

Mechanisms underlying salicylic acid on root parameters and antioxidant enzyme activity of potato plant under *Lelliottia amnigena* stress

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ABSTRACT

Salicylic acid (SA) plays many roles in plant physiology. In addition to pathogenesis-related resistance, SA is involved in the response to biotic stress. There are no adequate studies on mechanism underlying SA-mitigated *Lelliottia amnigena* infestations in potato (*Solanum tuberosum* L.) plant. Therefore, this study evaluated the mechanisms of SA on antioxidant enzyme activity of potato plant roots under *L. amnigena* stress. The bacterial suspension (3.69×10^7 CFU mL⁻¹) at 0.3 mL was inoculated into potato plants through stem injection. After 24 h, different SA concentrations (0.5, 1.0, 1.5 and 2.0 mM) were sprayed to the potato plants, and water as control. The results showed that *L. amnigena* infestation decreased fresh weight, dry weight, and relative water content of roots by 44.8%, 11.8% and 34.8%, respectively, compared to the control. The potato plant treatment with SA increased fresh weight, dry weight, and relative water content by an average of 34.88%, 10.28% and 13.50%; increased superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) by 35.95%, 32.55%, 33.74%, 33.25%, and 38.90%; decreased malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) content in potato plant roots by 42.7% and 31.1%, respectively, under *L. amnigena* stress compared to control. Treatment with SA induced transcriptional levels of the SOD, POD, CAT and glutathione S-transferase (GST) gene expression across the four levels of SA by an average of 3.12-fold, 3.46-fold, 2.93-fold and 3.83-fold, respectively, compared to control. Salicylic acid increased antioxidant enzymes activity in roots of potato plant under *L. amnigena* stress.

Key words: Biocontrol, phytohormones, plant pathogens, reactive oxygen species, *Solanum tuberosum*.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important food source worldwide (Wijesinha-Bettoni and Mouillé, 2019). Potato plants have comparatively shallow root systems and are sensitive to stress conditions (Turtola et al., 2005). The influence of pathogens is an important factor affecting the sustainable production of the crop (Obidiegwu et al., 2015). When plants are under pathogen stress, several physiological processes are disrupted by the production of elevated levels of reactive oxygen species (ROS), leading to oxidative stress within plant cells and thus photosynthetic activity, chlorophyll (chl) biosynthesis, hormonal balance, ion homeostasis, membrane stability, ATP production, lipid peroxidation, DNA damage, and imbalances in nutrient and water relationships, leading to cell abnormalities and death (Hasanuzzaman and Fujita, 2012). Plants deal with pathogen toxicity through various physiological and biochemical processes. Among the latter, plants endogenously activate both enzymatic and non-enzymatic antioxidant defense systems to scavenge ROS. Enzymatic antioxidants involved either directly or indirectly in scavenging ROS under pathogen-loaded conditions include superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL)

and peroxidase (POD) (Rahman et al., 2016). Exogenous application of salicylic acid (SA) results in the induction of signaling cascades that can enhance plant growth and productivity under various environmental stress conditions by enhancing and scavenging antioxidant activities ROS (Khan et al., 2015). Salicylic acid is one of the phenolic compounds with a hydroxyl or derivative group formed by plants (Cetinkaya and Kulak, 2019); it acts as a ubiquitous growth regulator that can regulate various physiological and metabolic processes and plays an important role in the defense mechanism against toxicity (Zhang et al., 2015).

It has been reported that SA triggers various gene expressions related to defense, either directly or through the H₂O₂-mediated signal transduction pathway regulating mitogen-activated protein kinase (MAPK) (Chai et al., 2014). *Lelliottia amnigena*, which is a Gram-negative, plant-pathogenic bacterium belonging to the family Enterobacteriaceae is a new species identified as a causal agent of soft rot of potato (Abd-Elhafeez et al., 2018). There are no adequate studies on the mechanism underlying SA-mitigated in *L. amnigena* infestations in potato plants. The objectives of this study were to evaluate the effect of SA on the physiological traits of potato plant roots under *L. amnigena* stress, and determine the effect of SA on molecular parameters of potato plant roots under *L. amnigena* stress.

