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Selenium Regulates Antioxidant Capacities and Diterpenoid Biosynthesis in the Medicinal Plant *Isodon rubescens*

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ABSTRACT

Dōng líng cǎo, the dried aboveground parts of *Isodon rubescens* (Hemls.) Hara., is commonly consumed as a medicinal decoction or tea beverage. Natural beverages can be an important source of human dietary selenium (Se). However, how *I. rubescens* plants respond to exogenous Se remains unknown. In this study, a pot cultivation experiment was employed to investigate the phenotypic and physiological responses of *I. rubescens* plants exposed to Se. Fifteen days after applying different concentrations of sodium selenate to the soil, the Se enrichment capacity, growth indices, antioxidant capacities, and the content of flavonoids and diterpenoids were measured in the plants. Further, the oridonin content was quantified using the high-performance liquid chromatography method, and the expression levels of key diterpenoid synthesis genes were analyzed by quantitative real-time PCR (qRT-PCR). *I. rubescens* plants efficiently accumulated Se, with the Se content increasing proportionally to the applied dose, reaching levels of nearly 200 mg·kg^{-1} dry leaves as Se concentration increased. None of the three Se treatments significantly altered the phenotypic indices, except a longer root length occurred in the 3 μM·kg^{-1} Se group. Among three Se doses, 6 μM·kg^{-1} Se gave the highest accumulation of flavonoids, diterpenoids, and oridonin, with the increase of 2.0-, 1.8-, and 1.9-fold in aboveground parts, respectively. Selenium application boosted the activities of antioxidant enzymes and antioxidant capacities according to 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric reducing/antioxidant power, and tea brewing color experiments. Four key synthase genes were upregulated significantly by 6 μM·kg^{-1} Se treatment, notably 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IrDXR), with a 5-fold increase, and kaurene synthase-like 4 (IrKSL4), with a 6-fold increase. Thus, Se application in *I. rubescens* cultivation may be a potential biofortification method to supplement Se while increasing flavonoid and diterpenoid contents.

KEYWORDS

Flavonoids; oridonin; physiological indicators; quantitative real-time PCR; selenium

1 Introduction

Since first discovered in the human body in 1817 [1], selenium (Se) has been extensively studied for its importance to human health [2−4]. Humans mainly acquire Se from foods and natural beverages [5,6]. Plants can absorb inorganic Se from the soil and metabolize it into organic forms [7]. The magnitude of Se enrichment by plants is directly associated with the Se abundance and forms in the soil [8−10]. However, under anticipated climate change and Se soil decreases, deficient dietary Se uptake is expected in approximately 0.7 billion people [11]. Accordingly, irrigation [12], foliar spraying [13], and seed priming [14] with exogenous Se are efficient ways to biofortify plant-based foods.

When exposed to exogenous Se, plants manifest multifaceted responses, including phenotypic, biochemical, and stress-resistance responses [15,16]. In addition, Se enrichment has been shown to modulate the secondary metabolisms of plants [17,18]. In recent years, as concerns regarding dietary nutrients have increased, the secondary metabolites in plants treated with Se have garnered broad research attention. In particular, Se can regulate flavonoid and terpenoid accumulation in plants [19,20]. However, these studies have mostly been conducted in crops, and the associated response mechanisms have not been elucidated.

