Zebra Chip Disease and Potato Biochemistry: Tuber Physiological Changes in Response to ‘*Candidatus Liberibacter solanacearum*’ Infection Over Time

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ABSTRACT

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Zebra chip disease, putatively caused by the bacterium ‘*Candidatus Liberibacter solanacearum*’, is of increasing concern to potato production in Mexico, the United States, and New Zealand. However, little is known about the etiology of this disease and changes that occur within host tubers that result in its symptoms. Previous studies found that increased levels of phenolics, amino acids, defense proteins, and carbohydrates in ‘*Ca. L. solanacearum*’-infected tubers are associated with symptoms of zebra chip. This study was conducted to quantify variations in levels of these biochemical components in relation to the time of infestation, symptom severity, and ‘*Ca. L. solanacearum*’ titer. Levels of phenolics,

peroxidases, polyphenol oxidases, and reducing sugars (glucose and, to some extent, fructose) changed during infection, with higher levels occurring in tubers infected at least 5 weeks before harvest than in those infected only a week before harvest and those of controls. Compared with the apical tuber ends, greater levels of phenolics, peroxidases, and sucrose occurred at the basal (stolon attachment) end of infected tubers. With the exception of phenolics, concentrations of the evaluated compounds were not associated with ‘*Ca. L. solanacearum*’ titer. However, there were significant associations between biochemical responses and symptom severity. The lack of a linear correlation between most plant biochemical responses and ‘*Ca. L. solanacearum*’ titer suggests that shifts in metabolic profiles are independent of variations in ‘*Ca. L. solanacearum*’ levels.

Additional keywords: defense-associated proteins, potato psyllid, *Solanum tuberosum*.

Pathogen infections trigger plant physical and biochemical defense mechanisms which may limit pathogen multiplication by making the host environment unsuitable for pathogen growth or directly targeting and eliminating the invading microorganism (5). A clear understanding of the underlying mechanisms of host–pathogen interactions is essential for developing genotypes that can withstand infection and the damage it causes to the host (19). This is of particular importance in monocultured agricultural systems, where the use of more tolerant or resistant cultivars may be a cost-efficient approach to minimize economic losses due to disease (8).

The vector-borne phloem-limited bacterium ‘*Candidatus Liberibacter solanacearum*’ is associated with disease in a wide range of solanaceous hosts, including potato, tomato, pepper, and eggplant, and is transmitted by the potato psyllid *Bactericera cockerelli* Sulc. (Hemiptera: Trioizidae) (6,11). In potato (*Solanum tuberosum* L.), ‘*Ca. L. solanacearum*’ causes a condition known as zebra chip (ZC). Since its first detection in the United States in 2000, the potato industry has experienced significant economic loss due to ZC (15). Recently, the presence of ZC was confirmed in major potato-growing regions of Idaho, Washington, and Oregon (4). In addition to reducing potato yield, ZC affects the marketability of tubers due to reduced quality, evidenced by brown discoloration of vascular and medullary rays, which appear to be unequally distributed within diseased potato tubers (14).

Furthermore, this brown discoloration becomes more pronounced in fried tuber slices.

Symptoms of ZC likely are not directly due to the presence of ‘*Ca. L. solanacearum*’ itself but rather a result of pathogen induction of host defense responses, including shifts in tuber biochemistry (25). Specifically, in earlier studies, ZC-diseased potato tubers possessed greater levels of amino acids, carbohydrates, phenolics, and defense-associated proteins (including peroxidases and polyphenol oxidases) than nondiseased or noninfected tubers (2,16,25). Symptom severity was associated with amino acid, phenolic, and defense-associated protein levels (25).

To date, symptomatic tubers have been collected in the field from obviously diseased plants with little or no knowledge of when the plant was infected with ‘*Ca. L. solanacearum*’ (16,25). Knowledge about changes in plant physiology over the time duration following infection could provide key information, useful in developing management plans for ZC. For instance, if physiological changes which impact tuber quality take several weeks to develop after infection by ‘*Ca. L. solanacearum*’, then insecticide applications for vector management could be stopped several weeks before harvest or, in areas with overwintering vector populations, could be limited in frequency. Moreover, heterogeneity of disease symptom distribution within infected tubers raises the question of whether physiological responses are localized around symptomatic areas. Although seed potato pieces infected by ‘*Ca. L. solanacearum*’ are known to have a significantly lower germination rate compared with their healthy counterparts (7), heterogeneity in the intensity of ‘*Ca. L. solanacearum*’-induced physiological changes may differentially impact sprouting in individual dormant buds (tuber eyes). If variations in biochemical responses are localized, tuber germination success may

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remain unchanged from noninfected tubers in areas not exhibiting ZC symptoms.

