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EFFECT OF EXOGENOUS APPLICATION OF SALICYLIC ACID ON THE SEVERITY OF TOMATO LEAF CURL DISEASE

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ABSTRACT

Salicylic acid (SA) is a natural plant hormone involved in natural plant defense against diseases by acting as the signaling molecule for triggering systemic acquired resistance. In the absence of the plant innate natural defense, the resistance can be induced through exogenous application SA or its functional analogue. In this study, the effect of SA treatment on the severity of leaf curl disease of tomato (*Solanum lycopersicum* L.) was evaluated under screen house conditions in two experimental trials at the Crop Protection Cluster, University of the Philippines Los Baños from 2012 to 2013. The study sought to determine the concentration of SA applied at different time of induction which can effectively reduce the severity of the disease. Healthy seedlings of susceptible tomato variety, Apollo White were treated by spraying with 50, 250 or 500µM SA at 5, 10 or 15 days before inoculation (dbi). At induction time of 5 dbi, treatment with 250µM SA had lowest leaf curl infection compared with the untreated control, while at 10 and 15 dbi, leaf curl infection was lowest with treatment of 50µM SA. Likewise, treatment with 50µM SA regardless of induction time had consistently delayed and reduced the severity of leaf curl disease. Generally, plants treated with 50µM SA had reduced amount of disease (AUDPC values), lower symptom severity score and lower disease index (DI) than the untreated control. The severity of the disease was also reduced with 250 and 500 µM SA treatment but the effect was more consistent with 50µM.

Key words: induced resistance, systemic acquired resistance

INTRODUCTION

In the Philippines, tomato leaf curl, the most destructive virus disease of tomato is caused by different whitefly-transmitted *Begomovirus* species with the *Tomato leaf curl Philippines virus* (ToLCPV) (Kon et al. 2002; Fauquet et al. 2008; Tsai et al. 2011) being the most prevalent. The disease is widespread and greatly affects the tomato production in the country causing up to 65% reduction in crop yield or even complete loss of the crop with severe infections (Mendoza 2005). Several practices have been employed to manage tomato leaf curl including the control of insect vector, mulching, early planting, seedling protection, seedling treatment, host-free period, sanitation and use of resistant varieties (Ioannou 1987; Ellsworth and Carillo 2001; Polston and Lapidot 2007). However, effective management of the disease remains a great challenge because of the limitations of these methods. The use of resistant varieties would be the most practical and effective means to manage virus diseases including leaf curl disease. However, there are limited virus resistant varieties, and only few tolerant are commercially available. Thus, other approaches for leaf curl management must be explored. The strategy is not necessarily to prevent infection but to reduce the severity of the leaf curl disease.

One approach, known as induced resistance (IR), has been shown to have potential for conferring resistance against plant viruses (Agrios 1988). The resistance is in the form of systemic acquired resistance (SAR) which can be triggered by exposing the plant to virulent, avirulent, or nonpathogenic microbes, or it can be induced artificially by a chemical agent (Vallad and Goodman 2004). The other form is induced systemic resistance (ISR) which is potentiated by a growth promoting rhizobacteria. In both SAR and ISR, the plant defense is preconditioned by prior infection or treatment with the inducer that results in resistance (or tolerance) against subsequent infection by a pathogen (Vallad and Goodman 2004).

Salicylic acid (SA) is a natural plant hormone (Khan 2010), and is known to be involved in natural plant defense against diseases. It acts as the signaling molecule for triggering SAR (Durrant and Dong 2004; An and Mou 2011). The induction of SAR is mostly associated with the accumulation of SA (Sticher et al. 1997;