Medicinal plants are usually consumed as decoctions or tea beverages for the uptake of bioactive compounds, providing another approach to supplement human dietary Se uptake. *Isodon rubescens* (Hemls.) Hara., with its aboveground parts called dōng líng cǎo in Mandarin, is a plant in the family Labiatae with medicinal and food value. This plant has a long history of being used as a wild vegetable and tea beverage in China [21,22]. Dōng líng cǎo has been proven effective in treating various diseases, including sore throat, tonsillitis, bronchitis, chronic hepatitis, joint rheumatism, and various cancers [23]. Owing to its high efficacy and broad usage, dōng líng cǎo-derived products have an annual commercial value reaching nearly $100 million in China [22]. Chromatographic analysis revealed that diterpenoids, phenolic acids, and flavonoids are the major components of dōng líng cǎo [24]. The ent-kaurane diterpenoid oridonin has been identified as a particularly important monomeric compound due to its anti-cancer and anti-inflammatory activities [22]. Oridonin biosynthesis in *I. rubescens* is regulated by methyl jasmonate [25], suggesting that it responds to external stimuli. Previous research has mostly focused on field crops, with a limited understanding of how Se enrichment affects secondary metabolisms in medicinal plants like *Isodon rubescens*. As a pillar-like medicinal plant, it is still unclear whether *I. rubescens* plants could take up and become enriched in Se as field crops. Whether Se enrichment is involved in diterpenoid metabolism regulation also needs to be investigated. Thus, the influence of external Se on *I. rubescens* plants may facilitate the creation of Se-biofortified dōng líng cǎo with more bioactive compounds, further improving the cultivation efficiency of this medicinal plant.

The hypothesis of this study is that *I. rubescens* accumulates more secondary metabolites while becoming enriched from exogenous Se. Thus, the present study investigated the response of *I. rubescens* plants to Se by measuring the resulting biochemical indicators and content of flavonoids, terpenoids, and oridonin. In addition, the mRNA levels of key synthase genes for terpenoid biosynthesis were quantified to explain the possible regulation mechanisms involved in Se enrichment. The results will help to develop high-quality dōng líng cǎo with high added value.

2 Materials and Methods

2.1 Materials and Reagents

I. rubescens seeds were purchased from Xiaxian County Shuanglian Traditional Chinese Herb Cultivation Specialized Cooperative, Yuncheng City, Shanxi Province, China.

Sodium selenate and standard Se were purchased from companies as described in our previous study [14]. Standard references for oridonin and rutin were obtained from Chengdu Purechem-Standard Co.,

Ltd. (Chengdu, China). Unless otherwise mentioned, all reagents used were the products of China National Pharmaceutical Industry Co., Ltd. (Beijing, China).

2.2 Experimental Design and Culture Conditions

The experiment was performed in the greenhouse facilities of Anhui Provincial Engineering Research Center for Efficient Utilization of Featured Resource Plants, Anhui, China (E116.8, N34.0). The surface-sterilization of seeds were carried out with 0.1% aqueous mercuric chloride solution (w/v). Then the seeds were spread on a seedling tray filled with nutrient soil (turf: coir: sandy loam: perlite: vermiculite, 5:3:3:1:1 [v:v:v:v:v], Huai'an Zhonghe Agriculture Co., Ltd., Huai'an, China). The culture conditions were set at 25°C and 60% relative humidity. The illumination condition was 16 h light/8 h dark, with a light intensity of 50 μmol photons $m^{-2}s^{-1}$ provided by cool white fluorescent lights.

At an approximate height of 10 cm, the seedlings were moved to 4 L-pots (20 cm in height) with commercial nutrient soil. Each pot contained 25 seedlings. Two weeks later, Se treatment was conducted by adding Na_2SeO_4 solution (150 mL) to the soil to reach final soil Se contents of 3, 6, and 9 $μM \cdot Kg^{-1}$ Se, with other seedlings irrigated with an equal volume of water as controls. The Se concentration range was set according to previous studies [12,15]. Plants were given 150 mL of running water to prevent Se from leaching out of the soil. After 15 d of growth, the plant materials were analyzed. After measuring the lengths of shoots and roots, some of the samples were vacuum-dried at 50°C to determine the dry weight and flavonoid and diterpenoid contents. The other samples were immediately used for measurement or stored at −80°C for physiological and gene expression analysis.

2.3 Assay of Antioxidant Capacities

The activities of antioxidant enzymes were determined as described previously [26]. The fresh leaf powder was extracted with an ice-cold extraction buffer to obtain a plant enzyme extract, and the protein content was quantified following the Bradford method [27]. Enzymatic activity was measured in the plant extract solution. Antioxidant capacities were assayed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) as described previously [28].

A tea brewing experiment was further conducted to visualize the antioxidant capacity. For each treatment, 0.2-g samples of dried leaves were immersed in 100 mL of drinking water for 12 h. Then, the final color obtained was observed and imaged using a Nikon D700 camera (Nikon, Tokyo, Japan).

