EFFECT OF BORON NUTRITION ON RESISTANCE RESPONSE OF TOMATO AGAINST BACTERIAL WILT CAUSED BY *RALSTONIA SOLANACEARUM*

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SUMMARY

This study investigated the effect of boron nutrient on tomato bacterial wilt caused by *Ralstonia solanacearum* and the regulation mechanisms. Plants, cultured in nutrient solution and treated with three concentrations of B (0.05, 0.50 and 2.50 mg l⁻¹), were inoculated with *R. solanacearum* by the method of root dip. Severity of disease development, plant nutrient uptake, hydrogen peroxide (H₂O₂) activity, enzymes like peroxidase (POD, EC 1.11.1.7), polyphenol oxidase (PPO, EC 1.10.3.2) activities in tomato leaves were analyzed. Disease severity of low, medium and high B treatments were 95.2%, 72.6% and 63.4% respectively. There was no significant difference in plant dry weight, indicating no B toxic or deficiency phenomenon in all treatments. Tomato plants absorbed significantly more Ca and B with the level of B in the nutrient solution increased. In addition, H₂O₂ level in high B treatment rose faster and reached a higher peak with 11.94 μM gFW⁻¹ (96.7% greater than low B plants). The activities of POD and PPO also have a greater increase in high B treatment with 97.12 U gFW⁻¹ and 94.00 U gFW⁻¹ compared to 39.16 U gFW⁻¹ and 70.51 U gFW⁻¹ in low B treatment. These results suggested that the regulation mechanism of B was to increase the Ca and B concentration, improve the rate and the amount of H₂O₂ accumulation, and increase the activities of POD and PPO in tomato.

**Key words**: boron, tomato, resistance response, regulating mechanisms, *Ralstonia solanacearum*

INTRODUCTION

*Ralstonia solanacearum* (previously named *Pseudomonas*) is a major cause of wilt disease in tomato (*Solanum lycopersicon* L.) around the world (Hayward, 1991). Various strategies have been developed for the control of bacterial wilt, including the development of resistant varieties (McGarvey et al., 1999), use of chemical fumigants (Rose et al., 2003), soil drainage (van Elsas et al., 2001), and biocontrol with beneficial microorganisms (Xue et al., 2009). However, due to a variety of reasons, these current methods each have their own limitations.

It was reported that all the essential nutrients can affect disease severity (Huber and Graham, 1999). Boron (B) is an essential nutritional element for plant and it also plays an important role in plant disease resistance system (Graham, 1983). B would affect plant resistance against pathogen, and previous studies already have demonstrated the mitigating effect of the application of B on disease management (Kataria and Grover, 1987; Arreola et al., 2008). However, little is known about the effect of B on tomato bacterial wilt caused by *R. solanacearum*.

The defense mechanisms regulated by B against pathogen challenge are not very clear. As pathogenic fungi are supposed not to have a B requirement, excess B may be toxic to them (Pratt, 2000). B plays an important role in maintaining the integrity of the cell wall (Hu and Brown, 1994). Therefore, it would help reduce pathogenesis. Garcia et al. (2011) reported that combined application of carbendazim and B increased SOD, GPX, CAT, and APX activities of tobacco, which may signify an additional tolerance mechanism to pathogenic infection. However, further investigation is required to discover the physiological regulation mechanisms of B on plant disease resistance.

Since B affects physiological and biological mechanisms in plants to alleviate disease, the aim of the present study was to investigate the effect and regulating mechanism of B on the resistance of tomato against bacterial wilt. Our objectives were 1) to investigate the effect of B on plant growth; 2) the relation of nutrient uptake to the severity of disease development; 3) the hydrogen peroxide (H₂O₂) concentration and resistance-related enzyme activities in...
tomato; and 4) identification of the regulatory mechanisms underlying tomato bacterial wilt resistance that are altered by B.

