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Research Article
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Hemin-induced increase in saponin content contributes to the alleviation of osmotic and cold stress damage to *Conyza blinii* in a heme oxygenase 1-dependent manner

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Abstract: Hemin can improve the stress resistance of plants through the heme oxygenase system. Additionally, substances contained in plants, such as secondary metabolites, can improve stress resistance. However, few studies have explored the effects of hemin on secondary metabolite content. Therefore, the effects of hemin on saponin synthesis and the mechanism of plant injury relief by hemin in *Conyza blinii* were investigated in this study. Hemin treatment promoted plant growth and increased the antioxidant enzyme activity and saponin content of *C. blinii* under osmotic stress and cold stress. Further study showed that hemin could provide sufficient precursors for saponin synthesis by improving the photosynthetic capacity of *C. blinii* and increasing the gene expression of key enzymes in the saponin synthesis pathway, thus increasing the saponin content. Moreover, the promotion effect of hemin on saponin synthesis is dependent on heme oxygenase-1 and can be reversed by the inhibitor Zn-protoporphyrin-IX (ZnPPIX). This study revealed that hemin can increase the saponin content of *C. blinii* and alleviate the damage caused by abiotic stress, and it also broadened the understanding of the relationship between hemin and secondary metabolites in plant abiotic stress relief.

Key words: Hemin; Saponin; *Conyza blinii*; Heme oxygenase; Abiotic stress

1 Introduction

Natural heme is a ubiquitous molecular complex of iron and tetrapyrrole to porphyrin IX (Ishikawa et al., 2001). Hemin is a purified natural heme found in animal blood (Wang and Doré, 2007), which is nontoxic to organisms and the environment, and is widely used in pharmacological research and clinical treatment (Shamloul and Wang, 2006; Luo et al., 2018). As a porphyrin, heme is similar in substance to chlorophyll (Velini et al., 2005), and chloride has chemical properties similar to those of natural heme (Gheisari et al., 2010). In the field of drug manufacturing, in addition to being used as one of the synthetic raw materials of bilirubin (BR), hemin also has applications in the field of new cancer drugs (Müllebner et al., 2015). In the food manufacturing industry, as the natural pigment in red blood, which produces a ruddy and fresh appearance in meat, it can replace certain synthetic pigments and nitrite in order to reduce their use (Becker et al., 2011).

Hemin can be used as an exogenous additive in the process of plant cultivation. It can significantly enhance the antioxidant capacity of plants, regulate their basic metabolic intensity, and promote the accumulation of effective substances (Fu et al., 2011). For example, when radish sprouts were treated with exogenous hemin and its different metabolites, the anthocyanin content increased and decreased to varying degrees (Liu et al., 2016). Compared with the control group, the expression intensities of the regulatory genes polynucleotide adenylyltransferase (*PAP1*), phenylalanine ammonia lyase (*PAL*), chalcone-flavonone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), chalcone synthase (*CHS*),

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and dihydroflavonol-4-reductase (*DFR*) in the anthocyanin synthesis pathway were significantly enhanced, which promoted the accumulation of target substances (Liu et al., 2016).

Heme oxygenase (HO) is a general term for the initiation and rate-limiting enzymes of heme degradation in plants (Troxler, 1972). There are three subtypes of HO, HO-1, HO-2, and HO-3 (Maines and Panahian, 2001). Heme oxygenase-1 (HO-1, EC 1.14.14.18) is an oxidoreductase that can degrade heme to produce BR, carbon monoxide (CO), and ferrous ions (Fe^{2+}) in animals and plants (Wagener et al., 2003; Baudouin et al., 2004). Some studies have shown that HO-1 is the degradation product and performs the main functions. For example, suspended cells of *Betula platyphylla* and the mycelium culture system of *Phellinus igniarius* were treated with one of the bioproducts of hemin, CO, and it was found that the gene expression levels of key enzymes in triterpene synthesis were upregulated in both species (Jiang et al., 2019; Wang et al., 2019). CO in plants is mainly produced by the degradation of heme by HO-1 (Otterbein et al., 2003), indicating that hemin can increase the accumulation of terpenoids. However, there are few experiments that can verify this.

Conyza blinii, also known as bitter wormwood and bear gall grass, is a biennial deciduous herb of the licorice genus Compositae (Sun et al., 2014). It is distributed in the Panxi area of Sichuan Province, central and southern Yunnan Province, and parts of Guizhou Province, China (Liu et al., 2017). As a Chinese herbal medicine, *C. blinii* is widely used in clinical treatment because of its anti-inflammatory, antitussive, expectorant, and antiasthmatic effects (Ma et al., 2017a; Sun et al., 2018).

C. blinii saponins are the main effective components of this plant. As early as 1989, an animal experiment with *C. blinii* in Chengdu City (China) showed that saponins had certain therapeutic effects in respiratory system diseases (Yang et al., 1989). They promoted the clearance of foreign bodies in the tracheal ciliary mucus system of rabbits (Geng et al., 2013). Further clinical experiments have shown that saponins are effective in the treatment of chronic bronchitis, with few side effects (Su et al., 2001). In addition, Liu et al. (2011) found that saponins inhibited the growth of cervical cancer and lung cancer cells. On this basis, Ma et al. (2017b) studied the mechanism, by which saponins inhibit cancer. The results showed that saponins caused mitochondrial apoptosis by destroying mitochondrial membrane potential, inhibited the migration and invasion of cancer cells, caused S-phase arrest of cancer cells, and inhibited the nuclear factor-κB (NF-κB) signaling pathway. In addition to these four main effects, saponins have been reported to have preventive and therapeutic effects on gastric ulcers in rats (Sun et al., 2014). Ma and Liu (2014) found that saponins could protect gastric mucosa from ethanol-induced gastric ulcers; in addition, a *C. blinii* saponin aqueous solution inhibited the growth of *Staphylococcus aureus* and *Staphylococcus albus*.

