overexpression of *LeNHX4* improved yield, fruit quality and salt tolerance in tomato plants(*Solanum lycopersicum* L.). Molecular Biology Reports. 47(4), 4145–4153. <https://doi.org/10.1007/s11033-020-05499-z>.
 30. Debnath, B., Hussain, M., Irshad, M., Mitra, S., Li, M., Liu, S., Qiu, D.L., 2018. Exogenous melatonin mitigates acid rain stress to Tomato plants through modulation of Leaf ultrastructure, photosynthesis and antioxidant potential. Molecules. 23(2), 388. <https://doi.org/10.3390/molecules23020388>.
 31. Wang, D., Zhang, W.R., Zhang, R.Q., Tao, N., Si, L., Guo, C.H., 2023. Phytotoxicity of nitrobenzene bioaccumulation in rice seedlings: Nitrobenzene inhibits growth, induces oxidative stress, and reduces photosynthetic pigment synthesis. Plant physiol bioch. 204, 108096. <https://doi.org/10.1016/j.plaphy.2023.108096>.
 32. Li, R.Q., Wang, Y.X., Sun, Y.L., Zhang, L., Chen, A.P., 2022. Effects of salt stress on leaf morphology and anatomical structure of *Bromus inermis* seedlings. Acta Agrest Sin. 30(6), 1450–1459. <https://doi.org/10.11733/j.issn.1007-0435.2022.06.016>.
 33. Dong, X.J., Zhang, X.S., 2001. Some observations of the adaptations of sandy shrubs to the arid environment in the Mu Us sandland: Leaf water relations and anatomic features. J Arid Environ. 48(1), 41–48. <https://doi.org/10.1006/jare.2000.0700>.

34. Ramakrishnan, V., 2002. Ribosome structure and the mechanism of translation. *Cell*. 108(4), 557–572. [https://doi.org/10.1016/s0092-8674\(02\)00619-0](https://doi.org/10.1016/s0092-8674(02)00619-0).
35. Jacoby, R.P., Taylor, N.L., Millar, A.H., 2011. The role of mitochondrial respiration in salinity tolerance. *Trends Plant Sci*. 16: 614–623. <https://doi.org/10.1016/j.tplants.2011.08.002>.
36. Cao, J.J., Wang, C.H., Hao, N., Fujiwara, T., Wu, T., 2022. Endoplasmic reticulum stress and reactive oxygen species in plants. *Antioxidants (Basel)*. 11(7), 1240. <https://doi.org/10.3390/antiox11071240>.
37. Wu, J.T., Gao, T., Hu, J.N., Zhao, L., Yu, Chang., Ma, F., 2022. Research advances in function and regulation mechanisms of plant small heat shock proteins (sHSPs) under environmental stresses. *Science of The Total Environment*. 825, 154054. <https://doi.org/10.1016/j.scitotenv.2022.154054>.
38. Fu, C., Liu, X.X., Yang, W.W., Zhao, C.M., Liu, J., 2016. Enhanced salt tolerance in tomato plants constitutively expressing heat-shock protein in the endoplasmic reticulum. *Genet. Mol. Res*, 15 (2), 15028301. <https://doi.org/10.4238/gmr.15028301>.
39. Tsvetkova, N.M., Horváth, I., Török, Z., Wolkers, W.F., Balogi, Z., Shigapova, N., Crowe, L.M., Tablin, F., Vierling, E., Crowe, J.H., Vigh, L., Small heat-shock proteins regulate membrane lipid polymorphism. *Proc Natl Acad Sci USA*. 99(21), 13504–13509. <https://doi.org/10.1073/pnas.192468399>.
40. Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., 2006. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr Opin in Plant Biol*. 9(4), 436–442. <https://doi.org/10.1016/j.pbi.2006.05.014>.
41. Hagen, G., Guilfoyle, T., Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Mol Biol.*, 49(3–4), 373–385. <https://doi.org/10.1023/A:1015207114117>.
42. Guo, Y., Jiang, Q.Y., Hu, Z., Sun, X.J., Fan, S.J., Zhang, H., 2018. Function of the auxin-responsive gene *TaSAUR75* under salt and drought stress. *Crop J*. 6(2), 181–190. <https://doi.org/10.1016/j.cj.2017.08.005>.
43. Wang, X.Y., Wei, X.H., Zhao, W., Li, X.M., Dong, S.K., 2024. Elucidating hormone transduction and the protein response of soybean roots to drought stress based on ultra performance liquid chromatography–tandem mass spectrometry and four-dimensional data-independent acquisition. *Environmental and Experimental Botany*. 224, 105820. <https://doi.org/10.1016/j.envexpbot.2024.105820>.
44. Liang, L.Y., Ze, M., Yang, J., Xu, Q., Du, C.M., Hu, X.H., Dong, M., Zou, L.J., Qi, T., 2024. Physiological and transcriptomic response reveals new insight into manganese tolerance of *Celosia argentea* Linn.. *Journal of Hazardous Materials*. 465, 133079. <https://doi.org/10.1016/j.jhazmat.2023.133079>.
45. Kováčik, J., Klejdus, B., 2008. Dynamics of phenolic acids and lignin accumulation in metal-treated *Matricaria chamomilla* roots. *Plant Cell Rep*. 27(3), 605–615. <https://doi.org/10.1007/s00299-007-0490-9>.

