Metabolites from Wild Potato Inhibit Virulence Factors of the Soft Rot and Blackleg Pathogen *Pectobacterium brasiliense*

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Potato (*Solanum tuberosum* L.) is the primary vegetable crop consumed worldwide and is largely affected by bacterial pathogens that can cause soft rot and blackleg disease. Recently, resistance to these diseases has been identified in the wild potato *S. chacoense*, and the mechanism of resistance is unknown. Here, it was hypothesized that *S. chacoense* stems or tubers have unique chemistry that confers resistance to the pathogen *Pectobacterium brasiliense* through bactericidal, bacteriostatic, or antivirulence activity. Stem and tuber metabolite extracts were collected from *S. chacoense* and tested for effects on *Pectobacterium* bacterial multiplication rates, and activity and expression of known exoenzymes and virulence genes using *S. tuberosum* extracts as a comparative control. Comparatively, the *S. chacoense* extracts did not affect bacterial multiplication rate; however, they did reduce pectinase, cellulase, and protease activities. The chemical extracts were profiled using a bioassay-guided fractionation, and a non-targeted metabolomics comparison of *S. chacoense* and *S. tuberosum* stems and tubers was performed. The data showed that selected alkaloids, phenolic amines, phenols, amines, and peptides are integrative chemical sources of resistance against the bacteria.

Keywords: *Pectobacterium*, potato, potato metabolomics, soft rot resistance

Potato (*Solanum tuberosum*) is an adaptable plant, a nutritional food, and the most common vegetable crop consumed worldwide (Jansky et al. 2019; Levy and Veilleux 2007). Cultivated potato is susceptible to many diseases, including blackleg, aerial stem rot, and soft rot caused by *Pectobacterium* spp. (Ma et al. 2007; Mansfield et al. 2012). Blackleg disease occurs when potato stems sprout from a *Pectobacterium*-

infected tuber, resulting in stem necrosis, leaf chlorosis, and wilting that decimates the crop within 2 months of planting. Aerial stem rot is infection of potato vines and is usually associated with previous physical vine damage, while soft rot is the decay of tubers infected with *Pectobacterium* spp. that can occur in seed tubers or in daughter tubers during plant growth, or during storage and transit. There are at least 18 *Pectobacterium* spp., and some species are more often associated with blackleg or with soft rot (Charkowski 2018; Khadka et al. 2020; Li et al. 2019).

Genus *Pectobacterium* is a broad-host-range necrotrophic pathogen that infects many crops in addition to potato (Ma et al. 2007) and, because of the large losses caused by this pathogen and the threat it imposes to agriculture, it was listed among the top 10 most relevant plant-pathogenic bacteria (Mansfield et al. 2012). Disease control measures in potato and other crops include limited generation production schemes, seed indexing, copper sprays, and sanitation. Other methods such as antibiotics, inorganic and organic salts, chlorine-based compounds, heat, and biological control have been proposed but they do not provide economical and effective disease management (Czajkowski et al. 2011; Motyka et al. 2017).

The major virulence factors of *Pectobacterium* spp. are secreted enzymes that rapidly degrade plant cell walls; specifically, pectinases, hemicellulases, and proteases (Charkowski et al. 2012). These enzymes degrade the middle lamella and primary plant cell walls, causing the plant cell to lyse (due to turgor pressure), resulting in the wilting and rotting symptoms typical of *Pectobacterium* infection. Therefore, the bacterial cells are necrotrophic, because they metabolize the plant cell wall components and intracellular contents. Furthermore, *Pectobacterium* spp. tightly regulate production of plant cell wall-degrading enzymes via quorum sensing (QS) (Liu et al. 2008). Moreover, plant cell wall-degrading enzyme activity is induced by plant phenolics, pectin fragments, and DNA-damaging agents (Charkowski et al. 2012; Liu et al. 1997). Disease caused by *Pectobacterium* spp. initiates when the pathogen develops into a virulent state, and this process is controlled through interactions with external signals from the host or neighboring pathogens.

Although resistance to *Pectobacterium* in *Solanum* spp. is limited, it is possible that plants restrict disease through production of phytoalexins or phytoanticipins. *Solanum* spp. produce rishitin, a terpene phytoalexin that modifies the outer surface of *Pectobacterium* cells and, at higher concentrations, rishitin causes holes to form in bacterial cell membranes (Robertson et al. 1985). Much remains to be learned about

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the role of phytoalexins in conferring resistance and much of the available data remains correlative (Hildenbrand and Ninnemann 1994). For example, three novel plant flavonoids are induced in *Pectobacterium*-resistant *Ornithogalum* lines after inoculation with *Pectobacterium* spp., but the function of these compounds in disease resistance remains unknown (Yedidia et al. 2019).

Solanaceous species also produce phytoanticipins, which are preformed metabolites (small molecules, generally less than 1,500 Da) that are toxic to many pathogens and pests. Potato plants produce these protective metabolites in both tubers and stems, including steroidal alkaloids (glycoalkaloids), steroidal glycosides (saponins), phenolics, amino glycosides, polyamine alkaloids (kukoamines), and tropane alkaloids (calystegines) (Chaparro et al. 2018; Friedman 2006). Phytoanticipins act as bioactive metabolites and provide protection by either acting as toxins or interrupting virulence pathways. For example, glycoalkaloids and saponins are directly toxic toward pests and herbivores by inhibiting proteinase activity and interrupting the integrity of animal gut membranes (Hussain et al. 2019). In contrast, phenolics and their derivatives are generally non-lethal but, instead, interfere with virulence signaling pathways of species of *Phytophthora*, *Verticillium*, *Pectobacterium*, *Burkholderia*, *Aliivibrio*, *Agrobacterium*, *Chromobacterium*, *Pectobacterium*, and *Dickeya* (Joshi et al. 2015, 2016; Muñoz-Cazares et al. 2017). In these pathogens, potato phenolics can specifically affect microbial pigment, biofilm, motility, exoenzyme, and QS pathways (Compean and Ynalvez 2014; Joshi et al. 2015; Othman et al. 2019; Silva et al. 2016). Likewise, alkaloids and terpenoids were shown to have antimicrobial and antivirulence activity against many gram-negative and gram-positive bacteria (Cushnie et al. 2014; Guimarães et al. 2019; Silva et al. 2016).