Our goal in this study was to investigate the effects of hemin treatment on saponin content in *C. blinii*, as well as its relationship to plant abiotic stress relief.

2 Materials and methods

2.1 Plant materials and hemin treatment

Seeds of *C. blinii* were obtained from Sichuan Agricultural University, Ya'an, China. The conditions of seed germination and seedling culture were described in our previous study (Zhan et al., 2021). When the seedlings were two months old, we put them into drought and low-temperature conditions and treated them with hemin.

Suspension cells were cultured in the new configuration (with every 40 mL containing 0.1896 g Murashige-Skoog's (MS) medium, 1.2 g sugar, 4 μL 6-benzylamino purine, 40 μL 2,4-dichlorophenoxyacetic acid, 10 μL 6-furfurylamino-purine (kinetin), and 2.186 g mannitol, and the pH was adjusted to 5.5–5.8) in the mother liquor. Then, 1 mL of *C. blinii* suspension cells were blended with *C. blinii* suspension cell sap at 25 °C and 120 r/min and placed on a thermostatic cultivation table for 5 d.

A series of 40 μL, 400 μL, 1 mL, and 2 mL of 1 mmol/L hemin were absorbed and added to 40 mL of *C. blinii* suspension cell solution so that the final concentrations were 1, 10, 25, and 50 μmol/L, respectively. All suspension cell solutions were incubated on a shaking table at 25 °C and 120 r/min for 4 d. The plants were treated with the same concentration of hemin and cultured in the greenhouse for 7 d, and the culture medium (supplemented with the corresponding concentration of hemin) was changed every 3 d. The control

group (CK group) consisted of suspension cell culture medium and *C. blinii* plants with only the mother liquor and no hemin.

Polyethylene glycol 6000 (PEG6000) was used to induce osmotic stress. PEG6000 was added to the culture medium for a final concentration of 10% (volume fraction). *C. blinii* was then treated with the culture medium containing 10% PEG6000 for 7 d. The culture medium was changed every 3 d, adding PEG6000 again.

At 21:00 every day, *C. blinii* was put into a 4 °C refrigerator for cold-stress treatment, and the plants were removed from the refrigerator at 9:00 the next day. Then, the plants were placed at room temperature to alleviate the freezing damage to the plants so that they would not freeze to death. This process was repeated daily for one week, at which point the physiological indices were measured.

The concentrations of the three metabolites of hemin were obtained by concentration gradient screening: 10 μmol/L hemin, BR, Fe^{2+}, carbon monoxide-releasing molecule 3 (CORM3), and 100 μmol/L inhibitor Zn-protoporphyrin-IX (ZnPPIX) (Chen et al., 2018).

2.2 Determination of physiological indices

Plant height, fresh weight, dry weight, photosynthetic pigment content, and photosynthetic parameters, including the net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r), were measured via the same methods mentioned in our previous study (Zheng et al., 2020). The photosynthetic pigment content was detected by extraction with an acetone-ethanol mixture (Gitelson et al., 2003).

2.3 Extraction of saponins and content analysis

Drawing the standard curve of total saponins: First, 20 mg of oleanolic acid standard was accurately weighed, and then 50 mL of a methanol reagent was used to dissolve the oleanolic acid solution to a concentration of 0.50 mg/mL. After reaching a constant volume, the standard solution was obtained by blending. Then, 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL of the oleanolic acid standard solutions were placed in test tubes. All of the solutions in the tubes were dried in a fume hood. Then, vanillin (5%, mass fraction)-glacial acetic acid solution (0.3 mL) and 0.8 mL of perchlorate were mixed into each tube. All test tubes were made airtight and shaken for 25 min at 65 °C. The absorption value of the chromogenic solution was measured at a wavelength of 544 nm with an ultraviolet (UV) spectrophotometer. The mass was used as the abscissa and the absorbance was plotted as the ordinate to plot the standard curve of the total saponins with the measured data.

2.4 Preparation and content determination of total saponins to be measured

The *C. blinii* leaves from the experimental group and the CK group were washed after being treated with different concentrations of hemin and then dried at 55 °C to a constant weight. After grinding into a powder, 0.1 g of *C. blinii* powder was put into a 10-mL centrifuge tube filled with 1.5 mL of 60% (volume fraction) ethanol. Extraction was performed for half an hour under the extraction conditions of 65 °C and 350 W of ultrasonic power. After extraction, the solution was centrifuged at 12 000 r/min for 5 min at low temperature, and the supernatant was absorbed. The subsequent steps were consistent with the determination of the standard saponin solution mentioned above. The absorbance value was determined at a wavelength of 544 nm and measured in parallel three times. Then, the total saponins in the sample solutions treated with different concentrations of hemin were determined by comparison with the standard curve. The culture medium of the *C. blinii* suspension cells in the experimental group and the medium of the CK group treated with hemin at different concentrations were centrifuged at 12 000 r/min for 5 min (Xiong et al., 2017). The supernatant was poured out and then color development was carried out with the same method used to prepare the curve, and the corresponding absorption value was measured.

2.5 RNA extraction and qRT-PCR analysis

After treatment, all the leaves of the samples were immediately put into liquid nitrogen. Subsequently, RNA was extracted using an EasySpin plant RNA Rapid Extraction kit (Adlai Biological Co., Ltd., Beijing, China) (Ekhtari et al., 2019) and complementary DNA (cDNA) was synthesized using reverse transcription kits (HiScript III RT SuperMix for qPCR (+gDNA wiper), Vazyme, Nanjing, China). A PrimeScript™ RT Reagent kit with genomic DNA (gDNA) Eraser (Vazyme) was used for reverse transcription.

