

## Responses of woody plant *Dalbergia odorifera* treated with glycine betaine to drought and cold stresses: involvement of the alternative oxidase

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### Abstract

Woody plants faced multiple abiotic stresses in forest plantation that can influence their growth and development. *Dalbergia odorifera* T. Chen is a vulnerably endangered tree species, and references about its responses to abiotic stresses are very rare in literature. Furthermore, the mechanisms underlying the abiotic stress tolerance in plants induced by exogenous glycine betaine (GB) remains unclear. Indeed, the alternative oxidase (AOX) is one of the major components of antioxidant enzymatic machinery, and there are no studies that focused on the effects of GB on the amount of AOX protein in plants under drought or cold stresses. Thus, the aim of this study was to investigate the effect of exogenous GB on the phenotype, osmoprotectants, photosynthetic pigments, and antioxidant systems in *D. odorifera* under cold and drought stresses. The layout of the trial was a factorial experiment in a completely randomized design using two factors including abiotic stress (drought and cold) and GB. Moreover, the principal component analysis based on the measured parameters manifested how the selected ROS scavengers were dispersed throughout the treatments. The results showed huge beneficial impacts of GB on the phenotypic traits of *D. odorifera*; GB also influences positively the antioxidant machinery, photosynthetic pigments, redox-homeostasis, and water status in *D. odorifera* seedlings under both stresses. Moreover, exogenous GB affects more the AOX pathway in *D. odorifera* under cold stress than under drought stress.

**Keywords:** AOX, APX, CAT, chilling, chlorophyll, *Dalbergia odorifera*, drought, glycine betaine, homeostasis, oxidative stress, peroxidase, ROS, SOD.

### Introduction

With global climate change, the research about the response of a plant to abiotic stresses is one of the most active themes in plant science. Indeed, climate change appears to have increased in recent years, and more changes are expected in the next decades concerning plant growth and adaptation to their environments (Cernoch and Kopecky 2020). Abiotic stresses such as drought or cold stress affect plants by harming the metabolic machinery;

it can also perturb the equilibrium of the production and scavenging of reactive oxygen species (ROS) and malondialdehyde (MDA) in the plant cell, which alters negatively plant growth and development (Sharma *et al.* 2019). Stress tolerance in plants is correlated with different physiological changes including the accumulation of osmoprotectants and the increase of antioxidant activities (Yancey *et al.* 1994). Some of these adjustments are needed for enhancing the drought or cold tolerance in plants, comprise changes in genes expression and in membrane

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**Abbreviations:** APX - ascorbate peroxidase; AsA - ascorbic acid; Car - carotenoid; CAT - catalase; Chl *a* - chlorophyll *a*; Chl *b* - chlorophyll *b*; *c*<sub>i</sub> - intercellular CO<sub>2</sub> concentration; GB - glycine betaine; GPX - glutathione peroxidase; MDA - malondialdehyde; POD - peroxidase; RC - relative conductivity; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase; WP - water potential (measured by dew-point method).

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composition and behaviour (Wanner and Junttila 1999, Sharma *et al.* 2019, Kumar 2020). Indeed to cope with the environmental stresses, the plant can reduce its growth rate or store diverse types of osmoprotectants such as glycine betaine (GB), proline, or sugars. And due to the contribution of GB to osmotic adjustment and protection of proteins and membranes from damages caused by the abiotic stresses, plentiful experiments of application of GB on its low accumulator and non-accumulator plant species have been done (Annunziata *et al.* 2019). In fact, GB as a quaternary ammonium compound is one of the most well-known osmoprotectants and is able to provide stress tolerance in various plant species. In their life cycles, plants can accumulate GB to regulate the disturbance of the osmotic homeostasis caused by environmental stresses such as salinity, drought, or cold stress (Xu *et al.* 2018, Kumar *et al.* 2019). The roles of GB in plants under stresses have been studied in numerous plant species. Plants sprayed with GB exhibited drought stress tolerance in tomato (Ma *et al.* 2007), soybean (Agboma *et al.* 1997), turnip rape (Mäkelä *et al.* 1997), and rice (Farooq *et al.* 2008). The increase of chilling tolerance by exogenous GB in tomato (Park *et al.* 2006), wheat (Allard *et al.* 1998), and maize (Chen *et al.* 2000) was also reported. Indeed many studies have proven the implication of exogenous GB in preserving the photosystem II (PS II) and its capacity to increase the net photosynthetic rate and stomatal conductance during abiotic stresses (Annunziata *et al.* 2019, Mäkelä *et al.* 1999). Exogenous GB is able to inhibit the MDA and ROS over-accumulation in plants under environmental stresses and activate the expression of oxidative stress response genes (Annunziata *et al.* 2019). Exogenous GB has a major role in regulating the antioxidant activities in plants under drought, salinity, or cold stress (Kumar 2021a,b). Several related reports have shown the effectiveness of GB in increasing the activities of catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (POD), or ascorbate peroxidase (APX) in various plants subjected to different abiotic stresses (Ma *et al.* 2006, Malekzadeh 2015, Shan *et al.* 2016). However, as far as we know, there is no previous research that has investigated the effect of exogenous GB on alternative oxidase activity (AOX) in plants under drought or cold stress. Furthermore, few studies have focused on the effect of exogenous GB on the woody plants, and most of them have relied on crops.

*Dalbergia odorifera*, also named fragrant rosewood, is an endemic species to China, which is a highly-valued species suffering from vulnerability due to over-development for wood and medicine (Li *et al.* 2018). Further, there is a huge lack of studies concerning this species and particularly its responses to abiotic stresses even with its high profits. Thus, the purpose of this present work was to study the responses of *D. odorifera* against drought and cold stress as affected by GB treatment. The AOX is a ubiquinol oxidase located in plant mitochondria, it can protect against unexpected inhibition of the cytochrome *c* oxidase pathway and plays an important role in stress tolerance (Berthold *et al.* 2000, Mróz *et al.* 2015). The aim of our work was to analyze the antioxidant enzymes in

*D. odorifera* seedlings under drought and cold stress and investigate whether the application of exogenous GB is able to increase the AOX protein content in plants under such stresses. Indeed, in our previous study, we reported the increase in the amount of AOX protein in salt-tolerant *D. odorifera* seedlings (Cisse *et al.* 2021a) and Annunziata *et al.* (2019) showed the ability of exogenous GB to increase the activities of antioxidant enzymes in diverse plant species. Furthermore, the principal component analysis was used to analyze the association or dispersion of the ROS scavengers throughout the experiment.