**MATERIALS AND METHODS**

**Plant growth.** The greenhouse experiments were conducted three times with the same procedure in the year of 2012 and 2013. Seeds of tomato (*Solanum lycopersicum* L.) cv. Shanghai 906 susceptible to bacterial wilt were sown and seedlings were grown in a mixture of vermiculites and perlites (1:1 v/v) in a greenhouse maintained between 15°C to 25°C under natural light. Seedlings were fertilized twice weekly with Hoagland’s solution one week after sowing. At the five- or six-leaf stage, seedling roots were washed gently in distilled water and plants were transplanted into each plastic pot containing 3 liters of the specified nutrient solution. Seedlings were supported with polyurethane foam in a plexiglass plate, and treated with three levels of B: 0.05, 0.50, and 2.50 mg l−1, representing low, medium and high concentrations, respectively. The nutrient solution for the medium B level is commonly used in commercial greenhouse production and consisted of macro-nutrients (in mM): N, 15.0 [5.0 KNO₃, 5.0 Ca(NO₃)₂], P, 1.0 (KH₂PO₄), K, 6.0 (5.0 KNO₃, 1.0 KH₂PO₄), Ca, 5.0 [Ca(NO₃)₂], Mg, 2.0 (MgSO₄); micronutrients (in mg liter⁻¹): Fe, 3.0 (Fe-EDTA), Mn, 0.5 (MnCl₂), B, 0.5 (H₃BO₃), Zn, 0.05 (ZnSO₄), Mo, 0.01 (Na₂MoO₄), and Cu, 0.02 (CuSO₄). For the low B level, H₃BO₃ in the solution was adjusted to 0.05 mg l⁻¹, and for the high B level, the solution was adjusted to 2.50 mg l⁻¹ H₃BO₃. The pH of all solutions was adjusted to 6.0 with 2 M NaOH, and all the solutions were changed twice a week.

**Pathogen inoculation and disease assessment.** *Ralstonia solanacearum* strain ZJ3721 (Li *et al.*, 2010) was grown for 2-3 days on YPGA (per l: yeast extract 5 g, bacto peptone 5 g, glucose 10 g and agar 15 g) agar or YPGA broth at 28°C. After incubation, the suspension population was counted by a bacterial counting chamber and adjusted to 10⁸ cells ml⁻¹. After seedlings had grown for thirty days, plants were inoculated by dipping the roots in the bacterial suspension for 15 min. The roots were then rinsed by distilled water and returned to the pots (Poya, 1993). Disease development was scored every other day for 20 days after inoculation by visual observation and a rating scale of 0 to 4, in which 0 = no symptoms observed; 1 = light mottling and a few thin yellow veins; 2 = mottling and vein clearing unevenly distributed on the leaf; 3 = mottling, leaf distortion, and stunting; and 4 = severe mottling, leaf curling, and stunting (Roberts *et al.*, 1988). There were three replicates with 36 seedlings per treatment. Disease severity was calculated as follows:

\[
\text{Disease severity} = \left(\frac{\Sigma (\text{the number of diseased plants in this index}) \times \text{disease index}}{\text{total number of plants investigated} \times \text{the highest disease index}}\right) \times 100 \%
\]

**Plant growth and nutrients analysis.** When the tomato plants had grown for 30 days in nutrient solution, the plant height, stem diameter, leaf thickness, leaf area and biomass of each treatment were measured. The non-inoculated tomato plants were harvested, washed with distilled water and dried at 70°C for 2 h, and 105°C for 48 h. Amounts of 100 mg of each dried sample were digested with a nitric acid-perchloric acid mixture (HNO₃-HClO₄). Nitrogen was analyzed using the total Kjeldahl nitrogen method. The filtrate was analyzed for phosphorus (P), potassium (K), calcium (Ca) and boron (B) using an atomic absorption spectrometer (model AA-670, Shimadzu Co., Kyoto, Japan).