For quantitative real-time polymerase chain reaction (qRT-PCR) analysis, three parallel assays were performed for each gene in each sample. All of the systems and conditions of RNA extraction and qRT-PCR are described in detail in our previous report (Zheng et al., 2020). Finally, the $2^{-\Delta\Delta C_T}$ method was used to calculate gene expression and for analysis.

2.6 Statistical analysis

Statistical analysis was performed using SPSS statistics 17.0 software (SPSS Inc., Chicago, IL, USA). All data were analyzed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test, and statistical significance was determined as a *P*-value of <0.05 using GraphPad Prism 7 statistical software (GraphPad Prism Inc., USA).

3 Results

3.1 Effects of hemin on the growth of *C. blinii* under osmotic stress and cold stress

To investigate the effects of different hemin concentrations on the growth of *C. blinii* under drought and cold stress, we used 1, 10, 25, and 50 μmol/L of hemin to treat *C. blinii*. As shown in Fig. 1, hemin treatment significantly promoted the growth of *C. blinii* under osmotic stress even at inappreciable doses. Under osmotic stress, 25 μmol/L hemin treatment best promoted the development of root length (Fig. 1a), plant height (Fig. 1b), and fresh weight (Fig. 1c), increasing them by 46.67%, 9.37%, and 6.67%, respectively, compared with the CK group. As with osmotic stress, hemin could effectively alleviate the growth inhibition of *C. blinii* caused by low temperature. In the experiment with different hemin concentrations, the development of a promoted peak occurred at 25 μmol/L hemin, in which root length, plant height, and fresh weight increased by 38.46%, 17.24%, and 42.86%, respectively, compared with the CK group.

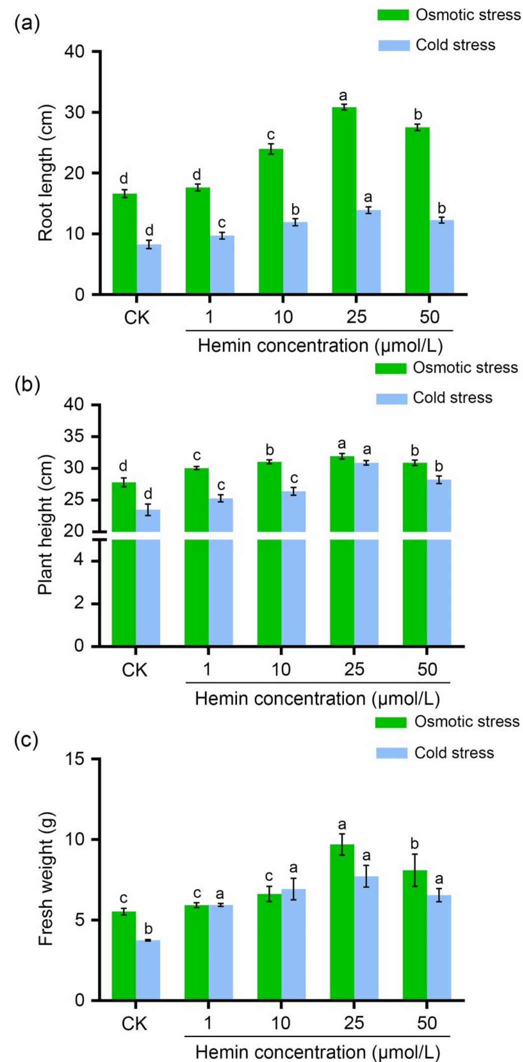

Fig. 1 Effects of hemin on the growth of *Conyza blinii* under osmotic stress and cold stress. The changes are shown in *C. blinii* root length (a), plant height (b), and fresh weight (c) after treatment with different concentrations of hemin under osmotic stress and cold stress. The control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), *n*=3. Different letters are significantly different among the groups (*P*<0.05).

3.2 Effects of hemin on antioxidant enzyme activity of *C. blinii* under osmotic stress and cold stress

When plants are subjected to environmental stress, antioxidant enzymes perform key roles in the reactive oxygen-scavenging system and can clear reactive oxygen species (ROS) within and outside of the cells to protect tissues from oxidative damage. As shown in Fig. 2a, the catalase (CAT) enzyme activity of *C. blinii* under osmotic stress increased with the continuous increase in hemin concentration. When the hemin concentration reached 25 μmol/L, the maximum increase was 1.09% compared with the CK group. Similarly, as shown in Figs. 2b and 2c, peroxidase (POD) and superoxide dismutase (SOD) activity also significantly increased when the hemin concentration reached 25 μmol/L,

with increases of 39.13% and 8.33%, respectively. Similar to the effects of hemin under cold stress, the CAT enzyme activity of *C. blinii* reached a maximum of approximately 2.12% at 25 μmol/L hemin (Fig. 2a). Likewise, the activity of the POD and SOD enzymes increased by 66.67% and 30.76%, respectively (Figs. 2b and 2c).

3.3 Effects of hemin on the saponin content of *C. blinii* under osmotic stress and cold stress

To explore the effects of hemin on the saponin content of *C. blinii* under osmotic stress and cold stress, we used different hemin concentrations to treat *C. blinii*. As expected, under drought and cold stress, the saponin content increased once *C. blinii* was subjected to imperceptible concentration (1 μmol/L) of hemin. To a certain extent, the increasing trend in saponin content was similar to that of hemin, regardless of drought or cold stress. When the hemin concentration reached 25 μmol/L, the saponin content increased 86.67% (Fig. 3). When the hemin concentration reached 50 μmol/L, the saponin content began to decrease; at its maximum, the increase was 84.61% compared with the CK group. The change in *C. blinii* saponin content after 25 μmol/L hemin treatment under cold stress was consistent with that under osmotic stress, with the greatest improvement effect of 85.29% (Fig. 3).