In this study, concentrations of phenolic compounds, peroxidases, polyphenol oxidases, chitinases, free amino acids, and sugars (sucrose, glucose, and fructose) were analyzed. These biochemical components were selected because they were observed to be associated with infection by '*Ca. L. solanacearum*' (2,16,25). Here, the primary objectives were to (i) evaluate biochemical changes that occur in potato tubers infected with '*Ca. L. solanacearum*' over time and (ii) quantify changes in biochemical properties at the basal (stolon or stem attachment area) and apical (budding end) ends of infected tubers.

MATERIALS AND METHODS

Experimental design. Field experiments were conducted at the Texas A&M AgriLife Research and Extension Center, Bushland. Seed potato pieces ('FL 1867') were planted in the field in April 2011 and covered with cages prior to emergence. Each cage contained four plants.

Potato psyllids from a '*Ca. L. solanacearum*'-positive greenhouse population were used to infest the experimental cages. Insects were maintained in the greenhouse for several generations and were reared on four to five potato plants (FL 1867) in 60-by-60-by-60-cm bugdorm mesh and plastic cages (BioQuip Products, Rancho Dominguez, CA). To initiate insect colonies, potato psyllids, of the central haplotype (23), were kindly provided by Dr. Christian Nansen, Texas A&M AgriLife Research and Extension Center, Lubbock. These potato psyllids were originally collected by Dr. Joe Munyaneza from ZC-infected plants growing in commercial fields in Texas. For the first two infestation events, status ('*Ca. L. solanacearum*' presence) of the greenhouse colony was verified by testing 10 randomly selected psyllids individually. '*Ca. L. solanacearum*' in our colony was determined to be a mix of biotype 1 and 2 (26). Thereafter, the presence of '*Ca. L. solanacearum*' was confirmed on a weekly basis by testing two to three composite samples of 10 insects with quantitative polymerase chain reaction (qPCR) (described below). The frequency of '*Ca. L. solanacearum*'-positive psyllids in our colony estimated to be as high as 97% in an independent study, conducted 3 months later.

Five times during the growing season, beginning 21 May and continuing every 2 weeks until harvest on 22 July 2011 (i.e., 9, 7, 5, 3, and 1 week prior to harvest), cages were infested by releasing 30 potato psyllids from the '*Ca. L. solanacearum*'-positive colony at the base of a single plant in each cage. Two-week infestation intervals not only provided the opportunity to evaluate effects of early-season and late-season infections but also allowed quantifying relative changes in tuber biochemistry over time. In this study, the term "season" reflects time period between planting and harvesting of the experimental plants. There were four cage-replicates for each of the infestation dates arranged in a randomized complete block design. In each infestation date, plants were exposed to the infective psyllids for a 1-week period. After the 1-week exposure time, plants were thoroughly sprayed, with a mix of Movento (Bayer CropScience, Durham, NC), Agri-Mek, and Fulfill (Syngenta Crop Protection, LLC, Greensboro, NC); insecticides were reapplied 1 week later. Tubers from the plants under which the psyllids were released were used for the biochemical analysis. At harvest, all tubers from the caged plants were collected and transferred to the laboratory for processing. The total number of harvested potato tubers ranged from 5 to 14 tubers/plant. Tubers from psyllid-free cages were used as negative controls. A thin slice, ≈ 5 mm, was removed from the basal end (stolon attachment end) and tubers were scored for symptom severity on a 0-to-3 scale, where 0 = asymptomatic and 3 = extensive browning (Fig. 1). An additional slice, 2 to 3 mm thick, was taken and stored at -80°C for later quantification of '*Ca. L. so-*

lanacearum' using qPCR, as described below. Sampled tubers were stored at 4.4°C for 2 months. After 2 months, potato tubers were removed from storage and analyzed for phenolic, amino acid, defense protein (peroxidase, polyphenol oxidase, and chitinase) and carbohydrate (sucrose, glucose, and fructose) content, according to procedures described below.

DNA extraction and '*Ca. L. solanacearum*' quantification.

All of the '*Ca. L. solanacearum*' extractions and quantifications were conducted at Texas A&M AgriLife Research, Bushland. Tuber extractions were performed following a slightly modified DNeasy Plant Mini Kit (Qiagen, Valencia, CA) protocol. Immediately following their removal from -80°C storage, finely chopped tuber samples (100 to 200 mg) were placed in liquid nitrogen and then ground in a hard Tissue Grinder (VWR, Sugar Land, TX), for 3 min (2 $\times$), using a 5-mm stainless-steel ball-bearing bead. Plant extractions were performed following DNeasy Plant Mini Kit (Qiagen) protocol. Final DNA product was eluted in 100 μl of the elution buffer.

