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Full Length Research Paper

Changes in photosynthetic properties and antioxidative system of pear leaves to boron toxicity

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Seedlings of the Cuiguan cultivar of the Asian pear (*Pyrus pyrifolia*) were used to study the effects of boron toxicity on leaf photosynthetic properties and lipid peroxidation. The plants were grown hydroponically and treated with four concentrations of boron: 10 (CK), 100, 300 and 500 μmol·L^{-1}. After 16 weeks of treatment, we measured the leaf contents of boron and chlorophyll (Chl), the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular carbon dioxide concentration (Ci), the concentrations of malondialdehyde (MDA), H_2O_2, ascorbic acid (ASA) and glutathione (GSH) and the enzymatic activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR). Treatment with high levels of boron significantly increased the boron content of the pear leaves and reduced Chla, Chlb and carotenoid (Car) contents. Boron stress also reduced the Pn and Gs but increased the Ci. Furthermore, the leaf concentrations of MDA and H_2O_2 increased with increasing concentrations of boron, whereas the enzymatic activities of SOD, APX, CAT and GR and the ASA and GSH levels first increased and then decreased. These results indicate that boron toxicity reduced the photosynthetic capacity of the pear plant, resulting in the accumulation of reactive oxygen species (ROS) and increased membrane lipid peroxidation. Moderate boron stress can therefore improve the vitality of a plant's ROS scavenging system, but high concentrations will eventually overcome the system.

Key words: Pear, boron toxicity, photosynthetic rate, lipid peroxidation.

INTRODUCTION

Boron is an essential element required by higher plants to maintain normal growth and development. Because boron is a trace element, the concentration range suitable for crop growth is very small. Boron toxicity is often induced as a result of the extensive use of boron-rich fertilizer, irrigation water, sewage or dust in crop production. Therefore, boron toxicity has been an important issue affecting agricultural production in many parts of the world (Nable et al., 1997; Parks and Edwards, 2005).

High boron stress is very destructive to a plant's photosynthetic system. Under high boron stress, the edge of the rape leaf dies (Fang, 2001), the photo synthetic area is reduced and the chlorophyll content decreases, resulting in a reduced photosynthetic rate. High boron stress and the consequent reduction in photosynthetic rate is a major factor hindering the growth of kiwi plants (Sotiropoulos et al., 2002). Han et al. (2009) showed that the photosynthetic rate of citrus leaves decreased significantly under high boron stress. As with other ions, boron stress can also lead to the formation of a large amount of reactive oxygen species (ROS) such as $O_2^{\bullet -}$ and H_2O_2, leading to cell membrane damage; in severe cases, ROS accumulation can cause cell death. In barley leaves, boron stress leads to the accumulation of ROS and increased membrane permeability (Karabal et al., 2003), and one tomato study showed that leaf levels of malondialdehyde (MDA) and H_2O_2 increased under high boron stress (Mittler, 2002; Blokhin et al., 2003). There are two types of antioxidant systems in the plant cell that protect the plant from ROS damage: One is

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the enzymatic antioxidant system, which includes enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX), and the other is the non-enzymatic antioxidant system, which involves ascorbic acid (ASA) and glutathione (GSH) (Mittler, 2002; Blokhin et al., 2003; Luis et al., 2007). Studies on apples and grapes (Molassiotis et al., 2006; Gunes et al., 2006) have shown that boron stress induced SOD, CAT and APX accumulation in the leaf enzymatic antioxidant system. Fewer studies have investigated the relationship between boron toxicity and the non-enzymatic antioxidant system. A study of apple rootstock (Sotiropoulos et al., 2006) found that when the boron concentration increased, the activity of the non-enzymatic antioxidant system also increased. However, another report has demonstrated that boron stress inhibited GSH formation in sunflower leaves (Ruiz et al., 2003). Two studies have shown that boron is closely related to ASA metabolism (Blevins and Lukaszewski, 1998; Brown et al., 2002). Ascorbic acid is an important antioxidant in the plant's subcellular structure, ASA can be used as a substrate to remove H_2O_2 by APX in the ASA-GSH cycle (Nakano and Asada, 1981). When plants face adversity (including boron toxicity), they will increase their ASA levels to increase resistance (Smirnoff, 2000; Keles et al., 2004), while decreases in ASA levels lead to the accumulation of reactive oxygen species like H_2O_2.

