Pest Management in Horticultural Ecosystems
Vol. 28, No.1 pp 152-160 (2022)

DOI number : 10.5958/0974-4541.2022.00028.5

Induction of systemic acquired resistance in *Capsicum annuum* L. against *Chilli Veinal Mottle Virus* by foliar spray of salicylic acid

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ABSTRACT: Management of viral diseases requires an integration of several methods aimed at preventing or delaying infection of crops, as the viruses overcome the resistance due to their genome plasticity. Plants have evolved a variety of active and passive mechanisms to defend themselves against viral pathogens. One such mechanism is systemic acquired resistance; a safekeeping system a plant has preserved to combat various pathogen attacks. The possibility of inducing resistance in plants against viruses with chemicals or beneficial microorganisms deserves more interest. This study was conducted to evaluate the efficacy of salicylic acid (SA) foliar spray against *Chilli Veinal Mottle Virus* (ChiVMV) infecting *Capsicum annuum* L. which induces systemic acquired resistance. Salicylic acid was used as a foliar spray in different concentrations against ChiVMV with reference to disease incidence, percentage inhibition of virus, virus quantification, plant growth and yield parameters, and internal SA accumulation, under pot conditions. Minimum disease incidence and maximum percentage inhibition were recorded when plants were sprayed with 100 ppm salicylic acid 24 h before challenge inoculation. SA 100 ppm foliar spray not only delayed the virus symptoms but also reduced the virus concentration with enhanced growth and yield. Exogenous SA increased the internal SA accumulation, a key factor for SAR.

Keywords: Pepper, systemic acquired resistance, induced resistance, *Capsicum annuum, Chilli Veinal Mottle Virus*, salicylic acid

INTRODUCTION

Both hot and sweet peppers (*Capsicum annuum* L.) are of commercial importance worldwide. Known for their traditional medicinal properties, they are used as fresh vegetables, dried spices, food colorants, and flavorants (Kenyon *et al.* 2014). After tomatoes, they are the world's second most wholesome consumed vegetable (Nkansah *et al.* 2017). Despite the economic and rich nutritional value of *Capsicum* spp., its production is severely hampered by various pathogens, which not only reduces yield and fruit quality but also increases the production of clean planting materials (Arogundade *et al.* 2020). Amongst these, Potyvirus is a large genus (Family: *Potyviridae*) comprising several important species that are the most prevalent viral pathogens causing economically devastating diseases in diverse tropical and subtropical fruits and vegetables including peppers (Kenyon *et al.* 2014). At least, eleven species of potyvirus are predominant viruses that affect peppers globally, among which *Chilli Veinal Mottle Virus* (ChiVMV) is the most prevalent virus infecting peppers in Asia (Tsai *et al.* 2008). First reported by Brunt *et al.* (1996), ChiVMV is a linear, positive-sense ssRNA of around 10 kb with a VPg structure at its 5' end and a poly (A) tract at its 3' end. RNA is encapsulated by a single type of coat protein and the open reading frame encodes a large polyprotein, co-translated into ten functional proteins (Banerjee *et al.* 2016). The virus particles are flexuous filaments of around 680 - 900 nm long which are transmitted mechanically and by several aphid species in a non-persistent manner. ChiVMV characteristic symptoms include dark green vein banding, mettling leaf, small and distorted leaves, and stunted growth (Anindya *et al.* 2004) causing more than 50% yield loss in quality and quantity (Riaz *et al.* 2021).

Current management prospects for ChiVMV infection in peppers are by the integration of several methods like protected nurseries, removal of solanaceous weeds, application of insecticides to control the aphids, early identification and timely disposal of infected plants, use of resistant varieties (Kalimuthu *et al.* 2022) But the conventional phytosanitary practices are inefficient as ChiVMV are rapidly spread by several aphids and have a broad host range. Enhancing plant resistance using elicitors, is an alternative, cheaper, and more fruitful approach to combat plant diseases (Yu *et al.* 2022). Plants have evolved several complex mechanisms to fight pathogen attacks by inducing various defense

responses. One of the conserved mechanisms is systemic acquired resistance (SAR) involving various defense-related genes. Succeeding the primary infection, the plant becomes resistant to subsequent pathogen attacks. This phenomenon, called SAR, has attracted scientific attention for more than 60 years (Zhu *et al.* 2014). First depicted by Ray and Beauverie independently (1901), SAR is a "whole-plant resistance". It is also called "Broad-spectrum resistance", as it confers a long-lasting guard against a wide range of pathogens (Conrath 2006).