MATERIALS AND METHODS

Study area and source of materials

The experiment was conducted in a greenhouse at Gansu Agricultural University in Lanzhou, Gansu, China, in summer 2020. In this experiment, potato (*Solanum tuberosum* L.) 'Atlantic' was used. Mini tubers of this potato variety were purchased for the experiment from Gansu Haofeng Seed Company Limited, Lanzhou, China. The pure bacterial strain PC3 (*Lelliottia amnigena*) was obtained from the Plant Pathology Laboratory of Gansu Agricultural University, Lanzhou, P.R. China.

Experimental design and treatments

The experimental design was completely randomized with two factorial arrangements having two controls, positive control (plants inoculated with strain PC3), negative control (plants inoculated with distilled water without PC3) and salicylic acid (SA) treatment (plants inoculated with strain PC3 and treated with 0.5, 1.0, 1.5, and 2.0 mM SA). Each of these groups consisted of three replicates. For each procedure, three plastic pots were used (9 cm in diameter and 10.5 cm in depth), each containing 1 kg sterile substrate. Fittings were inserted in the bottom of each plastic pot to allow water drainage and to allow air into plastic pots. Eighteen (18) uniform healthy mini tubers were sown 1 cm deep in the substrate and covered. The pots were arranged in a complete randomized design with three replicates. Plants were irrigated every 24 h and maintained at a constant temperature of 25 ± 0.5 °C, with supplemental day/night lighting of 16/8 h and relative humidity of 65%. Two independent experiments were performed.

Preparation of SA solutions and inoculation of the pathogen

Salicylic acid (SA; 2-hydroxybenzoic acid; Sangon Biotech Co. Ltd., Shanghai, China) solutions at different concentrations (0.5, 1.0, 1.5, and 2.0 mM) (pH 6.0-6.5) were prepared with distilled water containing 0.01% Tween-20 as surfactant according to the method of Cao et al. (2013) with some modification.

The bacterial suspension (0.3 mL ; $3.69 \times 10^7 \text{ CFU mL}^{-1}$) was inoculated into potato plants through stem injection. The potato plants were sprayed with solutions of 0.5, 1.0, 1.5, and 2.0 mM SA 24 h after inoculation of the pathogen. The SA treatments were repeated every 1 wk and control plants were sprayed with distilled water. This procedure was repeated for 60 d.

The roots of the sampled plants were washed and cut from the shoots and weighed immediately for measurement of fresh weight. To determine dry weight, all samples were oven-dried at 80 °C till constant weight. Relative water content (RWC) was calculated using a formula described by Tian and Philpot (2015): $\text{RWC}(\%) = (\text{FW} - \text{DW})/\text{FW} \times 100$; where FW is fresh weight and DW is dry weight.

Oxidants and antioxidants determination

The content of malondialdehyde (MDA), a product of lipid peroxidation produced by the thiobarbituric acid reaction and an indicator of oxidative damage to a biological system, was measured according to the manufacturer's protocol/kit (Malondialdehyde (MDA) Content Assay Kit; Beijing Solarbio Science and Technology, Beijing, China) (BC0025). The absorbance of each sample was measured at 600, 532, and 450 nm. The content of MDA was expressed as $\mu\text{mol g}^{-1}$ FW. The content of H_2O_2 in potato leaves was determined according to the manufacturer's protocol/kit (hydrogen peroxide (H_2O_2) content assay kit; Beijing Solarbio Science and Technology) (BC3595). In brief, 0.1 g fresh potato leaf was crushed in liquid nitrogen and placed on an ice bath in 1 mL acetone. The absorbance of each sample was measured at 415 nm.

