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Effect of calcium nutrition on resistance of tomato against bacterial wilt induced by *Ralstonia solanacearum*

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Abstract This study investigated the effect of calcium nutrition on tomato bacterial wilt caused by *Ralstonia solanacearum* and the regulation of resistance mechanisms. Plants cultured in nutrient solution with calcium concentrations of 0.5, 5.0, and 25.0 mM, were inoculated with *R. solanacearum* by the root dip method. Severity of disease development, Ca concentration in tomato root and shoot tissues, hydrogen peroxide (H_2O_2) concentration, peroxidase (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.2) in tomato leaves were analyzed. Disease severities of low, medium and high Ca treatments were 100 %, 77.1 % and 56.8 % respectively. Plant growth in high Ca treatment was significantly better than those in low Ca treatment in height, stem diameter and biomass. Tomato plants absorbed significantly more Ca in roots and shoots as the level of Ca in the nutrient solution increased. In addition, H_2O_2 level in high Ca treatment rose faster and reached a higher peak with 10.86 μM gFW^{-1}(31.32 % greater than medium Ca plants). The activities of POD and PPO also have a greater increase in high Ca treatment with 99.09 U gFW^{-1} and 107.24 U gFW^{-1} compared to 40.70 U gFW^{-1} and 77.45 U gFW^{-1} in low Ca treatment. A negative correlation was found between Ca concentration, level of H_2O_2, POD, PPO in tomato, and disease severity, indicating that they played an important role in resistance of tomato to this disease. These results suggested that Ca was involved in the regulation of H_2O_2 concentration, and activity of POD and PPO in tomato.

Keyword Calcium · Tomato · *Ralstonia solanacearum* · Resistance · Regulating mechanisms

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Introduction

Ralstonia solanacearum (pseudonym *Pseudomonas solanacearum*) causes a serious wilt disease in tomato (*Solanum lycopersicon* L.) and other crops around the world (Hayward 1991). It has caused yield reductions of several greenhouse crops in China and, with an increasing number of field sites infested with *R. solanacearum*, it has dramatically increased the threat to tobacco crops (Huang et al. 2009). Like other soil-borne diseases, various strategies have been developed for controlling bacterial wilt, including the development of resistant or partially resistant (tolerant) cultivars of tomato (McGarvey et al. 1999; Yamazaki and Hoshina 1995), chemical fumigants (Rose et al. 2003), soil drainage (Elsas et al. 2001), tillage practices

(Rivard et al. 2010), however, there is still no fully effective way to control this disease.

Various studies have reported that all of the essential nutrients can affect disease severity (Huber and Graham 1999). Nutrients are essential for plant growth and development as well as for pathogens, and are important factors in disease control (Agrios 2005). In particular, nutrients can affect the disease tolerance or resistance of plants to pathogens by affecting their rate of growth and their state of readiness to defend themselves against pathogenic attack (Linus-Muriithi and Irungu 2004). Silicon nutrition could reduce the severity of cucumber root rot caused by *Phytophthora melonis* (Mohaghegh et al. 2011).

Calcium is an important nutritional element that plays a major role in plant disease resistance system, and various studies have demonstrated the mitigating effect of calcium for disease management (Bateman and Lumsden 1965; Volpin and Elad 1991). Calcium affects the stability and function of plant membranes (Wallace et al. 1966), activates the wall-bound acid phosphatases (Sugawara et al. 1981), and has an important role in signal transduction during defence responses (Bush 1995; Ishihara et al. 1996; Gelli et al. 1997). Previous reports show that Ca can down-regulate H_2O_2 levels in plants by stimulating catalase (Yang and Poovaiah 2002). The effects of Ca on other plant enzymes correlated with resistance to pathogens also have been reported (Schober and Vermeulen 1999).

