Full Length Article

Foliar Spraying of Glycine Betaine Lowers Photosynthesis Inhibition of *Malus hupehensis* Leaves under Drought and Heat Stress

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Abstract

Apple is often affected by environmental stresses, particularly the drought and heat stress in the field in northern China. Rootstocks play a major role in tree resistance, and *Malus hupehensis* Rehd. var. *pinyiensis* Jiang (Pingyi Tiancha, PYTC) is a widely used rootstock for apple cultivars in China. In recent years, exogenous glycine betaine (GB) has been used to increase crop stress resistance. In this study, the impact of drought and heats stress applied alone and in combination on photosynthesis of PYTC seedlings was evaluated under GB (10 mM) application to find out the possible physiological and biochemical mechanisms of GB in improving the resistance of PYTC seedlings to these stresses. Potted seedlings (sand culture - water culture) were exposed to conditions of drought (30.0% PEG-6000(w/v), 25°C, 3 days), heat stress (42°C, 3 h) and their combination. Results showed that under combined drought and heat stresses, the photosynthesis inhibition degree of the leaves of PYTC was significantly higher than that under drought or heat stress alone. The increased inhibition of photosynthesis by the combined stresses was not simply the additive stress effect of separate heat- and drought treatments; different responses in plant physiology to drought and heat stresses were also found. Heat stress decreased the chlorophyll contents, net photosynthetic rate, apparent quantum efficiency and carboxylation efficiency of leaves more than drought stress. Drought stress decreased the transpiration rate, stomatal conductance and intercellular CO_2 concentration more than heat stress. In conclusion, the foliar-applied GB improved the photosynthesis of leaves of PYTC not only under single drought or heat stress but also under their combination. The improvement of water status and antioxidant enzyme activities were the possible mechanisms related to the promotion photosynthesis under stress conditions by GB application. © 2020 Friends Science Publishers

Keywords: Glycine betaine; *Malus hupehensis* Reld.; Membrane lipid peroxidation; Photosynthesis; Water status

Introduction

Apple (*Malus domestica* Rehd.) is one of the important cash crops and is mostly cultivated in hills and mountains in China. Its growth and development are vulnerable to various stresses, such as drought and heat stress, due to its special grown environments. Previous studies showed that under drought stress, apple growth is reduced due to leaf wilt, direction changed and angle changed and branch thickening (Wang 2017). Moreover, under drought stress, the net photosynthetic rate of plants decreased, and the ultra-structure of chloroplasts is also damaged (Hussain *et al.* 2018). Moreover, under high temperature stress, denaturation, degradation, synthesis and assembly of membrane proteins to be blocked, and cell membrane lipid peroxidation take place leading to reduced CO_2 fixation (Mathur *et al.* 2014; Hussain *et al.* 2018). According to statistics, from 1952 to 2006, only the drought caused a loss of 380 million yuan in apple-producing areas of Xianyang

City (Ma *et al.* 2008). Meanwhile, due to the greenhouse effect, the number of high temperature days increased significantly (Zhou *et al.* 2011). The decrease of photosynthesis caused by drought stress or heat stress is an important reason for crop yield reduction. With global warming, summer drought and high temperature has become the limiting factors of apple development in China (Wang 2017).

In recent years, the development of plant stress resistance research has played an important role in enhancing crop yield under stress. Studies have found that plants respond differently to drought, heat stress and their combination. Transcription mode, metabolic mode and molecular reaction are obviously different (Rizhsky *et al.* 2002, 2004; Shulaeva *et al.* 2008). However, most of these studies are single-stress adaptation studies under laboratory control conditions, while field stresses are more complex than indoors where often multiple stresses occur at the same time such as drought and heat stress, or drought and cold

stress (Mittler *et al.* 2001; Moffat 2002). Mittler (2006) proposed that in order to improve the tolerance of plants to natural environmental conditions, studies should focus on the tolerance of environmental conditions coexisting with multiple adversities, especially the simulation of field environment.

