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Antimicrobial Activity of Glycoalkaloids From *Solanum* spp. and Their Potential for Control of *Dickeya solani* and *Pectobacterium brasiliense*

Anna Grupa-Urbańska | Dorota Sołtys-Kalina | Renata Lebecka

Plant Breeding and Acclimatization Institute—National Research Institute, Radzików, Młochów Division, Młochów, Poland

Correspondence: Anna Grupa-Urbańska (a.grupa@ihar.edu.pl)

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ABSTRACT

Bacterial pathogens such as *Dickeya solani* and *Pectobacterium brasiliense* pose a significant threat to global food security by affecting major crops such as potato (*Solanum tuberosum*). Understanding the interaction between plant-derived molecules and bacterial virulence mechanisms is crucial for disease management strategies. This study investigated the effects of glycoalkaloids (GAs) extracted from the leaves of various potato (*Solanum* spp.) forms both directly (on bacterial growth and viability) and indirectly (on pectinolytic activity, biofilm formation and quorum-sensing [QS] gene expression). In vitro tests revealed that GAs significantly decreased bacterial cell multiplication factors and increased their death, which consequently inhibited pectinolytic activity and biofilm formation in *D. solani* and *P. brasiliense*. GAs from *Solanum chacoense* and cv. Tajfun were associated with significantly reducing QS-regulated gene expression, specifically in the *expI*, *expR* and virulence factor-modulating (Vfm) QS genes. GAs from the potato DG 00-683 showed the strongest association with the inhibition of biofilm formation. In addition, the greening of tubers cv. Tajfun, a process that increases the concentration of GAs, resulted in a significant reduction in tuber maceration after inoculation with bacteria, confirming their significant effect on pectinolytic bacteria. This study highlights the potential of *Solanum*-derived GAs as natural pesticides that enhance defence mechanisms in potato tubers against pectinolytic bacteria.

1 | Introduction

Bacterial plant pathogens are a major threat to global food security. In particular, *Dickeya solani* (Ds) and *Pectobacterium brasiliense* (Pcb), which belong to the *Pectobacteriaceae* family, are responsible for significant annual yield losses in major crops such as potato (*Solanum tuberosum*) (Del Mar Martínez-Prada et al. 2021; Devaux et al. 2020) but also affect a wide range of other crops, including onion (*Allium cepa*), sugar beet (*Beta vulgaris*), cabbage (*Brassica oleracea* var. *capitata*), broccoli (*B. oleracea*, var. *italica*), cucumber (*Cucumis sativus*) and tomato (*Solanum lycopersicum*) (Ozturk 2022; van der Wolf et al. 2021).

Members of the soft rot *Pectobacteriaceae* (SRP; *Pectobacterium* and *Dickeya*) cause two main potato diseases: blackleg (blackening at the stem base, wilting and plant collapse) and soft rot (aqueous maceration of tuber tissue pre- or postharvest). During the growing season, SRP-infected potato plants may also exhibit nonemergence, chlorosis, haulm desiccation and other blackleg-related symptoms (Hélias et al. 2000). Collectively, SRP rank among the top 10 most damaging plant-pathogenic bacteria (Mansfield et al. 2012).

SRP are present worldwide in soil and irrigation water. No curative measures are available for diseases caused by these

pathogens. Control strategies currently in use, whether in the field or during storage, have limited effectiveness in restricting their spread (van der Wolf et al. 2021). The identification of effective control strategies for crop diseases is crucial to mitigating their impacts on crop yields. Therefore, the pursuit of sustainable sources of disease resistance is a pivotal focus in plant biology research. The symptoms of these diseases, including rot, wilting and blackening, are primarily caused by plant cell wall-degrading enzymes (PCWDEs) secreted by pathogens, which are key virulence factors. These enzymes break down pectin, a primary component of the plant cell wall, facilitating tissue maceration during infection (Su et al. 2022; Toth et al. 2003). The synthesis and secretion of virulence factors in bacteria are regulated by a process called quorum sensing (QS). This process allows bacteria to monitor their population density and coordinate group behaviour, affecting the expression of critical genes involved in virulence, biofilm formation, motility and epiphytic fitness (Gutiérrez-Pacheco et al. 2019). Autoinducers (AIs) are small signalling molecules produced by bacteria during the stationary growth phase that mediate QS. These AIs regulate gene expression by reflecting the bacterial population density, thereby controlling the release of virulence factors. Notably, *N*-acyl-homoserine lactones (AHLs) accumulate as bacterial growth progresses and are synthesised by AHL synthase (ExpI) and sensed by a sensory protein (ExpR). Once the threshold level is reached, AHL-ExpR complexes form, initiating the expression of QS-regulated bacterial genes (Boo et al. 2021). Ds has two distinct QS systems that are conserved in *Dickeya* species: an AHL-based system (ExpIR system) and a specific virulence factor-modulating (Vfm) QS system (Liu et al. 2022). The latter system comprises 26 genes (*vfmA* to *vfmZ*) that are essential for the biosynthesis, transport and induction of the Vfm signal. Among these genes, *vfmE*, an AraC-type transcriptional regulator, plays a pivotal role. It is activated by the *vfmI-vfmH* system upon the detection of the Vfm signal, triggering the transcription of PCWDE genes and the Vfm operon. This process results in a self-amplifying feedback loop that accelerates signal accumulation (Nasser et al. 2013). In Pcb, QS is primarily regulated by the ExpI-ExpR system, where ExpI synthesises AHLs, including 3-oxo-C6-HSL and 3-oxo-C8-HSL, and ExpR serves as a response regulator. This system plays a crucial role in regulating virulence factors such as PCWDEs, motility and biofilm formation (Põllumaa et al. 2012; Pun et al. 2021). The Rsm system acts as a key regulator of virulence in Pcb, linking QS with the repression of PCWDE gene expression. At low cell density, ExpR activates *rsmA* transcription, leading to repression of virulence genes. As AHL accumulates, *rsmA* expression decreases, allowing the activation of virulence factors and increasing bacterial pathogenicity (Valente et al. 2017). Unlike Ds, which uses both the ExpI-ExpR and Vfm QS systems, Pcb relies primarily on ExpI-ExpR for virulence regulation (Liu et al. 2022). Recent studies have also highlighted the role of the ExpR2 receptor in *Pectobacterium carotovorum* (Pcc), which interacts with AHLs and modulates virulence-related gene expression (Alymanesh et al. 2024). Furthermore, QS inhibition by plant-derived compounds, such as phloretin, has been shown to attenuate Pcb virulence by disrupting AHL biosynthesis (Pun et al. 2021; Naga et al. 2023).

Potato is a major noncereal staple crop cultivated worldwide (Aksoy et al. 2021). Potato naturally synthesises glycoalkaloids (GAs), a group of secondary plant metabolites crucial for plant defence against pathogens, including bacteria, fungi, viruses and insects (Dahlin et al. 2017; Friedman 2006; Wolters et al. 2023). The amount of GAs increases significantly after pathogen and insect attacks. More than 90 different GAs have been identified in *Solanum* species (Friedman et al. 1997; Omayio et al. 2016). Three major classes of GAs have been recognised in potato species: those composed of the aglycon solanidine, leptines and leptinines. The richest sources of GAs are berries, sprouts and leaves. In cultivated potato tubers, approximately 95% of GAs consist of α-solanine and α-chaconine (Friedman 2006).