## Materials and methods

**Plants and experimental design:** *Dalbergia odorifera* T. Chen seeds used in this experiment were collected from the plantation forest at Ledong county, Hainan Province in China (18° 42' 57.91" N, 108° 52' 18.65" E) in March 2019. Sterilized and healthy seeds were selected and sown in plastic bags (10 × 10 × 10 cm), and the substrate was composed of 70 % red soil and 30 % sand. Each plastic bag contained 2 - 3 seeds pre-treated with hot water (60 °C) for 10 min and warm water (28 °C) for 10 h. The pots were placed into an artificial incubator QHX-400BHS-III (CIMO Medical Instrument Manufacturing Co., Shanghai). The seeds were germinated under alternating temperature and humidity of 25/28 °C and 85/75 % with a 12-h photoperiod and with photosynthetic photon flux density of 67.56  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Pu *et al.* 2021).

After 90 d (15 September - December 2019), the selected seedlings of the same size were placed in an artificial incubator (AI) and exposed to two stresses (cold and drought). For drought stress, the plants were well-watered 15 h before the beginning of the treatment. Indeed a low amount of water has been administered to the seedlings to a final soil water content of 40 % between 2 - 3 d. To maintain the mild drought conditions in *D. odorifera* seedlings the mass of the plastic bags was measured every day and clean water was added to a final water content of 40 % until the final harvest point as described by Wikberg and Ögren (2007). The seedlings under chilling stress were well-watered during the experiment and placed in a growth chamber set up at 20/4 °C temperatures with a 16/8 h photoperiod. In each of the 5 days at the end of the 4 °C treatment, the fresh leaves in the middle were harvested, then quickly frozen in liquid N<sub>2</sub> and stored at -80 °C for different biochemical parameter analyses. Well-watered plants were used as controls; two groups of seedlings (6 replicates/4 seedlings) were exposed to different conditions. The first group was placed in natural conditions (December - January) and submitted to an average day/night temperatures of 22/17 °C (Nc). Meanwhile, the second group of seedlings was transferred to a growth chamber with day/night temperatures of 28/25 °C (AI). The glycine betaine (50 mM) treatment was applied firstly on the soil (1.5 cm<sup>3</sup>) and then sprayed on the leaves (0.5 cm<sup>3</sup>) every two days (one time) according to our previous experiment (Cisse *et al.* 2021a). Following GB application or not, the experiment presented 7 groups

with 24 seedlings each: 1) control (Ck): well-watered plants (AI); 2) Ck + GB: well-watered plants + 50 mM GB (AI); 3) Nc: well-watered plants in natural conditions; 4) drought: soil water content of 40 % (AI); 5) drought + GB: soil water content of 40 % + 50 mM GB (AI); 6) cold: 20/4 °C (AI); 7) cold + GB: 20/4 °C + 50 mM GB (AI). The Ck: well-watered condition (AI) was set up as a group of control for the comparison of the results in the present study since it was submitted approximately to the optimal conditions of *Dalbergia odorifera* growth.

**Measurement of the roots membrane permeability:** Cell death in root samples which indicate the loss of plasma membrane integrity was measured spectrophotometrically using the absorption of Evans blue solution (Baker and Mock 1994). The intensity of the colour is proportional to the number of dead cells. Three root ends (1 cm) are cut and then incubated in 0.025 % (m/v) Evans blue solution for 30 min. Then the roots were rinsed for 15 min and crushed in 800 mm<sup>3</sup> of a solution of 50 % MeOH (v/v) and 1 % (m/v) sodium dodecyl sulphate (SDS). The homogenate was incubated in a water bath at 50 °C for 15 min and then centrifuged at 10 000 g for 15 min. The absorbance of the supernatant was read at 600 nm.

**Electrolyte leakage, water potential, and relative water content determination:** Electrolyte leakage was determined according to Cisse *et al.* (2021b) by using 5 discs from fresh leaves that were rinsed in a Petri dish containing distilled water (C1). The relative conductivity was calculated by using the following formula  $EL = C1 \times 100 / C2$  according to Fan *et al.* (2015) where C2 was conductivity of samples placed in a boiling water bath for 20 min and then cooled to room temperature. The dew point water potential (DWP) was determined with a DWP meter (WP4C, Gene Company, America) in accordance with the manufacturer's instructions. The relative water content was determined in fresh leaves as described by Yang *et al.* (2010).

**The content of chlorophylls and carotenoids** was determined by the colorimetric method. Approximately 0.2 g of fresh leaves was ground in 10 cm<sup>3</sup> of 80 % (v/v) acetone, then the extract was centrifuged at 10 000 g and 4 °C for 10 min, and the supernatant was used for spectrophotometer readings at 663, 646, and 470 nm. The content of pigments in each sample was calculated according to Lichtenthaler and Wellburn (1983).

**Determination of malondialdehyde (MDA) and hydrogen peroxide:** MDA content was determined by the method described by Yang *et al.* (2010) with some modifications. About 200 mg of sample was homogenized in 5 cm<sup>3</sup> of 5 % (m/v) trichloroacetic acid (TCA) and then centrifuged at 10 000 g for 10 min. Then 4 cm<sup>3</sup> of 20 % (m/v) TCA containing 0.5 % (m/v) thiobarbituric acid (TBA) and 1 cm<sup>3</sup> of the supernatant were incubated at 95 °C for 30 min, cooled on ice, centrifuged at 10 000 g for 15 min and absorbance was read at 532 nm and the content of MDA was expressed in [nmol g<sup>-1</sup>(f.m.)].

The hydrogen peroxide was quantified as described by Yang *et al.* (2010) with some modifications. Fresh leaves samples (200 mg) were grounded and homogenized in 0.1 % trichloroacetic acid (m/v) then centrifuged at 10 000 g for 15 min. A supernatant was incubated in the presence of potassium iodide added to a 10 mM buffer solution. The absorbance was determined at 390 nm, and the content of H<sub>2</sub>O<sub>2</sub> was estimated *via* a standard curve.