The total activities of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), and polyphenol oxidase (PPO; E.C. 1.14.18.1) were determined according to the manufacturer's protocol. One unit of SOD (YX-W-A500-WST-8, Beijing Solarbio Science and Technology) activity was measured as the amount of crude enzyme extract that inhibited the reduction of β -nitroblue tetrazolium chloride by 50% at 560 nm in the spectrophotometer. The CAT (YX-W-A501, Beijing Solarbio Science and Technology) activity was monitored in the spectrophotometer by calculating the degradation of H_2O_2 at 240 nm. The POD (YX-W-A502, Beijing Solarbio Science and Technology) activity, expressed as U mg^{-1} FW, was determined spectrophotometrically by measuring the increased absorbance at 470 nm. One unit of PPO was defined as the change in absorbance by 0.1 units per minute under assay conditions. The antioxidant kits were provided by Beijing Solarbio Science and Technology.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA extraction and analysis of 100 mg roots treated with different concentrations of SA was performed according to the methods of Xie et al. (2013) and using PureLink RNA Mini Kit (Tiangen Biotechnology, Beijing, China). The quantity and quality of isolated RNA were analyzed using a Nano-Drop spectrophotometer at the absorbance of 230 and 260 nm. The A260/A280 ratio indicated that the RNA was free from protein contamination. First-strand cDNA synthesis was performed using Revert Aid TM First Strand cDNA Synthesis Kit (Tiangen Biotechnology). Specific primers for the SOD, POD, CAT and glutathione S-transferase (GST) genes and the internal control actin gene were used to amplify amplicons specific for potato leaves. For quantitative real-time PCR (RT-qPCR), 2 \times SYBR Green qPCR Master Mix was used. Analysis 20 μL reaction mixture consisting of 10 μL 2 \times SYBR Green qPCR Master Mix, deionized water (6.6 μL), diluted cDNA (1 μL), ROX reference dye (0.4 μL), and 1 μL each primer. The relative expression of SOD, POD, CAT, GST and actin genes was determined using the $2^{-\Delta\Delta\text{Ct}}$ formula of Livak and Schmittgen (2001). The RT-qPCR primers used in this study are listed in Table 1.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, Illinois, USA) followed by one-way ANOVA. Experimental results were expressed as means (\pm SE) of two independent experiments with three replicates. Means were separated by the Duncan multiple range test at a probability level of 5%.

Table 1. Genes and primers sequences used for RT-PCR.

Genes	Primers	Sequence (5'-3')
Superoxide dismutase (SOD)	F	GCTGGTGCTAGAGTAGCCTG
	R	TCAAAGTTATCTGCCGTTCTCCA
Peroxidase (POD)	F	GTTGCCTTATCAGGTTTGTTCCTT
	R	CGGACGTGTTTGAATCAACTT
Catalase (CAT)	F	TTTCAGGAGATGTGCAGCGT
	R	ATGTATGAGGCTTTTATGCTGCT
Glutathione S-transferase (GST)	F	AGCTGGTGCCCATCAATCTC
	R	CCCTCTGAGACTCCTAATGATCC
Actin	F	GCTCCTAGAGCTGTATTCCCAAGT
	R	CAGTCGAAACGTGGTATCTTGACT

RESULTS

Effect of SA on growth parameters

The application of SA resulted in a significant ($p < 0.05$) increase in fresh root weight, dry root weight, and relative water content. There was a significant decrease in fresh and dry root weight in plants inoculated with *L. amnigena*. Under *L. amnigena* infestation, root fresh weight of potato plants was decreased by 44.80% compared to control plants (Figure 1A). However, the application of SA across the four concentrations (0.5, 1.0, 1.5, and 2.0 mM) increased fresh weight by 18.1%, 36.6%, 37.8%, and 47.0%, respectively, compared to the control (Figure 1A). In the case of *L. amnigena* infestation, root dry weight and RWC also decreased by an average of 11.8% and 34.80%, respectively, compared to the control. Application of SA across the four concentrations increased dry weight and RWC by 4.2%, 9.3%, 10.5%, 17.1% and 2.4%, 14.9%, 15.0%, 21.7%, respectively, compared to the control (Figures 1B, 1C).

