Salicylic acid effect on the mechanism of *Lelliottia amnigena* causing potato soft rot

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ABSTRACT

Salicylic acid (SA) plays an important role in protecting plants from biotic stresses. *Lelliottia amnigena* is a newly identified potato soft rot pathogen and there are no adequate studies on this soft rot pathogen. Therefore, this paper focussed on the effect of SA on the mechanism under which *L. amnigena* causes potato soft rot. *L. amnigena* was examined and detected to secrete pectinase, proteases, pectin lyase and cellulase, which are the most important pathogenic enzymes involved in the production of plant diseases. Sterilised healthy potato tubers were inoculated with 0.2 mL of *L. amnigena* suspension ($3.69 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$). After 24 h, 200 μL of four different SA concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) were used to treat the tubers. Co-culture of *L. amnigena* and SA significantly reduced the activity of pectinase, protease, pectin lyase and cellulase by an average of 33.8%, 43.4%, 67.7% and 46.9%, across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM), respectively, compared to the control. The average disease index was reduced by 54.7% across the four SA concentrations. Treatment with SA induced transcriptional levels of the superoxide dismutase, peroxide, catalase and glutathione S-transferase across the four levels by an average of 3.87, 3.25, 3.97 and 3.94-fold, respectively, compared to control. Based on our results, we could state that SA could reduce the activities of these extracellular enzymes produced by *L. amnigena* by modulating both enzymatic and non-enzymatic antioxidant activities and gene expression that induce natural resistance in plants against bacterial infections.

KEYWORDS: antioxidant enzymes, disease, extracellular enzymes, pathogen, gene expression

INTRODUCTION

Potato (*Solanum tuberosum*) is the fourth most important food crop in the world after rice, wheat and maize according to Dutt et al. (2017) and Rahaman and Shehab (2019). It is used worldwide to produce various food substances such as chips, crisps, potato flour, vegetable salad, canned food and feed for livestock (Manzira, 2010). China is the leading potato producer in the world, accounting for 26.3% of global potato production, followed by Russia and India (Wang et al., 2018). However, the production and quality of potato tubers

are affected by many different biotic and abiotic stresses including soft rot-causing pathogens (*Pectobacterium* and *Dickeya*) and other diseases such as blackleg, scab and late blight. Mostly, soft rot occurs during production in the field and storage (Pérombelon, 2002; Czajkowski et al., 2011). The virulence and pathogenicity of these bacteria are due to their ability in secreting large amounts of extracellular plant cell wall-degrading enzymes (PCWDE), including pectin lyases, proteases, pectinase and cellulases, leading to extensive tissue maceration,

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rot and subsequent death of the entire plant (Barras et al., 1994; Toth et al., 2003). They cause extensive tissue damage, invasiveness, dissemination, colonisation, and can promote the destruction of the plant cell wall. In addition, they break down plant substances into smaller molecules that are absorbed easily and utilised by pathogen for their growth and energy. The symptoms of soft rot are caused by the cumulative action of pectinases (especially pectate lyases) which destroy pectin, the adhesive material of the plant cell wall.

Many physical methods such as the production of certified seed, rigorous inspections, seed testing systems, good sanitation during harvest, sorting and grading of tubers, and the requirement for farmers to use varietal resistance seed have been used to control potato soft rot, but these procedures are expensive, time-consuming and do not eliminate pathways or avenues through which pathogenicity becomes established (Mauch-Mani et al., 2017; Silva et al., 2018). Chemical methods are also used to control bacterial pathogens. Synthetic bactericides are not the preferred method for controlling plant pathogens due to their negative effects on humans and the environment and the possibility of the selection of multidrug-resistant bacterial strains (Abd El-Kahir, 2004; Jess et al., 2014). As a promising alternative to the use of synthetic bactericides, phytohormones such as salicylic acid (SA) that induces natural resistance in plants against bacterial infections can be considered (Koo et al., 2020). They serve as chemical mediators to control cellular activities in higher plants (Kazan, 2015; Aymen, 2018).

Salicylic acid is one of the phenolic compounds formed by plants with a hydroxyl or derivative group (Yousif, 2018). Plant phenols are commonly referred to as specialised metabolites involved in important functions such as biosynthesis of lignin and allelopathic compounds that control plant responses to living stimuli (Kubalt, 2016), thermoregulation (Klessig et al. 2018) and defence signalling activity in plants (Kubalt, 2016). It also stimulates morphological, physiological and biochemical pathways of general plant defence (Wang and Li, 2006; Vlot et al., 2009). In inducing disease tolerance in plants, SA also controls ion uptake and antioxidant defence (Jayakannan et al., 2015). Due to its contribution to plant protection response under biotic and abiotic stresses, SA has received much attention.

