RESPONSES OF PHOTOSYNTHESIS, CHLOROPLAST ULTRASTRUCTURE, AND ANTIOXIDANT SYSTEM OF *MORINDA OFFICINALIS* HOW. TO EXOGENOUS 2, 4-EPIBRASSINOLIDE TREATMENTS UNDER HIGH TEMPERATURE STRESS

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Abstract. The present study attempts to evaluate the effects of EBR (2, 4-epibrassinolide) on photosynthetic parameters, biochemicals, antioxidant systems, and chloroplast ultrastructure in the leaves of *Morinda officinalis* under high temperature (HT). HT stress significantly reduced the net photosynthetic rate (P_N), stomatal conductance (G_s), and transpiration rate) (T_r), photosynthetic pigments, and inhibited photochemical activity. Besides, the application of EBR alleviated the decrease in chlorophyll contents and the inhibition of photosynthesis induced by HT stress and improved photosystem II efficiency. Furthermore, HT stress markedly increased reactive oxygen species levels and lipid per-oxidation, while the application of 0.5-1.0 mg L^{-1} EBR inhibited their increase and enhanced the activity of anti-oxidative enzymes. Microscopic analyses revealed that HT stress induced damages to chloroplasts and thylakoid membranes, displaying chloroplast envelopes disrupted, grana lamellae blurred and stroma lamellar disordered, while 0.5-1.0 mg L^{-1} EBR treatment effectively protected the thylakoid membrane structure from HT stress, maintained the typical shape of chloroplasts, and promoted the formation of grana. It can be concluded that 0.5-1.0 mg L^{-1} EBR can reduce the harmful effects of HT on *M. officinalis* seedlings by improving photosynthesis and protecting the photosynthetic membrane system from oxidative damage through up-regulating the capacity of antioxidant system.
Keywords: *photosynthetic parameters, chlorophyll, photosystem II, lipid per-oxidation, thylakoid membrane*

Abbreviations: BRs - brassinosteroids; EBR - 2, 4-epibrassinolide; P_N - net photosynthetic rate; G_s - stomatal conductance; T_r - transpiration rate; C_i - intercellular carbon dioxide concentration; *Chl* - chlorophyll; *Car* - carotenoids; F_0 - minimal fluorescence; F_m - the maximum fluorescence; F_v/F_m - maximal photochemical efficiency; Φ_{PSII} - actual photochemical efficiency of PSII; NPQ - non-photochemical quenching; qP - photochemical quenching; APX - ascorbate peroxidase; CAT - catalase; MDA - malondialdehyde; POD - peroxidase; SOD - superoxide dismutase; GR - glutathione reductase; GSH - reduced glutathione; ROS - reactive oxygen species; O_2^- - superoxide radical; H_2O_2 - hydrogen peroxide; EL - electrolyte leakage; *ChM* - chloroplast membrane; *SG* - starch granule; *OS* - osmiophilic plastoglobuli; *GT* - grana thylakoid; *SL* - stroma lamellae; *GL* - grana lamellae; control - normal temperature with distilled water; HT - high temperature with distilled water; HB1 - high temperature with spraying 0.10 mg L^{-1} EBR; HB2 - high temperature with spraying 0.50 mg L^{-1} EBR; HB3 - high temperature with spraying 1.00 mg L^{-1} EBR

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Introduction

Temperature is the most important environmental factor affecting plant growth and development. Excessive temperature is considered to be one of the most severe abiotic stresses restricting plant distribution, growth, and productivity (Jin, 2011; Niu and Wan, 2008). This stress often leads to molecular, biochemical, and physiological modifications which negatively affect the metabolism, reducing the growth and development of plants (Chen et al., 2017; Zhou et al., 2019; Tarin et al., 2020a). Photosynthesis is a key plant physiological process most sensitive to HT stress (Mathur et al., 2014). The thylakoid lamellae of photochemical reaction sites and chloroplast matrix of carbon metabolism sites are the main sites of damage under HT stress (Wise et al., 2010). Photosystem II (PSII) is the most sensitive link to HT during photosynthesis (Crafts-Brandner and Law, 2000). HT not only causes the significant changes in the number, morphology, and structure of organelles such as chloroplasts and mitochondria in plant leaves (Havaux and Tardy, 1996), it also changes the photosynthetic rate by changing the stomatal structure of plant leaves (Zheng et al., 2013), reducing chlorophyll content (Habap et al., 2014) and affecting the electron transfer process of PSII (He et al., 2017; Zushi et al., 2012). Furthermore, HT stress often destroys the balance of reactive oxygen species (ROS) metabolism in plant leaves, causes the accumulation of ROS free radicals, causes membrane lipid peroxidation, protein degeneration, cell membrane damage, resulting the increase of cell membrane permeability or even disintegration, and hindrance in normal metabolism of cells (Khanna-Chopra, 2012; Sedigheh et al., 2011; Silva and Asaeda, 2017). Simultaneously, it also promotes the activities of antioxidant enzymes such as SOD, POD, CAT, APX and the contents of non-antioxidant enzymes such as ASA and GSH to scavenge reactive oxygen species and alleviate the damage of HT stress to plants (Djanaguiraman et al., 2010; Gupta et al., 2013; Wu et al., 2014). In recent past, rise in temperature globally (Hansen et al., 2010; Virginia and Ebi, 2012), many provinces and regions in China have been experiencing prolonged summer with HT (Liu et al., 2015; Peng, 2014), particularly in the vast areas of southern China, which have experienced severe heat waves (Liu et al., 2017; Peng, 2014; Zuo et al., 2016).

Morinda officinalis How. is a vine shrub of Rubiaceae family, mainly grows in tropical and subtropical mountainous areas and forests and is distributed in Fujian, Guangdong, Guangxi, Hainan, and other southern provinces of China (Chen and Taylor, 2011). More than 100 compounds have been isolated from the flesh roots of *M. officinalis*, including anthraquinones, iridoid glycosides, phytosterols, polysaccharides, and oligosaccharides (Chen and Xue, 1987). The compounds extracted from the root of this plant can be used to relieving a wide spectrum of diseases, such as impotence (Wu et al., 2015), osteoporosis (Zhu et al., 2008), depression (Li et al., 2004), rheumatoid arthritis (Shin et al., 2013), dermatitis (Zhang et al., 2013a), and many other diseases. Furthermore, the roots of this plant have long been used as a tonic for kidney and yang, strengthening muscles and bones, eliminating rheumatism, and improving immunity, which has become one of the most common traditional medicines in China and even in Northeast Asia (Zhang et al., 2018). The suitable growth temperature of *M. officinalis* is 20-25 °C, in which the wild growth environment is a shrub or sparse forest edge on a hillside at an altitude of about 300 m approximately. An increase in the market demand for wood has put enormous pressure on the natural plantation *M. officinalis.* Recently, efforts have been made on the artificial plantation of *M. officinalis* by expanding the planting area in Fujian and Guangdong provinces covering almost 5000 ha in total (Zhang et al., 2016). *M. officinalis* is normally planted for 5-6 years before the final harvest. Most of the planting areas are low mountains

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and hills with an altitude of 300-700 m and a slope of 20-40 degrees. Previous reports have shown that *M. officinalis* is susceptible to destruction in stem rot resulted from HT stress in summer (Ding and Xu, 2003; Huang, 1982). HT in summer has seriously affected the normal growth and quality of *M. officinalis*. Therefore, there is a need to explore, how to improve the heat tolerance of *M. officinalis* which has become a serious problem in cultivation.