46. Sui, Z.W., Luo, J., Yao, R.L., Huang, C.L., Zhao, Y.C., Kong, L.Y., 2019. Functional characterization and correlation analysis of phenylalanine ammonia-lyase (PAL) in coumarin biosynthesis from *Peucedanum praeruptorum* Dunn.. *Phytochemistry*. 158: 35–45.
<https://doi.org/10.1016/j.phytochem.2018.11.006>.
47. Xin, J.P., Li, Y., Zhao, C., Tian, R.N., 2022. Transcriptome Sequencing in the Leaves of *Pontederiacordata* with Cadmium Exposure and Gene Mining in Phenylpropanoid Pathways. *Biotechnology Bulletin*. 38(6), 198–210. <https://doi.org/10.13560/j.cnki.biotech.bull.1985.2021-1151>.
48. Dong, Q., Wu, Y.J., Li, B., Chen, X., Peng, L., Sahito, Z.A., Li, H.X., Chen, Y.L., Tao, Q., Xu, Q., Huang, R., Luo, Y.L., Tang, X.Y., Li, Q.Q., Wang, C.Q., 2023. Multiple insights into lignin-mediated cadmium detoxification in rice (*Oryza sativa*). *Journal of hazardous materials*. 458, 131931.<https://doi.org/10.1016/j.jhazmat.2023.131931>.
49. Li, X.J., Zhao, Y., He, S.L., Meng, J., Lu, Y.C., Shi, H.N., Liu, C.L., Hao, B., Tang, Q.Y., Zhang, S. Y., Zhang, G.H., Luo, Y., Yang, S.C., Yang, J.L., Fan, W., 2024. Integrated metabolome and transcriptome analyses reveal the molecular mechanism underlying dynamic metabolic processes during taproot development of *Panax notoginseng*. *BMC Plant Biology*. 24(1), 170. <https://doi.org/10.1186/s12870-024-04861-8>.
50. Zhang, X.H., Peng, Q., Yan, Z., 2023. Transcriptome sequencing analysis of different sweet potato varieties under salt stress. *Acta Agronomica Sinica*. 49 (5): 1432–1444.
<https://doi.org/10.3724/SP.J.1006.2023.24143>.