Elicitors like DL-β-aminobutyric acid (BABA), 2, 6 dichloroisonicotinic acid (INA), N-cyanomethyl-2-chloroisonicotinamide (NCI), azelaic acid, benzothiadiazole (BTH), jasmonic acid, salicylic acid mimic microbial attack and results in physiological changes in plants inducing SAR. (Faoro and Gozzo 2015). The objective of this study is to evaluate plant defense response using salicylic acid (SA) as a foliar spray against ChiVMV infection in sweet pepper.

MATERIALS AND METHODS

All the experiments were carried out at the Division of Crop Protection, ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru, India during 2019-21. All the chemicals used for the experiments were purchased from Sigma-Aldrich, USA molecular biology grade.

Source of the virus, sap inoculation, and culture maintenance

ChiVMV symptomatic leaf samples were collected from the infected *Capsicum* plants around IIHR and were used as a source of inoculum for mechanical sap inoculations. *Datura stramonium* was used as a primary and propagation host. Simultaneously, a month-old *C. annuum* (Susceptible Cultivar: Arka Mohini) seedlings were mechanically sap inoculated. For inoculum preparation, 1g of leaf sample was pulverized in 0.1 M phosphate buffer (pH 7.0) with 0.01 M mercaptoethanol in a pre-chilled mortar and pestle (W/V). Carborundum powder, abrasive was dusted on the leaf surface pre-inoculation and sap inoculation was made by gently rubbing the prepared inoculum on the leaf surface followed by a water wash. Plants were maintained in an insect-proof greenhouse at a temperature range of 28-30 ^{o}C and relative humidity of 60-80%.

Confirmation of Virus

On the expression of symptoms, leaf samples were collected and were tested for the presence of the virus by DAC-ELISA using ChiVMV polyclonal antibodies (DSMZ, Germany). ELISA positive samples were further leaf dip prepared on the grid and examined under HT7700 transmission electron microscope (TEM) for confirmation of virus particles. RNA was extracted using TRI Reagent according to the manufacturer's instructions. Extracted RNA was reverse transcribed and the cDNA copies were amplified by RT-PCR followed by PCR using ChiVMV-specific primers (forward primer: ChVMF1133 5'-CACGCTGGAATGAACACCATG -3'; reverse primer: ChVMR2480 5'-CAGATGGGCGATAAACTGATCTC) (PCR conditions: Initial denaturation at 94 °C for 4 min followed by 35 cycles of 45 Sec denaturation at 94 °C, 1 min annealing at 56 °C and extension for 90 sec at 72 °C and a final extension of 20 min at 72 °C). Total RNA extracted from a healthy plant was used as a negative control. Amplified DNA fragments were subjected to gel electrophoresis on 1% agarose gel and visualized under a UV transilluminator. Eluted products were sent for sequencing at the sequencing facility of Medauxin, Bengaluru. Database search for ChiVMV sequences was carried out by the NCBI-BLAST program for confirmation of virus isolate.

Distribution of the treatments and experimental Design

Pepper seedlings were raised in portrays initially and were transplanted to pots after a month under controlled conditions. Salicylic acid (SA-mol.wt.: 132) in different concentrations (50, 100, 150 ppm) was used for foliar treatment, and water was used as a control (Table 1). After 24 hours of spray, the plants were challenge inoculated with ChiVMV (50 plants per treatment). Leaf samples were collected at 1, 3, 5, and 15 days post-inoculation (DPI) for further analysis.