The application of exogenous phytohormones is the most effective and commonly used method to improve plant tolerance under abiotic stress. Brassinosteroids (BRs) are a class of natural products widely existing in plants, which are similar to animal and insect steroids, which have been identified as the sixth major plant hormone regulating plant growth and development (Clouse and Sasse, 1998). Brassinolide is the first BR isolated and identified (Grove et al., 1979). BRs can regulate plant growth (Que et al., 2018), stimulate different plant metabolic processes (Sasse, 2003), reduce oxidative stress, alleviate photosynthesis inhibition, improve plant resistance and yield under adverse conditions, such as drought stress (Lima and Lobato, 2017), high and low-temperature stress (Qu et al., 2011; Zhang et al., 2013b), heavy metal stress (Ramakrishna and Rao, 2013), salt stress (Karlidag et al., 2011), and other biotic or abiotic stresses.2, 4-epibrassinolide (EBR) is a synthetic high-activity analogue of brassinolide. It has been widely used in production because of its good stability and low cost (Bajguz and Hayat, 2009). Zhang et al. (2014) reported that exogenous 2, 4-EBR (0.5-1.5 mg L^{-1}) alleviated the growth inhibition induced by HT stress. 1.0 mg L^{-1} 2, 4-EBR could increase the antioxidant enzyme activity, soluble protein and free proline content of melon under HT stress, and reduce the content of malondialdehyde. Spraying 0.05-0.2 mg L^{-1} EBR could improve photosynthetic efficiency and antioxidant enzyme system of eggplant seedlings under HT stress, and inhibit the accumulation of reactive oxygen species and lipid peroxidation (Wu et al., 2014). Pretreatment of *Robinia pseudoacacia* seeds with 1.04 μM 2, 4-EBR improved the stability of chloroplast cell membrane and thylakoid membrane, decreased the concentration of sodium ion in leaves, significantly reduced photosynthesis inhibition, and maintained a higher net photosynthetic rate (Yue et al., 2019).

However, BRs-mediated stress response occurs in different tissues, and the mechanism of BRs-induced tolerance is not fully understood. There are few reports about the effect of BRs on heat tolerance of woody plants (Li et al., 2018). Therefore, this study aims to investigate the impact of exogenous 2, 4-EBR on chloroplast ultrastructure, gas exchange, chlorophyll fluorescence, and antioxidant system of *M. officinalis* seedlings exposed to HT stress, and to reveal how EBR acting to alleviate HT stress on *M. officinalis* seedlings.

Materials and methods

Description of the study area, plant material, and treatments

The present experiment was started from April to September 2018 in Fujian Agriculture and Forestry University (26°08'N, 119°24'E), Fujian Province, China. Five-month-old healthy cutting seedlings of *M. officinalis* of uniform size (height: 20 cm) were obtained from Nanjing County, Hexi Town, Zhangzhou City, Fujian Province, China. The well-growing seedlings of *M. officinalis* were grown in plastic containers (17 cm in height and 15 cm in diameter) in a completely randomized design (*Fig. A1* in the *Appendix*), with a mixture of red soil and peat (9:1 volume ratio). Two plants per container were placed in Greenhouse with 60% to 70% relative humidity at 28/22 °C (day/night) for 8 weeks. The pre-culturing of cutting seedlings of *M. officinalis* were carried out in the plant incubator

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(60 cm × 60 cm × 50 cm) under the following controlled conditions; a 12-h photoperiod, the temperature of 28/22 °C (day/night), the photosynthetic photon flux density 600 μmol m^{-2} s^{-1} with 60 to70% relative humidity. All plants were irrigated every two days and NPK nutrients solution containing 1.0 mM Ca $(NO_3)_2$, 9.0 mM KNO_3, 0.5 mM KH_2PO_4 was applied once a week.

The 2, 4-epibrassinolide (EBR, sigma, USA) was dissolved in a minimum volume of ethanol and made to be a storage solution with distilled water, stored at 4 °C. Then the storage solution was diluted into the concentrations required for the test with distilled water, and adding tween 20 with a volume ratio of 0.5% as the surfactant. The EBR and HT treatment started after ten days of pre-culturing. Plants were divided into two groups; normal temperature (28 °C/22 °C) and HT (38 °C/30 °C), before exposure to the HT treatment under the similar condition of a-12 h photoperiod and 600 μmol m^{-2} s^{-1} photosynthetic photon flux density. Five different treatment combinations were made; (1) normal temperature + distilled water as control, (2) HT + distilled water as HT, (3) HT + spraying 0.10 mg L^{-1} EBR as HB1, (4) HT + 0.50 mg L^{-1} EBR as HB2, and (5) HT + 1.00 mg L^{-1} EBR as HB3 (Wu et. al., 2014). The pots were arranged in a completely randomized design with three replicates for each treatment (96 plants) with 480 plants in total for all five treatments. All the plants were sprayed with 25 ml of distilled water or EBR once in every two days. On the third day after spraying (the 10^{th} day under HT stress), leaves from the middle part of plant for each replicate were selected for the measurement of photosynthetic parameters and the third leaf of plant for each replicate from the five treatment were sampled for chloroplast ultrastructure observation, then the others were sampled for determination of chlorophyll contents, ROS and antioxidant systems as described under.

Measurement of gas exchange parameters

The net photosynthetic rate (P_N), transpiration rate (T_r), stomatal conductance (G_s) and intercellular CO_2 concentration (C_i) of the third or fourth fully expanded leaf from the top were measured by using an infrared gas analyzer portable photosynthesis system (LI-6400 XT, LI-COR Inc., Lincoln, NE, USA) from 09:00 to 11:30 h local time as described by Tarin et al. (2019b). During the measurements, the photosynthetic photon flux density was set to 800 μmol m^{-2} s^{-1}, the air relative humidity was from 60 to70%, the leaf temperature was maintained at 25 °C and the ambient CO_2 concentration was about 400 μmol mol^{-1}. Photosynthesis measurement was made once for each leaf and five leaves from different plants per treatment and repeated three times in each treatment.

Determination of chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured by using a portable pulse-modulated fluorometer (PAM-2500, Walz, Effeltrich, Germany). The data were taken once on the fourth fully expanded leaf of each plant, and six leaves of each treatment were chosen and numbered basipetally. The minimum fluorescence (F_0) and the maximum fluorescence (F_m) induced by a saturation pulse under a weak modulated light were determined after 30 min of dark adaption. Then the minimum fluorescence (F_0') and the maximum fluorescence (F_m')induced by a second saturation pulse in the light-adapted state, and the steady-state fluorescence (F_s) were recorded after light adaption at actinic light of 600 μmol m^{-2} s^{-1}. The maximum photochemical quantum yield of PSII(F_v/F_m), the effective photochemical quantum yield of PSII($\Phi_{PS\,II}$), non-photochemical quenching (NPQ), and

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photochemical quenching (qP) were calculated as the following formula, respectively (Genty et al., 1989; Roháček, 2002).

$$F_v/F_m = \frac{F_m - F_0}{F_m} \quad \text{(Eq.1)}$$

$$NPQ = \frac{F_m}{F_m'} - 1 \quad \text{(Eq.2)}$$

$$\Phi_{PSII} = \frac{F_m' - F_s}{F_m'} \quad \text{(Eq.3)}$$

$$qP = \frac{F_m' - F_s}{F_m' - F_0'} \quad \text{(Eq.4)}$$

Estimation of photosynthetic pigment determination

The photosynthetic pigment concentrations were extracted from 0.2 g leaves with 80% acetone (v/v) in the dark. The absorbance of the extracts was recorded by spectrophotometric measurements at 645, 663, and 470 nm, and the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, respectively were calculated according to the methodology of Lichtenthaler (1987).

Determination of ROS production and lipid peroxidation

The contents of hydrogen peroxide (H_2O_2) were determined by the absorbance of the peroxide-titanium complex at 410 nm as described by Patterson et al. (1984). The determination of the superoxide radical (O_2^-) production rate was based on the method of Elstner and Heupel (1976). The content of superoxide radical (O_2^-) was calculated from the standard curve of nitrite formed from the chemical reaction of hydroxylamine and superoxide radical (O_2^-).

