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Moderate Drought Stress Interferes with the Physiological State and Promotes the Accumulation of Isoflavone in Reproductive *Iris domestica* Rhizomes

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Abstract: Drought stress is one of the main factors affecting the growth and secondary metabolism of plants. *Iris domestica*, rich in isoflavones, is a common herbal medicine in China. In this study, the effects of drought stress and rehydration on resistance physiological characteristics and the secondary metabolism of two-year-old *I. domestica* during the vegetative and reproductive growth period were investigated. The results showed that the dry weight and fresh weight of rhizomes and roots under severe drought stress were significantly decreased, while those under moderate drought stress were not significantly changed. Meanwhile, the SOD activities, POD activities, MDA content and Pro content increased to resist drought at D1 and D2. In the vegetative growth period, the changes in isoflavone concentration and the expression levels of genes in isoflavone synthesis were more dramatic. Isoflavone accumulation was promoted, to some extent, in the reproductive growth period under the D1 drought treatment. In the actual production process, different measures, namely short-term stress regulation in the vegetative growth period and moderate drought stress (13.44% < soil water content ≤ 16.8%) in the reproductive growth period, need to be adopted to regulate isoflavone biosynthesis.

Keywords: drought stress; *Iris domestica*; physiological characteristics; qRT-PCR

Citation: Ai, Q.; Dai, A.; Han, M.; Yang, L.; Liu, C. Moderate Drought Stress Interferes with the Physiological State and Promotes the Accumulation of Isoflavone in Reproductive *Iris domestica* Rhizomes. *Agronomy* **2024**, *14*, 1730. https://doi.org/10.3390/agronomy14081730

Academic Editor: Michael Fuller

Received: 29 June 2024
Revised: 27 July 2024
Accepted: 2 August 2024
Published: 6 August 2024

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1. Introduction

Iris domestica (L.) Goldblatt et Amberley [syn. *Belamcanda chinensis* (L.) DC.] is a perennial herb of the family Iridaceae, and its dried rhizomes are recorded in the Chinese Pharmacopoeia as a traditional Chinese medicine "ye gan" [1]. There are as many as 13 traditional prescriptions, including Mahuang Decoction, Yegan Xiaodu Decoction, Huangqi Yegan Decoction, etc., containing *I. domestica* herbs, whose traditional indications include treating sore throat by eliminating heat, toxins and phlegm, and dyspnea, and dissolving sputum and relieving wheezing [2]. At present, there are about 45 kinds of Chinese patent medicines containing ye gan with an approval number, including Yegan Oral Liquid, etc., which are mainly used for soothing throat, eliminating phlegm and as an anti-virus [3,4]. The pharmacological effects are mainly due to the isoflavone compounds from *I. domestica*. After investigation, more than 40 kinds of isoflavone monomer compounds have been isolated from *I. domestica* [2,4–6]. Among them, tectoridin, tectorigenin, iridin, irigenin and irisflorentin are the main bioactive components [7]. Moreover, the content of irisflorentin is the quality control index of ye gan medicinal materials stipulated in the Chinese Pharmacopoeia [1]. Importantly, the compounds not only have good clinical efficacy, but also play an important role in plant adaptation to the external environment and stress resistance [8–10].

I. domestica is native to countries in Southeast Asia, such as China and Japan. In China, *I. domestica* is distributed from south to north, and from west to east, where the average annual precipitation of various regions has gradually decreased from 1600 mm to

400 mm [11]. Interestingly, the growth and development of *I. domestica* are good, indicating that *I. domestica* has strong adaptability to water [2]. Therefore, precipitation may be an important ecological factor causing the difference in yield and quality of *I. domestica*. In fact, *I. domestica* isoflavones are an important class of phenylpropanoid secondary metabolites, and the biosynthesis of these compounds is regulated by external elicitors (such as water) [12,13]. Many works have been conducted, proving that a certain degree of drought stress can promote the accumulation of secondary metabolites in medicinal plants [14]. For example, the experiments of Cheng and Yang showed that drought promoted the accumulation of baicalin and saikosaponin [15,16]. Therefore, drought stress is an important tactic to improve the resource creation and quality control technology of *I. domestica* and to expand the production of high-quality *I. domestica*.

When plants are in drought, they are often accompanied by changes in the expression levels of genes, including genes related to the synthesis of phenols [17,18]. For example, drought improves the expression levels of the glycyrrhizin biosynthesis pathway genes, which, in turn, promotes the biosynthesis of root glycyrrhizin [19]. The expression of these key enzyme genes in the metabolic pathway plays an important role in the synthesis of baicalin [20]. Interestingly, Yang found that drought stress did not cause consistent up-regulation of saikosaponin-related genes, and different gene expression patterns differ in different root growth stages [16]. The effects of drought stress on medicinal plants can not only promote gene levels and the accumulation of secondary metabolites, but may also lead to yield reduction [21–23]. So, it is crucial to find a balance point where quality is promoted and yield is minimally affected. Fortunately, in a previous study, a balance point was found in the response of *I. domestica* to drought stress [24], laying a foundation for further in-depth research on *I. domestica*.

In addition, studies have shown that rich isoflavones also exist in the roots, which has potential medicinal value [6,7], but there is no research on water regulation of *I. domestica* roots. Because the traditional harvesting period of Chinese *I. domestica* is in the autumn [2], the dynamic changes in isoflavones in *I. domestica* during the vegetative growth period have not been studied. Therefore, it is of great significance to systematically study the accumulation mechanism of isoflavones in rhizomes and roots under water stress in different growth periods. In this study, the resistance physiological changes (such as osmo-regulators and protective enzymes) and accumulation of isoflavones (tectoridin, iridin, tectorigenin, irigenin, and irisflorentin) were determined during drought stress and rehydration, and the effects of drought stress on the expression of *I. domestica* isoflavone biosynthetic pathway-related genes (*PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F6H* and *IFR*) were analyzed to provide a theoretical basis for the efficient cultivation of *I. domestica* in ecologically suitable areas. This paper is the first comprehensive study on the isoflavones of *I. domestica* rhizomes and roots.

2. Materials and Methods

2.1. Plant Materials

After being identified by Professor Mei Han from Jilin Agricultural University (JLAU), seedlings of *I. domestica* were collected from the Medicinal Botanical Garden of JLAU. The biennial, healthy, and disease-free seedlings were transplanted into planting pots with an inner diameter of 20.5 cm and a pot depth of 14.5 cm. These pots with three plants per pot were placed in a rain-proof shed (43°48′ N, 125°25′ E, JLAU) with a natural photoperiod (approximately 12 h light) from July to August 2021. The consistent growth conditions were as follows: average humidity: 67.81%, average high temperature: 28 °C, average low temperature: 21 °C. The soil used in the experiment was sandy loam containing 248 mg kg^{-1} of available nitrogen, 17 mg kg^{-1} of available phosphorus, and 140 mg kg^{-1} of available potassium. Before the experiment, 150 mL of tap water was pooled into each pot every day for four months.