Glycine betaine (GB) is considered as a very effective osmotic adjusting substance and stress-resistance factor (Hussain *et al.* 2008; Kurepin *et al.* 2015). It plays an important role in improving plant resistance to single stress such as drought or heat stress (Hussain *et al.* 2008; Anjum *et al.* 2011). However, these studies indicated that GB might play an important role in enhancing plant tolerance to individual drought and heat stress, and these research on GB stress resistance mainly focuses on annual plants and a few woody plants (Ahmad *et al.* 2013; Li *et al.* 2013, 2014). There is still limited data on GB's improvement of plant resistance to the combination of drought and heat stress.

Moreover, what is the content of GB in apple (*Malus*)? Is GB also synthesized and accumulated in apple under drought, heat and other environmental stresses? What is the relationship between GB and stress resistance of *Malus*? So far, there is little research. The PYTC is a widely used rootstock variety for apple cultivars in China. Understanding the resistance of PYTC to stress and improving its resistance to stress are important for the development of apple industry. Therefore, this study was conducted to evaluate the impact of drought and heats stress applied alone and in combination on photosynthesis of PYTC seedlings. Moreover, GB was applied to find out the possible physiological and biochemical mechanisms of GB in improving the resistance of PYTC seedlings to the three stresses.

Materials and Methods

Test materials

The experiment was carried out with PYTC seeds, which were bought from Shuohang Fruit Professional Cooperative of Mengyin County, Shandong Province. The source of PYTC seeds is certified by Mr. Ximin Song. The material used is belonged to Maloideae, *Malus* genus and *Malus hupehensis* Rehd. var. *pinyiensis* Jiang. The PYTC tree height was 2.5-3 m, and the tree diameter was 15-20 cm. The experimental PYTC seeds were collected in November 2017 in PYTC tree base of Shuohang Fruit Professional Cooperative of Mengyin County, Shandong Province. The planting area is about 26680 square kilometers, about 5000 PYTC tree are planted. This base is located in the warm temperate zone. The climate is mild. The details were shown in Table 1.

PYTC seeds were laminated mixed with fine sand in a refrigerator at 4°C for 20 days after being saturated with water. Then, the germinating seeds were planted orderly into

Table 1: The growth information of PYTC seeds used in this study

Category	Information
Sapling height (cm)	250-300
Sapling diameter (cm)	15-20
Collection location	Shuohang Fruit Professional Cooperative of Mengyin County, Shandong Province, China
Planting area (km^2)	26680
Total growth amount of plants (tree)	5000
Average temperature (°C)	8.6-12.8
Sunshine time (h)	>7
Shading rate (%)	40-50

Note: PYTC: Pingyi Tiancha (Chinese name of *M. hupehensis* var. *pinyiensis* Jiang)

plastic pots (height 8 cm, diameter 10 cm) containing quartzite, with a density of four plants per pot. The plants were grown in an artificial chamber at 25/20°C with a photon flux density of 300-400 μmol m^{-2} s^{-1}, a relative humidity of 65~70%, and a photoperiod of 14/10-h light/dark. The plants were first cultured with distilled water to the three-leaf-and-one-leaflet period, then distilled water was replaced with 1/2 Hoagland nutrient solution. Post-1 week, 1/2 Hoagland nutrient solution were replaced with whole Hoagland nutrient solution. When the 6th leaf was expanded fully, the plants were used for experiment treatment.

Treatments details

Before stresses were imposed, an aqueous solution of 10.0 mM GB (+GB) (optimal concentration according to pre-test) (Wang *et al.* 2014) and 0.1% (v/v) Tween 20 (1 mL of Tween 20 was added to 1 L of distilled water) (control, -GB) were sprayed on leaves until runoff was detected. The solution was sprayed twice a day at 6:00 a.m. and 6:00 p.m. for 3 days. After GB application, some of the GB treated and control treated plants were subjected to drought, heat stress and the combination of the drought and heat stress. Drought was induced with 30% (*w/v*) PEG-6000 (osmotic potential of about -1.85 MPa) until reaching relative water content (RWC) of 80 to 87% (to moderate drought stress level, 96 h; control (normal watering and normal temperature): 94.1 to 94.3%). A combination of drought and heat stress was performed by subjecting drought-stressed plants to a high temperature 42°C for 3 h (illumination 300-400 μmol/(m^2·s), humidity 65~70%), Single heat stress was applied by raising the temperature in artificial chamber to 42°C for 3 h at the same time when stress combination was executed. Thus, all treatments included single drought stress (DS), single heat stress (HS), the combination of drought and heat stress (DS+HS) and normal watering and normal temperature (WW). All the experiments were performed in triplicate parallels and all the determinations were repeated three times. The physiological parameters involved in stress tolerance were observed as below.