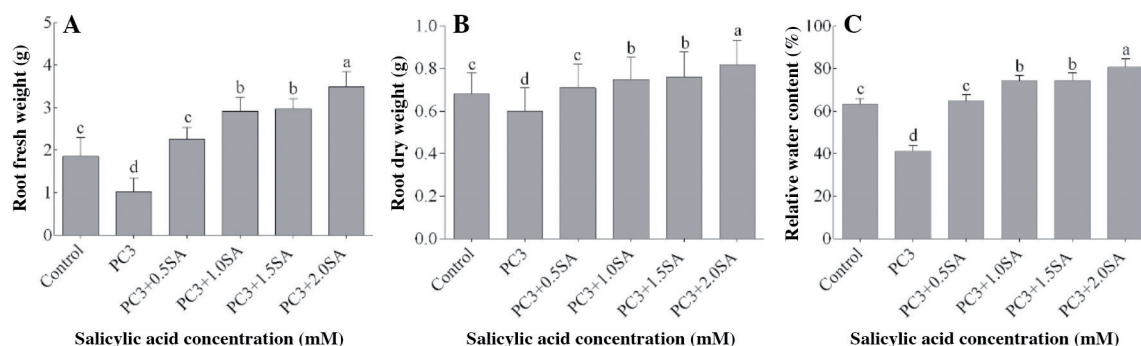
Effect of SA on MDA and H₂O₂ content

In response to *L. amnigena* infestation, lipid peroxidation (MDA) and hydrogen peroxide (H₂O₂) content in the root increased significantly ($p < 0.05$) by 40.9% and 42.8%, respectively, compared to the control (Figure 2). However, application of SA across the four concentrations significantly decreased MDA and H₂O₂ content by 18.4%, 39.8%, 48.7%, 63.7% and 8.7%, 27.9%, 29.8%, 57.9%, respectively, compared to the control (Figures 2A, 2B).

Effect of SA on antioxidant enzyme activities

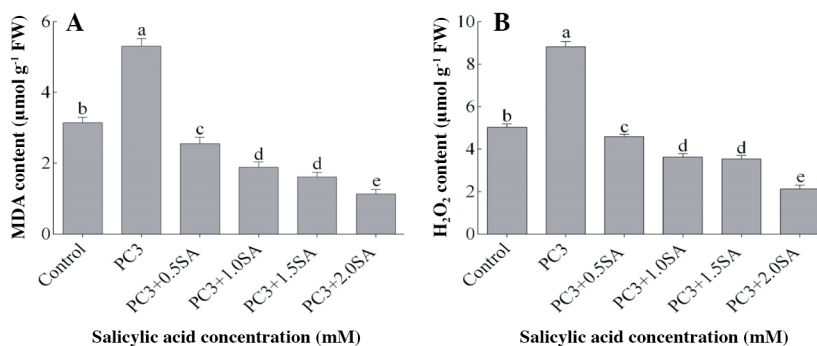
To inhibit ROS in potato plants under *L. amnigena* stress, their antioxidant system was activated and enzymes such as SOD, POD, CAT, PPO and phenylalanine ammonia-lyase (PAL) were significantly ($p < 0.05$) increased under SA treatments (Figure 3). Under normal conditions, the infestation of *L. amnigena* increased the activities of SOD, POD, CAT, PPO

Figure 1. Effect of salicylic acid (SA) on root fresh weight (A), root dry weight (B) and relative water content (C) of roots under *Lelliottia amnigena* stress (PC3 strain).