3.4 Effects of hemin on the biomass of *C. blinii*

The results showed that the fresh weight of *C. blinii* (Fig. 4a) increased the most significantly in the 25 μmol/L hemin treatment group compared with the CK group, with an increase of 26.07%. However, when the hemin concentration continued to rise to 50 μmol/L, the improvement effect on the fresh weight decreased, with a slight increase of 11.58% compared with the CK group. Without exception, synchronous changes in the dry weight of *C. blinii* (Fig. 4b) showed a similar trend and increased the most when the hemin concentration was 25 μmol/L.

3.5 Effects of hemin on the photosynthetic capacity of *C. blinii*

We studied the effects of hemin on the photosynthetic capacity of *C. blinii* with regard to the photosynthetic pigment content and photosynthetic capacity index. After the treatment of *C. blinii* with different

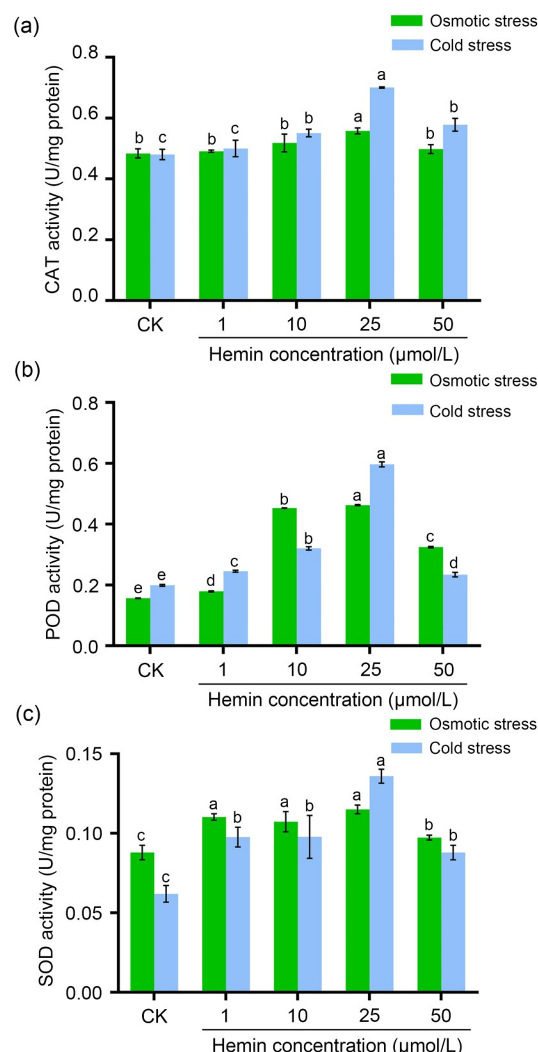

Fig. 2 Effects of hemin on antioxidant enzyme activity of *Conyza blinii* under osmotic stress and cold stress. The changes are shown in *C. blinii* catalase (CAT) enzyme activity (a), peroxidase (POD) enzyme activity (b), and superoxide dismutase (SOD) enzyme activity (c) after treatment with different concentrations of hemin. The control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), *n*=3. Different letters are significantly different among the groups (*P*<0.05).

hemin concentrations, a change in chlorophyll content was detected (Fig. 5a). When the hemin concentration reached 25 μmol/L, the chlorophyll content showed the greatest increase, reaching 50.02% compared with the CK group. The change in carotenoid content was similar to that in the chlorophyll content (Fig. 5b). When the hemin concentration reached 25 μmol/L, the carotenoid content increased by 44.60%, which was the highest increase.

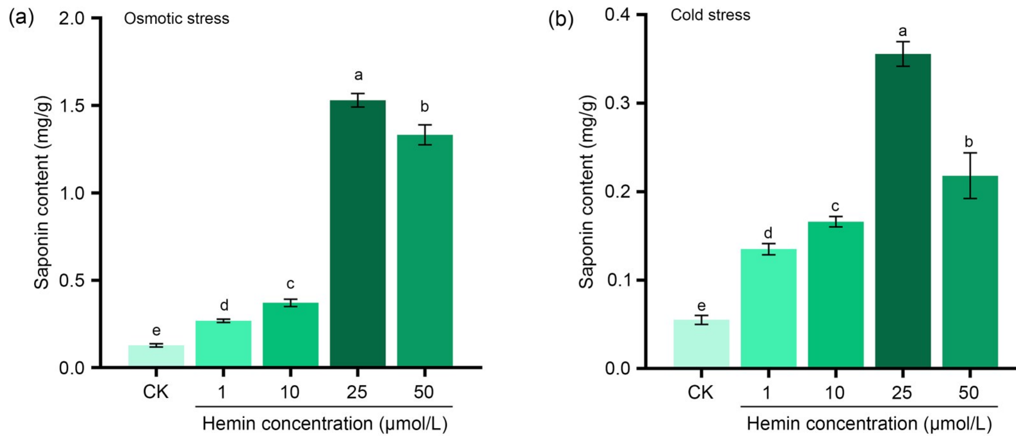

Fig. 3 Effects of hemin on the saponin content of *Conyza blinii* under osmotic stress (a) and cold stress (b). The control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), n=3. Different letters are significantly different among the groups (P<0.05).