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). Many crops, including apple, carrot, lettuce, onion, radish, strawberry and sugar beet, have been reported to be affected by *L. amnigena*, resulting in economic losses in agricultural production (Al-Kharousi et al. 2016; Hungund et al. 2010; Liu et al. 2016). Soft rot bacteria of the *Lelliottia* species produce and secrete PCWDE that causes the rotting of potatoes in the field and storage. Because they can produce a wider range of enzymes/isoenzymes more quickly

and in higher quantities than pectolytic saprophytic bacteria, they can penetrate living plants more easily and induce infection (Bateman 2012; Collmer et al. 2012). In contrast to viruses specialised to infect a confined host range, the mechanisms that have evolved in the soft rot *L. amnigena* appear to allow a significant degree of plasticity in their metabolism. However, there is no adequate information on the SA effect on the mechanism of *L. amnigena* causing potato soft rot. Therefore, this study focussed on the SA effect on the *L. amnigena* mechanism of causing potato soft rot.

MATERIALS AND METHODS

The pathogen, strain PC3 (*L. amnigena*) with GenBank accession number of the 16S rRNA SUB10508072 PC3 OK447935 was obtained from the Plant Pathology Laboratory of Gansu Agricultural University, Lanzhou, P.R. China. *L. amnigena* was previously identified to cause potato soft rot in Egypt by Abd-Elhafeez et al. (2018) and onion decay in China by Liu et al. (2016). Strain PC3 was grown on nutrient agar (NA) at 37 °C for overnight. Bacteria suspension was prepared to a final concentration of $3.6 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$ using a serial dilution method described by Ben-David and Davidson (2014). SA (SA; 2-hydroxybenzoic acid), obtained from Sangon Biotech Co. Ltd, Shanghai, China, was used for the study. A 5×2 factorial experiment in a randomised complete block design with three replications was used both in the laboratory and greenhouse. Physiological and molecular parameters were determined after plant treatments.

Potato tubers treatment

Potato tubers (*Solanum tuberosum* L., Atlantic variety) were obtained from the local market, Lanzhou, P.R. China. Atlantic variety was selected due to its susceptibility to various rot infections and also a popular mid-season chipping potato cultivar in China. Matured healthy tubers of similar size with no visible physical injuries or infection were used. The tubers were surface sterilised by immersion in 0.5% sodium hypochlorite (NaOCl) solution for 15 min, washed with sufficient sterile water and then air-dried overnight. The sterilised tubers were cut in halves, washed by rinsing in sterile water for 3 min, followed by surface sterilisation with 75% ethanol for 1 min and air drying. The treatments were: five levels of SA concentrations (0.0 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) and two modalities of *L. amnigena* inoculation (*L. amnigena* inoculation and no *L. amnigena* inoculation). The treatment combinations were presented in Table 1. A total of 18 pots (6 treatments with 3 replicates) were used for each experiment. The negative control (plants growing in loamy soil under control conditions without *L. amnigena* inoculation and SA treatment) whereas positive control (plants growing in loamy soil under conditions with *L. amnigena* inoculation).

Table 1. Treatment combinations.

Group	Name	Treatment
T1	Negative control	Distilled water
T2	Positive control	Strain PC3 (<i>L. amnigena</i>)
T3	PC3 + 0.5 mM SA	Inoculation of strain PC3 + 0.5 mM SA
T4	PC3 + 1.0 Mm SA	Inoculation of strain PC3 + 1.0 mM SA
T5	PC3 + 1.5 Mm SA	Inoculation of strain PC3 + 1.5 mM SA
T6	PC3 + 2.0 Mm SA	Inoculation of strain PC3 + 2.0 mM SA

SA, salicylic acid.

Preparation of SA solution

Salicylic acid – 2-hydroxybenzoic acid obtained from Sangon Biotech Co. Ltd., Shanghai, China, was used for the study. SA solutions at different concentrations (0 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) with pH at 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 modification. These ranges of concentrations were chosen because low or high SA levels were strictly correlated to a lower and higher oxidative stress according to Mateo et al. (2006).

Impact of SA on *in vitro* extracellular enzyme production of *L. amnigena*

0.2 mL of *L. amnigena* (strain PC3) ($3.6 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$) was inoculated into 10 mL of NA and allowed to grow at 37 °C for 12 h (overnight). Overnight cultures of the strain were re-suspended to the final concentration of $3.6 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$ in bacterial liquid with or without SA. Different concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) of SA were used. Culture supernatants from the growths at 37 °C were collected and analysed for the activity of protease ($\text{U} \cdot \text{mL}^{-1}$), pectinase ($\text{U} \cdot \text{mL}^{-1}$), pectin lyase ($\text{U} \cdot \text{mL}^{-1}$) and cellulase ($\text{U}/104 \text{ cell}$) according to the manufacturer's protocol/kit (Solarbio Science and Technology Company Ltd., Beijing, China). The activity of pectinase, protease, pectin lyase and cellulase were determined in a dual-wavelength spectrophotometer at the absorbance of 540 nm, 450 nm and 235 nm, respectively. The experiment was repeated three times.