In our recent paper (Sołtys-Kalina et al. 2023), we found that GAs isolated from the leaves of potato cultivars exhibit bacteriostatic and bactericidal properties against Ds and Pcb, with varying inhibitory effects depending on the GA composition. We also found that the exposure of potato tubers to light resulted in chlorophyll accumulation (greening) and this was associated with an increase in GA concentration and altered GA composition. While both processes can occur simultaneously, they follow distinct metabolic pathways (Sołtys-Kalina et al. 2023). Although the increase in GA concentration varied among cultivars, our study provided preliminary evidence that elevated levels of these compounds in tubers could contribute to reduced blackleg incidence without compromising yield (Sołtys-Kalina et al. 2023). Despite the long-standing knowledge of the relationship between GAs and their antimicrobial activity, the nature of these interactions remains poorly understood. Building on the foundation of QS in pathogenicity, we hypothesise that GAs derived from potato possess unique abilities to attenuate the virulence of Ds and Pcb, potentially through interference with QS-regulated pathways. This effect is expected to depend on the type and amount of GAs. In this study, we selected different potato forms, namely wild species, interspecific hybrids and cultivars, based on their ability to inhibit Ds and Pcb growth at similar levels, and we investigated the composition of GAs isolated from the leaves of these potato forms, and their antimicrobial properties on Ds and Pcb along with the secondary effects of their activity. The study involved an in vitro screen for multiplication, bacterial viability, pectinolytic activity, and selected GAs, biofilm formation and the expression of QS genes. In addition, the maceration of potato tubers of cv. Tajfun, with increased GA contents due to greening, was evaluated after inoculation with bacteria.

2 | Materials and Methods

2.1 | Bacterial Strains

Two bacterial strains known for their high aggressiveness towards potato, *P. brasiliense* Pcb3M16 (from our own collection) (Lebecka and Michalak 2020) and *D. solani* IFB0099, which is synonymous with IPO2276 (stored in the collection at Plant Research International in Wageningen, Netherlands) (Golanowska et al. 2015), were used in this study.

2.2 | Glycoalkaloid Isolation

GAs were isolated from the leaves of eight potato forms: potato hybrids DG 00-683 and DG 08-305 and *S. chacoense* (accession number 333133) originate from the in vitro collection

maintained in Plant Breeding and Acclimatisation Institute—National Research Institute, Młochów; *Solanum maglia* (accession number 401878) and *Solanum garciae* (accession number 401634) originate from the National Centre for Plant Genetic Resources, Polish Gene Bank, Radzików, Poland; and potato cultivars Tajfun, Owacja and Mieszko originate from the Potato Gene Bank in Bonin. Forty tubers per potato form were planted individually in pots filled with a commercial peat-based potting mix. Plants were grown under greenhouse conditions (16 h light/8 h dark; 22°C/18°C) and watered regularly to keep the substrate evenly moist, avoiding waterlogging. After 3 weeks, 20 plants of the same size were placed in a climatic chamber under controlled conditions (14 h of daylight at 20°C). Four weeks later, leaves from the middle and upper part of plants were collected, mixed within each plant genotype and divided into 20 g portions. Leaves were immediately frozen in liquid nitrogen and stored at −80°C.

GA extraction was performed as described by Sołtys-Kalina et al. (2023) who used a modified version of the method originally developed by Andreu et al. (2001). Briefly, 20 g of ground and frozen leaves were extracted in 1 L of 2% acetic acid for 24 h. Then, the extract was filtered with filter paper and a sterilising filter (0.2 μm) to remove plant debris and microorganisms. GAs were precipitated with a 5 M ammonium hydroxide solution and centrifuged at 3548 *g* for 30 min. After extraction, GAs were dissolved in 75% ethanol to obtain a concentration of 50 mg/mL. The GA composition in each extract was analysed using HPLC–MS exactly as described by Szajko et al. (2021, 2023). A semiquantitative analysis of the GA content was performed by calculating the chromatographic peak area (C) using the following scale: 0, C = 0; 1 = C < 25,000; 2 = 25,000 < C < 50,000; 3 = 50,000 < C < 75,000; 4 = 75,000 < C < 100,000; and 5 = C > 100,000.

2.3 | Bacterial Growth and Viability

To analyse bacterial growth in the presence of GAs isolated from potato forms, a multiplication factor (MF) was determined (Lebecka et al. 2018). MF represents the fold increase in bacterial density over time and was calculated as the ratio of the optical density (OD) measured after 24 h to the OD at the outset of the experiment (including dilutions). Bacterial cultures of Ds and Pcb were grown on Luria Bertani (LB) agar plates in the incubator at 30°C for 24 h, then suspended in LB broth and grown to an $OD_{600} = 1$ (10^9 CFU/mL), as measured at wavelength of 600 nm using a spectrophotometer (Hitachi U-1900). The bacterial suspension was diluted 100 times in an LB broth medium and 200 μL of the bacterial suspension was added to the wells of a flat-bottomed sterile 96-cell culture plate (Nest Biotechnology Co. Ltd). GA extracts isolated from the eight potato forms were diluted in the bacterial suspension culture to a final concentration of 0.8 mg/mL. The final concentration of GAs in the growth medium was chosen experimentally using a series of GA dilutions from potato cv. Irys and analysis of Ds and Pcb MF. Final GAs concentration of 0.8 mg/mL significantly lowered the value of the MF; however, it did not induce total inhibition of bacterial growth. A concentration of 0.8 mg/mL of GAs was used in all in vitro experiments. Control samples contained the same volume of 75% ethanol used as a solvent for the GAs. The growth of bacteria was monitored using a microplate reader F50 (Tecan) by measuring the OD at a wavelength of 620 nm, both at the starting point and after 24 h of incubation at 25°C with shaking at 150 rpm. The experiment was repeated twice. In each experiment, three biological and three technical replicates were performed, along with four control samples.

Bacterial viability was analysed using a CyFlow Space flow cytometer (Sysmex Partec GmbH) equipped with a blue laser (488 nm); data were processed with FlowMax software (Sysmex Partec GmbH). The number of dead cells was determined using the CyStain BacCount Viable kit (Sysmex Partec GmbH) after 48 h of bacterial incubation with GAs at 28°C in LB broth. The bacterial cultures were suspended in LB broth to an $OD_{600} = 0.1$ (10^8 CFU/mL), and 200 μL of this suspension was distributed into 0.5 mL sample tubes. GAs extracted from the eight different forms of potato were added to give a final concentration of 0.8 mg/mL. These procedures strictly adhered to previously described protocols (Sołtys-Kalina et al. 2023).

2.4 | Pectinolytic Activity of Bacteria

This study investigated the impact of GAs on the pectinolytic activity of two bacterial strains in crystal violet pectate (CVP) medium with and without the addition of GAs. The CVP medium was prepared as follows: 2 g/L $NaNO_3$, 5 g/L trisodium citrate dihydrate, 1 g/L tryptone, 1.6 g/L $MgSO_4$, 2 mL of crystal violet (CV) (0.075% aqueous solution), 13.6 mL of freshly prepared 10% $CaCl_2.2H_2O$, 4 g/L agar and 18 g/L sodium polypectate (PGA). GAs at a final concentration of 0.8 mg/mL were added to the sterilised CVP medium after autoclaving, while the medium was still warm, to prevent potential degradation of GAs at high temperatures. CVP medium without GAs served as a control. The final pH was determined to be approximately 7. The medium was then poured into Petri dishes in a laminar flow cabinet. A bacterial suspension with an initial concentration of $OD_{600} = 1$ (10^9 CFU/mL) was spotted onto CVP medium using a sterile inoculation needle. The Petri dishes were incubated at 30°C for 48 h. The pectinolytic activity of the bacterial strains was evaluated by measuring the volume of the depressions (cavities) surrounding their colonies on CVP medium. To measure cavity volume, each well was manually filled with CV-stained liquid using an automatic pipette until full. The pipette, Eppendorf model Multipette Stream, was set to a 10 μL increment so that the precision of each measurement was 10 μL. The results were expressed as a percentage relative to the control. The experiment included three biological replicates, each with four technical replicates.

