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Zebra chip-diseased potato tubers are characterized by increased levels of host phenolics, amino acids, and defense-related proteins☆

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ABSTRACT

Little is known about specific host chemistry effects on zebra chip disease symptom development in potatoes (*Solanum tuberosum*). This research compared chemical profiles and defense-related enzyme levels between non-symptomatic and zebra chip-symptomatic potato tubers. Levels of phenolics, five amino acids, peroxidases, polyphenol oxidases, chitinases, and β-1,3-glucanases were greater in symptomatic tubers than non-symptomatic tubers, and many of these compounds also were positively correlated with zebra chip disease severity. '*Candidatus* Liberibacter solanacearum' was consistently present in symptomatic tubers. However, the lack of associations between titers and tuber chemistry suggests a complicated relationship between this bacterium and zebra chip symptoms.

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1. Introduction

Zebra chip is an increasing threat to potato (*Solanum tuberosum* L.) production in North America and elsewhere. Incidence of this disease has increased beyond where it was first discovered in Mexico in 1994 to the United States by 2000 [1–3]. It is now also present in New Zealand [4]. The eponymous symptomology of zebra chip is dark discoloration of vascular tissues and medullar rays of tubers, increased browning when fresh tubers are cut, and banding of fried tuber slices for potato chip production [5,6]. Zebra chip disease is associated with infections by a fastidious bacterium, '*Candidatus* Liberibacter solanacearum', which is transmitted by the tomato-potato psyllid [*Bactericera cockerelli* (Sulc) (Hemiptera:Triozidae)], via infected tubers, and by grafting of vegetative propagated-material from zebra chip-symptomatic plants [6–9]. This bacterium has also been associated with diseases of other solanaceous crops such as tomato, pepper, and tamarillo, as well as in carrots (*Apiaceae*) [7,10–12]. However, Koch's postulates have yet to be completed due to the inability to culture this bacterium.

Both biotic and abiotic stresses could induce potatoes to undergo changes in secondary metabolite levels, including phenolic compounds [13–16]. Such shifts in secondary metabolism could directly or indirectly lead to zebra chip disease symptoms to develop, as greater phenolic levels would result in increased browning when fresh tubers are cut. Navarre et al. have already reported that increased total phenolic levels were associated with zebra chip tubers [5]. However, these changes were not correlated with zebra chip symptom severity, nor was the association of zebra chip with '*Ca.* Liberibacter solanacearum' established when the work by Navarre et al. was performed [5]. Programmed cell death, which will increase phenolic levels, also had just recently been reported in zebra chip-symptomatic tubers [17].

Pathogen infections also increase the levels of certain enzymes within potato, including those that could attack cell walls of pathogens (i.e. chitinases and β-1,3-glucanases) and those that create/maintain environments unsuitable for pathogens (i.e. peroxidases and polyphenol oxidases) [16,18]. Increases in polyphenol oxidase levels would result in increased browning in freshly cut potatoes as the enzyme oxidizes phenolic compounds into brown-colored products. Potatoes encountering biotic or abiotic stress also may undergo changes in primary metabolism. For instance, increases in amino acid production could occur. In potato tubers, increased amino acid content could result in increased browning when slices were fried, as amino acids are acrylamide precursors and acrylamide is a brown product [19–21]. Navarre et al. concluded that increases of tyrosine within zebra chip-affected potatoes might have resulted in observed banding symptoms of fried potato slices [5].

This work compares levels of phenolic compounds, amino acids, peroxidases, polyphenol oxidases, chitinases, and β-1,3-glucanases

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in non-symptomatic and zebra chip-symptomatic tubers to determine which of these are associated with zebra chip symptoms. Greater levels of phenolic compounds, amino acids, and defense-associated enzymes are hypothesized to be in greater levels in zebra chip-symptomatic tubers than in tubers lacking symptoms. This work expands upon that of Navarre et al. [5] by identifying and quantifying a greater number of amino acids than reported previously, adds additional insight into the relative activity levels of enzymes perceived to be involved in zebra chip symptom development, and attempts to find associations with '*Ca.* Liberibacter solanacearum', the likely causal agent for zebra chip disease. Compound and enzyme levels were correlated with zebra chip disease severity to further confirm potential roles in the formation of zebra chip symptoms. This study also assesses the potential role of '*Ca.* L. solanacearum' in zebra chip disease symptom development by correlating bacterial titers with symptom severity and host compound/enzyme levels. Thus, the goal of the study was to examine if zebra chip symptoms form as a result of host induced defense responses against the microbe '*Ca.* L. solanacearum'.

2. Material and methods

2.1. Sample preparation

Twenty tubers of a breeding line potato cultivar were obtained in August 2010 from a field where zebra chip was present. These were sliced in cross sections to assess zebra chip severity visually based on symptoms within the vascular tissue. Two cross section slices (labeled 'A' and 'B') were made from each tuber at proximal and distal ends of the tuber, and were assigned a symptom severity rating of zero (no symptoms) to three (severe symptoms) (Fig. 1).