**Measurement of proline, saccharides, and total phenols:** Proline content was determined by the colorimetric assay as described by Bates *et al.* (1973) with some modifications according to Cisse *et al.* (2021b).

Content of saccharides was estimated by the anthrone method (0.2 % anthrone) according to Yemm and Willis (1954) with some modifications following the description of Cisse *et al.* (2021b). The absorbance was read at 627 nm and a standard curve was used to calculate the content of the saccharides in each sample and expressed in [ $\mu\text{g g}^{-1}$ (f.m.)].

Phenolic compounds were determined according to the Folin-Ciocalteu reagent method (Swain and Hillis 1959). The phenolic compounds were extracted using 80 % (v/v) ethanol at 80 °C for 15 min and then the extract was concentrated by evaporating the ethanol. The residue was dissolved in 20 cm<sup>3</sup> of distilled water and to 1 cm<sup>3</sup> of aliquot, it was added 7.5 cm<sup>3</sup> of distilled water and 0.5 cm<sup>3</sup> of Folin's reagent. After 3 min, 1 cm<sup>3</sup> of saturated Na<sub>2</sub>CO<sub>3</sub> solution (40 %) was added to the tube and after 1 h absorbance was read at 725 nm. The content of phenols was determined according to the following formula:  $A_{725} \times \text{initial volume/fresh mass}$ .

**Measurement of proteins and thiols:** Soluble protein content was quantified by the Bradford method (1976). The absorbance was recorded at 595 nm and the content of protein was determined by using a standard curve.

Thiols react with 5,5'-dithiobis (2-nitrobenzoic acid) DTNB to form 2-nitro-5-thiobenzoic acid (TNB), which turns the colour of the solution to yellow in an alkaline medium and absorbance was read at 412 nm (Ellman 1959). The content of thiols was assayed and determined according to Cisse *et al.* (2021a).

**Measurement of antioxidant enzyme activities and AOX protein content:** Peroxidase (POD) activity was quantified following the method described by Fielding and Hall (1978) with some modifications. An amount of 0.5 g of fresh leaves was washed with distilled water, weighed, and triturated in a mortar at 4 °C by using 5 cm<sup>3</sup> of 0.1 M phosphate buffer solution (pH 7.0) containing 0.1 mM EDTA, 0.1 mM ascorbate, and 1 % polyvinylpyrrolidone (PVPP). The sample was centrifuged at 10 000 g and 4 °C for 15 min and the supernatant was used to assay the enzymatic activity of POD. The increment in absorption induced by guaiacol oxidation by H<sub>2</sub>O<sub>2</sub> was measured at 470 nm. Catalase (CAT) activity was determined by colorimetric assay at 240 nm by measuring the decomposition of H<sub>2</sub>O<sub>2</sub> according to the procedure described by Aebi (1984) with

some modifications. A sample of 0.3 g fresh leaves was homogenized with 0.1 mM potassium phosphate buffer (pH 7.8) on ice. The extract was centrifuged at 10 000 g and 4 °C for 15 min and then the supernatants were used to assay the CAT activities.

ELISA kit (*JL22749 Plant AOX ELISA KIT*; Jianglai Industrial Company, Shanghai, China) was used to determine the content of AOX protein in plant leaves according to [Cisse et al. \(2021a\)](#). A standard curve was used to estimate the content of AOX protein which was expressed in [ng g<sup>-1</sup>(f.m.)]. The protein formation of AOX does not directly determine the activity of the enzyme, but the increase of AOX protein is related to the AOX enzyme activity ([Saha et al. 2016](#)).

**Statistical analyses:** The results were expressed as mean ± standard error, and *Graph Pad Prism 9.0.0* software was used to draw the graphs and analyze the data. All data were subjected to analysis of variance for a factorial experiment in a completely randomized design. A two way ANOVA Tukey's HSD (honestly significant difference) test was used to determine the significance of our data at  $P \leq 0.05$ . The principal component analysis (PCA) was performed by *Graph Pad Prism 9.0.0*.

## Results

The group of seedlings submitted to optimal conditions represents the control group in this current experiment (Ck). Drought and cold stress significantly decreased the plant height, the diameter of the stem, and leaf thickness in *D. odorifera* seedlings (Table 1 Suppl.). The roots length was slightly diminished by the drought and cold stress. Generally, under both stresses, the seedlings subjected to GB treatment exhibited a significant increase of root length, plant height increment and diameter of the stem in comparison with the cold or drought-stressed seedlings (Table 1 Suppl. and Fig. 1 Suppl.). The leaf thickness was only increased (45.16 %) by GB under drought stress compared to only drought-stressed seedlings. The seedlings that were subjected to drought without GB treatment, showed a significant decrease of RWC (22.85 %), and a

decrease (22.85 %) of WP (Table 1 Suppl.). Meanwhile, GB did not influence significantly the RWC and WP in seedlings under cold stress. Fig. 2 Suppl. showed that after 2 d the seedlings submitted to cold stress exhibited injuries that can lead to the death of the plant after 7 d (Fig. 3 Suppl.); however, the cold-stressed plants treated by GB showed fewer injuries and stay alive beyond 7 d. Under non-stress conditions, the seedlings subjected to optimal conditions (Ck) showed better growth parameters compared to those under natural conditions (representing cold season). The addition of GB did not significantly increase the growth traits under non-stressed conditions.

The total chlorophyll (Chl<sub>a+b</sub>) content in *D. odorifera* leaves was strongly decreased by 80 % in the cold and drought treatments, compared to the control group (Ck,  $P \leq 0.001$ ). The carotenoid (Car) content in cold-stressed seedlings was significantly higher (19.35 %) compared to drought-stressed seedlings. Under drought or cold stress, the Chl content in seedlings was slightly increased by the GB treatment, but it was not significantly different from that without GB treatment. The Car content in cold-stressed seedlings was higher by 6.06 % compared to the GB cold-stressed seedlings (Table 1). Under non-stress conditions, the content of photosynthetic pigments was similar in Ck, Ck + GB and Nc groups.

Table 1 also shows the effect of drought and cold stress treatment combined with GB or not on root cell integrity. The absorption of the Evans blue solution was significantly higher in drought (63.02 %) and cold-stressed (62.03 %) seedlings compared to Ck. GB treatment reduced by 18.53 % the absorption of the blue Evans solution in the GB cold-stressed seedlings compared to the only cold-stressed seedlings. Under normal conditions, GB treatment improved significantly the membranes of the roots compared to Ck and Nc groups based on the lower absorption of the blue Evans solution in Ck + GB.