Vlot et al. 2009), and the production of pathogenesis-related (PR) proteins. In the absence of plant's innate natural defense, the resistance can be induced artificially by exogenous treatment with SA or its functional analogue**,** 2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Sticher et al. 1997). The success of inducing resistance depends on the right concentration and proper timing of induction. In the induction of resistance, a certain period of time between the treatment of the inducer and inoculation or exposure to the invading pathogen is also required for the establishment of SAR, and these corresponds to the time required for the coordinated accumulation of PR proteins and SA throughout the plant (Uknes et al. 1992; Cameron et al. 1994). Some studies have shown the role of SA for inducing resistance to plant viruses including *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV) and *Pepper golden mosaic virus* (PGMV) (Van Loon and Antoniw 1982; Malamy et al. 1990; Metraux et al. 1990; Anfoka 2000; Vallad and Goodman 2004; Vlot et al. 2009; Trejo-Saavedra et al. 2013). The resistance induced is either through the application of SA or BTH. This study aimed to manage tomato leaf curl through the induced resistance approach using SA as the inducer. Thus, the efficacy of exogenous application of SA in reducing the severity of tomato leaf curl was evaluated as an initial study to determine the potential of induced resistance for tomato leaf curl management. The objectives were to determine the concentration of SA and the proper timing of induction which can effectively reduce the severity of the disease.

MATERIAL AND METHODS

The effect of SA treatment on the severity of leaf curl disease was evaluated using Apollo white tomato variety in two experimental trials under screen house conditions at the Crop Protection Cluster, University of the Philippines Los Baños (UPLB). Trial 1 was conducted in the wet season (August-November 2012), while trial 2 in the dry season (March-June 2013). The effect of SA was determined by spraying the plants with varying concentrations at different time of induction. The study was conducted in a 4 x 3 factorial experiment, consisting of four concentrations (factor A) (0 or untreated control, 50, 250 and 500 µM) of SA, and three induction time (factor B) (5, 10 and 15 days before inoculation). The experiment was laid out following the randomized complete block design with three replications, and with six plants per replication.

Salicylic Acid Treatment

The plants were treated by spraying the healthy tomato seedlings with solution of each concentration of SA applied at different induction time. Spraying was done until the SA solution was already dripping. The induction time refers to the time period when SA was applied on the plant several days (5, 10 and 15 days) before challenged inoculation (dbi). For induction time of 15 dbi, SA was applied on 20 day-old seedlings, while 10 dbi and 5 dbi on 25 day-old and 30 day-old seedlings, respectively. This ensured that all seedlings were of the same age (35 day-old) during inoculation.

Virus Inoculation Using the Whitefly Vector

The leaf curl infected tomato plants served as the source of virus inoculum. The whiteflies were confined in screened nets, and allowed to feed and build up their population on the infected plants for about 30 days. Inoculation was subsequently conducted by exposing the SA treated and the untreated 35-day-old healthy tomato seedlings on the viruliferous whiteflies. In order to ensure uniform inoculation, the leaf curl infected tomato plants that served as the virus source were distributed in between rows for each treatment. The inoculated plants were observed for the development of the disease.

Disease Assessment

The effect of SA was assessed in terms of delay in disease development and reduction of disease severity. The delay in disease development was measured based on the leaf curl infection taken at different time period during the infection, and expressed as the disease progress curve. The reduction of disease severity was measured using parameters such as percent infection, symptom severity, Area under the disease progress curve (AUDPC) values and disease index (DI).

Leaf curl infection and disease progress curve. The presence of leaf curl disease was determined by visual observation of disease symptoms in the inoculated plants. Leaf curl infection was computed as the proportion of plants displaying symptoms over the total number of inoculated plants. The disease progress curve represented the percentage leaf curl infection plotted against time of 1, 2, 3 and 4 week post inoculation (wpi).

The area under the disease progress curve. The amount of disease was determined based on AUDPC values and computed using the following formula:

$$\text{AUDPC} = \sum_{i}^{n-1} \left(Yi + Yi + \frac{1}{2}\right)(ti + 1 - ti)$$

Where:
AUDPC =area under the disease progress curve (percent-days or proportion-days); *n* = number of assessment times; *y* = disease incidence; *t* = time

Symptom severity. The symptom severity was determined at 4 wpi, following the rating scale presented in Table 1.