Plants exposed to biotic and environmental stresses are under oxidative stress that results from the generation of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) (Foyer et al. 1997). Hydrogen peroxide is reported to be bactericidal as well as a key element involved in ROS (Levine et al. 1994). It is also a potent activator of certain MAP kinase cascades which are components of pathogen defence signalling (Bolwell 1999). Peroxidase (POD, EC 1.11.1.7) participates in oxidative burst, and play an active role in plant disease resistance system to pathogen (Silva et al. 2008). Polyphenol oxidase (PPO) is a systemic acquired resistance (SAR) related enzyme in plants, and also plays an active role in plant disease resistance to pathogens (Thipyapong et al. 2004; Bhonwong et al. 2009).

Resistance to bacterial wilt of a resistant tomato cultivar was correlated with increased Ca uptake (Yamazaki et al. 1996). Calcium increased the resistance of tomato to bacterial wilt and decreased the population of the pathogen in the xylem (Yamazaki et al. 2000). The physiological regulatory mechanisms involving Ca nutrition for tomato disease resistance induced by *R. solanacearum* are not known.

Since Ca can affect physiological mechanisms in plants to alleviate disease, the objective of the present study was to investigate the effect of Ca on the resistance of tomato to bacterial wilt caused by *R. solanacearum*. This objective involved: (i) investigation of the effect of Ca rate on plant growth, Ca concentration in tomato roots and shoots, H_2O_2 concentration, resistance-related enzyme activity, and severity of disease development in tomato; and (ii) identification of the regulatory mechanism involving Ca in bacterial wilt resistance.

Materials and methods

Plant growth

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 1 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 l of the specified nutrient solution. Seedlings were supported with polyurethane foam in a plexiglass plate, and treated with three levels of Ca: 0.5, 5, and 25 mM, representing low, medium and high concentrations, respectively. The nutrient solution for the medium Ca level is commonly used in commercial greenhouse production and consisted of macronutrients (in mM): N, 15.0 [5.0 KNO_3, 5.0 $Ca(NO_3)_2$], P, 1.0 (KH_2PO_4), K, 6.0 (5.0 KNO_3, 1.0 KH_2PO_4), Ca, 5.0 [$Ca(NO_3)_2$], and Mg, 2.0 ($MgSO_4$); and micronutrients (in mgl^{-1}) Fe, 3.0 (Fe-EDTA), Mn, 0.5 ($MnCl_2$), B, 0.5 (H_3BO_3), Zn, 0.05 ($ZnSO_4$), Mo, 0.01 (Na_2MoO_4), and Cu, 0.02 ($CuSO_4$). For the low Ca level, 4.5 mM $Ca(NO_3)_2$ in the solution was replaced with 9.0 mM $NaNO_3$, and for the high Ca level, the solution was adjusted with 20.0 mM $CaCl_2$. Although the low and high Ca solutions contained extra sodium or chloride ions, we confirmed in a preliminary experiment that excess

sodium and chloride ions did not affect bacterial wilt development. 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

R. solanacearum strain ZJ3721 (Li et al. 2010), highly virulent on tomato, was used in this study. A single colony of the virulent strain ZJ3721 was grown on YGPA (per litre: yeast extract 5 g, acto-peptone 5 g, glucose 10 g and agar 15 g) agar at 28 °C for 48 h. Then the inoculum was grown in YGPA (per litre: yeast extract 5 g, acto-peptone 5 g, glucose 10 g) broth on a rotary shaker (180 rpm) for 48 h at 28 °C. After incubation, the suspension population was counted by a bacterial counting chamber and adjusted to 10^8 cells ml^{-1}. After seedlings had grown for 30 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 (Poysa 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:

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

Plant growth and calcium analysis

Thirty days after transplanted into the respective nutrient solution, the plant height, stem diameter and biomass of each treatment were measured. Plant biomass was measured after dried at 70 °C for 2 h, and 105 °C for 48 h. The non-inoculated tomato plants were harvested and separated into roots and shoots (three replicates for each treatment). Plant materials were washed with distilled water and dried at 70 °C for 2 h, and 105 °C for 48 h. One hundred mg of each dried sample was digested with HNO_3-$HClO_4$ and the calcium concentration was analyzed with an atomic absorption spectrometer (model AA-670, Shimadzu Co., Kyoto, Japan).