Despite the extensive research on the effects of boron toxicity on plant photosynthesis and reactive oxygen damage, its mechanism of action is still unclear, and further investigate research needs to be conducted. The pear tree is one of the most highly cultivated fruit trees in China and is often subjected to boron stress. However, the effects of boron toxicity on the physiological metabolism of the pear plant, especially the effects on photosynthesis and active oxygen metabolism, have not been thoroughly investigated. We therefore used seedlings of a common type of fruit tree, the Cuiguan cultivar of the Asian pear *Pyrus pyrifolia*, in these experiments to study high boron stress conditions, pear leaf photosynthetic characteristics, ROS metabolism and changes in the antioxidant enzymatic system, with the aim of providing references for plant boron toxicity and information on how pear trees react to this type of stress.

MATERIALS AND METHODS

Experimental materials

One-year-old graft seedlings of Cuiguan pear trees (*P. pyrifolia*) were used as experimental materials (Bunge as rootstock) and were cultured hydroponically. The modified Hoagland nutrient solution formula was used as a standard nutrient solution (Han et al., 2009). The nutritional formula is as follows: 6 mmol·L^{-1} KNO_3, 4 mmol·L^{-1} $Ca(NO_3)_2$, 2 mmol·L^{-1} $NH_4H_2PO_4$, 1 mmol·L^{-1} $MgSO_4$, 50 μmol·L^{-1} KCl, 10 μmol·L^{-1} H_3BO_3, 2 μmol·L^{-1} $MnSO_4$, 2 μmol·L^{-1} $ZnSO_4$, 0.5 μmol·L^{-1} $CuSO_4$, 0.065 μmol·L^{-1} $(NH_4)_6Mo_7O_{24}$ and 40 μmol·L^{-1} Fe-EDTA. The boron concentration in the standard nutrient solution was used as the control (10 μmol·L^{-1}). The high boron treatments were set at 100, 300 and 500 μmol·L^{-1} and were repeated three times. Before the pear seedlings were placed into hydroponic cultures, three to five full buds were left for cutting, and root pruning was performed. Eight to ten centimeters of roots were preserved. Seedlings were cultured in a dark plastic box with a light intensity of 400 to 500 μmol·m^{-2}·s^{-1}. The day and night temperatures were 30 and 22°C, respectively; the relative humidity was 80 to 90%. The nutrient solution was changed once every seven days. The pH was adjusted to 6.50 ± 0.10. Samples were measured after 16 weeks of culture.

Indicator measurement

Measurement of boron and photosynthetic pigment

The measurement of Chla, Chlb and carotenoid (Car) levels was performed by the method of Lichtenthaler et al. (1987). Each measurement was repeated five times. For the microwave digestion method, the deionized water was kept at a volume of 25 ml. An inductively coupled plasma emission spectrometer (ICP method) was used to measure boron content. These measurements were repeated three times.

Gas exchange parameters

The net photosynthetic rate (Pn), stomatal conductance (Gs) and intercellular CO_2 concentration (Ci) were measured by the CIRAS21 photosynthetic measurement system (British PP Systems Co.). Measurements were made between 10:30-12:00 in sunny weather; the light intensity was 1300±35 μmol·m^{-2}·s^{-1}, and the leaf temperature and ambient atmospheric pressure were 27 ± 1.0°C and 1.7 ± 0.1 kPa, respectively. One leaf was counted as one sample; six to ten samples were measured per treatment.

Measurement of MDA and H_2O_2 content

MDA measurements were performed by the methods of Heath and Packer, (1968) and Fu and Huang, (2001). The H_2O_2 measurement was performed by the method of Mukherjee and Choudhuri, (1983). Each measurement was repeated three times.

Measurement of antioxidant enzymes

Extraction of SOD, CAT, APX and GR was performed as in Chen and Cheng (2003). Measurements of SOD activity were performed by the method of Giannopolitis and Rice (1977). CAT, APX and GR activities were measured using Chen and Cheng's method (2003). Each measurement was repeated four times.

Measurement of ASA and GSH content

Chen and Cheng's method (2003) was used to measure the ASA levels. The GSH level was measured according to Griffith's (1980) method. Each measurement was repeated four times.

Data analysis

SPSS and Excel were used for statistical analysis. A one-way ANOVA

Table 1. The effect of boron toxicity on concentrations of boron and photosynthetic pigments in pear leaves.