The contents of malondialdehyde (MDA) were measured as described by Guo et al. (2006a) by monitoring the thiobarbituric acid reactive products. Electrolyte leakage was determined by the method of Gong et al. (1998). The electrical conductance value (EC_1) was measured by inserting the electrode of the conduct meter into the exosmosis solution of leaves samples, then heating at 95 °C for 20 min to release the electrolytes. After cooling, the electrical conductance value (EC_2) was measured again. The percentage of electrolyte leakage was calculated by the following the formula:

$$\text{Electrolyte leakage (\%)} = \frac{\text{electrical conductance value of fresh sample}}{\text{electrical conductance value of sample cooked}} \times 100 \quad \text{(Eq.5)}$$

Determination of antioxidant enzyme activity and reduced glutathione (GSH) content

The activity of superoxide dismutase (SOD) was analyzed by the NBT method of Giannopolitis et al. (1977). One unit of SOD activity is the amount of enzyme required to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) by 50% at 560 nm. Catalase (CAT) activity was measured according to Havir and Mchale (1987).

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The activity of catalase was defined as the decrease in the absorbance at 240 nm for 1 min following the consumption of H_2O_2.

The activity of ascorbate peroxidase (APX) was determined according to the method of Nakano and Asada (1987) by monitoring the decrease of ascorbate peroxidase and measuring the change in absorbance at 290 nm for 1 min. Peroxidase activity (POD) was estimated by using the method of Kochba et al. (1977) by following the rate of guaiacol oxidation at 470 nm. The content of reduced glutathione (GSH) and the production rate of glutathione reductase (GR) both were determined by the method given by Foyer and Halliwell (1976).

Determination of free proline and soluble proteins content

Free proline contents were determined according to the method of Bates et al. (1973) by monitoring the ninhydrin colorimetric reaction. The contents of the total soluble proteins were determined by using the method of Bradford (1976). The absorbance was measured at 595 nm using bovine serum albumin as a standard.

Observation of chloroplast ultrastructure

Samples were collected from the middle of the main vein of the third leaf below the apical bud, cut into pieces of 1~2 mm^2. The cut leaves were immediately fixed in a solution containing 3% (v/v) glutaraldehyde in a 0.1 mol L^{-1} phosphate buffer (pH 7.2, 4 °C) for 2 h followed by 2% (w/v) osmium tetroxide in the same buffer for 2 h. After dehydration in a gradient series of ethanol and embedding in Epon 812, ultrathin sections (50~60 nm thick) were made with using an ultramicrotome, then stained with uranium acetate and lead citrate according to the method of Sun et al. (2011), and examined under a Tecnai G2 Spirit Bio Twin transmission electron microscopy (TECNAI, American) at an accelerating voltage of 120 KV.

Statistical analysis

Three replicates were chosen for all biochemical analyses, the data were expressed as the mean ± standard deviation (SD) with a minimum of three replicates. Statistical analysis was performed by one-way ANOVA with SPSS 17.0 (SPSS, Chicago, USA), the significant differences between the means were determined by using Duncan's multiple range comparison tests at 0.05 level of significance.

Results

Effects of 2, 4-EBR on the gas exchange parameters under HT stress

Compared to control treatment, HT stress significantly decreased P_N (*Fig. 1A*), T_r (*Fig. 1C*) and G_s (*Fig. 1D*) by 58.54%, 21.60%, and 50.96% respectively and significantly increased C_i (*Fig. 1B*) by 50.42% ($P < 0.05$). The application of 0.5-1.0mgL^{-1} EBR during stress significantly increased P_N (*Fig. 1A*), G_s (*Fig. 1C*) and T_r (*Fig. 1D*), and significantly decreased C_i (*Fig. 1B*) ($P < 0.05$) compared to HT treatment. The 0.1 mg L^{-1} EBR also significantly increased P_N, T_r, and decreased C_i ($P < 0.05$), but the increase in G_s was not significant. The highest P_N (3.37 μmol CO_2 m^{-2} s^{-1}), G_s (0.03 mol H_2O m^{-2} s^{-1}) and T_r (1.02 mmol H_2O m^{-2} s^{-1}), and lowest C_i (239.5 μmol CO_2 mol^{-1}) were observed in HB2 treatment, which were 135.73%,

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62.57%, 61.03% higher ($P < 0.05$), and 31.20% ($P < 0.05$) lower than HT treatment, respectively. Whereas the P_N and C_i under HB2 treatment, no significant difference as compared to control treatment (*Fig. 1*).

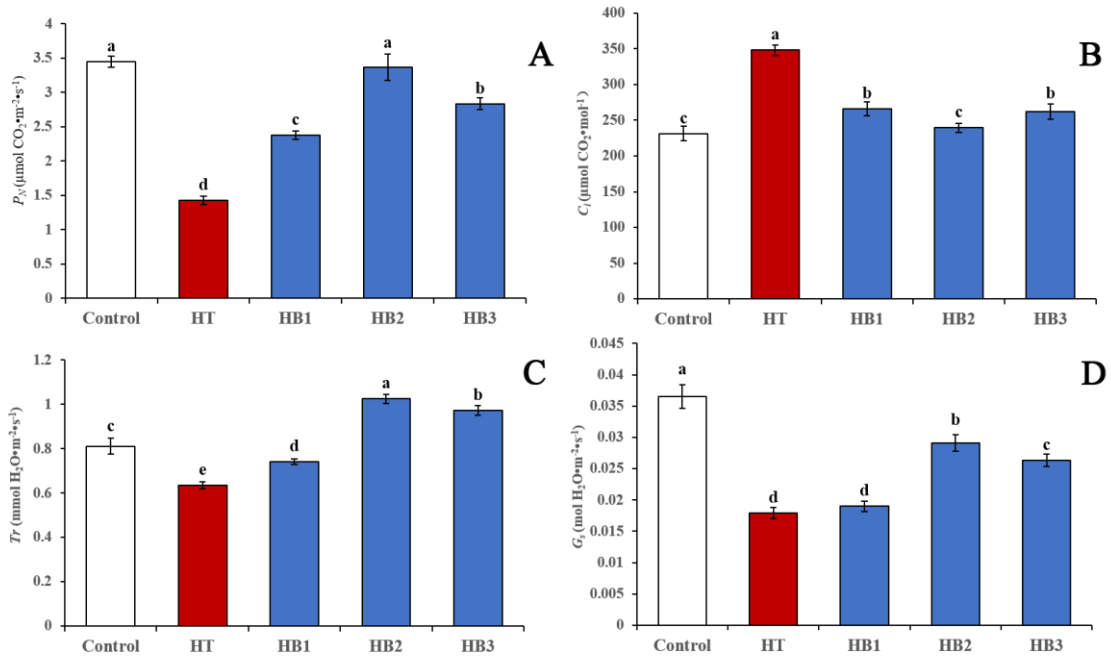

Figure 1. *Effects of EBR on gas exchange parameters in leaves of M. officinalis under HT stress. (A)* P_N*: Net photosynthetic rate, (B)* C_i*: Intercellular carbon dioxide concentration, (C)* T_r*: Transpiration rate, (D)* G_s*: Stomatal conductance. Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg* L^{-1} *EBR, HB2; High temperature with spraying 0.50 mg* L^{-1} *EBR, HB3; High temperature with spraying 1.00 mg* L^{-1} *EBR. Different letters indicate significant differences (*$P < 0.05$*) among various treatments with vertical bars as standard deviations*

Effects of 2, 4-EBR on chlorophyll fluorescence parameters under HT stress

Compared to control treatment, the F_m (*Fig. 2B*), F_v/F_m (*Fig. 2C*), $\Phi_{PS\,II}$ (*Fig. 2D*) and qP (*Fig. 2E*) of *M. officinalis* seedlings were significantly decreased by 25.48%, 18.68%, 34.86% and 20.23% ($P < 0.05$), whereas F_0 (*Fig. 2A*) and NPQ (*Fig. 2F*) were increased by 25.59% and 81.35% respectively ($P < 0.05$) under HT. The application of 0.1-1.0 mg L^{-1} EBR treatments significantly increased F_m, F_v/F_m, $\Phi_{PS\,II}$ and qP (*Fig. 2B-E*), and decreased F_0 (*Fig. 2A*) and NPQ (*Fig. 2F*) compared with HT treatment, and HB2 treatment caused the largest increase in F_m, F_v/F_m, $\Phi_{PS\,II}$ and qP by 21.09%, 20.56%, 42.50% and 18.85% ($P < 0.05$) respectively, while the biggest decrease in F_0 and NPQ by 18.59% and 43.98% ($P < 0.05$) than that of HT treatment (*Fig. 2*).