2.4 Measurement of Total Flavonoids

Flavonoid content was measured following a previously published method [29]. The flavonoids were extracted by mixing 100 mg of the powder derived from the vacuum-dried aboveground parts with methanol. After 10 min of extraction at room temperature with vortexing (100 $r \cdot min^{-1}$), the mixture was centrifuged to isolate the extract supernatants. The pellets were subjected to a second extraction with methanol. Two replicates were combined for flavonoid analysis. The extracts were reacted with $NaNO_2$ and $AlCl_3$ to measure total flavonoids, and the reaction was terminated with NaOH before recording the absorbance of the sample at 510 nm (A_{510}) with a spectrophotometer. The content was calculated by substituting A_{510} into a calibration curve for rutin based on a gradient concentration series, and the results were recorded as mg rutin equivalents per 1 g sample ($mgRE \cdot g^{-1}DW$).

2.5 Quantification of Diterpenoids

Total diterpenoids were measured by ultraviolet spectrophotometry [30]. For each sample, diterpenoids were extracted from 200 mg of powder obtained from the vacuum-dried aboveground parts by dissolving it in 3 mL of 95% ethanol. The absorbance of the extract solution at 238 nm was determined to calculate the total diterpenoid content against a calibration curve based on the oridonin standard.

High-performance liquid chromatography (HPLC) was used to quantify oridonin content based on our previously published method for measuring diterpenoids [31]. The measurement was done with an Agilent Technologies 1260 Infinity Series HPLC system (Agilent Technologies, Santa Clara, CA, USA). Solvent A was 0.1% acetic acid in water (*w:w*), and solvent B was 100% methanol. The gradient elution was adjusted linearly from 40% to 45% B from 0–6 min, held at 45% B from 6–35 min, and adjusted linearly from 45% to 95% B from 35–45 min. The oridonin content was calculated by comparing the peak area with that of the oridonin standard by linear regression and presented as a percentage of dry weight (DW). Each treatment contained three parallel replicates.

2.6 Determination of Se Content by Atomic Fluorescence Spectroscopy

The Se content in the roots, stems, and leaves of *I. rubescens* plants was determined following a previously published method [14]. Plant Se content was obtained by comparing the fluorescence intensity of the plant extract with a Se standard curve.

2.7 qRT-PCR Analysis

The mRNA levels of key diterpenoid synthase genes, 1-deoxy-d-xylulose 5-phosphate synthase (*IrDXS*), 1-deoxy-d-xylulose 5-phosphate reductoisomerase (*IrDXR*), copalyl diphosphate synthase 5 (*IrCPS5*), and kaurene synthase-like 4 (*IrKSL4*), were quantified by real-time quantitative PCR (qRT-PCR). mRNA isolation, cDNA synthesis, and qRT-PCR were carried out according to a previously published method [31]. The *actin* gene was used to normalize the expression levels. The primers and gene sequences listed in Table S1 were obtained from previously published references [32,33].

2.8 Data Analysis

The experiment contained three parallel replicates. Statistical analyses to identify differences between the control treatment without Se (CK) and Se treatments were conducted by analysis of variance (ANOVA) in IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA) using Duncan's multiple-range test ($p < 0.05$).

3 Results

3.1 Isodon rubescens Plants Accumulate Exogenous Se

When treated with 3–9 $\mu M \cdot Kg^{-1}$ Se, *I. rubescens* plants differentially accumulated Se in the roots, stems, and leaves. A two-way ANOVA showed a significant interaction between Se dose and plant tissue (Table S2). Se-treated *I. rubescens* plants had 10.8–23.1-fold, 27.3–45.8-fold, and 13.5–27.2-fold increases in Se content in the roots, stems, and leaves, respectively, compared with control ($p < 0.05$) (Fig. 1). Concerning various tissues, Se accumulation capacity was greatest in the roots, which was higher than that of leaves and stems ($p < 0.05$). While the relative Se content was not significantly different between stems and leaves treated with CK and 3 $\mu M \cdot Kg^{-1}$ Se ($p > 0.05$), it was higher in leaves than stems treated with high doses of Se ($p < 0.05$). Se accumulation increased with increases in Se treatment concentrations, with the relative Se content reaching 194.7 $mg \cdot kg^{-1}$ DW in leaves treated with 6 $\mu M \cdot Kg^{-1}$ Se and 247.7 $mg \cdot kg^{-1}$ DW with 9 $\mu M \cdot Kg^{-1}$ Se treatment. It suggests that exogenous Se application during *I. rubescens* cultivation is a potential approach to increasing human dietary Se uptake.