Table 1. **Details of experimental treatments**

	Treatments
C	Healthy negative control
I	Infected positive control
SA100	100 ppm SA sprayed/uninoculated
SAC50	50 ppm SA sprayed/Inoculated
SAC100	100 ppm SA sprayed/Inoculated
SAC150	150 ppm SA sprayed/Inoculated

Disease incidence (DI)

DI, the percentage of plants that developed symptoms in each treatment compared to control, was scored visually at 15, 30, and 45 DPI.

$$\text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total plant population}} \times 100$$

Percentage disease inhibition (PDI)

The effect of SA on the plants was quantified based on the symptom expression after 30 days post-inoculation. The percent disease inhibition over control was calculated by using the formula given by Vincent (1947).

$$PDI\ (\%) = (C - T) \times 100 / C$$

Where, C = Percent disease in untreated control, T = Percent disease in treatment

Quantification of virus infection

Leaf samples were collected at 1, 3, 5, and 15 DPI. Virus infection in the treated and control plants was quantified by DAC-ELISA (Hobbs *et al.* 1987) using ChiVMV specific antibodies (DSMZ, Germany) at 405 nm using VERSA max microplate reader (Molecular Devices). The amount of virus infection was calculated from the calibration curve using the mean absorbance values of the respective sample (Khedhair 2016).

Plant growth and yield parameters

Plant growth and yield parameters like plant height (cm), number of fruits per plant, individual fruit weight (g), and fresh fruit yield (g/plant) were recorded after 90 days from 10 plants per treatment.

Quantification of salicylic acid (SA) by LCMS

SA was extracted according to the procedure described by Pan *et al.* (2008). 1g leaf sample was homogenized in hormone buffer containing 1-propanol/ H_2O/concentrated HCl (2:1:0.002, v/v/v), sonicated for 30 min, and incubated overnight at 4 °C. An equal volume of dichloromethane was added to the homogenate, sonicated for 30 min, and then centrifuged at 12,000 rpm for 10 minutes. Water traces from the bottom layer was removed with the help of sodium sulphate and evaporated using a flash evaporator. After completely dried, the sample was dissolved in 1m L of methanol-0.05% formic acid (1:1, v/v). The solution was filtered using a nylon filter paper and injected into LCMS (Waters Acquity UPLC H class coupled with TQD MS/MS) for further analysis.

Statistical Analysis

Values presented are of at least three independent replicates. The significance of differences was determined using analysis of variance (ANOVA), one way ANOVA (Holm-Sidak's multiple comparisons test) for PDI, and two-way ANOVA (Tukey's multiple comparisons test) for DI, ELISA, and growth and yield parameters, using GraphPad V8.0.1 for Windows 10.

RESULTS

Culture maintenance and virus confirmation

Virus culture was successfully sap inoculated and maintained on *D. stramonium* and *C. annuum* L. (Fig. 1). Inoculated plants showed characteristic ChiVMV symptoms of dark green vein banding, mosaic, mottling, and distorted leaves. Infected samples exhibited a strong positive reaction to ChiVMV-specific antisera in the DAC-ELISA test. O.D. values of infected samples were 3 times higher than that of healthy and buffer control samples. Leaf dip preparation of virus-infected leaf extract observed under TEM showed the presence of flexuous filaments of around 650 nm in size (Fig. 2). RT-PCR/ PCR resulted in amplification of 1.2 kb DNA fragment from infected plants, but not healthy control plants (Fig. 3). Cloned and sequenced PCR products were subjected to a Blast search which showed nucleotide identity with ChiVMV – Bangalore isolate.

Fig. 1. Mechanical sap inoculation
Pepper plant showing characteristic ChiVMV symptoms of dark green vein banding, mosaic, mottling and distorted leaves.

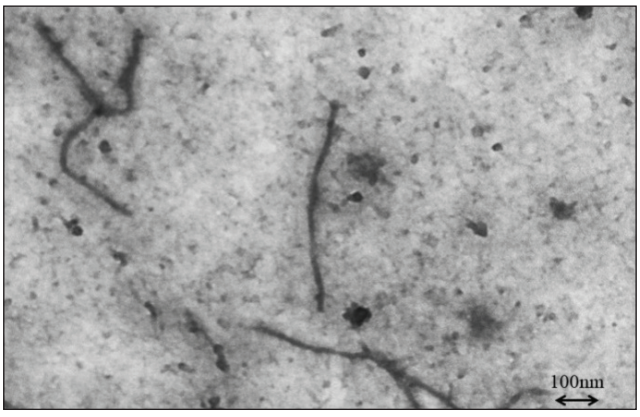

Fig. 2. Virus confirmation on TEM
TEM image depicting flexuous filaments of around 650 nm in size.