Each potato slice then was pulverized with a mortar and pestle, and 0.1 g of pulverized tissue was weighed out into two different tubes. Remaining material was used for PCR analyses. These were flash-frozen in liquid nitrogen and kept at −20 °C for subsequent analysis.

2.2. PCR analysis

In this study, traditional PCR was used for qualitative analysis to determine bacterial presence or absence, and quantitative PCR to determine bacterial titers. DNA was extracted from the pulverized potato tuber tissue using the DNeasy Plant Mini Kit (Qiagen, Valencia CA) following manufacturer's guidelines. For both types of PCR reactions (traditional and quantitative), primer set OA2 (GCGCTTATTT TTAATAGGAG CGGCA)/OI2c (GCCTCGCGAC TTCGCAACCC AT) was used [7], and each reaction had the same cycling conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s, and72 °C for 30 s.

The traditional PCR reaction mix consisted of 2.5 μl of 10X Buffer, 2.5 μl of 2.5 mM dNTP's, 0.2 μl of 5 U/μl Takara Taq Hot-Start Version (Clontech, Mountain View CA), 0.5 μl 10 μM OA2 primer, 0.5 μl 10 μM OI2c primer, 2 μl of extracted tuber DNA and 16.8 μl of H_2O for a total of 25 μl. This reaction was run on an S1000 Thermal Cycler (BioRad, Hercules CA) and PCR products were visualized with UV light after ethidium bromide staining.

For quantitative PCR, the reactions consisted of 12.5 μl of 2X iQ SYBR Green Supermix (BioRad, Hercules CA), 0.5 μl of 10 μM OA2 primer, 0.5 μl of 10 μM OI2c primer, 2 μl of extracted tuber DNA, and 9.5 μl H_2O for a total of 25 μl. The reactions were monitored using an Opticon 2 Real Time PCR System (BioRad, Hercules CA). C(t) values were calculated by the software and results tabulated.

2.3. Phenolic analysis

Methanol (0.5 ml; Thermo-Fisher Inc., Pittsburgh, PA, USA) was added to one of the 0.1 g tissue sub-samples from all 40 samples (the 'A' and 'B' samples from all 20 tubers). These were left overnight at 4 °C. The samples were then centrifuged at 10,000 × g and the supernatant removed to clean, labeled tubes. The pellet was extracted again in 0.5 mL of methanol, left overnight at 4 °C, and the centrifuged again. The two supernatants were combined and referred to as methanol extract (1 ml). Methanol extracts were analyzed via high-performance liquid chromatography (HPLC) using a Shimadzu (Columbia, MD, USA) LC-20AD pump system with an SPD-20 photodiode array detector for compound quantification. The system was equipped with a Shimadzu Shim-Pack XR-ODS 4.6 X 100 mm column, used a Shimadzu CTO-20A column oven (set at 50 °C), and used a Shimadzu SIL-20A HT cooled auto-sampler (set at 4 °C). The flow rate was set a 1 mL/min. For each sample, 50 μL was injected and a binary gradient was performed whereby a mixture of 95% solvent A (water with 2% acetic acid) was gradually replaced with 100% solvent B (methanol with 2% acetic acid), based on the method from Wallis et al. [22]. In brief, following a 2 min hold with 95% solvent A, the composition of solvent B first reached 52.5% over 20 min, then reached 100% over the next 10 min, followed by a 3 min hold at 100% solvent B. The system then reset to 95% solvent A over the final 5 min to prepare for the next run. The total runtime per sample was 40 min.

Compounds were identified based on matching UV/Vis spectra and retention times with commercially-available standards (Sigma–Aldrich, St. Louis, MO, CA), if available. Additional compounds were putatively identified by matching UV/Vis spectra and relative retention times of potato tuber phenolic compounds previously reported [5]. Compounds were quantified as peak areas measured at 280 nm as this wavelength was observed to give the best separation of the peaks. Serial dilutions of commercial standards of chlorogenic acid (Sigma) and kaempferol (Sigma) were used to convert peak areas to μg/g FW for compounds belonging to the chlorogenic acid/phenolic acid and flavonoid groupings, respectively. Due to chlorogenic acid breaking down in methanol into two distinct peaks, chlorogenic acid and its isomers were summed together in analyses [23]. Other compound groups derived were chlorogenic acids and other coumaric acid derivatives, flavonoids, and total phenolics, as these all form from distinct pathways [22].

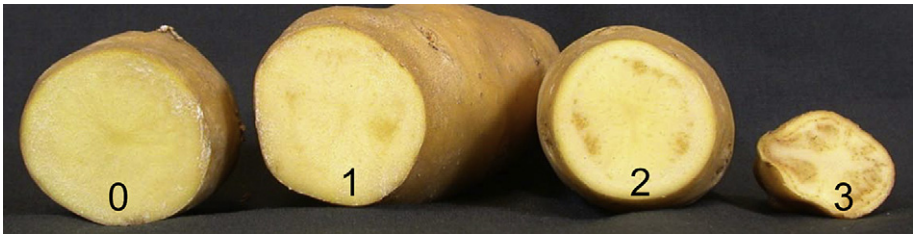

Fig. 1. Examples of potato zebra chip disease severity ratings utilized in this study. The range goes from 0, which represents no symptoms, to 3, which represents severe symptoms.

