



Article Effects of Combined Application of Salicylic Acid and Proline on the Defense Response of Potato Tubers to Newly Emerging Soft Rot Bacteria (*Lelliottia amnigena*) Infection

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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Abstract: Potato soft rot, caused by the pathogenic bacterium Lelliottia amnigena (Enterobacter amnigenus), is a serious and widespread disease affecting global potato production. Both salicylic acid (SA) and proline (Pro) play important roles in enhancing potato tuber resistance to soft rot. However, the combined effects of SA and Pro on defense responses of potato tubers to L. amnigena infection remain unknown. Hence, the combined effects of SA and Pro in controlling newly emerging potato soft rot bacteria were investigated. Sterilized healthy potato tubers were pretreated with 1.5 mM SA and 2.0 mM Pro 24 h before an inoculation of 0.3 mL of *L. amnigena* suspension (3.69×10^7 CFU mL⁻¹). Rotting was noticed on the surfaces of the hole where the L. amnigena suspension was inoculated. Application of SA and Pro with L. amnigena lowered the activity of pectinase, protease, pectin lyase, and cellulase by 64.3, 77.8, 66.4 and 84.1%, and decreased malondialdehyde and hydrogen peroxide contents by 77.2% and 83.8%, respectively, compared to the control. The activities of NADPH oxidase, superoxide dismutase, peroxide, catalase, polyphenol oxidase, phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase, 4-coumaryl-CoA ligase and cinnamate-4-hydroxylase were increased in the potato tubers with combined treatments by 91.4, 92.4, 91.8, 93.5, 94.9, 91.3, 96.2, 94.7 and 97.7%, respectively, compared to untreated stressed tubers. Six defense-related genes, pathogenesis-related protein, tyrosine-protein kinase, Chitinase-like protein, phenylalanine ammonia-lyase, pathogenesisrelated homeodomain protein, and serine protease inhibitor, were induced in SA + Pro treatment when compared with individual application of SA or Pro. This study indicates that the combined treatment of 1.5 mM SA and 2.0 mM Pro had a synergistic effect in controlling potato soft rot caused by a newly emerging bacterium.

Keywords: antioxidant enzymes; extracellular enzymes; pathogen; reactive oxygen species; systemic acquired resistance

1. Introduction

According to Lim et al. [1], potato is the fourth most important food crop and staple food in the world. Potato tubers are in most cases stored for 3–6 months before being processed and/or consumed by humans. The average annual loss due to potato soft rot is estimated to be between 6 and 25%, with up to 60% of tubers in some cases being damaged [2,3]. One of the most common potato diseases that reduce tuber quality during storage is soft rot caused by *Pectobacterium carotovorum* subsp. *Carotovorum* [4]. Postharvest diseases in fruits and vegetables are caused by bacteria during storage. Throughout the growing season and during storage, members of the families *Enterobacteriaceae* and *Pectobacteriaceae* are the primary cause of potato soft rot [4]. These pathogens cause infection either during the preharvest stage in the field or after harvesting during storage and transportation. Soft rot bacteria degrade pectate molecules, which bind plant cells together, eventually causing the plant structure to fall apart. Potato wounds or damage are the primary sources of soft rot bacteria invasion. This usually happens during harvesting and grading, allowing bacteria to invade the tuber [5]. When this is combined with water on the tuber's surface, the bacteria can overcome the tuber's natural defenses and initiate tuber rot. Soft rot pathogens produce many enzymes capable of degrading the plant cell wall other than saprophytic pectolytic bacteria. These enzymes include pectinases, cellulases, proteases, pectin lyase, and xylanases, each with its own set of properties [6]. Due to its ability to synthesize a broader variety of isoenzymes faster and in greater amounts than pectolytic saprophytic bacteria, *L. amnigena* enters tissues more easily and causes infection [7,8].

Chemical control is an important method of controlling plant diseases. However, because of their negative effects on humans and the environment, synthetic bactericides are not the preferred method for controlling plant pathogens [9]. As a result, an eco-friendly alternative strategy for bacterial soft rot management must be developed. Increased natural defense system of plants is one of the promising environmentally friendly methods for postharvest disease control [10,11]. The activation of plant defense systems that help to delay the spread of various pathogens can help to protect the plant from bacterial pathogens [11]. Induced disease resistance in plants is a viable option for preventing the invasion of bacterial pathogens and an appealing disease control strategy [12].

Salicylic acid (SA) and proline (Pro), which induce natural resistance in plants to bacterial infections and can provide year-round protection, can be considered a promising alternative to the use of synthetic bactericides [13]. SA is essential for plant growth, development, and defense responses, and can also be used to inhibit microbial growth [14]. Plant resistance to pathogens is induced by SA application via mechanisms such as oxidative burst, cell wall reinforcement, and gene expression regulation [13]. SA is important in adaptable interactions, in which a small amount of SA controls the expression of a collection of defense-related genes, resulting in a defense-like response [15]. Several studies [16,17] show that applying SA to tomatoes can improve their resistance to *Ralstonia solanacearum* in the greenhouse or the field. According to previous studies, SA is an essential elicitor that triggers plant resistance to pathogens such as bacteria and fungi [18,19]. SA has been identified to mediate resistance in a variety of plant-pathogen interactions. Depending on the pathogen, SA can prevent pathogen proliferation and cell-to-cell or long-distance pathogen migration [20]. As a result, SA has been demonstrated to be a potential compound for inhibiting postharvest fungal pathogens and thus improving fruit postharvest quality. For example, SA treatments effectively controlled postharvest damage in the cases of Colletotrichum gloeosporioides on mango [21], Penicillium expansum on sweet cherry [22] and peach [23], Botrytis cinerea on peach [24], and Monilinia spp. on sweet cherry [22], apricot [25], and nectarine [26].