2.2. Experimental Design

According to the classification of drought [25], the plant was under moderate drought stress when the soil water content was between 13.44% and 16.8%, and the plant was under extreme severe drought stress when the soil water content was lower than 10.08%. In this experiment, 14.0% and 6.5% soil water content drought points were adopted. The drought–rehydration experiment on two-year-old *I. domestica* began on 15 July 2021 and ended on 15 August 2021 (Table 1). Before the experiment, *I. domestica* was divided into 4 plots with a total of 48 pots, with 2 plots in the vegetative growth period (24 flowerpots) and 2 in the reproductive growth period (24 flowerpots). Plants in the vegetative growth period were randomly divided into normal growth group (CK) and treatment group (D1 and D2 drought groups, R1 and R2 rehydration groups). A total of 18 plants (6 pots) were used for each treatment. The CK group was watered with 150 mL tap water every day to keep the soil water content at about 25%, ensuring its normal growth, while the treatment group was watered with 150 mL at one time before the experiment, and then the soil water content naturally decreased, and no more water was added. The soil moisture in the D1 and D2 drought groups gradually decreased to 14% for 2 days (Scheme 1) and 6.5% for 16 days (Scheme 2). The R1 (Scheme 1) and R2 (Scheme 2) rehydration groups slowly recovered for 2 days and 3 days to 25% on the basis of the D1 and D2 drought groups, respectively. When I. domestica grew naturally to August 1, 2021, its growth state was reproductive growth. The same drought–rehydration treatment as that used for I. domestica of the vegetative growth period was implemented. During the experiment, soil water content was measured with a HH2 Soil Moisture Meter (Delta-T Devices Ltd., Cambridge, UK) every day until the end of the experiment. CK, D1, D2, R1 and R2 samples were collected from 8:00 to 9:00 in the morning. Three pots were sampled each time, and each pot represents a biological repeat. The collected samples were placed in ice cubes and quickly brought back to the laboratory. After removing the surface dust and soil, the samples were immediately frozen with liquid nitrogen and stored in a −80 °C refrigerator until use.

Table 1. The soil water content of experimental scheme.

Scheme	Start	D1	R1	D2	R2
CK	25.0	25.0	25.0	25.0	25.0
Scheme 1	25.0	14.0	25.0	-	-
Scheme 2	25.0	14.0	-	6.5	25.0

Note: - stands for no processing.

2.3. Determination of Fresh Weight and Dry Weight

After washing and dry-wiping all parts of the tissues, all samples were weighed using an electronic balance (AUY220, SHIMADZU, Kyoto, Japan) and their fresh weight (FW) was recorded. Subsequently, the roots and rhizomes were placed in a 105 °C oven (XMTD-8222, JINGHONG, Shanghai, China) for 30 min, and then placed in a 60 °C oven to dry to constant weight and the final dry weight (DW) was recorded.

2.4. Determination of Resistance Physiological Indexes

Subsequently, 0.50 g of fresh samples from each group was put in a 10 mL centrifuge tube. Adding an appropriate amount of phosphate-buffered solution (PBS for SOD and POD) or solvent (TCA solution for MDA test and sulfosalicylic acid for Pro test), the mix was fully ground with a manual grinder (D160, DLAB, Beijing, China) for 2 min. After adding PBS or solvent to a final volume of 5 mL, the crude extract was mixed evenly and centrifuged at 12,000 r min^{-1}, 4 °C for 10 min at low temperature. Indicators of the liquid supernatant were measured with a microplate reader (Spectra Max 190, Molecular Devices, LLC, San Jose, CA, USA). SOD activities, POD activities, MDA content and Pro content were analyzed with Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method, catalog number: A001-1), Peroxidase assay kit (catalog number: A084-3-1), Malondialdehyde (MDA) assay kit (Thibabituric acid method, catalog number: A003-3-1), and proline assay

kit (catalog number: A107-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The unit of SOD activities was defined as one SOD activity per milliliter of reaction solution when the SOD inhibition rate reached 50% unit. The unit of POD activities was defined as the amount of enzyme catalyzing 1 μg of substrate per gram of tissue per minute at 37 °C as one unit of enzyme activity. Abscisic acid (ABA) concentration was determined by enzyme-linked immunosorbent assay kit (catalog number: ml002458, mlbio, Shanghai, China) at 450 nm. The absorbance (Y) of ABA standard was used as the ordinate, and the concentration (X) of ABA standard was used as the abscess. The results were in the range of 2.5~80 ng mL^{-1}, and the regression equation was $Y = 0.016X + 0.03333$, $R^2 = 0.9974$. The concentration of ABA was calculated according to the standard curve.

2.5. qRT-PCR on Genes in the Isoflavone Synthesis

Total RNA from *I. domestica* rhizomes and roots was extracted with a Fast Plant RNA Kit For Polysaccharides & Polyphenolics-Rich (catalog number: ZP411C, ZOMANBIO, Beijing, China), following the manufacturer's protocol. RNA purity and concentration were assessed by a P330 nanophotometer (catalog number: P330, Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized by the PrimeScript™ RT Reagent Kit (catalog number: RR047A, Takara Biomedical Technology, Beijing, China). The reaction system and procedures with SYBR Premix Ex TaqII (catalog number: RR820W, TaKaRa, Dalian, China) referred to those described by Cheng [15]. The elongation factor 1 beta (*EF1β*) was used as a reference gene to standardized target gene [26]. A Mx 3000 quantitative PCR instrument (Mx3000P, Agilent, Santa Clara, CA, USA) was used to perform the qRT-PCR assay and calculate the relative expression of the *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F6H* and *IFR* genes using the $2^{-\Delta\Delta Ct}$ method [27], three biological replicates and three technical replicates. These gene sequences are derived from transcriptome data [24]. Primers for each gene were designed by Primer Premier 5.0 and the primers' efficiency is shown in Supplementary Table S1.

2.6. Determination of Isoflavone Concentration

The *I. domestica* dried rhizomes and root powder (0.1000 g) were placed in a conical flask (50 mL), and 25 mL of 75% methanol solution was added. Ultrasonic treatment (frequency 40 kHz, temperature 25 °C) was performed for 30 min. The solution was centrifuged at 3000 rpm min^{-1} for 10 min, filtered. A constant volume of the filtrate to 25 mL was accomplished with 75% methanol solution. The final solution was filtered by 0.22 μm membrane and analyzed by high-performance liquid chromatography (HPLC, 1100, Agilent, Santa Clara, CA, USA). The HPLC analysis was conducted as previously described to determine tectorigenin, tectoridin, irigenin, iridin and irisflorentin contents [28], which were calculated according to the standard curve equation (Supplementary Table S2).

2.7. Statistical Analyses

All results are represented as means ± SE (standard error). All indicators were measured with 3 replicates. Excel 2019 was used for data processing. SPSS software (Version 19.0 for Windows, SPSS, Chicago, IL, USA) was used for principal component analysis (PCA) and Pearson's correlation analysis. As for PCA analysis, SPPS software automatic calculated and standardized the raw data to eliminate dimensional or order-of-magnitude effects. The data were analyzed by analysis of variance (ANOVA) using the least significant differences (LSD), and a $p < 0.05$ was considered significant. DPS software (Version 12.01 for Windows, DPS, Shenzhen, China) was used for Duncan's single-factor variance analysis. A simple graph was drawn with Origin 2018.