Several accessions of wild potato species have been demonstrated to be resistant or tolerant to *Pectobacterium* diseases. Here, we hypothesized that tubers and stems of domestic and wild potato differ in their profile of antimicrobial metabolites, and that metabolites contribute to resistance to soft rot disease by acting as toxins (e.g., bactericidal or bacteriostatic), or affect the virulence of these pathogenic bacteria. The lines tested in this study were chosen for their relationship to a diploid potato breeding system that was developed to facilitate genetic mapping and introgression of traits in *Solanum* species (Leisner et al. 2018; The Potato Genome Sequencing Consortium 2011). As part of this effort, the double monoploid potato line DM1 was generated from *S. tuberosum* group *phureja* (Paz and Veilleux 1999). DM1 has multiple disabled genes and deleterious alleles (The Potato Genome Sequencing Consortium 2011) and is susceptible to blackleg and soft rot. In contrast, the diploid wild potato *S. chacoense* line M6 is resistant or tolerant to many pathogens and it exhibits high tolerance to *Pectobacterium* spp. (Chung et al. 2017). We compared metabolite extracts from M6 to those from DM1 for toxicity to *Pectobacterium* spp. and inhibition of its virulence-related phenotypes to identify potential potato phytoanticipins.

RESULTS

S. chacoense M6 had reduced *Pectobacterium* colonization but metabolite extracts did not reduce *Pectobacterium* multiplication.

An initial experiment was performed to validate phenotypes in planta, and *S. tuberosum* line DM1 (susceptible potato) and *S. chacoense* M6 (resistant potato) stems were inoculated with red fluorescence protein (RFP)-tagged *Pectobacterium brasiliense* Pb1692. Microscopic analysis validated living bacteria in both resistant and susceptible lines; however, DM1 experienced

active infections whereas, in M6 plants, the bacterial colonization was comparatively minor (Fig. 1A).

Next, an in vitro experiment was performed to understand whether these infection phenotypes were bactericidal or bacteriostatic and due to the metabolite differences between the two lines. *P. brasiliense* Pb1692 was cultured in a metabolite extract of DM1 or M6 to determine whether M6 extracts reduced the multiplication rate of *P. brasiliense*. Bacteria were able to multiply using metabolites extracts of both DM1 and M6 (stem and tuber) as metabolic substrates. Except for one time point (15 h) in the M6 stem extract, *P. brasiliense* multiplication rates were similar in both M6 and DM1 extracts (Fig. 1B).

Metabolite extracts of *S. chacoense* M6 reduced *P. brasiliense* exoenzyme activity and virulence gene expression.

Effects of metabolite extracts on virulence factors were measured with enzymatic activity and gene expression assays. Compared with DM1 as a susceptible control, M6 stem and tuber metabolites reduced bacterial protease (Prt) and cellulase (Cel) activities, and M6 tuber extracts reduced pectate lyase (Pel) activity (Pel) (Fig. 2A). To investigate the mechanism by which metabolite extracts affected synthesis of these exoenzymes, we assessed the effect of M6 metabolites on the expression of selected genes: *pel1* and *pel2* with Pel, *prt1* and *prtE* with Prt, *prtW* and *inh* with metalloprotease and Prt inhibitor, and *secA* with the protein translocate subunit. The expression of *prt1*, *pel1*, and *pel2* was reduced in M6 metabolite

Fig. 1. Colonization and multiplication of *Pectobacterium brasiliense* Pb1692. **A**, Example microscopic observation (×20 magnification) of DM1 and M6 potato stem inoculated with red fluorescence protein-tagged *P. brasiliense* Pb1692. Images were taken after 2 h of incubating 1-month-old stem cuttings in bacteria suspended in water. **B**, Multiplication of *P. brasiliense* Pb1692 in metabolite extracts of M6 (blue triangle) or DM1 (red square) stem or tubers or water (black circles) at different time points. Bacteria cultured overnight were adjusted to concentration of 10⁶ CFU ml⁻¹ using sterile water and mixed with metabolite extracts (10 μg ml⁻¹) from stem and tuber. Bacteria were then incubated for 15 h at 28°C under continuous shaking. After 0, 3, 6, 9, 12, and 15 h of incubation, bacterial cells were counted using a serial dilution method and population was expressed as CFU ml⁻¹. Data shown are the combination of two independent experiments with four replicates in each experiment and presented as mean ± standard error of the mean. An asterisk (*) indicates significant difference (Student's *t* test, *P* < 0.05) in CFU counts between treatments (M6 and DM1 metabolite extracts).

extracts compared with DM1 extracts (Fig. 2B). In contrast, *prtW*, *inh*, and *secA* gene expression was not different between M6 and DM1 metabolite extracts. Similarly, *prtE* had lower expression in M6 tuber extracts but the differential expression was not observed in stem extracts (Fig. 2B).

Metabolite extracts of *S. chacoense* M6 reduced *P. brasiliense* Pb1692 N-acyl homoserine lactone synthesis.

N-acyl homoserine lactone (AHL) is a signaling molecule used for a bacterial cell-to-cell communication system (i.e., QS) and is required by *Pectobacterium* spp. for exoenzyme synthesis (de Kievit and Iglewski 2000). To study the effect of metabolite extracts on QS, we assessed the effect on *Pectobacterium* production of AHL with reporter strain *Chromobacterium violaceum* CV026. In the presence of external AHL, the reporter strain produced the purple pigment violacein (McClellan et al. 1997). Supernatant obtained by growing Pb1692 in M6 metabolite extracts had low AHL compared with DM1, illustrated by lower intensity of purple color in the reporter strain (Fig. 3A). AHL in Pb1692 is synthesized by *expI*;

therefore, the effect of metabolite extracts was also studied on *expI* gene expression. M6 metabolite extracts reduced the expression of the gene compared with DM1 metabolites (Fig. 3B).