Effects of 2, 4-EBR on photosynthetic pigments under HT stress

The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in leaves of *M. officinalis* seedlings under HT stress were significantly lower than that of control treatment by 40.78%, 38.99%, 40.33%, and 33.98%, respectively ($P < 0.05$;

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Table 1). Application of EBR at 0.1~1.0 mg L^{-1} significantly increased the contents of chlorophyll a and chlorophyll b under HT stress ($P < 0.05$). The effect of different concentrations of EBR treatment on the contents of photosynthetic pigments was different. As compared to the HT treatment, HB2 treatment had the best effect on increasing the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids by 58.49%, 57.62%, 58.27%, and 45.30%, respectively ($P < 0.05$; *Table 1*).

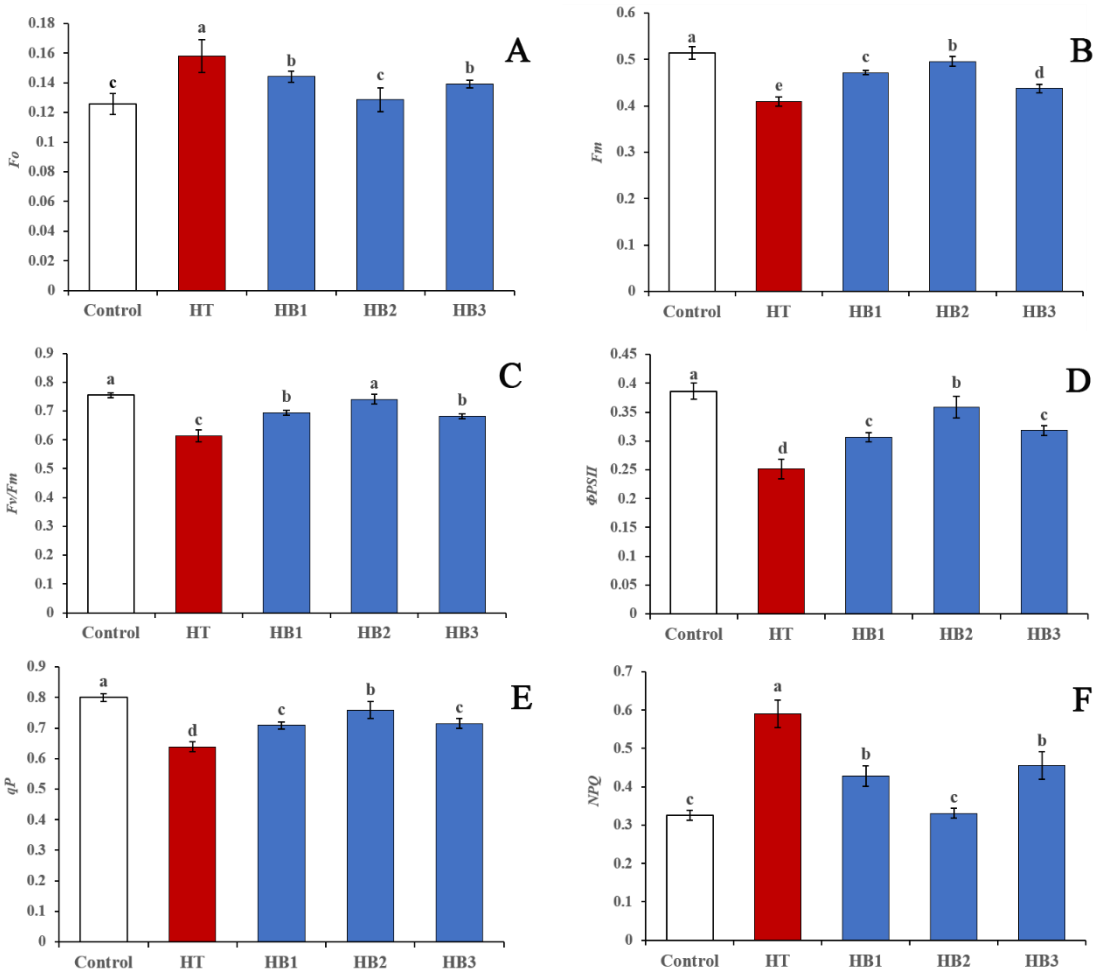

Figure 2. *Effects of EBR on chlorophyll fluorescence parameters in leaves of M. officinalis under HT stress. (A)* F_0*: Minimal fluorescence, (B)* F_m*: The maximum fluorescence, (C)* F_v/F_m*: Maximal photochemical, (D)* Φ_{PSII}*: Actual photochemical efficiency of PSII efficiency, (E)* qP*: Photochemical quenching, (F)* NPQ*: Non-photochemical. Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg* L^{-1} *EBR, HB2; High temperature with spraying 0.50 mg* L^{-1} *EBR, HB3; High temperature with spraying 1.00 mg* L^{-1} *EBR. Different letters indicate significant differences (*$P < 0.05$*) among various treatments with vertical bars as standard deviations*

Effects of 2, 4-EBR on the producing rate of O_2^-, contents of MDA and H_2O_2, electrolyte leakage under HT stress

HT stress caused a remarkable increase in the content of H_2O_2 and the rate of O_2^- production of *M. officinalis* seedlings by 2.03 and 3.46 times, respectively ($P < 0.05$)

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(*Fig. 3A, B*), as compared to the control treatment. While the application of 0.1-1.0 mg L^{-1} EBR significantly reduced the levels of H_2O_2 content and O_2^-producing rate of *M. officinalis* leaves in the HT stress, and the content of H_2O_2 and the rate of O_2^- production both contributed u-shaped curves with the increase of EBR concentration (*Fig. 3A, B*). The lowest levels of H_2O_2 content and O_2^- production rate were observed in HB2 treatment, which was lower than that of HT treatment by 50.49% and 53.65%, respectively ($P < 0.05$).

Table 1. *Photosynthetic pigments in M. officinalis seedlings splayed with EBR under HT stress*

Treatment	*Chl. a* (mg g^{-1} FW)	*Chl. b* (mg g^{-1} FW)	*Total Chl* (mg g^{-1} FW)	*Car.* (mg g^{-1} FW)
Control	2.04 ± 0.037 a	0.68 ± 0.017 a	2.72 ± 0.022 a	0.49 ± 0.011 a
HT	1.21 ± 0.017 e	0.42 ± 0.026 c	1.62 ± 0.039 d	0.33 ± 0.007 c
HB1	1.87 ± 0.022 c	0.64 ± 0.011 a	2.51 ± 0.028 b	0.43 ± 0.010 b
HB2	1.92 ± 0.010 b	0.65 ± 0.029 a	2.57 ± 0.032 b	0.47 ± 0.007 a
HB3	1.76 ± 0.015 d	0.52 ± 0.035 b	2.28 ± 0.021 c	0.45 ± 0.013 b

Chl a: Chlorophyll a, *Chl b*: Chlorophyll b, *Total Chl*: Total Chlorophyll, *Car:* Carotenoids. Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg L^{-1} EBR, HB2; High temperature with spraying 0.50 mg L^{-1} EBR, HB3; High temperature with spraying 1.00 mg L^{-1} EBR. Different letters indicate significant differences ($P < 0.05$) among various treatments with ± as standard deviations