3. Results

3.1. Fresh Weight and Dry Weight of Rhizomes and Roots

The changes in soil water content under drought and rehydration conditions are shown in Figure 1. With the decrease in soil water content, the greater the impact of

drought on the dry and fresh weight of *I. domestica* (Figure 2). The D1 drought treatment (approximately 14.0% soil water content) had little effect on the FW and DW of rhizomes in both growth periods ($p > 0.05$, Figure 2A,B). And as the soil water content decreased to about 6.5%, the D2 drought treatment had a significant effect on that (Figure 2A,B). The FW decreased by 14.6% and 33.1% in the vegetative growth period and the reproductive growth period compared with the CK group plants, respectively, and the DW decreased by 9.8% and 24.7%, respectively. The rhizomes results were similar, as the growth of roots was significantly inhibited during the D2 drought period ($p < 0.05$). Interestingly, rehydration had less effect on rhizomes and roots FW and DW (Figure 2C,D).

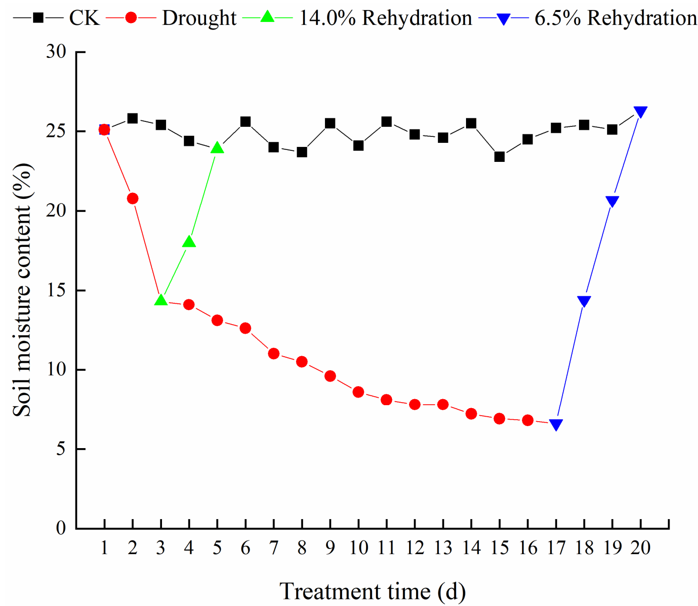

Figure 1. Changes in soil moisture content for control (black squares), drought-stressed (red circles) and rehydration groups (green triangle representing 14.0% rehydration, blue triangle representing 6.5% rehydration).

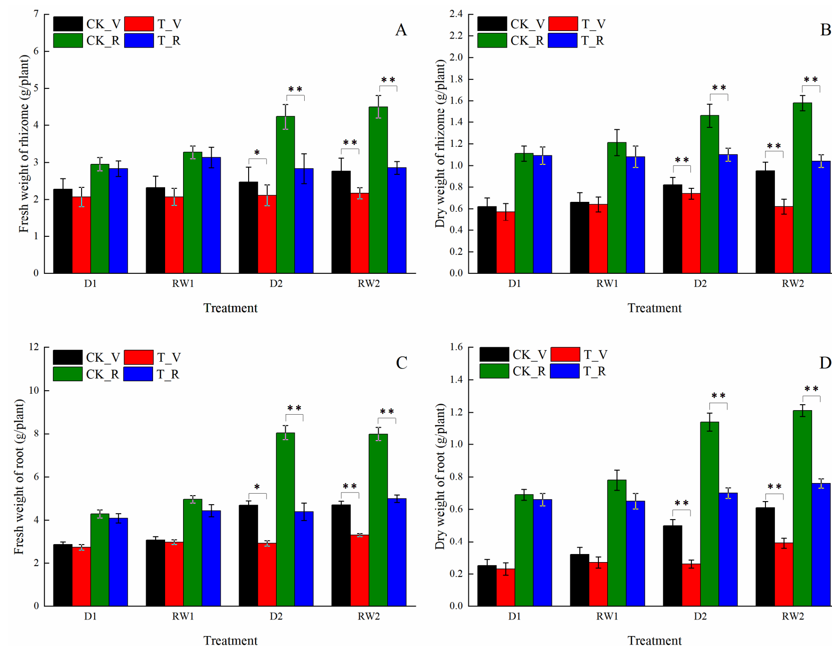

Figure 2. Changes in FW and DW for control, drought-stressed (D1, D2) and rehydration groups (RW1, RW2). (**A**,**B**) are FW and DW of rhizome. (**C**,**D**) are FW and DW of root. The data are expressed

as means ± SDs (n = 3). * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis. CK_V, control plants during vegetative growth (black bar); CK_R, control plants during reproductive growth (green bar); T_V, treatment plants during vegetative growth (red bar); T_R, treatment plants during reproductive growth (blue bar).

3.2. Physiological Responses of Rhizomes and Roots

Compared with the CK group, the ABA concentration in rhizomes at the vegetative growth period changed non-significantly ($p > 0.05$). Both drought and rehydration have significant effects on SOD activities, POD activities, and MDA and Pro accumulation in rhizomes: they significantly increased under drought stress and decreased after re-watering at both growth stages (Figure 3).