3.2 Se Does not Affect the Phenotype of I. rubescens

Isodon rubescens plants treated with Se grew normally (Fig. 2A), except scattered grayish spots were observed on the leaves of plants treated with 9 $\mu M \cdot kg^{-1}$ Se. Se treatment did not change the plant height (Fig. 2B), shoot weight (Fig. 2D), or root weight (Fig. 2E) ($p > 0.05$). However, increased root length was observed under 9 $\mu M \cdot kg^{-1}$ Se treatment (Fig. 2C) ($p < 0.05$) compared with the controls. As leaf spots appeared under 9 $\mu M \cdot kg^{-1}$ Se treatment, plants were inferred to be impaired by the highest level of

exogenous Se tested. Thus, a suitable Se concentration would not compromise *I. rubescens* plant growth but might improve plant vigor by increasing root length.
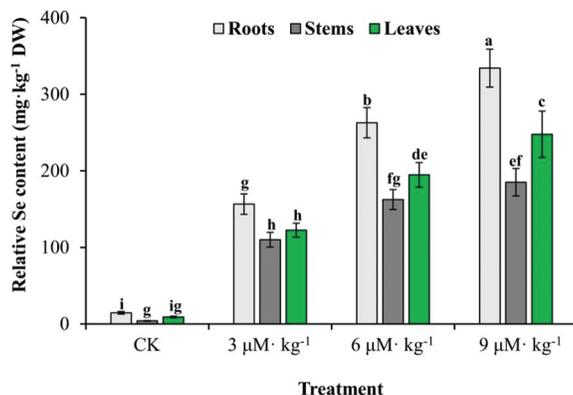

Figure 1: Se accumulation in various tissues of Se-treated and non-treated (CK) *Isodon rubescens* plants. Error bars indicate the standard error of the mean (n = 3). Values marked by different lowercase letters differ significantly using Duncan's multiple range test ($p < 0.05$)
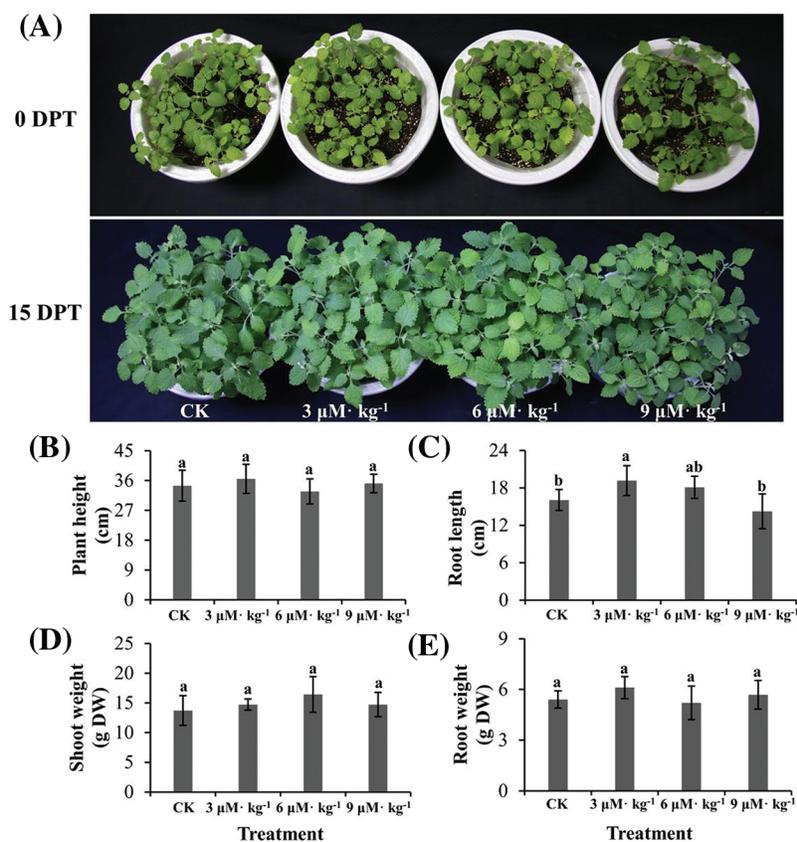