Impact of SA on bacterial soft rot of potato tuber

This experiment checked the effect of treating potato tubers with SA on the severity of soft rot disease. The experiment was conducted in the laboratory. Matured healthy potato tubers were surface sterilised with 75% ethanol for 1 min and then rinsed 3 times in sterile distilled water. The tubers were allowed to dry at room temperature. Ten tubers were used for each replicate. A total of 30 tubers were used for each treatment. A hole (0.5 cm in diameter and 1 cm deep) was created in the

centre of each sterilised tuber using a sterile cork borer (BML505-15 mm, Wuhan Servicebio Technology Co. Ltd., China). A solution of bacterial suspension ($3.69 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$ at 0.2 mL) was put in the hole of the tubers. After 24 h, tubers were treated with SA at different concentrations (0 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM at 200 μL) through spraying with sterile distilled water as a negative control. Treated tubers were packed in clean, sterilised plastic containers, each lined with sterilised damp cotton and stored at room temperature for 14 days. Three replicates were used for each treatment. The experiment was repeated three times.

Inoculation of tubers and disease assessment

Inoculation of 0.2 mL of *L. amnigena* suspension was performed in the laboratory 24 h before treatment with SA as previously described. Disease index (DI) was recorded 7 days and 14 days after inoculation (DAI). The DI was scored by visually assessing the maceration area of each tuber using a visual scale of 0–4, (Scherf et al., 2010) where 0 = no rot, 1 indicated that 1–25% of the tubers were rotted, 2 indicated that 26–50% of the tubers were rotted, 3 indicated that 51–75% of the tubers were rotted and 4 indicated that 76–100% of the tubers were rotted. The DI was calculated as:

$$\text{DI (\%)} = \left(\frac{\text{S}[\text{number of diseased tubers in this index} \times \text{di}]}{\text{[total number of tubers investigated} \times \text{highest di}]} \right) \times 100$$

The action of SA on the growth of potato plant

Salicylic acid concentrations (0 mM, 0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) were sprayed using Handheld Pressure Sprayer (301-B, Pinghu Kaixin Plastic Industry Co., Ltd., China) on the foliage of the plants 24 h after inoculation of the bacterial suspension ($3.69 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$) through stem injection ($0.2 \text{ mL} \cdot \text{plant}^{-1}$). Briefly, this part of the experiment was conducted in a greenhouse (day temperature 25–35 °C, night temperature 18–22 °C, daytime relative humidity 45–55% and light intensity 15,000–18,000 lx). One tuber per pot was sown per plastic pots (12.5 cm diameter, 9.5 cm deep) filled with 2 kg of loamy soil. SA treatments were repeated every 1 week and control plants were sprayed with distilled water. This procedure was repeated for 60 days. Treatments were arranged as randomised complete blocks with 30 potato plants. The experiment was repeated three times with three replications. After 60 days of treatment, plant height (cm), the number of leaves per plant, leaf area (cm^2) and plant fresh weight (g) traits were determined. The plant dry weight (g) was guided in an electric oven (YLD-3000, Shanghai Yuejin Medical Equipment Co., Ltd., China) at 105 °C until a constant weight was reached and then determined. Data were compared with plants under control conditions (plants without pathogen inoculation). The entire experiment was conducted three times under the same conditions with similar results and the data from the three experiments were pooled.

Measurement of chlorophyll and carotenoid content in leaves

Total chlorophyll and carotenoids were extracted with 100% acetone (10000418, Sinopharm Chemical Reagents Co. Ltd., China, $\geq 99.5\%$ purity) according to the method of Lichtenthaler (1987). In brief, 0.2 g of fresh leaves was ground to powder in liquid nitrogen and homogenised with 5 mL of acetone and then centrifuged (BT16R, OLABO Scientific Co. Ltd, Jinan, China). The chlorophyll and carotenoid content were determined in a dual-wavelength spectrophotometer (EPOCH2 Plate Reader, BioTek, USA) at the absorbance of A_{661.6} nm at 644.8 nm and 470 nm.