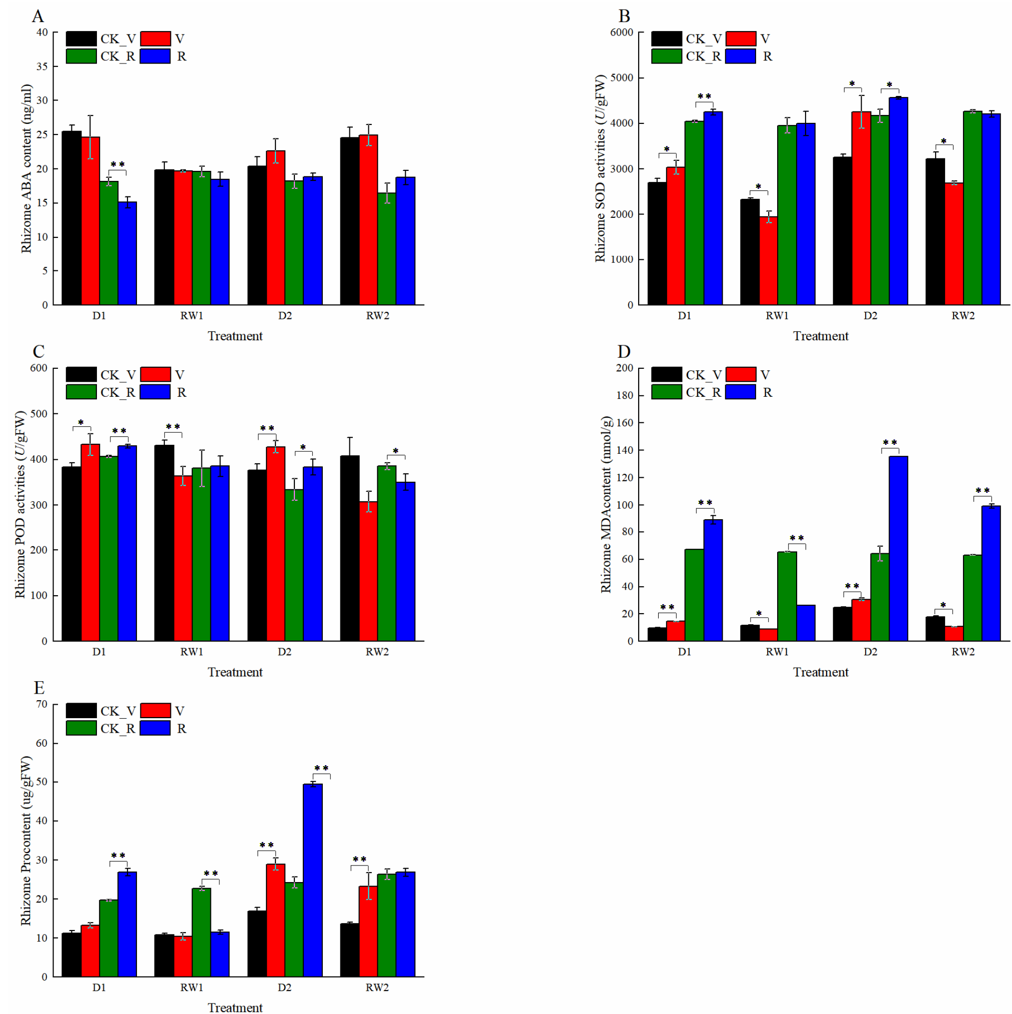

Figure 3. Physiological changes in *I. domestica* rhizome. (**A**), ABA (abscisic acid); (**B**), SOD (superoxide dismutase); (**C**), POD (peroxidase); (**D**), MDA (malondialdehyde); (**E**), Pro (proline); the data are expressed as means ± SDs (n = 3). * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis. CK_V, control plants during vegetative growth (black bar); CK_R, control plants during reproductive growth (green bar); T_V, treatment plants during vegetative growth (red bar); T_R, treatment plants during reproductive growth (blue bar).

For roots, the ABA concentrations increased significantly under the D1 drought point and decreased after re-watering. The trends of roots SOD and POD activities were similar to those of rhizomes during the two growing phases: drought promoted their accumulation,

while rehydration led to their return to normal levels. As regards MDA accumulation, D1 and RW1 treatments have no significant effect on MDA accumulation. More severe droughts (D2 drought point) can promote the accumulation of roots' MDA, but their levels decreased after rehydration. As for vegetative growth roots, Pro accumulation varied between growth periods. During the reproductive period, the roots accumulated proline significantly at the beginning of the drought, while during the vegetative period, they did not (Figure 4).

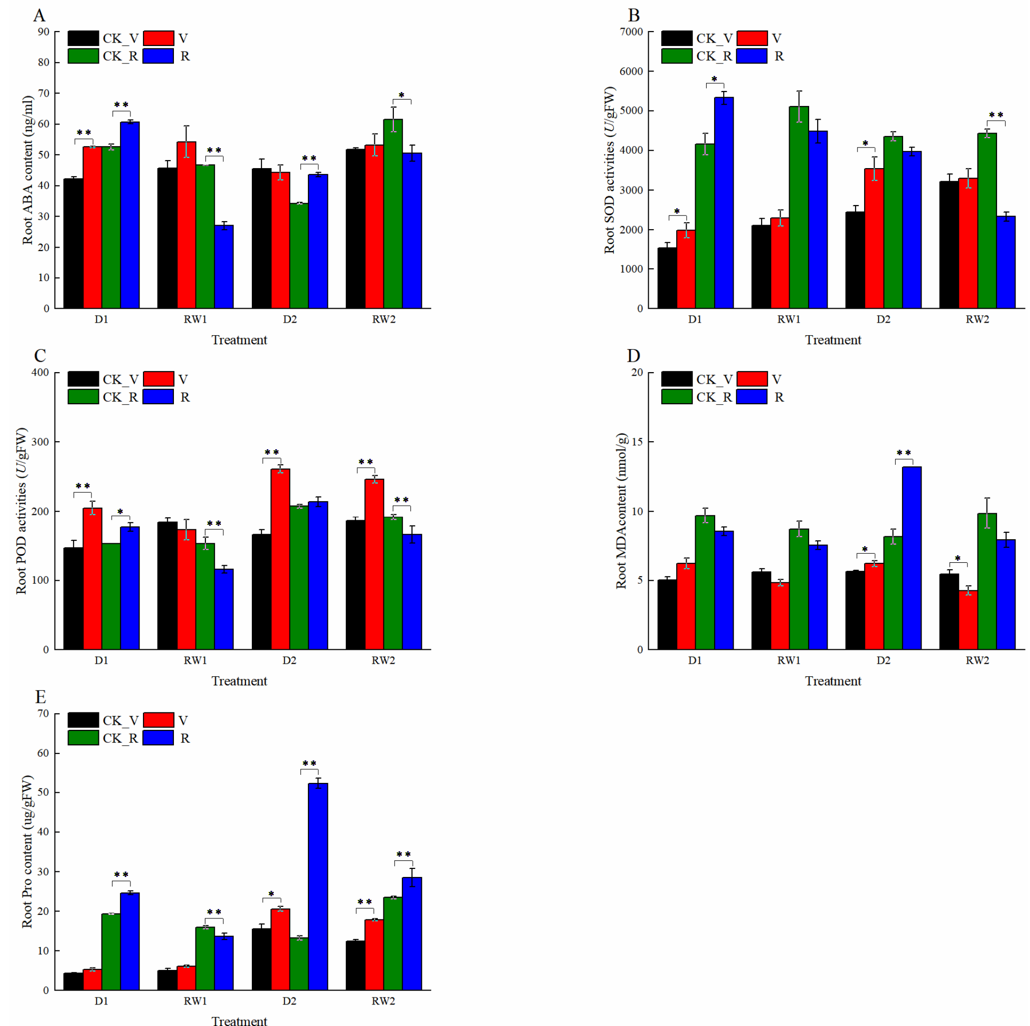

Figure 4. Physiological changes in *I. domestica* root. (**A**), ABA (abscisic acid); (**B**), SOD (superoxide dismutase); (**C**), POD (peroxidase); (**D**), MDA (malondialdehyde); (**E**), Pro (proline); the data are expressed as means ± SD (n = 3, SD means standard deviation). * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis. CK_V, control plants during vegetative growth (black bar); CK_R, control plants during reproductive growth (green bar); T_V, treatment plants during vegetative growth (red bar); T_R, treatment plants during reproductive growth (blue bar).