Pro is a water-soluble amino acid that is essential and multifunctional in plants, and accumulates in high contents under stress [27,28]. Pro as a proteinogenic amino acid naturally increases in response to biotic and abiotic stress by increasing Pro synthesis or decreasing Pro degradation [27]. Pro is essential for maintaining osmotic balance, preserving the structure of enzymes and membranes in key proteins, protecting photosynthetic products, and scavenging free radicals [29,30]. Pro application improves plant resistance to both biotic and abiotic stresses according to many studies [31,32]. Pro is a widely studied and used osmoprotectant under stress conditions, and it has been found to mitigate the effect of stress in plants [31,33–35]. Many studies on the effects of Pro on various crops under oxidative stress have been carried out. For example, Pro application in rose, rice seedlings, chickpeas, and citrus improved stress tolerance by increasing the activity of antioxidant enzymes, decreasing membrane lipid peroxidation, and retaining ascorbic acid content as non-enzymatic components of the antioxidant system [36]. As far as we know, many reports have been published evaluating the impact of SA and Pro applied as single treatments on crops growing under biotic and abiotic stress environments. However, there is no study on SA and Pro treatment combinations to ameliorate the effects of L. amnigena on potato tubers. We hypothesized that SA and Pro can control potato soft rot bacteria, L.

amnigena due to their antibacterial properties. As a result, this study aimed to evaluate the combined effects of SA and Pro in controlling potato soft rot caused by a newly emerging bacterium (*L. amnigena*).

2. Materials and Methods

2.1. Source of Materials

Potato tubers, var. Atlantic, were obtained from the production field in Lanzhou, Gansu Province, China. *Lelliottia amnigena* (PC3) was obtained from the Plant Pathology Laboratory, Gansu Agricultural University, Lanzhou, China [37]. *Lelliottia amnigena* was cultured on a nutrient agar (NA) medium (3.0 g peptone, 4.0 g glucose, 9.0 g agar, 1.5 g beef extract, and 500 mL water) in Petri dishes for 2 d at 28 °C. The bacterial inoculum was prepared from the 2-d-old cultured bacteria according to the method of Ben-David and Davidson [38]. The bacterial inoculum (3.69 × 10⁷ CFU mL⁻¹) was quantified and stored at 4 °C. SA and Pro were purchased from Sangon Biotech Company Limited, Shanghai in China. 1.5 mM SA was chosen based on our previous study [39], and 2.0 mM Pro concentration was prepared following the method described by Perveen and Nazir [40], with a few modifications.

2.2. Experimental Design

The two independent experiments were arranged in a randomized complete block design using a factorial experiment with three replications of each treatment. The treatments were: (i) sterilized distilled water (negative control potato tubers treated with water without *L. amnigena* and SA and Pro treatments); (ii) *L. amnigena* (PC3) (positive control potato tubers treated with *L. amnigena* without SA and Pro treatments); (iii) *L. amnigena* + 1.5 mM SA; (iv) *L. amnigena* + 2.0 mM Pro; and (v) *L. amnigena* + 1.5 mM SA + 2.0 mM Pro. SA and Pro were purchased from Sangon Biotech Co., Ltd., Shanghai and Sigma (C₅H₉NO₂, CAS No. 147-85-3) Shanghai, China, respectively.

2.3. Effect of SA and Pro on Extracellular Enzyme Production by L. amnigena

The *L. amnigena* culture was inoculated into 250 mL of bacterial liquid (1.5 g peptone, 2.0 g glucose, 0.75 g beef extract, and 250 mL water) with and without SA and Pro, and kept for 2 d at 37 °C in an Honour Instrument Shaker Machine (HNYC-202T, Guangdong, China). One mL for each test of cultured bacterial liquid was taken and centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants were collected to perform the production levels of protease, pectinase, pectin lyase, and cellulase, following protocol assay kits (Solarbio Science and Technology Co., Ltd., Beijing, China). Pectinase, protease, pectin lyase, and cellulose were measured at 540, 450, 235 and 540 nm, respectively, using a spectrophotometer (EPOCH2 Plate Reader, BioTek, Santa Clara, CA, USA). The activities were expressed as U mL⁻¹. Each activity was carried out three times.

2.4. Effect of SA and Pro on Potato Soft Rot

The combined effect of SA and Pro on the severity of potato soft rot was investigated in this experiment. The experiment was carried out in a laboratory. Uniform healthy potato tubers were sterilized for 1 min with 75% ethanol before being washed three times in distilled water. The tubers were dried at room temperature. Using a sterile cork borer (BML505-15 mm, Wuhan Servicebio Technology Co., Ltd., Wuhan, China), a hole (about 5 mm in diameter and 10 mm in depth) was made in the center of each sterilized tuber. The tubers were pretreated with 1.5 mM SA and 2.0 mM Pro by pipetting them into the holes of the tubers using a 1000 μ L Filtered Pipette Tip (ISO9001:2015, Wuhan Servicebio Technology Co., Ltd., Wuhan, China) 1 d before inoculation with the *L. amnigena* inoculum. A total of 0.3 mL of the *L. amnigena* inoculum was pipetted into the holes of the pretreated and untreated tubers with a 1000 μ L Filtered Pipette Tip, and water as a control (CK). The inoculated tubers were placed in sterilized sealed plastic containers packed with sterilized moist cotton (moderate) and kept for 7 d at room temperature. The experiment was repeated with three replicates and for each experiment; 15 tubers were used for all the treatments.