Fractionation of M6 metabolite extracts and effect on *Pectobacterium* spp.

A bioassay-guided fractionation study was used to reduce the complexity of the M6 metabolite extract and identify sets of candidate metabolites that inhibit virulence. In these assays, the M6 stem and tuber extracts were fractionated using solid-phase extraction (between 20 and 100% acetonitrile [ACN] eluents),

Fig. 2. Effects of M6 and DM1 metabolite extracts on exoenzyme activity and gene expression of *Pectobacterium brasiliense* Pb1692. **A**, Enzymatic activity assays after growing bacterial cells (10^6 CFU ml^{-1}) in metabolite extracts ($10 \mu\text{g ml}^{-1}$) for 12 h at 28°C under continuous shaking. Data shown are the combination of two independent experiments with four replicates in each experiment and presented as mean $\pm$ standard error of the mean. An asterisk (*) indicates differences between M6 and DM1 treatments (Student's *t* test, $P < 0.05$). **B**, Gene expression analysis of *P. brasiliense* Pb1692 in response to incubation with metabolite extracts of M6 (blue) and DM1 (red). Bacterial cells (10^6 CFU ml^{-1}) were grown in metabolite extracts ($10 \mu\text{g ml}^{-1}$) for 12 h at 28°C and transcript level of *pel1* and *pel2* (pectate lyases), *prt1* and *prtE* (proteases), *prtW* and *inh* (metalloprotease and protease inhibitor), and *secA* (secretion system) were determined by quantitative reverse-transcription PCR. The level of transcripts was normalized to housekeeping gene *recA* and expressed relative to DM1 ($\log_2$ [relative expression]). Data shown are the combination of two independent experiments with three replicates in each experiment and presented as mean $\pm$ standard error of the mean. An asterisk (*) indicates differences between M6 and DM1 treatments (Student's *t* test, $P < 0.05$).

Fig. 3. Effect of DM1 and M6 metabolite extracts on N-acyl homoserine lactone (AHL) of *Pectobacterium brasiliense* Pb1692. **A**, Purple color exhibited by CV026 as a response to AHL present in Pb1692 grown supernatant. Pictures were taken after incubating the plate at 30°C for 18 h. Experiment was repeated three times with similar results. **B**, *expI* gene expression analysis of *P. brasiliense* Pb1692 in response to incubation with metabolite extracts of M6 (blue) and DM1 (red). Bacteria were incubated for 15 h and transcript levels were determined using quantitative reverse-transcription PCR and expressed relative to DM1 ($\log_2$ [relative expression]). Expression was after normalizing to housekeeping gene *recA*. Data shown are the combination of two independent experiments with three replicates in each experiment and presented as mean $\pm$ standard error of the mean. An asterisk (*) indicates differences between M6 and DM1 treatments (Student's *t* test, $P < 0.05$).

Fig. 4. Exoenzyme activity of *Pectobacterium brasiliense* Pb1692 in M6 metabolite fractions. Activity assays were performed after growing bacterial cells (10^6 CFU ml^{-1}) in metabolite extracts for 12 h at 28°C under continuous shaking. Treatments consisted of 5 mg of each M6 fraction (20 to 100%) added to 5 mg of the DM1 metabolite extracts for a final concentration of extract at 10 mg/ml. Data shown are the combination of two independent experiments with $n = 3$ replicates in each experiment and presented as mean $\pm$ standard error of the mean. An asterisk (*) indicates differences between DM1 + M6 fractions and control DM1 treatments (Dunnett's test, $P < 0.05$).

adding portions of the M6 fraction back to DM1, and testing for effects on exoenzyme activity. All M6 fractions eluted at 60 and 80% ACN significantly inhibited Prt, Pel, and Cel activity (Fig. 4).

Metabolomics analysis of *S. chacoense* extracts using liquid chromatography mass spectrometry.

A comparative metabolomics experiment was performed to identify pools of metabolites associated with the antivirulence activity in M6 stem and tuber fractions, relative to the metabolite content of DM1. The 60 and 80% ACN fractions from stem and tuber (denoted S60 and S80 for stem and T60 and T80 for tuber) were evaluated using liquid chromatography mass spectrometry (LC-MS). The metabolomics analysis detected a total of 4,576 metabolites among the four sample types (Fig. 5; Supplementary Material). Principal component analysis (PCA) was performed to characterize metabolites that distinguish M6 and DM1 (Fig. 5A). In total, seven principal components (PCs) were generated that explained 93.2% of the variation. The first two PCs (52.7%) demonstrated metabolite variation influenced by the three main experimental factors: PC1 was mostly influenced by fraction (60 or 80) and mildly driven by tissue (stem or tuber), and PC2 was largely influenced by genotype (DM1 or M6).

Because the 60 and 80% ACN M6 fractions exhibited similar responses to exoenzyme activity (Fig. 3B), and similar response were observed in both stems and tubers extracts (Fig. 3B), metabolites were considered of interest based on the following thresholds: they were (i) common to stems and tubers, (ii) common in the 60 and 80% fractions, and (iii) more abundant in all M6 tissues and fractions compared with all DM1 samples. Metabolites that exhibited this pattern in the data set were determined by correlation-scaling (corr) the PCA loadings, and focused on highly negative corr values for PC2 and loading values centered around zero for PC1. Of the 4,576 metabolites detected in this experiment, 108 metabolites met the thresholds for significance. The 108 metabolites were subsequently annotated and categorized into 11 ontological classes (Table 1) based on their chemistry. Several classes are known to contain compounds with demonstrated antimicrobial activity, including steroidal alkaloids (herein synonymous with “glycoalkaloids” discussed in the potato plant system), steroidal saponins, tropane and other alkaloids, cyclic peptides, and phenolics.