2.4. Amino acid and protein analyses

All buffers and substrate chemicals for protein analyses were supplied by Sigma, unless indicated. To one 0.1 g aliquot of potato tissue, 0.5 mL of 0.01 M sodium phosphate buffer (with pH adjusted to 6.8) was added and allowed to incubate overnight at 4 °C. The samples were then centrifuged at 10,000 × g for 5 min and the supernatant was then transferred into clean tubes. This supernatant (sodium phosphate buffer extract) then was used for all subsequent amino acid and protein analyses.

Levels of amino acids were examined by processing 100 μL of this extract using the GC-FID based EZ:FAAST amino acid kit from Phenomenex (Torrance, CA, USA) following all instructions provided and using the column included in the kit. Norvaline (at 200 nmol/mL) was used as the internal standard, and compound levels were expressed as nmol per g fresh weight.

Total protein content was assessed using the Lowry Protein Reagent Kit from Sigma using 100 μL of protein extract in each reaction and following the protein precipitation protocol before commencing the quantification protocol. Absorbance of the final samples was read at 630 nm with a Biotek ELx800UV 96-well microplate reader (Winooski, VT, USA).

Defense-associated enzyme assays were performed according to the methods of Barto et al. [24], Cipollini et al. [25], and/or Abeles and Forrence [26], with some modifications. In brief, to assess peroxidase activity, 15 μL of the protein extract was added to 150 μL of 0.25% guaiacol in 0.1 M sodium phosphate (dibasic, anhydrous) buffer pH 6.0, containing 0.3% hydrogen peroxide in a microplate. After incubation for 5 min at room temperature the microplate was then read at 450 nm. To assess polyphenol oxidase activity, 30 μL of the protein extract was added to 150 μL of 3 mM caffeic acid in 0.05 M sodium phosphate (dibasic) buffer pH 8.0 in a microplate. The microplate was then read at 450 nm after incubation for 5 min at room temperature. To assess chitinase activity, 20 μL of the protein extract was added to 200 μL of 0.05 M potassium phosphate buffer plus 0.150 M sodium sulphate (PPSS) pH 6.0, and 100 μL of *p*-nitrophenyl-β-*N*-acetylglucosaminide in PPSS (10 mg per 100 mL) in a microplate. This mixture was incubated for 6 h at 40 °C, and then read with a microplate reader at 405 nm. Finally, to assess β-1,3-glucanase, 20 μL of extract was added to 200 μL of laminarin in 0.05 M PPSS, and the solution was incubated for 2 h at 50 °C. The conversion of laminarin to glucose was measured following the protocols of a glucose kit from Sigma, with 100 μL of this protein extract/laminarin mix used in glucose reaction mixture.

2.5. Statistical analyses

All statistics were performed using SPSS ver. 19.0 (IBM, Armonk, NY, USA), with $\alpha = 0.05$. Prior to analyses, data were analyzed via SPSS Explore feature for normality and statistical outlier analysis (i.e. data greater than 2 standard errors of the mean). Independent samples *t*-tests were conducted to determine if significant differences in compound levels existed between healthy and zebra chip-symptomatic tubers with chlorogenic acids and other coumaric acid derivatives, chlorogenic acid derivatives, flavonoids, and total phenolics used as compound classes for analyses. Unknown compounds were excluded from groupings. *T*-tests did not assume equality of variance when Levene's tests indicated such. Mean compound levels from tubers with different disease ratings were also compared using univariate ANOVAs followed by LSD multiple comparison tests. Spearman correlations were performed to correlate compounds, compound classes, and protein levels with potato disease severity ratings. Pearson correlations were used to find associations between the different proteins analyzed. Independent samples *t*-tests with equal variances assumed or not assumed (as determined by Levene's test for equality of variances, due to concerns over the low number of non-infected samples) were performed to observe potential differences between tubers that tested PCR-positive or PCR-negative for '*Ca.* L. solanacearum'. Spearman correlations were also performed to find associations of '*Ca.* L. solanacearum' titers with disease severity ratings and chemistry.

3. Results

3.1. Methanol-soluble compounds significantly increased in symptomatic tubers

A total of 21 compounds were quantified by HPLC in extracts from both zebra chip-symptomatic and non-symptomatic tubers. These were putatively identified as eight chlorogenic acid derivatives and other lignin precursors, nine flavonoids, and four unknown compounds (Table 1). All of these compounds occurred at significantly greater levels (1.5–18 times) in samples from zebra chip-symptomatic tubers than in samples from non-symptomatic tubers, and were significantly positively associated with zebra chip severity ratings (Table 1).

Summing compound peaks together, symptomatic tubers had a 5-fold increase of chlorogenic acid derivatives and other phenolic acids over non-symptomatic tubers, a 3-fold increase in total flavonoids, and a 3.5-fold increase in all phenolic compounds combined (Table 1). These compound groups also were significantly positively associated with the disease severity ratings (Table 1).