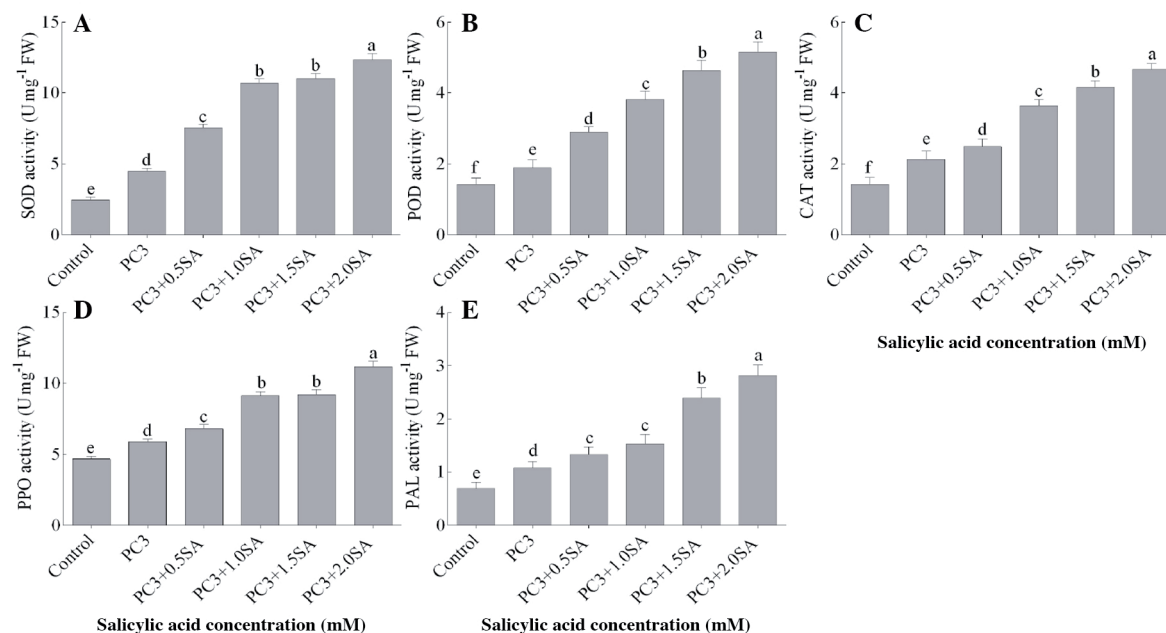
Data represent means \pm SE of three replicates. Lowercase letters denote significant difference between treatments according to LSD test ($p < 0.05$).

Figure 2. Effect of salicylic acid (SA) on malondialdehyde (MDA) (A) and hydrogen peroxide (H₂O₂) (B) content of potato roots under *Lelliottia amnigena* stress (PC3 strain).



Data represent means \pm SE of three replicates. Lowercase letters denote significant difference between treatments according to LSD test ($p < 0.05$).

Figure 3. Effect of salicylic acid (SA) on superoxide dismutase (SOD) (A), peroxidase (POD) (B), catalase (CAT) (C), polyphenol oxidase (PPO) (D) and phenylalanine ammonia-lyase (PAL) (E) activities in roots of potato plants under *Lelliottia amnigena* stress (PC3 strain).



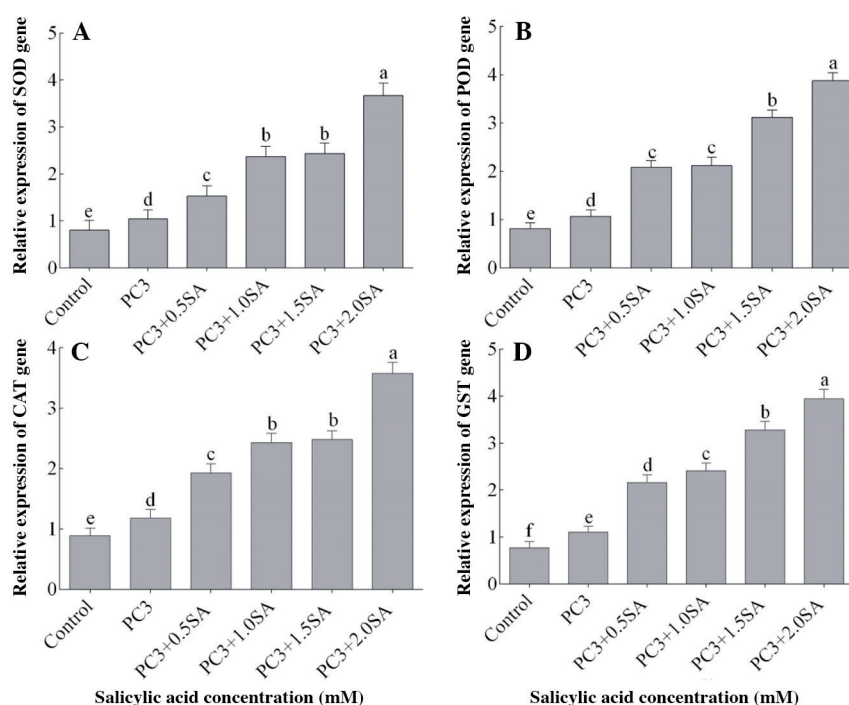
Data represent means \pm SE of three replicates. Lowercase letters denote significant difference between treatments according to LSD test ($p < 0.05$).

