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EFFECTS OF SULFUR ON KIWIFRUIT CANKER CAUSED BY *PSEUDOMONAS SYRINGAE* PV. *ACTINIDAE*

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Abstract

The inhibition effect of sulfur on kiwifruit canker, caused by *Pseudomonas syringe* pv. *actinidiae* (*Psa*), by using turbidimetry and the inhibition zone method was investigated. It determined the control effects of sulfur on kiwifruit canker in pot and field experiments, as well as the effect of sulfur on defense enzyme activities in kiwifruit leaves. The results showed that sulfur had an inhibition role during the logarithmic phase of *Psa* growth and could inhibit the division and proliferation of the pathogen. However, toxicity was weak and its EC_{50} value was 1326.99 mg/l. When *Psa* was inoculated onto potted kiwifruit vines, the incidence rate was lowest on kiwifruit vines with the 1.5 kg/m^3 sulfur treatment. The control effect was up to 73.34% better than those that had not been treated with sulfur. The incidence rate was lowest when the kiwifruit plants were treated with 2.0 kg/m^3 sulfur. Furthermore, the control effect was up to 76.67% at two years after sulfur treatment. Sulfur could improve superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and phenylalanine ammonia lyase (PAL) activities in kiwifruit leaves when they were treated with 0.5 - 3.0 kg/m^3 sulfur, and kiwifruit plant disease resistance was enhanced to some extent. The pot experiment indicated that SOD, CAT, POD, and PAL activities, and disease resistance in kiwifruit leaves were highest when the plants were treated with 1.5 kg/m^3 sulfur. However, the field experiment showed that SOD, CAT, POD, and PAL activities, and disease resistance in kiwifruit leaves were highest when the leaves were treated with 2.0 kg/m^3 sulfur. These results demonstrated that moderate applications of sulfur and organic fertilizer can significantly improve defense enzyme activity in kiwifruit leaves and increase plant resistance to disease.

Introduction

Kiwifruit (*Actinidia deliciosa*) has a unique flavor and is the most nutrient dense of all fruits (Cassano *et al.* 2004). It is often called 'the king of fruits' due to its good reputation. In recent years, damage caused by kiwifruit canker at planting time has become more serious year after year which has posed a threat to the production of the fruit (Prencipe *et al.* 2016). Kiwifruit canker is caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*) and has become one of the most destructive kiwifruit orchard diseases in the United States (Rees-George *et al.* 2010, Zhao *et al.* 2015), Japan (Takikawa *et al.* 1989), New Zealand (Everett *et al.* 2011), South Korea (Koh *et al.* 2010), Greece (Holeva *et al.* 2015), and other countries (Balestra *et al.* 2009). The first case of kiwifruit canker disease in China was reported at Dongshanfeng farm, Hunan Province. Since then, kiwifruit canker has occurred in many places in China, including Shanxi, Sichuan, Hunan and Guizhou, and has been listed as a national forest plant quarantine disease (Gao *et al.* 2012). Over the past several years, kiwifruit canker has occurred so frequently and seriously that considerable amounts of chemical pesticide have been applied, which has led to pathogen resistance against the bactericides (Frampton *et al.* 2014). Therefore, alternative management strategies to effectively control kiwifruit canker need to be investigated.

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Plant nutrition levels are highly correlated with defense mechanisms and resistance (Amtmann *et al.* 2008, Elmer and Datnoff 2014). Many studies have shown that a large number of mineral elements had positive effects on defense responses against pathogens (Miles *et al.* 2009, Garcia-Mina 2012, Huber and Jones 2013). For example, the application of sulfur-containing fertilizer to soil can stimulate the sulfur metabolism in plants, which can often enhance plant disease resistance. This process is called sulfur-induced resistance (Rausch and Wachter 2005). The aim of this study was to investigate the effect of different concentrations of sulfur on kiwifruit canker prevention and control, and to provide a new approach to control kiwifruit canker.

Materials and Methods

The infected branches were collected from Yizhong orchard, Xiuwen County, Guizhou Province. The kiwifruit vines were severely infected with canker. The bacterial strains were isolated and purified from the branches using the grinding separation method, and then the isolates were tested using Koch's postulates. The physiological and biochemical characteristics of the isolates were investigated and the molecular biological characteristics were identified. Then the strains were preserved in cryogenic vials filled with sterile water at room temperature.