All phenolic compounds levels were generally significantly lower in tubers with the "0" disease rating than the other tubers, with the exception of sinapic acid, quercetin-3-β-D-galactoside, and unknown compound 1 (Table 2). Phenolic compound groups generally exhibited the same trends as individual phenolic compounds, with significantly lower levels in tubers with the 0 disease rating than the other ratings, and tubers with a "3" disease rating also greater than those with a "1" rating (Fig. 2).

3.2. Changes in amino acid levels and enzyme activity within zebra chip-symptomatic tubers

Nineteen amino acids were quantified by the GC-based EZ:FAAST kit, including all the amino acids used in protein synthesis except arginine (which cannot be detected by this kit) and cysteine. Valine, leucine, isoleucine, proline, and tryptophan were present at greater levels in zebra chip-symptomatic tuber samples than non-symptomatic tuber samples (Table 3). Increased valine, leucine, and isoleucine levels were positively correlated with zebra chip disease symptoms (Table 3). Leucine ($F = 14.362$; $P < 0.001$; $N = 32$) and isoleucine ($F = 7.872$; $P = 0.001$; $N = 32$) occurred at significantly lower levels in tubers with the "0" rating than the all of the other ratings. Valine ($F = 3.371$; $P = 0.033$; $N = 31$) was present in significantly lower levels in tubers with the "0" rating than those of the "1" and "3" ratings only.

Zebra chip-symptomatic tubers possessed more peroxidase (8-fold increase), polyphenol oxidase (2-fold increase), chitinase (2-fold increase), and β-1,3-glucanase (2-fold increase) than tubers without disease symptoms (Fig. 3). Zebra chip-free potatoes had greater levels of total protein (60% increase) than zebra chip-affected tubers ($t = 2.171$; $P = 0.037$; $N = 34$). All the enzymes were positively correlated with disease ratings, although calculated total protein levels were not (Table 4). Peroxidase ($F = 7.951$; $P < 0.001$; $N = 35$), polyphenol oxidase ($F = 5.126$; $P = 0.005$; $N = 35$), and chitinase ($F = 3.814$; $P = 0.020$; $N = 35$) were at significantly lower levels in tubers with a "0" rating than all other

Table 1
Mean (±SE) concentrations of phenolic compounds in non-symptomatic and zebra chip-symptomatic potato tubers, with *t*-test statistics given. Statistics for Spearman correlations between compounds and disease severity ratings are also given.

Compound class	Compound	Non-symptomatic	Symptomatic	*t*	*Spearman ρ*	*N*
Total phenolics		2.170 ± 0.170	10.100 ± 1.100	7.091***	0.805***	33
Chlorogenic Acid derivatives and other phenolic acids (μg/g FW)	Total Chlorogenic and Phenolic Acids	1.700 ± 0.150	8.630 ± 0.980	6.978***	0.807***	33
	Total Chlorogenic Acids[a]	0.824 ± 0.053	5.700 ± 0.690	4.550***	0.820***	33
	neochlorogenic acid[a]	0.326 ± 0.032	1.710 ± 0.160	8.741***	0.677***	32
	chlorogenic acid, peak 1[a]	0.176 ± 0.025	0.593 ± 0.063	6.161***	0.835***	30
	chlorogenic acid, peak 2[a]	0.174 ± 0.024	2.980 ± 0.530	5.265***	0.816***	31
	cryptochlorogenic acid[a]	0.150 ± 0.021	1.090 ± 0.160	5.864***	0.797***	33
	coumaric acid	0.248 ± 0.032	0.520 ± 0.086	2.962**	0.496**	33
	coniferyl alcohol	0.109 ± 0.021	0.402 ± 0.066	4.248***	0.744***	33
	sinapyl alcohol	0.444 ± 0.059	0.844 ± 0.085	3.853**	0.522**	33
	sinapic acid	0.218 ± 0.043	0.637 ± 0.103	3.743**	0.544**	27
Flavonoids (μg/g FW)	Total Flavonoids	0.468 ± 0.040	1.500 ± 0.140	7.276***	0.811***	33
	flavan-3-ol 1	0.260 ± 0.036	0.876 ± 0.081	6.913***	0.734***	33
	flavan-3-ol 2	0.062 ± 0.005	0.096 ± 0.009	3.160**	0.496**	33
	genistin	0.022 ± 0.005	0.113 ± 0.022	4.086***	0.750***	33
	quercetin−3-β-D-galactoside	0.035 ± 0.007	0.068 ± 0.010	2.778**	0.433*	33
	naringin	0.021 ± 0.004	0.045 ± 0.007	2.830**	0.603***	33
	naringenin	0.026 ± 0.004	0.106 ± 0.014	5.611***	0.735***	32
	luteolin	0.020 ± 0.009	0.039 ± 0.004	2.119*	0.528**	29
	genistein	0.020 ± 0.004	0.057 ± 0.008	4.221***	0.647***	29
	kaempferol	0.018 ± 0.003	0.100 ± 0.014	5.756***	0.563**	31
Unidentified Compounds (peak areas, mAU * min)	unknown 1	82,300 ± 8000	151,000 ± 12000	2.958**	0.520**	30
	unknown 2	353,000 ± 18000	837,000 ± 47000	9.263***	0.724***	32
	unknown 3	45,000 ± 2000	179,000 ± 23000	5.598***	0.675***	30
	unknown 4	12,000 ± 1000	40,000 ± 5000	5.188***	0.527**	29