Table 2: Effects of foliar-applied glycinebetaine (GB) on the GB contents in leaves of PYTC seedlings subjected to drought stress, heat stress and their combination

Treatment	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
GB content (µmolog^{-1}DW)	75.2 ± 4.6d	130.6 ± 5.2b	110.6 ± 3.5c	151.3 ± 4.3a	28.2 ± 2.3gh	41.8 ± 3.6ef	36.7 ± 5.4c	52.4 ± 3.3e

Note: DW: dry weight of the leave; WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought stress and heat stress. Values are the means ± standard errors (*S.E.*) of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

Table 3: Chlorophyll (Chl) and carotenoid (Car) contents in leaves of PYTC subjected to drought stress, heat stress and their combination

Treatments	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
Chl (mg·g^{-1}DW)	8.75 ± 0.38a	8.61 ± 0.18a	8.07 ± 0.53ab	7.82 ± 0.25b	8.67 ± 0.27a	8.49 ± 0.20a	5.93 ± 0.26c	5.45 ± 0.21cd
Car (mg·g^{-1}DW)	1.43 ± 0.10a	1.43 ± 0.04a	1.39 ± 0.01ab	1.25 ± 0.11c	1.46 ± 0.06a	1.26 ± 0.03c	1.04 ± 0.04d	0.73 ± 0.03e

Note: DW: dry weight of the leave; WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought and heat stress. Values are the means ± standard errors (*S.E.)* of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

Table 4: Net photosynthetic rate (P_n), transpiration rate (Tr), stomatal conductance(Gs), intercellular CO_2 concentration (Ci), the apparent quantum yield (AQY) and the carboxylation efficiency of photosynthesis (CE) of PYTC seedlings subjected to drought stress, heat stress and their combination

Treatments	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
Pn (µmol . m^{-2}.s^{-1})	13.9 ± 0.4a	11.9 ± 0.b5	9.3 ± 0.2c	3.3 ± 0.1f	13.7 ± 0.1a	8.6 ± 0.3cd	6.3 ± 0.1e	1.0 ± 0.3g
Tr (mmol. m^{-2}.s^{-1})	3.2 ± 0.0a	2.3 ± 0.3bc	2.5 ± 0.1b	1.6 ± 0.1f	3.1 ± 0.0a	2.0 ± 0.2d	2.3 ± 0.2bc	1.2 ± 0.1e
Gs(mmol. m^{-2}.s^{-1})	137.0 ± 9.4a	68.0 ± 4.6d	102.0 ± 5.6bc	46.5 ± 3.6e	143.0 ± 8.0ab	99.0 ± 6.8be	124.0 ± 5.5d	60.0 ± 9.0f
Ci(µmol . mol^{-1})	193.0 ± 12.5bc	134.0 ± 14.3d	185.0 ± 9.5c	196.0 ± 10.3bc	214.0 ± 11.4ab	142 ± 11.9d	226.0 ± 10.6ab	261.0 ± 18.0a
AQY(CO$_2$.photon^{-1})	0.032 ± 0.0003a	0.026 ± 0.0006c	0.016 ± 0.0004e	0.012 ± 0.0001f	0.030 ± 0.0005ab	0.020 ± 0.0006d	0.010 ± 0.0005fg	0.003 ± 0.0001h
CE(µmol. m^{-2}.s^{-1})	0.041 ± 0.006a	0.028 ± 0.005c	0.019 ± 0.004d	0.012 ± 0.003e	0.039 ± 0.004ab	0.020 ± 0.003d	0.011 ± 0.003e	0.005 ± 0.002f

Note: WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought and heat stress. Values are the means ± standard errors (*S.E.*) of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

Table 5: Changes in maximal efficiency of PSII photochemistry (F_v/F_m) , actual efficiency of PSII (Φ_{PSII}), the activities of Ca^{2+}-ATPase and Mg^{2+}-ATPase in the thylakoid membrane of PYTC seedling leaves subjected to drought stress, heat stress and their combination