2.5 | Biofilm Formation

A microtitre plate assay with CV was used to assess biofilm formation, with modifications according to previous studies (O'Toole 2011; Nykyri et al. 2013). One microlitre of the bacterial suspension (10^8 CFU/mL) was added to 100 μL of LB medium supplemented with and without GAs (final concentration of 0.8 mg/mL) from the selected three potato forms (cv. Tajfun, DG 00-683 and *S. chacoense*) in a 96-cell culture plate

(Nest Biotechnology Co. Ltd). After 6 h of incubation at 30°C without shaking, 25 μL of 1% CV was added to each well for a 15-min incubation at room temperature. After the plates were washed three or four times with water, 150 μL of 96% ethanol was added to each well for 15 min at room temperature to dissolve the CV. The biofilm density was quantitatively determined by measuring the OD at a wavelength of 560 nm using a microplate reader (Tecan; Infinite F50). The experiment was conducted with three biological replicates, each with four technical replicates.

2.6 | Expression of the QS Genes

Expression of the following QS genes was analysed for Ds: *expI*, encoding the autoinducer synthase genes; *expR* encoding transcriptional regulator genes; and *vfmA* and *vfmE*, encoding virulence factor modulation genes. For Pcb, *expI* and *expR* were analysed, which encode the autoinducer synthase and transcriptional regulator genes, respectively. Ten microlitres of bacterial suspension (10^8 CFU/mL) was added to 190 μL of LB medium in each well (sterile 24-multiwell; Lummox) followed by the addition of GAs from cv. Tajfun, DG 00-683 and *S. chacoense* to achieve a final concentration of 0.8 mg/mL. The control consisted of LB medium supplemented with bacteria and the same amount of ethanol as that provided with the GA extract. The bacteria were incubated at 30°C and shaken at a speed of 150 rpm for 8 h. The experiments were conducted in three biological replicates, each with three technical replicates. Total RNA was isolated using the Zymo Research Direct-zol RNA Miniprep Kit with TRI Reagent, according to the manufacturer's protocol. The quality and quantity of RNA were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). RNA samples (1 μg) were extracted using the Direct-zol RNA Miniprep kit (Zymo Research), which includes an on-column DNase I digestion step to remove genomic DNA before reverse transcription (RT). cDNA was synthesised using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's recommendations. Each reaction was diluted fivefold for further analyses. The quality of the obtained cDNA was verified by quantitative PCR (qPCR) analysis of a reference gene, confirming the successful removal of gDNA. The reaction mixture was prepared with 5 μL of 2× PCR mix Plus, 1 μL of primer mix, and 1 μL of fivefold diluted cDNA, and the volume was increased to 10 μL with water. The PCR conditions were an initial 3-min incubation at 95°C; 40 cycles of 95°C for 10 s, 62°C or 65°C for 20 s, and 72°C for 30 s; with a final 72°C for 10 min. The PCR products were then analysed on a 2% agarose gel and visualised under UV light. The expression of selected genes was examined using real-time qPCR. The composition of the reaction mixture (10 μL) included 5 μL of (1) LightCycler 480 SYBR Green I MasterMix (Roche), 1 μL of diluted cDNA template, 1 μL of specific primer mix (20 μM each), and 3 μL of water. All analyses were performed in three technical replicates. For each primer pair, no-template control (NTC) reactions were also performed. The reaction was performed using a LightCycler 480 instrument (Roche). The PCR conditions for reagent (1) were as follows: 95°C for 5 min; 40 cycles of 95°C for 10 s, 65°C for 20 s and 95°C for 30 s; then 95°C for 5 s and 68°C for 60 s with a ramp of 0.5°C every 10 s up to 95°C; then a final hold at 40°C. The PCR conditions for reagent (2) were as follows: 95°C for 2 min, 95°C for 5 min; 40 cycles of 95°C for 10 s, 95°C for 60 s; then 95°C for 5 s at 65°C with a ramp of 0.5°C every 10 s up to 95°C; then a final hold at 40°C. The relative expression level was calculated using the formula $2^{-\Delta Ct}$, where $\Delta Ct = C_t(\text{target gene}) - C_t(\text{reference gene})$. For the study of the expression of selected genes, primers were designed using PRIMER3 software. The *lpxC* gene was selected as a reference gene due to its stable expression in pectinolytic bacteria, as reported by Hommais et al. (2011). For primer details, refer to Table S1.

2.7 | Evaluation of Soft Rot in Inoculated Tubers of the Cultivar Tajfun

The tuber maceration test was conducted as previously described (Lebecka, Wasilewicz-Flis, et al. 2021). A total of 80 tubers of cv. Tajfun were used: 40 were artificially greened for 10 days under 24 h of continuous light at 19°C, and 40 were nongreened as a control, kept at the same temperature for 10 days but in complete darkness. Each of the four replicates included five tubers (20 tubers total per treatment). Treatments included greened tubers inoculated with Ds (20 tubers), nongreened tubers inoculated with Ds (20 tubers), greened tubers inoculated with Pcb (20 tubers) and nongreened tubers inoculated with Pcb (20 tubers), giving a total of 80 tubers. Prior to inoculation, the tubers were washed, air-dried, wounded with a steel rod and inoculated with a 10 μL bacterial suspension at 10^6 CFU/mL. The holes were sealed with Vaseline and a piece of Parafilm. The tubers were sprayed with water and then enclosed in boxes. The tubers were maintained in a climatic chamber at 27°C for 3 days as described by Lebecka, Śliwka, et al. (2021). At the end of the incubation period, tubers were weighed before and after removal of the decayed tissue from the tuber. Disease severity was expressed as the weight of decayed tissue.

2.8 | Statistical Analysis

Statistical analyses were performed using Statistica 13 software (Statsoft Inc.). The analysis of variance (ANOVA) and Duncan test were used for determining differences between treatments. For laboratory experiments, the graphical presentation of basic statistics was performed using Microsoft Excel for Microsoft 365 MSO, R Studio (v. 4.3.1), Inkscape and BioRender (https://app.biorender.com/biorender-templates).

3 | Results

3.1 | Glycoalkaloid Composition in Potato Forms

We have identified GAs from leaves of eight potato forms using high-performance liquid chromatography-mass spectrometry (Table 1). Four steroidal GAs (solasonine, solamargine, α-solanine and α-chaconine) and two leptine GAs (leptinine I and leptinine II) were identified. All potato forms contained α-solanine and α-chaconine. In *S. maglia* and *S. chacoense*, α-solanine and α-chaconine were the only two recognised GAs. In *S. garciae*, all six GAs were present (Table 1).