Determination of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) in leaves

The content of 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 (BC0025, Solarbio Science and Technology Company Ltd., Beijing, China). The absorbance of each sample was measured at 600 nm, 532 nm and 450 nm, respectively. The content of MDA was expressed as $\mu\text{mol} \cdot \text{g}^{-1}$ fresh weight ($\mu\text{mol} \cdot \text{g}^{-1}$ FW). The content of hydrogen peroxide (H₂O₂) in potato leaves was determined according to the manufacturer's protocol/kit (BC3595, Solarbio Science and Technology Company Ltd., Beijing, China). In brief, 0.1 g of fresh potato leaf was crushed in liquid nitrogen (Henan Boss Liquid Nitrogen Container Co., Ltd., China, 78.03% by volume, 75.5% by weight) and placed on an ice bath in 1 mL of acetone. The absorbance of each sample was measured at 415 nm using a spectrophotometer (EPOCH2 Plate Reader, BioTek, USA).

Antioxidants enzymatic activities in potato leaves

The total activities superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), peroxide (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) 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 (EPOCH2 Plate Reader, BioTek, United States). CAT (YX-W-A501) activity was monitored in the spectrophotometer by calculating the degradation of H₂O₂ at 240 nm. POD (YX-W-A502) activity, expressed as $\text{U} \cdot \text{mg}^{-1}$ FW, was determined spectrophotometrically by measuring the increased absorbance at 470 nm. One unit of PPO was defined as the change in absorbance by $0.1 \text{ U} \cdot \text{min}^{-1}$ under assay conditions. The antioxidant kits were provided by Solarbio Science and Technology Company Ltd., Beijing, China.

Extraction of total RNA and analysis of gene expression

Total RNA extraction and analysis of 100 mg fresh potato leaves sprayed with different concentrations of SA were 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 analysed using a Nano-Drop spectrophotometer (Thermo Scientific, USA) at the absorbance of 230 nm 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, Beijing, China). 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 as presented in Table 2. For quantitative real-time PCR (qRT-PCR), 2× SYBR Green qPCR Master Mix was used. Analysis 20 μL reaction mixture consisting of 10 μL 2× SYBR Green qPCR Master Mix, deionised water (6.6 μL), diluted cDNA (1 μL), ROX reference dye (0.4 μL) and 1 μL of 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). Three biological repeats were performed for each experiment.

Statistical analysis

The data were tested in each experiment included SA controlling *L. amnigena* and breaking the mechanism of infection. Data were analysed using two-way ANOVA in SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), and mean comparisons were made using Duncan's new multiple range test and the significance was considered at $p < 0.05$.

RESULTS

The mechanism under which *L. amnigena* causes soft rot

The mechanism under which *L. amnigena* causes soft rot was determined in an *in vitro* on *L. amnigena*. Our result shows that *L. amnigena* produced extracellular enzymes such as pectinase, protease, pectin lyase and cellulase that contribute to the development of potato soft rot (Table 3).

The impact of SA on *L. amnigena* mechanism of infection

Our results showed that SA had a significant ($p < 0.05$) impact on the production of pectinase, protease, pectin lyase and cellulase. The addition of SA across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM)

Table 2. Gene description and primers sequences used for the qRT-PCR.

Gene symbol	Description	Primer sequence (5'–3')	Experiment
SOD	Manganese superoxide dismutase	F-GCTGGTGCTAGAGTAGCCTG R-TCAAAGTTATCTGCCGTTCTCCA	Antioxidant activity test
POD	Peroxidase	F-GTTGCCTTATCAGGTTTGTCTTT R-CGGACGTGTTTGAATCAACTT	Antioxidant activity test
CAT	Catalase	F-TTTCAGGAGATGTGCAGCGT R-ATGTATGAGGCTTTTATGCTGCT	Antioxidant activity test
GST	Glutathione S-transferase	F-AGCTGGTGCCCATCAATCTC R-CCCTCTGAGACTCCTAATGATCC	Antioxidant activity test
ACT	Actin-related protein Actin	F-GCTCCTAGAGCTGTATTCCCAAGT R-CAGTCGAAACGTGGTATCTTGACT	Antioxidant activity test

CAT, catalase; GST, Glutathione S-transferase; POD, Peroxidase; qRT-PCR, quantitative real-time PCR; SOD, superoxide dismutase.

Table 3. Production of extracellular enzymes by suspensions ($3.6 \text{ CFU} \cdot \text{mL}^{-1} \times 10^7 \text{ CFU} \cdot \text{mL}^{-1}$) from *L. amnigena*.