*$P < 0.050$; **$P < 0.010$; ***$P < 0.001$.
[a] For comparison to other methods chlorogenic acid and its derivatives are summed together [23].

tubers; whereas β-1,3-glucanase ($F = 3.050$; $P = 0.046$; $N = 30$) levels were at significantly lower levels in tubers with a "0" level than those at the "3" level only.

3.3. Associations between disease symptoms, tuber chemistry, and 'Ca. L. solanacearum' titers

'*Ca*. L. solanacearum' was detected by conventional PCR in all symptomatic tuber samples, as well as in six tuber samples not exhibiting zebra chip symptoms. These qualitative results from tradition PCR were confirmed by the results of qPCR. PCR results revealed that *'Ca.* L. solanacearum' was present in all symptomatic tubers; however, calculated titers by qPCR were not significantly associated with disease severity ($\rho = 0.100$; $P = 0.567$; $N = 35$).

Tuber samples PCR-positive for *'Ca.* L. solanacearum' (including both those that were symptomatic and those non-symptomatic but positive for the bacterium) had greater levels of all examined phenolic compounds, leucine, and isoleucine than those testing negative for the bacterium (Table 5). It appeared that tubers which tested PCR-positive for the bacterium (both symptomatic and non-symptomatic) possessed significantly greater levels of most of the same host compounds observed to be significantly increased in zebra chip-symptomatic tubers, likely because all symptomatic tubers tested PCR-positive with an additional six non-symptomatic tubers also positive.

Table 2
Mean (±SE) concentrations of individual phenolic compounds in potato tubers with different disease severities (Fig. 1), with univariate ANOVA statistics given. Letters represent significant differences by LSD tests.

Compound class	Compound	0	1	2	3	*F*	*N*
Chlorogenic Acid Derivatives and Other Phenolic Acids (μg/g FW)	neochlorogenic acid[a]	0.326 ± 0.032b	1.560 ± 0.210a	2.010 ± 0.260a	1.640 ± 0.340a	12.537***	32
	chlorogenic acid, peak 1[a]	0.176 ± 0.025c	0.407 ± 0.068b	0.734 ± 0.089a	0.798 ± 0.123a	19.152***	30
	chlorogenic acid, peak 2[a]	0.174 ± 0.024c	1.860 ± 0.630bc	2.720 ± 0.940ab	4.630 ± 1.030a	7.318***	31
	cryptochlorogenic acid[a]	0.150 ± 0.021c	0.650 ± 0.167b	1.270 ± 0.260a	1.500 ± 0.330a	10.806***	33
	coumaric acid	0.248 ± 0.032b	0.296 ± 0.075b	0.658 ± 0.168a	0.690 ± 0.182a	4.597**	33
	coniferyl alcohol	0.109 ± 0.021b	0.167 ± 0.044b	0.453 ± 0.045a	0.659 ± 0.139a	14.584***	33
	sinapyl alcohol	0.444 ± 0.059b	0.693 ± 0.135ab	0.975 ± 0.122a	0.927 ± 0.173a	4.364*	33
	sinapic acid	0.218 ± 0.043	0.594 ± 0.231	0.537 ± 0.073	0.765 ± 0.185	2.137	27
Flavonoids (μg/g FW)	flavan-3-ol 1	0.260 ± 0.036b	0.766 ± 0.141a	0.907 ± 0.155a	0.990 ± 0.130a	9.611***	33
	flavan-3-ol 2	0.062 ± 0.005b	0.080 ± 0.014ab	0.108 ± 0.015a	0.106 ± 0.020a	2.957*	33
	genistin	0.022 ± 0.005c	0.043 ± 0.014bc	0.104 ± 0.022b	0.209 ± 0.044a	14.640***	33
	quercetin−3-β-D-galactoside	0.035 ± 0.007	0.053 ± 0.017	0.088 ± 0.013	0.070 ± 0.017	2.795	33
	naringin	0.021 ± 0.004b	0.023 ± 0.005b	0.053 ± 0.008a	0.066 ± 0.017a	6.727***	33
	naringenin	0.026 ± 0.004b	0.068 ± 0.013b	0.128 ± 0.021a	0.138 ± 0.031a	9.458***	32
	luteolin	0.020 ± 0.009b	0.026 ± 0.005b	0.050 ± 0.008a	0.048 ± 0.008a	3.997*	29
	genistein	0.020 ± 0.004c	0.038 ± 0.006bc	0.068 ± 0.016ab	0.071 ± 0.018a	4.757**	29
	kaempferol	0.018 ± 0.003c	0.078 ± 0.013b	0.139 ± 0.026a	0.094 ± 0.031ab	0.563**	31
Unidentified Compounds (peak areas, mAU * min)	unknown 1	82300 ± 8000	146000 ± 21000	152000 ± 17000	160000 ± 29000	2.809	29
	unknown 2	353000 ± 18000c	739000 ± 78000b	813000 ± 66000ab	994000 ± 83000a	12.623***	28
	unknown 3	45000 ± 2000b	176000 ± 45000a	184000 ± 49000a	153000 ± 15000a	4.599**	32
	unknown 4	12000 ± 1000b	43000 ± 10000a	38000 ± 8000a	37000 ± 9000a	4.527**	29