Figure 2: Phenotypic analysis of Se-treated and non-treated (CK) *Isodon rubescens* plants. (A) Plants without Se treatment or treated with 3, 6, or 9 $\mu M \cdot kg^{-1}$ Se. DPT, days post-treatment. (B) Plant height. (C) Root length. (D) Shoot weight. (E) Root weight. The data were collected on day 15 of treatment. Error bars indicate the standard error of the mean (n = 3). Values marked by different lowercase letters differ significantly within each panel ($p < 0.05$)

3.3 Se Improves Flavonoid and Diterpenoid Accumulation in I. rubescens

Selenium treatment significantly increased total flavonoid accumulation in *I. rubescens* plants, with increases of 1.8-, 2.0- and 1.5-fold by 3, 6, and 9 $\mu M \cdot kg^{-1}$ Se treatments, respectively ($p < 0.05$) (Fig. 3A). This indicates that Se regulates flavonoid metabolism in *I. rubescens* plants.
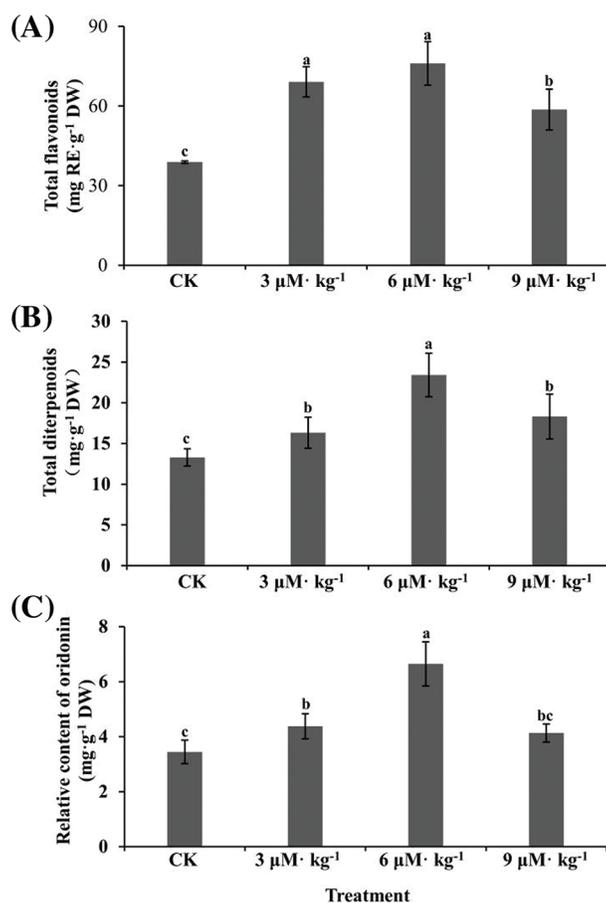

Figure 3: Total flavonoids (A), total diterpenoids (B), and relative oridonin content (C) in the aboveground parts of Se-treated and non-treated (CK) *Isodon rubescens* plants. Error bars indicate the standard error of the mean (n = 3). Values marked by different lowercase letters differ significantly within each plot ($p < 0.05$)

Similarly, the total diterpenoid content was also enhanced by all three Se treatments ($p < 0.05$) (Fig. 3B), with a 1.8-fold increase by 6 $\mu M \cdot kg^{-1}$ treatment. The content of the major diterpenoid oridonin was quantified by HPLC (Fig. S1). Treatment with 3 and 6 $\mu M \cdot kg^{-1}$ Se significantly promoted oridonin accumulation (Fig. 3C), with a 1.9-fold increase by 6 $\mu M \cdot kg^{-1}$ Se treatment but not 9 $\mu M \cdot kg^{-1}$ Se treatment. Thus, suitable concentrations of exogenous Se could modulate the content of flavonoids and diterpenoids in *I. rubescens*.