Treatment ($\mu mol \cdot L^{-1}$)	Boron content ($\mu g \cdot g^{-1}$)	Chla ($mg \cdot g^{-1}FW$)	Chlb ($mg \cdot g^{-1}FW$)	Car ($mg \cdot g^{-1}FW$)	Chla/b
10	80.27±0.41^{d}	1.94±0.06^{a}	0.64±0.05^{a}	0.45±0.04^{a}	3.02±0.17^{a}
100	106.40±2.10^{c}	1.81±0.04^{a}	0.61±0.04^{a}	0.43±0.03^{a}	2.95±0.12^{ab}
300	155.70±5.55^{b}	1.52±0.11^{b}	0.52±0.05^{b}	0.4±0.02^{ab}	2.92±0.07^{ab}
500	204.96±6.48^{a}	1.19±0.06^{c}	0.42±0.03^{c}	0.35±0.03^{b}	2.79±0.04^{b}

Different letters in the same column indicate significant differences ($P < 0.05$).

Table 2. The effects of boron toxicity on photosynthetic rate, stomatal conductance and intercellular carbon dioxide concentration in pear leaves.

Treatment ($\mu mol \cdot L^{-1}$)	Net photosynthetic rate (Pn) $\mu mol \cdot m^{-2} \cdot s^{-1}$	Stomatal conductance (Gs) $mmol \cdot m^{-2} \cdot s^{-1}$	Intercellular CO_2 (Ci) $\mu mol \cdot mol^{-1}$
10	12.64±0.27^{a}	218.9±12.5^{a}	180.7±4.56^{c}
100	12.23±0.18^{a}	200.8±9.86^{a}	192.1±6.87^{c}
300	10.62±0.12^{b}	176.4±5.63^{b}	229.4±8.65^{b}
500	9.76±0.09^{c}	150.6±9.32^{c}	246.5±7.31^{a}

Different letters in the same column indicate significant differences ($P < 0.05$).

was used to determine the significance of the differences between treatments. Duncan's method was used for multiple comparisons.

RESULTS

The effect of boron toxicity on pear photosynthesis

Boron and leaf photosynthetic pigments

Table 1 shows that the boron content of the leaves tended to increase with increasing concentrations of exogenous boron. The differences between treatments were significant as compared to the control. In the leaves treated with 100, 300 or 500 $\mu mol \cdot L^{-1}$, the boron level increased by 32.0, 93.9 and 155.3%, respectively.

Under boron stress conditions, leaf levels of Chla, Chlb, Car and Chla/b decreased (Table 1). This reduction in Chla and Chlb content was significant relative to the control for boron concentrations higher than 300 $\mu mol \cdot L^{-1}$. Likewise, the reduction in Car was significant relative to the control when the boron concentration was 500 $\mu mol \cdot L^{-1}$. Chla/b and Car showed similar patterns.

Photosynthetic rate, stomatal conductance and intercellular CO_2 concentrations

Boron stress inhibited pear leaf photosynthesis (Table 2). The leaf Pn showed a decreasing trend with increasing boron concentrations. When the concentration exceeded 300 $\mu mol \cdot L^{-1}$, the difference between the experimental condition and the control was significant. The Pn and Gs demonstrated similar patterns. The intercellular CO_2 concentration increased with increasing boron concentrations. The differences between the 300 and 500 $\mu mol \cdot L^{-1}$ treatments and the control were significant.

The effect of boron toxicity on pear leaf membrane lipid peroxidation

MDA and H_2O_2 content in leaves

MDA is the major product of lipid peroxidation and can be used to estimate the level of lipid peroxidation in a given tissue. Hydrogen peroxide (H_2O_2) can also serve as an indicator of damage by ROS in leaves. Figure 1 shows that the MDA content in pear leaves increased with increasing concentrations of boron. In the leaves treated with 100, 300 or 500 $\mu mol \cdot L^{-1}$, MDA levels were higher than the control by 10.03, 67.82 and 133.09%, respectively. For concentrations exceeding 300 $\mu mol \cdot L^{-1}$, the difference between the treated and control leaves was significant. The level of H_2O_2 in the leaves also increased with increasing concentrations of boron, and the difference between treatments was significant. These data indicate that boron stress exacerbated the extent of lipid peroxidation.