Table 1. Rating scale used in evaluating the symptom severity of tomato leaf curl.

Symptom severity score	Symptom description
0	No leaf curl disease symptom
1	Leaf curl disease symptom on the shoot apex
2	Leaf curl disease symptom on the shoot apex, and on the first and second leaf petioles
3	Leaf curl disease symptom on upper half portion of the plant
4	Leaf curl disease symptom on the whole plant
5	Leaf curl disease symptom on the whole plant with severe stunting

Disease index (DI). It is a measurement of disease severity based on the proportion of plants with different symptom severity score, and computed as:

$$\frac{[n(1)+n(2)+n(3)+n(4)+n(5)]}{t(n)}$$

where n(1), n(2), n(3), n(4), n(5) = number of plants showing a reaction scale (1), (2), (3),(4), (5), respectively and t(n) = total number plants scored.

Statistical analysis

The Analysis of Variance (ANOVA) for leaf curl infection (%) and AUDPC values were analyzed using the Statistical Tool for Agricultural Research (STAR version 2.0.1). The treatment means were compared using the Least Significant Difference Test (LSD, $p \leq 0.05$) and the symptom severity score data was analyzed by Non-Parametric Analysis.

RESULTS AND DISCUSSION

Leaf Curl Infection

Salicylic acid treatment generally reduced leaf curl infection compared with the untreated control. The efficacy was affected by the concentration, and by the induction time depending on the concentration of SA (Fig. 1). In trial 1, leaf curl infection at 2 wpi was lower in plants treated with varying concentrations of SA at 5, 10 or15 dbi compared with the control (Fig. 1A). Infection also varied with varying concentrations of SA. At induction time of 5 and 10 dbi, treatment with 50µM SA resulted to lower infection (both 11%) compared to 250µM (22% and 16%) and 500 µM (22 and 33%). However, at induction time of 15 dbi, infection was not yet observed with 250 and 500µM treatments, while 6% of the 50µM treated plants were already infected. At 4 wpi, treatment with 250µM at 5 dbi was slightly lower (72%) than with 50µM (78%), while the 500µM treated and the untreated control had comparable infection (83%) (Fig. 1B). However, treatment at 10 and 15 dbi with 50µM resulted to lower infection (66% and 61%) than 250µM (100 and 83%) and 500µM (83 and 77%). In trial 2, infection at 2 wpi was also lower in SA treated plants than the untreated control (Fig. 1C). Both 250 and 500µM treatments at 5 dbi had infection of 55%, while 50µM had 66%. However, infection was slightly lower with 50µM treatment at 10 (38%) and 15 dbi (38%), while 250µM had 44% at 10 dbi and 66% at 15 dbi. At 4 wpi, treatment with 250µM at 5 dbi had the lowest infection (83%); however, at 10 and 15 dbi, the 50µM treatment had the lowest with 77% and 55%, respectively.

The effect of varying concentrations of SA was also observed when the mean infection regardless of induction time was considered in the analysis. Generally, SA treatment regardless of concentration resulted in lower infection as well as delayed infection compared with the untreated control (Fig. 2). The effect was more

apparent with treatment of 50 µM than 250 and 500 µM. In both trials, the development of the disease shown as the disease progress curve was delayed in the SA treated plants (Fig. 2). In trial 1, the SA treated plants regardless of concentration had significantly lower infection (less than 10%) at 1 wpi than the untreated control (35%) (Fig. 2A). At 2 wpi, the disease did not progress rapidly wherein the treated plants remained to have significantly lower infection (9-18%) than the untreated plants (50%). However, at 3 wpi, only the 50µM treated plants had lower infection (44%) than the control (69%). Infection increased rapidly in the 250µM (74%) and 500µM (70%) treated plants comparable with the untreated control (69%). At 4 wpi, only the 50µM treated plants had significantly lower infection (68%) than the untreated control (87%). In trial 2, similar result was observed at 1-2 wpi, wherein the SA treated plants had lower infection than the control. Infection started to increase at 3 wpi in plants treated with 500 µM (89%), but not with 50µM (62%) and 250µM (65%) (Fig. 2B). At 4 wpi, only those treated with 50µM SA (75%) had significantly lower infection than the control (100%).