3.3. Expression of the Isoflavone-Related Key Enzyme Genes

The synthesis of *I. domestica* isoflavones requires two metabolic pathways (the phenylpropanoid pathway and the flavonoid pathway). In the phenylpropanoid pathway, these enzyme genes (*PAL*, *C4H* and *4CL*) of vegetative growth rhizomes could quickly respond to drought stress, and their expression levels at the D1 stage maintained a high level. Compared with vegetative rhizomes, the expression levels of these genes at the D1 drought point were inhibited to varying degrees during the reproductive period (Figure 5, square). In the flavonoid pathway, whether it was vegetative growth or reproductive growth, the

expression of *CHS*, *CHI*, and *F6H* genes was down-regulated at D1, while the expression of *IFR* genes during the vegetative period was up-regulated, which may mean that mild drought stress prompts the flow of flavonoids' metabolic pathways to other branches (Figure 5, square).

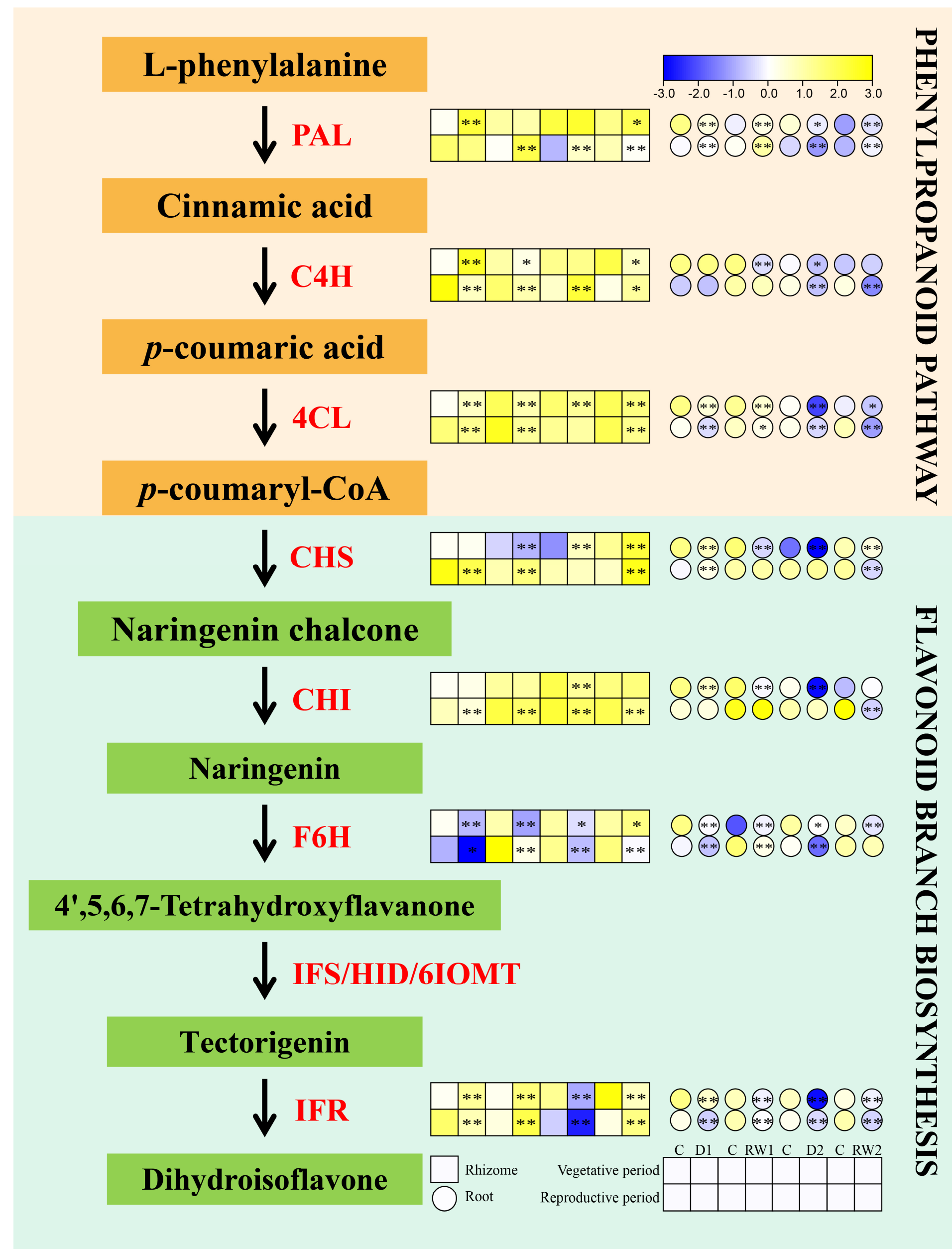

Figure 5. Expression heatmap of rhizome (square) and root (circle) isoflavone-related key enzyme genes (*PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F6H*, *IFR*). Yellow color represents up-regulation, whereas blue color shows down-regulation respect to the control treatment. C, control plants; D1, plants during 14.0% soil moisture stress; RW1, rehydration plants from 14.0% soil moisture; D2, plants during 6.5% soil moisture stress; RW2, rehydration plants from 6.5% soil moisture. *PAL*, phenylalanine ammonia lyase; *C4H*, cinnamate 4-hydroxylase; *4CL*, *p*-coumaroyl coenzyme A ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F6H*, flavonoid 6-hydroxylase; *IFR*, isoflavone reductase. Scale is the $\log_2$ of the mean expression values after normalization (n = 3). Absolute values as well as $\log_2$ values of the transcript expression can be found in Supplementary Table S3. * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis.

For *I. domestica* roots, phenylpropanoid pathway genes were down-regulated under drought and rehydration, except *PAL*, the expression of which was promoted significantly under rehydration (Figure 5, circle). For the phenylpropanoid pathway genes, their expression levels were suppressed in most treatment (Figure 5, circle). In general, rhizomes' isoflavone-synthesis-related genes were up-regulated in response to drought at the D1 drought period, while for roots, it was the opposite, showing differences in response to drought.

3.4. Isoflavone Concentration in Rhizomes and Roots

The medicinal components in *I. domestica* are mainly isoflavones. The changes in five isoflavones, including tectoridin, tectorigenin, iridin, irigenin and irisflorentin, are shown in Figure 6. The D1 and RW1 treatments promoted the accumulation of isoflavones in the vegetative period rhizomes, and the tectoridin, tectorigenin and irigenin were significantly improved. For the reproductive period rhizomes at the D1 point, the contents of each isoflavone compound except iridin were synthesized positively. Instead, the accumulation of five isoflavones levels was hindered by drought and rehydration (Figure 6). And isoflavone amounts in rhizomes, especially under the D2 point, decreased significantly compared to the control.