2.5. Disease Assessment

The disease index (DI) of the tubers was measured up to 7 d after inoculation with PC3 inoculum. The disease index was determined using a method described by Scherf et al. [41], with a 5 degrees scale (0–4), where 0 = no disease, 1 = trace to 25% of the tubers were rotted, 2 = 26%–50% of the tubers were rotted, 3 = 51%–75% of the tubers were rotted, and 4 = 76%–100% of the tubers were rotted. The DI was determined using the formula DI (%) = [\sum (number of diseased tubers × disease index)/(total number of tubers investigated × highest disease index)] × 100.

2.6. Sampling

Tissues were collected from inoculated tubers according to Zhang et al. [42] after 7 d of treatment with SA and Pro using a sterilized sharp blade. Tissues of untreated tubers were collected. Liquid nitrogen (Henan Boss Liquid Nitrogen Container Co., Ltd., Dongtai, China, 78.0% by volume, 75.5% by weight) was used to freeze the collected tissues, ground with a pestle and mortar into a powdery form, and then stored at -80 °C until use.

2.7. Malondialdehyde and Hydrogen Peroxide Content in Potato Tuber

The malondialdehyde (MDA) content was determined following the assay kit provided (BC0025, Solarbio Science and Technology Co., Ltd., Beijing, China). In brief, 0.1 g of frozen potato tuber was ground in liquid nitrogen (Henan Boss Liquid Nitrogen Container Co., Ltd., Dongtai, China, 78.03% by volume, 75.5% by weight). One mL of extract solution was added and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Regents were added according to the manufacturer's instructions. MDA was measured at 600, 532 and 450 nm, and expressed as µmol kg⁻¹ FW. This was repeated three times. The hydrogen peroxide (H₂O₂) content was estimated following the protocol of the assay kit provided (BC3595, Solarbio Science and Technology Co., Ltd., Beijing, China). In brief, 0.1 g of frozen potato tuber was crushed in liquid nitrogen and placed on an ice bath in 1 mL of acetone. One mL of the extract solution was added and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Other regents were added according to the manufacturer's instructions at 415 nm and expressed as µmol kg⁻¹ FW. All the results are expressed on a fresh weight (FW).

2.8. Assay of Some Enzymatic Activities

NADPH oxidase (NOX; EC 1.6.3.1) activity was measured following the protocol of the assay kit provided (BC0630, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen. 1 mL of extract solution was added to 0.1 g of frozen powder of potato tuber and centrifuged at $600 \times g$ for 5 min at 4 °C. The supernatant was transferred to another centrifuge tube and centrifuged at $11,000 \times g$ for 10 min at 4 °C. The various reagents were added as instructed by the manufacturer. The absorbance (OD) value was determined at 600 nm, then NOX activity was expressed as U g kg⁻¹ FW. Peroxidase (POD; EC 1.11.1.7) activity was measured using the instructions of the assay kit provided (BC0090, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen. One mL of the extract solution was added to 0.1 g of frozen powder of potato tuber and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Reagents were added as instructed. The OD value was measured at 470 nm using a spectrophotometer and POD activity was expressed as U g kg⁻¹ FW.

Catalase (CAT; EC 1.11.1.6) activity was analyzed following the instructions of the assay kit provided (BC0200, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen. One mL of the extract solution was added to 0.1 g of frozen powder of potato tuber and centrifuged at $8000 \times g$

for 10 min at 4 °C, and the supernatant was collected. Reagents were added as instructed. The OD value was performed spectrophotometrically at 240 nm and CAT activity was expressed as U g kg⁻¹ FW. Superoxide dismutase (SOD; EC 1.15.1.1) activity was analyzed using the instructions of the assay kit provided (BC0170, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen and 1 mL of the extract solution was added, then centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Reagents were added as instructed by the manufacturer. The OD value of SOD was measured at 560 nm and SOD activity was expressed as U g kg⁻¹ FW. Polyphenol oxide (PPO; EC 1.14.81.1) activity was analyzed according to the protocol of the assay kit provided (BC0195, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen. One mL of the extract solution was added and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Reagents were added as instructed by the manufacturer. The OD value of SOD was measured in liquid nitrogen. One mL of the extract solution was added and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Reagents were added as instructed by the manufacturer. The OD value of PPO was measured at 420 nm and PPO activity was expressed as U g kg⁻¹ FW.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was determined using the protocol of the assay kit provided (BC0210, Solarbio Science and Technology Co., Ltd., Beijing, China). 0.1 g of the frozen potato tuber was ground in liquid nitrogen, 1 mL of the extract solution was added and centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. The OD of PAL was measured spectrophotometrically at 290 nm, then expressed as U g kg⁻¹ FW. Cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) activity was determined according to the instructions of the assay kit provided (BC4170, Solarbio Science and Technology Co., Ltd., Beijing, China). One mL of extract solution was added to 0.1 g of frozen powder of potato tuber, then centrifuged at $10,000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Other reagents were added as instructed by the manufacturer. The OD was determined at 340 nm and CAD activity was expressed as U g kg⁻¹ FW. 4-coumaryl-CoA ligase (4CL; EC 6.2.1.12) activity was assayed following the instructions of the assay kit provided (BC4220, Solarbio Science and Technology Co., Ltd., Beijing, China). One mL of the extract solution was added to 0.1 g of frozen powder of potato tuber, then centrifuged at $8000 \times g$ for 10 min at 4 °C, and the supernatant was collected. Other reagents were added as instructed by the manufacturer. The OD was measured at 333 nm and 4CL activity was expressed as U g kg⁻¹ FW. Cinnamate-4hydroxylase (C4H; EC 1.14.13.11) activity was determined using a kit provided (BC4080, Solarbio Science and Technology Co., Ltd., Beijing, China). One mL of the extract solution was added to 0.1 g of frozen powder of potato tuber, then centrifuged at $12,000 \times g$ for 15 min at 4 °C, and the supernatant was collected. Regents were added as instructed The OD was measured at 340 nm and expressed as U g kg⁻¹ FW.