H_2O_2 concentration measurment

Plant leaf samples were collected 0 (just before inoculation), 6, 12, 24, 48, 72 and 96 h after inoculation (four replicates for each treatment). The concentration of H_2O_2 in tissue was determined following the method used by Kar and Choudhuri (1987). Hydrogen peroxide was isolated from 1 g leaf tissue in ice-cold acetone. 5 % (w/v) of a titanium sulphate and NH_4OH solution was added to form the peroxide-titanium complex that precipitated. The precipitate was collected after centrifugation at 3,000 rpm for 10 min, and dissolved in 2 M H_2SO_4. The absorbance of the solution was determined by a spectrophotometer (DU800, Beckman, USA). The H_2O_2 concentration was calculated from a standard curve prepared in the same way.

Enzyme assay

Plant leaf samples were collected 0 (just before inoculation), 6, 12, 24, 48, 72 and 96 h after inoculation (four replicates for each treatment). 1.0 g of leaf tissue was homogenized in 5 ml of phosphoric acid extracting buffer (0.05 M phosphate with a pH of 7.0) in ice bath. The homogenates were filtered through four layers of cheese cloth and then collected after centrifugation 12,000 rpm for 20 min at 4 °C, and the supernatants were used for the assays of enzyme activities. The POD activity was assayed in a 500 μl reaction mixture containing 0.1 M phosphate buffer (pH=7.0), 5 mM guaiacol, 3 mM H_2O_2 and 50 μl of crude enzyme extract. We measured the rate of change in absorbance at 420 nm with a spectrophotometer. The POD activity in the extract was calculated by a standard curve prepared from horse radish peroxidase (Murage and Masuda 1997). Every minute's change of O.D. value 0.01 was defined as a unit (U). The PPO

activity was determined by the following methods that a mixture of 21 μl of the enzymatic extract, 36 μl of 0.1 M phosphate buffer and 1 μl of 10 mM catechol was added in the assay tube, and it was incubated for 30 min at 30 °C. The addition of 8 μl 2 N perchloric acid was used to stop the reaction. The absorbance was then measured at 395 nm (Soares et al. 2005). Every minute's change of O.D. value 0.01 was defined as a unit (U).

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

Disease severity

There were dynamic differences in disease severity with the different treatments (Fig. 1). Medium Ca plants began to wilt 4 days after inoculation, and their disease severity rating was 77.1 % 20 days after inoculation. Plants in low Ca treatment also began to wilt 4 days after inoculation but the disease progressed more rapidly and all plants were dead 14 days after inoculation, with the disease significantly (22.9 % greater) more severe than the plants in the medium Ca level solution. Plants in high Ca treatment began to wilt 6 days after inoculation, 2 days later than the medium or low Ca treatments and the disease progressed slowly so that the disease severity was only 56.8 % 20 days after inoculation.

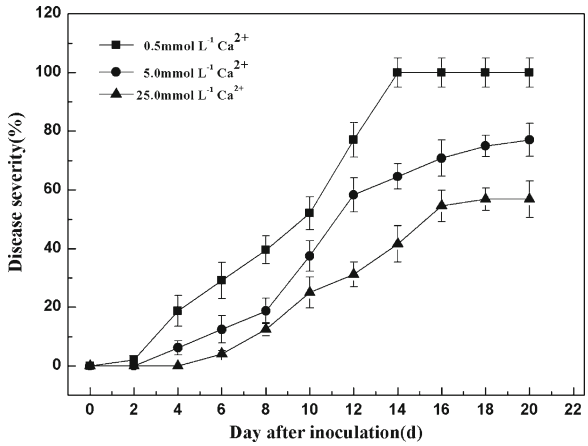

Fig. 1 Disease development after inoculation of tomato with *R. solanacearum*

Plant growth

Plant height for low Ca treatment was 55.31 cm, significantly lower than the medium and high Ca treatments, which were 60.13, and 63.21 cm, respectively (Table 1). Plants in the medium and high Ca treatments also had larger stem diameters (3.39 and 4.02 cm, respectively) than those in the low Ca treatment. In addition, plant biomass in the low Ca treatment (3.87 g) was significantly lower than the medium (4.71 g) and high Ca treatments (5.10 g). Although growth values continued to increase as Ca increased, there were no significant differences in plant height, stem diameter and biomass between the medium and high Ca treatment, suggesting that plant physiological sufficiency was met by the high Ca rate.

