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Original article

Salicylic acid-mediated enhancement of resistance in tomato plants against *Xanthomonas perforans*

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ABSTRACT

Introduction: Bacterial spot disease of tomato was identified a few decades ago. The varying point of entry for the pathogen has enhanced the complexities for designing an effective disease management method. Recent advances in the use of inducers to elicit immune response in order to prepare the plant for a future attack by the pathogen are similar to the vaccination that is undertaken in humans and is a trending technology.
Objectives: The present study aims at understanding the effect of salicylic acid (SA) for management of bacterial spot disease caused by *Xanthomonas perforans.*
Methods: The study evaluated the effects of SA on the disease incidence along with seed germination and seedling vigor in two cultivars of tomato namely, Sun hybrid- resistant variety (R) and Quality- highly susceptible variety(HS)
Results: The germination and seedling vigor, which was reduced in case of HS variety in comparison with that of the R variety, was notably improved after seed priming in both the cultivars. The antioxidant enzyme activities of ascorbate peroxidise (APx), catalase (CAT) and glutathione reductase (GR) were assessed which showed that the R variety reached maximum activity at 18 h post inoculation (hpi), 24 hpi and 21 hpi, respectively, whereas the HS variety reached maximum at 30 hpi for APx and 36 hpi for CAT and GR activities. The transcript accumulation using qRT-PCR was also evaluated showing mRNA accumulation was maximum in the R cultivar after SA priming at 1.5 mM concentration.
Conclusion: the present study demonstrates the potential benefits of seed priming with SA to effectively elicit defence response in tomato seedlings against the bacterial spot disease.

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1. Introduction

Tomato (*Solanum lycopersicum* Mill.) is an economically important crop with a production of 12.5 million hectare costing about $1.67 billion in the year 2017 (Cooksey and Azad, 1992). Among the several infections encountered by the plant namely, bacterial spot caused by *Xanthomonas* sp., bacterial speck by *Pseudomonas* sp. and bacterial canker by *Clavibacter*sp., bacterial spot disease has caused the greatest economic loss and therefore demands attention in managing this disease. This seed-borne pathogen remains as an epiphyte in seedlings as well as mature plants until it attains ambient conditions (Yang et al., 2005). The pathogen enters the plant through small wounds or lesions, which appear as necrosis (Morales et al., 2005). However, it was noted that the *Xanthomonas* strains developed resistance to this antibiotic and therefore withdrawn. This eventually led to the use of copper (Cu)-based bacteriocides, which was in itself harmful to the soil (Donici et al., 2019). In addition, Cu-tolerant strains were detected as early as in 1960s, which was characterized in 1983 (La Torre et al., 2018). Later discovery of the effectiveness of a combination of Cu-mancozeb application to prevent foliar diseases led to its extensive usage. Yet, it is not the optimized management method owing to the harmful effects of metal accumulation in soil (Pietrzak and McPhail 2004).

Chemical agents that induce the activation of defense pathways are gaining focus owing to the development of innate response by plants against infectious pathogens. These chemical agents are known to induce two types of responses namely, induced systemic resistance (ISR) and systemic acquired resistance (SAR). While ISR is generated in response to the plant growth-promoting rhizobacteria, SAR is produced in response to a range of necrotic pathogens. Induced resistance exploits natural defense of the plant itself and therefore can be used as an alternative and non-conventional and way of dealing with plant diseases (Edreva, 2004). Different chemicals like acibenzolar-S-methyl, Probenazole, β- aminobutric acid (BABA) (Ovadia et al., 2000), Nitric oxide (Song and Goodman, 2001), Dipotassium phosphate (K_2HPO_4) (Orober et al., 2002) are commercially used as resistance inducing agents. On the same lines, the role of SAR plant activator- Salicylic acid (SA) in effectively inducing pathogen response (Ali et al., 2018). Studies have proven the benefits of SA in inducing transcriptional reprogramming to counter biotic and abiotic stress (Kang et al., 2014). Application of SA both endogenously and exogenously was seen to regulate the antioxidant metabolism and ensure protection against ROS.

With this background, the present study aims to evaluate the ability of SA to induce resistance in tomato seedlings and its efficacy in controlling the bacterial spot disease. In our previous study, the seed germination and seedling vigor were remarkably reduced by the infection with *X- perforans* in the susceptible variety when compared with that of the resistance one (Chandrashekar and Umesha, 2014). Therefore, the effect of seed priming using SA on the seed germination and seedling vigor was also assessed in this study. Additionally, SA-mediated defense gene expressions in response to the inducer upon challenge inoculation with the pathogen were assessed using quantitative real-time polymerase chain reaction (qRT-PCR).