The 108 metabolites were then analyzed for fold-change (FC) and pairwise comparisons to quantify the magnitude differences between M6 and DM1 (Table 1; Fig. 5B). Because potato tubers are swollen underground stems, and these metabolomics data were not heavily influenced by tissue type (Fig. 5A), we combined the stem and tuber metabolite data from each fraction independently in both potato species prior to further analysis. Overall, 45% (2,060 of 4,576) of the features were more abundant ($P \leq 0.05$; $\log_2 FC > 1$) in the 60% M6 fraction compared with 60% DM1 fraction (Fig. 5B). Similarly, 44% (2,003 of 4,576) of the features were more abundant in the 80% M6 fraction (Fig. 5C). Most of the 108 exoenzyme-associated metabolites determined from the PCA were also observed as significant in this univariate analysis (95 of 108).

The 108 metabolites were further analyzed for known antimicrobial activities. In total, three α - and β -steroidal alkaloids were detected and ranged between 2.89- to 7.01-fold higher in M6, and no γ forms were detected (α , β , or γ denote three, two, or one saccharide residue conjugated to an alkaloid backbone, respectively). All other classes exhibited similar ranges of FC; for example, saponins (4.36 to 6.39), phenolic amines (1.3 to 5.51), phenolics (0.92 to 6.02), organic acid and amines (1.27 to 4.92), peptides (1.12 to 6.11), saccharides (1.63 to 6.86), lipids (1.74 to 7.06), and others and unknowns (0.61 to 6.82).

DISCUSSION

Genus *Pectobacterium* causes huge annual losses worldwide in the potato industry (Mansfield et al. 2012). Some wild potato lines, including *S. chacoense* M6, are resistant or tolerant to *Pectobacterium* spp. (Leisner et al. 2018). In many wild relatives of cultivated crops, disease resistance is correlated with higher content of phenolics, tropane alkaloids, and alkaloids (Friedman 2006; Othman et al. 2019). To define *Pectobacterium* resistance mechanisms in *S. chacoense* M6, plant metabolite extracts and their effects on *P. brasiliense* Pb1692 multiplication, enzyme activity, and gene expression were studied and compared with extracts from susceptible *S. tuberosum* DM1. Because the pathogen grew as well or better in metabolite extracts from the resistant potato as the susceptible potato, the M6 extracts do not appear to contain bacterial multiplication inhibitors or toxins that affect cell viability. Interestingly, the M6 stem extract had a higher saccharide content than the DM1 stem extract, which may account for the higher bacterial multiplication observed at the last time point of this experiment.

Fig. 5. Principal component analysis (PCA) and volcano plot analysis of potato metabolite fractions. **A**, Metabolites detected by liquid chromatography mass spectrometry are presented as a biplot of correlated-scaled scores (triangles = stem [S] and tuber [T] 60 and 80% fractions) and loadings (circles). Ellipses denote [0.5], [0.75], and [1.0] correlation values. Data were generated based on 4,576 metabolites among 16 samples. Metabolites represented by blue color (108) were considered to meet the correlation threshold of -0.8 for principal component (PC)2 (positively associated with M6) and centered around 0 for PC1 (0.2 threshold to indicate a higher comparative abundance in both stems and tubers, in both 60 and 80% fractions). **B** and **C**, Volcano plot for the differential abundance ($\log_2$ M6/DM1, x-axis) and significance ($-\log_{10} P$ value, y-axis) of 4,576 metabolites (gray dots) of M6 and DM1 potato. Colored circles represent highly correlated M6 metabolites (0.8/0.2) as determined from PCA. Vertical dashed lines are threshold of M6/DM1 ($\log_2$ M6/DM1 < -1 or > 1); horizontal dashed line is the threshold for P value at 0.05. FC = fold change. Volcano plots were created for **B**, 60% and **C**, 80% acetonitrile elution.

Table 1. Metabolites putatively associated with inhibiting virulence of *Pectobacterium brasiliense* Pb1692