SOD, CAT, GR and APX activity in leaves

Figure 2 shows that under increasing concentrations of boron, SOD activity first increased and then decreased. As compared with the control, SOD activity was significantly increased after treatment with 100 or 300

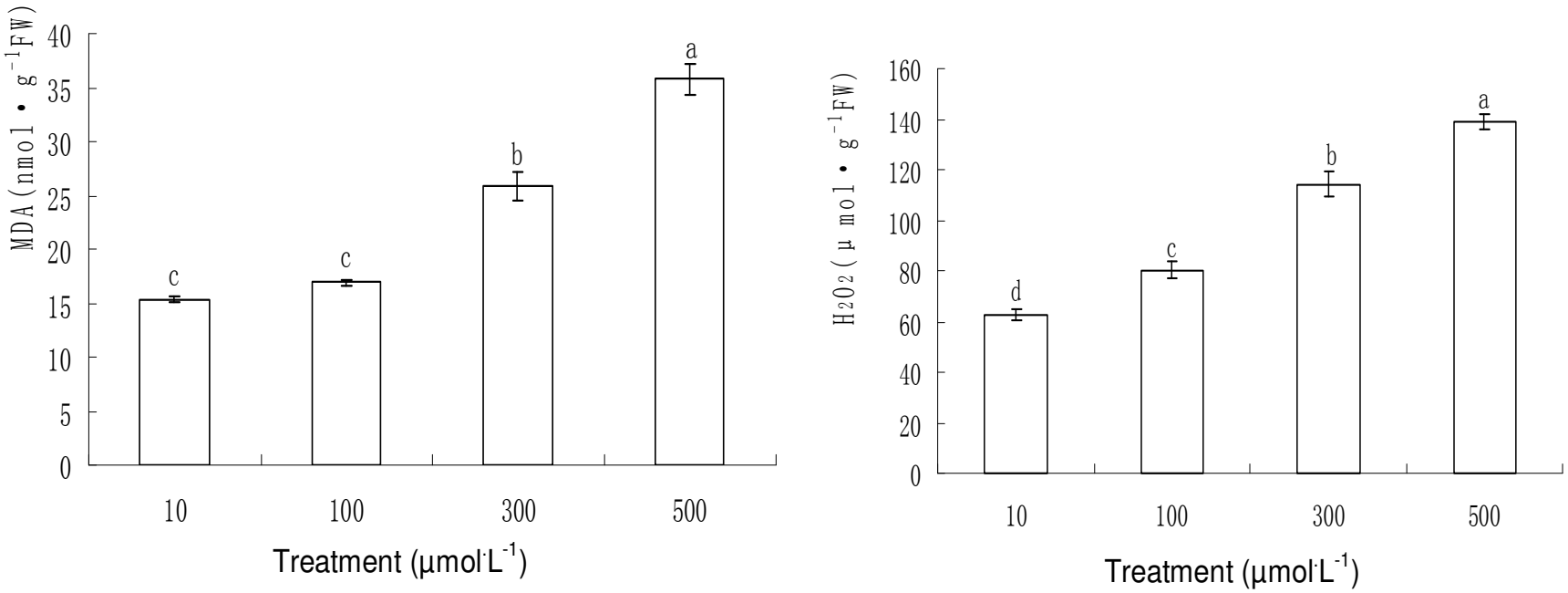

Figure 1. The effect of boron toxicity on MDA and H_2O_2 contents in pear leaves. Different letters represent significant differences between treatments ($P < 0.05$).

μmol·L^{-1} boron (22.79 and 82.56% higher, respectively). Treatment with 500 μmol·L^{-1} boron resulted in significantly decreased SOD activity (by 23.43%) ($P<0.05$). Changes in CAT activity under boron stress conditions were similar to those of SOD.

GR and APX activities also increased and then decreased as the concentration of boron increased. GR activity significantly increased in the 100 and 300 μmol·L^{-1} treatments relative to the control. In the 500 μmol·L^{-1} boron treatment, GR activity was significantly lower than in the 300 μmol·L^{-1} treatment but still higher than the control. APX activity presented a similar pattern, but the difference between the 500 μmol·L^{-1} treatment and the control was not significant.