2. Materials and methods

2.1. Host and pathogen preparation

Different tomato cultivars (*viz.,* Sun hybrid- resistant variety (R) and Quality- highly susceptible variety (HS) seeds) were taken from a private seed distributor (Akash Agro agency and Annadatha Kendra, Mysore). Seeds were subjected to sodium hypochlorite treatment for surface sterilization followed by thorough washing using distilled water. Further, the collection of the organism (*X. perforans*) and inoculum preparation was carried out as mentioned previously.

2.2. Inducer preparation and treatment

Salicylic acid (SA), the inducer (Sigma, USA), was procured and a solution was prepared by dissolving it in a minimal quantity of distilled water until homogenized without granules formation. The stock solution was made up to 20 mM and from that working solutions of varying concentrations (0.5, 1.0, 1.5 and 2 mM) were prepared for the experiments. Hundred seeds of the tomato cultivar were taken in quadruplicates each from R and HS category and dipped in 100 ml of the inducers suspension at 25 ± 2 °C with constant stirring.

2.3. Effect of X. perforans on seed germination and seedling vigor under laboratory conditions

Seeds from both the tomato cultivars were exposed to *X. perforans* at a culture load of $1x10^8$ colony forming units/ml. Exposure was carried out by dipping the seeds in the bacterial suspension and incubated in a shaker for 12 h. A batch of control seeds immersed in sterile distilled water was also taken. After the incubation period, the germination rates were established by placing the air-dried seeds on wet blotting papers as per the blotters experiment. The vigor index was also calculated by "(mean root length + mean shoot length) × percent germination".

2.4. Seed quality and disease incidence as a result of seed-priming

The seed priming using R and HS tomato cultivars was performed as per the procedure given by Chandrashekar and Umesha (2014). Briefly, the seeds soaked in varying concentrations of SA were allowed to germinate to assess the germination potential and seedling vigor. Parameters such as root length, seed germination and shoot length were assessed after successful germination of the seeds. The formula "(mean root length + mean shoot length) × percent germination" was taken for the calculation of vigor index. The experiments were conducted in triplicates with each study having 100 seeds. Further studies were based on the optimal concentration of SA obtained as a result of this assay.

2.5. Enzyme studies

The SA primed and pathogen-challenged tomato seedlings from both the cultivars were used for this assay. The 8-day-old seedlings were treated exposed to the pathogens and harvested at intervals of 0, 3, 6, 9, up to 72 h post-inoculation (hpi) and stored at −80 °C for further enzymatic assays. The seedlings were used for the extraction of Ascorbate peroxidases (APx), Catalsae (CAT) and Glutathione reductase (GR) enzymes.

The above said enzyme assays were carried out as per the procedure given by Chandrashekar and Umesha (2014). In addition, the enzyme assay for GR was performed as per Smith et al. (1988).

2.6. Semi-quantitative RT-PCR for the analysis of defence genes

Seedlings treated with SA were grown and 8-day-old were taken for total RNA isolation from R (18 hpi for APx; 24 hpi for CAT and 21 hpi for GR) and HS (30 hpi for APx; 36 hpi for CAT and 36 hpi for GR) cultivars on the basis of in vitro enzyme assays for (APx, CAT and GR). RNA using the GeneJET Plant RNA purifica-

Table 1
Genes and primers used for the gene expression studies.

Genes	Forward	Reverse
APx	5′-TGGGAGGGTGGTGACATATTTT-3′	5′ TTGAAGTGCATAACTTCCCATCTTT 3′
CAT	5′- ATGGAAGGTTTTGGCGTTCA- 3′	5′ - CGCGTGGCTATGATTAGTACCA-3′
GR	5′-GATTGGAGCACCCTCATTGC-3′	5′-GAGTTTACCATCCACATCCACTGTA-3′
18s rRNA	5′- GTGCATGGCCGTTCTTAGTTG −3′	5′-CAGGCTGAGGTCTCGTTCGT −3′

tion Mini Kit (Thermo Scientific, India), as per the manufacturer's instructions. RNA was further treated with RNase-free DNase (Fermentas Mumbai, India) to remove contaminating DNA and further quantified spectrophotometrically and using agarose gel.

RNA (4 μg) from each sample was used for semi- quantitative RT-PCR. The primers for the study were designed using primer express ver 3.0 (Applied Biosystems, USA) and assessed using BLAST for confirmation of its specificity. The constitutive gene in this study was 18S rRNA. The gene expression studies for CAT, GR and APx were carried out by semi-quantitative RT-PCR. Oligonucleotide primers used for each of the gene is given in Table 1.

The assay conditions were; initial 4 min denaturation at 94 °C, followed by 94 °C for 1 min; 55 °C for 2.3 min, and 72 °C for 1 min and a final extension at 72 °C for 5 min- 16 cycles for APx and CAT, 18 cycles for GR and 21 cycles for 18S rRNA genes. The SYBR green PCR master mix was used for the assay and the primers were taken at 5 μM concentration. The specificity of the reaction was evaluated by melting curve studies (65-95 °C with a heating rate of 0.5 °C/s) performed at the end of each cycle.