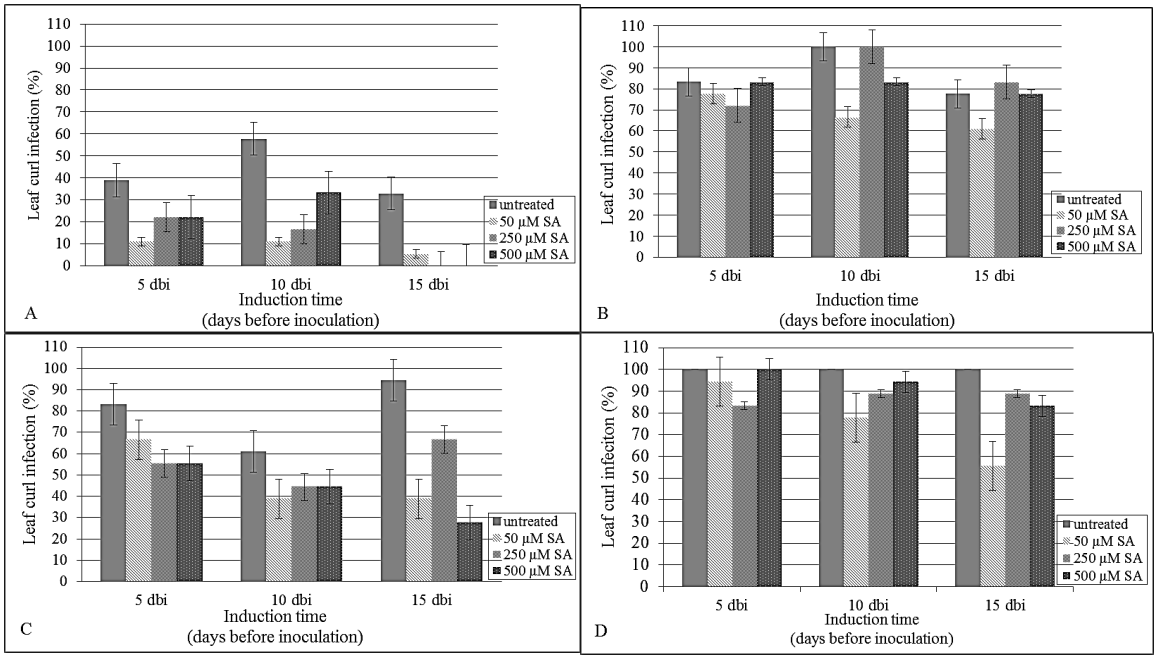

Fig. 1. Leaf curl infection of tomato plants treated at different induction time with varying concentrations of salicylic acid. A-B) Trial 1 at 2 and 4 weeks post inoculation (wpi); and C-D) Trial 2 at 2 and 4 wpi.