Extracellular enzymes	Enzyme activity ($\text{U} \cdot \text{mL}^{-1}$)
Pectinase	$77.27 \pm 3.441 \text{ b}$
Protease	$91.25 \pm 4.672 \text{ a}$
Pectin lyase	$2.22 \pm 0.102 \text{ c}$
Cellulase	$0.08 \pm 0.009 \text{ c}$

Data are presented as mean $\pm$ standard error (SE) of three independent experiments performed in triplicate. Different letters indicate a significant difference according to Duncan's multiple range test ($p < 0.05$).

significantly reduced the activity of pectinase, protease, pectin lyase and cellulase by an average of 33.8%, 43.4%, 67.7% and 46.9%, respectively, compared to control (Figure 1A–1D).

Effect of SA on severity and reduction of soft rot of potato tubers

Our results showed that SA-treated tubers had <30% rot 2 weeks after inoculation compared to untreated tubers, with a disease incidence of 44.40% (Figure 2B). When compared to strain PC3-treated tubers, SA-treated tubers exhibit a decrease in DI of 27.80% and 44.40% after 7 and 14 DAI, respectively (Figure 2G). When compared to control, SA treatments across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) reduced DI by an average of 47.5% and 54.7% at 7 days and 14 days, respectively (Figure 2H).

The action of SA on the growth of potato plant

Our results showed that strain PC3-treated plant reduced plant height, the number of leaves, leaf area, fresh weight and dry weight by 13.7%, 19%, 25%, 15% and 27%, respectively, compared to control (Figure 3A). However, foliar spraying of SA across the four concentrations increased plant height, the number of leaves, leaf area, fresh weight and dry weight by 47.8%, 22.8%, 13.5%, 16.3% and 18.3%, respectively, compared to control (Figure 3A–3E).

Chlorophyll and carotenoid content in leaves

Our results show that plants inoculated with strain PC3 reduced chlorophyll *a* and *b*, total chlorophyll and carotenoid contents by 38.6%, 67.3%, 50.7% and 61.8%, respectively, compared to control (Figure 4A–4D). However, foliar application of SA across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) significantly ($p < 0.05$) increased chlorophyll *a* and *b*, total chlorophyll and carotenoid contents by an average of 45.3%, 50.7%, 52.7% and 71.8%, respectively, compared to control (Figure 4A–4D).

Effects of SA on MDA and H_2O_2 content in the leaves of potato plants

Our results show that plants treated with strain PC3 increased MDA and H_2O_2 by 29% and 45%, respectively compared to control. However, foliar application of SA to plants inoculated with *L. amnigena* significantly ($p < 0.05$) reduced MDA and H_2O_2 content across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) by an average of 47.0% and 47.5%, respectively compared to control (Figure 5A–5B). The correlation matrix among chlorophyll, carotenoid, MDA and H_2O_2 contents is presented in Table 4. H_2O_2 showed a significant and negative correlation with chlorophyll ($r = -0.948$), and carotenoid ($r = -0.884$). MDA was significantly and negatively correlated with chlorophyll ($r = -0.985$) and carotenoid ($r = -0.951$). Moreover, carotenoid showed a significant and positive correlation with chlorophyll ($r = 0.987$), MDA was significantly and positively correlated with H_2O_2 ($r = 0.975$).

Antioxidant enzyme activities in potato leaves

Our results show that the plants treated with strain PC3 initially increased the activities of SOD, POD, CAT and PPO by 32.1%, 27.1%, 38.6% and 47.5%, respectively, compared to control. The activity of SOD, POD, CAT and PPO across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and 2.0 mM) was significantly ($p < 0.05$) increased by an average of 69.9%, 59.3%, 63.9% and 68.6%, respectively, compared to control (Figure 6A–6D).

Figure 1. Effect of SA on (A) pectinase, (B) protease, (C) pectin lyase and (D) cellulase production of *L. amnigena* (Strain PC3) stress. Data are presented as mean $\pm$ SE. Different letters indicate a significant difference according to Duncan's multiple range test ($p < 0.05$). SA, salicylic acid.

Effect of SA on antioxidant gene expression

Our results show that inoculation of *L. amnigena* to potato plants initially increased gene expression of SOD, POD, CAT, and GST. However, treatment with SA induced transcriptional levels of the SOD, POD, CAT and GST gene expression across the four concentrations (0.5 mM, 1.0 mM, 1.5 mM and, 2.0 mM) by an average of 3.87, 3.25, 3.97 and 3.94-fold, respectively, compared to control (Figure 7A–7D).