Calcium nutrient uptake

Tissue Ca was lowest for the low Ca compared with the medium and high Ca treatment (3.58, 12.35, 17.15 g $kgDW^{-1}$ in roots, and 6.75, 19.20, 24.19 g $kgDW^{-1}$ in shoots, respectively). Thus, the Ca in plants receiving the low Ca was 71.01 % (in roots) and 64.84 % (in shoots) less than in plants receiving the medium level of Ca, but it was significantly higher in plants receiving the highest Ca (38.87 % in roots and 25.99 % in shoots) (Fig. 2).

H_2O_2 concentration in tomato tissue

The H_2O_2 concentration in tomato leaves was similar for all treatments before inoculation with *R. solanacearum*; however, it increased rapidly following inoculation and rose to the highest point in the first 24 h after inoculation (Fig. 3). The H_2O_2 in leaves of plants receiving the low, medium and high Ca were 6.53, 8.27, 10.86 μM gFW^{-1} (189 %, 236 %, and 311 % more than it was before inoculation, respectively). From 24 h to 96 h after inoculation, H_2O_2 concentration declined slowly to the original level in the low and medium Ca treatments, but it was still 4.54 μM gFW^{-1}

Table 1 Effect of Ca on growth of tomato plants

Treatment	Height (cm)	Diameter (cm)	Biomass (g)
Low Ca	55.31±1.15a	3.07±0.15a	3.87±0.30a
Medium Ca	60.13±1.93b	3.39±0.11b	4.71±0.33b
High Ca	63.21±2.64b	4.02±0.23b	5.10±0.51b

The data were expressed as the mean±standard deviation (SD). Different letters in a column denote significant differences as determined by the LSD test (P=0.05)

in the high Ca treatment (72 % greater than it was before inoculation). At 24 h after inoculation, H_2O_2 in low Ca plants increased slower and only reached 6.53 μM gFW^{-1} (21.04 % less than the medium Ca plants) whereas H_2O_2 levels increased faster and reached 10.86 μM gFW^{-1} in the high Ca plants (31.32 % greater than medium Ca plants).

Resistance-related enzymes

POD activity was influenced by Ca rate and increased to the highest point in the first 24 h after inoculation (Fig. 4a). POD activity was lowest with the low Ca, intermediate in the medium Ca and highest with the high Ca (40.70, 72.35, 99.09 U gFW^{-1} or 86 %, 306 %, and 307 % greater than it was before inoculation, respectively). POD activity declined slowly to the original level from 24 h to 96 h after inoculation. When POD activity reached the highest point, it was 40.70 U gFW^{-1} with the low Ca treatment or 43.75 % less than with medium Ca. POD activity was 99.09 U gFW^{-1} in high Ca treatment (36.96 % greater than the medium Ca).

During the first 12 h after inoculation, PPO activity increased sharply and remained high compared to before inoculation (Fig. 4b). PPO activity with the low, medium and high Ca at 12 h after inoculation were 77.45, 88.29, 107.24 U gFW^{-1} (109 %, 27 %, and 316 % higher than it was before inoculation, respectively). PPO activity reached the highest point 12 h after inoculation when it was 77.45 U gFW^{-1} with the low Ca treatment (12.28 % less than the medium Ca) in contrast to 107.24 U gFW^{-1} (21.46 % greater than the medium) with the high Ca treatment.