3.4 Se Enrichment Increases Antioxidant Capacity in I. rubescens

SOD activity was increased by all three Se treatments ($p < 0.05$) by 1.3–2.0-fold compared to CK treatment (Fig. 4A). Similar patterns were observed in POD (Fig. 4B) and CAT activities (Fig. 4C). The antioxidant capacity under all three Se treatments increased significantly compared with CK treatment, as indicated by the DPPH (Fig. 5A) and FRAP assays (Fig. 5B). The tea brewing color under all three Se treatments was lighter than that of CK treatment, particularly that of 6 $\mu M \cdot kg^{-1}$ Se treatment (Fig. 5C).
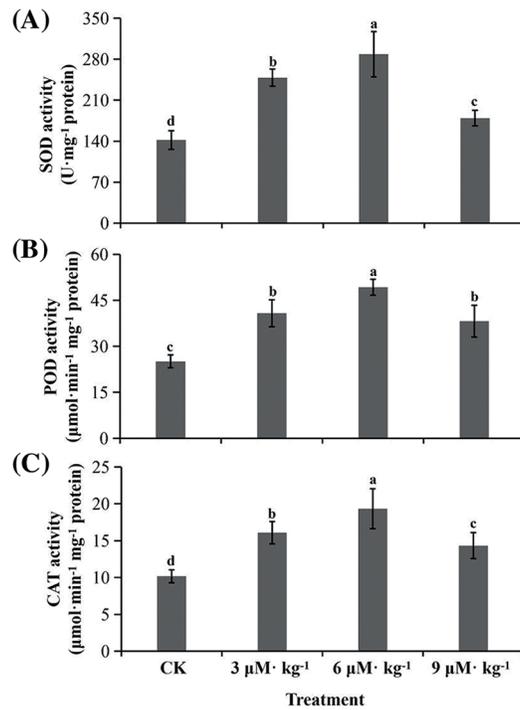
Figure 4: Total flavonoids and antioxidant enzymatic activity in Se-treated and non-treated (CK) *Isodon rubescens* plants. (A) Superoxide dismutase (SOD) activity. (B) Peroxidase (POD) activity. (C) Catalase (CAT) activity. Error bars indicate the standard error of the mean (n = 3). Values marked by different lowercase letters differ significantly within each plot ($p < 0.05$)

Figure 5: Antioxidant capacity assay of Se-treated and non-treated (CK) *Isodon rubescens* plants. (A) 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay results. (B) Ferric reducing/antioxidant power (FRAP) assay results. (C) Tea brewing after 12 h. Error bars indicate the standard error of the mean (n = 3). Values marked by different lowercase letters differ significantly within each plot ($p < 0.05$)
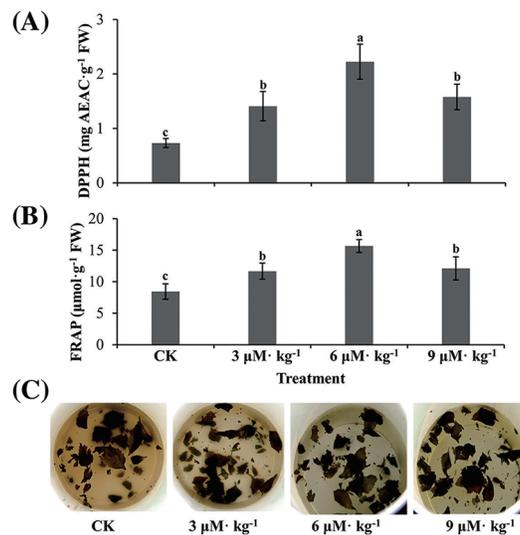
3.5 Se-Caused Diterpenoid Accumulation Is Associated with Expression of Key Synthase Genes