3.2 | Impact of GAs on the Bacterial Multiplication Factor

We explored the effects of GAs from potato forms on the MF of Ds and Pcb (Figure 1). The results of two-way ANOVA showed a significant effect of GAs ($p < 0.001$), but no significant effect of bacterial strain ($p = 0.059$) on MF value. There was a significant effect of the interaction between bacterial strain and GAs ($p < 0.001$); therefore, one-way ANOVA was performed for each of the bacterial strains. All tested GAs significantly inhibited bacterial growth compared with the control without GAs. The effect of inhibition was found to be significantly greater for Pcb than for Ds, when the MF value was reduced from 67% to 87% of the control, in comparison with Ds, for which the MF was reduced from 53% to 71%. The GAs extracted from *S. maglia* exhibited the strongest effect on both the Pcb and Ds bacterial strains, significantly reducing MF to an average of approximately 1.1 (reduced by 87% compared with the control) and 1.6 (reduced by 71% of the control), respectively.

3.3 | Impact of GAs on Bacterial Viability

The results of the two-way ANOVA indicated a significant effect of GAs, treatment, and the GAs × treatment interaction on the number of dead cells of two bacterial pathogens. The range of bacterial cell death rates for Pcb following 48 h of incubation with and without GAs was found to be between 2.4% and 17.8% and between 1.1% and 2.7%, respectively. For Ds, the range was between 2.6% and 8.5% and between 1.7% and 3.7%. GAs from *S. chacoense* caused significantly the highest cell death rate, reaching 17.8% and 7.5% for Pcb and Ds, respectively. The effect of the

TABLE 1 | Composition and amounts of glycoalkaloids isolated from the leaves of different potato forms.

	Glycoalkaloid[b]					
Potato form[a]	**Leptinine I**	**Leptinine II**	**Solasonine**	**Solamargine**	**α-Solanine**	**α-Chaconine**
DG 08-305	0	0	1	2	3	4
DG 00-683	1	0	0	0	2	2
Solanum maglia	0	0	0	0	4	4
Solanum chacoense	0	0	0	0	2	5
Solanum garciae	2	1	1	1	4	5
Tajfun[c]	0	0	0	0	4	4
Owacja[c]	0	0	0	0	3	4
Mieszko[c]	1	0	0	0	3	3

[a]Includes potato hybrids, wild *Solanum* species and cultivars used in this study.
[b]The quantity of each glycoalkaloid was determined via HPLC–MS using a semiquantitative method. The peak area scale used was C = 0; 1 = C < 25,000; 2 = 25,000 < C < 50,000; 3 = 50,000 < C < 75,000; 4 = 75,000 < C < 100,000; and 5 = C > 100,000.
[c]The data for these cultivars were previously published in Sołtys-Kalina et al. (2023).

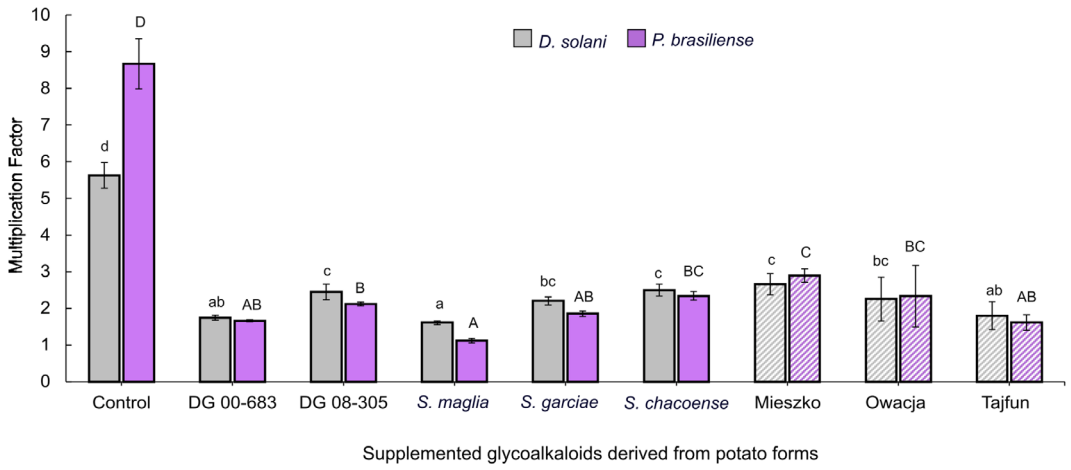

FIGURE 1 | Multiplication factors (MFs) of *Dickeya solani* and *Pectobacterium brasiliense* 24 h after an incubation in Luria Bertani medium (control) or Luria Bertani medium supplemented with glycoalkaloids isolated from the leaves of different potato forms. The results are expressed as means ± standard error (SE). Different lowercase letters indicate significant differences among *D. solani* treatments, while different uppercase letters indicate significant differences among *P. brasiliense* treatments (Duncan's test, $p < 0.05$). Data for cvs. Tajfun, Owacja and Mieszko (shown with diagonal hatching) were previously published in Sołtys-Kalina et al. (2023). MF is defined in the Materials and Methods section.

TABLE 2 | Percentage of dead *Dickeya solani* and *Pectobacterium brasiliense* cells after 48 h of incubation in Luria Bertani medium with or without the addition of glycoalkaloids.

	D. solani		*P. brasiliense*	
Glycoalkaloids from leaves of potato forms[a]	**Control**	**Glycoalkaloids**	**Control**	**Glycoalkaloids**
DG 08-305	1.8	8.5*	1.1	2.4 ns
DG 00-683	3.7	6.1*	2.7	9.3*
Solanum maglia	3.1	7.8*	2.1	11.5*
Solanum garciae	2.1	4.7*	1.5	5.4*
Solanum chacoense	2.8	7.5*	2.6	17.8*
Tajfun[b]	1.7	2.6 ns	1.7	6.1*
Owacja[b]	2.4	3.4 ns	2.3	4.0 ns
Mieszko[b]	2.6	4.1*	2.2	2.9 ns

Note: Difference according to Duncan's test (in comparison with control).
[a]Includes potato hybrids, wild *Solanum* species and cultivars used in this study.
[b]The data for these cultivars were previously published in Sołtys-Kalina et al. (2023).
*$p \leq 0.05$, ns, not statistically significant.

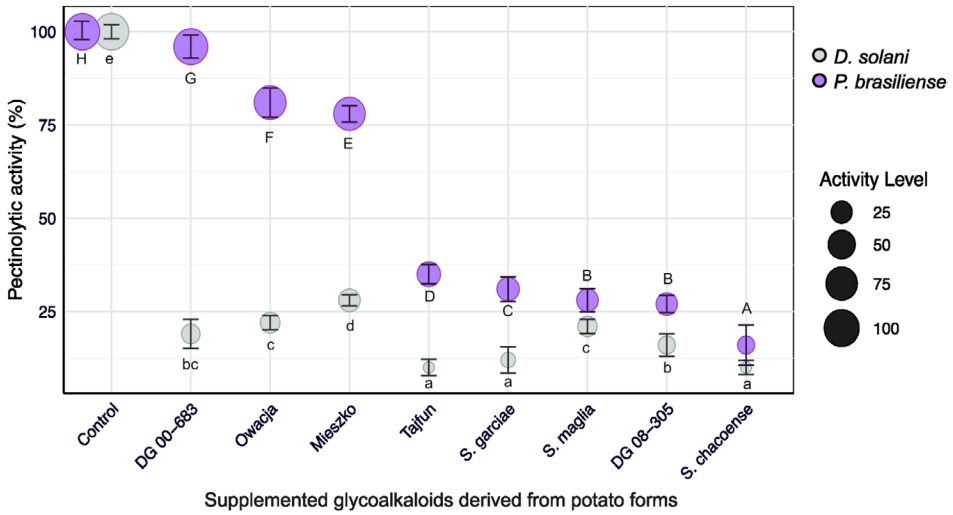

FIGURE 2 | Evaluation of the pectinolytic activity of *Dickeya solani* and *Pectobacterium brasiliense*. Pectin-degrading capacity of bacterial strains, measured as a percentage relative to the control based on the volume of cavities in crystal violet pectate (CVP) medium. Control samples received the same volume of 75% ethanol as used for glycoalkaloids dissolution, but without the addition of glycoalkaloids. The results are expressed as means ± standard error (SE). Different lowercase letters indicate significant differences among *D. solani* treatments, while different uppercase letters indicate significant differences among *P. brasiliense* treatments (Duncan's test, $p < 0.05$).

interaction of GAs with bacterial strains was most pronounced for GAs from the hybrid DG 08-305, reaching 8.5% cell death for DS, while the lowest cell death rate of 2.4% for Pcb was not significant (Table 2).