Discussion

Previous studies have reported that Ca could suppress diseases caused by several pathogens (Berry et al. 1988; Eraslan et al. 2007). Yamazaki (2001) reported that increased Ca concentration in the nutrient solution could reduce bacterial wilt severity in a moderately resistant tomato cultivar; however, little information is available on the regulatory mechanism influenced by Ca related to tomato bacterial wilt caused by *R. solanacearum*. In the present study, we investigated the

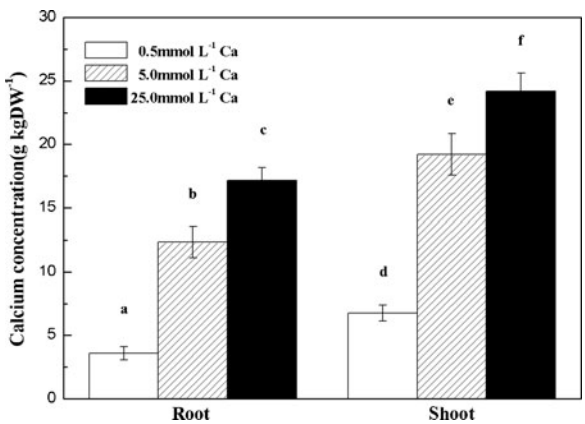

Fig. 2 Ca concentration in tomato roots and shoots. Bars with a different letter are significantly different at P<0.05 by ANOVA

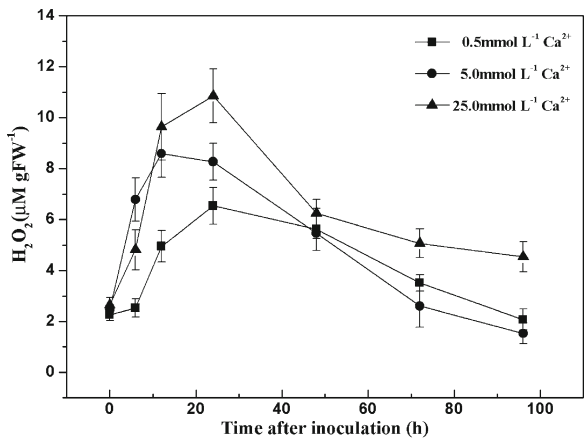

Fig. 3 Changes in H_2O_2 concentration in tomato leaves 96 h after inoculation with *R. solanacearum*

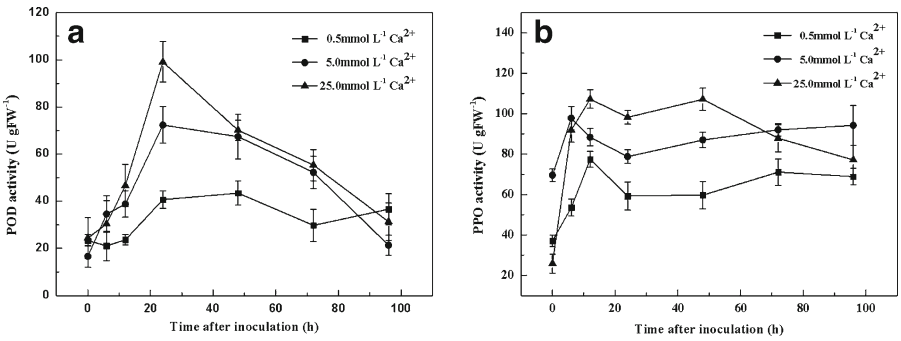

Fig. 4 Changes in POD (**a**) and PPO (**b**) activity in tomato leaves 96 h after inoculation with *R. solanacearum*

effect of Ca on severity of tomato bacterial wilt, Ca uptake, and physiological responses involving H_2O_2, POD, and PPO activity.

Increasing Ca nutrition to near the level of physiological sufficiency significantly reduced the severity of tomato wilt caused by *R. solanacearum*. Bacterial wilt resistant tomato cultivars have been characterized by high Ca uptake (Yamazaki et al. 1996) similar to our results with increased Ca availability for a susceptible cultivar. Increased Ca in root and shoot tissues were associated with less disease. This is in agreement with Sugimoto et al. (2008) who reported that disease reduction was related to the increased Ca uptake by plants. We also found a significant negative correlation between the severity of bacterial wilt and Ca in tomato roots (correlation coefficient of −0.999, $P<0.05$) (Table 2) and shoots ($R^2=-0.993$); however, the correlation in shoots was not significant. These results indicate that the reduction in disease severity by Ca was mainly due to increased Ca in tomato roots. Since bacterial wilt caused by *R. solanacearum* is a soilborne disease where plant roots are infected first, Ca may function in the cell wall to prevent the invasion of pathogens. Conway et al. (1992) reported that Ca reduced disease severity partly through increased levels of calcium in the cell wall region.