Figures

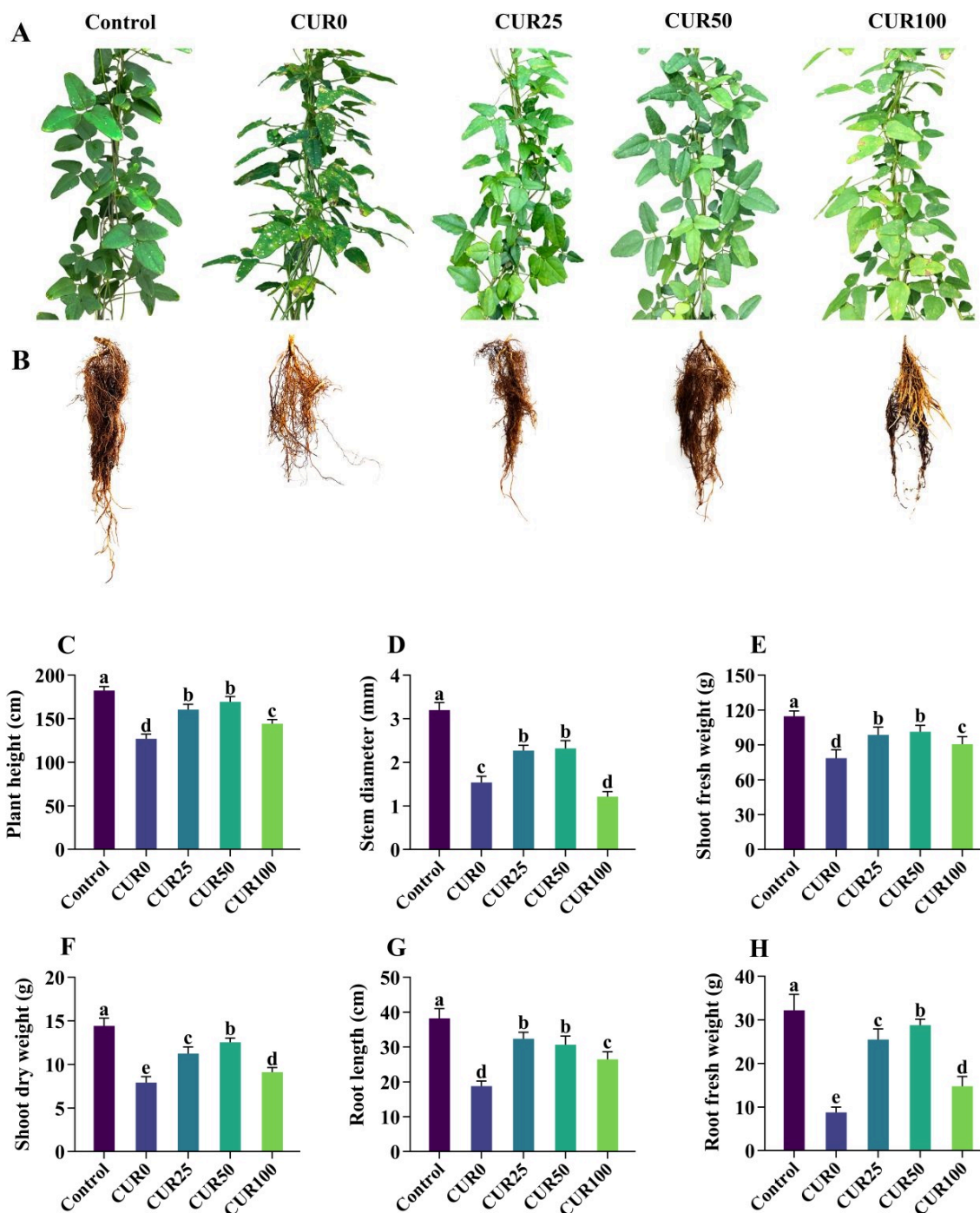


Figure 1

Morphological features of *A. trifoliata* plants treated with different concentrations of CUR. A: morphology of aboveground part; B: morphology of root system; C–H: alterations in morphological indicators. One-way ANOVA was performed to compare the significant changes between treatments, and the results are displayed as means $\pm$ SDs ($n = 4$). control group is Control; CUR0: be treated with simulated acid rain having a pH of 2.0; CUR25: be treated with simulated acid rain of pH 2.0 and 25

$\mu\text{mol/L}$ curcumin; CUR50: be treated with simulated acid rain of pH 2.0 and 50 $\mu\text{mol/L}$ curcumin; CUR100: be treated with simulated acid rain of pH 2.0 and 100 $\mu\text{mol/L}$ curcumin. There are significant variations between the little letters in the bar chart at the $P < 0.05$ level. The same applies hereinafter