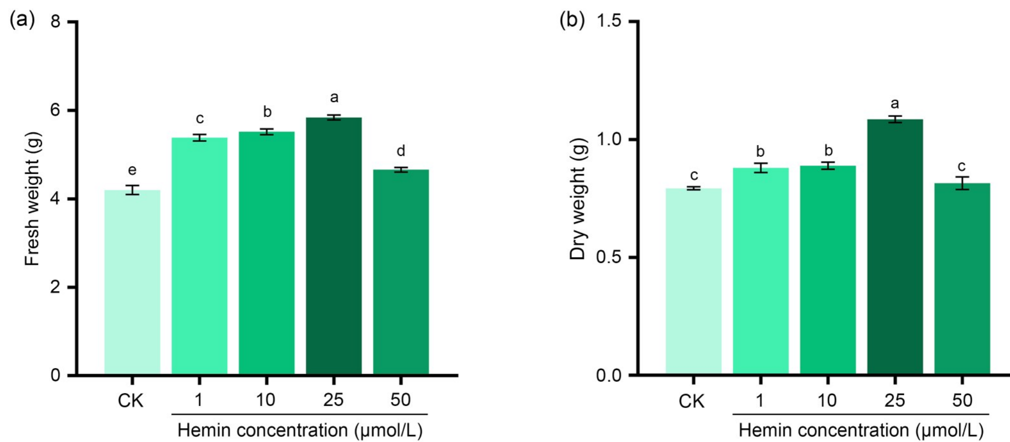

Fig. 4 Effects of hemin on the biomass of *Conyza blinii.* The changes are shown in fresh weight (a) and dry weight (b) after treatment with different concentrations of hemin. The *C. blinii* plants were treated without osmotic stress or cold stress, and the control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), n=3. Different letters are significantly different among the groups (P<0.05).

Subsequently, we explored the P_n, T_r, G_s, and C_i (Figs. 5c–5f). When the hemin concentration reached 25 μmol/L, P_n, T_r, G_s, and C_i synchronously reached their peak, increasing by 21.10%, 42.86%, 45.57%, and 26.05%, respectively, compared with the CK group.

3.6 Effects of hemin on the synthesis of *C. blinii* saponins

In order to explore the influence of hemin on saponin synthesis in *C. blinii*, different concentrations of hemin were used to treat the suspension cells and plants of *C. blinii*. The saponin content, in both the suspension cells and plants, significantly increased under 25 μmol/L hemin treatment, with increase rates of 47.69% and 87.09%, respectively, compared with the CK group (Figs. 6a and 6b).

Moreover, we detected the expression levels of key enzyme genes in the saponin synthesis pathway of *C. blinii* (Fig. 6c) under different hemin concentrations, and the results showed that when the hemin concentration reached 25 μmol/L, the key enzyme genes in the saponin synthesis pathway, including *CbHMGR*, *CbFPPS*, *CbSQS*, *CbSQE*, and β-amyrin synthase from *C. blinii* (*CbβAS*) (Fig. 6d), were upregulated. We also tested the expression level of the heme oxygenase 1 (*CbHO-1*) gene in *C. blinii* and found that hemin treatment increased the expression of the *CbHO-1* gene; the enhancement effect was most significant when the hemin concentration was 25 μmol/L.