DISCUSSION

Our study has shown that *L. amnigena* secretes extracellular enzymes (pectinase, protease, cellulase and pectin lyase) to cause potato soft rot in tubers. Our results are in agreement with Reeleder and Brammal (1994) and Prathyusha and Suneetha (2011), who revealed that many phytopathogens produce extracellular enzymes to invade plants, causing soft rot diseases both in the field and storage. Again, the secretion of the aforementioned enzymes, by *L. amnigena* contributed to the maceration of parenchyma cells and this finding is in line with the study by Popović et al. (2017) who discovered *Pectobacterium atrosepticum* causing bacterial soft rot on calla lily via exoenzyme production. However, when the pathogen was treated with SA, a reduction was noticed in the secretion of the extracellular enzyme. The

colonies of *L. amnigena* in the absence of SA were flat with a rough appearance displaying irregular colony edges. Bacteria that were grown with the SA were incapable of producing flat and long zones, twitching zones and had round, smooth, regular colony edges, and short colony growth which may reduce their mode of infection and spreading within tissues. Therefore treatment of the pathogen with SA could reduce/block the pathway in which *L. amnigena* secretes these extracellular enzymes.

Our results showed that the application of 1.5–2.0 mM SA solution significantly reduced the severity of soft rot in potato tubers 7 days and 14 DAI compared to the untreated tubers, confirming the induction of systemic resistance. Our results again indicated that treatments of potato soft rot disease with SA are a result of the association between the extracellular enzymes and the inhibitor sensitivity of SA. The reduced activity of extracellular enzymes decreased the potato soft rot and induced potato tuber resistance to *L. amnigena*. Our finding is consistent with Bawa et al. (2019) who reported that application of SA reduced disease severity and induced resistance to *Fusarium solani* in soybean seedlings. A study by Li and Zou (2017) indicated that foliar spraying of tomato plants with SA at a concentration of 2.0 mM led to a significant reduction in disease severity. Similarly, our study also showed that

Figure 2. Symptoms of artificial infection with strain PC3 14 DAI. Tubers were treated with SA 24 h before inoculation with strain PC3. (A) Control (CK), (B) strain PC3 (C) 0.5 + PC3, (D) 1.0 + PC3, (E) 1.5 + PC3, (F) 2.0 + PC3. (G) DI (H) Disease reduction. Data are presented as mean $\pm$ SE. Different letters indicate a significant difference according to Duncan's multiple range test ($p < 0.05$). Potato tubers were treated with different concentrations of SA for 24 h before inoculation with strain PC3. The treatments were water (negative control), strain PC3 (positive control), PC3 + 0.5 mM SA, PC3 + 1.0 mM SA, PC3 + 1.5 mM SA, PC3 + 2.0 mM SA. Strain PC3: *L. amnigena*, DAI. Figure 2A, tuber treated with distilled water showed no signs of rot, Figure 2B tuber inoculated with *L. amnigena* without SA treatment showed the highest signs of rot. Figure 2C–2F, tubers inoculated with *L. amnigena* and treated with different concentrations of SA showed minor signs of rot with Figure 2F showing very little sign of rot. DAI, days after inoculation; DI, disease index; SA, salicylic acid.

the application of SA at 2.0 mM induces better disease resistance of potato tubers against *L. amnigena*. Our findings suggest that SA can greatly improve disease resistance in potato tubers and that SA treatment can replace the use of synthetic bactericides in the control of soft rot in potato tubers.

Foliar application of SA significantly increased plant height, leaf area, leaf number and fresh and dry weights compared to untreated plants. It was found that inoculation of *L. amnigena* decreased the growth parameters of the potato plants. However, co-inoculation of SA with the pathogen improved the growth parameters compared to the control plants. These findings were due to the bactericidal effect of SA through the mechanism of growth promotion and inhibition of *L. amnigena* extracellular enzymes production. In the same context, a study by Al-Jeboori et al. (2017) showed that spraying potato plants with SA at a concentration of $100 \text{ mg} \cdot \text{L}^{-1}$ resulted in the highest significant plant height values. Our findings are consistent with Amin et al. (2008) who reported that SA increases cell division in the meristem of wheat seedlings and improves plant growth.

Photosynthesis is an important physiological mechanism for enhancing plant survival by utilising light energy and synthesising organic compounds in plants (Xu et al., 2014). The effectiveness of photosynthesis depends on chlorophyll content, but reactive oxygen species (ROS) can cause a noticeable decrease in chlorophyll content in plants due to their sensitive nature (Van Oosten et al., 2017). Total chlorophyll content can be used as an indicator in the determining the effects of supplements on plant metabolism. In previous reports, SA application could cause an increase in total chlorophyll content (Baghizadeh et al., 2014). Our results are in agreement with these findings with an increase in chlorophyll content by SA application. Therefore, plant survival, development and production are primarily dependent on improved chlorophyll content. Our results showed that foliar application of SA significantly increased chlorophyll and carotenoid content under control and *L. amnigena* stress. The infection of *L. amnigena* decreased the leaf number, leaf area, water and nutrient uptake which directly affected the chlorophyll and carotenoid content. However, the SA treatment through

Figure 3. Effect of SA on (A) Plant height, (cm) (B) number of leaves, (C) leaf area (cm²), (D) fresh weight (g) and (E) dry weight (g) under *L. amnigena* (Strain PC3) stress, where CK represents control treatment with distilled water. Data represent mean $\pm$ SE of three replicates. Lower case letters indicate statistical significance between treatments by least significant difference (LSD) test ($p < 0.05$). SA, salicylic acid.

