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CHANGES IN ANTIOXIDATIVE AND PHOTOSYNTHETIC PROPERTIES SYSTEM OF FRENCH BEAN (*Phaseolus vulgaris*) TO BORON TOXICITY

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ABSTRACT

Seedlings of the S-9 cultivar of the French bean (*Phaseolus vulgaris*) were used to study the effects of boron toxicity on lipid peroxidation leaf and photosynthetic properties. The plants were grown hydroponically and treated with four concentrations of boron, 50, 100 and 300 µM After 48h 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 bean 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 bean seedlings, 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.

Keywords: French bean, 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 $O2^{\bullet -}$ 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 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 French bean is one of the most highly cultivated vegetable crop grown all over the world and is often subjected to boron stress. However, the effects of boron toxicity on the physiological metabolism of the French bean, especially the effects on photosynthesis and active oxygen metabolism, have not been thoroughly investigated. We therefore used seedlings S-9 cultivar of the French bean *(Phaseolus vulgaris)*, in these experiments to study high boron stress conditions, bean seedlings 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 French bean crop react to this type of stress.

MATERIALS AND METHODS

Experimental materials

Six day old seedlings of French bean (*P. vulgaris*) were used as experimental materials and were cultured hydroponically according to modified Hoagland nutrient solution (Han *et al*., 2009). The boron concentration in the standard nutrient solution was used as the control (10 µM). The high boron treatments were set at 50, 100 and 300 µM and were repeated three times. Seedlings were cultured in a dark plastic box with a light intensity of 400 to 500 $\mu mol \cdot m^{-2}/s^{-1}$. The day and night temperatures were 30 and 22°C, respectively; the relative humidity was 80 to 90%. The pH was adjusted to 6.50 ± 0.10. Samples were measured after 48 h of stress treatment.

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 20 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 in sunny weather; the light intensity was 1300±35 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 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 Wang *et al*. Different letters in the same column indicate significant differences ($P < 0.05$) were 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 French bean photosynthesis Boron and leaf photosynthetic pigments

Figure-1 and Table-1 showed 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 µM, 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 µM. Likewise, the reduction in Car was significant relative to the control when the boron concentration was 300 µM. Chla/b and Car showed similar patterns.

Table-1. The effect of boron toxicity on concentrations of boron and photosynthetic pigments in French bean.

Treatment µmoles	Boron content µg/g	Chla µg/g FW	Chlb µg/g FW	Chla/b µg/g FW	Car µg/g FW
50	80.27±0.41[d]	1.94±0.06[a]	0.64±0.05[a]	3.02±0.17[a]	0.45±0.04[a]
100	106.40±2.10[c]	1.81±0.04[a]	0.61±0.04[a]	2.95±0.12[ab]	0.43±0.03[a]
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]

Different letters in the same column indicated significant difference (P 0.05)

Figure-1. Effect of boron toxicity on French bean: six days old seedlings subjected to boron stress for 48 h. Control [C], stressed [S].

Photosynthetic rate, stomatal conductance and intercellular CO_2 concentrations

Boron stress inhibited Bean leaf photosynthesis (Table-2). The leaf Pn showed a decreasing trend with increasing boron concentrations. When the concentration exceeded 300 µM, 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 µmol/L^{-1} treatments and the control were significant.

Table-2. The effect of boron toxicity on photosynthetic rate, stomatal conductance and intercellular carbon dioxide.

Treatment µmoles	Net photosynthetic rate (Pn) µmoles m-2.s^{-1}	Stomatal conductance (Gs) µmoles. m-2.s^{-1}	Intercellular CO_2 (Ci) µmole/mol^{-1}
50	12.64±0.27[a]	218.9±12.5[a]	180±4.56[c]
100	12.23±0.18[a]	200.8±9.86[a]	192±6.87[c]
300	10.62±0.12[b]	176.4±5.63[b]	229.4±8.65[b]

Different letters in the same column indicated significant difference (P 0.05).

The effect of boron toxicity on French bean 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 showed that the MDA content in French bean leaves increased with increasing concentrations of boron. In the leaves treated with 100, 300 or 500 µM, MDA levels were higher than the control by 10.03, 67.82 and 133.09%, respectively. For concentrations exceeding 300 µM, 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

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 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 were not significant.