Electrolyte leakage (EL) is an important indicator related to leaf senescence and cell membrane deterioration. The results of the experiment confirm that the drought and cold stress applied to *D. odorifera* seedlings increased significantly the EL by 31.81 and 52 %, respectively, compared to the control (Table 1). Moreover, under cold

Table 1. Variations of content of chlorophylls (Chl) and carotenoids (Car), Evans blue absorption, electrolyte leakage (EL), content of phenolic compounds and thiols in *Dalbergia odorifera* leaves under drought and cold stresses and treated by GB, (for more detail see Materials and methods). Means ± SEs,  $n = 6$ ; different letters indicate significant differences between treatments ( $P < 0.05$ ) based on Tukey's multiple comparison tests.

Treatments	Chl <sub>a</sub> [mg g <sup>-1</sup> (f.m.)]	Chl <sub>b</sub> [mg g <sup>-1</sup> (f.m.)]	Car [mg g <sup>-1</sup> (f.m.)]	Evans blue [%]	EL [%]	Phenols [mg g <sup>-1</sup> (f.m.)]	Thiols [mg g <sup>-1</sup> (f.m.)]
Ck	1.1 ± 0.17 a	0.32 ± 0.05 ab	0.28 ± 0.03 a	23.8 ± 7.6 bc	14.8 ± 4.5 b	21.6 ± 0.89 b	1.02 ± 0.02 c
Ck+GB	1.3 ± 0.23 a	0.31 ± 0.11 ab	0.21 ± 0.05 ab	14.6 ± 3.9 c	16.5 ± 3.4 b	26.2 ± 1.67 a	1.21 ± 0.07 b
Nc	1.1 ± 0.26 a	0.41 ± 0.10 a	0.18 ± 0.02 abc	32.4 ± 2.8 b	15.6 ± 5.3 b	18.3 ± 0.83 cde	1.05 ± 0.02 c
Drought	0.5 ± 0.08 b	0.16 ± 0.09 c	0.14 ± 0.01 c	45.7 ± 10.4 a	20.4 ± 2.8 ab	19.7 ± 1.61 bcd	1.24 ± 0.08 b
Drought+GB	0.6 ± 0.14 b	0.17 ± 0.04 c	0.14 ± 0.02 c	45.2 ± 6.3 a	19.8 ± 1.7 ab	20.4 ± 0.19 bc	1.54 ± 0.02 a
Cold	0.4 ± 0.13 b	0.18 ± 0.11 c	0.17 ± 0.03 abc	33.6 ± 8.7 ab	25.2 ± 6.1 a	17.3 ± 1.42 e	1.04 ± 0.06 c
Cold+GB	0.7 ± 0.10 b	0.20 ± 0.08 bc	0.16 ± 0.01 bc	27.9 ± 3.5 b	21.9 ± 2.8 ab	17.7 ± 0.37 de	0.97 ± 0.03 c

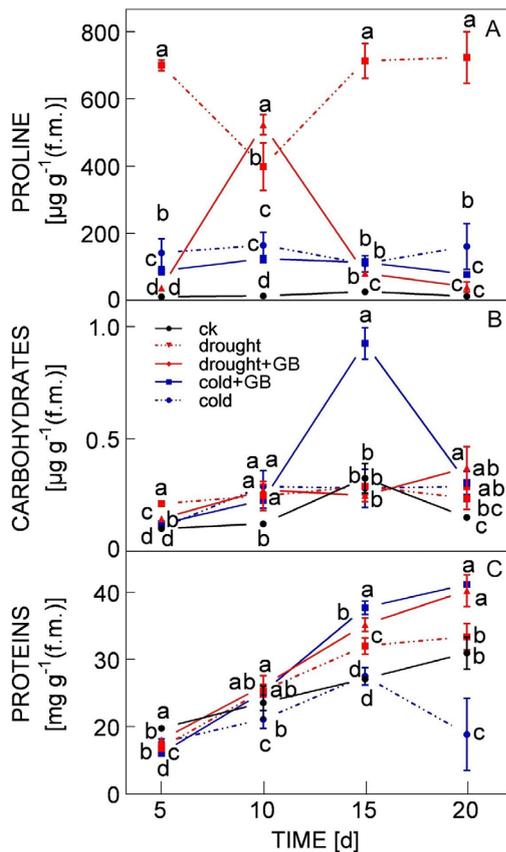


Fig. 1. Effects of glycine betaine (GB) on the content of proline (A), sugars (B) and proteins (C) in *Dalbergia odorifera* under drought and cold stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control.

stress, GB treatment reduced significantly (14.01 %) the EL in GB cold-stressed seedlings compared to the seedlings that were not subjected to GB treatment.

Our data in Table 1 show a significant increase in the content of phenolic compounds by GB treatment in *D. odorifera* seedlings under stress. However, the amount of phenolic compounds was strongly higher (14.17 %) in seedlings subjected to drought than in those under cold stress. Moreover, the thiols content in drought-stressed seedlings was significantly higher (17.54 %) than in cold-stressed plants. The content of thiols did not show significant differences between Ck seedlings and those submitted to cold stress. Without stress, the thiols and phenolic compounds were significantly enhanced by GB treatment in *D. odorifera* seedlings compared to Ck and Nc groups.