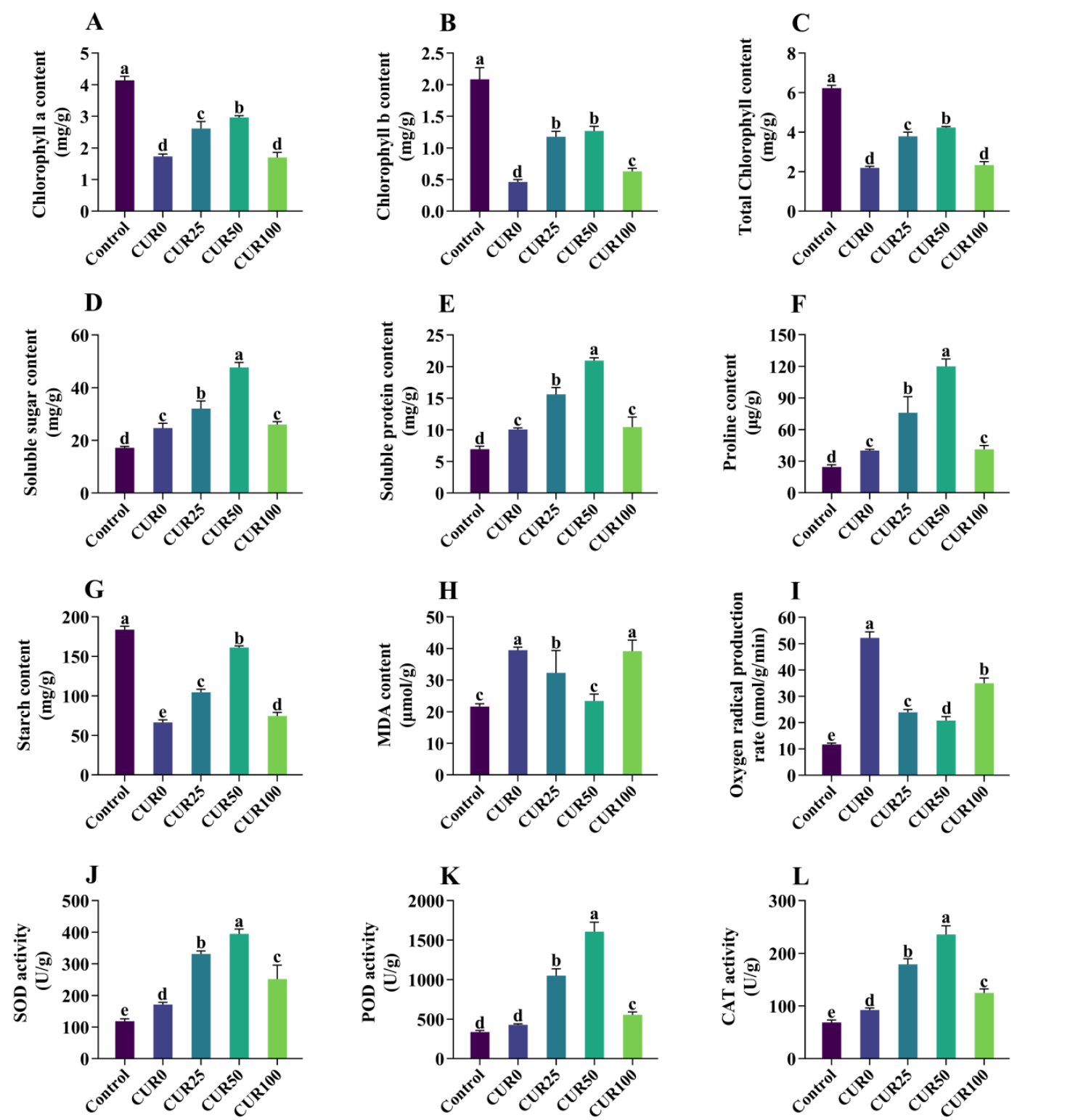


Figure 2

Physiological indicators of leaves of *A. trifoliata* treated with different concentrations of CUR. A–C: chlorophyll content; D–G: content of osmotic adjustment substances; H–I: content of membrane lipid

peroxides; J–L: activity of antioxidant enzymes.