Treatments	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
F_v/F_m	0.840 ± 0.008a	0.832 ± 0.035ab	0.697 ± 0.021d	0684 ± 0.033de	0.849 ± 0.012a	0.799 ± 0016c	0.635 ± 0.013ef	0.611 ± 0.017f
Φ_{PSII}	0.475 ± 0.022a	0.326 ± 0.006b	0.314 ± 0.015b	0.249 ± 0.027d	0.476 ± 0.027a	0.298 ± 0.011c	0.247 ± 0.022d	0.208 ± 0.007e
Ca^{2+}-ATPase	127.7 ± 7.7a	115.3 ± 7.0b	92.2 ± 3.4d	82.4 ± 8.8de	126.2 ± 7.2a	106.0 ± 2.5c	73.1 ± 9.7e	55.0 ± 5.1f
Mg^{2+}-ATPase	42.7 ± 0.9a	30.1 ± 0.7c	22.0 ± 1.5d	20.8 ± 2.5de	40.6 ± 1.7ab	21.3 ± 1.5d	13.9 ± 2.0f	9.1 ± 2.2g

Note: WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought and heat stress. Values are the means ± standard errors (*S.E.*) of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

Measurements

Chlorophyll, carotenoids and GB contents: The GB content was determined using high performance liquid chromatography (HPLC) (*LC-6A, Shimadzu Corp.*, Kyoto, Japan) according to the procedure described by Ma *et al.* (2007). Chlorophyll and carotenoid contents were estimated following Zhao *et al.* (2002a) with few modifications. Using 80% (v/v) acetone to extract, a spectrophotometer to compare color, was applied and proposed formula was applied to calculate the contents of each pigment in the extract.

Photosynthetic gas-exchange parameters: The net photosynthetic rate (P_n), stomatal conductance (Gs), transpiration rate (Tr), intercellular CO_2 concentration (Ci), apparent quantum yield (AQY) and carboxylation efficiency of photosynthesis (CE) were carried out with 6th fully expanded attached leaves using a portable infrared gas analyzer (*Ciras-2, PP Systems*, Norfolk, U.K.). The light-saturating photosynthetic rate was recorded at a CO_2 concentration of 360 µL L^{-1} and temperature of 25°C with relative humidity of 80% and saturating light (800 µmol m^{-2} s^{-1}). The initial slope of the reaction curve was obtained with the linear regression of data with light intensity below 250 µmol·m^{-2}·s^{-1} in the P_n-PPFD reaction curve as the AQY; and the initial slope obtained by linear regression of data below 250 ppm CO_2 in the P_n-Ci reaction curve was the CE. The photosynthetic parameters were measured for about 10 min, during which no significant recovery was detected.

Chl a fluorescence analysis: Photosystem (PSII) chlorophyll a fluorescence measurements were performed using an *FMS-2* portable pulse modulated fluorometer (*Hansatech*, U.K.). The leaves were first adapted to darkness for 20 min, the fluorescence parameters under dark adaptation were measured, where F_o was the initial fluorescence, F_m was the maximum fluorescence, and

Table 6: Leaf relative water content (RWC), osmotic adjustment (OA) of PYTC seedlings subjected to drought stress, heat stress and their combination

Treatments	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
RWC (%)	94.1 ± 2.1a	86.9 ± 1.2bc	92.9 ± 2.3a	82.4 ± 1.8d	94.3 ± 2.1a	80.1 ± 1.1d	89.4 ± 1.1ab	70.1 ± 1.1e
OA (Mpa)	0.0 ± 0.0d	0.19 ± 0.01de	0.15 ± 0.00cd	0.22 ± 0.01a	0.0 ± 0.0d	0.15 ± 0.00 c	0.13 ± 0.01f	0.16 ± 0.01c

Note: WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought and heat stress. Values are the means ± standard errors (*S.E.*) of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

Table 7: Leaf total soluble sugars and proline contents of PYTC Seedlings subjected to drought stress, heat stress and their combination