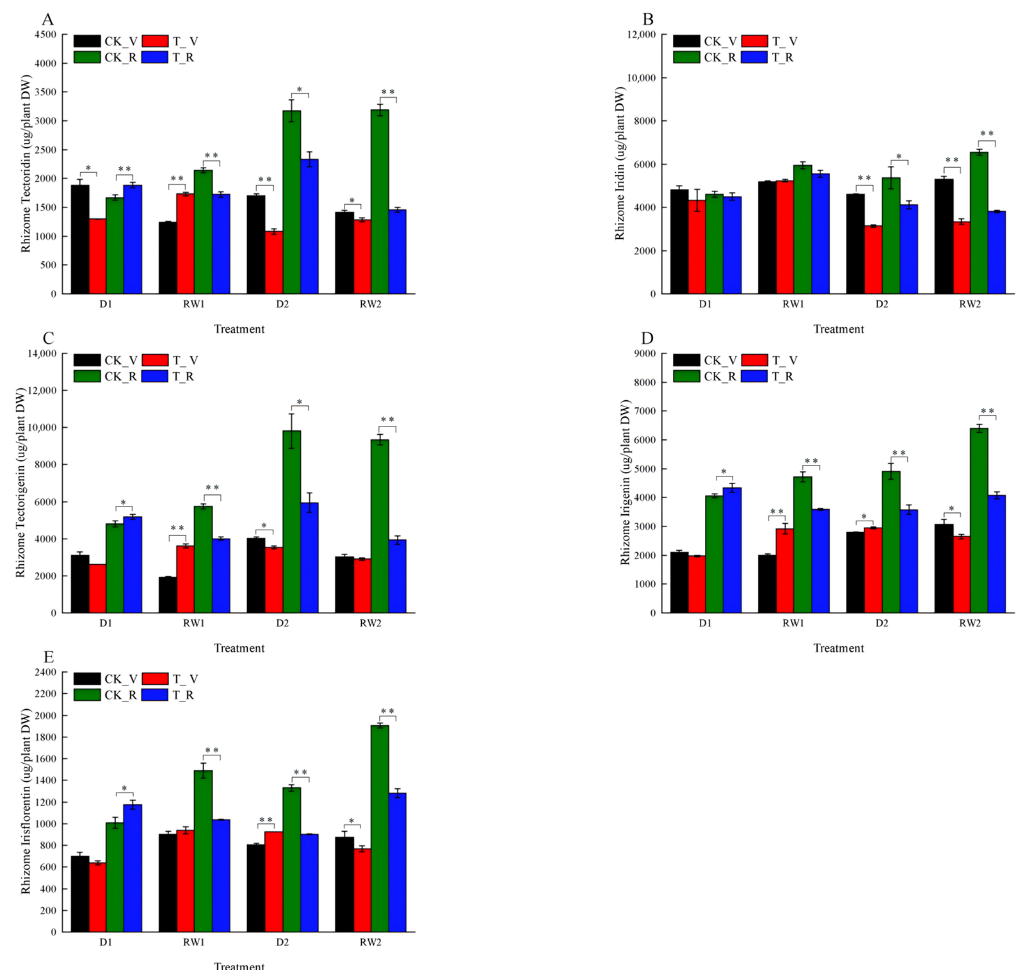

Figure 6. Changes in rhizome isoflavone content. (**A**), tectoridin; (**B**), iridin; (**C**), tectorigenin; (**D**), irigenin; (**E**), irisflorentin. The data are expressed as means ± SD (n = 3). * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis. CK_V, control plants during vegetative growth (black bar); CK_R, control plants during reproductive growth (green bar); T_V, treatment plants during vegetative growth (red bar); T_R, treatment plants during reproductive growth (blue bar).

Studies have shown that *I. domestica* roots are also rich in isoflavones [7]. Therefore, this study also discussed the dynamic changes in roots' isoflavones under drought–rehydration. As shown in Figure 7, we found that the content of five isoflavones in the roots was severely suppressed by drought, and even if the water was rehydrated, it had little effect on its content. Interestingly, although there were differences in the effects of drought and rehydration on the isoflavone content of the rhizomes and roots, there was still a clear pattern: as growth time increased, untreated tissues had higher levels of isoflavones. The above results showed that drought can only promote the accumulation of isoflavones in *I. domestica* to some extent.

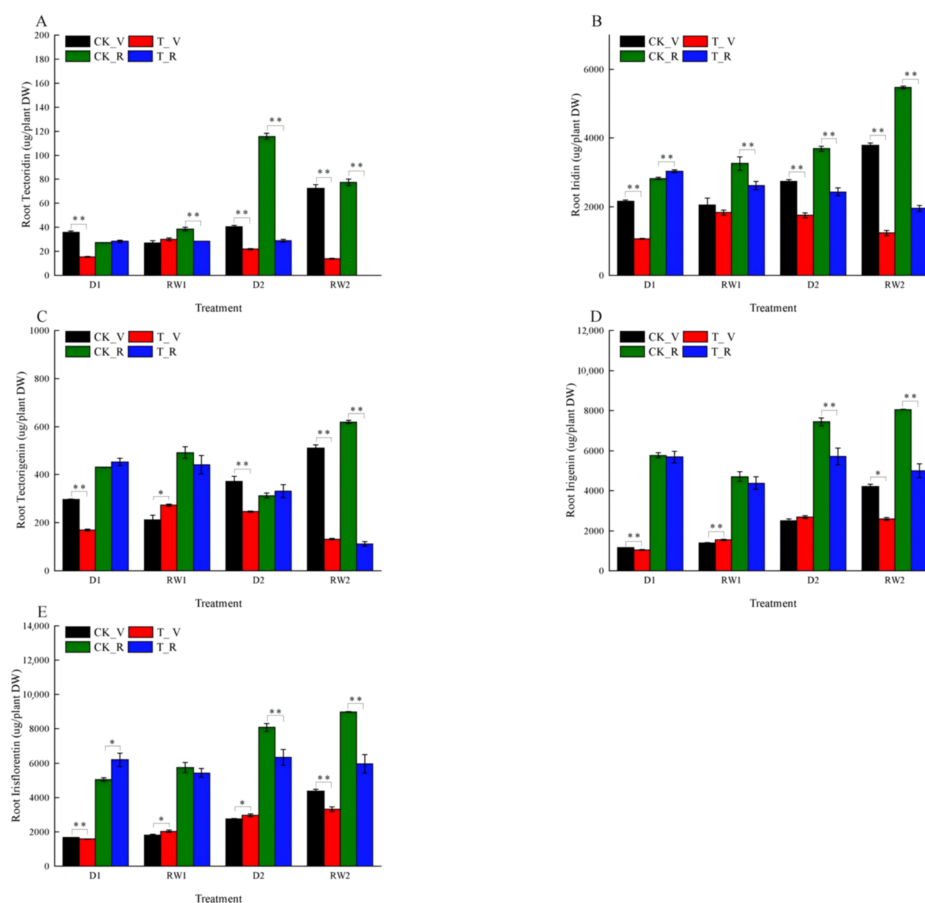

Figure 7. Changes in root isoflavone content. (**A**), tectoridin; (**B**), iridin; (**C**), tectorigenin; (**D**), irigenin; (**E**), irisflorentin. The data are expressed as means ± SD (n = 3). * represents the 0.05 significance level, Duncan's single-factor variance analysis. ** represents the 0.01 significance level, Duncan's single-factor variance analysis. CK_V, control plants during vegetative growth (black bar); CK_R, control plants during reproductive growth (green bar); T_V, treatment plants during vegetative growth (red bar); T_R, treatment plants during reproductive growth (blue bar).