The NA medium (consisted of 5.0 g/l beef extract, 10.0 g/l peptone, and 5.0 g/l NaCl) was adjusted to pH 6.8 - 7.0 and sterilized by autoclaving at 121°C for 20 mins.

The sulfur treatment was a 50% sulfur suspension (HebeiShuangji Chemicals Company, Hebei, China) and the sulfur based bactericide contained 95% sulfur (w/v). The total nutrient content in the refined organic fertilizer was greater ≥ 4% and the organic matter content was ≥ 30% (w/v) (Guizhou Jilong Ecological Technology Company, Guiyang, China).

Indoor toxicity measurements was carried out by microtiter plate assay (Delvigne *et al.* 2006). The sulfur solution was diluted to six different concentrations (0, 10, 20, 40, 80 and 160 mg/l) with sterile water and then the *Psa* fermentation liquor was mixed with one of the different sulfur based bactericide solutions at a concentration of 0.1% (v/v). Cell growth was evaluated by measuring the optical density of the culture at 600 nm with a microplate spectrophotometer (Thermo Fisher Scientific Incorporated.). The pathogen culture without the sulfur based bactericide was used as the control and cultured under the same conditions. Each treatment concentration was prepared in triplicate and the experiment was performed three times. Growth curves for *Psa* subjected to the bactericide treatments were constructed (Delvigne *et al.* 2006).

Another indoor toxicity measurements was carried out by inhibition zone method (Liu *et al.* 2014). The Petri dish containing the bacterial cells was cultured in a constant temperature oven at 25°C for 36 hrs, and the crossing method was used to measure the inhibitory zone diameter The regression equation for toxicity of the median effective concentration (EC_{50}) and the correlation coefficient (R) were calculated using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

The pot experiment for bactericide effects was carried out at the Guizhou University (Guiyang, China) teaching experiment farm from December, 2014 to April, 2015. The kiwifruit variety used was 'Hongyang' and the plants were one year old. The kiwifruit plants were transplanted into plastic buckets that had been filled with soil containing organic fertilizer and different concentrations of sulfur powder. The pot experiments contained seven sulfur concentration treatments. Every treatment consisted of soil, 10 kg organic fertilizer and a different concentration of sulfur powder. The sulfur concentrations were 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kg/m^3 and the sulfur powder was added to 1 m^3 soil. The treatment containing soil, 10 kg organic fertilizer and 0 kg/m^3 sulfur powder was used as the control. One kiwi vine was planted in each pot, which represented an independent replicate. Every treatment was repeated 10 times. The plants were allowed to grow for three months and then they were inoculated with *Psa* by using the green stem

stab method (Hoyte *et al.* 2013). The disease incidence was observed at 28 days. The disease index, incidence rate, and control effect were calculated. The standard classifications for disease level were described by Hoyte *et al.* (2013).

The investigation on the effectiveness of sulfur as a bactericide was carried out in a kiwifruit orchard from December, 2014 to April, 2016. The orchard belongs to Yizhong farm and is located in Maguan village, Xiuwen County, Guiyang Province. The soil type in the orchard was a loam soil with medium soil fertility. The kiwifruit variety used was 'Miliang-1' and the vines were 18 years old. The tree vigor was moderate and the management level of the tree was medium. The experiment had a randomized block design and contained seven treatments. Each treatment consisted of soil, 10 kg organic fertilizer and a different concentration of sulfur powder. The sulfur concentrations were 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 kg/m^3. The sulfur powder was added to 1 m^3 soil. The treatment containing soil, 10 kg organic fertilizer and no sulfur powder was used as the control. Every treatment contained 30 kiwifruit plants. The management regime for every treatment was the same throughout the experiment. The field was managed according to the cultivation practices. The disease incidence was assessed in early April, 2015 and 2016. The incidence rate, disease index, and control effects were calculated in the following way:

Incidence rate (%) = (The number of plants showing disease symptoms / the total number of investigated plants) × 100

Disease index = ∑(Number of plants infected with pathogenic bacteria × Representative value of corresponding class) / (Total number of investigated plants × Representative value of the highest class) × 100.