3.4 | Impact of GAs on Bacterial Pectinolytic Activity

Studies on the pectinolytic activity of Ds and Pcb showed significant inhibitory effects of GAs isolated from different potato forms (with the exception of the nonsignificant effect of GAs from DG 00-683 on Pcb bacteria) (Figure 2). The mean pectinolytic activity of Ds after treatment with GAs was 17% and ranged from 10% to 28% of that of the control. GAs from *S. chacoense* most significantly reduced the pectinolytic activity of Ds to 10% and that of Pcb to 16% (Figure 2).

3.5 | Impact of GAs on Bacterial Biofilm Formation

For biofilm formation and QS gene expression studies, GAs isolated from three representative potato forms were selected: a cultivar (cv. Tajfun), a hybrid (DG 00-683) and a wild species (*S. chacoense*). The selection was based on their relatively low MF values as well as their overall antimicrobial potential, including strong inhibitory effects on pectinolytic activity and cell

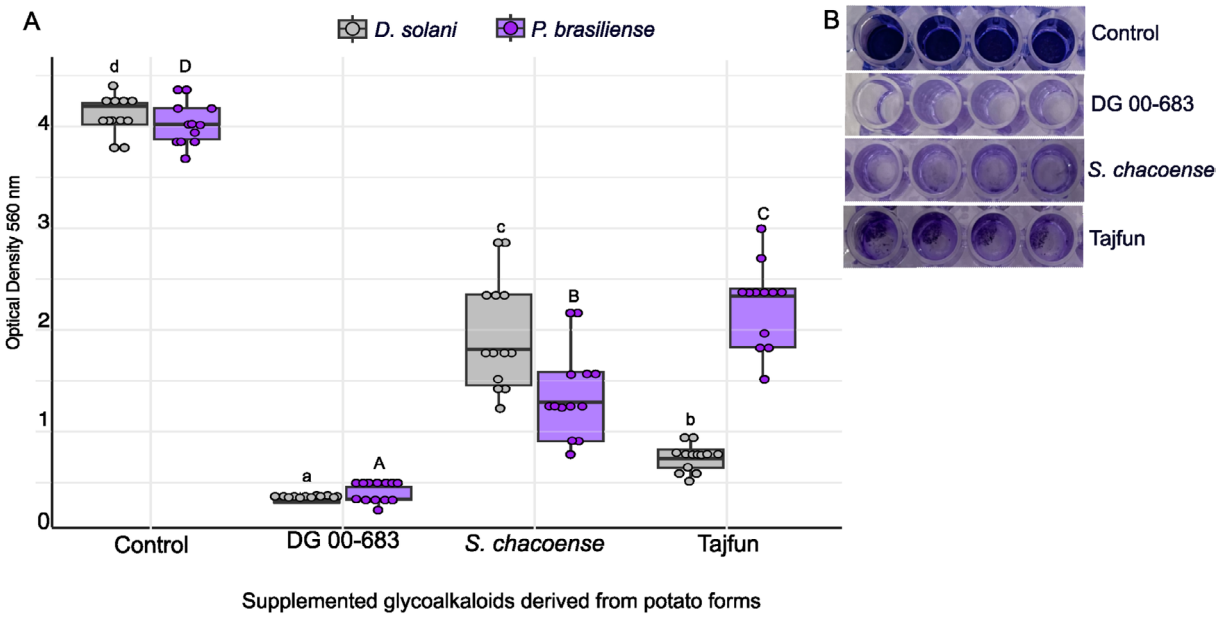

FIGURE 3 | Crystal violet-based assay for the assessment of bacterial biofilm formation by *Dickeya solani* and *Pectobacterium brasiliense* after 6 h of incubation in Luria Bertani medium with or without the addition of glycoalkaloids isolated from the leaves of three different potato forms. (A) Average optical density at 560 nm ($OD_{560\ nm}$) from 12 replicates ± SD. Different lowercase letters indicate significant differences among *D. solani* treatments, while different uppercase letters indicate significant differences among *P. brasiliense* treatments (Duncan's test, $p < 0.05$). (B) Representative image of wells stained with crystal violet, where the violet colour intensity reflects the amount of biofilm formed by *P. brasiliense*.

viability. Although *S. maglia* showed the lowest MF among wild species, *S. chacoense* was chosen due to its more consistent antimicrobial effects across assays, including the most significant reduction in pectinolytic activity and the highest cell death rate in Pcb.

GAs from selected potato forms, DG 00-683, *S. chacoense* and cv. Tajfun, exhibited various effects on the biofilm formation of Ds and Pcb (Figure 3A,B). All GAs tested significantly inhibited biofilm formation compared with the control. The GAs from DG 00-683 produced the most profound inhibition of both Ds and Pcb, as evidenced by the lowest OD values (mean $OD_{560} = 0.32$) (Figure 3A,B). Interestingly, the GAs from cv. Tajfun significantly impeded biofilm formation in Ds (mean $OD_{560} = 0.73$) but had less of an effect on Pcb (mean $OD_{560} = 2.20$).

3.6 | Impact of Selected GAs on the Expression of Quorum-Sensing Genes

Panel A of Figure 4 shows the major QS genes in Ds and Pcb, helping to visualise the systems targeted in our analysis. In this schematic, we delineate the central QS genes that were selected for our analysis, highlighting the complexity of the QS networks within these bacterial species. The analysed genes included *expI*, *expR*, *vfmA* and *vfmE* in Ds, and *expI* and *expR* in Pcb. Notably, the diagram underscores the presence of two distinct QS systems in Ds as opposed to a single system in Pcb. For Ds, GAs derived from *S. chacoense* and cv. Tajfun exerted an inhibitory effect on QS genes, with pronounced inhibitory effects on the expression of the *expI* gene (relative expression levels of 0.33 and 0.36, respectively) (Figure 4B). Interestingly, GAs from DG 00-683 induced the expression of *expI* (relative expression level = 2.0), an AHL synthase gene, in Pcb but inhibited the expression of the receptor protein-encoding gene *expR* (relative expression level = 0.08); in contrast, in Ds, the opposite effect was observed. This result indicates that GAs from the hybrid DG 00-683 specifically induced the expression of the investigated *vfm* genes in Ds (Figure 4B).