2.7. Statistical analysis

The data obtained were expressed as Mean ± SD. Least significant difference (LSD) test was performed with $p \leq 0.05$ between treatments. The study was carried out in triplicates and represented in figures as mean ± standard errors.

3. Results

3.1. Priming with salicylic acid on seed quality parameters and disease incidence

Fifteen day-old-seedlings, Resistant (R) and Highly susceptible (HS) cultivars were evaluated to study the effects of seed priming on seed quality and disease incidence. It was noted that maximum germination (90%) was observed in the resistant (R) variety with maximum seedling vigor (1030) at an SA concentration of 1.5 mM after a treatment for 12 h. Conversely, in the highly susceptible (HS) variety at the same concentration of SA for the same period showed a maximum germination of 88% and seedling vigor of 994. However, at all other concentrations the germination and seedling vigor were relatively lower (Tables 2 and 3). These findings suggested that the optimal concentration of SA for seed priming was 1.5 mM and therefore chosen for further experiments.

Table 3
Salicylic acid priming of tomato seeds on bacterial spot disease incidence.

Concentrations SA (mM)	Disease incidence (%)			
	Green house condition		Field condition	
	R	HS	R	HS
Control	9^{e}	45^{e}	7^{c}	34^{e}
0.5	7^{d}	25^{d}	5^{b}	28^{d}
1.0	5^{c}	23^{c}	5^{b}	25^{c}
1.5	3^{a}	15^{a}	3^{a}	13^{a}
2.0	4^{b}	17^{b}	4^{a}	15^{b}

This study demonstrates the effects of the seed-borne pathogen- *X. perforans* on germination and seed vigor of tomato seeds, both of which reduced significantly in the HS variety when compared to the R variety. Further, seed priming using SA ensured lower susceptibility of the seedlings to the pathogen attack (see Fig. 1).

After seed-priming using SA, tomato seeds were evaluated for the presence of bacterial spot disease *in plants*. The study comprised of 20–25 plants each taken in 10 replicates and the whole experiment was repeated twice. The values expressed herein are represented as mean ± SE.

3.2. Enzymes studies

In the present study, the temporal changes produced by the seed priming were evaluated in terms of susceptibility of tomato seedlings towards *X. perforans*. The harvesting of samples for enzyme assays were carried out over a period of 72 hpi (hours after pathogen inoculation). The activities varied between that of the samples before and after inoculation. It was noted that the seed-

Table 2
Salicylic acid priming of tomato seeds on seed development parameters such as seed germination and vigor.

Concentrations SA (mM)	Germination (%)		MSL (cm)		MRL (cm)		VI	
	R	HS	R	HS	R	HS	R	HS
Control	73.50^{a}	73.0^{a}	3.10^{a}	3.10^{a}	5.20^{a}	4.70^{a}	624.0^{a}	578.0^{a}
0.50	80.0^{b}	77.50^{b}	3.10^{a}	3.10^{a}	5.20^{a}	4.70^{a}	680.0^{b}	632.0^{b}
1.0	85.0^{c}	80.0^{c}	4.10^{b}	4.00^{b}	5.60^{b}	5.30^{b}	825.0^{c}	744.0^{c}
1.50	90.0^{e}	88.0^{e}	5.00^{d}	4.80^{d}	6.70^{d}	6.50^{d}	1030.0^{e}	994.0^{e}
2.0	87.0^{d}	85.0^{d}	4.80^{c}	4.40^{c}	6.40^{c}	6.0^{c}	974.0^{d}	844.0^{d}

Values defined are mean of two independent experiments of quadruplicates of 100 seeds each. The mean values in the same column with diverse superscripts are significantly diverse ($p \leq 0.05$) as separated by DMRT (Duncan's multiple range test). "MSL: mean shoot length; MRL: mean root length; VI; vigor index. R: Resistant (cv. Sun hybrid); HS: Highly susceptible (cv. Quality seeds)".

Fig. 1. Effect of SA priming on the seed quality parameters of tomato. Tomato seedswere treated with SA (1.5 mM) and subjected to standard blotter method for theassessment of seed germination and seedling vigor. Increased seedling vigor can beobserved with SA primed seeds when compared to control. C-Control; T-Treatment.

lings pre-treated with SA and further challenged with the pathogen demonstrated highest activity. Temporal pattern study of APX activity revealed that the activity was initiated at 9 hpi and reached its maximum at 18 hpi. The SA treated seedlings revealed maximum APX activity at 18 h (2.3 units) where seedlings treated with SA and uninoculated had the APX activity of 1.3 units at the same 18 hpi. Seedlings treated with distilled water inoculated and uninoculated also showed APX activity of 0.8 units and 0.3 units, respectively but did not reach the peak as that shown by SA treated seedlings challenged with *X. perforans*. In highly susceptible tomato cultivar, APX enzyme revealed the maximum activity at 30 hpi (0.6 units) when compared to its corresponding control (0.05 units (Fig. 2).