2.9. Quantitative Real-Time (qRT) PCR Analysis

Quantitative RT-PCR (ABI 7500, Applied Biosystems, Foster City, CA, USA) was conducted to evaluate gene expression (NOXB, PAL, CAD, 4CL, and C4H) and plant defense-related genes [pathogenesis-related protein (PR1), tyrosine-protein kinase (PR2), phenylalanine ammonia-lyase (PAL), Chitinase-like protein (CTL1), pathogenesis-related homeodomain protein (PRH3), and serine protease inhibitor (SPI1)] in the potato tubers subjected to SA and Pro treatments under L. amnigena stress. A PureLink® RNA Mini Kit (Tiangen Biotechnology, Beijing, China) was used to extract total RNA. A Nano-Drop spectrophotometer was used to measure the quantity and quality of isolated RNA at absorbances of 230 and 260 nm. The A_{260}/A_{280} ratio showed that the RNA was not contaminated with proteins. The Revert AidTM First Strand cDNA Synthesis Kit was used for first-strand cDNA synthesis (Tiangen Biotechnology, Beijing, China). The 23 μ L reaction of cDNA contained 0.5 μ L of RNA, 2 μ L oligo (dT), 4 μ L 5 \times M-MLV buffer, 1 μ L dNTPs, $0.5 \,\mu\text{L}$ RNasin, 1 μL M-MLV, and 14 μL of ddH₂O. The primer sequences and NCBI gene IDs are presented in Table 1. qRT–PCR was determined using $2 \times$ SYBR Green qPCR Master Mix (Shanghai LZ Biotech Co., Ltd., Shanghai, China). The 20 µL reaction mixture contained 1 μ L of each primer, 1 μ L of cDNA, 0.4 μ L of ROX reference dye, 10 μ L 2 \times SYBR

Green qPCR Master Mix, and 6.6 μ L of ddH₂O, and was determined. Using potato actin, relative expression levels were calculated using the formula $2^{-\Delta\Delta CT}$ [43]. For each gene, three biological replicates were used.

Gene Symbol	Description	Primer Sequence (5'-3')	Gene ID	Activity
Act	Actin	F: ACAATGCTTGCACGTTTCCTC	102605823	Antioxidant
		R: TTAGCTGGGACCATTGCCTG		
NOXB	NADPH oxidase	F: CATTGCTTCTTCAGGCTCCG	111509039	Antioxidant
		R: CCACAAAGCCATCACCCAAA		
PAL	Phenylalanine ammonia-lyase	F: GAGGAGTATAGGAAGCCGG	102596017	Antioxidant
		R: CTCATCCCTTCCATCACCCA		
CAD	Cinnamyl alcohol	F: GGCCTGATGATGTGCAAGTC	102584791	Antioxidant
	dehydrogenase	R: CCAACAAGCAATCCAACTCCA		
4CL	4-counmaryl-CoA ligase	F: GCCCTGAATTTGTGTTTGCG	102596056	Antioxidant
		R: CCTTCACTTTCCCCGCAAAA		
C4H	Cinnamate-4-hdroxylase	F: AGTCTGAGGCTGCTAGTGT	817599	Antioxidant
		R: GAGTCTGAGGCTGCTAGTGT		
PR 1	Pathogenesis-related protein	F: GCCAATCCAGGCTGTAGCA	102580826	Plant defense
		R: AGTGGGGAAGAAGAATGTGGAC		
PR 2	Tyrosine-protein kinase	F: ACCGCCTTCGAGAACTAGAG	111517981	Plant defense
		R: CCACAAACTTGCCATATCACCA		
PRH3	Pathogenesis-related homeodomain protein	F: GCAAAGGGGAAGCTGGGTAA	102596310	Plant defense
		R: TGTTACTTTCAGCTGCATCCTCT		
CTL1	Chitinase-like protein 1	F: ATTACGGTCGTGGTGCCTTG	102595303	Plant defense
		R: ATCTGCAACTGCTTTCCGTG		
PAL	Phenylalanine ammonia-lyase	F: TGGTGGTGCCCTTCAAAAAG	102596017	Plant defense
		R: CGTAGCTTGTTATGTCATGATGAT		
SPI1	Serine protease inhibitor-1	F: TAGGTGGCCAGAACTGGTTG	823839	Plant defense
		R: TGTGTTAGCGATTGTCCTTCGA		
Act	Actin	F: ACAATGCTTGCACGTTTCCTC	102593148	Plant defense
		R: TTAGCTGGGACCATTGCCTG		

Table 1. Gene description, primers sequences, and NCBI gene ID for the genes used for the qRT–PCR.

2.10. Statistical Analysis

The data were subject to one-way ANOVA using the SPSS package (SPSS V16.0; SPSS, Inc., Chicago, IL, USA). Treatment effects were determined using Duncan's multiple range test and significant results were expressed at p < 0.05.

3. Results

3.1. Effect of SA and Pro on Extracellular Enzyme Production by L. amnigena

The results of our study show that SA and Pro affected the synthesis of pectinase, protease, pectin lyase, and cellulase, which are virulence factors in *L. amnigena*. The application of SA and *L. amnigena* (PC3) (SA + PC3) lowered the production of pectinase, protease, pectin lyase, and cellulase by 55.6, 73.1, 55.1, and 62.5%, respectively, compared to the control (Figure 1). In addition, co-cultured Pro and PC3 (Pro + PC3) decreased pectinase, protease, pectin lyase, and cellulase by 40.7, 64.5, 53.3, and 34.1%, respectively. However, the combined SA and Pro with PC3 (SA + Pro + PC3) reduced pectinase, protease, pectin lyase, and cellulase by 64.3, 77.8, 66.4, and 84.1%, respectively, compared to the control (Figure 1).