Leaf ASA and GSH contents

ASA and GSH are non-enzymatic antioxidants present in plants that are important parts of the free radical scavenging system. Figure 3 shows that with increasing concentrations of boron, the level of ASA first increased and then decreased. The ASA levels in leaves exposed to 100 or 300 μmol·L^{-1} boron were significantly increased relative to the control. In the 500 μmol·L^{-1} boron treatment, the leaf ASA level was significantly lower than in the 100 and 300 μmol·L^{-1} treatments but not significantly different from the control. As the severity of boron stress increased, the GSH level also increased and then decreased. In the 100 and 300 μmol·L^{-1} treatments, the GSH level was higher than in the control, whereas in the 500 μmol·L^{-1} boron treatment, it drastically declined to a level lower than the control. Differences among the treatments were significant (Figure 3).

DISCUSSION

A study by Dannel et al. (1997) suggested that the supply of exogenous boron determines the boron levels in the plant. Consistent with these previous results, our study found that treatment with nutrient solutions containing increasing concentrations of boron resulted in increased absorption of boron by the plant. The normal level of boron in the pear tree is generally around 30 to 100 μg·g^{-1};

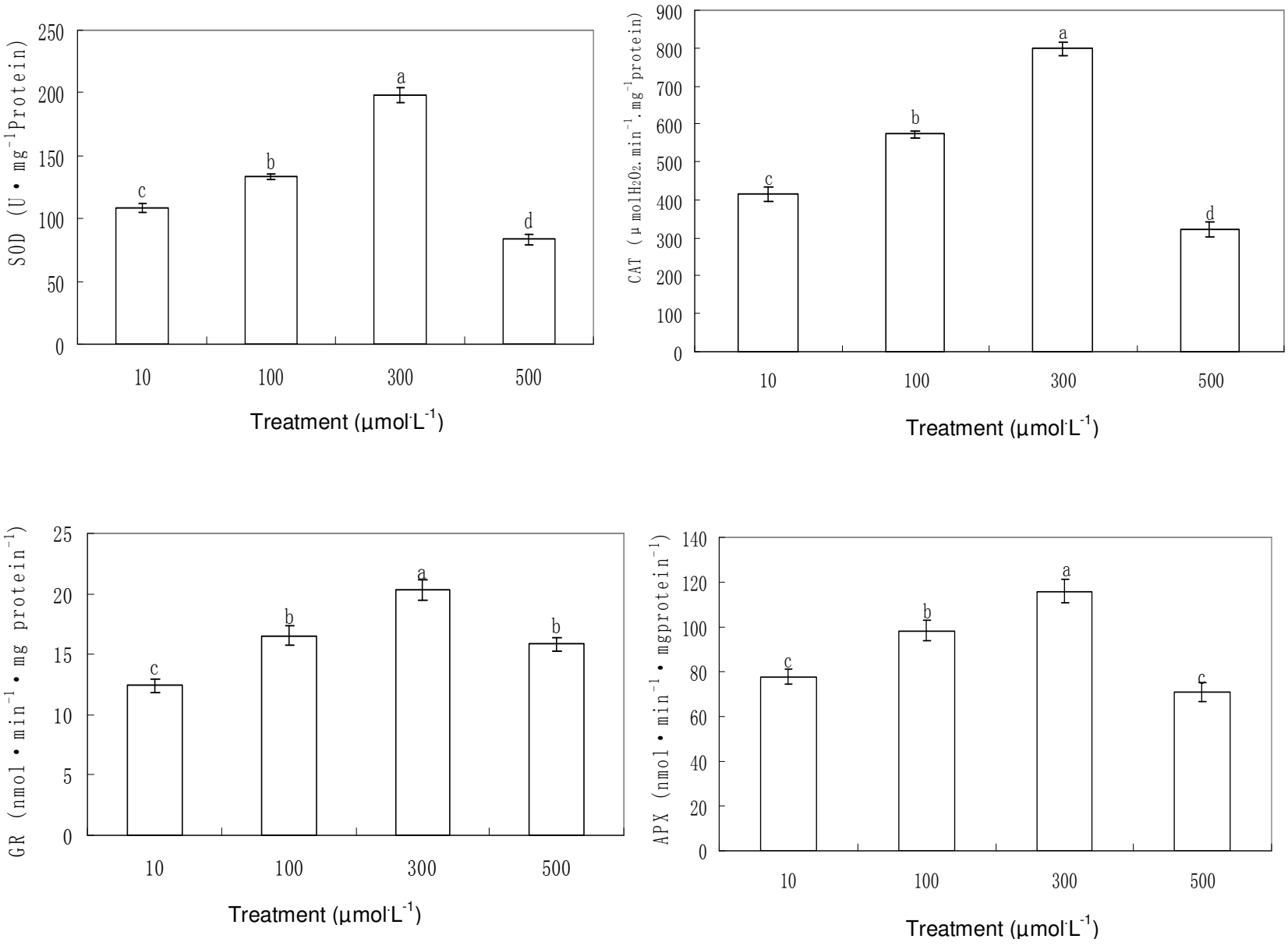

Figure 2. The effect of boron toxicity on SOD, CAT, GR and APX activities in pear leaves.