Further, Catalase activity in R cultivar seeds treated with SA and inoculated with the pathogen recorded maximum CAT activity at 24 hpi (4.2 units) whereas the seeds treated with SA but un-inoculated recorded a CAT activity of 1.7 units. The same cultivars when treated with the pathogen only showed the CAT activity of 0.85 units and whereas the control showed 0.25 units and these values never reached the level of the activity shown by the seeds treated with SA and challenge inoculated with the pathogen. In HS tomato cultivar, SA treated seedlings exhibited CAT enzyme activity of 2.2 units at 36 hpi when compared to 0.03 units recorded by its control. The level of CAT activity in water treated seedlings inoculated and un-inoculated had CAT activity of 0.05 units and 0.03 units, respectively, but did not reach the level of

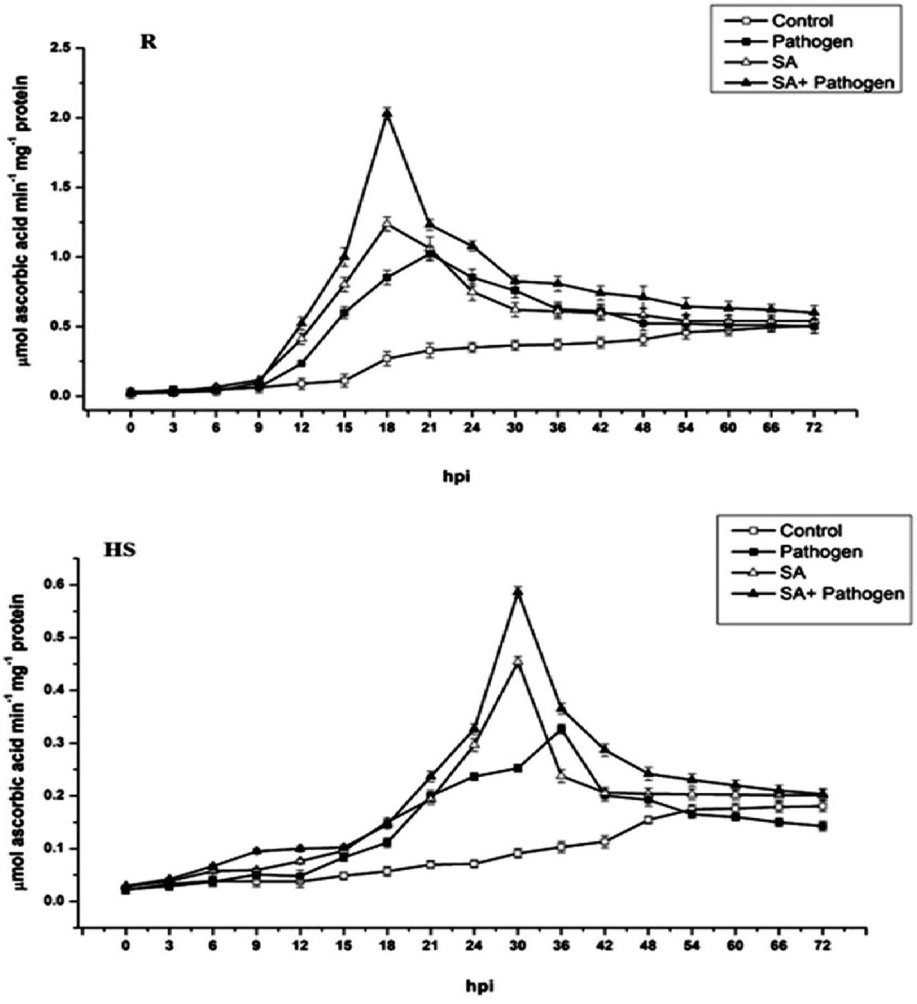

Fig. 2. Variations in the temporal pattern exhibited by resistant (R) and highly susceptible (HS) tomato cultivars in terms of APx activity. The data representation is the average of triplicates.