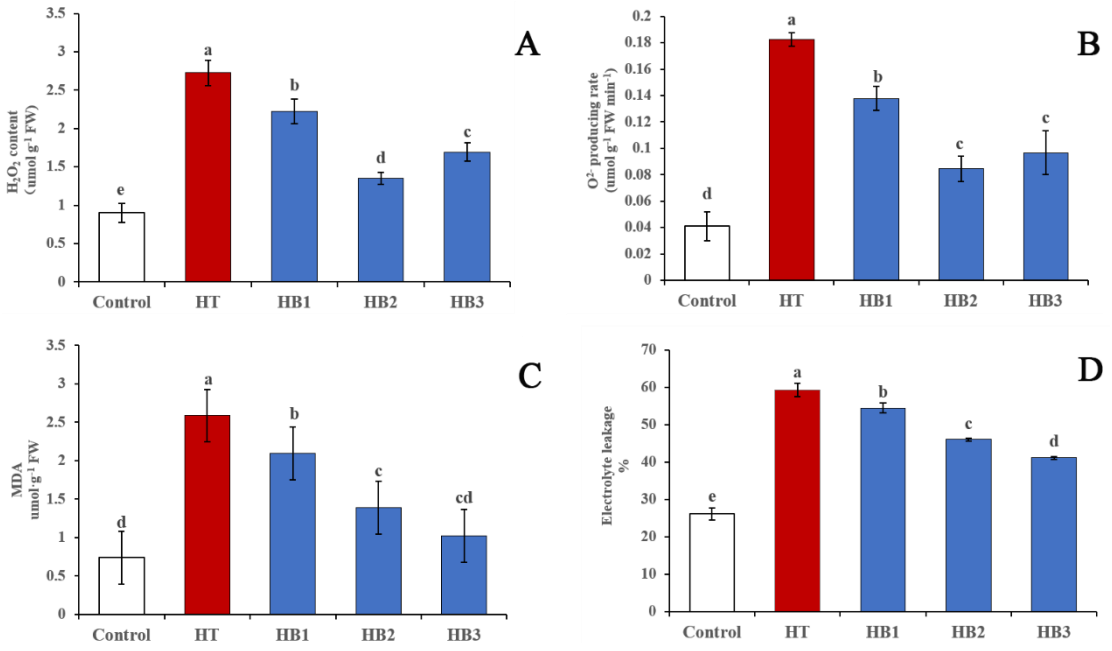

Figure 3. *Effects of EBR on (A) Hydrogen peroxide (H_2O_2) content, (B) Superoxide anion (O_2^-) producing rate, (C) Malondialdehyde (MDA) content, (D) Electrolyte leakage in leaves of M. officinalis under HT stress. Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg L^{-1} EBR, HB2; High temperature with spraying 0.50 mg L^{-1} EBR, HB3; High temperature with spraying 1.00 mg L^{-1} EBR. Different letters indicate significant differences ($P < 0.05$) among various treatments with vertical bars as standard deviations*

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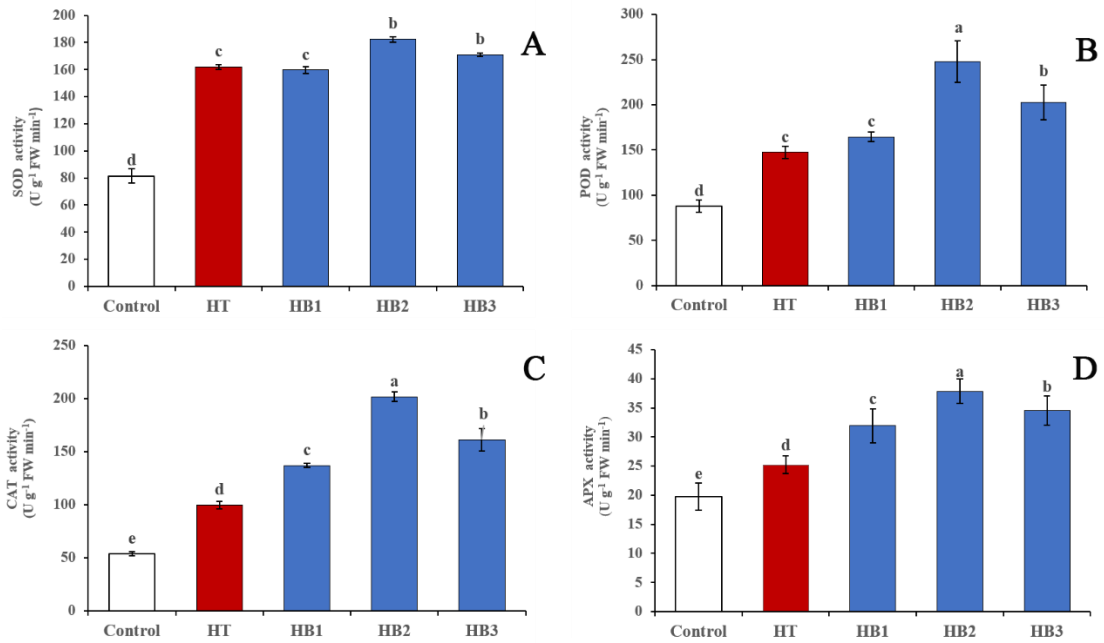
Compared with control treatment, HT stress resulted in a significant increase in the MDA content and electrolyte leakage of seedlings by 2.50 and 1.27 times ($P < 0.05$) (*Fig. 3C, D*), respectively. However, the application of EBR decreased the MDA content and electrolyte leakage of seedlings under stress gradually with the increase of EBR concentration in comparison with HT treatment (*Fig. 3C, D*). HB3 treatment significantly decreased the MDA content and the electrolyte leakage by 60.46% and 30.62% ($P < 0.05$), respectively as compared to HT treatment. Moreover, for MDA contents, no significant difference was observed between HB3, HB2, and control treatment, respectively.

Effects of 2, 4-EBR on the activities of the antioxidant enzyme under HT stress

HT stress significantly induced the activities of SOD, CAT, POD, APX, and GR by 98.82%, 85.70%, 67.61%, 27.72%, and 89.32% higher, respectively than that of the control treatment ($P < 0.05$) (*Fig. 4A-E*). Compared with HT treatment, 0.5-1.0 mg L^{-1} EBR remarkably enhanced the five enzyme activities during stress, while 0.1 mg L^{-1} EBR treatment significantly increased the activities of CAT, APX, and GR during stress, and had no significant difference on the activities of SOD and POD under HT treatment (*Fig. 4A-E*). HB2 treatment was found most effective for increasing the activities of SOD, CAT, POD, APX, and GR by 12.56%, 102.41%, 68.14%, 50.01%, and 116.09% respectively ($P < 0.05$), as compared to those under HT.

Effects of 2, 4-EBR on reduced glutathione content under HT stress

The GSH content in leaves of *M. officinalis* under HT stress increased remarkably by 1.11 times in comparison to control treatment ($P < 0.05$; *Fig. 4F*). Compared with HT treatment, the application of 0.5-1.0 mg L^{-1} EBR significantly increased the GSH content under stress, while HB1 treatment showed no influence on the GSH content (*Fig. 4F*). Moreover, HB2 treatment exhibited the highest increase in the contents of GSH by 99.65% ($P < 0.05$) higher than that of HT treatment (0.85 mg·g^{-1} FW), with no significant difference over HB3 treatment.