Treatments	+GB				-GB			
	WW	DS	HS	DS+HS	WW	DS	HS	DS+HS
soluble sugar content (mg.g^{-1} DW)	11.0 ± 1.0fg	33.6 ± 1.5b	14.6 ± 1.3e	39.7 ± 4.2a	10.5 ± 0.9fg	25.0 ± 2.0d	12.3 ± 2.3ef	30.3 ± 1.9be
Proline content (μg.g^{-1} DW)	15.8 ± 1.8g	34.3 ± 2.0b	25.5 ± 2.7d	50.7 ± 3.9a	14.2 ± 1.0gh	22.6 ± 1.8de	19.7 ± 1.8ef	30.9 ± 2.6c

Note: WW: well watered control; DS: drought stress; HS: heat stress; DS+HS: combination of drought and heat stress. Values are the means ± standard errors (*S.E.*) of three replicates. Means in a row followed by the different letters indicate significant difference at *P* < 0.05

F_v/F_m= （F_m-F_o）/F_m. Thereafter, the leaves were exposed to light intensity of 300 μmol·m^{-2}·s^{-1}, and the fluorescence parameters such as maximum fluorescence (F_m') and steady-state fluorescence (F_s) under light adaptation were measured, the actual PSII efficiency under irradiance (Φ_{PSII}) were calculated according to the formula: $\Phi_{PSII} = (F_m' - F_s)/F_m'$.

Water status, free proline and soluble sugar contents of the leaves: The relative water content (*RWC*) of the leaves was determined according to Ma *et al.* (2007). Osmotic adjustment (OA) was determined using a vapour pressure osmometer (*5520, WESCOR*, America) according to the method as was described by Wang *et al.* (2010). Referring to the method of Zhao *et al.* (2002b), the proline contents were measured with the ninhydrin method. The soluble sugar contents were measured with the anthrone colorimetry.

Ion leakage and MDA levels: The ion leakage from the cellular membrane was determined via conductivity measurement according to Fan *et al.* (1997). Malondialdehyde (MDA) level was assayed according to the method that was described by Zhao *et al.* (2002b).

Superoxide anion radical (O_2^-) production rate and H_2O_2 contents: The production rate of O_2^- was measured according to the method described by Wang and Luo (1990). The H_2O_2 contents were measured according to the method of Sairam and Srivastava (2002).

Antioxidant enzyme activities: Total superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) were determined as described by Bartoli *et al.* (1999). A total of 0.5 g of leaves were placed in a pre-cooled mortar, and 5 mL of pre-cooled phosphate buffer was added for ice bath and grinding, and centrifuged at 12,000g at 4°C for 10 min. The supernatants were collected and used to assay antioxidant enzymatic activities. Results were expressed by enzyme activity based on unit protein.

Statistical analysis

All experiments were repeated 3 times, the presented values
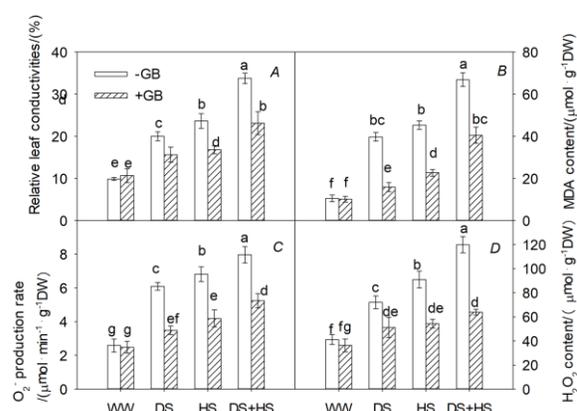
Fig. 1: Effect of GB application on relative leaf conductivities (**A**), MDA content (**B**), the production rate of O_2^- (**C**) and H_2O_2 content (**D**) of PYTC plants subjected to lone and combined drought and heat stress

Means in a row followed by the different letters indicate significant difference at *P* < 0.05 (Values are the means ± standard errors (S.E.) of three replicates)
DW: Leaf dry weight; WW: Well-watered; DS: Drought stress; GB: Glycine betaine
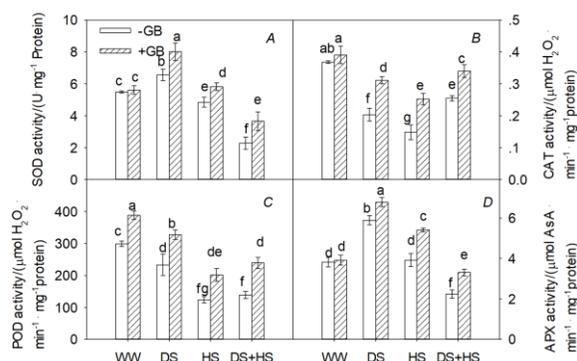
Fig. 2: Effect of GB application on activities of superoxide dismutase (SOD, **A**), catalase (CAT, **B**), peroxidase (POD, **C**), and ascorbate peroxidase (APX, **D**) in leaves of PYTC subjected to alone and combined drought and heat stress