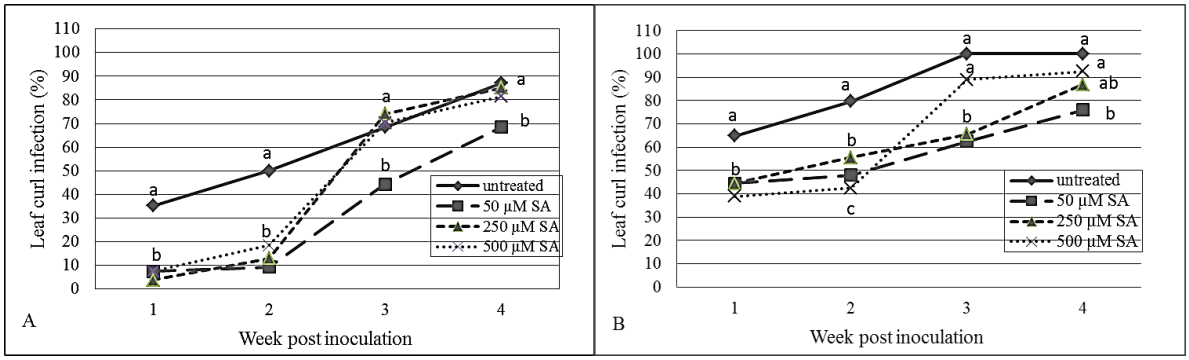

Fig. 2. Disease progress curve for tomato leaf curl infection in plants treated with varying concentrations of salicylic acid in Trial 1 (A) and Trial 2 (B). At each time point (weeks post inoculation), values with the same letter are not significantly different at 5% Least Significant Difference.

Area Under Disease Progress Curve (AUDPC)

The amount of leaf curl disease measured as AUDPC values was reduced with SA treatment (Table 2). In both trials, SA treatment regardless of concentration had significantly lower AUDPC values than the untreated control. In trials 1 and 2, plants treated with 50µM SA had AUDPC value of 641%-days and 1199%-days, respectively which were significantly lower than the control (1257%-days and 1834%-days). In trial 1, treatment with 50µM SA had significantly lower AUDPC value (641%-days) than 250 (907%-days) and 500µM

(933%-days). In trial 2, the AUDPC values were not significantly different among the 50, 250 and 500µM SA treatments. However, AUDPC value was lowest (1199%-days) with 50µM SA treatment.

Table 2. Area Under Disease Progress Curve (AUDPC) based on percentage leaf curl infection of plants treated with varying concentrations of salicylic acid.

Salicylic acid concentration (µM)	AUDPC(%-days)[1,2]	
	Trial 1	Trial 2
0	1,257 ± 332 a	1,834 ± 107 a
50	641 ± 108 c	1,199 ± 118 b
250	907 ± 125 b	1,307 ± 92 b
500	933 ± 257 b	1,380 ± 216 b

[1]In a column, values with the same letter are not significantly different at 5% LSD
[2] (±) standard deviation for each treatment

Symptom Severity

The treatment with lower concentration of SA reduced the symptom severity of leaf curl disease. At 4 wpi, significant differences among the treatments with varying concentrations of SA was found, but not with different induction time. Analysis of symptom severity score of treated plants regardless of induction time showed that those treated with 50µM had consistently lower symptom severity score than the untreated control in both trials (Table 3). In trial 1, plants treated with 50µM had significantly lower symptom severity score (1.6) than the control (ss=2.8). Likewise, symptom severity of the 250µM SA treated plants (ss=2.1) was significantly lower than the control, but not those treated with 500µM (ss=2.4). In trial 2, similar reduction in symptom severity at 4 wpi was observed with SA treatment at lower concentration but not at higher concentration. Treatment with 50µM SA resulted in consistently lower symptom severity score than the control. Plants treated with 50µM had symptom severity score of 2.1 which was significantly lower than the control (ss= 2.7). However, symptom severity scores of plants treated with 250 (ss=2.5) and 500µM (ss=2.7) were not significantly different with the untreated plants (Table 3). The 50 µM SA treated plants with mean severity score of 2.1 was closest to severity score=2, wherein most of the treated plants had symptoms on the shoot apex, and on first and second leaf petioles (Fig. 3B). The untreated plants had severity score closest to ss=3, wherein the upper half portion of the plant had already leaf curl disease symptoms (Fig. 3 C).

Table 3. Symptom severity score and disease index for tomato leaf curl at 4 weeks after inoculation of plants treated with varying concentrations of salicylic acid.