Hydrogen peroxide (H_2O_2), as an important reactive oxygen species (ROS), is always generated in the early events following infection by pathogens (Goodman and Novacky 1994) and can facilitate oxidative cross-linking of cell wall components by inducing an array of protective genes (Lamb and Dixon 1997; Wojtaszek 1997). In the present experiment, we observed a burst of H_2O_2 generation in the first 24 h after inoculation in all treatments, indicating an active role of H_2O_2 in the tomato defence system against *R. solanacearum*. Since Ca nutrition significantly enhanced the generation rate and level of H_2O_2 in tomato, and the negative correlation ($R^2=-0.972$) between H_2O_2 concentration and disease severity (Table 2), it provides further confirmation that the reduction in disease severity was due to the increased H_2O_2 concentration stimulated by Ca. Calcium may act as an important regulating factor of the ROS process in plants since blocking calcium signaling prevents elicitation of the oxidative burst by oligogalacturonides (Chandra and Low 1997).

An excess of active oxygen species may alter plant metabolism by structurally modifying proteins. POD is an important antioxidant in plant cells, and POD is involved in the detoxification of ROS (Mittler et al. 2004), while PPO catalyze the oxygen-dependent oxidation of phenols to quinones, and participates in plant systemic acquired resistance. Previous reports have pointed out that POD and PPO participate in plant defence reactions against pathogens (Tyagi et

Table 2 Pearson correlation analysis between disease severity[i] and Ca content, H_2O_2 concentration, and resistance-related enzymes in tomato

	Root Ca	Shoot Ca	H_2O_2[ii]	POD[iii]	PPO[iv]
r	−0.999*	−0.993	−0.972	−0.997*	−0.961

r Indicates correlation coefficient

[i] Disease severity of tomato 14 days after inoculation; [ii] H_2O_2 concentration of tomato 24 h after inoculation; [iii] POD activity of tomato 24 h after inoculation; [iv] PPO activity of tomato 12 h after inoculation

*Correlation is significant at the 0.05 level (2-tailed)

al. 2000; Pourcel et al. 2007). In the present experiment, we observed a progressive increase in POD activity with all treatments during the first 24 h after inoculation, indicating that POD may play an active role in the tomato defence system against *R. solanacearum*. Since high Ca nutrition significantly increased POD activity in tomato and there was a significant negative correlation (R^2=−0.997) between POD activity and disease severity (Table 2), the reduction in disease severity could be due to increased POD activity stimulated by high Ca (full physiological sufficiency) in tomato. Calcium may play an active role in regulating POD activity in plants,, which is in agreement with Li et al. (2003) who reported that external Ca increased POD activity in plant cells during stress. Yang and Poovaiah (2002) also found that Ca could stimulate POD activity in plants in response to biotic and abiotic stresses.

The rapid increase in PPO activity during the first 6 h of disease development in all treatments indicated that PPO also may play an active role in the tomato defence system against *R. solanacearum*. Balogun and Teraoka (2004) found that host-pathogen interactions always led to an increase in PPO activity. Li and Steffens (2002) also reported that overexpression of PPO in tomato led to significantly increased resistance to bacterial disease. This is consistent with our finding that Ca significantly increased PPO activity in tomato along with a negative correlation (R^2=−0.961) between PPO activity and disease severity (Table 2). These results showed that the reduction of disease severity was associated with increased PPO activity which was stimulated by Ca. Ruiz et al. (2003) reported that Ca exerted a positive influence on PPO involved in the metabolism of phenolics.

Conclusion

In this study, we have presented data on the physiological regulatory mechanism underlying the role of Ca nutrition in bacterial wilt resistance. High Ca nutrition significantly increased the Ca in tomato tissues of roots and shoots, improved the rate and amount of H_2O_2 accumulation, and increased the activity of POD and PPO in tomato, thus leading to the reduction of the severity of tomato wilt. Further research at the molecular level is needed to fully understand the specific role of Ca in resistance of tomato against *R. solanacearum*.

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