Figure 4. Effect of SA on (A) chlorophyll a (B) chlorophyll b, (C) total chlorophyll a + b, (D) carotenoid under *L. amnigena* (Strain PC3) stress, where CK represents control treatment with distilled water. Data represent mean $\pm$ SE of three replicates. Lower case letters indicate statistical significance between treatments by LSD test ($p < 0.05$). SA, salicylic acid.

Figure 5. Effect of SA on (A) MDA content, (B) H₂O₂ content of potato leaves under *L. amnigena* (Strain PC3) stress, where CK represents control treatment with distilled water. Data represent mean $\pm$ SE of three replicates. Lower case letters indicate statistical significance between treatments by LSD test ($p < 0.05$). H₂O₂, hydrogen peroxide; MDA, malondialdehyde; SA, salicylic acid.

Table 4. Pearson's correlation matrix among some parameters measured in the plant.

Traits	Chlorophyll	H ₂ O ₂	MDA	Carotenoid
Chlorophyll	–			
H ₂ O ₂	–0.948**	–		
MDA	–0.985**	0.975**	–	
Carotenoid	0.987**	–0.884*	–0.951**	–

*Means significant difference at 5%

**Means significant difference at 1%.

H₂O₂, hydrogen peroxide; MDA, malondialdehyde.

spraying reversed the aforementioned effects of the pathogen. Our results suggest that the application of SA decreased oxidative stress and increased the leaf surface area, and improved the closure and opening of the stomata which modulated carbon dioxide absorption. These facilitated photosynthetic activities of the plant. At the same time, a reduction in the activation of chlorophyll-degrading enzymes was observed in previous studies using SA to enhance plant photosynthesis under different conditions (Van Oosten et al., 2017). The reduction of carotenoid content under stress has been associated with the degradation of β -carotene (Sultana et al., 1999). Under stress, the reduction of carotenoids could be related to their protective function in the photosynthetic apparatus, as carotenoids are responsible for scavenging ROS, preventing lipid peroxidation and ultimately mitigating oxidative stress (Koyro, 2006). Our results are in agreement with the findings of Shafiei et al. (2019) who reported that the application of 2 mM SA mitigated the negative effects of stress in young olive trees by improving chlorophyll content, increasing total soluble carbohydrate and proline content of leaves, which may lead to osmotic adjustment.

MDA and H₂O₂ levels increased significantly in the treatment with *L. amnigena* compared with the water treatment and the treatments with SA in this study. Foliar application of SA reduced MDA and H₂O₂ levels in all cases when compared with the corresponding *L. amnigena* treatment. *L. amnigena*-stressed potato plants showed a higher accumulation of ROS content and thus suffered oxidative damage. Consequently, the increased H₂O₂ formation resulted in higher MDA content. While ROS can help improve the resistance of plant tissues to disease, high ROS levels can cause lipid peroxidation and lead to loss of membrane integrity of plant organs. Previous studies have demonstrated the effectiveness of SA treatment in reducing the lipid peroxidation level of plant tissues under stress compared to control plants (Hayat et al., 2007). Based on all the above results, the beneficial effects of treatments with SA could be attributed to the reduction in the accumulation of H₂O₂ and MDA levels, possibly by increasing the activity of antioxidant enzymes. Our results are in agreement with those of Sayyari et al. (2013) and Estaji (2020) who reported that application of SA significantly decreased MDA accumulation and H₂O₂ under drought stress.

The correlation results of our study showed that the physiological and molecular responses in potato plants under *L. amnigena* stress treated with SA have both positive and negative associations. MDA and H₂O₂ content in the leaves were found to be considerably higher, lowering chlorophyll and carotenoid contents. The content of MDA and H₂O₂ was shown to be strongly inversely related to the content of chlorophyll and carotenoid. Our results are consistent with those of Wei et al. (2021) who found that higher MDA and H₂O₂ content in wheat plants resulted in lower chlorophyll and carotenoid contents.

Figure 6. Effect SA on (A) SOD activity, (B) POD activity, (C) CAT activity and (D) PPO activity of potato leaves under *L. amnigena* (Strain PC3) stress, where CK represents control treatment with distilled water. Data represent mean $\pm$ SE of three replicates. Lower case letters indicate statistical significance between treatments by LSD test ($p < 0.05$). CAT, catalase; POD, peroxide; PPO, polyphenol oxidase; SA, salicylic acid; SOD, superoxide dismutase.