Means in a row followed by the different letters indicate significant difference at *P* < 0.05 (Values are the means ± standard errors (S.E.) of three replicates)
DW: Leaf dry weight; WW: Well-watered; DS: Drought stress; GB: Glycine betaine

were the means ± standard errors (*S.E.*) of three replicates. The test results were plotted with Sigmaplot 12.0 and the statistical analysis was conducted using Data Processing System (*DPS*; Zhejiang University, China). Differences among the GB-pretreated and the control treated plants or treatments were compared using Duncan's multiple range tests at 0.05 probability levels.

Results

Chlorophyll, carotenoid and GB contents

The contents of chlorophyll (Chl) and carotenoid (Car) in the leaves of GB-pretreated plants and control treated plants were determined (Table 2). DS had only caused a decrease in Car content in the control treated plants. HS significantly decreased the contents of Chl and Car in the leaves of PYTC, DS + HS further redused the Chl and Car contents in the leaves of PYTC ($P < 0.05$). Foliar application of GB alleviated the decreases of Chl and Car contents under the three stresses. GB content of the GB pre-treated plants (+GB) was significantly ($P < 0.05$) higher than that of the control treated plants (-GB) when no stress treatment was given (Table 2) due to absorption of exogenous GB by leaves. When subjected to DS, HS and DS+HS, GB contents in leaves of GB pre-treated and control treated plants increased significantly ($P < 0.05$) than that of WW, DS+HS induced a greater level of GB than individual DS or HS, and the DS induced the greater level of GB than HS. GB pre-treated plants, showing that the PYTC plants sensitively responded to these stresses by accumulating more GB in their leaves (Table 2).

Gas exchange traits

The effects of foliar-applied GB and the stresses on the CO_2 assimilation and other gas exchange parameters were significant (Table 3). The net photosynthetic rates (P_n) of the leaves of PYTC were significantly inhibited by DS and HS and were dramatically decreased upon exposure to DS+HS. The degree of inhibiion in HS was more than that in DS, and the degree in co-stress of DS and HS was more serious than that in single DS or HS. Similar results were observed in apparent quantum yield (AQY) and carboxylation efficiency (CE) were essentially the same as the P_n. However, the effects of diferent stress on transpiration rate (Tr) and stomatal conductance (Gs) were different from the P_n, AQY and CE. The degree of inhibition of Tr and Gs in DS was more than that in HS, .and when HS was accompanied by DS, the degree of inhibition decreased again. Additionally, DS alone decreased Ci in the plants, when the plants were subjected to a combination of DS and HS; however, Ci was increased significantly. The data in Table 4 also revealed that foliar-applied GB improved P_n and various gas exchange parameters under all stresses. In particular, the most evident effect of GB is on Gs, then on

AQY, and then on CE (Table 3).

Activity of functional proteins in thylakoid membrane

Individual DS had little effect on F_v/F_m, however, HS and DS+HS decreased it significantly; all three stress decreased Φ_{PSII}, the DS+HS resulted in a more drastic decline of F_v/F_m and Φ_{PSII} than each stress alone. Compared to control treated plants, foliar-applied GB alleviated the decrease of both F_v/F_m and Φ_{PSII} under stress conditions. At the same time, the ATP activity of the thylakoid membrane was also detected (Table 4). DS and HS had significantly reduced the activity of Ca^{2+}-ATPase and Mg^{2+}-ATPase in thylakoid membrane, the combined stress had a more drastic decline of these enzyme activities than with application of each stress type alone. Foliar-applied GB reduced the decrease of the ATP activity under all three stresses (Table 4).