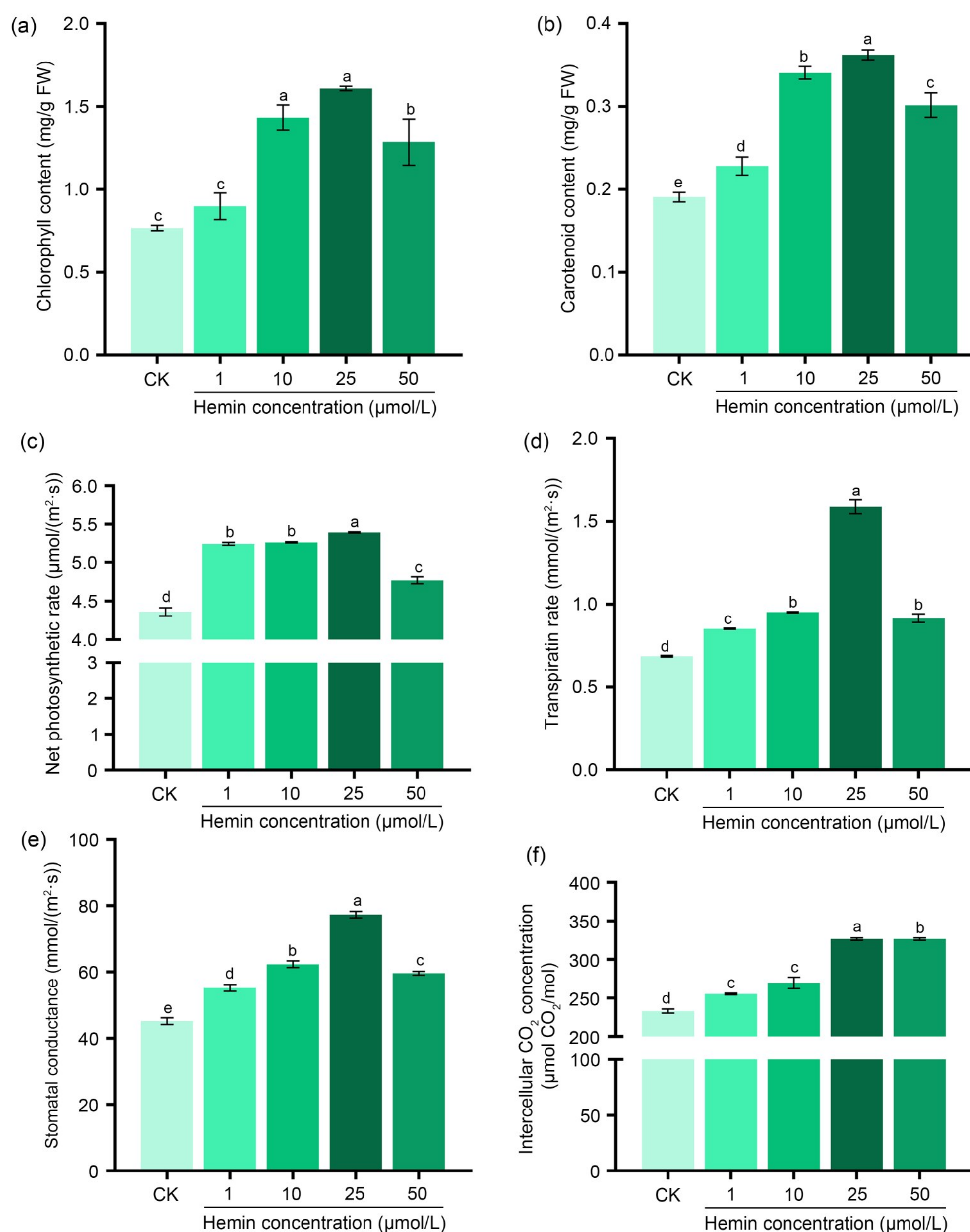

Fig. 5 Effects of hemin on the photosynthetic capacity of *Conyza blinii*. The changes are shown in chlorophyll content (a), carotenoid content (b), P_n (c), T_r (d), G_s (e), and C_i (f) of *C. blinii* under different concentrations of hemin. The *C. blinii* plants were treated without osmotic stress or cold stress, and the control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), *n*=3. Different letters are significantly different among the groups (P<0.05). P_n: net photosynthetic rate; G_s: stomatal conductance; C_i: intercellular CO_2 concentration; T_r: transpiration rate; FW: fresh weight.

3.7 Effects of hemin and its decomposition products and inhibitors on the saponin content of *C. blinii*

The decomposition products of hemin in plants are BR, Fe^{2+}, and CO. We investigated the effects of these different decomposition products on *C. blinii* saponin synthesis, and the results showed that BR had the most significant effect on *C. blinii* saponin synthesis; it also had the most similar effect to hemin (Fig. 7). Moreover, we found that when ZnPPIX, an inhibitor of the *CbHO-1* gene, was added along with hemin treatment, the enhancement effect of hemin on saponin content and gene expression in the mevalonate (MVA) pathway disappeared.

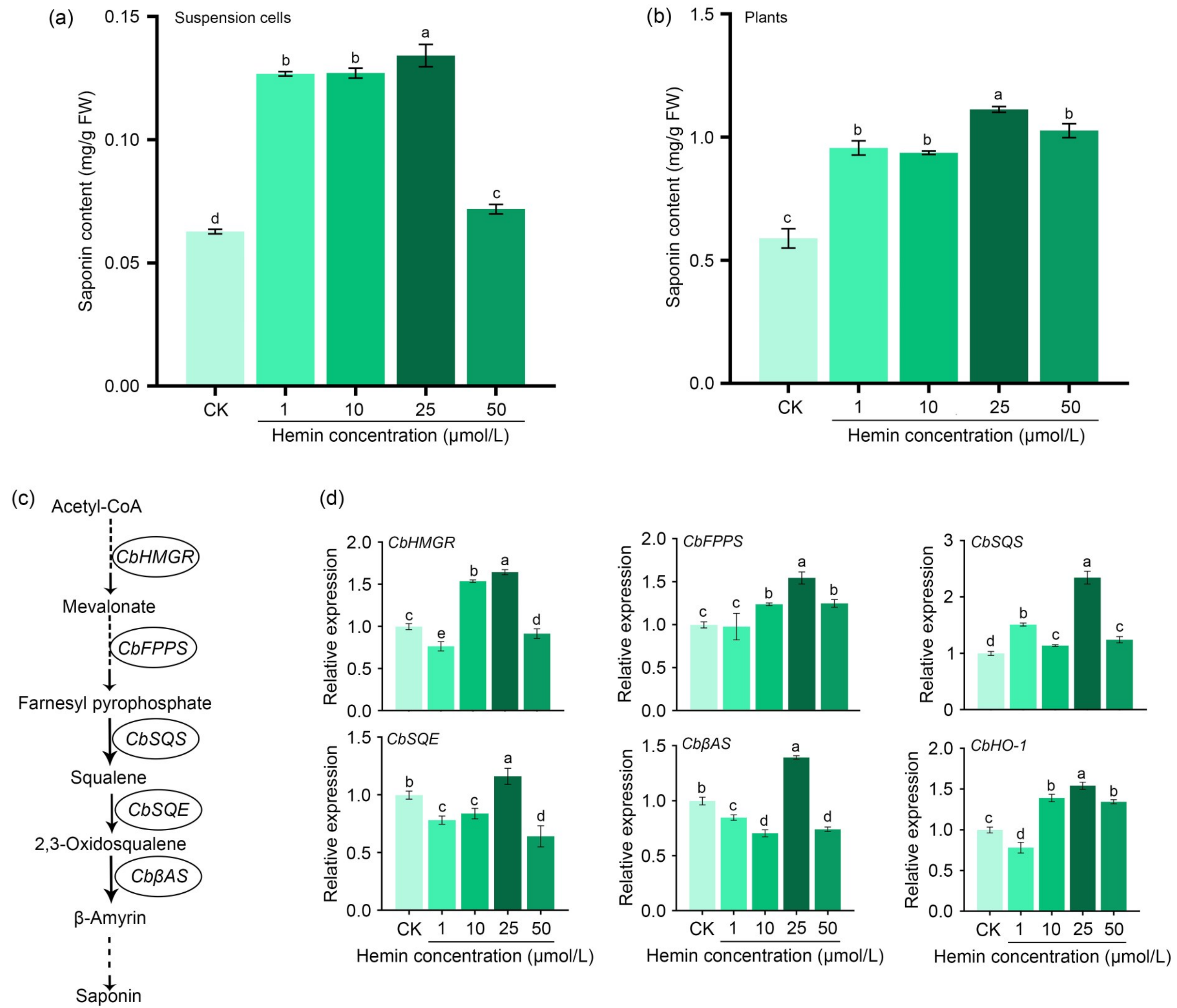

Fig. 6 Effects of hemin on saponin synthesis in *Conyza blinii*. The changes are shown in the saponin contents of *C. blinii* suspension cells (a) and plants (b) under treatment with different hemin concentrations. (c) The diagram of saponin biosynthesis pathway and some key enzyme genes, including *CbHMGR*, *CbFPPS*, *CbSQS*, *CbSQE*, and *CbβAS*. (d) Relative expression levels of key enzyme genes in the saponin synthesis pathway, including *CbHMGR*, *CbFPPS*, *CbSQS*, *CbSQE*, and *CbβAS*, and heme oxygenase 1 (*CbHO-1*), after treatment with different concentrations of hemin. The *C. blinii* plants were treated without osmotic stress or cold stress, and the control (CK) group did not receive additional hemin. The values are expressed as mean±standard deviation (SD), *n*=3. Different letters are significantly different among the groups (*P*<0.05). FW: fresh weight.