Control effect (%) = (Disease index of the control – Disease index of the treatment)/Disease index of the control × 100.

A total of 0.5 g kiwifruit leaves were collected from the kiwifruit plants at the full-bloom stage from the pot and field experiments. The leaves, together with a little of polyvinyl pyrrolidone quartz sand and an appropriate amount of 0.1 mol/l buffer solution, were placed in a precooled mortar and a homogenate was obtained by grinding the sample in an ice bath. The homogenate was centrifuged below 4°C at 4000 rpm for 10 min. The supernatant, which contained the crude enzyme, was preserved at –20°C in a freezer. The superoxide dismutase (SOD) activity was determined using the nitroblue tetrazolium (NBT) reduction method (Xia *et al.* 2016). The guaiacol colorimetric method was used to measure the peroxide dismutase (POD) activity (Kochba *et al.* 1977). The catalase (CAT) activity was determined using the methods developed by Xia *et al.* (2016), and the phenylalanine ammonia lyase (PAL) activity was determined using the methods developed by Koike and Nanbu (1997).

Data are expressed as means ± standard deviation (Sd) and statistically significant differences between treatments were tested using one-way ANOVA. All statistical tests were conducted using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). p values of less than 0.05 were considered to be statistically significant.

Results and Discussion

The bacterial concentration was determined by measuring the optical density of the cell suspension at 600 nm (Fig. 1). There was no clear change in the OD values of the cell suspensions at 1 - 8 hrs after the control group had been inoculated with the kiwifruit canker pathogen. The data indicated that the bacteria grew slowly in their lag growth phase. The OD values at 600 nm increased after 8 h of culturing, which indicated that the bacterial concentration had increased. The growth rate was rapid between 8 and 20 hrs, which indicated that *Psa* had entered its logarithmic growth phase. Twenty hours after *Psa* inoculation, the OD values at 600 nm showed no significant

change, which showed that the bacteria growth had entered its stationary phase. In the samples taken from groups that had been treated with sulfur, the logarithmic phase was delayed and the bacterial concentration only increased after 10 hrs rather than 8 hrs. The growth curve for *Psa* in the sulfur-treated groups was below that of the pathogen in the control group. This showed that sulfur inhibited *Psa* growth.

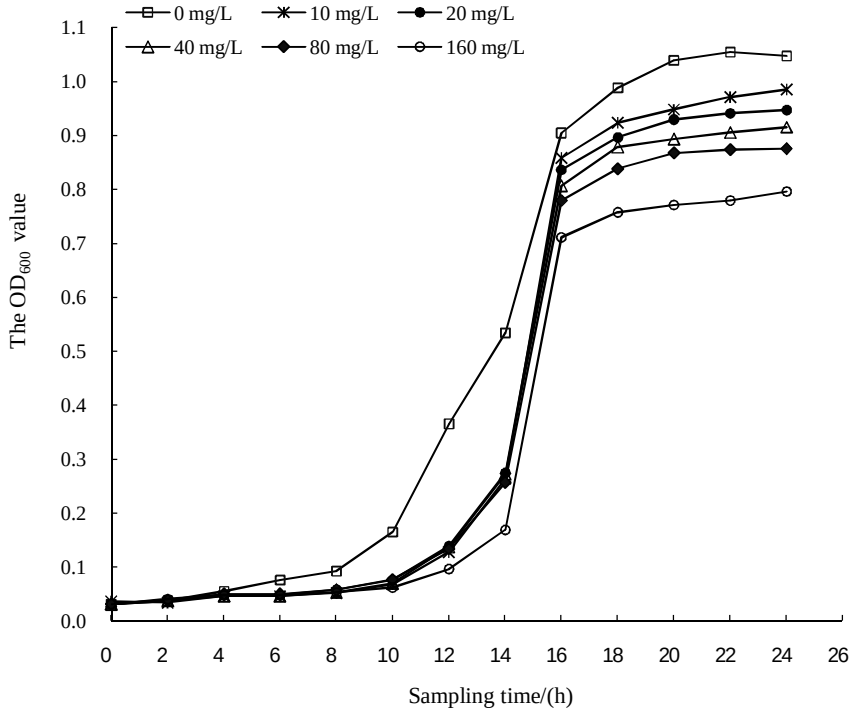

Fig.1. Effects of sulfur on the growth of *Pseudomonas syringae* pv. *actinidiae.*