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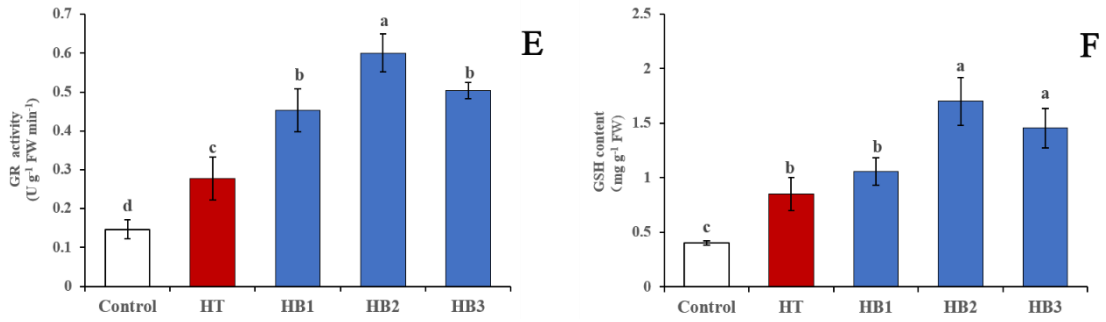

Figure 4. *Effects of EBR on activities of (A) Superoxide dismutase (SOD), (B) Peroxidase (POD), (C) Catalase (CAT), (D) Ascorbate peroxidase (APX), (E) Glutathione reductase (GR), and (F) Contents of reduced glutathione (GSH) in the leaves of M. officinalis under HT stress. Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg L^{-1} EBR, HB2; High temperature with spraying 0.50 mg L^{-1} EBR, HB3; High temperature with spraying 1.00 mg L^{-1} EBR. Different letters indicate significant differences ($P < 0.05$) among various treatments with vertical bars as standard deviations*

Effects of 2, 4-EBR on the content of proline and soluble protein under HT stress

Soluble protein content in leaves of *M. officinalis* under HT stress decreased significantly by 22.24% as compared with the control treatment (208.41 μg g^{-1} FW) ($P < 0.05$; *Table 2*). EBR treatment increased the content of soluble protein under HT stress, but there was no significant difference between EBR treatments for soluble protein contents (*Table 2*). The soluble protein content at HB2 treatment did not show a more significant difference than that of the control treatment (close to the control level; *Table 2*). While the proline contents under HT increased 1.24 times in comparison to the control treatment (30.25 μg g^{-1} FW) ($P < 0.05$). Compared with HT treatment, EBR treatment remarkably enhanced the proline content under stress, and with the increase in EBR concentration, the content of proline showed an increasing trend first and then decreased. The maximum accumulation of proline at HB2 treatment (108.39 μg g^{-1} FW) was observed 59.89% ($P < 0.05$) higher than that of HT treatment (67.79 μg g^{-1} FW).

Effects of 2, 4-EBR on the ultrastructural changes of chloroplasts and thylakoids under HT stress

The ultrastructure of chloroplast showed thylakoids with grana under control treatment in (*Fig. 5A, B*). HT stress caused remarkable changes in the ultrastructure of chloroplasts and thylakoids (*Fig. 5C, D*). Compared with the control, the chloroplasts of high-temperature stress were swollen with increasing the number of plastoglobuli, and chloroplast envelopes partly were destroyed as becoming indistinct. Furthermore, the number of grana lamellae decreased in some chloroplast under stress in comparison to the control, and the thylakoid matrix was swelling with distorted and loosened grana lamellae (*Fig. 5C, D*). Under HT stress, the chloroplast in mesophyll cells showed no significant swelling, the chloroplast envelope was intact and the fabric of thylakoid lamellar was maintained by applying the EBR (*Fig. 5E, F*). The chloroplast ultrastructure had no noticeable change under HT by the application of 0.5-1.0 mg L^{-1} EBR as compared with the control, and the obvious decreased number of plastoglobuli

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and the stacked tightly grana lamellae was observed, as granum were well-arranged and had the smooth thylakoid membranes (*Fig. 5F*). Most of the chloroplasts maintained the typical shape at 0.1 mg L^{-1} EBR treatment under stress, while few thylakoid grana lamellae were slightly swollen and the number of plastoglobuli were much more than that of the control (*Fig. 5E*).

Table 2. *Content of proline and soluble protein in M. officinalis seedlings splayed with EBR under HT stress*

Treatment	Proline content μg g^{-1} FW	Soluble protein content μg g^{-1} FW
Control	30.25 ± 1.44 d	208.41 ± 10.83 a
HT	67.79 ± 4.22 c	162.05 ± 9.33 c
HB1	80.22 ± 5.85 b	173.62 ± 14.92 bc
HB2	108.39 ± 9.24 a	191.74 ± 5.15 ab
HB3	89.81 ± 7.49 b	183.49 ± 6.97 b

Treatments include: Control; Normal temperature with distilled water, HT; High temperature with distilled water, HB1; High temperature with spraying 0.10 mg L^{-1} EBR, HB2; High temperature with spraying 0.50 mg L^{-1} EBR, HB3; High temperature with spraying 1.00 mg L^{-1} EBR. Different letters indicate significant differences ($P < 0.05$) among various treatments with ± as standard deviations

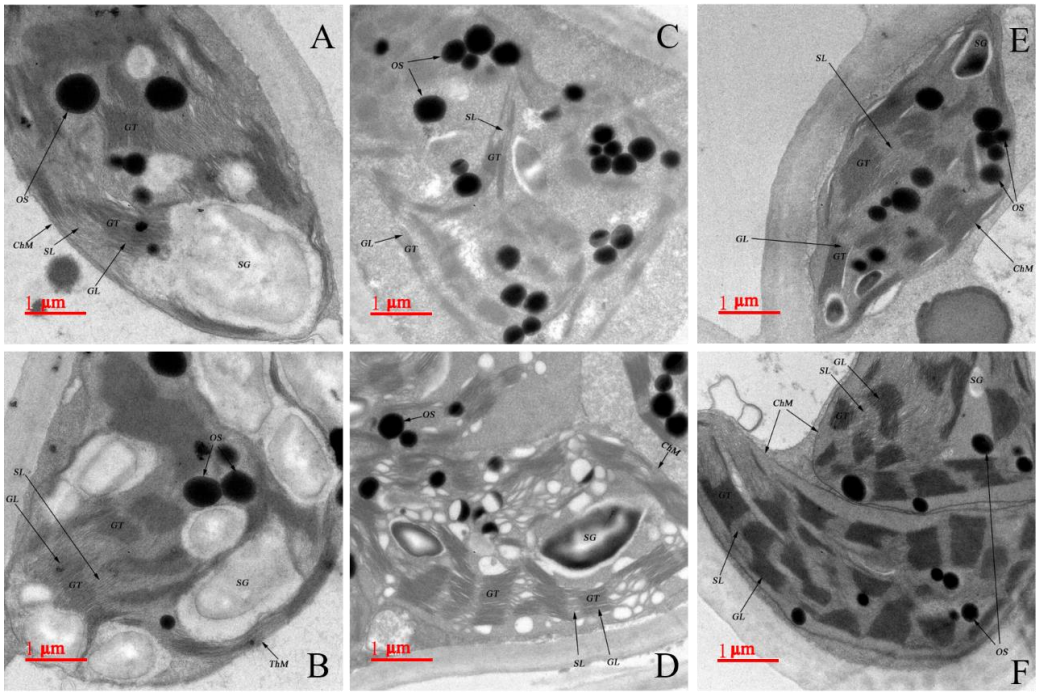

Figure 5. *Effects of HT on the ultrastructure of chloroplasts and thylakoids in the leaves of M. officinalis with or without EBR treatment. (A, B) The ultrastructure of chloroplasts and thylakoids under control treatment, (C, D) The ultrastructure of chloroplasts and thylakoids under high temperature treatment, (E) The ultrastructure of chloroplasts and thylakoids under high temperature with spraying 0.10 mg·L^{-1} EBR, (F) The ultrastructure of chloroplasts and thylakoids under high temperature with spraying 0.50mg·L^{-1} EBR, ChM: Chloroplast membrane, SG: Starch granule, OS: Osmiophilic plastoglobuli, GT: Grana thylakoid, SL: Stroma lamellae, GL: Grana lamellae*