and PAL by 25.7%, 24%, 25.7%, 25.8%, and 26.8%, respectively, compared to the control. However, application of SA across the four concentrations significantly increased SOD by 30.6%, 35.6%, 36.7%, and 40.8%, respectively, compared to the control (Figure 3A). Also, application of SA across the four concentrations significantly increased POD by 28.4%, 30.2%, 34.0% and 36.4%, respectively, compared to the control (Figure 3B). In addition, SA across the four concentrations significantly increased CAT by 29.6%, 31.7%, 33.8% and 39.8%, respectively, compared to the control (Figure 3C). Application of SA across the four concentrations also increased PPO by 29.8%, 30.5% 31.2% and 41.5%, as well as PAL by 34.9%, 36.7%, 38.1% and 45.9%, respectively, compared to the control (Figures 3D, 3E).

Effect of SA on antioxidant gene expression

The results showed that the level of expression of SOD, POD, CAT and GST genes in the roots of potato plants under *L. amnigena* stress was increased by treatment with SA compared to control plants (Figure 4). Treatment with SA induced transcriptional levels of the SOD and POD gene expression across the five levels by 2.12-fold, 2.92-fold, 3.32-fold, 3.50-fold, 3.74-fold and 2.10-fold, 3.41-fold, 3.46-fold, 3.90-fold and 4.43-fold, respectively, compared to the control. In addition, application of SA induced transcriptional levels of the CAT and GST gene expression across the five levels by 1.90-fold, 2.82-fold, 2.98-fold, 3.08-fold, 3.85-fold and 2.80-fold, 3.48-fold, 3.82-fold, 4.21-fold and 4.84-fold, respectively, compared to the control. However, treatment of SA showed the highest expression level for all antioxidant enzyme genes, especially when treated with 2.0 mM (Figures 4A-4D). This indicates that treatment with SA modulated biotic stress-responsive gene expression in potato plants, which in turn increased their tolerance to *L. amnigena* infestation. Significantly ($p < 0.05$), all antioxidant enzyme genes were relatively up-regulated in the plants treated with SA at all concentrations compared to the plants not treated with SA, indicating the important role of SA and antioxidant enzymes under biotic stress.

Figure 4. Effect of salicylic acid (SA) on gene expression of superoxide dismutase (SOD) (A), peroxidase (POD) (B), catalase (CAT) (C) and glutathione S-transferase (GST) (D) in roots of potato plants under *Lelliottia amnigena* stress (PC3 strain).



Data represent means \pm SE of three replicates. Lowercase letters denote significant difference between treatments according to LSD test ($p < 0.05$).

DISCUSSION

The results of our study showed that plants inoculated with *L. amnigena* reduced fresh and dry root weight compared to control plants. The application of SA significantly increased fresh weight of roots of potato plants (Zhang et al., 2011). The role of SA in plant growth can be attributed to its effect on increasing cell division (Gunes et al., 2007) and we can say that the increase in fresh and dry root weight of the plant is attributed to the application of SA to trigger the negative effects of stress caused by *L. amnigena* and improving plant growth. Arfan et al. (2007) reported improved fresh and dry root weight of stressed maize plants treated with SA. Salicylic acid is reported to counteract the adverse effects of stress by enhancing root growth (Aldesuquy et al., 2012). Relative water content (RWC) is a suitable factor to assess the physiological water status of stressed plants (Kadioglu et al., 2011). In this study, RWC decreased in potato plants under pathogen stress conditions, but the treatment of SA significantly increased RWC in these plants. Other studies have shown that SA can lead to an increase in RWC under stress (Alam et al., 2013; Ali et al., 2020); these results are consistent with our observations in potato plants. The RWC was reduced under stress, which is consistent with the results in *Nigella sativa* (Kabiri et al., 2014). Treatment with SA caused an increase in RWC under stress, which is similar to the observations of Farooq et al. (2009). Salicylic acid caused increased accumulation of compatible osmolytes in plants followed by increased water uptake.