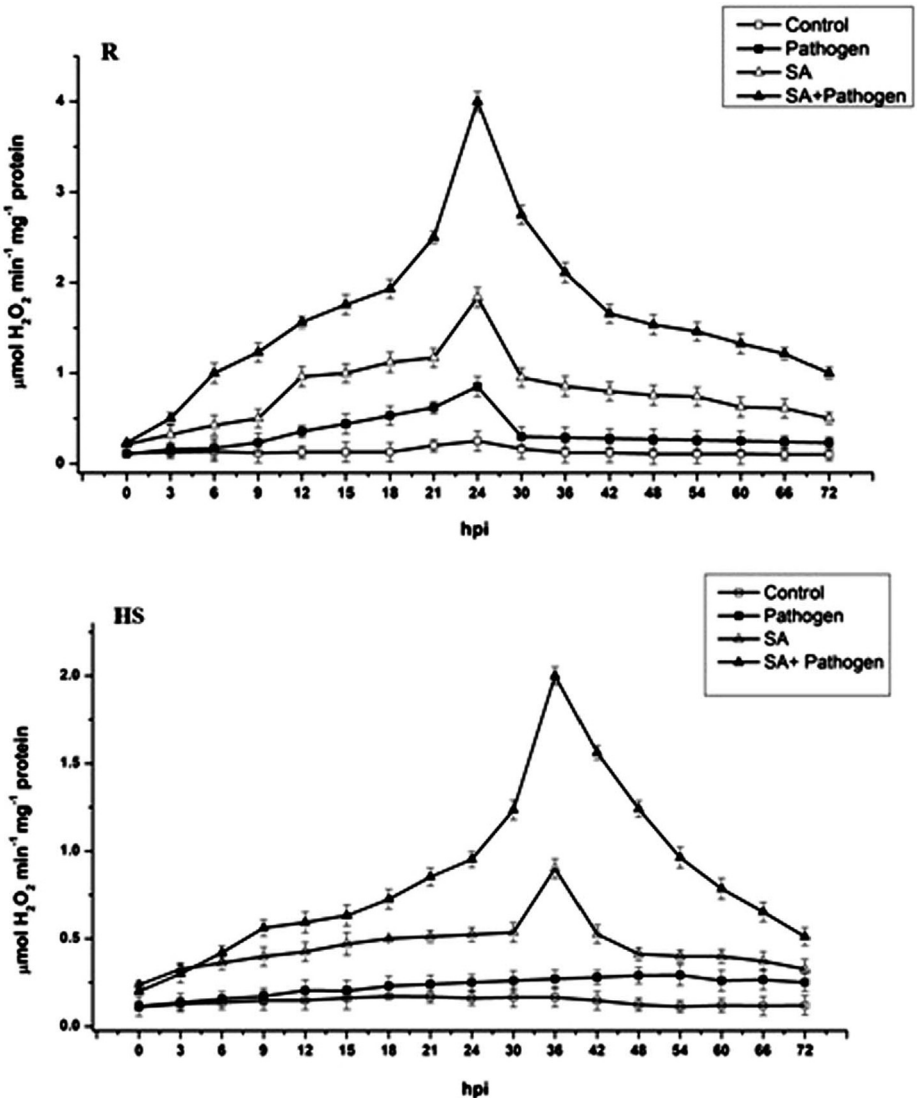

Fig. 3. Variations in the temporal pattern exhibited by resistant (R) and highly susceptible (HS) tomato cultivars in terms of CAT activity. The data representation is the average of triplicates.

activity as in SA treated seedlings. The R cultivars also showed an increase in CAT activity upon treatment with SA and inoculated with the pathogen. The activity ranged from 3.8 to 4.2 units, which was not evident in any other treatments. The susceptible group of cultivars also recorded a significant increase in CAT activity which ranged from 2.3 to 2.6 units when the seeds were treated with SA and challenge inoculated with the pathogen. This difference in enzyme activity was not observed in any other treatments. SA activity of HS cultivar significantly (p = 0.05) increased which ranged from 1.3 to 0.9 units (Fig. 3).

Glutathione reductase activity in resistant cultivar seeds treated with SA and inoculated with the pathogen recorded maximum GR activity at 21 hpi (4.3 units) whereas the seeds treated with only SA recorded the activity of 2.8 units. The same cultivars when treated with pathogen only showed the GR activity of 2.3 units, where as the control showed 1.0 unit and these values never reached the level of the activity shown by the seeds treated with SA and inoculated with the pathogen. In highly susceptible tomato cultivar, GR enzyme showed maximum activity of 2.2 units at 36 hpi when compared to 0.03 units recorded by its control (Fig. 4).

3.3. Semi-quantitative RT-PCR

Seed-priming with SA and inoculated with *X. perforans* exhibited APx gene levels on par with that of the R cultivar suggesting the potential of priming on inducing a remarkable resistance to the HS variety similar to that of the R variety. Similarly, the control groups from both the cultivars also demonstrated accumulation of genes although not statistically significant. Gene expression analysis for CAT and GR also exhibited a significant increase after seed priming by SA in the HS cultivar in comparison with that of the R variety. Conversely, the house-keeping gene expression (18 s rRNA) remained unaltered among both the varieties (Figs. 5–7). The findings from this study demonstrate the resistance induced by the seed-priming by SA in preventing infection by *X. perforans*.