Superclass, subclass, metabolite ^a	Identifier ^b	Log ₂ FC (M6/DM1) ^c		Activity ^d
		60%	80%	
Alkaloids				
Steroidal alkaloids				
α-Solamarine 01	PHpos_C455	3.33	3.1	Cushine et al. 2014
α-Solamarine 02	PHneg_C0282	5.7	4.57	Cushine et al. 2014
α-Solanine 01	PHpos_C126	3.24	3.86	Cushine et al. 2014
α-Solanine 02	PHpos_C593	2.89	4.45	Cushine et al. 2014
β-Chaconine 01	HIneg_C0490	5.01	6.34	Cushine et al. 2014
β-Chaconine 02	HIneg_C0572	5.31	7.01	Cushine et al. 2014
β-Chaconine 03	PHpos_C413	2.43	5.37	Cushine et al. 2014
Tropane alkaloids				
Calystegine A3/A6/A7	HIneg_C1183	3.93	3.37	Christudas et al. 2012
Calystegine B2/B5	HIpos_C328	3.24	3.44	Christudas et al. 2012
Phenolic amines and related				
(2E)-N-(4-acetamidobutyl)-3-(4-hydroxy-3-methoxyphenyl) acrylamide	PHpos_C322	2.3	2.17*	Bouarab-Chibane et al. 2019
4-(2-Nitroethyl) phenyl primeveroside	HIneg_C0563	2.93	1.3*	Bouarab-Chibane et al. 2019
N-transferuloyloctopamine 01	HIneg_C0009	3.02	2.48	López-Gresa et al. 2011
N-transferuloyloctopamine 02	PHpos_C055	3.22	1.65*	López-Gresa et al. 2011
N-transferuloyloctopamine 03	PHneg_C865	3.91	2.73*	López-Gresa et al. 2011
Phenylbutyrylglutamine	HIneg_C0290	5.51	5.48	Bouarab-Chibane et al. 2019
Other alkaloids				
Alkaloid 01	HIneg_C1021	2.71	2.9	Cushine et al. 2014
Clavepictine	HIpos_C085	4.87	6.46	Cushine et al. 2014
Amines				
Amino acids				
Acetylucine	HIneg_C1309	1.27	1.46	...
Arginine	HIneg_C0878	2.8	1.58*	...
Proline 01	HIpos_C265	1.51*	3.13	Zhang et al. 1999
Proline 02	HIpos_C332	2.3*	2.3	Zhang et al. 1999
Proline 03	HIneg_C0833	4.72	3.96	Zhang et al. 1999
Valine	HIpos_C527	3.01	3.57	...
Lipid amines				
N-oleoyl phenylalanine 01	PHpos_C454	3.4	3.52	...
N-oleoyl phenylalanine 02	PHpos_C639	5.33	4.52	...
Others				
Similar to ecalcidene	HIpos_C560	1.81	3.75	...
Inosine monophosphate	Phneg_C0614	3.23	2.49	...
Organic acids				
Organic acids				
L-ascorbic acid-2-glucoside	PHneg_C1316	4.08	1.56*	Verghese et al. 2017
Malic acid	HIneg_C0134	4.62	4.92	Raybaudi-Massilia et al. 2009
Phenolics and related				
3-(2-Methylpropanoyloxy)-8-(2-methylbutanoyloxy)-9,10-epoxy-p-mentha-1,3,5-triene	HIpos_C137	5.97	6.52	Bouarab-Chibane et al. 2019
Coumaric acid	HIneg_C1434	4.13	3.42	Lou et al. 2012
Coumarin	PHpos_C562	3.25	1.8*	Bouarab-Chibane et al. 2019
Derricin	PHneg_C1039	6.02	4.19	Bouarab-Chibane et al. 2019
Epicatechin methyl gallate	PHneg_C0051	4.52	2.3	Nikoo et al. 2018
Ferulic acid	HIneg_C0079	3.15	3.5	Borges et al. 2013
Glucocaffeic acid	PHneg_C0877	3.34	1.57	...
Melanin	HIneg_C0485	3.56	2.1	...
Phenolic	PHpos_C325	2.7	0.92*	Bouarab-Chibane et al. 2019
Shikimic acid	HIneg_C1391	2.95	2.18*	Bai et al. 2015
Terezine B/C 01	PHpos_C776	4.61	3.11	Abdou et al. 2020
Terezine B/C 02	HIneg_C0185	5.13	4.76	Abdou et al. 2020
Terpenes				
Steroidal saponins				
Graecunin	PHpos_C276	4.33	3.96	...
Saponin 01	PHneg_C0221	0.86*	2.13	Soetan et al. 2006
Saponin 02	PHneg_C0608	5.24	5.08	Soetan et al. 2006
Saponin 03	PHneg_C959	2.71	2.53	Soetan et al. 2006
Saponin 04	HIpos_C142	3.77	3.88	Soetan et al. 2006
Torvoside	PHneg_C0112	6.39	5.97	Soetan et al. 2006

(Continued on next page)

^a Metabolite annotation based on interpretation of mass spectrometry data. Isomers are indicated by identical spectra but different retention times, denoted as 01, 02, and so on, or potentially the same compound detected on different platforms. Level 3 annotations (denoted simply by a class) are when a spectrum indicates a chemical class but a specific metabolite could not be confidently assigned. M = neutral mass and nd = annotation could not be determined.

^b PH = phenyl hexyl, HI = hydrophilic interaction liquid chromatography, pos = positive mode, and neg = negative mode.

^c Log₂ fold-change (FC) values for 60 and 80% acetonitrile. An asterisk indicates $P > 0.05$ and values without an asterisk indicate significant differences between M6 and DM1 at $P < 0.05$.

^d Reported antimicrobial activity.

Table 1. (Continued from previous page)