The content of sugars and proline of *D. odorifera* leaves was higher in seedlings under drought or cold stress compared to the Ck (Fig. 1A,B). Under drought stress, GB treatment mostly decreased the proline content in *D. odorifera* seedlings (Fig. 1A). Meanwhile, a comparison of cold-stressed and GB cold-stressed seedlings show a slight difference. Furthermore, the content of proline in *D. odorifera* leaves was strongly higher in seedlings

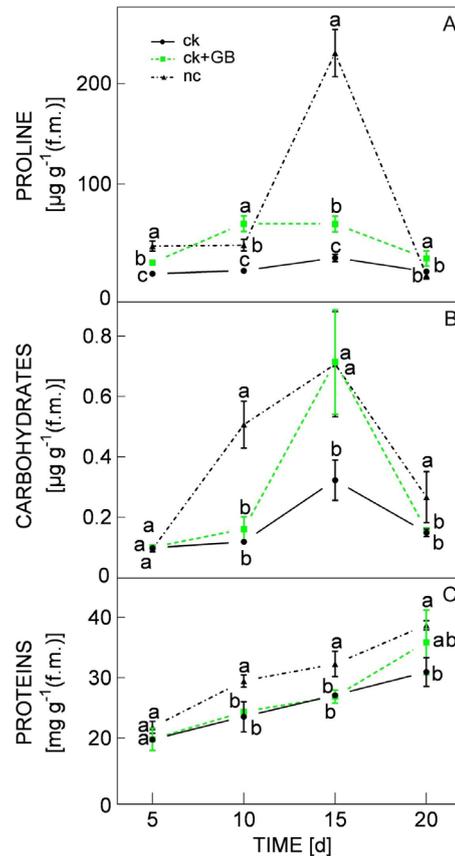


Fig. 2. Effects of glycine betaine (GB) on the content of proline (A), sugars (B) and proteins (C) in *Dalbergia odorifera* without stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control, nc - natural conditions.

that were subjected to drought compared to those under cold stress. Nonetheless, at day 10 the proline content strongly increased by 171.68 % in seedlings subjected to GB treatment under drought stress as compared to the drought-stressed seedlings. The results also showed that GB treatment decreased significantly (37.97 %) the sugar content in seedlings under drought compared to the drought-stressed seedlings, however, the sugar content was increased (15.96 %) in plants treated by GB under chilling stress at day 5 (Fig. 1B). However, under drought stress, the GB treatment enhanced significantly the sugar content in *D. odorifera* leaves only after day 20. Generally, under normal conditions, GB treatment (Ck + GB) increased slightly sugar content in seedlings compared to the Ck group; meanwhile, it increased significantly the proline content in *D. odorifera* seedlings (Fig. 2A,B). The soluble protein content of *D. odorifera* seedling leaves was significantly increased by GB treatment under drought and cold stress (Fig. 1C). The seedlings under cold stress without GB treatment exhibited a significant decrease in protein content during the experiment compared to drought-stressed seedlings. The fluctuation of proline and sugars in seedlings subjected to natural conditions (cold season) were similar to those in Ck + GB group at

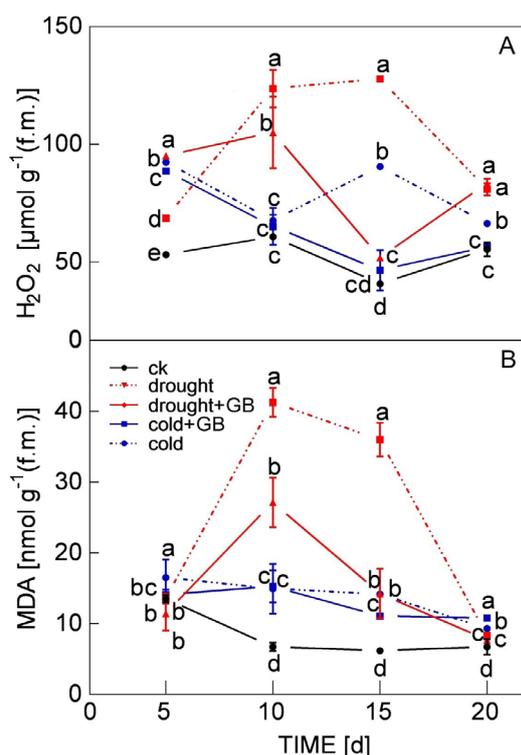


Fig. 3. Content of  $\text{H}_2\text{O}_2$  (A) and MDA (B) in *Dalbergia odorifera* seedlings subjected to glycine betaine (GB) treatment under drought and cold stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control.

different times (Fig. 2A,B). Meanwhile, without stress, the protein content was higher in seedlings exposed to natural conditions compared to Ck and Ck + GB groups, while the GB increased significantly the protein content at day 20 compared to Ck (Fig. 2C).

As shown in Fig. 3, during the cold and drought treatment,  $\text{H}_2\text{O}_2$  and MDA content in *D. odorifera* seedlings were significantly higher compared to Ck. The  $\text{H}_2\text{O}_2$  content in *D. odorifera* seedlings was strongly lower at days 5 and 15 in those grown under natural conditions compared to Ck and Ck + GB (Fig. 4A). Generally, after 10 d the drought-stressed seedlings showed a significant increase (75.55 % at day 15 and 17.97 % at day 20) in  $\text{H}_2\text{O}_2$  content as compared with the seedlings that were submitted to chilling stress. Moreover, during both stresses, the application of GB treatment decreased strongly the content of  $\text{H}_2\text{O}_2$  in *D. odorifera* seedlings compared to the stressed seedlings (Fig. 3A).

During this experiment, the GB treatment decreased strongly the MDA content in drought-stressed seedlings between 10 (45 %) to 15 (62.28 %) days (Fig. 3B). Meanwhile, under cold stress, the GB treatment reduced slightly the MDA content in *D. odorifera* seedlings (day 15). Without stress, the MDA content was similar in the control groups, but at day 15 the MDA content was significantly higher in seedlings subjected to GB treatment. During the treatments, the seedlings that were exposed to natural conditions showed an enhancement in MDA content compared to Ck and Ck + GB at days 5, 10,

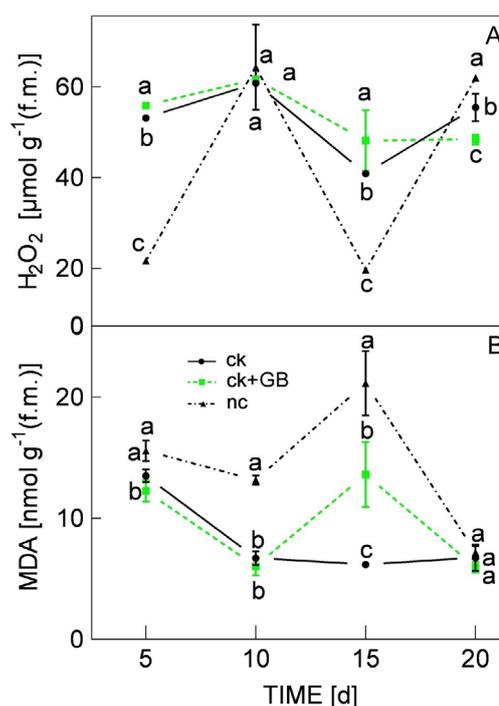


Fig. 4. Content of  $\text{H}_2\text{O}_2$  (A) and MDA (B) in *Dalbergia odorifera* seedlings subjected to glycine betaine (GB) treatment without stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control, nc - natural conditions.

and 15 (Fig. 4B).