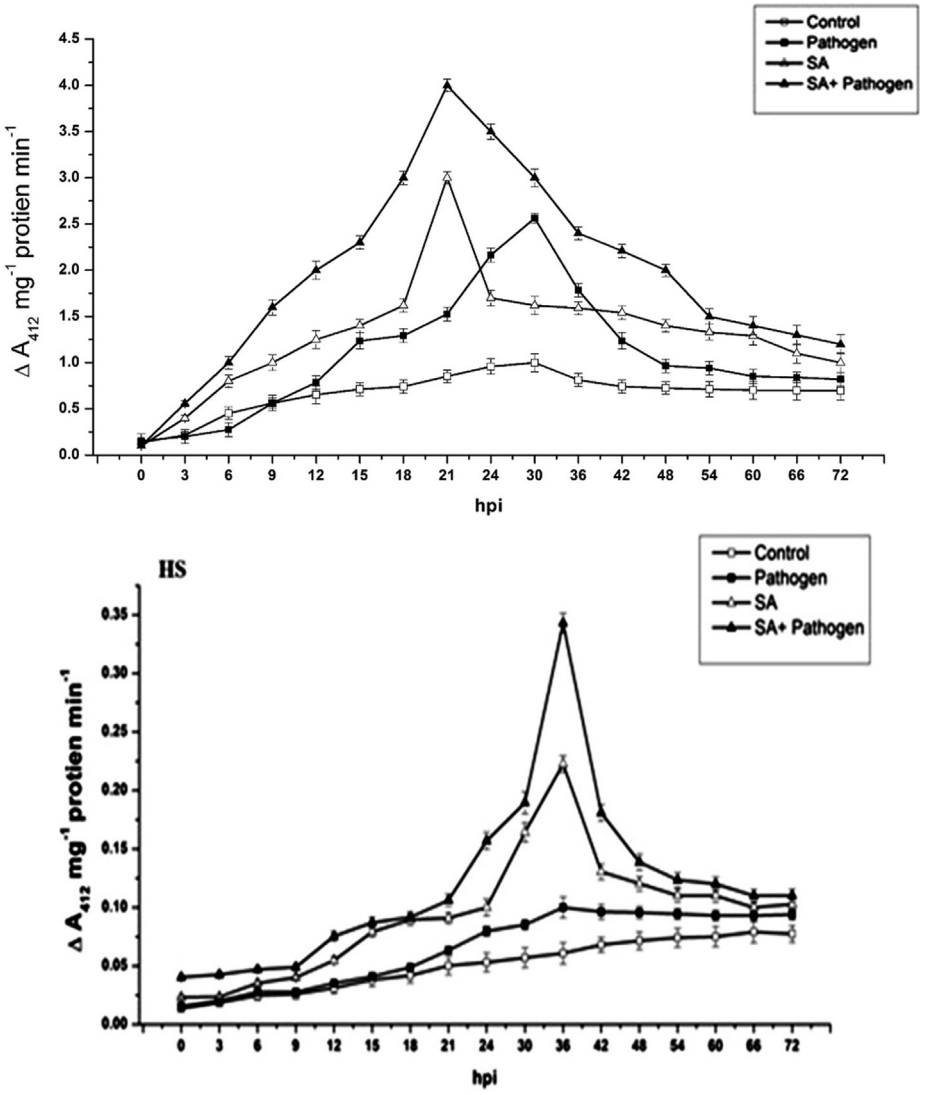

Fig. 4. Variations in the temporal pattern exhibited by resistant (R) and highly susceptible (HS) tomato cultivars in terms of GR activity. The data representation is the average of triplicates.

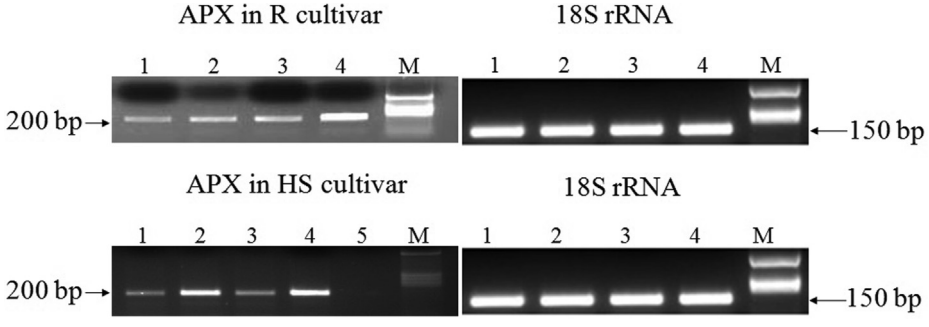

Fig. 5. Semi-quantitative PCR to study the APx gene expression upon 1.5 mM SA priming. Lane 1: Control; Lane 2: 1.5 mM SA; Lane 3: Pathogen only; Lane 4: Pathogen + 1.5 mM SA; Lane 5: ND-Non DNA template; Lane M: DNA ladder (1 Kb). R: Resistant HS: Highly susceptible.