3.7 | Response of Greened cv. Tajfun Tubers to Inoculation With *D. solani* and *P. brasiliense*

Tajfun was selected for this study because the GAs extracted from its leaves showed the strongest in vitro antimicrobial activity against both Ds and Pcb (Sołtys-Kalina et al. 2023). GA levels in various tuber zones (skin, flesh and whole tuber) of cv. Tajfun were studied. The highest level of GAs in the greened tubers was observed in the skin, 2601 mg/kg dry weight, while in the nongreened tubers, it was 504 mg/kg dry weight as measured spectrophotometrically (Figure 5A). No significant differences in GA content were observed for the flesh or whole tubers (Figure 5A). A visible difference in pigmentation between greened and control tubers is presented in Figure 5B. The results of two-way ANOVA indicated significant effects of greening and bacterial strain on disease severity. In general, greened tubers exhibited fewer rotting symptoms than nongreened tubers. Figure 6A shows symptoms of rot in the tubers of cv. Tajfun after inoculation with the two bacterial strains: Ds and Pcb. The mean weight of the rotten tissue of the nongreened tubers inoculated with Ds was 2.5 g, while that of the greened tubers was 1.8 g (Figure 6B). Similarly, nongreened tubers inoculated with

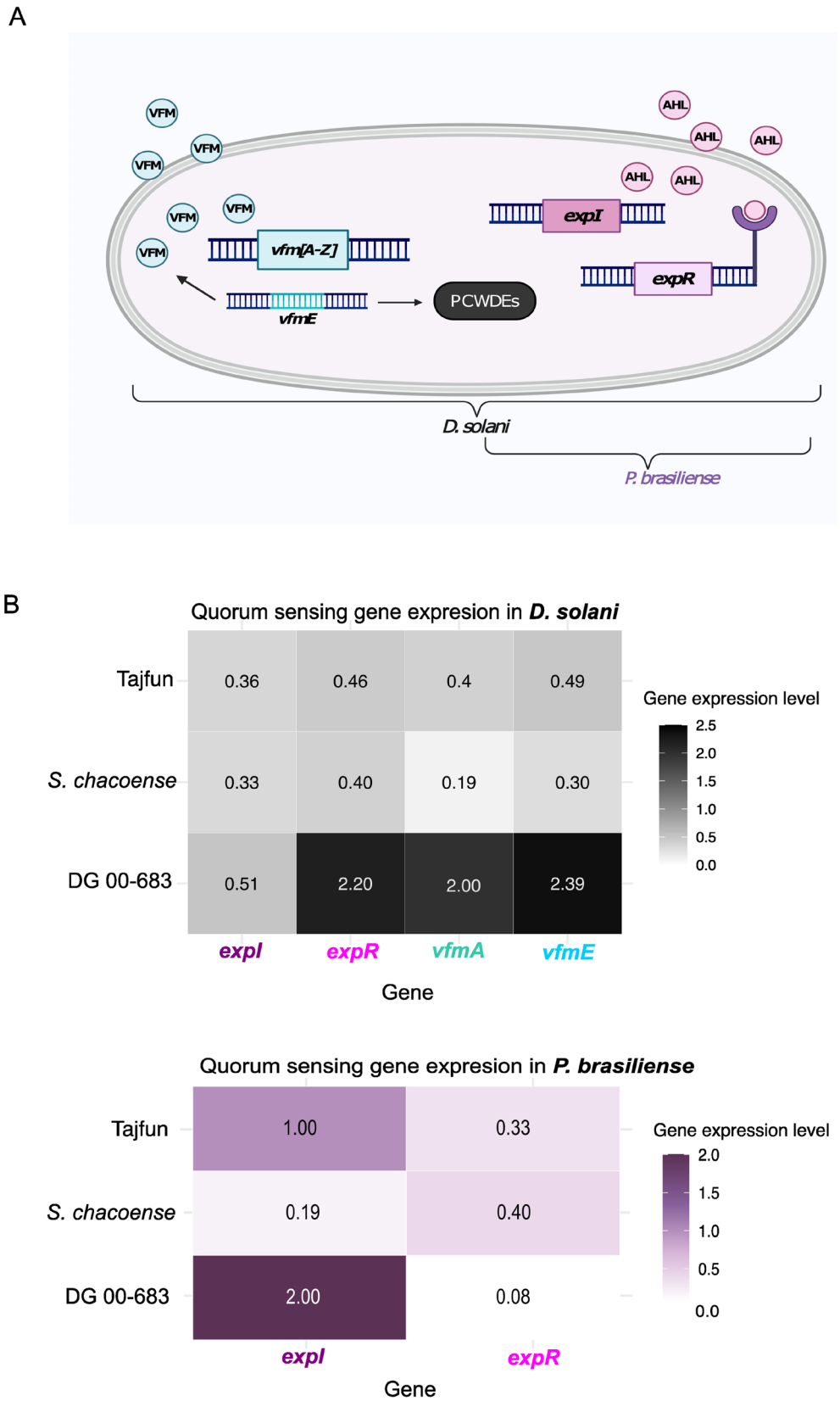

FIGURE 4 | (A) Quorum-sensing (QS) systems in *Dickeya solani* and *Pectobacterium brasiliense*. Brackets highlight that *D. solani* possesses two QS systems, whereas *P. brasiliense* is equipped with only one. (B) Relative gene expression in *D. solani* and *P. brasiliense* after 8 h of incubation in Luria Bertani medium supplemented with glycoalkaloids derived from the leaves of the potato cv. Tajfun, the wild species *Solanum chacoense*, and the *Solanum* spp. hybrid DG 00-683. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method, with $\Delta C_t = C_t(\text{target gene}) - C_t(\text{reference gene})$. The analysed QS-related genes were *expI*, *expR*, *vfmA* and *vfmE* in *D. solani*, and *expI* and *expR* in *P. brasiliense*. The *lpxC* gene was used as the reference gene. The image in panel A was created with BioRender.com.

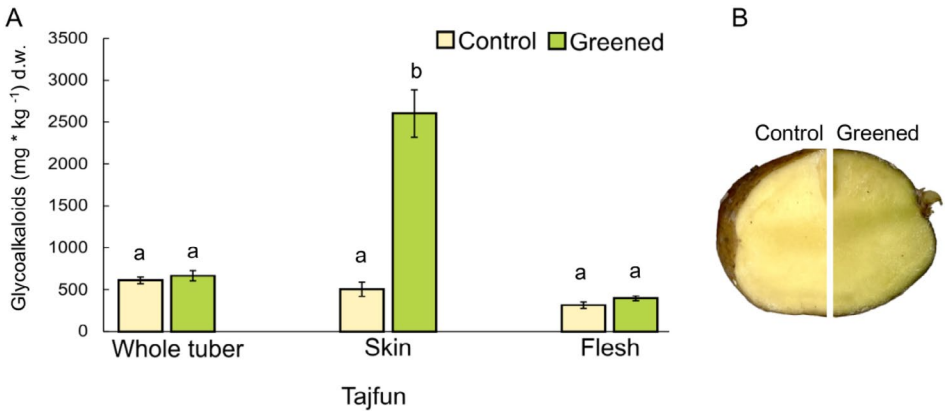

FIGURE 5 | Glycoalkaloid levels in the tuber zones of cv. Tajfun after 14 days of greening under light exposure. (A) Quantitative analysis of glycoalkaloid levels in cv. Tajfun tubers, comparing whole tubers, skins and flesh between control (nongreened) and greened samples. (B) Visual comparison of control (left panel) and greened (right panel) potato tubers, highlighting the external and internal colour differences.