The toxicity test using the inhibition zone method indicated that sulfur could inhibit *Psa* growth (Table 1), but the virulence was relatively weak. The regression equation for toxicity was y = 3.35 + 0.53x, The EC_{50} value for sulfur effects on *Psa* was 1326.99 mg/l, and the R value was 0.97. The present study suggests that sulfur is reputed to have no 'in vitro' effect on *P. syringae* in the laboratory (McLaren *et al.* 2005). It must also be mentioned that sulfur mainly played an inhibition role during the *Psa* logarithmic infection phase and could inhibit the division and proliferation of the pathogen when the measurement method was adopted for the microtiter plate assay.

As shown in Table 2, sulfur improved the control of kiwifruit canker in pot experiment. Kiwifruit canker was comparatively effectively prevented and controlled when *Psa* was exposed to 0.5~2.0 kg/m^3 sulfur, whereas prevention and control was not effective when the sulfur content exceeded 2.0 kg/m^3. The control effect was 73.34% when *Psa* was treated with 1.5 kg/m^3 sulfur and was 66.67% when the pathogen was treated with 1.0 kg/m^3 sulfur, which suggested that kiwifruit canker could be effectively prevented and controlled at moderate sulfur application rates.

Table 1 Inhibition effect of sulfur on *Pseudomonas syringae* pv. *actinidae.*

Sulfur conc. (mg/l)	Diameter of inhibition zone (cm)				Inhibition rate (%)
	Repetition I	RepetitionII	Repetition III	Mean	
CK	–	–	–	–	–
50	0.76	0.80	0.70	0.75	20.43±0.34
100	0.89	0.83	0.80	0.84	28.70±0.29
200	0.96	0.92	0.90	0.93	35.22±0.37
400	0.99	1.00	1.03	1.01	40.43±0.51
800	1.08	1.02	1.06	1.05	43.04±0.21

The diameter of sterile filter papers was 0.6 cm.

Table 2 Control effect of sulfur on kiwifruit canker in the pot and field experiments.

Sulfur content (kg/m^3)	Pot experiment (young plants)			Field experiment[a] (18 year old vines)		
	Disease index	Incidence rate (%)	Control effect (%)	Disease index	Incidence rate (%)	Control effect (%)
0	41.67±1.87	66.67±1.14	–	39.47±1.11	66.67±1.41	–
0.5	19.44±1.95	38.89±0.89	53.35±1.13c	21.05±1.08	22.22±0.65	46.67±0.56e
1.0	13.89±0.44	27.78±0.54	66.67±1.16b	18.42±0.21	44.44±0.84	53.33±0.54d
1.5	11.11±0.12	22.22±0.54	73.34±1.37a	15.79±0.95	33.33±0.65	59.99±1.03c
2.0	18.06±0.86	33.33±0.68	56.66±0.96c	9.21±0.46	16.67±0.36	76.67±1.37a
2.5	22.22±1.22	44.44±1.21	43.34±0.44d	13.16±0.86	27.78±0.56	66.66±0.66b
3.0	30.56±1.65	55.56±2.15	26.66±1.98e	15.79±0.65	22.22±0.84	59.99±1.07c

[a]These data were investigated after treating with sulfur two years. All data were obtained from a representative assay, which was the mean of ten identical replicates ± Sd. Different letters indicate statistically significant differences ($p = 0.05$) between groups in each column.