4 Discussion

4.1 Hemin can alleviate the inhibitory effects of morphogenesis under osmotic stress and cold stress

Hemin affects plants in many ways, such as in the formation and elongation of roots and in supporting plant resistance to abiotic stress (Xuan et al., 2008; Chen et al., 2013; Jin et al., 2016). A large number of reports have shown that the tolerance of plants to a variety of abiotic stresses, including heavy metal stress and UV-B stress, can be greatly improved after exogenous hemin is applied (Noriega et al., 2004; Yannarelli et al., 2006; Li et al., 2012). The results of this experiment showed that the growth of *C. blinii* under osmotic stress and cold stress was not inhibited but instead promoted by the application of exogenous hemin. Moreover, the promotion of morphological development was efficient (even at a low dose of hemin) in a concentration-dependent manner.

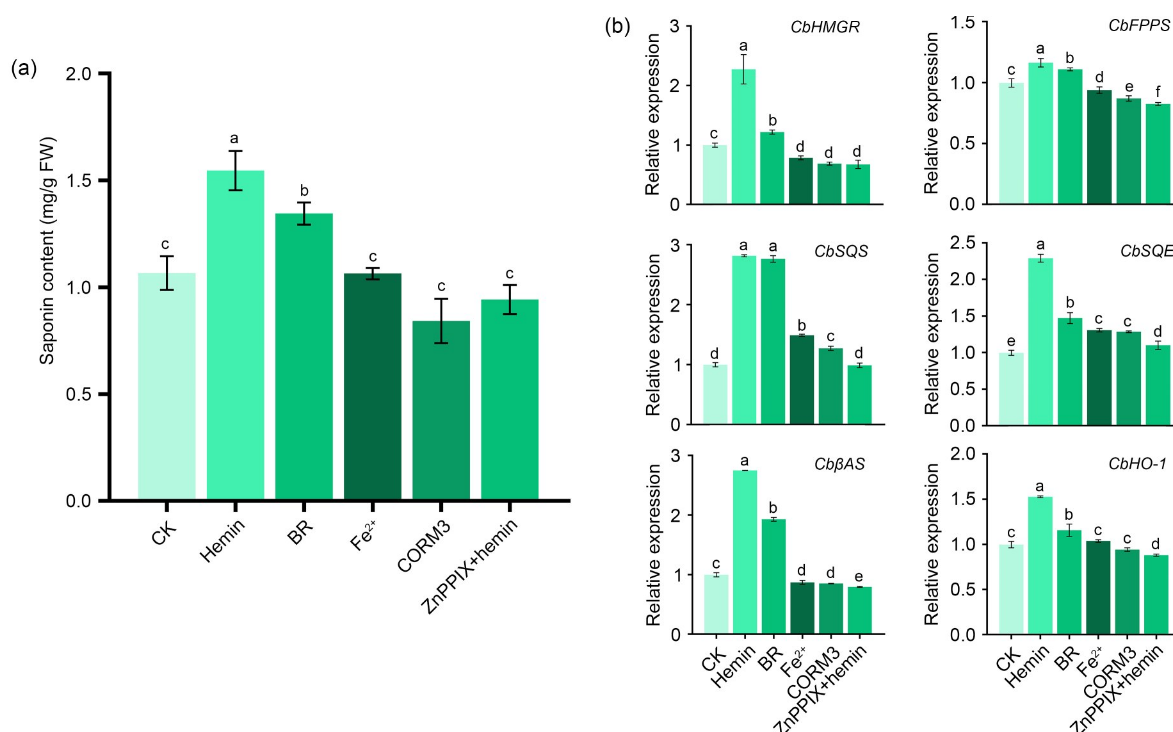

Fig. 7 Effects of different hemin substances on saponin synthesis in *Conyza blinii*. The changes are shown in saponin content (a) of *C. blinii* treated with different substances, in which CORM3 is a CO donor and ZnPPIX is a CbHO-1 inhibitor, and in the expression levels of key enzymes (b) of the *C. blinii* saponin synthesis pathway (*CbHMGR*, *CbFPPS*, *CbSQS*, *CbSQE*, and *CbβAS*) and *CbHO-1* after different treatments. The *C. blinii* plants were treated without osmotic stress or cold stress, and the control (CK) group did not receive additional hemin. The values are expressed as mean± standard deviation (SD), *n*=3. Different letters are significantly different among the groups (*P*<0.05). BR: bilirubin; CORM3: carbon monoxide (CO)-releasing molecule 3; ZnPPIX: Zn-protoporphyrin-IX; FW: fresh weight.