3.5. Principal Component Analysis of Responses in I. domestica to Drought Stress

For rhizome PCA analysis, the distribution of each index was relatively scattered (Figure 8). PCA1 and PCA2 explained 46.1% and 18.2% of the total variability, respectively. The PCA1 axis was positively correlated with tectorigenin, while PCA2 was positively correlated with the remaining four isoflavones, suggesting that these indicators may be involved in the response to drought stress in *I. domestica*. In addition, the rhizomes accumulated more tectoridin, irisflorentin, and iridin under the V_CK_D1, R_D1, R_RW1 treatment from the results of the second quadrant, while other indicators (SOD Pro and MDA) were the opposite.

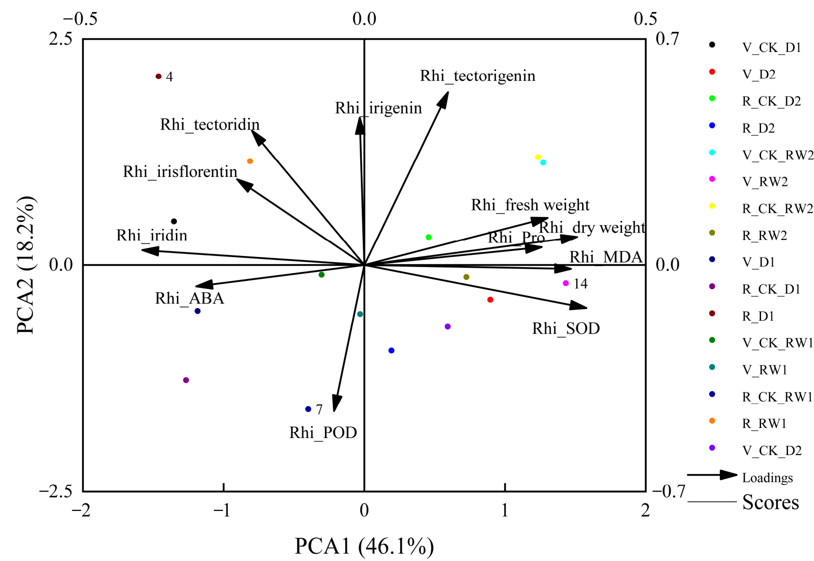

Figure 8. Rhizome principal component analysis (PCA) biplot of the first two PCA axes for proline (Pro), abscisic acid (ABA), malondialdehyde (MDA), superoxide dismutase (SOD), peroxidase (POD), fresh weight (FW), dry weight (DW), tectoridin, iridin, tectorigenin, irigenin, and irisflorentin. Differently colored dots represent different plant groups.

For root PCA analysis, the distribution of each indicator is relatively concentrated. PCA1 and PCA2 explained 50.5% and 15.3% of the total variability, respectively (Figure 9). The PCA1 axis was positively correlated with irigenin and irisflorentin, and the PCA2 axis was positively correlated with tectoridin and iridin. In addition, tectorigenin, iridin, and tectoridin indicators were located in the third quadrant, farther away from other treatment groups, indicating that the treatments were not conducive to the accumulation of these compounds. And similar to the results of rhizomes, both SOD, Pro and MDA were clustered in the positive axis of PCA1 and strongly correlated with each. This evidence indicated that osmotic regulators and the protective enzyme system were important factors for the strong drought resistance in *I. domestica*.

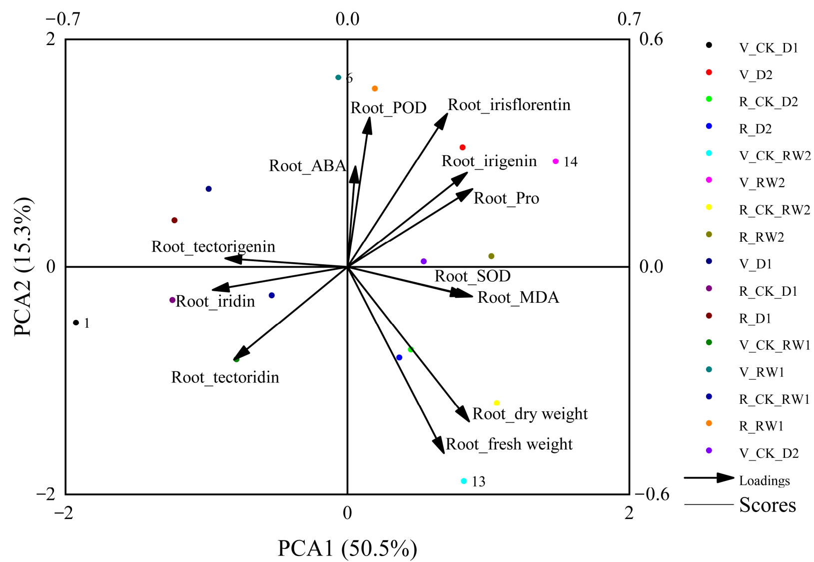

Figure 9. Root principal component analysis (PCA) biplot of the first two PCA axes for proline (Pro), abscisic acid (ABA), malondialdehyde (MDA), superoxide dismutase (SOD), peroxidase (POD), fresh weight (FW), dry weight (DW), tectoridin, iridin, tectorigenin, irigenin, and irisflorentin. Differently colored dots represent different plant groups.

4. Discussion

4.1. Differences in Dry and Fresh Weights in Response to Drought

Medicinal plants are rich in many secondary metabolites that can cure diseases, and have high economic value [29]. In medicinal plant cultivation, a higher yield may mean more secondary metabolites; so, yield is an important economic indicator for plants. In this study, D1 drought stress (moderate drought) had no significant effect on the FW and DW of rhizomes and roots of *I. domestica*, whether in the vegetative growth period or the reproductive growth period. Further, the FW and DW under D2 drought stress were significantly reduced. Therefore, excessive drought stress should be avoided in the actual production of *I. domestica* to reduce losses. Interestingly, drought had a greater effect on roots, as they had a greater reduction in yield at the D2 stage than rhizomes; the same results were also observed in another study [30]. In fact, the function of roots is to absorb soil nutrients and transport them to other organs. It is therefore hypothesized that when faced with an existential threat (extreme drought stress), plants are more willing to allocate resources to organs (rhizomes) for reproduction rather than to the roots [30].

4.2. Differences in the Synthesis of Isoflavonoids for Rhizomes and Roots

The accumulation and synthesis of multifunctional secondary metabolites are tissue-specific and spatiotemporally specific, thereby ensuring a high degree of adaptation to the environment or offsetting the costs incurred by secondary metabolites [31–33]. This also suggests that plant secondary metabolite constituents and concentration may vary at the individual and population levels, as well as across ontogeny stages and tissue types [31]. At present, many studies have confirmed this conjecture. For example, the total content of ginsenoside in the main roots of ginseng is generally higher than that in the lateral roots. And the total content of ginsenosides increased with ginseng age in roots [34]. Piper unripe pulp and ripe pulp had the highest alkenylphenol content and highest diversity, while the flowers had less than pulp; leaves and seeds had only a few detectable concentration of the compound [33]. There are more saponins and fewer flavonoids in *Bupleurum chinensis* [16]. In this study, the isoflavone content of different tissue parts of *I. domestica* was significantly different (Figures 6 and 7). In rhizomes, the content of tectoridin and tectorigenin was much higher than that in roots; the content of iridin was not much different; the content of roots' irigenin was slightly higher than rhizomes', and the content of roots' irisflorentin was much higher than rhizomes'. These results are more consistent with the work of Tian [7].