In 2013 and 2014, kiwifruit canker seriously affected the kiwifruit orchard where this experiment was undertaken. The results for April, 2015 indicated that kiwifruit vines that had been treated with sulfur showed only slight symptoms of kiwifruit canker. The kiwifruit vines had not absorbed all the sulfur after three months. Thus, the disease indices of the groups treated with 0.5 and 2 kg/m^3 sulfur were calculated to be 22.92 and 25.00, respectively, which was normal. In 2016, the results in Table 2 showed that the canker incidence rate was higher than 2015 at 66.67% for the kiwifruit vines not treated with sulfur, whereas 2 years after the kiwifruit vines had been treated with sulfur, the incidence rate significantly decreased and all treatments had an incidence rate of less than 45% ($F_{2,6} = 55.33$, $p < 0.001$). The results indicated that sulfur had a good control effect on kiwifruit canker. The best sulfur control effect was calculated to be 76.67% when kiwifruit vines were treated with 2.0 kg/m^3 sulfur. When kiwifruit vines were treated with 2.5 kg/m^3 sulfur, the control effect was calculated to be 66.66%. However, when the vines were treated with 0.5 kg/m^3, the control effect was only 46.67% (Table 2). Moderate sulfur applications can increase plant yields, improve the quality of agricultural products, and enhance plant disease resistance (Bloem *et al.* 2007, Bloem *et al.* 2015). McLaren *et al.* (2005 and 2006) also demonstrated that

sulfur showed potential as a replacement for copper in an apricot and nectarine spray programs for the control of bacterial blast, which is also caused by *P. syringae*. These results were similar to previous reports by Pavlista (2005) and Haneklaus *et al.* (2007). They suggested that moderate sulfur applications could reduce the incidence rate of garlic rust, potato scab, grape leaf spot, stem canker, and downy mildew.

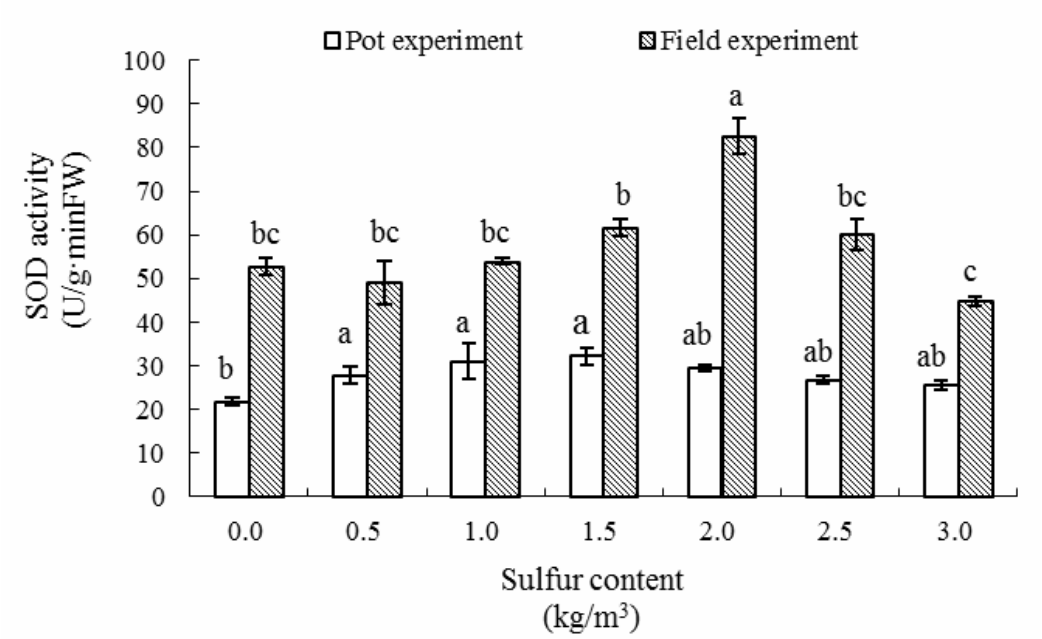

Fig. 2. Effect of sulfur on SOD activity in kiwifruit leaves. Different letters indicate statistically differences ($p = 0.05$) between groups. The same as follows.

As the sulfur content increased, the SOD activity in the potted kiwifruit leaves first rose up to 1.5 kg/m^3, but then declined (Fig. 2). However, all activities were above the control SOD activity rate. The highest activity occurred in kiwifruit leaves that had been treated with 1.5 kg/m^3 sulfur. Activity significantly increased by 47.70% compared to the control plants. Furthermore, the SOD activities of some kiwifruit leaves after treatment with sulfur in the field experiment were higher than the SOD activities in the untreated leaves. SOD activity in kiwifruit leaves subjected to the 2.0 kg/m^3 sulfur treatment was highest and showed a significant increase in activity of 56.58% compared to the control leaves.