Comparative cycle threshold method, also known as $\Delta\Delta\text{Ct}$ (20), was performed to determine '*Ca. L. solanacearum*' titers using an Applied Biosystems (Carlsbad, CA) 7500 Real-Time PCR System. The reaction mix contained TaqMan Universal Master Mix (Applied Biosystems), 0.3 μM forward primer LsoF (9), 0.3 μM reverse primer HLBr (10), and 0.25 μM HLBP TaqMan probe (10). Eukaryotic 18S rRNA (VIC/MGB probe, primer limited) (Applied Biosystems) was used as the endogenous control. The amount of target in each sample was normalized to the '*Ca. L. solanacearum*' endogenous reference and quantified in relation to a calibrator. The calibrator was created by amplification of a 1,168-bp fragment of the 16S rDNA region of '*Ca. L. solanacearum*' using primer set OA2/OI2C (12). The amplicon was cloned using a TA cloning Kit (Invitrogen, CA) following the manufacturer's protocols. Number of DNA molecules was calculated based on the average molecular weight of a pair of deoxyribonucleotide (660 Da) for the number of bases in the plasmid DNA with the following formula: nanograms of DNA $\times$ Avogadro constant (6.023×10^{23} molecules/mol)/base pair length (5,097 bp) $\times$ (1.0×10^9) $\times$ (660 Da). A single genome of '*Ca. L. solanacearum*' was estimated to be 5.586×10^{-9} ng. The calibrator was created by serial dilution of quantified plasmid DNA with healthy plant DNA to contain 50,000 genome copies.

Multiple plates were analyzed at the same time, using the plate study function of ABI default analysis software.

Biochemical analyses. All biochemical analyses were performed at the United States Department of Agriculture–Agricultural Research Services San Joaquin Valley Agricultural Sciences Center in Parlier, CA, with tuber samples shipped on ice overnight from Bushland, TX. Upon arrival, tubers were freshly sliced (to remove the oxidized surface from the prior sampling for '*Ca. L. solanacearum*' titer analyses), roughly 5 g of tissue was removed with a 12-mm cork borer on both apical and basal ends of the tubers and separately smashed using a mortar and pestle, and two 0.1-g aliquots of this smashed tissue were placed into separate 1.5-ml microcentrifuge tubes and immediately flash frozen in liquid nitrogen to prevent further oxidation. These tubes were kept at -20°C until further chemical analyses.

One aliquot of pulverized tissue was twice-extracted overnight with 0.5 ml of methanol (Sigma-Aldrich, St. Louis) for a combined 1 ml of methanol extract. This methanol extract was then analyzed for phenolics using a Shimadzu (Columbia, MD) high-performance liquid chromatography system (HPLC), as described in detail by Wallis et al. (25). The observed retention times and UV/Vis spectra at the peaks were compared with the available commercial standards for compound identification. The commercial standards used for quantification were chlorogenic acid (for all chlorogenic acid derivatives), kaempferol (for all flavonoids), coumaric acid (for all coumaric acid derivatives), and quinic acid. These were chosen due to similarity of UV/Vis spectra

across all similar compounds (25). Standard curves were derived by running a dilution series (1:1, 1:3, 1:5, and 1:7) of an original standard solution of methanol at 20 mg/ml. Liquid chromatography mass spectrometry analyses further confirmed compound identities (25).

The second aliquot of tuber tissue was twice-extracted overnight at 4°C in a phosphate-buffered saline (PBS) solution (pH 7.8), for a combined 1 ml of extract. From a portion of this extract, relative enzymatic activities of peroxidase (using guaiacol as a substrate in dilute hydrogen peroxide buffer), polyphenol oxidase (using caffeic acid as a substrate), and exo-chitinase (using *p*-nitrophenyl- β -*N*-acetylglucosaminide as a substrate) were quantified. This was done using a Biotek Epoch (Winooski, VT) microplate reader at 450 nm (peroxidase and polyphenol oxidase) or 405 nm (chitinase), according to Barto et al. (1) and Wallis et al. (25). An aliquot of 100 μ l of the remaining buffer extract was used to quantify amino acids, using a commercially available kit from Phenomenex (Torrance, CA), which used a Shimadzu 2010 GC-FID gas chromatograph and a Zebtron AAA column. Manufacturer protocols and conditions described by Wallis et al. (25) were followed for amino acid derivatization and quantification.

Sugars in an aliquot of the PBS buffer extracts also were analyzed by using the Shimadzu HPLC equipped with a 300-by-7.8-mm Supelco C-611 carbohydrate column (Sigma-Aldrich) and a refractive index detector (RID-10) (Shimadzu). The column was kept at 60°C by a CTO-20 column heater (Shimadzu), and the system also was equipped with a Shimadzu SIL-20AHT autosampler. In brief, 100 μ l of extract was injected into a system running 0.5 ml of 0.0001 N NaOH solvent for 40 min/sample. Peaks were identified and quantified by running different known concentrations and deriving standard curves (using original concentrations and then diluting 1:1, 1:3, 1:5, and 1:7 [original solution/water]) for commercially obtained sucrose (22.9 mg/ml original standard solution), glucose (24.3 mg/ml original standard solution), and fructose (60.7 mg/ml original standard solution) (Sigma-Aldrich).