**H₂O₂ concentration measurement.** Leaf samples were collected 0 (just before inoculation), 6, 12, 24, 48, 72 and 96 h after inoculation, with four replicates for each treatment. The concentration of H₂O₂ in the tissue was determined following the method described by Kar and Choudhuri (1987). H₂O₂ was isolated from 1 g leaf tissue in ice-cold acetone. 5% (w/v) of titanium sulfate and ammonium hydroxide solution was added to precipitate the peroxy-acid-titanium complex. The precipitate was collected in ice-cold acetone. 5% (w/v) of titanium sulfate and ammonium hydroxide solution was added to precipitate the peroxy-acid-titanium complex. The precipitate was collected in ice-cold acetone. The homogenates were filtered and treated with three levels of B: 0.05, 0.50, and 2.50 mg l⁻¹, representing low, medium and high concentrations, respectively. The nutrient solution for the medium B level is commonly used in commercial greenhouse production and consisted of macro-nutrients (in mM): N, 15.0 [5.0 KNO₃, 5.0 Ca(NO₃)₂], P, 1.0 (KH₂PO₄), K, 6.0 (5.0 KNO₃, 1.0 KH₂PO₄), Ca, 5.0 [Ca(NO₃)₂], Mg, 2.0 (MgSO₄); micronutrients (in mg liter⁻¹): Fe, 3.0 (Fe-EDTA), Mn, 0.5 (MnCl₂), B, 0.5 (H₃BO₃), Zn, 0.05 (ZnSO₄), Mo, 0.01 (Na₂MoO₄), and Cu, 0.02 (CuSO₄). For the low B level, H₃BO₃ in the solution was adjusted to 0.05 mg l⁻¹, and for the high B level, the solution was adjusted to 2.50 mg l⁻¹ H₃BO₃. The pH of all solutions was adjusted to 6.0 with 2 M NaOH, and all the solutions were changed twice a week.

**Enzyme assay.** Leaf samples were collected 0 (just before inoculation), 6, 12, 24, 48, 72 and 96 h after inoculation (four replicates for each treatment). Samples were immediately flash-frozen and stored in liquid nitrogen prior to analysis. 1.0 g of leaf tissue was homogenized in 5 ml of phosphoric acid extracting buffer (0.05 M phosphate, pH 7.0) in an ice bath. The homogenates were filtered.
through four layers of cheese cloth and collected after centrifugation at 12,000 rpm for 20 min at 4°C, and the supernatants used for the enzyme activity assays. The activity of the enzymes was assayed as described by Li et al. (2008).

Statistical analysis. Data were subjected to analysis of variance (ANOVA) and the least significant differences at P < 0.05 (Fisher LSD) was determined. Analyses were performed using the Statistics software package (SPSS 18.0). Correlation analysis was conducted by Pearson’s correlation (2-tailed, P = 0.05).

RESULTS

Data on plant disease severity, plant growth, plant nutrient uptake, leaf H₂O₂ concentration and leaf enzymes activity were combined, respectively, as there was no significant difference between the three trials.

Disease severity. There were dynamic differences in disease severity with the different treatments (Fig. 1). Medium B plants began to wilt 4 days after inoculation, and their disease severity rating was 72.6% 20 days after inoculation. Plants in low B treatment began to wilt 2 days after inoculation, 2 days earlier than medium B treatment, and the disease progressed more rapidly and was significantly (22.6% greater) more severe than on the plants grown in the medium B level solution. Plants in high B treatment began to wilt 6 days after inoculation, 2 days later than the medium B treatment and the disease progressed slowly so that the disease severity was 63.4% 20 days after inoculation.

Plant growth. Plant heights for low B and medium B treatments were 56.67 cm and 54.33 cm respectively, significantly higher compared to the high B treatment (48.67 cm, Table 1). The leaf area of high B treatment (73.55 cm²) was significantly lower than treatments of low (90.58 cm²) and medium B (85.50 cm²). Plants in the medium and high B treatments had larger stem diameters (6.41 and 6.61 mm, respectively) than those in the low B treatment. In addition, leaf thickness in the low B treatment (0.33 mm) was significantly lower than the medium (0.43 mm) and high B treatments (0.48 mm). However, there was no significant difference in biomass between all treatments.