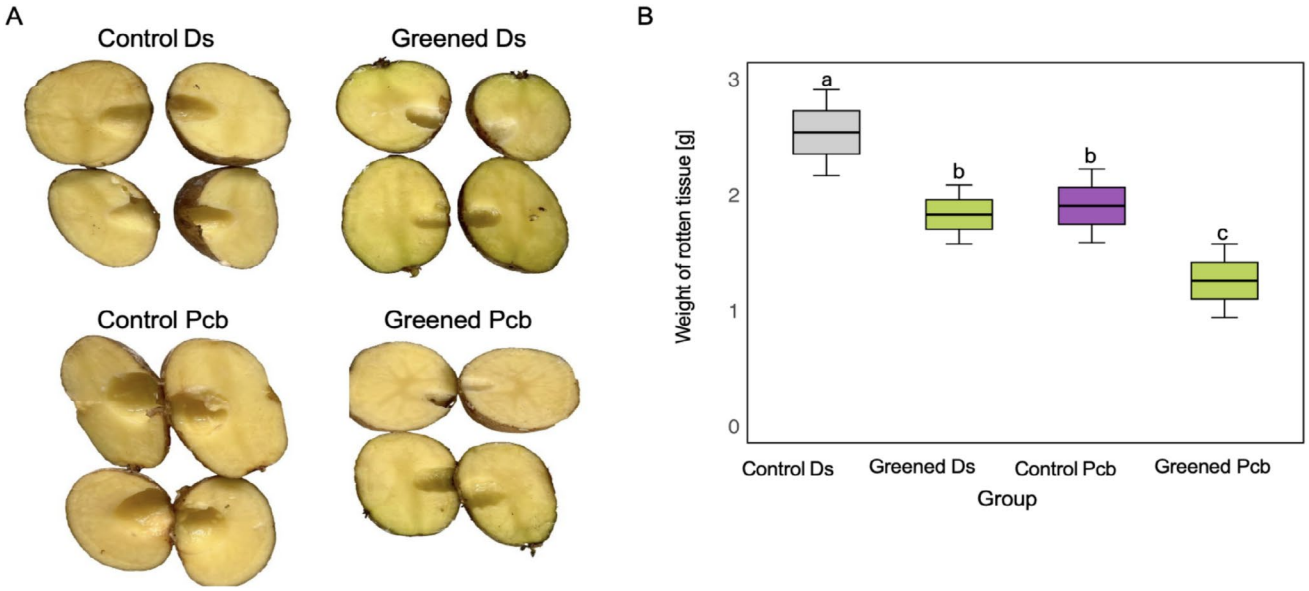

FIGURE 6 | Disease severity of nongreened (control) and greened potato tubers of cv. Tajfun inoculated with *Dickeya solani* (Ds) and *Pectobacterium brasiliense* (Pcb). (A) Visual appearance of tubers after removal of rotten tissue. (B) Mean maceration values from 20 replicates ± standard errors (SE). Different letters indicate significant differences among all treatments according to Duncan's test ($p < 0.05$).

Pcb showed greater maceration (1.9 g) than did greened tubers (1.2 g) (Figure 6B).

4 | Discussion

Potato is threatened by various plant pathogens, with bacteria of the genus *Pectobacterium* causing notable economic losses worldwide. Interestingly, certain wild relatives of cultivated potato exhibit greater resistance to pectinolytic bacteria than cultivated relatives, although the underlying mechanisms, potentially involving higher concentrations of phenolics and alkaloids, remain to be fully elucidated (Friedman 2006; Joshi et al. 2024; Lebecka, Śliwka, et al. 2021; Ma et al. 2022). In the European Union potato sector, blackleg and tuber soft rot caused by SRP (*Pectobacterium* and *Dickeya*) are estimated to incur approximately €46 million in annual losses (Dupuis et al. 2021). GAs, naturally occurring compounds in the *Solanum* genus, have been identified as potential contributors to this resistance. Our previous research (Sołtys-Kalina et al. 2023) demonstrated the effect of GAs from four potato cultivars on bacterial growth and viability. Additionally, it was shown that the greening process of potato tubers, which leads to an increased concentration of GAs, can significantly reduce the incidence of blackleg. However, while these findings suggest a protective role for GAs, the mechanisms by which GAs exert their effects whether—through direct antimicrobial activity, inhibition of virulence factors like pectinolytic enzymes, or other indirect effects are—not yet fully understood.

This present study aims to investigate the antimicrobial properties of GAs extracted from various potato forms against Ds and Pcb. Building on our previous work (Sołtys-Kalina et al. 2023), which focused on selected cultivars of *S. tuberosum*, the present study broadens the scope by including wild *Solanum* species and interspecific hybrids. This expanded approach enabled the identification of two additional GAs, leptinine II and solasonine, not previously detected in these potato genotypes (Sołtys-Kalina et al. 2023), thereby extending the known GA spectrum in these genotypes. Specifically, we examined how different compositions and proportions of GAs influence the virulence of these pathogens. We analysed GAs from potato forms encompassing up to six GAs, such as in *S. garciae*, to those possessing only two GAs (α-solanine and α-chaconine), such as in *S. maglia*, *S. chacoense* and DG 00-683. The GAs α-solanine and α-chaconine have been found to be the dominant GA compounds in cultivars of *S. tuberosum*, demonstrating their significance in cultivated species (Friedman 2006; Sołtys-Kalina et al. 2023). Interestingly, *S. garciae*, despite its broad GA profile, did not induce high levels of bacterial cell death; though it did reduce bacterial MF, suggesting that its GAs may inhibit proliferation rather than directly affecting cell viability. This observation highlights that greater GA diversity does not necessarily equate to stronger antimicrobial activity. Instead, specific GA combinations or concentrations may be more critical in determining antimicrobial effectiveness. The wide diversity of GA profiles studied here thus provides a valuable model to dissect the functional impact of GA composition on bacterial pathogens.

Building on the previously observed antimicrobial effects of GAs (Sołtys-Kalina et al. 2023), we investigated their influence on bacterial growth, as measured by MF and cell mortality. The most basic way to indirectly measure the concentration of bacteria is to measure its OD. An alternative approach involves the utilisation of a standard curve; however, this method is laborious and subject to bias from multiple factors that can influence the curve. These include the use of exceedingly minute dilutions and the enumeration of colonies based on the assumption that each colony is derived from a single viable cell. Therefore, the MF obtained by dividing the OD of the bacterial suspension after incubation by the OD at the start of the experiment allows comparison of different isolates and treatments regardless of the number of bacteria in the initial suspensions. A much more sensitive method is the analysis of bacterial viability using flow cytometry. Flow cytometry distinguishes between living and dead bacteria with a precision of one cell. The pronounced reduction in MF in both Ds and Pcb in the presence of GAs, both from potato cultivars (Sołtys-Kalina et al. 2023) and various potato forms (this study), confirms the potential of these compounds to act as bacterial growth inhibitors. The GAs from *S. maglia* significantly reduced MF, indicating a robust antimicrobial action that could be exploited to enhance the natural resistance of potato to bacterial pathogens.

The differential effect of GAs on bacterial viability, as indicated by the percentage of dead cells, provides important insights into the antimicrobial potency of potato-derived compounds. In this study, all GAs from potato forms caused an increase in the number of dead cells in comparison with the control, and the highest effect (17.8%) was observed for GAs from *S. chacoense* against Pcb. In our previous study (Sołtys-Kalina et al. 2023), a significant bactericidal activity on Pcb was observed for GAs from cv. Tajfun, Owacja and Mieszko. In the case of Ds, a significant effect was observed for GAs from cv. Mieszko and Irys. In general, these observations confirm the direct bactericidal and bacteriostatic effects of GAs from *Solanum* sp. on Ds and Pcb.