*$P < 0.050$; **$P < 0.010$; ***$P < 0.001$.
[a] For comparison to other methods chlorogenic acid and its derivatives are summed together in Fig. 2.

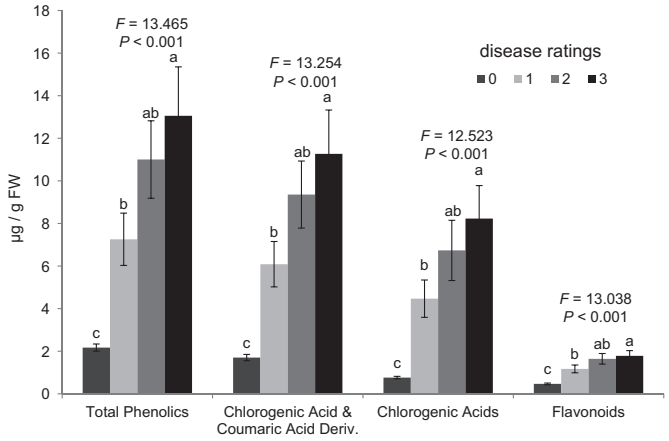

Fig. 2. Mean (±SE) amounts of total phenolic levels and levels of different phenolic compound groupings found in the tubers exhibiting the different zebra chip disease ratings. Univariate ANOVA statistics are given, with different letters representing significant differences between means. In all comparisons, $N = 33$.

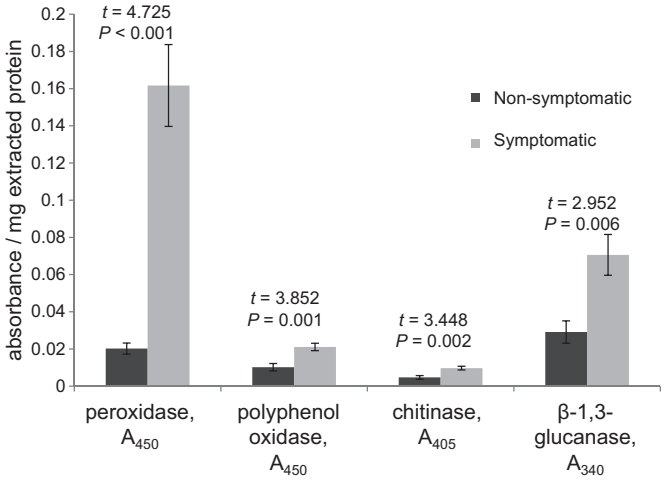

Fig. 3. Mean (±SE) protein levels of the four defense-related proteins analyzed in this study found in both non-symptomatic and zebra chip-symptomatic potato tubers. The wavelengths used to quantify these proteins are given below each of the enzyme names. *T*-test statistics are given. In all comparisons, $N = 34$.

In the six non-symptomatic tubers that were PCR-positive for *'Ca.* L. solanacearum', only quercetin-3-β-D-galactoside ($t = 2.521$; $P = 0.033$; $N = 11$) and genistein ($t = 2.887$; $P = 0.028$; $N = 8$) occurred in greater levels than in the non-symptomatic tubers that tested negative for the bacterium.

Bacterial titers were positively associated with unknown 3, and negatively associated with the amino acid glutamic acid (Table 5). Bacterial titers were significantly positively correlated with chitinase levels ($\rho = 0.410$; $P = 0.014$; $N = 35$). No other significant associations were observed between *'Ca.* L. solanacearum' titers and compound or defense enzyme levels.

4. Discussion

In this study, potato tubers with zebra chip disease symptoms consistently possessed greater levels of phenolics and defense-related enzymes than non-symptomatic tubers. In addition, increased levels of five amino acids were observed. Zebra chip disease symptoms could be explained from these findings. Enzymatic browning of freshly-cut potato tubers would be expected in symptomatic potatoes because they possessed greater polyphenol oxidase activity and phenolic compound levels than non-symptomatic tubers, and polyphenol oxidases convert phenolics into brown-colored melanins and benzoquinone [20].

Greater levels of phenolic compounds could result in the other symptoms of zebra chip disease as well. Stunting of zebra chip-symptomatic potato plants could be due to a shift of resources towards phenolic compound production and away from primary growth [27,28]. Discoloration of leaves and stems could be due to increased levels of particular phenolic compounds as well. Analyses of how zebra chip effects the metabolism of the above-ground tissues of potato are warranted to determine if this is the case.