Superclass, subclass, metabolite ^a	Identifier ^b	Log ₂ FC (M6/DM1) ^c		Activity ^d
		60%	80%	
Tuberoside 01	PHneg_C0111	6.27	5.27	Soetan et al. 2006
Tuberoside 02	PHneg_C0175	6.05	4.28	Soetan et al. 2006
Tuberoside 03	HIneg_C0851	4.46	3.33	Soetan et al. 2006
Tuberoside 04	HIneg_C1184	4.36	5.69	Soetan et al. 2006
Peptides				
Cyclic peptide similar to ustiloxin	PHpos_C543	3.05	1.69	Jakubczyk and Dussart 2020
N-acetyl-leucine/acetyl-isoleucine	HIneg_C1166	3.88	3.71	...
Peptide 01	PHneg_C0017	6.11	1.85	...
Peptide 02	PHneg_C0178	5.8	5.03	...
Peptide 03	PHneg_C0280	4.33	2.79	...
Peptide 04	PHneg_C0317	5.59	4.48	...
Peptide 05	PHneg_C1323	4.46	1.57*	...
Peptide 06	PHneg_C0727	2.73	2.23	...
Peptide 07	PHneg_C0732	1.12	2.12	...
Peptide 08	PHneg_C1032	1.38	1.62	...
Peptide 09	PHneg_C0214	5.47	4.54	...
Peptide 10	HIneg_C0263	2.32	2.43	...
Peptide 11	HIneg_C0226	1.38	1.62	...
Peptide similar to polyoxin B	HIneg_C0196	3.27	3.85	Keymanesh et al. 2009
Saccharides				
Monosaccharides				
Monosaccharide 01	HIneg_C0579	2.62	1.19*	...
Monosaccharide 02	HIneg_C1337	3.45	2.77	...
Disaccharides				
Disaccharide 01	PHpos_C010	4.83	2.57	...
Disaccharide 02	PHneg_C0092	6.86	4.23	...
Disaccharide 03	PHneg_C0106	4.3	2.54	...
Disaccharide 04	HIneg_C0005	4.34	2.84	...
Disaccharide 05	HIneg_C0340	2.97	1.63	...
Disaccharide 06	HIneg_C1338	4.03	1.91	...
Trisaccharides				
Trisaccharide-01	HIneg_C0577	4.62	3.53	...
Trisaccharides 02	HIneg_C1343	0.02*	0.19*	...
Oligosaccharides				
Oligosaccharide	HIneg_C1154	4.03	3.49	...
Sugar acids				
Glucuronic acid	HIneg_C0835	2.66	2.52	...
Others				
N-acetyl-6-O-L-fucosyl-D-glucosamine	HIneg_C1435	2.88	1.67	...
Lipids				
Glycerolipids				
PI (28:1)	HIneg_C0573	6.2	7.06	...
PI (38:4)	PHpos_C284	1.74	5.04	...
PS (34:6)	HIneg_C0697	5.11	6.95	...
PS (42:9)	PHpos_C404	2.35	4.31	...
Others				
1-Octen-3-yl glucoside	PHneg_C1287	4.03	4.39	...
Ethyl (S)-3-hydroxybutyrate glucoside	HIneg_C0753	4.35	3.82	...
Ethyl (S)-3-hydroxybutyrate glucoside	PHneg_C1040	5.64	4.09	...
Isopentyl gentiobioside	HIneg_C1422	3.62	3.94	...
1,2,10-Trihydroxydihydro-translinalyl oxide 7-O-beta-D-glucopyranoside	PHneg_C0619	4.09	2.7	...
Unknowns				
Unknowns				
[M - H] ⁻ = 327.1066	HIneg_C0437	1.79*	2.25	...
[M - H] ⁻ = 605.8024	HIneg_C1178	3.24	2.92	...
[M - H] ⁻ = 948.4741	PHneg_C1031	1.42	2.25	...
M = 1043.5921	PHpos_C274	1.11	3.32	...
M = 1050.5608	PHpos_C680	5.61	5.31	...
M = 1102.2589	HIneg_C0339	2.86	1.59	...
M = 1572.9533	PHneg_C0220	5.53	6.82	...
M = 294.1313	HIneg_C1424	3.25	2.17	...
M = 341.1231	PHneg_C1280	2.91	2.87	...
M = 344.1306	HIneg_C1444	4.36	2.76	...
M = 410.0514	PHneg_C1286	3.77	1.49	...
M = 657.3779	PHneg_C1181	1.66	3.38	...
M = 706.2142	PHneg_C0338	3.56	2.33	...
M = 708.2141	PHneg_C1072	3.71	1.54	...
M = 742.4245	HIneg_C1334	4.37	5.82	...
M = 743.4169	HIneg_C0852	4.24	5.33	...
M = 897.5098	PHneg_C0733	0.72	1.96	...
M = 959.5107	PHneg_C0516	0.61	1.41	...

(Continued on next page)

Table 1. (Continued from previous page)

Superclass, subclass, metabolite ^a	Identifier ^b	Log ₂ FC (M6/DM1) ^c		Activity ^d
		60%	80%	
M = 965.4969	PHneg_C0329	0.72	1.99	...
nd	HIneg_C1283	2.65	1.84*	...
nd	PHneg_C1036	2.6	1.59	...

M6 metabolite extracts did not reduce bacterial multiplication; therefore, we investigated effects of the extracts on bacterial exoenzyme activity. *Pectobacterium* spp. secrete Prt, Pel, and Cel to macerate the plant cell wall and to obtain nutrients (Pérombelon 2002). *P. brasiliense* grown in M6 metabolite extracts had significantly lower Prt, Pel, and Cel activity. Furthermore, gene expression of *pel1*, *pel2*, *prt1*, and *prtE* demonstrated that these enzymes were downregulated in the presence of M6 extracts, which corresponds with the lower activity observed in the enzyme assays. In addition, M6 metabolite extracts reduced or inhibited AHL synthesis. These results support the idea that M6 resistance to blackleg and soft rot is due, at least in part, to inhibition of bacterial exoenzyme expression via QS inhibition.

Plants are complex organisms producing more than approximately 300,000 metabolites (Wang et al. 2019). This complexity makes it challenging to understand and study specific groups of metabolites that contributed to lower activity of cell-wall-degrading enzymes in our study. Therefore, we fractionated the M6 metabolite extracts into five fractions based on polarity. As with whole extracts, none of the fractions reduced bacterial multiplication but only some inhibited exoenzyme activity, with the 60 and 80% ACN eluted fractions from M6 stems and tubers having the strongest inhibition (Fig. 3). Therefore, these two fractions from stem and tubers were studied independently for their composition using LC-MS. The M6 and DM1 chemical features are distinct from each other. Potato tubers are underground stems; thus, we also compared potato stem and tuber extracts within each potato line and did not find differences. Therefore, the stem and tuber extract data from M6 were combined, as were the stem and tuber extract data from DM1, to provide more statistical power in comparisons between potato lines.

The metabolites that were highly correlated with M6 extracts included saccharides, phenolics, phenyl amines, amino acids, steroidal saponins, alkaloids, peptides, lipids, and organic acids. There were several metabolites which could not be annotated or classified, and these were classified into a single group of unknown metabolites. Among the aforementioned chemical classes, steroidal saponins (torvoside and tuberoside), phenols (ferulic acid and coumarins), and alkaloids (chalconine and solanine) have demonstrated antivirulence or antimicrobial activity against many gram-negative and gram-positive bacteria (Joshi et al. 2015; Othman et al. 2019; Radulović et al. 2013). Further analysis of these metabolites revealed higher content of these chemical classes in M6 than DM1. However, as described above, our multiplication assays did not show evidence of antimicrobial activity but, instead, showed antivirulence properties in these extracts. Such observations might be because of the sublethal concentrations of compounds in the extracts that were used for growing bacteria.