The AOX protein content was considerably higher in seedlings under drought (129 and 171.52 %) or cold (71.85 and 163.25 %) stress and GB treatment at days 5 and 10 compared to Ck. Furthermore, under drought stress the GB treatment decreased significantly the AOX protein content, except for day 20 where the AOX protein content was greater in GB drought-stressed seedlings compared to only drought-stressed seedlings (Fig. 5A). Simultaneously, the GB treatment increased significantly the AOX protein content in seedlings under cold stress. Mostly, the activities of antioxidant enzymes in *D. odorifera* seedlings were significantly higher between days 5 and 10 in the cold and drought-stressed seedlings compared to Ck ( $P < 0.001$ , Fig. 5B,C). Generally, the peroxidase activity increased significantly with GB treatment under both stresses throughout the experiment (days 5, 10, 15, and 20) compared to the other treatments (Fig. 5C). Moreover, during the experiment, the POD activity was similar in seedlings under drought and cold stress without GB treatment. Furthermore, under cold stress, the seedlings subjected to GB treatment increased strongly the CAT activity at day 5 (9.21 %), 10 (40.16 %), 15 (21.73 %), and day 20 (50.06 %) compared to the cold-stressed seedlings. At day 20 in seedlings under cold stress without GB treatment, the CAT activity was strongly lower than in the Ck (Fig. 5B). Moreover, besides cold or drought stress the GB treatment increased strongly the AOX content in *D. odorifera* seedlings only at day 10 compared to Ck.

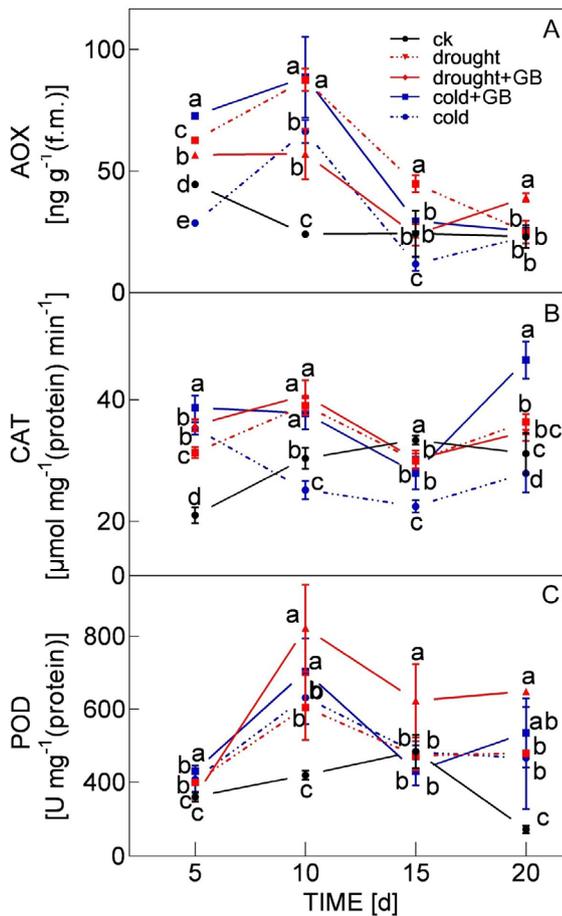


Fig. 5. Content of AOX protein (A) and activities of CAT (B) and POD (C) in *Dalbergia odorifera* seedlings treated with glycine betaine (GB) and under drought or cold stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control.

The seedlings that were exposed to natural conditions showed a significant increase in the AOX content at day 15 (Fig. 6A). Furthermore, under non-stress conditions the CAT activity was significantly enhanced in Ck + GB seedlings throughout the experiment compared to the Ck group, moreover, the POD activity was increased at days 15 and 20 by GB treatment (Fig. 6B,C).

The assessment of the correlation between different measured parameters has been done by principal component analysis. The total cumulative proportion of variance of the PC1 and PC2 factors corresponded to 62.44 %, and their eigenvalues were higher than 1. The proportion of variance of the first two principal components was 42.97 and 19.47 %, respectively. The heat map and loadings plot based on PC1 and PC2 was used to understand the data structure and measured parameters associations (Fig. 4 Suppl.). Fig. 5 Suppl. showed a strong dissociation between thiols and proteins; and a significant association among  $H_2O_2$  and root membrane permeability; POD, EL, sugars, and MDA; between root height, water potential, water content, and diameter of stem; between the photosynthetic pigments at the end of the experiment (day 20). The association among AOX, POD, and CAT was not strong as indicated

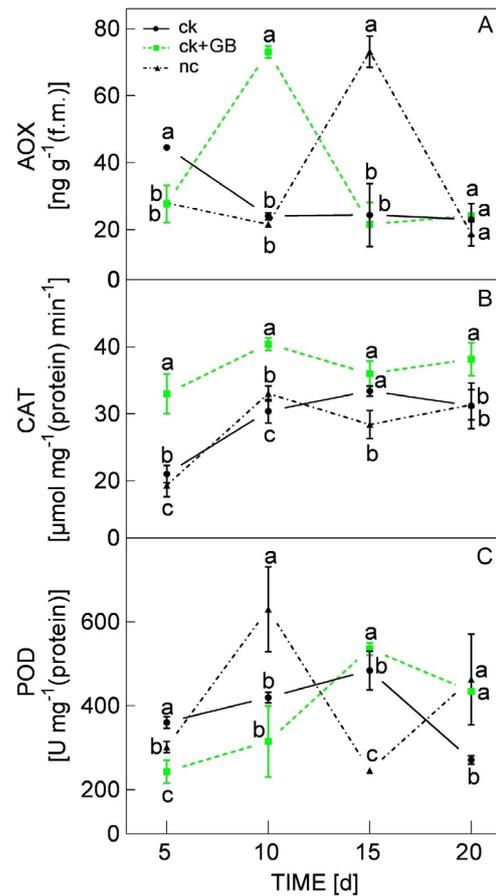


Fig. 6. Content of AOX protein (A) and activities of CAT (B) and POD (C) in *Dalbergia odorifera* seedlings treated with glycine betaine (GB) without stress. Means  $\pm$  SEs,  $n = 6$ , different letters indicate significant differences according to Tukey's multiple comparison test ( $P < 0.05$ ). ck - control, nc - natural conditions.