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Discussion

Numerous studies have shown that the application of exogenous 2, 4-EBR can regulate various physiological metabolic processes in plants and improve the stress resistance including abiotic stresses such as drought stress (Lima and Lobato, 2017), salt stress (Yue et al., 2019), flood stress (Wang et al., 2015), high or low-temperature stress (El-Bassiony et al., 2012; Shu et al., 2016). However, reports that contain the effects of 2, 4-EBR on the woody plant are scant. Wang et al. (2015) pointed out that the application of exogenous EBR protected the photosynthetic organelles of grape leaves from the adverse effects of water stress, and increased chlorophyll content, and reduced the limitation of stomatal and non-stomatal photosynthesis. Whereas Yue et al. (2019) observed that pretreatment of locust seeds and seedlings with exogenous 2, 4-EBR enhanced the activity of antioxidant system in leaves of locust seedlings under salt stress, stabilized chloroplast and thylakoid membrane ultrastructure, and related effects on sodium ion concentration and photosynthetic gas exchange parameters in leaves. In our study, it was found that appropriate concentration of exogenous EBR effectively enhanced the photosynthetic characteristics of *M. officinalis* leaves, reduce oxidative damage and protect the chloroplast ultrastructure of mesophyll in plants when subjected to HT stress.

HT stress inhibits the photosynthesis process by decreasing the photosynthetic rate and reducing light energy conversion efficiency. According to Farquhar et al. (1982), the decrease of photosynthetic rate may be contributed to stomatal or non-stomatal limitation, when the decrease of P_N is mainly caused by stomatal limitation, G_s and C_i decrease simultaneously, when the decrease of P_N is mainly caused by non-stomatal limitation, C_i increases or remains constant accompanied with a decrease in G_s. In this study, HT stress reduced P_N of plants without EBR treatment in parallel with decreased G_s and increased C_i, suggesting that HT stress affected non-stomatal limitation (*Fig. 1A-D*). While EBR treatment alleviated the decline of P_N and G_s in plants under HT stress and promoted the decrease of C_i and the increase of T_r at the same time (*Fig. 1A-D*), which is consistent with the results of Thussagunpanit et al. (2015b) and Zhang et al. (2014). It is possible that EBR treatment can mitigate the non-stomatal limitation to photosynthesis, increase the activity of Rubisco (Xia et al., 2009), and the absorption capacity of CO_2 in the Calvin cycle (Zhao et al., 2017) under HT. Meanwhile, the increase of G_s and T_r in EBR-treated plants may decrease the leaf temperature (Singh and Shono, 2005), which helps to reduce the damage from HT stress and maintain a high photosynthetic rate.

Studies have shown that HT stress accelerated the degradation of chlorophyll and inhibited the biosynthesis of chlorophyll, resulted in the reduction of chlorophyll content (Aien et al., 2011). In our study, HT stress significantly reduced chlorophyll a, chlorophyll b, total chlorophyll content and carotenoid content in leaves, while EBR treatment increased photosynthetic pigments content of *M. officinalis* seedlings (*Table 1*), which is similar to the previous studies (Thussagunpanit et al., 2015a; Zhang et al., 2013b). The possible reason is that EBR enhances the antioxidant enzyme system, effectively eliminates the accumulation of reactive oxygen species, and reduces the damage of membrane lipid peroxidation products (MDA) to chloroplast membrane (Lima and Lobato, 2017), promotes the biosynthesis of chlorophyll (Eriko et al., 2014). This indicated that EBR with appropriate concentration could alleviate the damage of HT to the chloroplast of plants, enhance the ability of light capture, lessen the non-stomatal limitation of HT on net photosynthetic rate, and improve photosynthesis effectively, which is in the line with studies of *Cucumis sativus* (Fariduddin et al.,

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2013). Carotenoids are considered to be used as antioxidants to resist lipid peroxidation, reduce the fluidity of thylakoid membranes, and increase the stability of membranes (Tang et al., 2007). Besides, the increase of the carotenoids contents helps to eliminate the accumulation of excess excitation energy, thus playing the role of photoprotection (Calatayud and Barreno, 2004).

Chlorophyll fluorescence is a subtle reflection of the primary response of photosynthesis. It is widely used to study the effect of environmental stress on photosynthesis (Sayed, 2003). In our study, the maximum photochemical efficiency of PSII as F_v/F_m, the effective photochemical quantum yield of PSII as Φ_{PSII}, and photochemical quenching of PSII as qP in *M. officinalis* leaves significantly decreased in response to HT stress (*Fig. 2C-E*). However, EBR-treated plants showed the less decrease in F_v/F_m ratio under HT (*Fig. 2C*), suggesting that exogenous EBR can alleviate the damage of HT to PSII reaction center, which may be because of exogenous EBR which improves the conversion efficiency of PSII primary light energy and reduces photosynthesis inhibition (Maxwell and Johnson, 2000; Zhang et al., 2014). Correspondingly, the changes pattern of Φ_{PSII} and qP are similar to those of F_v/F_m (*Fig. 2C-E*). Under HT, EBR treatment induced the increase of Φ_{PSII} (*Fig. 2D*), indicating that exogenous EBR promotes an enhancement of the carboxylation efficiency caused by downstream regulation mechanism (Zhang et al., 2014). The increase in qP suggested that an improvement in the rate of reductant consumption and ATP formation by noncyclic electron transport relative to the excitation rate of the open PSII reaction center (Nogués and Baker, 2000). Whereas, the increase in qP is conducive to the separation of electron charges in the reaction center to obtain a higher photochemical quantum yield of PSII and electron transfer rate (Guo et al., 2006b). NPQ is photochemical quenching of PSII, representing the energy absorbed by PSII antenna pigments which could not be used for energy dissipation of electron transfer in photochemistry (Vasil'Ev et al., 1998). The increase of NPQ in EBR treated plants with varying degrees under HT was smaller than that of untreated plants under HT stress (*Fig. 2F*), suggesting that the application of EBR protected the PSII reaction center, with accelerating the rate of photosynthetic electron transfer and reducing heat dissipation of excitation energy in PSII antenna under HT (Zhang et al., 2013b). Similar findings were observed by Ogweno et al. (2008) and Janeczko et al. (2011) both that EBR treatment could protect PSII against over-excitation and from the damage of thylakoid membrane induced by HT.

HT stress affects the utilization and transformation of light energy in plant leaves. Surplus light energy is converted to the excitation energy of Mehler reaction, and the improvement of the reaction of photosynthetic electron transfer in branch with molecular oxygen as its receptor, which accelerates the production of reactive oxygen species and destroys the dynamic balance between reactive oxygen species accumulation and antioxidant defense system in plants, and leads to the accumulation of reactive oxygen species (Grennan and Ort, 2007). Accumulation of reactive oxygen species (ROS) causes lipid peroxidation and electrolyte leakage, affecting the normal physiological function of cells. MDA is one of the end products of membrane lipid peroxidation which binds with proteins and enzymes on the membrane, resulting in the destruction of membrane integrity and loss of selective permeability, and increase of conductivity (Anjum et al., 2016). It has been further explained that exogenous EBR could induce the expression of related regulatory genes involved in defense and antioxidant response, thus increasing the tolerance of plants to oxidative stress and

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alleviating the damage of stress to cells (Wu et al., 2014). In our study, HT caused a significant increase of O_2^- production rate and H_2O_2 content in *M. officinalis* leaves (*Fig. 3A, B*), accelerated the accumulation of membrane lipid peroxide MDA and led to an increase in MDA content and electrolyte leakage (*Fig. 3C, D*), which indicates that HT had induced oxidative stress to *M. officinalis*.