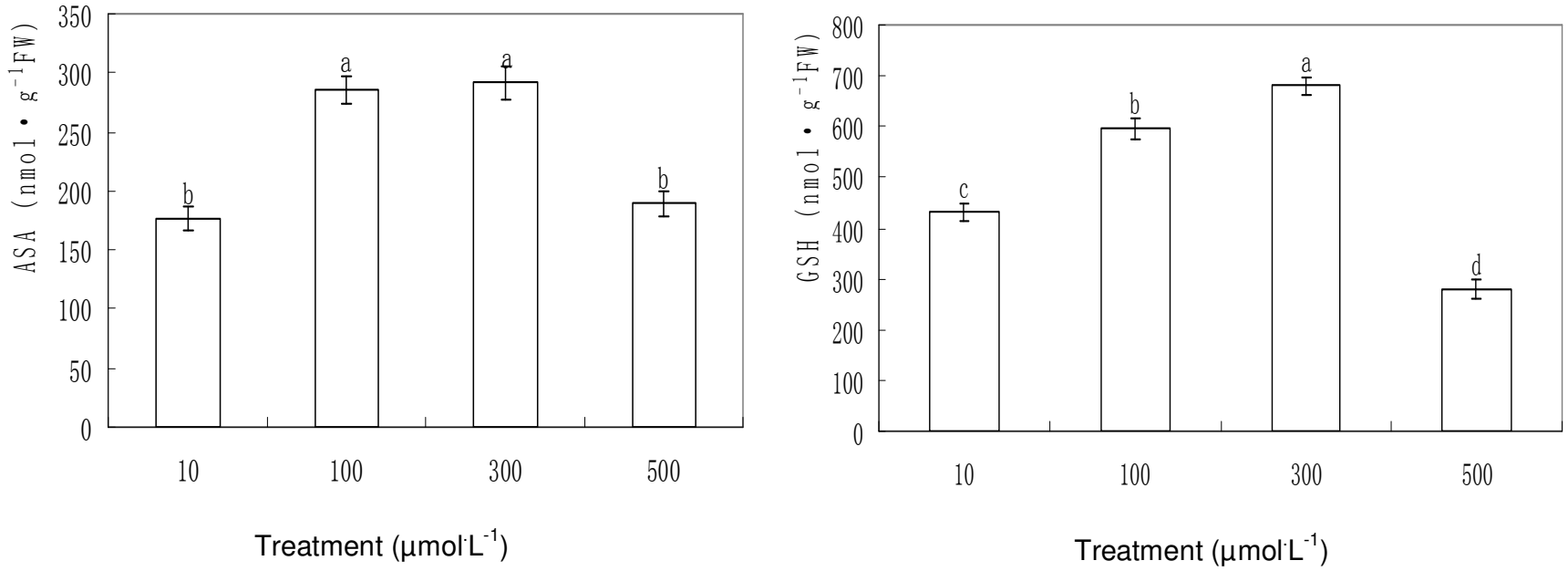

Figure 3. The effect of boron toxicity on ASA and GSH contents in pear leaves.

concentrations higher than 100 μg·g^{-1} can cause boron poisoning. The boron concentration in each experimental treatment in the present study was higher than 100 μg·g^{-1}. These elevated concentrations were significantly higher than the normal level and affected the normal growth and development of pear seedlings.