Disease incidence (DI)

Pre-treatment of salicylic acid foliar spray on *C. annuum* L. delayed and reduced the symptom severity of ChiVMV. The progress of the disease in the untreated/ challenge inoculated control plants were rapid and the symptoms appeared around 15 DPI with 95.6% mean disease incidence. But 50 ppm (SAC50) foliar spray delayed the symptoms by 3-5 days (18- 20 DPI) whereas 100 ppm (SAC100) and 150 ppm (SAC150) delayed it by around 8 to 10 days (23-25 DPI) **(Fig. 4)**. In 50 ppm SA sprayed plants the mean disease incidence was around 38.3%. 100 ppm and 150 ppm SA further decreased the disease incidence to around 25.3% and 26% **(Fig.5).**

Fig. 4. Delayed and reduced ChiVMV symptoms on pepper plants (a) 100ppm SA sprayed ChiVMV challenge inoculated pepper plant with delayed/ reduced symptoms and enhanced growth when compared to (b) ChiVMV challenge inoculated pepper plant showing symptoms.

Percentage disease inhibition (PDI)

In 50 ppm salicylic acid-treated and challenge inoculated plants the disease was inhibited by 58.50 % after 30 DPI. Whereas, 100 ppm and 150 ppm SA sprayed/ challenge inoculated plants showed 72.79% and 72.11 % disease inhibition respectively **(Fig. 6)**. Also, the symptoms were restricted and did not spread to other leaves.

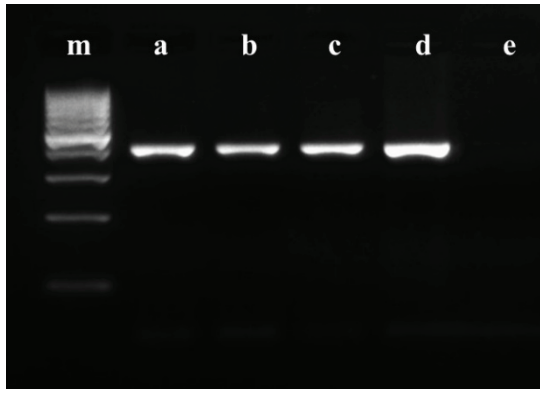

Fig 3. PCR confirmation of the virus (m) Lambda DNA/ECOR1+Hind III Marker; (a-d) 1.2 kb amplified fragment from symptomatic pepper samples specific to ChiVMV specific primer, (e) Healthy pepper leaves (Negative control);

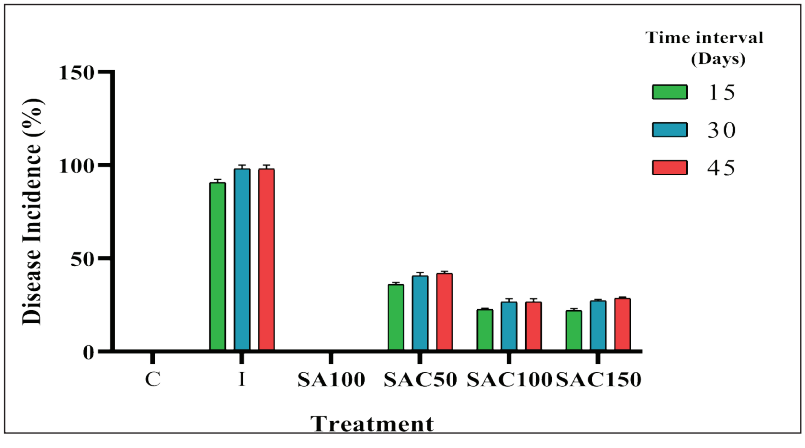

Fig. 5. Disease incidence at 15, 30, 45 DPI. The x-axis indicates different treatments. Vertical bars refer to mean ± SEm (n = 3), p-value <0.0001.