An active oxygen scavenging system plays an important role in protecting cells from photooxidation damage (Mittler, 2002). In our study, HT increased the activities of SOD, POD, APX, CAT, and GR in *M. officinalis* leaves (*Fig. 4A-E*), and EBR treatment further enhanced the activities of these five enzymes and also reduced the production rate of O_2^- and H_2O_2 content, MDA content and electrolyte leakage in the leaves of *M. officinalis* seedlings under HT stress (*Fig. 3A-D*). Recently, many studies have shown that EBR plays an important role in activating antioxidant defense system and scavenging reactive oxygen species under abiotic stresses. Lima1 et al. (2017) reported that EBR treatment significantly reduced the MDA content and electrolyte leakage in cowpea plants under drought stress, which could be attributed to the application of EBR to increase SOD, POD, APX and CAT activities under stress, and to reduce the rate of O_2^- production and H_2O_2 content. Arora et al. (2010) also showed that MDA contents in mustard leaves treated with EBR decreased significantly under zinc stress, while the activities of antioxidant enzymes (SOD, CAT, POD, APX, GR, MDhar and Dhar) increased significantly. The increase in activities of antioxidant enzymes after EBR treatment might be due to BR induces de novo synthesis or activate the enzymes mediated through transcription or translation of specific genes (Bajguz, 2000).

GSH is one of the important non-enzymatic antioxidants in the ASA-GSH cycle, which plays an important role in protecting plants from ROS damage (Yuan et al., 2013). EBR regulates the activities of antioxidant enzymes (APX, GR) involved in the ASA-GSH cycle, improves the circulation ability of ASA-GSH, and reduces oxidative stress damage (Yuan et al., 2013). Wu et al. (2014) described that exogenous EBR increased the content of non-enzymatic antioxidants ASA and GSH in the leaves of eggplant plants under the HT stress, and inhibited the production of free radicals and membrane lipid peroxidation, thereby enhancing the tolerance of plants to HT stress. In this study, 0.5 mg L^{-1} EBR significantly enhanced GR and APX activities in the leaves of *M. officinalis* under HT stress, increased GSH content, and enhanced the ability of scavenging ROS (*Fig. 4F*). Our results indicated that the application of EBR could eliminate ROS in time by increasing the activity of antioxidant enzymes and the content of non-enzymatic antioxidants, reduction in the damage of reactive oxygen species to the membrane, maintained the integrity and stability of the membrane structure and improved the resistance of *M. officinalis* seedling to HT oxidative stress.

Under abiotic stress, proline largely accumulates in plants as an important membrane stabilizer and scavenger of harmful free radicals, which plays an important role in protecting cell membranes, stabilizing protein structure, and improving cell water retention (Shamsul et al., 2012). However, abiotic stress has a negative impact on the protein biosynthesis of plants (Wahid et al., 2007). In our study, seedlings subjected to HT stress showed increased proline content and decreased protein content (*Table 2*) which was also reported in *Cucumis melo* under HT stress by Zhang et al. (2014). Moreover, 0.5-1.0 mg L^{-1} EBR treatment further increased proline contents and enhanced the accumulation of soluble protein of the leaves under HT stress (*Table 2*). Wu et al. (2014) indicated that the application of 0.05-0.2 μM EBR significantly increased the contents of proline and soluble protein in eggplant under HT stress. EBR

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application enhances the accumulation of proline may be attributed to stimulate the activity of delta-pyrrolidine-5-carboxylate synthase (a key enzyme in proline biosynthesis pathway) or inhibit the activity of proline dehydrogenase, thus promoting the biosynthesis of proline or enhancing the accumulation of proline. The increase in soluble protein contents in the present study might be due to EBR inducing or activating de novo polypeptide synthesis (Ramakrishna and Rao, 2012). Kulaeva et al. (1991) had earlier proposed that EBR activated the synthesis of protein in wheat leaves under HT stress, also improved the anti-stress ability of the protein synthesis system and the characteristics of the cell membrane. It seems to believe that EBR can induce plants to increase the accumulation of different compatible solutes to protect plants from oxidative damage caused by HT stress.

Chloroplasts are not only the main sites of photosynthesis, energy flow in plants, but also one of the most sensitive organelles to stress (Stoyanova and Yordanov, 2000). Studies have shown that oxidative stress induced by stress often leads to oxidative damage of organelles (Djanaguiraman et al., 2018). Austin et al. (2006) reported that the numbers of osmiophilic granules in chloroplasts were in dynamic equilibrium at normal temperature, lipids accumulated in thylakoid membranes, and released into plastoglobuli with the increase of temperature. Almeida et al. (2005) also indicated that the number of osmiophilic precipitates increased in chloroplasts of plants treated with hydrogen oxide. Yue et al. (2019) found that salt stress not only induced oxidative stress to *Robinia pseudoacacia* seedlings but also resulted in swelling of chloroplast and rupture of the thylakoid membrane in leaves. In this study, microscopic analysis showed that HT stress led to the chloroplast swelling, and some thylakoid grana lamellae became swollen and loosened as the number of osmiophilic granules increased (*Fig. 5C, D*), suggesting that HT stress had destroyed chloroplasts and thylakoids, which is consistent with the results of Djanaguiraman et al. (2018) in response to HT stress in wheat. Zhang et al. (2010) demonstrated that the oxidation of membrane lipids in chloroplasts or thylakoids induced by ROS resulted in the formation of osmiophilic granules, the size and number of them involved in the degree of membrane lipid peroxidation in chloroplasts.

The thylakoid membrane is the place of light-dependent reaction in photosynthesis, where chlorophyll is mainly located (Mathur et al., 2014). It is more sensitive to HT than chloroplast membrane or other organelles (Sayed et al., 1989), as the damage of HT stress occurring would lead to the degradation of chlorophyll and the decrease of photosynthetic rate (Jumrani et al., 2017). Almeida et al. (2005) reported that exogenous homobrassinolide increased the activities of antioxidant enzymes in potato plants under the condition of 100 mM H_2O_2 treatment, and significantly lowered the negative effects of H_2O_2 on mesophyll subcellular structure of the plants, with restoring the affected cell structure and reducing the symptoms of leaf injury. Our results showed that exogenous EBR alleviated the degradation of chloroplast subjected to HT (*Fig. 5E, F*). In particular, under HT stress the application of 0.5 mg L^{-1} EBR was beneficial to keep the integrity of chloroplast ultrastructure and normalize the overall morphology of thylakoids in chloroplasts (*Fig. 5F*), as promoting the synthesis of chlorophyll and maintaining the structural integrity and relative stability of photosynthetic membrane system (Dobrikova et al., 2014; Yue et al., 2019). This could be explained by the fact that exogenous EBR protected the photosynthetic membrane system from oxidative damage caused by HT stress through improving the antioxidant system and reducing lipid peroxidation. Simultaneously, it was confirmed with the decrease in H_2O_2 and MDA contents in *M. officinalis* plants treated EBR under HT stress.

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Conclusion

In conclusion, exogenous EBR reduced the inhibition of photosynthesis of *M. officinalis* seedlings under HT stress, enhanced antioxidant system and the content of photosynthetic pigments, decreased the accumulation of reactive oxygen species and the degree of membrane lipid peroxidation, stabilized the membrane's structure of chloroplast and thylakoid, and alleviated the oxidative damage of heat stress to the photosynthetic apparatus. EBR also had a dose-effect on alleviating the adverse effects of HT stress on *M. officinalis* seedlings, and EBR at 0.5 mg L^{-1} was the best concentration. However, further research is needed to focus on the elucidation of EBR-induced thermotolerance on molecular levels of plants that how EBR enhances the accumulation of heat-shock proteins and affects the transcriptional level of heat-shock proteins genes.

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APPENDIX

Figure A1. *View of seedlings showing different stages of M. officinalis How., arranged in completely randomized design*

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