Water status, soluble sugar content and proline accumulation

Data given in Table 4 showed that HS had no significant effect on the RWC of PYTC leaves. DS caused a significant decrease in RWC, and DS+HS deteriorated the RWC further. Foliar-applied GB significantly alleviated the decrease in RWC of PYTC leaves compared to the control treated plants under DS and DS+HS ($P < 0.05$). Compared with WW, under DS and DS + HS, the RWC of the GB-pretreated plants decreased by 7.7 and 12.5% respectively, while that of the control treated plants decreased by 15.1 and 25.7% respectively. Under stress, the PYTC leaves showed higher osmotic adjustment ability (Table 5). The response of OA to DS was greater than that to HS, and the greatest OA was observed under DS+HS. The OAs of GB-pretreated plants were greater than that in the control treated plants under the three stresses (Table 4). Soluble sugar and proline contents were increased significantly under DS, HS and DS+HS (Table 7). The contents of soluble sugar and proline were highest under DS+HS, than under the DS, and then under the HS. Moreover, the levels of them in the GB-pretreated group were dramatically higher than in the control treated plants, this was consistent with OA levels in Table 4.

Active oxygen scavenging system

Both DS and HS increased ion leakage significantly ($P<0.05$), with the increase being slightly greater under HS than DS, and the greatest increase was observed under DS+HS ($P < 0.05$) (Fig. 1A). Similar results were observed with MDA levels (Fig. 1B). All stresses increased $O_2^{\cdot-}$ and H_2O_2 productions. Compared to DS, HS increased them to a lesser extent, and the greatest increase was observed under stress combination. However, all these levels, including $O_2^{\cdot-}$ and H_2O_2 as well as MDA, were lower in GB-pretreated plants than that in the control treated plants (Fig. 1C).

Further, the activities of several key antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) under different stress conditions were investigated (Fig. 2). The responses of SOD and APX to DS were observed as increased activity, but that of CAT and POD were detected as decreased activity. When they were subjected to HS, however, almost all the antioxidant enzyme activities were decreased except for APX. A stress combination of drought and heat inhibited SOD and APX activities further, but pretreatment of DS increased the tolerance of CAT and POD to HS. Foliar-applied GB increased activities of all four antioxidant enzymes, especially POD, followed by CAT and APX (Fig. 2).

Discussion

Results confirmed that PYTC synthesizes and accumulates GB in vivo naturally; the amount of natural synthesis GB in PYTC is very low, which was substantially lower than that of resistant plants such as Chenopodiaceae and Poaceae (McCue and Hanson 1990; Ma *et al.* 2003). Foliar-applied GB was absorbed and accumulated in PYTC leaves; this was consistent with findings in other crop plants (Ma *et al.* 2006, 2007). Furthermore, stress-induced GB was significantly higher in GB-pretreated plants than that in the control treated plants. GB content reached 75.2–151.3 $\mu mol \cdot g^{-1}$ DW in GB pre-treated plants, but in GB pre-untreated plants it is 28.2-52.4$\mu mol \cdot g^{-1}$ DW (Table 2).

The responses of plants to different stress stimulation maybe different, especially, when they occur together, different stresses might accrue conflicting or antagonistic responses (Mittler 2006). In the present study, different responses were observed and interrogated the underlying mechanisms of P_n to drought, heat, and the combination in PYTC leaves. First, the stress combination resulted in a greater decrease in P_n of PYTC leaves than single drought stress or heat stress, and heat stress reduced P_n more than drought stress (Table 3). The negative effect of drought on P_n was due to decreased Gs that resulted in the decreased Ci; while heat stress and the combination of drought and heat stress decreased Gs and increased Ci (except for the HS stressed GB-pretreated plants), suggesting that there are different mechanisms underlying the decrease in P_n caused by drought stress and heat stress. It means that the decrease of P_n may result from the stomatal factors under drought stress, but that it may be due to non-stomatal factors under heat stress. Second, drought stress limited transpiration rate more seriously than heat stress (Table 3), this consistent with the higher RWC of the heat stressed plants (Table 4). However, the heat stress decreased the activity of some enzymes and the cell membrane integrity more effectively than the drought stress leading to reduced Pn (Fig. 2). These observations were consistent with those of Rizhsky *et al.* (2002).