SAPPCS

Figure 1. Effect of salicylic acid (SA) and proline (Pro) on Protease (A), Pectin lyase (B), Cellulase (C), and Pectinase (D) production by L. amnigena. Data are presented as mean \pm standard error (SE) of two independent experiments performed in three replicates. Means with the same lowercase letters are not significantly different at p < 0.05 according to Duncan's multiple range test. PC3–L. annigena.

203

SA PROPRCS

ProtPC3

3.2. Disease Assessment

SATROS

150_L

100

50

0

1.0_[(C) a

0.8

0.6

0.4

0.2

0.0

RC3

2°3

Protease activity (U/mL)

Cellulase activity (U/mL)

(A)

а

The results show that SA or Pro and their combined treatments reduced the disease index of potato soft rot. However, L. amnigena-treated tubers experienced a high incidence of soft rot compared to potato tubers treated with SA and Pro (Figure 2). The results show that applied SA reduced the disease index by 67.9% after 6 d of treatment. In addition, applied Pro reduced the disease index by 64.6% after 6 d of treatment. However, the combined application of SA and Pro reduced the disease index by 72.5% after 6 d of treatment. The combination of SA and Pro treatment provided better disease control than either SA or Pro application alone (Figure 2).



Figure 2. Effect of salicylic acid (SA) and proline (Pro) on the disease index of tubers inoculated with L. amnigena (PC3). Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at p < 0.05 according to Duncan's multiple range test.

40

20

n

d

SA PROPRCS

ProfPC3

3.3. Effects of SA and Pro on MDA and H_2O_2 Content in Potato Tubers Inoculated with L. amnigena

Our results in Figure 3 show that *L. amnigena* stress significantly increased MDA and H_2O_2 contents in potato tubers by 50.0 and 40.7%, respectively, compared to the control. In Figure 3, the beneficial impact of SA or proline or SA + Pro on decreasing oxidative stress and MDA and H_2O_2 is observed. These treatments resulted in a significant decrease in MDA and H_2O_2 , and the best treatment was SA + Pro (77.2 and 83.8%), followed by SA then Pro (Figure 3).



Figure 3. Effect of salicylic acid (SA) and proline (Pro) on the MDA (**A**) and H_2O_2 (**B**) content of potato tubers under *L. amnigena* (PC3) stress, where CK represents the control treatment with distilled water. Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at *p* < 0.05 according to Duncan's multiple range test.

3.4. Effects of SA and Pro on NOX, SOD, POD, PPO, and CAT Activity in Potato Tubers Inoculated with L. amnigena

The activities of NOX, SOD, POD, CAT, and PPO as stress indicators were higher in potato tubers exposed to *L. amnigena* than in controls. The data presented in Figure 4A–E show that antioxidant enzymes NOX, SOD, POD, PPO, and CAT activity significantly increased by 69.3, 80.9, 78.0, 86.0, and 83.0%, respectively, in potato tubers under *L. amnigena* stress treated with SA, respectively, compared with untreated stressed tubers. In addition, Pro-treated tubers increased NOX, SOD, POD, CAT, and PPO activity by 62.1, 69.8, 70.4, 66.9, and 73.9%, respectively, compared with untreated stressed tubers. However, the best results of NOX, SOD, POD, CAT, and PPO activity were recorded with SA + Pro treatment compared with untreated stressed tubers (Figure 4A–E).



Figure 4. Effect of salicylic acid (SA) and proline (Pro) on NOX (A), SOD (B), CAT (C), POD (D), and PPO (E) activities in potato under *L. amnigena* (PC3) stress, where CK represents the control treatment with distilled water. Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at *p* < 0.05 according to Duncan's multiple range test.

3.5. SA and Pro Treatment Increased PAL, CAD, 4CL, and C4H Activity in Potaato Tubers Inoculated with L. amanigena

In the current study, the results show that PAL, CAD, 4CL, and C4H activities increased significantly by exposure to *L. amnigena* stress (Figure 5). However, the application of SA led to an increase in PAL, CAD, 4CL, and C4H activities by 77.7, 73.6, 79.1, and 76.0%, respectively, compared with untreated stressed tubers. Additionally, application of Pro increased PAL, CAD, 4CL, and C4H activities by 70.4, 60.4, 72.1, and 64.9%, respectively, as compared to the control. Similarly, the activities of PAL, CAD, 4CL, and C4H were higher in the SA + Pro treatments than in the two single treatments (Figure 5).



Figure 5. Effect of salicylic acid (SA) and proline (Pro) on CAD (**A**), PAL (**B**), C4H (**C**), and 4CL (**D**) activities in potato tubers under *L. amnigena* (PC3) stress, where CK represents the control treatment with distilled water. Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at *p* < 0.05 according to Duncan's multiple range test.

3.6. SA and Pro Treatment Up-Regulated NOX, PAL, CAD, 4CL, and C4H Genes in Potato Tubers Inoculated with L. amnigena

When compared to untreated stressed tubers, the NOX, PAL, CAD, 4CL, and C4H transcripts were significantly induced in the SA, Pro, and SA + Pro treatments (Figure 6A–E). SA treatment increased the expression of NOX, PAL, CAD, 4CL, and C4H genes 2.7, 2.8, 1.8, 1.8, and 1.6-fold, respectively, compared with untreated stressed tubers. Similarly, Pro-treated tubers elevated the expression of NOX, PAL, CAD, 4CL, and C4H genes 2.5, 2.6, 1.7, 1.7, and 1.4-fold, respectively, compared with untreated stressed tubers. However, in the SA + Pro treatments, the transcriptional levels of NOX, PAL, CAD, 4CL, and C4H genes were significantly higher (3.1, 3.1, 2.2, 2.5, and 2.1-fold), respectively, compared to the control (Figure 6A–E).