The impaired ability of bacteria to multiply in the presence of GAs can have significant consequences on the severity of the bacterial community on the host (their aggressiveness) and how the bacteria communicate with each other. We observed indirect effects in pectinolytic activity, biofilm formation and bacterial communication via QS gene expression. Pectinolytic enzymes are central to the virulence of soft rot pathogens such as *Pectobacterium* and *Dickeya*, facilitating the breakdown of plant cell walls and the consequent release of nutrients necessary for bacterial proliferation (Barras et al. 1987; Garibaldi and Bateman 1971; Toth et al. 2021). Our research showed that GAs from various potato forms were associated with a significant reduction in the activity of these crucial enzymes, with a more pronounced effect observed on Pcb compared to Ds. Specifically, GAs from *S. chacoense* reduced the pectinolytic activity of Ds to 10% and that of Pcb to 16% relative to the control, indicating substantial inhibitory effects.

The modulation of bacterial biofilm formation by GAs from certain potato forms represents an additional strategy for addressing the pathogenicity of Ds and Pcb. The formation of biofilms plays an important role in the colonisation of vascular tissue by bacteria in blackleg disease (Moleleki et al. 2017). Our study showed that GAs from DG 00-683 significantly suppressed biofilm formation by both Ds and Pcb, achieving the lowest observed mean OD values. This suppression suggests the potential for GAs to disrupt the establishment and maintenance of biofilms, an important factor in the pathogenesis and persistence of these bacteria in agricultural environments. Building on our understanding of the role of GAs in inhibiting biofilm formation, we focused on QS, a sophisticated bacterial communication system orchestrating gene expression associated with virulence, pathogenicity and resistance. QS enables bacteria to sense and respond to the cell population density through a complex signalling mechanism. This regulatory function is mediated by signalling molecules such as AHL, which are synthesised by the *expI* gene (Pena et al. 2019). The schematic representation of the QS circuitry is delineated in Figure 4A, illustrating the presence of dual QS systems in Ds and a single system in Pcb; it provides a foundational context for our analysis. Our investigation of the QS systems of Ds and Pcb revealed that the expression of key QS genes was affected by GAs derived from *Solanum* spp. leaves. We observed a significant downregulation of the *expI* gene, responsible for AHL synthesis, when plants were treated with GAs from *S. chacoense* and cv. Tajfun. These compounds may 'delay' bacterial communication involved in virulence gene activation. GAs from DG 00-683 induced the expression of the *expI* gene in Pcb while suppressing the expression of the *expR* gene, suggesting differential effects on QS components. Parallel to our findings, the work of Joshi et al. (2021) indicated that phenolic compounds can reduce QS in *Pectobacterium* by inhibiting the expression of the *expI* and *expR* genes. This research supports our observations on the potential of plant-derived metabolites to interfere with bacterial QS systems. Our study extends this

paradigm by exploring the Vfm QS system that is unique to *Dickeya* species, particularly highlighting the inhibitory effect of GAs on the *vfmA* and *vfmE* genes, which further delineates the complex regulatory networks governing bacterial pathogenicity. This suppression of QS gene expression by GAs could be a critical factor in mitigating the virulence of Ds because QS is known to regulate a suite of virulence factors, including PCWDEs. Similarly, research has revealed the significant biocontrol potential of phloretin, an apple phytoalexin, against Pcb. Phloretin affects bacterial growth, motility, biofilm formation and the synthesis of QS-signalling molecules, indicating that similar to GAs, it impairs the virulence and fitness of the pathogen by interfering with its communication systems (Pun et al. 2021). Phenolic compounds, phytoalexins and GAs are all examples of plant secondary metabolites, although they differ in their chemical nature and mode of action. Unlike phytoalexins, which are synthesised only upon infection and localised at the damage site, GAs are often constitutively present in leaf tissues. This feature may enhance their role as intrinsic biocontrol agents by providing a continuous defence barrier.

Based on our previous research on potato cultivars and the results of our current research, we can conclude that the greatest antimicrobial activity is possessed by the cultivar Tajfun and the potato hybrid *S. chacoense*, in which the GA composition is limited to two GAs, α-solanine and α-chaconine, at a ratio of 1:1. The two GAs act in a synergistic manner at similar proportions, meaning that they have a pronounced biological effect when they act in combination rather than alone. Our findings are consistent with other studies on GAs in which synergistic interactions were detected against coleopteran insects of potato, and the fungi *Ascobolus crenulatus*, *Alternaria brassicicola*, *Phoma medicaginis* and *Rhizoctonia solani* (Fewell and Roddick 1993; Nenaah 2011). We demonstrated the bactericidal and bacteriostatic effects of GAs on Ds and Pcb via in vitro experiments. Our study investigated the process of greening, a phenomenon in which potato tubers synthesise chlorophyll and simultaneously increase their glycoalkaloid content when exposed to light. The greening process not only alters the tuber's appearance but also, more importantly, enhances its resistance to pathogens. We conducted an in vivo experiment on greened tubers of potato cv. Tajfun, which were inoculated with Ds and Pcb. The significant increase in the GA content within the skin of the greened tubers, up to fivefold compared with that of the control, confirmed the enhanced protective barrier against these pathogens. Previous studies have shown that the antibacterial effect of an extract derived from potato peels is more pronounced on gram-positive than on gram-negative bacteria (Al Kabee 2019). The present study revealed that this impact can also be observed on pectinolytic gram-negative bacteria. This result was confirmed by the reduction in the average weight of rotten tissue post-inoculation, with greened tubers exhibiting less damage from both Ds and Pcb under optimal conditions for bacterial growth and a high OD value of the inoculum.

This approach may be even more effective under field conditions, where the number of infecting bacteria is much lower, and weather conditions are less favourable.

In conclusion, our research demonstrated the antimicrobial properties of GAs, which effectively affect the bacteria's ability to multiply and induce cell death. The lower number of bacterial cells entails many indirect effects in the Ds and Pcb bacterial community, including impairment of critical virulence factors in Ds and Pcb. This attenuation is achieved by interfering with factors critical to pathogenesis, such as QS, biofilm formation and the secretion of PCWDEs. GAs from *Solanum* leaves show promise for specifically targeting the virulence factors of Ds and Pcb, offering a nonchemical method for managing these potato pathogens. There are at least two potential strategies for applying the knowledge regarding the observed variation in GA profiles across different potato varieties and GA composition-dependent antibacterial effects. First, using greened tubers of common cultivars as seed material. High GA content after greening may limit the proliferation of bacterial cells in the mother tuber and the migration of bacteria to the shoot and progeny tubers. In this case, tuber greening and high GA content are restricted only to the mother tuber. Second, using potato hybrids of known GA composition in leaves in breeding programmes. Specific GA composition may inhibit bacteria proliferation in the potato shoot and their migration to the progeny tubers as well. Both proposed applications suggest that selective breeding targeted at specific glycoalkaloid compositions could be a strategic approach to boost the resistance of potato to blackleg and soft rot.

Author Contributions

A.G.-U.: methodology, investigation, formal analysis and visualization, data curation, writing – original draft, writing – review and editing. D.S.-K.: conceptualization, methodology, investigation, data curation, writing – review and editing. R.L.: conceptualization, resources, writing – review and editing, supervision, project administration and funding acquisition

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Primers designed for the study of gene expression by reverse transcription-quantitative PCR.