Salicylic acid concentration (µM)	Mean symptom severity score [1,2]		Disease Index (%) [3]
	Trial 1	Trial 2	
0	2.8 ± 0.18 a	2.7 ± 0.18 a	78
50	1.6 ± 0.23 c	2.1 ± 0.54 b	36
250	2.1 ± 0.23 b	2.5 ± 0.16 a	55
500	2.4 ± 0.41 ab	2.7 ± 0.43 a	65

[1] Symptom severity score: 0-no leaf curl like symptoms; 1-leaf curl disease symptom on the shoot apex; 2- leaf curl disease symptoms on the shoot apex and on the first and second petioles of the plant; 3- leaf curl disease symptoms on upper half portion of the plant; 4- leaf curl disease symptoms on the whole plant; 5- leaf curl disease symptoms on the whole plant and severe stunting. In a column, values with the same letter are not significantly different at 5% LSD.
[2] (±) standard deviation for each treatment
[3] Mean of two trials

Fig. 3. Symptom severity for tomato leaf curl disease, A) severity score (ss) = 1, leaf curl symptom on the shoot apex; B) ss = 2, symptoms on the shoot apex and on the first and second leaf petioles; C) ss=3, leaf curl symptoms on upper half portion of the plant.

Disease Index

The SA treatment regardless of concentration resulted in lower disease index (DI) compared with the untreated control (Table 4). Among the SA concentrations, treatment with 50µM gave the lowest DI of 36%, while the untreated control had 78%. Treatment with 250µM and 500µM also resulted in lower DI, but the effect was more apparent with 50µM. Plants treated with 250µM SA had DI of 55%, while those treated with 500µM had 65% DI.

The efficacy evaluation conducted in this study showed that exogenous application of SA can delay the development and reduce the severity of tomato leaf curl disease. At shorter induction time of 5 dbi, treatment with 50 and 250µM SA effectively reduced leaf curl infection compared with the untreated control, but the reduction was greater with treatment of higher concentration (250µM) than lower concentration (50µM). However, at longer induction time of 10 and 15 dbi, reduction of leaf curl infection was highest with treatment of 50µM SA. Overall, reduction in the severity of tomato leaf curl was consistent with treatment of 50µM SA at 15 days before inoculation.

Since SA was applied on plants at different ages of 20, 25 and 30 day old for 15 dbi, 10 dbi and 5 dbi treatments, respectively, the difference in leaf curl infection cannot be attributed mainly to differences in the length of induction time. In this study, SA treatment was imposed at different ages of seedlings to allow the inoculation of the virus on seedlings of the same age (35 day-old). Thus, in future studies, the effect of seedling age must be evaluated in order to clearly determine the effect of induction time on the efficacy of SA treatment. In this case, the SA will be applied on seedlings of the same age, but the plants will be of different ages during inoculation. Considering the effect of SA concentration regardless of induction time, the result showed that treatment with 50µM SA compared to 250 and 500 µM delayed the development and reduced the severity of the disease. The disease progress curve clearly showed that the development of leaf curl infection was delayed in the SA treated compared with the untreated control. The effect was more consistent with treatment of 50µM, which resulted to delayed disease development from the early to later stages of infection (1-4 wpi), compared with the untreated control and 250 and 500µM treatments. Likewise, SA treatment had reduced the severity of the disease. The amount of leaf curl disease expressed as AUDPC values were consistently lower in the treated than the untreated control. The effect was more apparent with 50µM than 250 and 500µM concentration. The lower disease index of 50µM SA treated plants compared with those treated with 250 and 500µM, and the untreated control, showed that SA treatment at low concentration of 50 µM can reduce the severity of leaf curl disease.