Under conditions of boron stress, the amount of photosynthetic pigment in pear leaves was significantly reduced (Table 1), and the CO_2 assimilation rate appeared to reduce with the increase in the intercellular CO_2 concentration (Table 2). This indicates that boron stress reduced the photosynthetic capacity of pear leaves, consistent with the study of citrus plants by Han et al. (2009). Previous studies (Cave et al., 1981) have shown that stress conditions may lead to the accumulation of starch in leaves. Excess starch can destroy the structure of chloroplasts, thereby affecting the formation of photosynthetic pigments and lowering the photosynthetic rate. Another study (Schaffer et al., 1986) showed that hexose accumulation in leaves inhibited the expression of photosynthesis-related enzymes, causing feedback inhibition of photosynthesis. However, the specific cause of the boron-induced reduction in photosynthetic pigments and the photosynthetic rate is still unclear and requires further study.

MDA and H_2O_2 serve as measuring indices for leaf damage by ROS (Bowler et al., 1992). Mittler (2002) proposed that membrane lipid damage was related to *in vivo* H_2O_2 content, as high concentrations of H_2O_2 would accelerate the Haber-Weiss reaction, generating highly toxic •OH and thereby initiating lipid peroxidation. MDA is a product of lipid peroxidation. We found that the H_2O_2 and MDA levels were significantly increased under boron stress, indicating that boron toxicity increased lipid peroxidation in pear leaf cells, consistent with previous studies in apples (Molassiotis et al., 2006) and grapes (Gunes et al., 2006).

Increases in the antioxidant protection mechanism represent an adaptive strategy against ROS-induced damage. Studies have shown that the biosynthesis of ROS scavenging enzymes is affected by the regulation of cellular substrates: Over a certain concentration range, increases in the active oxygen concentration are accompanied by increases in enzymatic oxygen scavenging activity (Bowler et al., 1992). H_2O_2 is formed in the

reaction catalyzed by SOD. H_2O_2 in the cytoplasm can be cleared by CAT, while the conversion of H_2O_2 in chloroplasts relies on APX (Cakmak and Marschner, 1992). Through the ASA-GSH-NADPH catalytic oxidation cycle, APX can eliminate H_2O_2 and $O_2^{\bullet -}$. The oxidized ASA undergoes reduction by a GSH-mediated non-enzymatic reaction. GR promotes the reduction of oxidized glutathione (GSSG) to GSH. Thus, SOD, CAT, APX, GR, ASA and GSH play important roles in scavenging ROS in plant cells. We found that high B concentrations in the culture medium provoke oxidative damage such as the contents of H_2O_2 was increased, and also whether enzymatic antioxidants such as SOD, CAT, APX and GR activity (Figures 1 and 2) or non-enzymatic antioxidants such as ASA and GSH activity (Figure 3) increased. But when the boron concentration was greater than 500 $\mu mol \cdot L^{-1}$, enzymatic activity decreased. These patterns suggest that at relatively low levels of boron poisoning, pear trees can upregulate enzymatic anti-oxidant activity to remove ROS, whereas the protective enzyme system is overwhelmed when the boron concentration passes a certain threshold. Studies have shown that H_2O_2 plays a major role in the regulation of SOD and GR gene expression (Stanislaw, et al., 1993). In this way, certain concentrations of H_2O_2 can promote SOD, CAT and APX synthesis (Bowler et al., 1992) and increase CAT, APX and GR activity in wheat leaves (Feng et al., 1998). However, when the accumulated $O_2^{\bullet -}$ and H_2O_2 exceed the scavenging ability of the defense system, ROS will accumulate, causing lipid peroxidation and membrane damage. And perhaps, this is the cause why the scavenging enzymes were decreased by 500 $\mu mol \cdot L^{-1}$ boron treated in the present study.

In summary, boron toxicity led to decreases in photosynthetic pigment levels and CO_2 assimilation in pear leaves, resulting in an increase in ROS levels. The increase in lipid peroxidation induced a corresponding increase in the activity of protective enzymes and antioxidants such as SOD, CAT, APX, GR, ASA and GSH, which collectively act to clear ROS. However, when the boron concentration was raised to toxic levels, enzymatic activity decreased, and the lipid peroxidation of the cell membrane was further intensified.

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Abbreviations

Chl, Chlorophyll; **Pn,** net photosynthetic rate; **Gs,** stomatal conductance; **Ci,** intercellular carbon dioxide concentration; **MDA,** malondialdehyde; **ASA,** ascorbic acid; **GSH,** glutathione; **CAT,** catalase; **GR,** glutathione reductase; **SOD,** superoxide dismutase; **Car,** carotenoids; **APX,** ascorbate peroxidase; **ROS,** reactive oxygen species.

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