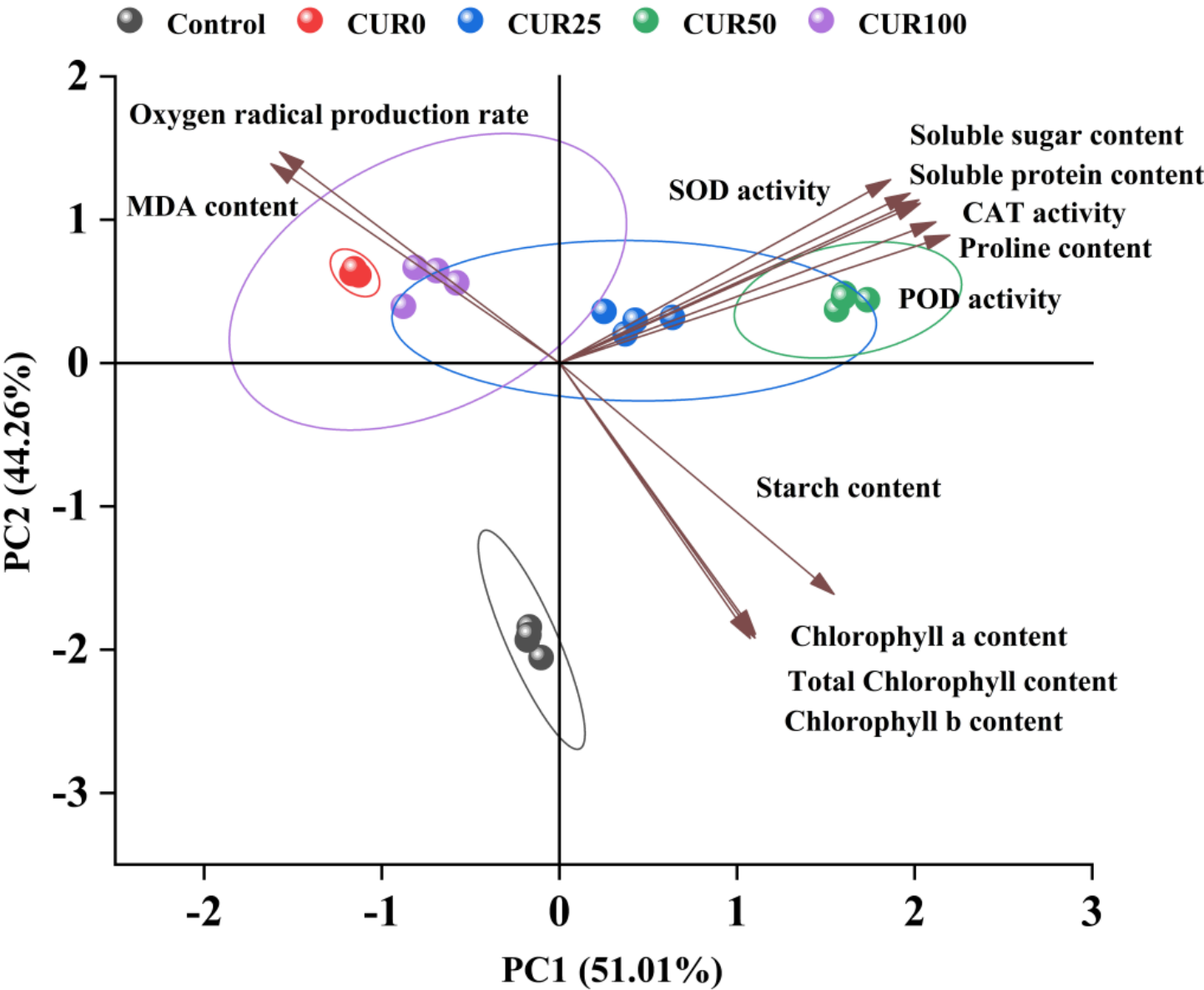


Figure 3

Results of principal component (PCA) comprehensive evaluation of physiological indexes of leaves of *A. trifoliata* treated with different concentrations of CUR. The confidence circle in the figure is at the 0.95 level. The same applies hereinafter

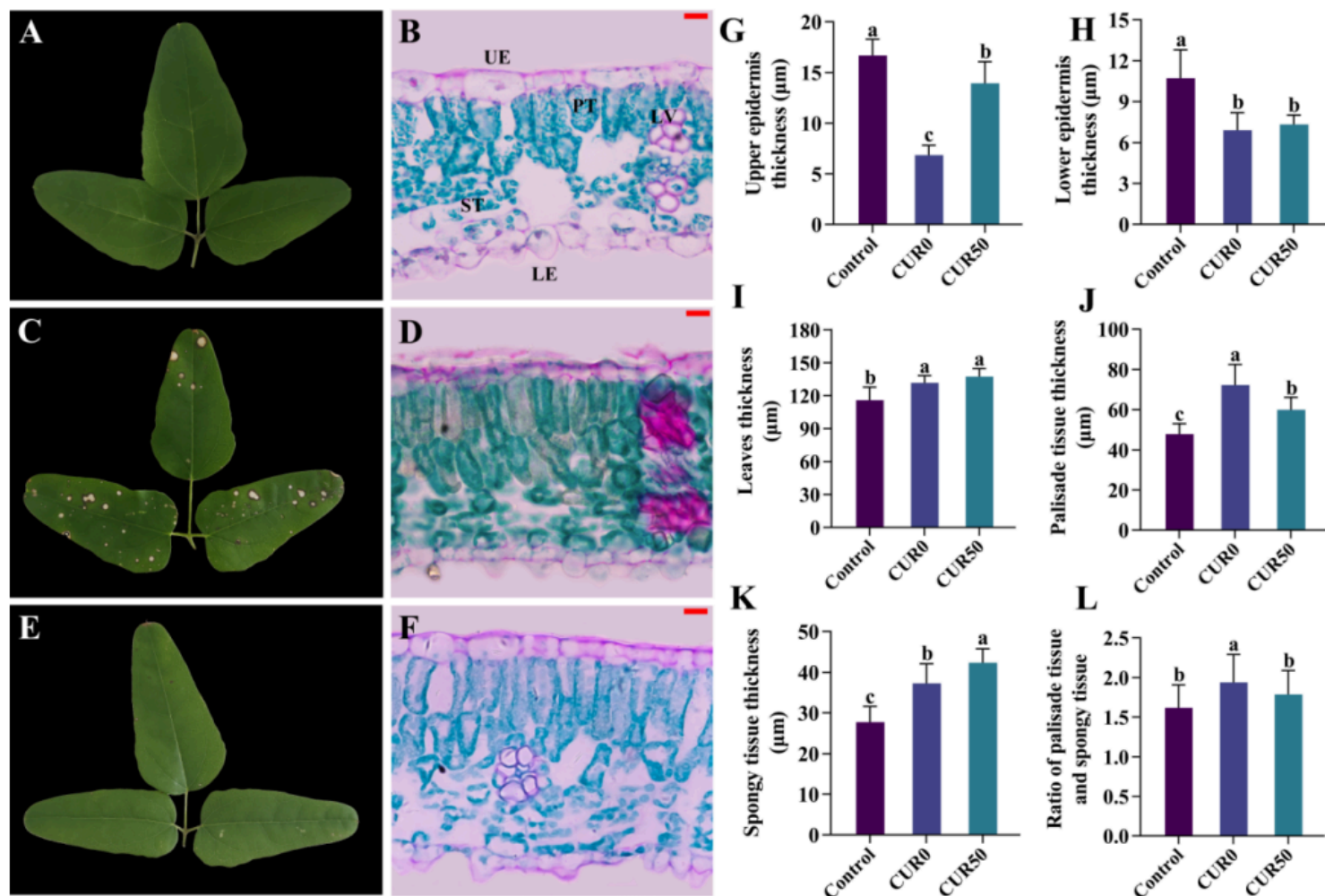


Figure 4

Leaf morphology and mesophyll anatomical structure of *A. trifoliata* leaves treated with different concentrations of CUR. A, C and E: leaf morphology; B, D and F: leaf mesophyll tissue; G–L: leaf mesophyll anatomical structure indexes. UE: upper epidermis; LE: lower epidermis; PT: palisade tissue; ST: spongy tissue; LV: Lateral veins. Scale bar: 20 μm