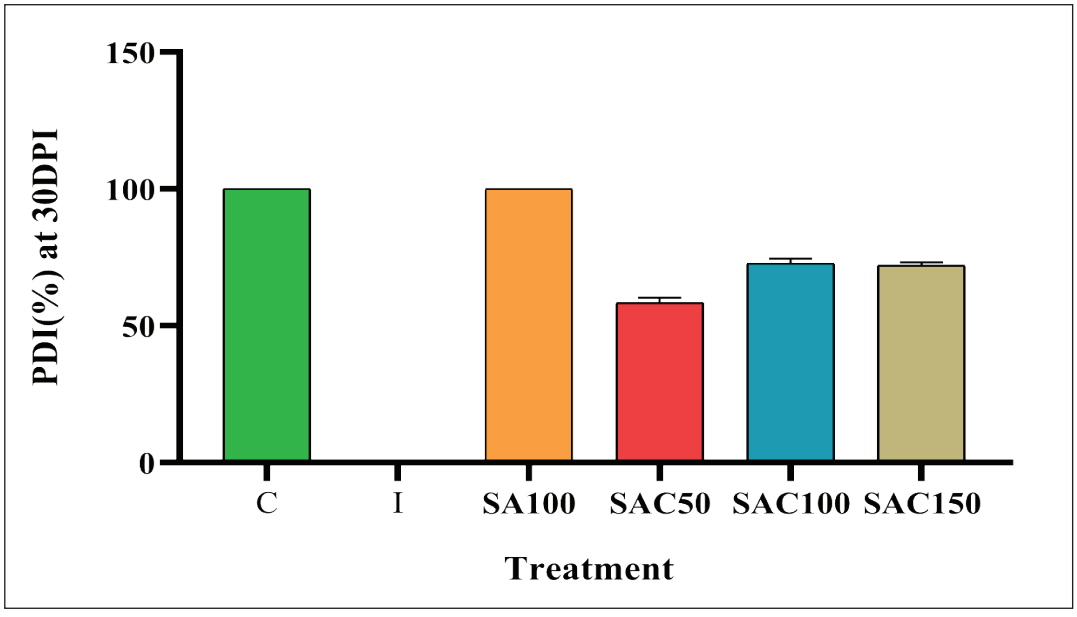

Fig 6. Percentage disease inhibition at 30 DPI. The x-axis indicates different treatments. Vertical bars refer to mean ± SEm (n = 3), p-value <0.0001.

Quantification of virus infection

The DAC-ELISA values of SA treated pepper seedlings showed a significant reduction in the viral concentration when compared with positive control plants. SAC100 significantly reduced the viral concentration over the period when compared to SAC50. There was no significant difference between SAC100 and SAC150. Healthy and SA100 seedlings contained no detectable ChiVMV **(Fig. 7)**.

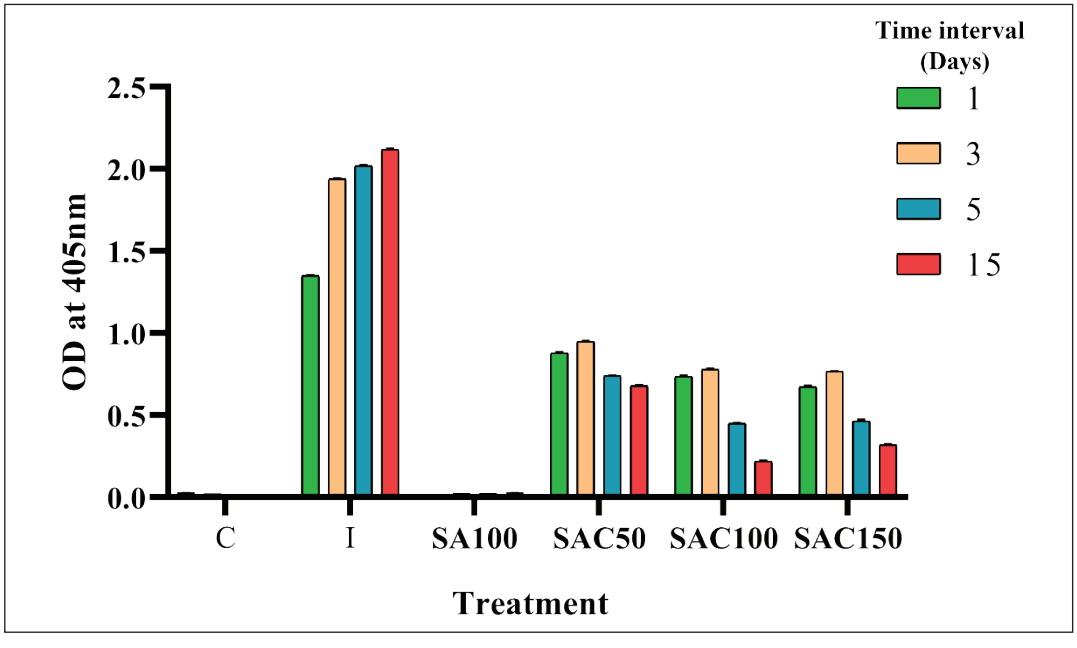

Fig 7. Quantification of virus infection. SA treated pepper plants showing significant reduction in the viral concentration when compared with positive control plants (I). The x-axis indicates different treatments. Vertical bars refer to mean ± SEm (n = 3), p-value <0.0001.