Figure 6. Effect of salicylic acid (SA) and proline (Pro) on the gene expression of PAL (**A**), NOX (**B**), 4CL (**C**), C4H (**D**), and CAD (**E**) in potato tubers under *L. amnigena* (PC3) stress, where CK represents the control treatment with distilled water. Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at *p* < 0.05 according to Duncan's multiple range test.

3.7. Effects of Combined SA and Pro Treatment on the Expression Levels of Plant Defense-Related Genes in Potato Tubers Inoculated with L. amnigena

SA, Pro, and SA + Pro treatments affected plant defense-related genes (PR1, PR2, CTL1, PAL, PRH3, and SPI1) (Figure 7A–F). The results show that PR1, PR1, CTL1, PAL, PRH3, and SPI1 genes were elevated by exposure to *L. amnigena* stress (Figure 7A–F). However, treatment of *L. amnigena*-infected tubers with SA increased the transcription levels of PR1, PR2, CTL1, PAL, PRH3, and SPI1 2.8, 2.0, 2.1, 2.1, 2.5, and 2.7-fold, respectively, compared to the untreated stressed tubers. Pro treatment also increased PR1, PR2, CTL1, PAL, PRH3, and SPI1 2.5, 1.9, 2.0, 2.0, 2.4, and 1.5-fold, respectively, compared to *L. amnigena* alone (Figure 7). However, treatment of combined SA and Pro further increased PR1, PR2, CTL1, PAL, PRH3, and SPI1 3.1, 2.5, 2.6, 2.4, 3.0, and 2.9-fold, respectively, compared to the control.



Figure 7. Effect of salicylic acid (SA) and proline (Pro) on the relative expression of pathogenesisrelated protein (PR1) (**A**), tyrosine-protein kinase (PR2) (**B**), Chitinase-like protein (CTL1) (**C**), phenylalanine ammonia-lyase (PAL) (**D**), pathogenesis-related homeodomain protein (PRH3) (**E**), and serine protease inhibitor (SPI1) (**F**) in potato tubers under *L. amnigena* (PC3) stress, where CK represents the control treatment with distilled water. Data are presented as mean \pm standard error (SE), based on two independent experiments with three replicates. Means with the same lowercase letters are not significantly different at *p* < 0.05 according to Duncan's multiple range test.

4. Discussion

Extracellular enzymes can be used as a primary mechanism by pathogens, including bacteria, to develop plant diseases. The activity of the extracellular enzymes permits the pathogen to penetrate host tissues by breaking down the host cells' defensive outer layers. Extracellular enzymes secreted by microorganisms play an important role in disease progression. These degrade plant materials into smaller particles that pathogens can easily absorb and use for growth and development. In this current study, pectinase, protease, pectin lyase, and cellulase were produced by *L. amnigena*, aiding it to cause potato soft rot. Application of SA or Pro alone to *L. amnigena* reduced the synthesis of pectinase, protease, pectin lyase, and cellulase. However, combined SA and Pro further reduced the production of these enzymes. This means that, while using SA and Pro alone can limit

extracellular enzyme production by *L. amnigena*, combining the two is the most effective. Similarly, Bandara et al. [44] discovered that applied SA had an impact on the secretion of protease and elastase by *Pseudomonas aeruginosa*, which is involved in the development of microbial keratitis. Plant pathogens have evolved complex penetration, invasion, and colonization tactics to disable susceptible hosts' plant defense mechanisms and cause disease [45]. The pathogen normally infects the plant by producing components that are utilized to manipulate plant tissue to get physical access to the tissues and to draw nutrients [46]. These factors allow the plant both to induce disease and to advance the infection into the tissue's core, resulting in a worsening of the disease situation. Pathogens attack plants in different ways. One mode necessitates that the plants and living plant tissues cooperate. Within the invaded plant tissues, the pathogen produces penetration structures and a network of flagella [47]. This mass of cell wall structures generated in the plant's intercellular gaps extracts nutrients from the plant.

The reduction in disease severity caused by SA and Pro could be the result of the antibacterial consequence of SA and Pro, which reduces L. amnigena production of extracellular enzymes. The results show that the treatment of SA and Pro significantly reduced potato soft rot induced by L. amnigena; we suggested that combined SA and Pro treatment in the present study has synergistic effects on the control of potato soft rot caused by L. *amnigena*. Our results could also be attributed to either a direct toxicity effect of SA and Pro on *L. amnigena* growth or an indirect plant defense-related effect by inducing resistance in infected tissues. Although the molecular mechanism of SA and Pro-induced *L. amnigena* resistance is unknown, it appears to include inhibition of pathogen-secreted extracellular enzymes. SA was found to be effective in generating localized acquired tolerance to Pectobacterium carotovora subsp. carotovora infection [48]. Lastochkina et al. [49] previously discovered that SA can boost potato resistance to *F. oxysporum* and *Phytophthora infestans* postharvest disease. Eshgpour et al. [50] also reported that applied SA reduced infection of *Pectobacterium carotovorum* causing potato soft rot in vitro studies, which are similar to the current study's findings. Our findings are comparable with those of Bawa et al. [51], who found that applying SA to soybean seedlings decreased disease severity and induced resistance to Fusarium solani. According to Li and Zou [52], foliar spraying tomato plants with SA at a dosage of 2.0 mM resulted in a substantial reduction in disease severity. According to Yao and Tian [22], the roles of SA in reducing brown rot may be due to the direct toxicity of SA on fungal mycelia and/or an indirect plant defense-related effect by activation of some defense enzymes that play an important role, such as: (i) breaking down the fungus cell wall (such as chitinase and -1,3-glucanase); (ii) saving the plant cell wall; or (iii) increasing antioxidants (such as PAL or POD). According to Cecchini et al. [53], Pro is a defense compound contributing to hypersensitive response and tolerance to diseases. Pro treatments have thus been shown in several investigations to reduce the negative impacts of environmental pressures such as pathogen infections [54]. Ben et al. [55] reported that Pro application induces plant resistance to various pathogens through mechanisms such as oxidative scavenger and gene expression regulation. Qian et al. [56] also found that applied Pro mitigated superficial scald incidence and index in pear fruit.