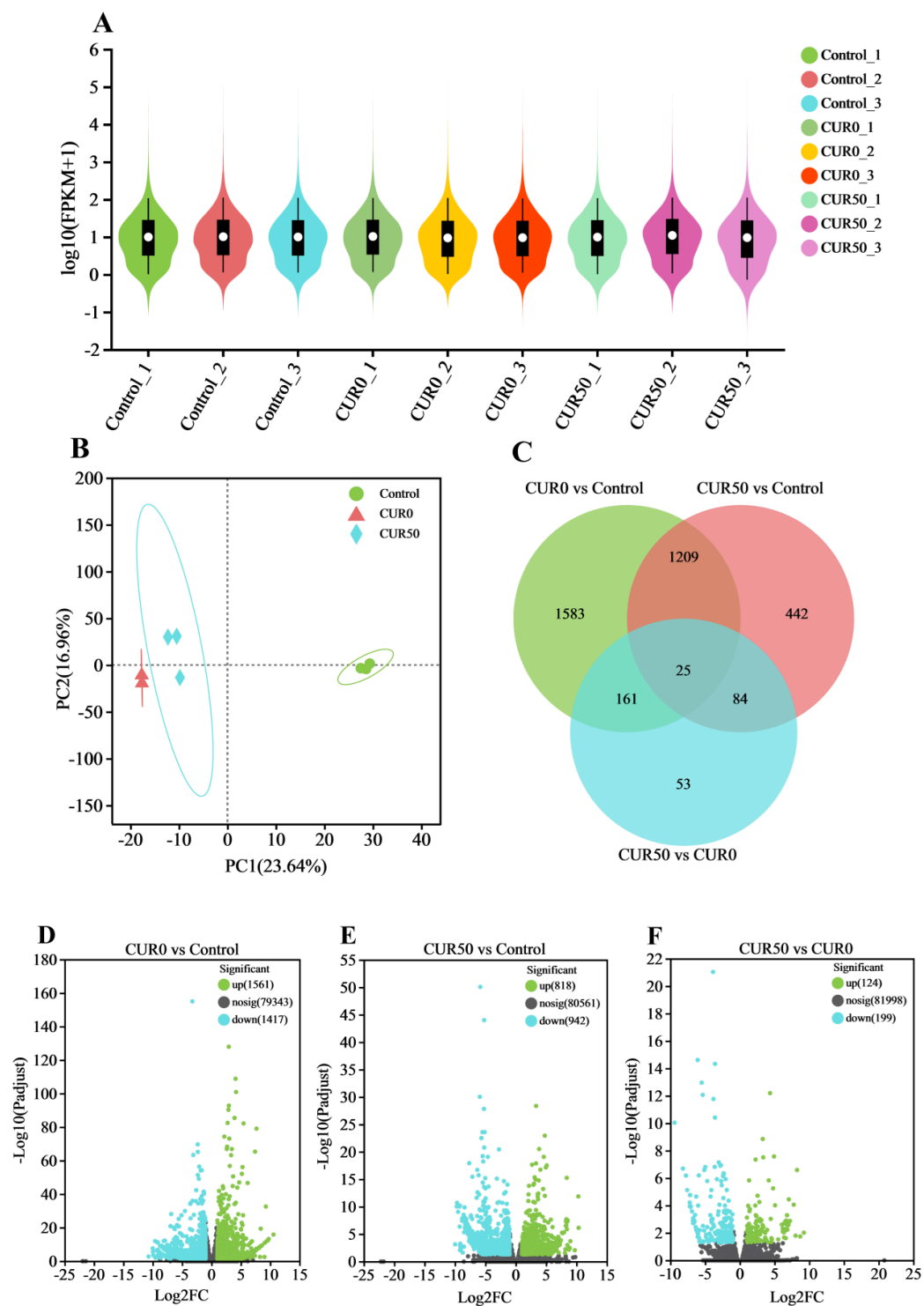


Figure 5

Changes in differentially expressed genes (DEGs) among different comparison groups. A: violin plot of FPKM quantity distribution; B: expression of different treatment groups in PCA analysis of sample quantity; C: venn diagram of DEGs between CUR0 vs Control, CUR50 vs Control and CUR50 vs CUR0 groups; D–F: volcano plots of DEGs between CUR0 vs Control, CUR50 vs Control and CUR50 vs CUR0 groups