Increased browning upon frying potato slices could be due to increased amino acid content, as amino acids combine with free sugars present in the tubers (that are presumably not limiting) to form acrylamide, which is linked to the browning Maillard reaction [19]. Although increased levels of antioxidants, such as flavonoids and essential amino acids, might make zebra chip-symptomatic tubers more nutritionally healthy, the formation of acrylamide (a known carcinogen) upon frying should make consumption undesirable if diseased potatoes are used for chips [21]. It is important to note that, in this study at least, only amino acids with non-polar, neutrally-charged side chains were found at significantly greater levels in symptomatic tissues.

The level of induction in multiple defense-related compounds and proteins suggests that infection by a pathogen(s) occurred and

Table 3
Mean (±SE) concentrations (µmol/g FW) of amino acids in non-symptomatic and zebra chip-symptomatic potato tubers, with *t*-test statistics given. Statistics for Spearman correlations between compounds and disease severity ratings are also given.

Amino acid	Non-Symptomatic	Symptomatic	*t*	*Spearman ρ*	*N*
alanine	5.88 ± 0.87	4.90 ± 0.39	1.017	−0.016	30
glycine	4.63 ± 0.63	5.26 ± 0.45	0.824	0.082	33
valine	23.7 ± 1.6	42.7 ± 4.2	4.234***	0.422*	31
leucine	3.38 ± 0.41	9.29 ± 0.84	6.351***	0.784***	32
isoleucine	3.63 ± 0.34	9.91 ± 0.96	6.167***	0.622***	32
threonine	4.42 ± 0.71	6.79 ± 0.95	1.821	0.214	30
serine	28.5 ± 7.4	35.9 ± 7.5	0.672	0.149	33
proline	18.4 ± 2.8	28.1 ± 3.7	2.088*	0.292	32
asparagine	140 ± 26	174 ± 31	0.777	−0.020	33
aspartic acid	1.21 ± 0.12	1.33 ± 0.18	0.567	−0.047	29
methionine	27.2 ± 5.7	17.8 ± 3.5	1.497	−0.289	33
glutamic acid	2.12 ± 0.25	1.85 ± 0.21	0.811	−0.057	30
phenylalanine	3.18 ± 0.39	3.83 ± 0.64	0.873	0.289	32
glutamine	56.2 ± 13.8	83.5 ± 20.8	1.094	−0.030	33
ornithine	3.34 ± 0.19	4.04 ± 0.33	1.795	−0.005	29
lysine	4.39 ± 0.44	6.34 ± 0.92	1.920	0.181	33
histidine	5.13 ± 0.77	4.67 ± 1.21	0.293	−0.330	17
tyrosine	3.88 ± 0.20	4.96 ± 0.64	1.594	−0.162	32
tryptophan	3.92 ± 0.22	5.33 ± 0.49	2.632*	0.151	32

*$P < 0.050$; **$P < 0.010$; ***$P < 0.001$.

Table 4
Spearman ρ- values between zebra chip disease severity ratings and observed values of defense proteins, and Pearson *R*-values between different defense proteins.

	Zebra chip rating	Total protein	Peroxidase	Polyphenol oxidase	Chitinase
Total protein, $N = 20$	−0.255				
peroxidase, $N = 20$	0.796***	−0.377			
polyphenol oxidase, $N = 20$	0.497**	−0.616**	0.872***		
chitinase, $N = 20$	0.494**	−0.654**	0.832***	0.977***	
β−1,3-glucanase, $N = 16$	0.493**	−0.417	0.714**	0.702**	0.751***

*$P < 0.050$; **$P < 0.010$; ***$P < 0.001$.

Table 5
Mean (±SE) concentrations of phenolic compounds and significantly different amino acids (those with $P < 0.05$) in non-infected and '*Candidatus* L. solanacerum'-infected potato tubers, with *t*-test statistics given. Statistics for Spearman correlations between compounds and '*Ca.* L. solanacerum' titer are also given.