Plant growth, and yield parameters

Salicylic acid 100 ppm foliar spray on healthy plants increased the plant height when compared with untreated **(Fig. 8)**. The infected plants had characteristic stunted growth. There was a significant increase in the plant height treated with salicylic acid/ challenge inoculated when compared to healthy control. But there was no significant difference between the different concentrations. The number of fruits per plant increased with SAC100 and SA100 when compared with healthy control. There was a significant difference between SAC50 and SAC100/ SAC150, but not between SAC100 and SAC150. The same was with fruit length and width. Fruit weight in SAC100 and SAC150 increased three times when compared to infected plants (I). SA100 enhanced the fruit weight even compared to healthy control. There was a significant yield enhancement in SAC100 when compared not only with infected, but with healthy control too (Table 2).

Fig. 8. Plant growth (a) 100ppm SA sprayed ChiVMV challenge inoculated pepper plant with increased plant height when compared to (b) ChiVMV challenge inoculated pepper plant.

Table 2. Effect of foliar spray of SA on growth and yield parameters of pepper plants at 90 days after transplanting, p-value <0.05

Treatment	Plant height (cm)	No. of fruits/ plant	Fruit length (cm)	Fruit width (cm)	Avg. fruit weight (g)	Yield/plant (kg)
C	71.1	4.7	6.8	4.9	106	0.59
I	32.2	2.1	1.8	1.6	42	0.23
SA100	76.3	5.6	7.8	5.5	118	0.73
SAC50	77.1	3.1	4.8	3.4	72	0.32
SAC100	76.7	4.9	7.6	5.2	116	0.62
SAC150	76.6	4.8	7.7	5.2	115	0.61
SEm±	7.28	0.54	0.97	0.62	12.69	0.08
CD at 5%	20.71	1.53	2.76	1.76	36.06	0.23

Quantification of total salicylic acid (SA)

Salicylic acid accumulation increased internally in the plants treated with exogenous SA when compared to the infected plants. SAC100 proved to be more significant than SAC50. Though there was no significant difference between SAC100 and SAC150, SAC100 treated plants showed a slightly higher concentration of internal SA accumulation. SA accumulation increased from day 1 to day 3 and gradually decreased. In all the treatments SA accumulation reached the maximum on day 3 and gradually decreased. Control plants showed a negligible amount of internal SA accumulation **(Fig 9).**

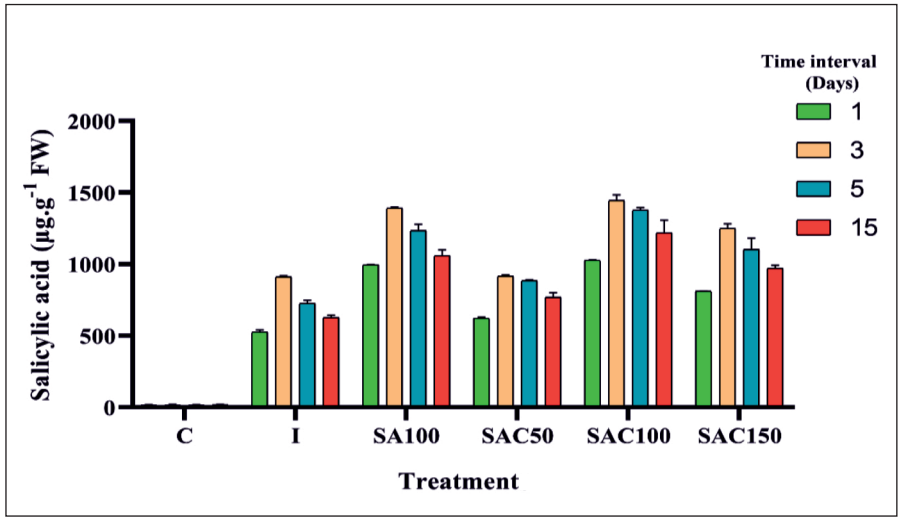

Fig 9. Quantification of total salicylic acid (SA) Effects of exogenous SA application on internal SA accumulation in pepper plants. The x-axis indicates the different treatments. Vertical bars refer to mean ± SEm (n = 3), p-value <0.001.