Several studies have demonstrated the efficacy of exogenous application of SA analogue (BTH) for controlling fungal and bacterial diseases (Siegrist et al. 1997; Cole 1999), the effect of which is in the form of induced resistance. However, induced resistance to viruses through exogenous application of SA or its functional analogue has been demonstrated in only few studies. The resistance of tobacco to subsequent infection of TMV is found to be enhanced by pre-treatment of plants with aspirin or SA (White 1979). Likewise, the application of 100µM BTH as a soil drench, 7 days before inoculation with CMV-Y, protected plants against the virus (Anfoka 2000). The resistance is expressed as decrease in disease incidence and severity in BTH-treated plants. At 21 days after challenge inoculation with CMV-Y, the disease incidence in plants did not exceed 12.5% while 91.7% of control plants are severely infected, and the development of the disease is delayed for 7 days. Resistance to PepGMV infection is also induced in pepper plants by BTH treatment (Trejo-Saavedra et al. 2013). Treatment of pepper plants with 150-300 mg L^{-1} BTH reduced the symptom severity and percentage of infected plants. The reduction is directly correlated with the concentration of BTH, and the time period between BTH application and the inoculation with the virus. The protection obtained with BTH treatment is less evident in plants inoculated 10 or 15 days compared with 5 days after the BTH treatment. In their result, it was shown that the efficacy of BTH protection decreases over time. Our results showed that 50µM SA applied at 15 dbi was the most effective treatment in reducing the severity of leaf curl disease. It appeared that protection was correlated with induction time but depending on the concentration of SA. In our study, the efficacy decreased at longer induction time (15 dbi) when plants were treated with 250µM SA, while the efficacy increased at longer induction time with lower concentration of 50µM. Among the SA concentrations, the 50µM compared with 250 and 500 µM can effectively reduce the disease severity. The 50µM was lower than the SA concentration of 1.5mM which is effective for inducing plant natural defenses to abiotic and biotic stresses (War *et al*. 2011). Resistance to CMV-Y in tomato was induced by treatment with 0.1mM BTH applied as soil drench at seven days before challenged inoculation with the virus (Anfoka 2000). Treatment of pepper with BTH at 300 mg-L^{-1} was found effective for inducing resistance to PepGMV (Trejo-Saavedra 2013).

The efficacy of SA in reducing the severity of leaf curl disease needs to be confirmed under field conditions, and may also be evaluated by comparing the response to SA treatment in susceptible and tolerant varieties. The resistance response can be further evaluated by measurement of the virus titre in plant. Although, the virus titre was not measured in this study, the observed reduction in disease severity would indicate resistance response to virus infection. The parameters that were used to assess disease severity such as percent infection, symptom severity score, AUDPC values and disease index have been used to assess resistance to virus infection (Alviar et al. 2012). The resistance to CMV in tomato induced by BTH treatment was also measured based on reduction of disease severity (Anfoka 2000). In this study, the efficacy of SA treatment was more apparent with induction time of 15 dbi compared with 5 and 10 dbi, and this may indicate an induced resistance response. In the induction of resistance, a certain period of time between the treatment of the inducer and exposure to the invading pathogen is required. However, in order to determine if the response observed is induced resistance, analyses of SA and PR protein accumulation need to be conducted in future studies. Resistance in tobacco against TMV as induced by BTH treatment is accompanied by the induction of SAR genes (Friedrich et al. 1996). Moreover, Lawton et al. (1996) showed that the resistance induced by BTH treatment on *Arabidopsis* plants to *Turnip crinkle virus* (TCV) is accompanied by PR protein accumulation. Resistance to a geminivirus PepGMV infection is also induced in pepper plants by treatment with BTH, and the resistance is through the activation of the SA pathway (Trejo-Saavedra 2013). SA is an endogenous signal for the activation of certain plant defense responses, including PR-gene expression and the consequent establishment of enhanced resistance (Klessig 2000). Moreover, the use of other inducer such as BTH, and the recently identified priming activators such as azelaic acid (AZA) and pipecolic acid (PA) can also be evaluated, as they may also have potential in providing protection against virus diseases (Conrath et al. 2015).

CONCLUSION

Exogenous application of SA can reduce the severity of tomato leaf curl disease. The efficacy was affected by the concentration, and by the induction time depending on the concentration of SA. Treatment with 50µM SA at induction time of 15 dbi reduced leaf curl infection more effectively than at 5 and 10 dbi. Likewise, 250µM SA also reduced the severity of the disease, but at shorter induction time of 5 dbi. Overall, treatment with 50µM SA is the most effective. The severity of the disease was also reduced with 250 and 500 µM SA treatments but the effect was more consistent with 50µM.

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