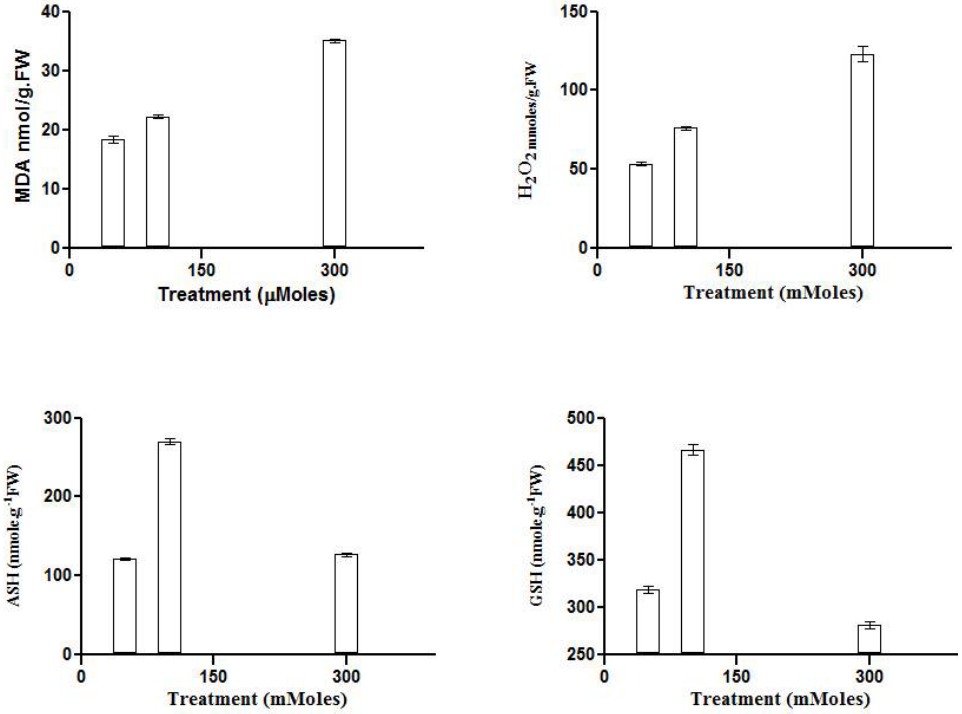

Figure-2. The effect of boron toxicity on MDA, H_2O_2, ASA, and GSH contents in French bean.

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 300 µM 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 µM treatments, the GSH level was higher than in the control, whereas in the 300 µM boron treatments, it drastically declined to a level lower than the control. Differences among the treatments were significant (Figure-3).

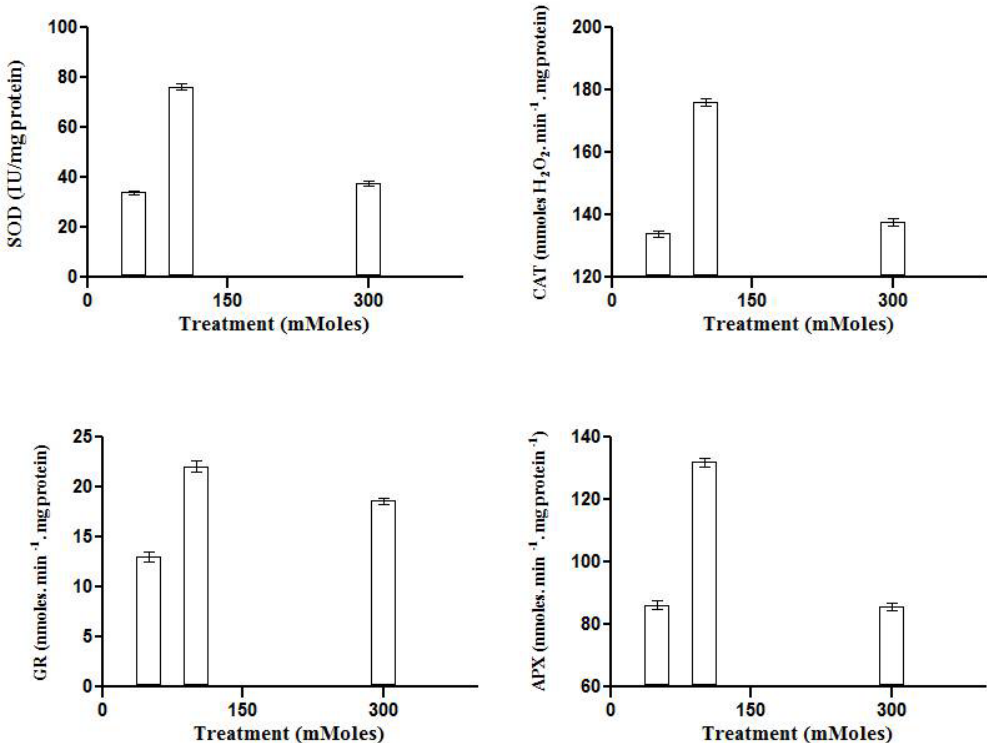

Figure-3. The effect of boron toxicity on antioxidant enzymes (SOD, CAT, GR, and APX) in French bean.

DISCUSSIONS

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 French bean is generally around 30 to 100 µg·g-1; 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 French bean seedlings. Under conditions of boron stress, the amount of photosynthetic pigment in French bean 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 French bean 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 French bean 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 $O2^{\bullet -}$. 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 µM, enzymatic activity decreased. These patterns suggest that at relatively low levels of boron poisoning, French bean can up-regulate enzymatic antioxidant 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 H2O2 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 $O2^{\bullet -}$ 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 µM boron treated in the present study.

In summary, boron toxicity led to decreases in photosynthetic pigment levels and CO_2 assimilation in French bean leaves, resulting in an increase in ROS levels. The increase in lipid peroxidation induced a corresponding increase in the activity of protective enzymes anntioxidants 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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