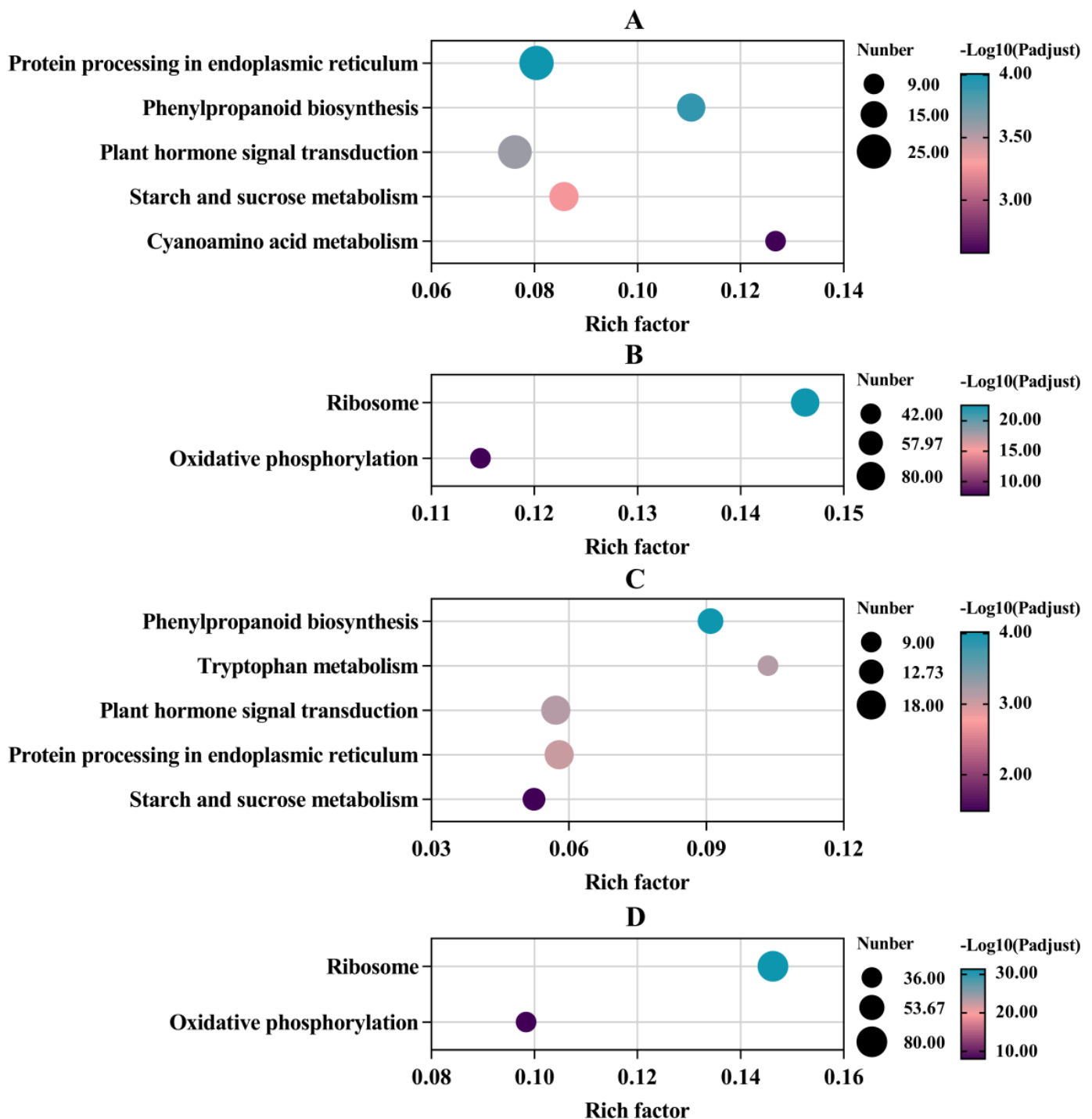


Figure 6

KEGG enrichment analysis of DEGs among different comparison groups. A: bubble plot of KEGG enrichment of up-regulated DEGs in CUR0 vs Control; B: bubble plot of KEGG enrichment of down-regulated DEGs in CUR0 vs Control; C: bubble plot of KEGG enrichment of up-regulated DEGs in CUR50 vs Control; D: bubble plot of KEGG enrichment of down-regulated DEGs in CUR50 vs Control