Compound class	Compound	Non-infected	Infected	*t*	*Spearman ρ*	*N*
Total phenolics		2.380 ± 0.310	8.380 ± 1.080	7.091***	0.246	33
Chlorogenic acid derivatives and other phenolic acids (μg/g FW)	Total Chlorogenic and Phenolic Acids	1.900 ± 0.290	7.110 ± 0.950	6.978***	0.262	33
	Total Chlorogenic Acids[a]	0.752 ± 0.117	5.100 ± 0.740	4.550***	0.270	27
	neochlorogenic acid[a]	0.312 ± 0.056	1.410 ± 0.160	8.741***	0.283	33
	chlorogenic acid, peak 1[a]	0.206 ± 0.034	0.487 ± 0.062	6.161***	0.135	31
	chlorogenic acid, peak 2[a]	0.171 ± 0.037	2.460 ± 0.480	5.265***	0.205	32
	cryptochlorogenic acid[a]	0.160 ± 0.027	0.089 ± 0.145	5.864***	0.327	34
	coumaric acid	0.245 ± 0.051	0.455 ± 0.072	2.962**	−0.084	34
	coniferyl alcohol	0.130 ± 0.045	0.335 ± 0.057	4.248***	−0.059	34
	sinapyl alcohol	0.551 ± 0.102	0.739 ± 0.078	3.853**	0.035	34
	sinapic acid	0.224 ± 0.056	0.581 ± 0.095	3.743**	0.061	28
Flavonoids (μg/g FW)	Total Flavonoids	0.482 ± 0.043	1.270 ± 0.140	7.276***	0.176	33
	flavan-3-ol 1	0.216 ± 0.035	0.752 ± 0.079	6.913***	0.199	34
	flavan-3-ol 2	0.066 ± 0.012	0.088 ± 0.008	3.160**	0.037	34
	genistin	0.027 ± 0.008	0.092 ± 0.019	4.086***	−0.008	34
	quercetin 3-β-D-galactoside	0.051 ± 0.011	0.058 ± 0.008	2.778**	−0.118	34
	naringin	0.029 ± 0.007	0.038 ± 0.006	2.830**	−0.042	34
	naringenin	0.027 ± 0.005	0.091 ± 0.013	5.611***	0.015	34
	luteolin	0.041 ± 0.019	0.033 ± 0.004	2.119*	−0.090	34
	genistein	0.027 ± 0.002	0.050 ± 0.008	4.221***	−0.037	33
	kaempferol	0.019 ± 0.005	0.087 ± 0.013	5.756***	0.208	30
Unidentified Compounds (peak areas, mAU * min)	unknown 1	89700 ± 8000	145,000 ± 13000	2.958**	0.067	29
	unknown 2	333,000 ± 18000	803,000 ± 52000	9.263***	0.060	28
	unknown 3	41,000 ± 3000	148,000 ± 20000	5.598***	0.388*	32
	unknown 4	12,000 ± 2000	34,000 ± 5000	5.188***	0.253	33
Amino Acids (μmol/g FW)	leucine	2.97 ± 0.71	8.03 ± 0.82	4.672***	−0.215	29
	isoleucine	2.93 ± 0.32	8.42 ± 0.91	5.695***	−0.204	29
	glutamic acid	1.63 ± 0.32	1.99 ± 0.19	0.640	−0.425*	27

*$P < 0.050$; **$P < 0.010$; ***$P < 0.001$.
[a] For comparison to other methods chlorogenic acid and its derivatives are summed together [23].

the host responded strongly to its presence, as evidenced by increases of defense-associated phenolic compounds and proteins [13]. In this study, all tubers with zebra chip disease symptoms tested positive for '*Ca.* L. solanacearum' by PCR, suggesting that this bacterium triggered disease responses resulting in zebra chip symptom development. However, bacterial titers were neither associated with symptom severities nor with phenolic/defense-associated enzyme levels. This could be due to the relatively low titers and uneven distribution of the pathogen that made qPCR analyses unable to accurately quantify titers in the tuber samples. Likewise, induction of host defense responses might not occur in a linear manner to pathogen infection, rendering linear correlation analyses ineffective at observing relationships. It also was possible that other unmeasured pathogens/pests or abiotic conditions may have triggered defense responses instead, and future studies are needed to identify other biotic/abiotic stressors that could be involved in zebra chip disease of potatoes.

The physiological changes observed in zebra chip-symptomatic tubers could provide targets for novel host response-based detection methods. Whether or not such methodology would be specific for zebra chip disease or generic for a variety of tuber diseases and/or other biotic/abiotic stresses remains unclear and warrants further research. Furthermore, the link between zebra chip and '*Ca.* L. solanacearum' needs to be further established, and the finding that non-symptomatic but PCR-positive tubers only had two compounds in greater amounts than non-infected tubers could prove problematic for chemistry-based detection approaches for the bacteria. Regardless, measurement of phenolic compounds or amino acids could be used in lieu of molecular genetics-based detection techniques in screenings for potentially zebra chip-diseased tubers and plants, as the association between zebra chip symptoms and chemistry is clear even if associations between chemistry and '*Ca.* L. solanacearum' remain unclear.

5. Conclusion

Zebra chip-symptomatic potatoes possessed chemical profiles consistent with those of hosts infected with a pathogen, i.e. greater phenolic, amino acid, and defense-associated enzyme levels. Greater phenolic and polyphenol oxidase levels could result in increased browning of freshly cut tubers, whereas increased amino acid levels could result in zebra chip banding patterns on fried potato chips. Although '*Ca.* L. solanacearum' was detected in all zebra chip-symptomatic tubers, neither symptom severity nor phenolic compound levels were correlated with bacterial titers, perhaps because hosts respond in a non-linear manner to pathogens. Yet it is equally possible that these results suggest that '*Ca.* L. solanacearum' may not be the sole causal agent of the disease. Other pathogens, pests, or abiotic conditions also could trigger tuber metabolism to change, which over time could result in zebra chip symptom formation.

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