A well-known side impact of stress is a buildup of oxidatives (reactive oxygen species, ROS) [57]. To minimize ROS damage effectively, plants have evolved scavenging systems like antioxidants. SOD, POD, PAL, and CAT, which are essential enzyme systems for ROS scavenging mechanisms, are essential metrics used for measuring plant resistance to stress. ROS scavenging mechanisms, are mediated by antioxidant enzymes and are the first edge of defense against stress. To reduce ROS generation and interference, as well as to alleviate the negative impacts of stress on plant growth and development, effective antioxidant capacity is required [57]. Protein denaturation occurs in plant cells due to oxidative stress, and significant amounts of MDA and H₂O₂ accumulate, which may act as primary stress mediators and triggers of plant defense systems [58]. While reactive oxygen species (ROS) can help improve plant tissue resistance to pathogen infections, high accumulations of ROS also lead to lipid peroxidation and loss of plant organ membrane

virtue. In this study, potato tubers responded to L. amnigena treatment by producing more oxidants such as MDA and H₂O₂. SA reduced oxidant content in tubers inoculated with L. amnigena by acting as an antibiotic against bacteria pathogens, which is consistent with the study of Mishra and Baek [59]. Bawa et al. [60] found a decrease in H_2O_2 and MDA levels in soybean plants treated with SA, compared to untreated soybean plants under biotic stress. Studies by Estaji [61] and Sayyari et al. [62] discovered that applying SA reduced oxidants such as MDA and H_2O_2 content in pepper and purslane, respectively, under drought stress. According to Naeem et al. [63], SA at 0.5 mM may be administered to tomato plants under saline conditions up to 90 mM, significantly alleviating the harmful effect of salt stress. Iqbal et al. [64] demonstrated that SA supplementation reduced the negative effects of salt stress on wheat cultivar development. The results show that the content of MDA and H2O2 in Pro-treated tubers compared to untreated inoculated tubers indicates that proline has efficacy in alleviation of *L. amnigena*-induced oxidative damage in potato tubers. This finding agrees with the findings of Hayat et al. [65], who discovered that applied Pro decreased MDA and H₂O₂ contents in pigeon peas subjected to cadmium stress. Pro application enhances plant resistance to biotic stress [66]. However, the combined therapy of SA and Pro with L. amnigena-infected tubers significantly reduced MDA and H_2O_2 content. De Carvalho et al. [67] discovered that applied Pro reduced the negative impacts of ROS as a result of increased SOD, POD, and CAT. The results of the current study collaborate with those of Abdelaal et al. [32], who found that combined SA and Pro lowered MDA and H_2O_2 levels in barley plants under drought stress. Sanchez-Rodriguez et al. [68] demonstrated that tomato plants treated with Pro reduced MDA and H₂O₂ levels, while increasing the activity of the antioxidant enzymes.

In a variety of plants, the role of antioxidant enzymes in plant defense against pathogen stress has been studied [69]. The current study found that either SA or Pro alone increased the activity of NOX, SOD, POD, CAT, and PPO in tubers subjected to *L. amnigena* stress. However, combined treatment of SA and Pro with L. amnigena-infected tubers further increased the activity of NOX, SOD, POD, CAT, and PPO, compared to the controls. The role of SA in the antioxidative system is widely assumed to be that of a signal molecule. This result suggests that SA treatment of L. amnigena-inoculated tubers may induce resistance because it activates the plant defense system by increasing some enzymes (NOX, SOD, POD, CAT, and PPO). Previous research has shown that, under stress, SA can maintain the activity of antioxidant enzymes to some extent, and it may also help to limit the impact of oxidative processes associated with disease development and spread, implying that SA may play an important antioxidant role in oxidative processes associated with plant defense responses [70,71]. Furthermore, SA-induced protein synthesis could be used to activate antioxidants [72]. The findings support the findings of Ma et al. [73], who found that during salt stress, SA raised the antioxidant enzyme activity in *Dianthus* superbus. Similar results were obtained in a previous work on Portulaca oleracea L., in which SA increased antioxidant enzyme activity, lowering ROS concentrations under diverse environmental conditions [74]. By adhering to hydrogen bonds, Pro can increase protein stability and safeguard membrane integrity [75]. Pro may also protect cells by enhancing water absorption capacity and promoting enzyme activity [76]. Pro, in addition to being an osmolyte, is a powerful antioxidative defense molecule, a metal chelator, a protein stabilizer, a ROS scavenger, and an inhibitor of programmed cell death [77,78]. Exogenous Pro has been shown in several studies to improve plant stress tolerance [7,79]. This type of treatment may activate stress avoidance systems by increasing stress tolerance and enhancing reactivity to stress triggers during a later stressful condition [54]. Pro contributes to the plant's stress response by enhancing antioxidant enzymes [65]. Pro was found to increase the enzyme's activity associated with the ascorbate–glutathione revolution [80], implying a role in improving cell antioxidant capacity. Abdelhamid et al. [81] discovered that applied Pro raised SOD, CAT, and POD in *Phaseolus vulgaris* L. plants subjected to salt stress. Furthermore, Tabssum et al. [82] reported that applied Pro at 50 mM increased SOD and CAT activity in rice under salt conditions. Ghaffari et al. [83] reported that