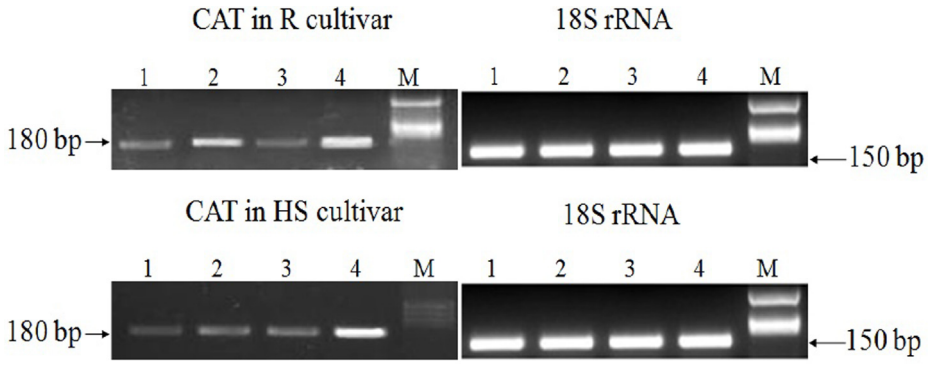

Fig. 6. Semi-quantitative PCR to study the CAT gene expression upon 1.5 mM SA priming. Lane 1: Control; Lane 2: 1.5 mM SA; Lane 3: Pathogen only; Lane 4: Pathogen + 1.5 mM SA; Lane M: DNA ladder (1 Kb).R: Resistant; HS: Highly susceptible.

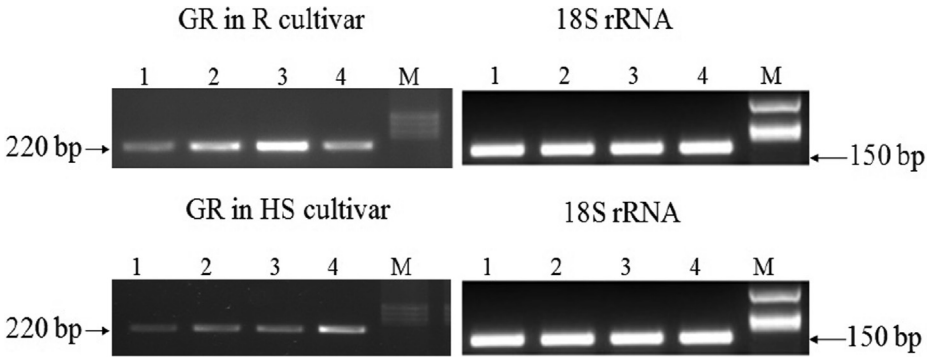

Fig. 7. Semi-quantitative PCR to study the GR gene expression upon 1.5 mM SA priming. Lane 1: Control; Lane 2: 1.5 mM SA; Lane 3: Pathogen only; Lane 4: Pathogen + 1.5 mM SA; Lane M: DNA ladder (1 Kb).R: Resistant; HS: Highly susceptible.

3.4. qRT PCR studies for the differential expression of defense genes

qRT-PCR studies for assessing the expression of defense-related genes such as APx, CAT and GR from both the cultivars revealed that 1.5 mM treatment using SA could potentially induce a significant upregulation of these genes. APx from the R cultivar showed (5.03 in the control group) an upregulation by 6.5 folds after seed priming. The inoculation with the pathogen *X. perforans*alone led to a downregulation of APx by 3.8 folds. Likewise, the gene expression in case of HS variety was upregulated by 4.01 folds in the SA-treated group from 1.08 folds in the control group (Fig. 8A).

Gene expressions for CAT genes also showed similar trend where the R variety showed a 3.4 fold upregulation in the SA-treated group from 2.1 fold increase in the control group. Even the HS cultivar showed upregulation by 1.5 fold in the control which was further elevated to 2.8 fold after SA seed-priming. However, the CAT genes were downregulated by 0.9 folds after the exposure to the pathogen *X. perforans*alone without seed priming (Fig. 8B). The relative expression of GR genes also exhibited similar upregulation. The control groups that recorded 1.25 fold increase showed an upregulation by 2.8 folds after the treatment with SA in R cultivar tomato seeds. Similarly, HS cultivar also showed an increase by 2.5 folds in the seed-primed group from that of 1.25 fold in the control groups. However, seeds without priming and treatment with the pathogen *X. perforans* showed a downregulation by −1.02 folds (Fig. 8C).