Nutrient uptake. No difference was shown in macro-element (N, P and K) between all treatments (Table 2). However, tissue Ca for medium B and high B treatments were 30.97 g/kg and 30.57 g/kg respectively, significantly higher than the low B treatment, which was 25.78 g/kg. Tissue B was lowest for the low B compared with the medium and high B treatment (36.16, 98.36 and 112.94 mg/kg, respectively). Thus, the B in plants receiving the low B was 63.24% less than in plants receiving the medium level of B, but it was significantly higher in plants receiving the highest B (14.82% greater than the medium B treatment).

H₂O₂ concentration in tomato tissue. H₂O₂ concentration in tomato leaves was similar for all treatments before inoculation with R. solanacearum; however, it increased rapidly following inoculation (Fig. 2). H₂O₂ concentration in plants receiving low B treatment rose slowly and reached the highest point with 6.07 μM gFW⁻¹ at 48 h after inoculation. While H₂O₂ concentration in plants receiving
medium and high B treatments rose much faster and reached the highest point with 8.68 and 11.94 μM gFW⁻¹ at 12 h after inoculation, earlier and higher than it in the low B treatment. When the H₂O₂ concentration reached its peak, H₂O₂ in low B plants was 30.06% lower than the medium B treatment, whereas H₂O₂ levels in high B treatment was 11.94 μM gFW⁻¹ (37.56% greater than the medium B plants).

Resistance-related enzymes. POD activity was influenced by B rate and increased slowly in the first 24 h after inoculation (Fig. 3). Then, POD activity of low B treatment declined slowly, while POD activity of medium B treatment kept increasing for the next 48 h slowly, and POD activity of high B treatment had a sharp increase and reached a much higher level. POD activity was lowest with the low B, intermediate in the medium B and highest with the high B (39.16, 69.35, 97.12 U gFW⁻¹ or 32%, 204%, and 242% greater than it was before inoculation, respectively). Then POD activity declined slowly to the original level.

During the first 72 h after inoculation, PPO activity increased continuously and reached the highest point in all treatments (Fig. 4). PPO activity of the low, medium and high B treatment, which was at its peak after inoculation, were 70.51, 82.90, 94.00 U gFW⁻¹ (111%, 156%, and 439% higher than it was before inoculation, respectively). When POD activity reached the highest point, it was 70.51 U gFW⁻¹ with the low B treatment (15.0% less than the medium B) in contrast to 94.00 U gFW⁻¹ (13.4% greater than the medium B) with the high B treatment.

DISCUSSION

Previous studies have reported that B applications could suppress plant diseases caused by several pathogens (Simoglou and Dordas, 2006; Deora, et al., 2011). Frenkel et al. (2010) have reported that B could decrease late blight severity of tomato. However, little findings are available concerning the regulation mechanism about B on tomato bacterial wilt caused by R. solanacearum. In the present study, we investigate the effect of B on severity of tomato bacterial wilt, nutrient uptake, and physiological response like H₂O₂, POD, and PPO activity in tomato.

There was no B toxicity (high B treatment) or deficiency (low B treatment) in all treatments, as plant biomass had no significant increase or reduction. We found that increased B could significantly reduce the disease severity of tomato wilt caused by R. solanacearum. It is known that the pathogen enters plant roots through natural openings or wounds, invades the xylem vessels and secrets a large amount of extra-cellular polysaccharides that prevent water transportation, leading to plant death. We found that B could enlarge the stem diameter, which was beneficial for nutrient and water transportation of plant. At the same time, B also caused a decreased leaf area, reducing plant transpiration. As a result, disease severity was decreased with a reduction of plant need for water. It was in agreement with Landi (2013), who also reported that B could decrease plant transpiration.