The biosynthetic pathway of diterpenoids is generally understood, although the functions of some key synthases remain unclear (Fig. 6A) [22,32,33]. Except for *IrDXR* under 3 μM·kg^{-1} Se treatment and *IrCPS5* under 3 and 9 μM·kg^{-1} Se treatments, all four genes were upregulated in Se-treated *I. rubescens* plants, particularly *IrDXS* by 5-fold under 9 μM·kg^{-1} Se treatment, *IrDXR* by 5-fold under 6 μM·kg^{-1} Se treatment, and *IrKSL4* by 6-fold under 6 μM·kg^{-1} Se treatment ($p < 0.05$) (Fig. 6B). Thus, expression increases induced in these genes may have contributed to the enhanced accumulation of diterpenoids observed in Se-treated *I. rubescens*.
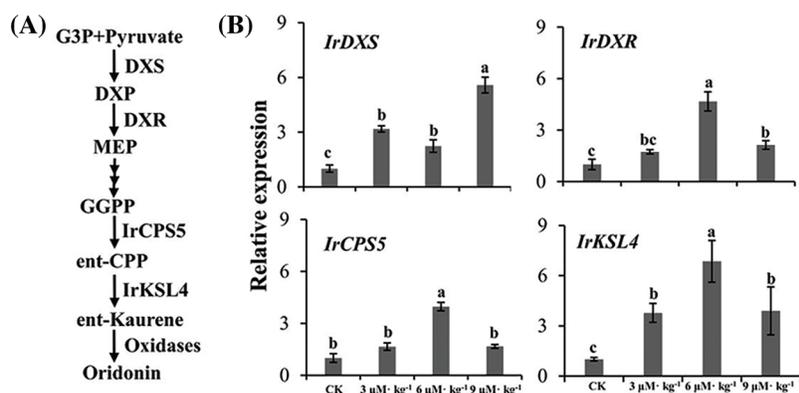

Figure 6: Expression of key biosynthase genes for diterpenoid accumulation in *Isodon rubescens* as determined by qRT-PCR. (A) A proposed pathway of diterpenoid biosynthesis. (B) Relative expression levels of genes based on qRT-PCR results. Different lowercase letters show significant differences within each plot ($p < 0.05$). IrDXS, *I. rubescens* 1-deoxy-d-xylulose 5-phosphate synthase; IrDXR, *I. rubescens* 1-deoxy-d-xylulose 5-phosphate reductoisomerase; IrCPS5, *I. rubescens* copalyl diphosphate synthase 5; IrKSL4, *I. rubescens* kaurene synthase-like 4

4 Discussion

The exogenous application of Na_2SeO_4 promoted the accumulation of Se, flavonoids, and diterpenoids (particularly oridonin) in *I. rubescens* but did not decrease the plant biomass. This promoting effect was likely attributable to the upregulated expression of key diterpenoid biosynthesis genes.

The safe Se range in humans is extremely narrow, with an intake of <40 μg·d^{-1} as deficient and an intake of >400 μg·d^{-1} as toxic [17]. The recommended daily allowance of Se is 60–70 μg [34], particularly close to the deficiency threshold, highlighting the importance of clarifying the Se sources and content in foods and beverages. The medicinal plant *I. rubescens* has clear application potential for Se supplementation in human diets since it is already consumed in the form of wild vegetables, medicinal decoctions, and tea beverages. Previous studies reported the successful preparation of Se polysaccharides based on the structural modification of polysaccharides extracted from *I. rubescens* [35], implying the value of utilizing *I. rubescens* for Se supplementation. When exposed to Na_2SeO_4, *I. rubescens* plants accumulated at least 17-fold more Se in their leaves than control plants, reaching nearly 200 mg·kg^{-1} DW. The Se accumulation capacity was similar to that of other medicinal and edible plants, including *Plantago ovata* [36], *Cardamine hupingshanesis* [37], *Brassica oleracea*, and *Triticum aestivum* [38]. None of the three Se doses tested affected the phenotypic indices or biomass of *I. rubescens*, in contrast to *P. ovata* and *Brassica rapa*, which were both phenotypically influenced by Se treatment [36,39]. This discrepancy is likely due to differences in Se tolerance among these plant species. The present results suggest that the

exogenous Se application to *I. rubescens* allows Se supplementation in human diets without compromising dōng líng cǎo yield.