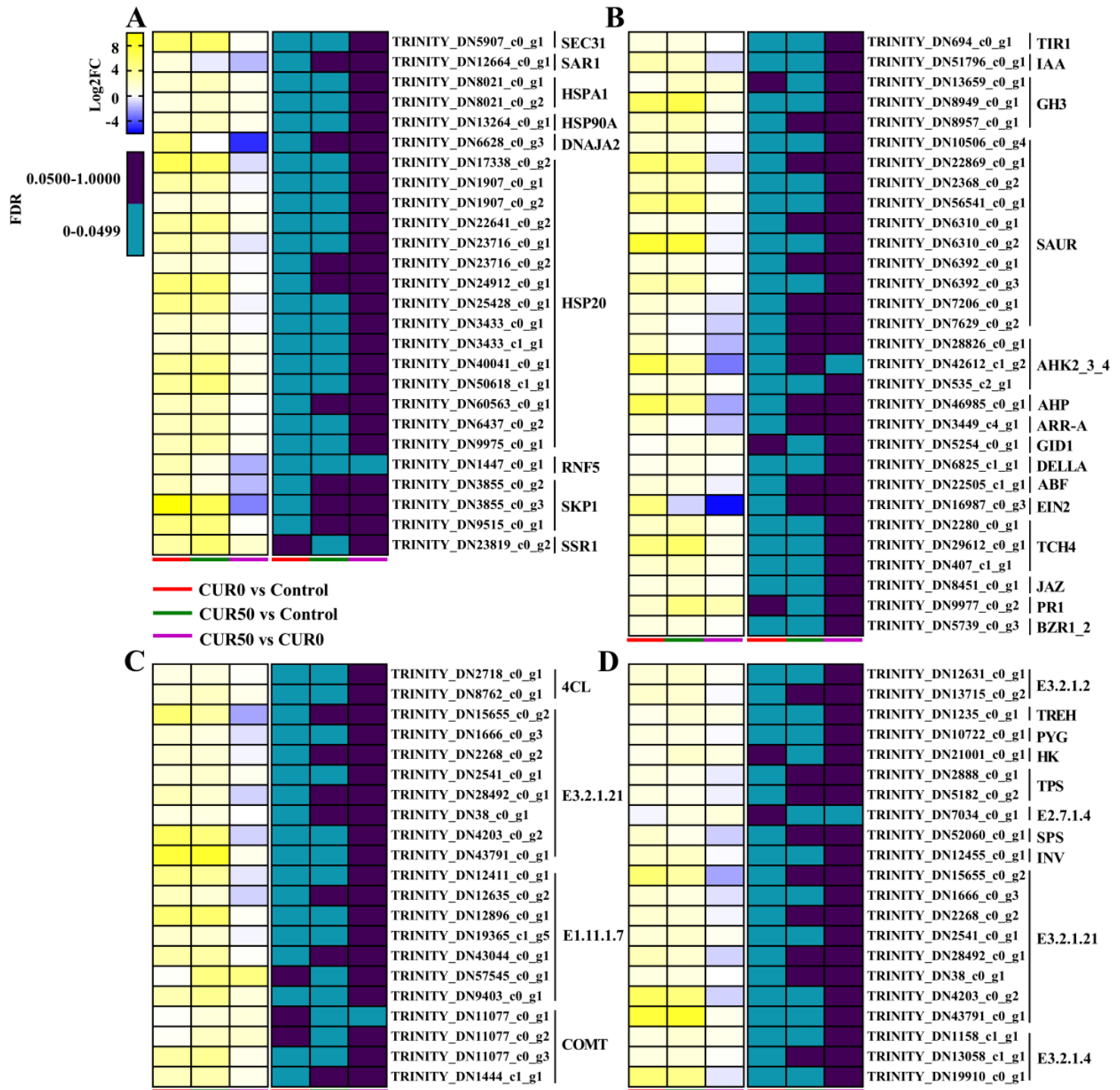


Figure 7

FPKM expression patterns of DEGs in the pathways of protein processing in endoplasmic reticulum (A), plant hormone signal transduction (B), phenylpropanoid biosynthesis (C), and starch and sucrose metabolism (D) among different comparison groups. SEC31: protein transport protein SEC31; SAR1: GTP-binding protein SAR1; HSPA1s: heat shock 70kDa protein 1/6/8; HSP90A: molecular chaperone HspG; DNAJA2: DnaJ homolog subfamily A member 2; HSP20: HSP20 family protein; RNF5: E3 ubiquitin-protein ligase RNF5/185; SKP1: S-phase kinase-associated protein 1; SSR1: translocon-associated

protein subunit alpha; TIR1: transport inhibitor response 1; IAA: auxin-responsive protein IAA; GH3: auxin responsive GH3 gene family; SAUR: SAUR family protein; AHK2_3_4: arabidopsis histidine kinase 2/3/4 (cytokinin receptor); AHP: histidine-containing phosphotransfer protein; ARR-A: two-component response regulator ARR-A family; GID1: gibberellin receptor GID1; DELLA: DELLA protein; ABF: ABA responsive element binding factor; ethylene-insensitive protein 2; TCH4: xyloglucosyl transferase TCH4; JAZ: jasmonate ZIM domain-containing protein; PR1: pathogenesis-related protein 1; BZR1_2: brassinosteroid resistant 1/2; 4CL: 4-coumarate-CoA ligase; E3.2.1.21: beta-glucosidase; E1.11.1.7: peroxidase; COMT: caffeic acid 3-O-methyltransferase; E3.2.1.2: beta-amylase; TREH: alpha, alpha-trehalase; PYG: glycogen phosphorylase; HK: hexokinase; TPS: trehalose 6-phosphate phosphatase; E2.7.1.4: fructokinase; SPS: sucrose-phosphate synthase; INV: beta-fructofuranosidase; E3.2.1.4: endoglucanase.