4. Conclusion

The present study clearly demonstrates that seed priming improves seed germination and seed vigor remarkably thereby preventing the harmful effects of infection by *X. perforans*. However, the precise mechanism of action is not known. Yet, the increase in defence-related genes as well as their corresponding enzyme activities showed that SA indeed elicits immune response in the plant leading to its beneficiary role in inducing resistance against the pathogenic attack. In addition, SA was found to be potent at a concentration as low as 1.5 mM, which is remarkable for its use in the greenhouse and field conditions. Therefore, the results from the present study uplift the understanding of using plant defence-inducers to deliver resistance to the plant and can be used as a potent disease management approach against bacterial spot disease of tomato. Further studies are underway to assess the role of SA in eliciting other pathways involved in plant defence.

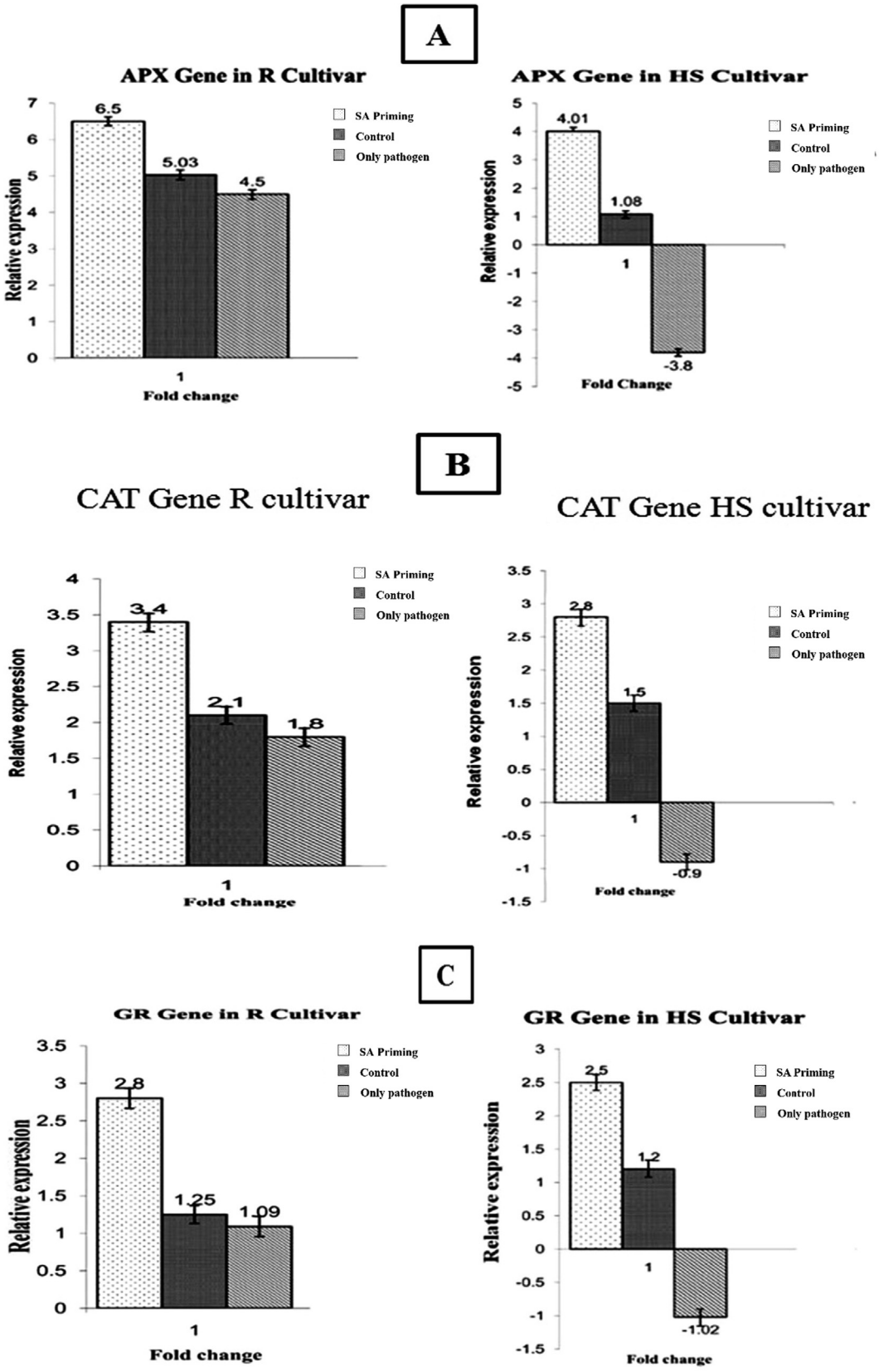

Fig. 8. Quantitative real time- PCR assay for relative expression levels of APx(A), CAT(B) and GR(C) genes in both resistant (R) and highly susceptible (HS) tomato cultivars. Values are the means of n = 3. R: Resistant (cv. *Sun hybrid*); HS: Highly susceptible (cv. *Quality seeds*).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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