In our study, treatment with SA increased the activities of SOD, POD, PPO and CAT. It has been shown that SA plays a significant role in the induction of systemic acquired resistance (SAR) of plants (Hayat et al., 2010). In support to our findings, other previous studies also found that enzymatic and non-enzymatic antioxidants were increased during SA treatments on crop plant (Sofy, 2015; Chen et al., 2016; Razmi et al., 2017; Moharramnejad et al., 2019). However, the SA and *L. amnigena* combined treatment increased enzymatic activities higher than the control. The corresponding mechanism was that SA could move freely between cells, tissues and organs as a long-range mediator (Kumar, 2014), to stimulate the activities of one or more antioxidant enzymes and then increase plant tolerance to overcome oxidative stress induced by various biotic and abiotic stresses (Idrees et al., 2011; Ibrahim et al., 2017). Therefore, SA reduced excess ROS by up-regulating antioxidant protection systems during *L. amnigena* stress. Our study is consistent with the study of Shen et al. (2014) and Ma et al. (2017), who reported that SA significantly increased antioxidant enzyme activities under drought and salt stress.

It has been reported that exogenous SA application induced the expression of defence-related genes that were systematically activated after infection by tobacco mosaic virus (TMV) (Nandi and Babu, 2013). Similarly, our results showed that the level of expression of SOD, POD, CAT and GST genes in potato leaves under *L. amnigena* was increased by SA treatment compared to the control. Also, the transcription levels of SOD, POD, CAT and GST genes were significantly increased and up-regulated under *L. amnigena* stress, following the corresponding antioxidant enzyme activity indicating their role against oxidative stress. SA is an endogenous signal for the activation of certain plant defence responses, including pathogenesis-related (PR) gene expression and the establishment of stress resistance (Klessig et al., 2018). A significant increase in gene expression after SA application under pathogen stress helped to reverse the effects of pathogen-induced ROS on growth and photosynthesis. Our results are consistent with previous studies by Morris et al. (2000), who reported that SA treatment increases gene expression during leaf senescence.

Figure 7. Effect of SA on expression patterns of SOD (A), POD (B), CAT (C), and GST (D) in leaves of potato plants under *L. amnigena* (Strain PC3) stress, where CK represents control treatment with distilled water. Data represent mean $\pm$ SE of three replicates. Lower case letters indicate statistical significance between treatments by LSD test ($p < 0.05$). CAT, catalase; GST, glutathione S-transferase; POD, peroxide; SA, salicylic acid; SOD, superoxide dismutase.

CONCLUSIONS

Our results suggest that SA is a plant growth-promoting hormone that can prime plant to tolerate stresses *in vitro* and *in vivo*, and induce defence in plant by attenuating the negative effects of pathogens. Rigorous physiological, biochemical and molecular assays used in the study allowed us to explore the possible mechanisms and pathways in which SA attenuates the suppressive effects of *L. amnigena* in both potato tuber and plant growth. The pathway include SA pathway. The mechanism involves are: (i) reduction in exoenzymes production, (ii) reduction of bacterial fission, growth and mobility (iii) antioxidant defence system and expression increase of plant growth. The reduced activity of extracellular enzymes from 1.5 mM to 2.0 mM SA also reduced pathogenicity in the tubers against soft rot, indicating that, the exogenously application of SA induces many

defence responses of the plant that affect the invasion of plant pathogens. Carotenoids are pigments distributed throughout the plant that is necessary for photosynthesis and absorb energy from the sun's blue-green spectrum and transfer it to chlorophylls, thereby increasing the wavelength range of light. According to our findings, *L. amnigena* stress caused significant decreases in chlorophyll and carotenoid contents. Based on the results, foliar application SA (1.5–2.0 mM) on potato plant leaves increased the chlorophyll and carotenoid content, also increased the activity of antioxidant enzymes and reduced oxidative stress (MDA and H_2O_2) thereby increasing plants defence stress. Management of soft rot disease in potato tubers caused by *L. amnigena* can be considered based on our results using a threshold from 1.5 mM to 2.0 mM SA before storage or planting. Extended field trials are needed to further optimise the use of SA to control soft rot caused by *L. amnigena*.

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AUTHOR CONTRIBUTIONS

All authors contributed extensively to the work presented in this article. R.O, C.Y. and S.B. designed the experiment. S.B and L.C. assembled input data and L.W. wrote code. R.O., M.J and S.B. analysed output data. C.Y. provided experiment material. R.O. administered the experiments and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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