Fig. 3 shows that an increase in the sulfur application rate led to a rise in CAT activity in kiwifruit leaves. All the sulfur treatments increased CAT activities compared to the control plants. CAT activity was highest when the kiwifruit plants were treated with 1.5 kg/m^3 sulfur in the pot experiment, which was significantly higher than the control plants. The CAT activity in the leaves was highest after they had been treated with 2.0 kg/m^3 sulfur in the field experiment, which was significantly higher than the control leaves. However, the CAT activity decreased at the highest sulfur application rates.

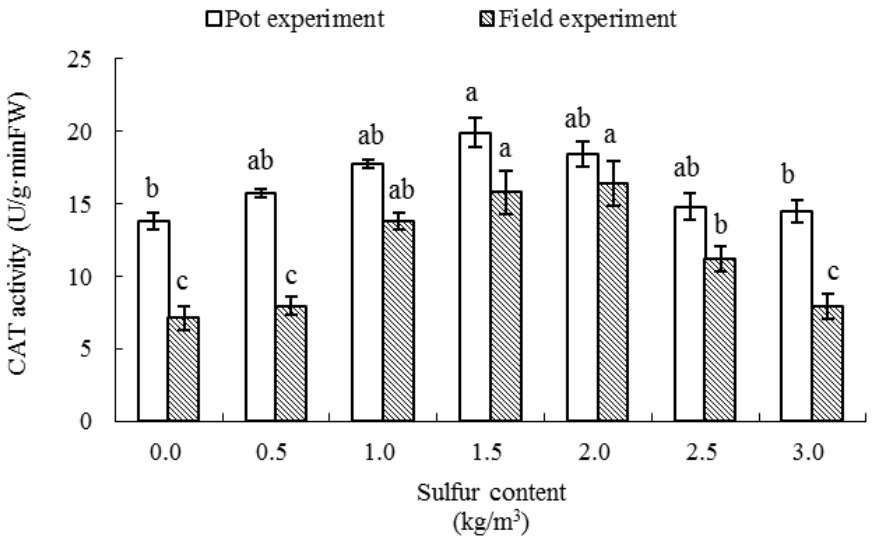

Fig. 3. Effect of sulfur on CAT activities in kiwifruit leaves.

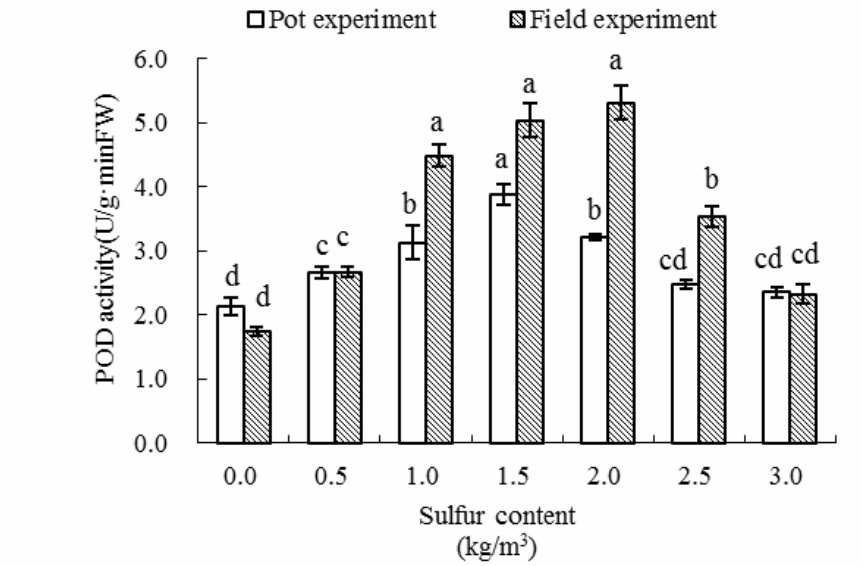

Fig. 4. Effect of sulfur on POD activity in kiwifruit leaves.

POD activity in the leaves increased linearly when the kiwifruit plants were treated with 0.5□ 1.5 kg/m^3 sulfur in the pot experiment (Fig. 4). However, POD activity decreased when the sulfur application rate exceeded 1.5 kg/m^3. When the sulfur application rate was below 2.0 kg/m^3 in the field experiment, the POD activity increased as the sulfur application rate rose. However, POD activity decreased gradually when the sulfur application rate exceeded 2.0 kg/m^3. The results indicated that moderate sulfur applications increased POD activity, but excessive use inhibited it.