Foliar application of GB increased P_n of PYTC

leaves under stresses, which was consistent with previous studies on tomato and wheat crops (Zhao *et al.* 2007; Li *et al.* 2013; Athar *et al.* 2015). But what was its mechanism? Results suggested that foliar application of GB can enhance the tolerance of PSII and ATPase activities to DS and HS (Table 4) and protect the structures of chloroplast and thylakoid from the damage by stress (data not shown). This was consistent with other reports as well (Mamedov *et al.* 1993). Furthermore, results also suggested that the foliar application of GB on the water status (Table 6) and the antioxidative defense system (Fig. 2) were involved in its mechanisms. Foliar-applied GB and increase in other metabolites (such as soluble sugars and free proline) reduced the osmotic potential, which facilitated cells' water absorption and stomata opening, it was speculated that foliar application of GB may facilitate the maintenance of aquaporin activity of PYTC leaves under the stresses, this may be beneficial to the maintenance of photosynthesis.

GB may maintain the photosynthetic capacity not only through increasing Gs but also by maintaining Rubisco activity (Sage and Kubien 2007) under stress. Correspondingly, foliar-applied GB improved those parameters also to a greater extent under drought than heat stress, and which may be related to the greater osmotic adjustment in GB-pretreated plants in drought condition. The greater OA in GB-pretreated plants under drought than heat stress resulted from the accumulation of GB and other solutes. This indicated that high OA value in the leaves of GB-pretreated plants may be related to more GB content. These observations aligned with the findings proposed by Wang *et al.* (2010) with exceptions. These differences involves the proline accumulation under heat stress which was inconsistent with that of Rizhsky *et al.* (2004) in Arabidopsis, and their results were that proline was not increased under heat stress. It was speculated that it may be related to different experimental materials and different stress conditions. In this study, heat stress condition was treating at 42°C for 3 h, while Rizhsky *et al.* (2004) treated at 38°C for 6 h. While different approaches were applied which can justify the discrepancies, future investigations are required to clarify such differences.

Cell membrane stability is often affected by lipid peroxidation caused by ROS under stress conditions (Sudhakar *et al.* 2001; Hussain *et al.* 2017), which results in the production of MDA as shown in Fig. 2B. Foliar application of GB can help to alleviate lipid peroxidation (Fig. 2B) and maintain the cell membrane stability (Fig. 2A) consistent with previous studies (Saneoka *et al.* 2004; Lv *et al.* 2007). GB acts as a potential scavenger of the toxic ROS produced during abiotic stress (Banerjee and Roychoudhury 2017). However, GB could not directly remove reactive oxygen (Hanson *et al.* 1985), and our results indicated that foliar application of GB increased antioxidant enzyme activity (Fig. 2), especially POD, then CAT and APX. The POD can transform some carbohydrates contained in tissues

into lignin and increase the degree of lignification; this may be helpful to keep plants upright under stresses. The CAT and APX both remove H_2O_2, which was consistent with the lower H_2O_2 content of the GB-pretreated plants (Fig. 1D), the enhanced activities of the antioxidative enzymes (Fig. 2) may be the major factors involved in the GB-mediated decrease of ROS (Fig.1C and 1D). Previous studies have shown that GB can stabilize the stereoscopic structure of enzyme to maintain enzyme activity under high salt, cold and heat stresses (Gorham 1995). Also it has been reported that GB can stabilize RuBP carboxylase activity (Yang *et al.* 2007). Interestingly, in present study, the results (Fig. 2) have showed that under normal conditions, the activities of POD and CAT in the leaves of GB-pretreated plants were higher than that of the control treated plants. Under stresses, it was speculated that GB was beneficial to stabilizing the antioxidant enzyme protein on one hand, and may promote the expression of relevant genes of POD and CAT to enhance their antioxidant activity on the other hand (Park *et al.* 2006).

Conclusion

Photosynthesis in PYTC leaves was affected by drought and heat stresses, effect was more sever under combined stresses, in different ways. For instance, inhibitions of net photosynthesis, apparent quantum efficiency and carboxylation efficiency were more in heat stress while drought effects were more severe on transpiration rate and stomatal conductance. Foliar-applied of GB increased the tolerance of plants by alleviating the inhibition of photosynthesis, due to improvement of water balance and antioxidant metabolism involved in the protection of the photosynthetic machinery. Moreover, GB, under stress conditions, application also improved osmotic adjustment function and relative water contents.

Acknowledgement

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