Pro-mediated changes in antioxidant enzymatic activities and the physiology of sugar beet under drought stress. Pro regulates the activity of SOD, CAT, and POX enzymes in plant cells, as well as their involvement in metabolic response development in response to environmental factors, according to Abdallah and El-Bassiouny [84]. The results of the present study are also in agreement with those of Abdelaal et al. [32], who discovered that combining SA and Pro increased drought resistance in barley plants through modulation of antioxidant activities. Based on the findings, it is proposed that the reduction of oxidative stress by activated antioxidant enzymatic systems may contribute to the synergistic effects of SA and Pro in the control of *L. amnigena*-caused potato soft rot.

Phenylpropanoid metabolism aids in the reduction of potato soft rot by providing substrates for the synthesis of phenolic and monolignin with antibacterial properties [85]. PAL is an important enzyme that initiates this metabolism by deaminating L-phenylalanine to trans-cinnamic acid [86]. In the current study, SA and Pro treatments significantly increased the production of phenylpropanoids such as PAL, CAD, 4CL, and C4H in potato tubers inoculated with *L. annigena*. This could be due to an increase in the expression levels of phenylpropanoid-related genes following treatment with SA or Pro, as previous research has shown that different types of elicitors increase the expression levels of phenylpropanoid-related genes and increase phenolic compound accumulation. Thus, SA and Pro treatment may promote the phenylpropanoid pathway by increasing enzyme activity and the synthesis of phenol compounds and lignin at the tuber's rotting site. SA may affect the phenylpropanoid pathway by inducing key enzymes such as PAL, CAD, 4CL, and C4H, resulting in phenolic compound accumulation. PAL, CAD, 4CL, and C4H also act as plant defense mechanisms, and the activities of these phenylpropanoids help the potato tubers to reduce the excess reactive oxygen species which is the source of oxidative stress during pathogen infection [87]. Plants can successfully induce PAL, CAD, 4CL, and C4H activity when infected with microorganisms such as bacteria pathogens [20,88]. Our results confirm this; PAL, CAD, 4CL, and C4H activities increased in L. amnigena-infected tubers treated with SA and Pro.

The important role of SA and Pro in protecting plants from stress may be due to their capability to increase the gene expression of *PR* proteins [89]. In this current study, potato tubers infected with *L. amnigena* increased relative expression of *NOX*, *PAL*, *CAD*, *4CL*, and *C4H* compared with the control. However, combined treatment of SA and Pro with *L. amnigena*-infected tubers significantly up-regulated *NOX*, *PAL*, *CAD*, *4CL*, and *C4H* genes. Lavrova et al. [90] reported that applied SA promotes gene expression in tomato plants infected with pathogens. These findings also support those of El-Esawi et al. [91], who found that *bZIP62*, *DREB2*, *ERF3*, and *OLPb* were increased in rosemary plants treated with SA, implying that applied SA-modulated genes enhanced the rosemary plants' resistance to the salinity condition. SA reduces oxidative damage caused by *L. amnigena* stress by up-regulating antioxidant defense mechanisms, making potato tubers more resistant to this type of biotic stress [92]. The Pro is a radical scavenger, in addition to being a suitable osmolyte [93]. As a result, Pro served as both an osmolyte molecule and an antioxidant [94]. Pro also serves as an antioxidative defense molecule and a signaling molecule during stress, in addition to being an effective osmolyte [93].

Plant defenses defined by pathogen gene-for-gene recognition by plants containing resistance genes have also been connected to the stimulation of SA-dependent R-genes. Higher expression of PR proteins has been linked to activated resistance attained with SA in earlier research [95]. In addition, SA-primed stressed-infected plants had 1.7, 2.9, 2.1, 2.5, and 2-fold *IAA27*, *MPK1*, *GPX*, chitinase, and 1,3-glucanase, respectively, than non-primed susceptibility inoculation controls. Since these genes are critical for developing resistance during the host–pathogen interaction, the high level of *IAA27*, *MPK1*, *GPX*, chitinase, and 1,3-glucanase correlates with disease protection studies. The increased stimulation of defense gene processes in potato tubers treated with SA or Pro alone suggests the molecular mechanisms governing both SA and Pro to alleviate *L. amnigena* infection in tubers. Combined treatment of SA and Pro with *L. amnigena*-infected tubers induced the

up-regulation of *PR1*, *PR2*, *CTL1*, *PAL*, *PRH3*, and *SPI1*, compared to the controls, which led to the most important effector genes for systemic acquired resistance (SAR) mediated by SA and Pro [96,97]. Chen et al. [98] discovered that applied Pro at 0.5 mM activated PR gene expression. The discovery that Pro raises the *OxyR* gene implies that Pro metabolism raises ROS scavengers. *OxyR* reacts with H₂O₂ to form a disulfide bond between *Cys199* and *Cys208*, resulting in *OxyR* regulon transcriptional activation [99].

5. Conclusions

The use of SA and Pro could help to reduce potato soft rot caused by *L. amnigena*. It could be attributed to the stimulation of antioxidant enzymes and the alleviation of oxidative damage caused by pathogenic infection, which stabilized intracellular redox homeostasis. SA and Pro treatment also increased the activities of *PR1*, *PR2*, *CTL1*, *PAL*, *PRH3*, and *SPI1* in the tuber, which may slow the growth of pathogenic bacteria (*L. amnigena*). These findings suggest that antioxidants and plant defense-related genes, particularly in potato tubers, are important in the defense response to pathogen infection.

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