by the angles between their vectors. There were two major groups of association following Fig. 5 Suppl.; the first was related to Chl  $a+b$ , Chl  $a$ , Chl  $b$ , and Car. The second group belonged to growth traits (diameters of stem and root length), WP, and WC. Fig. 6 Suppl. displayed the way that different parameters related to oxidative stress are associated through the duration of the present experiment. It showed a strong association between POD and AOX; among AOX and CAT based on the small angle of their vectors (at days 5 and 10, respectively). The association between the two osmoprotectants (proline and sugars) was strong at day 5 (Fig. 6A Suppl.). Indeed, the association between the different measured parameters responsible for the ROS scavenging split up following the duration of the experiment (Fig. 6C,D Suppl.).

## Discussion

The evaluation of the effects of abiotic stresses on plants can be based on their effects on water status, growth parameters, antioxidant enzyme activities, photosynthesis, and other parameters. The results of the present study

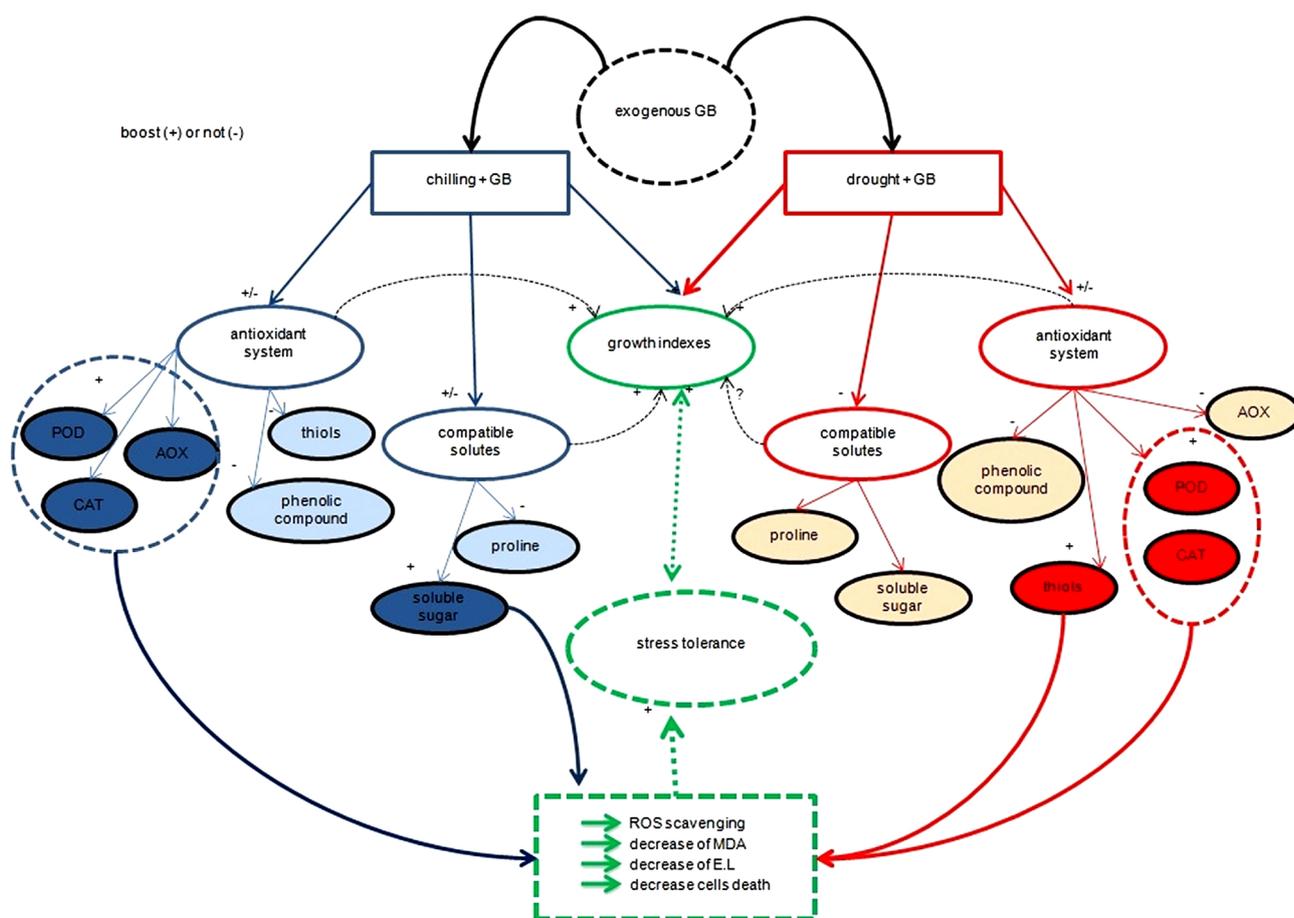


Fig. 7. A designed model showing different pathways that exogenous GB utilizes to enhance the drought and cold tolerance in *Dalbergia odorifera*. AOX - alternative oxidase, CAT - catalase, EL - electrolyte leakage, GB - glycine betaine, MDA - malondialdehyde, POD - peroxidase, ROS - reactive oxygen species.

confirmed the negative effects of drought and cold stresses on plant growth as also mentioned by Ritonga and Chen (2020). Nonetheless, GB treatment increased significantly the growth parameters in *D. odorifera* seedlings under drought and cold stresses. Indeed, GB can improve the growth and survival of plants counterbalancing metabolic dysfunctions caused by drought or chilling (Annunziata et al. 2019). Moreover, Malekzadeh (2015) reported the positive effect of GB treatment on growth traits in the soybean plant. Drought or cold stress can decrease the absorption of water that is followed by stomata closure (Sharma et al. 2020). Furthermore, numerous studies concerning low accumulators and non-accumulators of GB proved the beneficial effect of GB on photosynthetic pigments and on preserving the PS II function (Khan et al. 2014, Tian et al. 2017). The results of this work showed better photosynthetic pigments accumulation in seedlings treated with GB under drought and cold stress. Based on the previous study, that phenomenon can be explained by the fact that GB application to leaves can permit the translocation of GB to the meristematic tissues, particularly to flower buds and shoot apices, and then interact with photosynthesis apparatus (Mäkelä et al. 1996, Park et al. 2006, Annunziata et al. 2019).