The steroidal and tropane alkaloids detected in our study (calystegines) also have antimicrobial and antivirulence activity against phytopathogens (Friedman 2006; Petersson et al. 2013). These metabolites were found in higher amount in M6 potato. Along with these compounds, higher content of phenols or flavonoid derivatives (coumarins, ferulic acid, shikimic acid, and other phenols) were detected in M6 extracts. These

phenolics reduced the virulence of *Pectobacterium* and *Pseudomonas* spp. by interfering with the bacterial QS system (Borges et al. 2013; Bai et al. 2015; Joshi et al. 2016; Lou et al. 2012). Similarly, phenolic compounds such as catechin, cinnamaldehyde, coumarin, curcumin, eugenol, quercetin, resveratrol, and salicylic acid interrupt bacterial signaling systems, secretion systems, toxin production, motility, and extracellular enzymes of many gram-negative and gram-positive bacteria (Joshi et al. 2020; Muñoz-Cazares et al. 2017). The metabolites in these chemical classes are more abundant in M6 potato, which might contribute to soft rot resistance in M6. In addition, M6 has higher content of lipids, fatty acyls, and peptides. Lipids or fatty acyls destabilize the membrane properties of bacterial cells, leading to direct and indirect inhibitory effects (Yoon et al. 2018). This group of metabolites also improves antimicrobial activities of phenolic compounds when present together (Zhang et al. 2017). Altogether, the majority of the metabolites with antimicrobial and antivirulence properties were detected at a higher concentration in M6 extracts than in DM1 extracts and they may contribute to soft-rot and black leg resistance additively or synergistically by inhibition of plant cell-wall-degrading enzyme production or activity. Also, the biochemical assays performed using potato extracts can be a useful tool for breeders and plant pathologists to test resistance against *Pectobacterium* spp. and other microbes with similar modes of virulence.

MATERIALS AND METHODS

Plant materials, bacterial strains, and chemicals.

S. tuberosum (DM1) and *S. chacoense* (Hassl.) (M6) were grown in a greenhouse at 18 to 24°C with a 16-h day length. Plants were grown in ProMix Bx General Purpose mix, fertilized with Osmocote Plus 15-9-12 (Scotts-MiracleGro, U.S.A.), and irrigated to saturation every other day until used for assays. Aphids and other pests were managed with Botaniguard ES and Molt-X (BioWorks, U.S.A.), Entrust SC (Corteva, U.S.A.), Distance IGR (Valent Biosciences, U.S.A.), Judo and Azatin (OHP Inc., U.S.A.), Avid 0.15EC (Syngenta, U.S.A.), and Compass (Bayer, U.S.A.).

Tubers and 1-month old stems were used for extraction of metabolites. LC-MS-grade water, analytical-grade methanol, ACN, and hydrochloric acid were purchased from Fisher Chemicals (Thermo Fisher Scientific, U.S.A.). *P. brasiliense* Pb1692 was used for all experiments in this study (Duarte et al. 2004). Nutrient broth (NB), agar, skim milk powder, tryptone, Congo red, and NaCl were purchased from Difco Laboratories (Thermo Fisher Scientific). Bacteria were grown at 30°C under continuous shaking conditions.

Microscopy.

One-month-old DM1 and M6 potato plants were cut at the stem base and dipped into 10 ml of water containing RFP (p519::ntrp::ds.Red.T3)-tagged Pb1692 (10^6 CFU ml⁻¹) for 2 h. A cross section of stem was taken 5 cm above the dipped stem mark and observed under an Olympus IX83 Inverted Spinning Disk Confocal Microscope, using an RFP filter (Olympus, Japan).

Extraction and fractionation of metabolites.

Potato tubers and stems of both species under study were harvested, flash frozen in liquid nitrogen, and ground to a crude powder using mortar and pestle. Ground samples were lyophilized for at least 12 h (HarvestRight, U.S.A.). The freeze-dried stem and tuber tissues were then ground to a fine powder using a coffee grinder. For metabolite extraction, 2 ml of 70:30 methanol/water (vol/vol) was added to a 100 mg of tissue, agitated for 2 h at 4°C using a vortexer, then sonicated for 5 min at room temperature. The mixture was then centrifuged at $6,000 \times g$ for 20 min at 4°C and the supernatant was transferred to a new vial and dried under a stream of nitrogen gas (Organomation Associates Inc., U.S.A.). The dry matter (the metabolite extract) was weighed for each sample to normalize the doses used in the microbial studies.

Fractionated extracts were used for some assays. Metabolites were separated based on polarity using Discovery Solid Phase Extraction (SPE) C18 columns (Sigma-Aldrich Inc., U.S.A.) with an ACN gradient between 20 and 100%. SPE columns were attached to a vacuum trap, the flow rate was set at 0.5 ml min^{-1} , and columns were first activated using 5% methanol. For fractionation, the dried metabolite extracts were resuspended in 5% methanol and run through the activated columns. The initial eluent (unretained metabolites) was discarded and the columns were again washed in 5% methanol. After washing, a 20% ACN solution was run through the column and the eluent was collected in separate vials. Similarly, a gradient of ACN solution was used (40, 60, 80, and 100%) for eluting the compound bound in SPE-C18 columns with the same flow rate.

Bacterial multiplication and exoenzyme measurements.

Bacterial cultures were grown overnight in NB at 30°C under continuous shaking at 220 rpm. The overnight-grown culture was centrifuged and resuspended into sterile water, which was used as a source of inoculum for studying bacterial responses to metabolite extracts. The dried metabolite extracts were resuspended in sterile water at a concentration of 10 mg ml^{-1} and bacteria were inoculated at a concentration of 10^6 CFU ml^{-1} . Bacteria were then incubated for 15 h at 30°C under continuous shaking at 220 rpm. Bacterial multiplication was recorded at 3-h time points using a serial dilution method and results were recorded as CFU ml^{-1} . The culture tubes were then centrifuged ($8,000 \times g$, 5 min) to separate supernatant and Pb1692 cells. The bacterial pellets were used for RNA extraction and supernatant was filter sterilized and used to measure AHL (using reporter strain CV026) and the exoenzyme activity: Pel, Cel, and Prt. The activity assays of these enzymes were performed in plate assays as previously described (Chatterjee et al. 1995; Vijayaraghavan and Vincent 2013). For metabolite fractions, exoenzyme activity was measured using the same procedure with 1 ml of each M6 fraction (concentration of 5 mg ml^{-1}) added to 10 mg of DM1 total metabolite extractions.