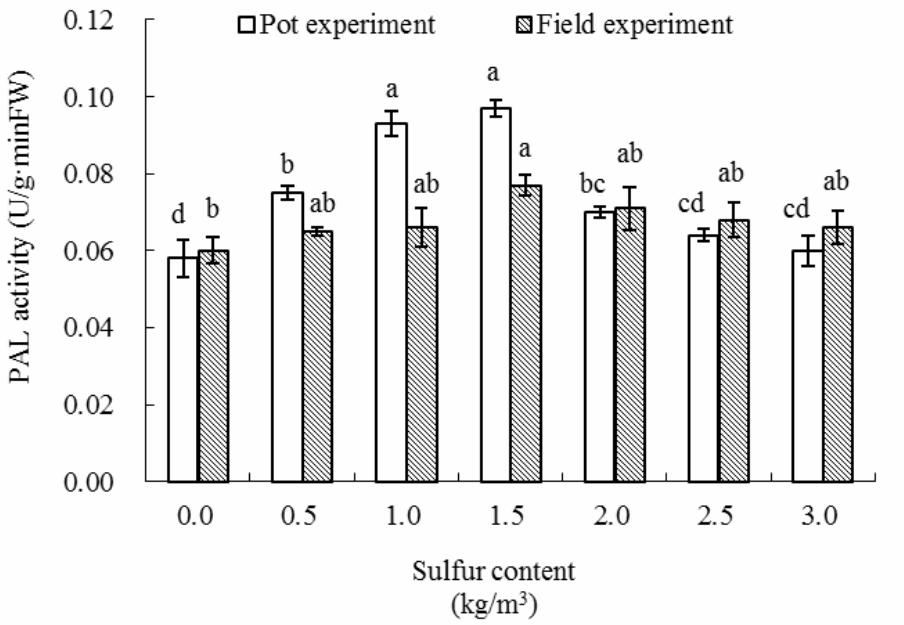

Fig. 5. Effect of sulfur on PAL activity in kiwifruit leaves.

Fig. 5 also shows that as the sulfur application rate increased, PAL activity in kiwifruit leaves first rose, but then declined. However, the PAL activities for all sulfur treatments were higher than in the control leaves. The PAL activity in kiwifruit leaves subjected to the 1.5 kg/m^3 sulfur treatment was highest in both the pot and field experiments, and showed significant increases of 67.24 and 28.33%, respectively, compared to the control leaves. However PAL activity began to be inhibited when the sulfur application rate exceeded 1.5 kg/m^3.

Inducing plant resistance to disease is a complex process. It requires a number of specific compounds and changes to enzyme activity (Haneklaus *et al.* 2007, Király *et al.* 2012). The enzymes that control plant resistance to disease include PAL, POD, CAT, SOD, plant protection factor, and phenols (Bloem *et al.* 2004, Clay *et al.* 2009, Swarupa *et al.* 2014). However, the sulfur mode of action is unclear since it has little effect on *P. syringae* in the laboratory, but the possibilities include both direct and indirect effects on the plant (McLaren *et al.* 2005).

In conclusion, the pot and field experiments in this study showed that sulfur could improve SOD, CAT, POD, and PAL activities in kiwifruit leaves when sulfur was applied at 1.0 to 2.5 kg/m^3. These application rates also increased disease resistance, which leads to improved kiwifruit canker disease prevention and control. These results were similar to those produced by studies on flue-cured tobacco and garlic reported by Kruse *et al.* (2007) and Zhu *et al.* (2008). Therefore, this study would have more hopeful prospects in applying sulfur during kiwifruit production to control or mitigate kiwifruit canker. However, the mechanism underlying sulfur control of the *Psa* needs further study. Future research should investigate the exact mode of action by which the sulfur supply affects bacterial pathogens, explore sulfur-induced-resistance gene expression in kiwifruit, and sulfur participation in the cellular signal transduction pathways from the molecular biology viewpoint.

Acknowledgements

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