4.2 Hemin, with dual status, is involved with ROS and in photosynthesis and metabolism

When plants are subjected to abiotic stress, a large amount of ROS is produced in the body, mainly in the form of superoxide anions and hydrogen peroxide (Matthus et al., 2019). These oxygen free radicals can cause significant damage to the organism and also cause wilt and plant death (Orrenius et al., 1992). The antioxidant enzyme system plays a key role in the effective scavenging of ROS in plants (Ozdener and Aydin, 2010). The plant antioxidant enzyme system contains several components, such as SOD, POD, CAT, and ascorbate peroxidase (APX) (Mei and Song, 2010). These enzymes cooperate with each other and participate in the removal process of ROS in vivo in an orderly manner when plants are subjected to abiotic stress (Shah and Nahakpam, 2012). Hemin is degraded by HO-1 into BR, Fe^{2+}, and CO, which function as secondary messengers to enhance antioxidant enzyme activity in plants and resist abiotic stress, a result that has been widely reported (Dennery et al., 2003). Of these, CO, which is as efficient as hemin, promotes the expression of *POD*, *SOD*, and other antioxidant enzyme genes (Jin et al., 2013). In this study, the activity of antioxidant enzymes, including CAT, SOD, and POD, was upregulated, which was similar to the results of Noriega et al. (2012) and Jung et al. (2016). Therefore, our findings indicate that hemin could initiate the activity of the antioxidant enzymes in *C. blinii* under osmotic stress and cold stress, and then multiple antioxidant enzymes in *C. blinii* could participate in the process of scavenging ROS in an orderly manner.

On the other hand, the stress adaptability of *C. blinii* was enhanced by hemin in addition to natural growth and development. Specifically, hemin treatment induced photosynthetic capability to an extraordinary degree and exhibited concentration dependence on the effects of enzyme activity. Photosynthesis is the basis of metabolism, providing energy and substrates for most redox reactions. Accordingly, we found that the saponin content of *C. blinii* increased

after hemin treatment under osmotic stress and cold stress. As secondary metabolites, triterpenoid saponins have good antioxidant capacity (Nzowa et al., 2010). Plant secondary metabolites are mostly synthesized based on primary metabolites to help plants resist multiple stresses, including abiotic stress and biotic stress (Bacon et al., 2015). Research has shown that hemin can promote the synthesis of anthocyanin under UV-B, thus avoiding further damage caused by UV-B (Zhu et al., 2019). Similar results in this study once again demonstrated that hemin can promote the synthesis of plant secondary metabolites, thereby participating in the process of ROS clearance and alleviating damage to plants.

4.3 Hemin promotes the synthesis of saponins by improving the photosynthetic capacity of *C. blinii* and the expression of key enzyme genes in the saponin synthesis pathway

We found that the content of saponins of *C. blinii* under normal conditions increased after hemin treatment, both in the plant and in its suspension cells. We also investigated the mechanism and found that the photosynthetic pigment content of *C. blinii* increased after hemin treatment; and the P_n, G_s, T_r, and C_i all showed an increasing trend, indicating that the photosynthetic capacity of *C. blinii* was significantly improved after hemin treatment. The synthesis of secondary metabolites requires a certain structural basis, mainly through photosynthetic synthesis of organic materials as the skeleton (Ghasemzadeh and Jaafar, 2011). Therefore, the greatly improved photosynthetic capacity leads to the ability of *C. blinii* to synthesize more organic substances, providing sufficient precursors for saponin synthesis. This was verified by the increases in fresh weight and dry weight after hemin treatment.

Moreover, we found that the expression levels of key enzyme genes in the saponin synthesis pathway were upregulated by exogenous hemin, which facilitated the synthesis of saponins. Therefore, *C. blinii* can be induced by hemin to synthesize more saponins by enhancing metabolic activity.

4.4 Increase in *C. blinii* saponins induced by hemin is dependent on *CbHO-1*

The relationship between hemin's effects on plants and the *CbHO-1* system in plants has been a hot research topic. Our study found that *CbHO-1* gene expression was significantly upregulated in *C. blinii* after hemin treatment. After adding ZnPPIX, an inhibitor of *CbHO-1*, to the hemin-treated group, the expression level of *CbHO-1* was lower than that of the CK group; that is, the expression level of *CbHO-1* was significantly reduced. Additionally, after ZnPPIX treatment, there was a decrease in saponin content and a decrease in the expression of key enzymes in the saponin synthesis pathway, which indicates that the increase in saponin synthesis induced by hemin is dependent on the expression of *CbHO-1*. After inhibition of *CbHO-1* expression, the positive effects of hemin on saponin synthesis disappeared or were even reversed.

Furthermore, we found that BR played a dominant role among the hemin decomposition components in facilitating the synthesis of saponins and in the expression of key enzyme genes in the saponin synthesis pathway. Some studies have found that CO plays a key role as a signal molecule, and Fe^{2+} is the major functioning component among the hemin decomposition components (Podkalicka et al., 2018). Here, BR played an equal and crucial role.

5 Conclusions

In this study, we found that after hemin treatment, the activity of the antioxidant enzymes of *C. blinii* increased as well as the synthesis of saponins, which improved the tolerance of *C. blinii* to osmotic stress and cold stress. Hemin can promote the synthesis of saponins by increasing the photosynthetic capacity of *C. blinii* and the expression of key enzyme genes in the saponin synthesis pathway, thus participating in resistance to abiotic stress. Overall, our study explored the mechanism of saponin synthesis in *C. blinii* in response to drought and cold stress induced by hemin and expanded the understanding of the effects of heme on plant secondary metabolites in response to abiotic stress.

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Author contributions

Junyi ZHAN and Tianrun ZHENG designed research; Junyi ZHAN, Tianrun ZHENG, Wenjun SUN, Ming YANG, and

Maojia WANG performed research; Wenjun SUN, Ming YANG, and Maojia WANG analyzed data; Junyi ZHAN, Tianrun ZHENG, and Zhi SHAN wrote the paper. Hui CHEN is responsible for writing, reviewing, editing, and ensuring that the descriptions are accurate. All authors have read and approved the final manuscript, and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Tianrun ZHENG, Junyi ZHAN, Ming YANG, Maojia WANG, Wenjun SUN, Zhi SHAN, and Hui CHEN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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