RNA extraction, cDNA preparation, and quantitative reverse-transcription PCR.

The Pb1692 bacterial cell pellet was used for RNA extraction using TRIzol reagent, following the user guide (Thermo Fisher Scientific). Extracted RNA was employed to prepare cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., U.S.A.). In total, $1 \mu\text{g}$ of RNA was used for cDNA preparation, following the manufacturer's instruction. The quantification of transcripts (*pel1* and *pel2* with Pel, *prt1* and *prtE* with Prt, *prtW* and *inh* with metalloprotease and Prt inhibitor, and *secA* with the protein translocate subunit) was performed as previously described (Joshi et al. 2016).

Detection of AHL molecules.

C. violaceum is a gram-negative bacterium that produces a purple pigment, violacein, in the presence of AHL. The reporter

strain CV026, which has a mini-Tn5 insertion in the *luxI* homolog, is used to detect the presence of AHL (McClellan et al. 1997). This mutant strain produces purple pigment violacein in the presence of AHL compounds with N-acyl C4 to C8 side chains. Therefore, CV026 was used to detect the level of AHL in this study.

Pcb1692 was cultured in metabolite extracts and the supernatant was separated as described above. The reporter strain CV026 was grown in fresh NB medium supplemented with kanamycin ($50 \mu\text{g ml}^{-1}$). Each supernatant ($50 \mu\text{l}$) was transferred to the center of nutrient agar plates inside a hole made with a 5-mm-diameter cork borer. Then, CV026 was spread with a microbial loop on four sides of the hole on the nutrient agar (Thermo Fisher Scientific) plates. The plates were incubated overnight (18 h) at 30°C and the intensity of the purple color exhibited by the reporter strain was assessed.

LC-MS metabolite detection, data processing, and metabolite annotation.

The fractions that were biologically active against Pb1692 were dried and resuspended in 50% methanol at a concentration of 4 mg ml^{-1} . The metabolomic profiling was performed on a Waters Acquity Classic ultrahigh-performance liquid chromatography system coupled to a Waters Xevo G2 time-of-flight mass spectrometer (Waters, U.S.A.). Two LCs were used for metabolite separation (in separate injections): reverse-phase chromatography (Waters Acquity CSH Phenyl Hexyl column, $1.7 \mu\text{M}$, $1.0 \times 100 \text{ mm}$) (Waters) and hydrophilic interaction chromatography (HILIC) (EMD Millipore ZIC-pHILIC, $5 \mu\text{M}$, $2.0 \times 150 \text{ mm}$) (EMD Millipore, U.S.A.). Samples were kept at 6°C and run in a randomized order using $3 \mu\text{l}$ of injection volume. The mobile phases for reverse-phase chromatography were (A) water with 2 mM ammonium formate and (B) ACN with 0.1% formic acid. A constant flow rate of 0.2 ml min^{-1} was used. The gradient started at 99% A and held for 1 min, changed to 98% B over 12 min, held at 98% B for 3 min, then returned to 99% A over 0.05 min and held at 99% A for 3.9 min. The column was held at 65°C. The mobile phases for HILIC were (A) water with 10 mM ammonium bicarbonate at pH 9.6 and (B) ACN. The gradient used was as follows: time = 0 min, 10% A, flow = 0.27 ml min^{-1} ; 1.5 min, 10% A, 0.27 ml min^{-1} ; 10 min, 38% A, 0.27 ml min^{-1} ; 14 min, 60% A, 0.24 ml min^{-1} ; 18 min, 80% A, flow = 0.2 ml min^{-1} ; 20 min, 95% A, 0.15 ml min^{-1} ; 21 min, 95% A, 0.15 ml min^{-1} ; 27 min, 10% A, 0.3 ml min^{-1} ; 28 min, 10% A, flow = 0.4 ml min^{-1} ; 36 min, 10% A, 0.4 ml min^{-1} ; 36.5 min, 10% A, 0.27 ml min^{-1} ; and 40 min, 10% A, 0.27 ml min^{-1} . The HILIC column temperature was held at 50°C. The data were acquired in both positive and negative mode on the mass detector with electrospray capillary voltage set at 2,200 V, source temperature at 150°C, and nitrogen desolvation temperature at 350°C with a flow rate of 800 liters h^{-1} . The detector was scanned over 50 to 1,200 m/z at 0.2 s/scan, alternating between MS (6 V collision energy) and MSE mode (15 to 30 V ramp). Calibration was performed using sodium iodide with 1 ppm mass accuracy. Quality control was performed by running quality control samples after every six experimental samples using a pooled sample approach, as previously described (Broadhurst et al. 2018).

For data processing, .RAW files were converted to .cdf format and processed as previously described (Yao et al. 2019) in R statistical software (R Core Team 2015). Briefly, peak detection, grouping, alignment, and filling were performed using XCMS in R (Smith et al. 2006), then deconvoluted and normalized to total ion current using the RAMClust package in R (Broeckling et al. 2014). Interpretation of spectra occurred using the R package Interpret MS spectrum and MS-Finder v3.0 (Lai et al. 2018; Tsugawa et al. 2016), which determined

molecular weights of fragments, chemical formulas, and structures, which were eventually searched and matched using external MS databases and tools such as METLIN (Montenegro-Burke et al. 2020), MetFrag (Wolf et al. 2010), and NIST library (Mikaia et al. 2014).

Statistical analysis.

Bacterial multiplication, exoenzyme, and gene expression data were analyzed with JMP-Pro software (version 5.0; SAS Institute Inc., U.S.A.) using Student's *t* tests to compare between treatments, with a *P* threshold of 0.05. Metabolomics data were analyzed in SIMCA software (version 15.0; Umetrics, Umea, Sweden) for PCA of spectral clusters using unit variance scaling. FC for metabolites was calculated using the mean relative abundance value of each M6 metabolite compared with the mean DM1 abundance for the same metabolite. All graphs were illustrated using Graph Pad Prism 8.

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