In this present study, MDA content increased in plants under *L. amnigena* stress, which is consistent with the results of Zafari et al. (2012). Salicylic acid significantly mitigated this effect; such an effect was also reported by Yadav et al. (2018). Moreover, lower MDA content in SA-treated plants could be correlated with the increased activities of antioxidant enzymes. Our result is consistent with the findings in wheat (Agarwal et al., 2005). Interestingly, the application of SA in this study significantly reduced MDA (ROS representative) and increased antioxidant content in *L. amnigena*-stressed plant roots. In this study, the H_2O_2 level was significantly increased under *L. amnigena* stress, which is in agreement with Zafari et al. (2012). The results in this current study showed that increasing concentrations of SA significantly decreased H_2O_2 content, with 2.0 mM SA having the lowest H_2O_2 content.

Salicylic acid has been significantly recognized as an endogenous natural signaling molecule involved in plant defense mechanisms by regulating both physiological and biochemical processes (Dong et al., 2015). All these researches have shown that SA-regulated abiotic and biotic stress tolerance in plants is involved in antioxidant responses, suggesting that the protection of plants from oxidative damage by SA is closely associated with an enhanced antioxidant system (Suruchi et al., 2014). In our study, the application of SA to plants under *L. amnigena* stress significantly increased the activities of antioxidant enzymes (SOD, POD, CAT, PPO, and PAL) compared to the control plants. Attenuation of oxidative damage by scavenging ROS via antioxidant enzymes is an important strategy of plants to protect them from stress. In addition, SA enhances enzymatic (SOD, POD, CAT, PPO, and PAL) and plays a major role in increasing plant stress tolerance and reducing oxidative stress (Mutlu et al., 2016), which in turn improves plant growth of pathogen-stressed plants. In short, CAT is the key enzyme in the removal of hydrogen peroxide (H₂O₂) and complements the action of superoxide dismutase (Sewelam et al., 2016). In this study, the activities of antioxidant enzymes were altered in response to SA treatment. A similar change in these enzymes was also observed by Kaur et al. (2012). However, exogenous SA restored the activities of the tested enzymes, reducing ROS and oxidative damage in potato roots. The data confirm that the key role of SA is to serve as a signaling molecule, thereby strengthening the defense system. Similar results were also observed by Singh et al. (2015). Our results suggest that exogenous SA regulates antioxidant enzymes, thereby lowering ROS and protecting potato plants from pathogen stress. In general, based on the increased activities of SOD, POD, CAT, PPO and PAL, it can be said that the treatment of SA effectively increased the antioxidants of potato plant roots to defend against the pathogen.

Exogenous supplementation of SA to *L. amnigena*-infested potato plants enhanced the up-regulation of enzymatic antioxidants in the roots. Importantly, exogenous application of SA up-regulated all the enzymes that were down-regulated under *L. amnigena* stress and further up-regulated the enzymes that showed increased activity under *L. amnigena* stress. Accordingly, by up-regulating enzyme activities such as SOD, POD, CAT, which play important roles in ROS scavenging, plants can avoid ROS injury (Abdelaal et al., 2020). Finally, SA scavenges excess ROS while *L. amnigena* stress-induced oxidative damage by upregulating antioxidant defense systems, making plants more resistant to this form of biotic stress (Mutlu et al., 2016). Results suggest that the application of SA enhances *L. amnigena*-induced stress resistance in potato plants by mitigating oxidative damage through up-regulation of the antioxidant defense system, thereby improving growth and productivity.

CONCLUSIONS

The results of the current study on potato plants showed that salicylic acid (SA) treatment plays an important role in plant-pathogen tolerance. Our findings revealed that the application of SA through the spraying induces an antioxidant system in the roots of potato plants under *Lelliottia amnigena* stress. Therefore, the results obtained in this study suggest that 2.0 mM SA could be used as bactericide for the management of *L. amnigena*. Based on our findings, we suggest that a concentration of 2.0 mM SA is the best for controlling *L. amnigena*.

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