Nutrients are essential for plant growth and are important factors in disease control (Agrios, 2005). Our results show that B had little effect on plant nutrient uptake of nitrogen (N), phosphor (P) and potassium (K). However, sufficient levels of B improved plant calcium uptake by 20.1%, which was an important nutritional element and played a major role in plant disease resistance system (Bateman and Lumsden, 1965; Volpin and Elad, 1991). Jiang et al. (2013) found that increased calcium concentrations in plants was associated with alleviated tomato bacterial wilt by 43.2%. We also found a negative correlation between the severity of bacterial wilt and Ca concentration in tomato (Table 3), indicating that the reduction in disease severity by B could be partly due to increased Ca concentration in tomato. Keane and Sackston (1970) also reported the effect of B in maintaining calcium nutrition within the cell wall, leading to improved plant resistance against pathogen.

Previous studies have reported that B excess (Esim et al., 2013) and deficiency (Mukhopadhyay et al., 2013) could affect plant antioxidant system.
the levels of $\text{H}_2\text{O}_2$, POD and PPO showed no significant difference among all three treatments before inoculation with pathogen, indicating that there was no B toxic or deficiency effect on plant physiology.

As an important reactive oxygen species (ROS), $\text{H}_2\text{O}_2$ is always generated in the earliest events after plants are infected by pathogens (Goodman and Novacky, 1994) and it plays a key role in plant disease resistance (Lamb and Dixon, 1997). In the present experiment, it was observed that there was a burst of $\text{H}_2\text{O}_2$ generation in the first 12 h after inoculation in all treatments, indicating that $\text{H}_2\text{O}_2$ played an active role in the tomato defense system against bacterial wilt. Since B nutrition significantly enhanced the generation rate and level of $\text{H}_2\text{O}_2$ in tomato, and there was a significant negative correlation ($R^2 = -0.999$) between $\text{H}_2\text{O}_2$ concentration and disease severity (Table 3). These results showed that the reduction of disease severity was due to the increased $\text{H}_2\text{O}_2$ activity, stimulated by B, in tomato. Aftab et al. (2010) also found the increased activities of $\text{H}_2\text{O}_2$ in response to increasing levels of B.

POD is involved in the detoxification of ROS (Mittler et al., 2004), and is an important antioxidant in plant cells. PPO catalyzes the oxygen-dependent oxidation of phenols to quinones. It was reported that POD and PPO participated in plant defense against pathogens (Tyagi et al., 2000; Pourcel et al., 2007). In this study, we observed a progressive increase of POD activity for the first 48 h and PPO activity for the 96 h after inoculation in all treatment, indicating that POD and PPO played an active role in the tomato defense system against *R. solanacearum*. Since high B nutrition significantly increased POD and PPO activities in tomato and there was a negative correlation between POD ($R^2 = -0.990$) and PPO ($R^2 = -1.000$) activity and disease severity (Table 3), the reduction in disease severity could be due to increased POD and PPO activities stimulated by high B (full physiological sufficiency) in tomato. B may play an active role in regulating resistance-related enzymes in plants, which is in agreement with Liu and Yang (2000) who reported that application of B to soybean would raise the activity of PPO. Keles et al. (2011) demonstrated that antioxidant enzyme activity, like superoxide dismutase (SOD) and catalase (CAT), increased with high B concentrations in tomato. Ghanati et al. (2005) also reported that increased B nutrient would increase of the activity of syringaldazine peroxidase.

In this study, we present a study into the physiological regulatory mechanism underlying the role of B nutrition in bacterial wilt resistance. The results pointed that sufficient B significantly increase the calcium and B concentration, stimulated $\text{H}_2\text{O}_2$ accumulation, and increase the activities of POD and PPO in plants, leading to a reduction of the severity of tomato wilt. Further research at the molecular level is needed to fully understand the specific role of B in tolerance of tomato against *R. solanacearum*.

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