DISCUSSION

Boundless economic losses occur due to virus diseases worldwide. Plants fight against them in assorted tactics. Treatment of plants with biotic or abiotic agents can stimulate the immune system and develops resistance which may further restrict the pathogen growth and decrease the disease severity. Inducing systemic acquired resistance in the plants against the viruses is a superlative approach to managing the viruses (Zhang *et al.* 2011). Foliar spray increases growth and yield both quantitatively and qualitatively. It also increases soil fertility, nutrient uptake, and nitrogen fixation. Several compounds are used for foliar fertilization amongst which salicylic acid is advantageous for plants in multiple ways.

Salicylic acid, a natural growth regulator is said to influence several physiological and metabolic processes. Exogenous application of SA is known to enhance plant growth and yield (Ibrahim *et al.* 2019). 150 ppm SA spray enhanced mungbean growth with the highest seed yield/ha (Ali and Mahmoud 2013). 75 mg/ L SA spray enhanced the plant growth and yield in a few chili cultivars grown in arid regions (Nafees *et al.* 2019). 1.5 g /L SA increased the fruit weight and yield by 15.9 to 27.7% in various sweet pepper cultivars (Ibrahim *et al.*, 2019).

The salicylic acid foliar spray may increase the yield by reducing stress-induced growth control. Foliar spray of SA is effective against various pests and fungi with increased plant growth and yield (Thakur *et al.* 2014; Dixit *et al.* 2018; Yousif 2018; Bakr *et al.* 2020). SA induced resistance in resistant and susceptible tomato cultivars against the *Tomato yellow leaf curl virus* (Li *et al.* 2019). In resistant and susceptible *Vigna mungo*, SA induced resistance against *Mungbean Yellow Mosaic Virus* with reduced disease incidence and increased seed yield (Sahni and Prasad 2021).

Our data showed that foliar application of salicylic acid on *C. annuum,* 24 hours before inoculation reduced the disease severity and enhanced the growth and yield. The highest percentage inhibition of 72.9% was with 100 ppm SA. SA spray also delayed the onset of symptoms on the pepper plants. SA foliar spray delayed the onset of CMV infection on squash plants due to the inhibition of cell-to-cell movement of the virus (Mayers *et al.* 2005). 100 ppm SA reduced the virus concentration as well. There was a significant difference between 50 ppm and 100 ppm, but no significant difference was observed in the tests between 100 ppm and 150 ppm. Though there was not much difference between the different concentrations concerning plant height, considering other growth and yield parameters 100 ppm was considered the desired concentration.

Salicylic acid, a defense-related hormone, accumulates internally and induces SAR, which is correlated with the expression of SAR genes, including pathogenesis-related (PR) proteins and defense-related genes. These PR proteins and internal SA accumulation play an important role in resistance responses (Zhang *et al.* 2011). Our data showed a significant increase in internal SA accumulation when treated with exogenous SA 100 ppm, thereby enhancing the plant host resistance to ChiVMV in pepper plants.

To conclude, 100 ppm salicylic acid foliar spray was effective against *Chilli Veinal Mottle Virus* management in *C. annuum* by inducing systemic acquired resistance. Exogenous application of SA delayed and also reduced the symptom severity. Also, SA 100 ppm enhanced the plant growth and yield under controlled conditions. There was a significant increase in the internal SA accumulation, a key activator of the SAR pathway which will provide long-term resistance against the pathogens.

ACKNOWLEDGMENT

The authors are grateful to the Director and SAIF facility, ICAR- Indian Institute of Horticultural Research, Bengaluru for providing the LCMS facility, research facilities, and support. The first author is thankful to Mr. Saravan Kumar Parepally, Research Scholar, Division of Crop Protection, ICAR-IIHR for the support provided in biostatistics work. The research was financially supported by the project on the "Consortium platform on Vaccines and diagnostics", the Indian Council of Agricultural Research, Government of India, New Delhi, India.

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MS Received: 18 April 2022
MS Accepted: 27 May 2022