Selenium enrichment, like environmental stress, activates plant instincts to protect their biological systems [21,40,41]. This process occurs through enzymatic and non-enzymatic approaches, thus helping plants to scavenge excess reactive oxygen species. In this study, Se treatments boosted the activity of three antioxidant enzymes, SOD, CAT, and POD, and enhanced the antioxidant capacity. Se accumulators usually have a higher antioxidant capacity than Se non-accumulators [42], consistent with the Se-regulated antioxidant capacity observed in the present study.

When absorbing exogenous Se, plants are invaded by a foreign matter that may disrupt their intrinsic metabolic systems. This imbalance may influence the secondary metabolism of plants, thus regulating the accumulation of secondary metabolites. Se treatment has been observed to regulate secondary metabolites in many other plants [18]. In the present study, when *I. rubescens* plants were treated with Na_2SeO_4, total flavonoids and diterpenoids were increased, consistent with the results of previous studies [14,42,43]. Moreover, higher antioxidant capacity was observed in *I. rubescens* plants treated with exogenous Se, which was likely explained by the increased antioxidant enzymatic activities and secondary metabolites observed. Since the diterpenoid oridonin is the major pharmaceutically active compound in dōng líng cǎo, it was further assayed by HPLC. The oridonin content increased by 1.9-fold in *I. rubescens* leaves treated with 6 $\mu M \cdot kg^{-1}$ Se. In contrast to previous studies that mostly focused on the content of total essential oils, our study revealed that exogenous Se induced the accumulation of oridonin, an important anti-cancer compound.

The expression levels of four genes encoding proteins involved in the diterpenoid biosynthesis pathway were quantified to explain why Se promoted the accumulation of diterpenoids (particularly oridonin) in *I. rubescens* plants. The four genes tested have each been functionally characterized [32,33], and all were upregulated by treatment with Na_2SeO_4, particularly exhibiting a 5-fold increase in *IrDXR* and a 6-fold increase in *IrKSL4* by 6 $\mu M \cdot kg^{-1}$ Se treatment. DXR is the second protein involved in the terpenoid synthesis pathway, while KSL catalyzes the last several steps of oridonin biosynthesis [32,33]. Thus, the genes encoding these proteins may contribute to the increased biosynthesis of diterpenoids and oridonin, in particular, in *I. rubescens* plants treated with Se. This study revealed key synthase genes involved in diterpenoid biosynthesis, but the response mechanism to Se needs further study. Transcriptomic analysis may provide insight into the response mechanism of plants to Se treatment [44]. Thus, combining transcriptomic analysis with the functional characterization of key synthase genes/transcription factors could reveal these molecular mechanisms, thus providing breeding targets for selecting high-quality *I. rubescens* germplasms.

5 Conclusions

The present results suggest that dōng líng cǎo consumed as a wild vegetable, medicinal decoction, or tea beverage has promising potential as a dietary source of supplemental Se, especially when plants are treated with exogenous Se. The observed Se-induced diterpenoid accumulation was explained by the upregulated expression of key synthase genes, particularly *IrDXR* and *IrKSL4*. These results advance the understanding and exploration of the full value of dōng líng cǎo as a medicinal resource and dietary supplement.

Author Contributions: The authors confirm their contribution to the paper as follows: study conception and design: Yongbo Duan and Fenglan Zhao; data collection: Fenglan Zhao, Shuwen Wu, and Xue Meng; analysis and interpretation of results: Jianping Xue; and manuscript writing: Fenglan Zhao and Yongbo Duan. All authors reviewed the results and approved the final version of the manuscript for submission.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

Supplementary Materials: The supplementary material is available online at https://doi.org/10.32604/phyton.2024.052287.

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