4.3. Dynamic Changes in Isoflavones at Different Growth Phenological Stages

In addition to the above, the concentration of secondary metabolites differed in different growth stages. In this paper, compared with the vegetative growth period, the concentration of tectoridin, tectorigenin, irigenin, and irisflorentin in rhizomes and that of irigenin, and irisflorentin in roots were all increased. And the only isoflavone copromoted between roots and rhizomes in the reproductive phase is irisflorentin. This is in contrast to the results of Yang, which showed no difference in secondary metabolites, such as saikosaponins during *Bupleurum chinense*'s vegetative growth and reproductive growth periods [16], indicating that the secondary metabolites of different medicinal plants are different in different phenological stages. Meanwhile, vegetative growth tissues contained more isoflavones than reproductive growth tissues, meaning plants invest more resources (chemical defense) in younger tissues, possibly because younger tissues are more susceptible to disturbance than older plants [35–37]. It is speculated that the distribution pattern of isoflavones in rhizomes and roots may be related to the expression of key enzyme genes for isoflavone synthesis, which requires further analysis [6].

4.4. Differences in Transcript Changes Associated with Isoflavone Synthesis

Rogers provided cell-type-specific transcriptomic profiles demonstrating that complex gene regulatory networks occur at the cellular level, resulting in cell-type-specific expression of many proteins involved in secondary metabolism [38]. Groenenboom et al. studied

the flavonoid pathway in tomato seedlings and found that changes in transcript abundance lead to changes in enzymes that affect the accumulation of metabolites [39]. Analyzing the response of rhizomes and roots to drought at the transcriptional level is helpful to better understand the mechanism of plant drought resistance, and to preliminarily elucidate the differences in isoflavone accumulation in rhizomes and roots. The drought response patterns of rhizomes (*PAL*, *C4H*, *4CL* genes, etc.) in different growth stages were different. The expression of the *PAL* gene, as well as that of *C4H* and *4CL*, in vegetative-growth-period rhizomes was up-regulated in the D1 drought stage, while it was down-regulated in D2. This may lead to differences in isoflavone accumulation in different tissues of *I. domestica*. Yang's research supported the results in this paper [16]. In addition, *I. domestica* roots in the vegetative growth period were more sensitive to drought. And the concentration of isoflavones and their synthesis-related enzyme genes was inhibited under moderate drought (D1 drought stage). In the rehydration stage (RW1), concentration and gene expressions can be promoted. During the reproductive growth period, the expression of the *PAL* and *CHS* genes in roots was up-regulated in response to drought. And the isoflavone content also increased correspondingly. However, rehydration was not conducive to gene transcription regulation and isoflavone biosynthesis. Two diametrically opposed results may be differential responses of *I. domestica* to drought. It seemed that this was inconsistent with the Growth/Differentiation Balance (GDB) hypothesis [40] because cells do not differentiate below drought by differentiating isoflavones under moderate drought.

At the same time, resistance physiological indicators (ABA, SOD enzyme, Pro) related to drought resistance were improved. Therefore, we speculate that isoflavones have a limited effect on the drought resistance of *I. domestica* (especially during the vegetative growth period). Although some studies have reported that *I. domestica* isoflavones have a strong scavenging ability of reactive oxygen species, these experiments are only in vitro experiments and cannot represent the plant body. Interestingly, some studies have reported that isoflavones have anti-trypanosome activity [41] and rust fungus [42], which further indicates that the role of *I. domestica* isoflavones is not only scavenging reactive oxygen species. On the other hand, this phenomenon may be one of the measures for plant secondary metabolites to reduce the cost of their production [43]. The PCA analysis results also showed that isoflavones were mainly positively correlated with the PCA2 axis, explaining a small part of the total variance, further indicating that isoflavones played a secondary role in drought resistance in *I. domestica*.

4.5. Resistance Physiological Changes in Drought and Rehydration

More studies show that ROS is a double-edged sword in that too much and too little are not conducive to the healthy growth of plants [44]. In most plants, plant cells accumulate reactive oxygen species under drought stress, leading to metabolic disorders [45]. Under long-term evolution pressure, plants adopt enzymatic systems (SOD, POD, etc.) and non-enzymatic systems (Pro, etc.) to scavenge reactive oxygen species to maintain normal cellular activity [46]. In this study, MDA content was significantly increased, which indicated that drought stress caused damage to plant cells. Moreover, SOD enzyme activity, POD enzyme activity and proline content were significantly increased under drought stress, which indicated that the above two systems existed in *I. domestica* to resist drought stress. These results are consistent with those of previous studies [9]. In addition to the role of clearing reactive oxygen species in rhizomes and roots, POD enzymes can also catalyze the reduction of H_2O_2 by taking electrons to phenolic molecules such as isoflavones. This may be the reason for the reduction in rhizomes' and roots' isoflavone levels due to severe drought stress. In addition, ABA may be involved in regulating the drought resistance mechanism of roots from the PCA results. The above research results will be helpful for the ecological planting of medicinal plant and the development and utilization of non-medicinal parts of *I. domestica*.

5. Conclusions

In this study, moderate drought stress (14% soil water content) balanced cell homeostasis with minimal plant growth impact, enhanced protective enzyme activity and osmotic substance levels, and increased isoflavone buildup. Therefore, soil moisture can be adjusted appropriately to put the herb into a stress state to increase the quality of medicinal materials during the cultivation of *I. domestica*. In addition, roots, rich in isoflavones, have great development prospects, and should be harvested together with rhizomes. The results lay a foundation for the study of stress cultivation and drought resistance of *I. domestica*. Unfortunately, only biennial plants were researched in this study, and whether triennial individuals have the same growth and development regularity has not been explored, which warrants further investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14081730/s1, Table S1: Primers used for qRT-PCR; Table S2: Regression analysis of rhizome and root isoflavones; Table S3: Absolute values as well as $\log_2$ values of the transcript expression.

Author Contributions: Conceptualization, L.Y. and M.H.; methodology, C.L. and A.D.; software, Q.A.; validation, Q.A., A.D. and C.L.; formal analysis, Q.A.; investigation, Q.A.; resources, M.H.; data curation, C.L.; writing—original draft preparation, Q.A.; writing—review and editing, A.D.; visualization, Q.A.; supervision, L.Y.; project administration, M.H.; funding acquisition, Q.A., C.L. and L.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Modern Agricultural Industrial Technology System Project (CARS-21), National Natural Science Foundation of China (31200178), Science and Technology Development Plan of Jilin Province (20210204184YY), and Talent introduction program SKYJ202303 (200425).

Data Availability Statement: The original contributions presented in the study are included in this article; further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors acknowledge Ping Di, Yingxin Sun, Meng Zhang, Zeliang LÜ for their support in this experiment.

Conflicts of Interest: The authors declare no conflicts of interest.

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