Moreover in this study exogenous GB relieved drought and cold stress damage to cell membranes by reducing the electrolyte leakage or roots membrane permeability in *D. odorifera* seedlings. However, the decrease of those two indexes was not significant under drought stress. Indeed, exogenous GB reduced significantly EL and root membrane permeability provoked by cold and drought stress in *D. odorifera* seedlings. The increase of the root membrane permeability and electrolyte leakage in stressed seedlings occurred together with a high accumulation of  $H_2O_2$ . Indeed, GB can decrease the EL and root membrane permeability via its ability to scavenge directly the ROS or might interact with the efflux of various ions involved in membrane stability under stress, thus reducing loss of membrane integrity and membrane damages.

The lipid peroxidation was reduced by GB treatment, particularly under drought stress. Meanwhile, during the experiment, the seedlings submitted to cold stress showed a slightly decreased MDA content. Indeed, the accumulation of GB in plant cells can adjust the disturbance of osmotic homeostasis caused by abiotic stresses such as cold or drought stress (Xu et al. 2018). Also, Chen and Murata (2008) suggested that one of the roles of GB might be to protect cell membranes. Furthermore,

the accumulation of GB in the pepper plant contributed to the protection of membrane damages caused by water deficit (Escalante-Magaña *et al.* 2019). Drought and cold as abiotic stresses can trigger oxidative stress in plants that is due to overproduction and accumulation of ROS. The accumulation of H<sub>2</sub>O<sub>2</sub> in the plant can provoke severe damages in cell components and cause their dysfunction (Demidchik 2015, Staniak *et al.* 2020). The results showed a significant decrease of H<sub>2</sub>O<sub>2</sub> in *D. odorifera* seedlings by GB treatment under abiotic stresses during our experiments. Previous studies showed the positive effect of GB treatment on H<sub>2</sub>O<sub>2</sub> scavenging in diverse plants such as rice (Demiral and Türkan 2004) or tobacco plant (Banu *et al.* 2010) under abiotic stresses. Among ROS, the choice of H<sub>2</sub>O<sub>2</sub> lies in the fact that H<sub>2</sub>O<sub>2</sub> is more stable than the others (Sies 1993). The results suggested that GB treatment scavenge directly the ROS in *D. odorifera* plant cells to relieve the damages caused by the oxidative stress. To cope with oxidase stress caused by abiotic stresses, plants can use diverse pathways to counteract the overaccumulation of ROS in plant cells. Under abiotic stresses, plants can accumulate osmolytes such as proline or sugars to counterbalance the disturbed homeostasis provoked by abiotic stress and maybe directly scavenge the ROS (Sharma *et al.* 2019). The results showed that GB can increase the sugar content under chilling stress and also in controls. Different studies reported a positive correlation between soluble sugar content and exogenous GB in plants under abiotic stress. The increased amount of photosynthetic pigments in this present study might explain the positive effect of GB on the biosynthesis of sugars as described by Osman (2015). Indeed, the application of GB in leaves of *Pisum sativum* under drought stress increased the growth traits and the content of photosynthates (Osman 2015).

The proline content did not increase in *D. odorifera* seedlings by GB treatment under chilling or drought stress. Thus, the results suggested that exogenous GB might regulate the ROS scavenging and cell homeostasis in *D. odorifera* by regulating the compatible solutes. The accumulation of exogenous GB in plant cells might provoke the regulation of proline and sugar content in *D. odorifera* leaves under abiotic stresses. Indeed, as an osmolyte, GB has the same function similarly to proline or sugars (Sharma *et al.* 2019). Moreover, this present work confirmed the report of Yancey (1994) that related a protective role of GB in plants protein content under abiotic stresses. The seedlings that were submitted to GB treatment exhibited an enhancement in protein content.

Furthermore against oxidative stress, the plant can accumulate diverse antioxidants such as the phenolic compounds or the thiols which can play a major role to relieve the damages caused by environmental stresses (Zagorchev *et al.* 2013, Varela *et al.* 2016). Indeed it was also reported that the phenols own an antioxidant activity which can scavenge the ROS under abiotic stress in *Hypericum pruinatum* (Caliskan *et al.* 2017). In the present work, the thiols were significantly more involved in providing stress tolerance in *D. odorifera* seedlings compared to the phenolic compounds and particularly during drought stress. Furthermore, the other component

of the antioxidant machinery has a major role to scavenge ROS (Das and Roychoudhury 2014). These studies focused on *Glycine max* (Malekzadeh 2015) or wheat plants (Ma *et al.* 2006, Raza *et al.* 2007, 2014). Moreover, exogenous GB provides chilling tolerance in *Prunus persica* by improving the antioxidant activities (Shan *et al.* 2016). Nonetheless, it is important to notice a lack of information about the effect of GB on AOX protein content. There is no study that focused on the effect of exogenous GB in plants AOX protein content under abiotic stresses. Despite the major role that AOX play against environmental stresses. In the present study, GB showed its ability to increase the CAT, POD activities and AOX protein content in *D. odorifera* seedlings. However, under drought stress, GB treatment did not increase the AOX protein content. But under cold stress, exogenous GB promotes the AOX protein content rather than POD activity (Fig. 7). The present work demonstrated how exogenous GB alleviated drought and cold stress in *D. odorifera* seedlings (Fig. 7).

## Conclusions

Abiotic stresses are able to induce a series of physiological dysfunctions in woody plants. To cope with environmental stresses in a plant, it is widely considered to use exogenous glycine betaine for conferring abiotic stress tolerance to diverse plant species. The present findings confirmed that the exogenous glycine betaine promoted efficiently drought and chilling tolerance in a woody plant *D. odorifera*. However, most theories or studies about the influence of exogenous GB in plants are focused on POD, CAT, and SOD. This study proves the involvement of exogenous glycine betaine in the regulation of the plant AOX protein content during environmental stresses. Furthermore, exogenous GB provides abiotic stress tolerance in different pathways.

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