Statistical analyses. For all analyses, SPSS software (version 20.0; IBM, Armonk, NY) was used. All data were assessed for normality by plotting residuals, and outliers (data greater than two standard errors of a mean) were removed. Relationships between the level of each biochemical group and symptom severity were examined by Spearman rho correlations. Multivariate analysis of covariance (MANCOVA) was used to examine effects of infection duration (the time between infestation and harvest) on all amino acids, phenolic compounds, carbohydrates, and defense enzymes, while controlling for the quantity of '*Ca. L. solanacearum*' (co-variable). Because pathogen quantity was a co-variable, non-infected controls were excluded from MANCOVAs. Likewise,

'*Ca. L. solanacearum*' was only quantified at the basal ends and, therefore, MANCOVA was not performed on apical tuber ends. Instead, separate multivariate analyses of variance (MANOVAs) were performed on basal and apical tuber ends to examine effects of infection duration on each of the four compound classes. Separate MANOVAs were used because biochemical levels at the basal and apical ends were not independent. Performing MANOVAs also allowed for the inclusion of the noninfected controls for comparison. In all multivariate models, plant replicate and infection duration were treated as categorical variables. Plant replicate was removed from the model, where its effect was largely nonsignificant (if $P > 0.1$). Analysis of variance (ANOVA) tests were performed subsequent to significant MANOVAs and were followed by Tukey's honestly significant difference tests for pairwise comparisons. Pearson's correlation was used to determine associations between chemical levels at basal and apical tuber ends.

RESULTS

Variations in titer level and symptom severity. There was a significant difference in symptom severities among tubers of plants which were infested on different dates (Kruskal-Wallis $\chi^2_4 = 22.01$, $P < 0.001$, $N = 40$) (Fig. 2). This variation was primarily due to the absence of ZC symptoms in tubers which had been infected for only a week (the last infestation period). Tubers from potato plants infected 9 weeks before harvest had the highest disease ratings (mean $\pm$ standard error (SE): 2.27 ± 0.17) (Fig. 2). ANOVA revealed a significant variation in pathogen quantity among different infestation dates ($F_{3,29} = 9.57$, $P < 0.001$). The highest mean '*Ca. Liberibacter solanacearum*' titer was detected in tubers which had been infested for 3 weeks (12.12 ± 2.93 SE) (Fig. 2). Indeed, a negative correlation was detected between symptom severity and titer levels (Spearman rho: $\rho = -0.432$, $P = 0.012$, $N = 33$). Tubers from plants infested 1 week before harvest were both asymptomatic (symptom score of 0) and tested negative for '*Ca. L. solanacearum*'. To avoid statistical bias, they were not included in ANOVA and correlation analyses.

Variations in amino acid levels. There was a significant effect of infection duration on the amino acids levels at the basal tuber ends (MANCOVA: Pillai's Trace [Λ] = 2.87, $F_{56,76} = 3.45$, $P < 0.001$). There was also a similar significant effect of plant replicate ($\Lambda = 2.11$, $F_{42,54} = 3.06$, $P < 0.001$). Amino acid levels were not influenced by the '*Ca. L. solanacearum*' titer ($\Lambda = 0.544$, $F_{14,15} = 1.27$, $P = 0.332$). To include noninfected controls, analysis was preceded with a MANOVA; amino acid levels were influenced by infection duration at the basal end ($\Lambda = 3.16$, $F_{70,130} =$

TABLE 1. Mean ($\pm 95\%$ confidence interval) amino acid concentrations (μ mol/g FW) at basal tuber ends from plants infested in different dates throughout the season^z

Compound	' <i>Candidatus Liberibacter solanacearum</i> ' infection duration (weeks before harvest)					
	9	7	5	3	1	Noninfected
Aspartic acid	44.5 (15.0) a	44.1 (11.6) a	43.5 (14.3) a	44.1 (15.2) a	33.6 (18.4) a	47.2 (94.7) a
Asparagine	396 (111) a	507 (56) a	447 (102) a	443 (124) a	465 (225) a	396 (106) a
Glutamic acid	12.9 (12.1) a	15.2 (9.7) a	14.9 (7.2) a	12.5 (5.4) a	23.0 (8.9) a	22.4 (4.6) a
Glutamine	124 (44) a	134 (29) a	128 (40) a	144 (100) a	86.6 (48.6) a	64.1 (24.5) a
Histidine	8.59 (2.12) a	7.75 (1.15) a	6.81 (1.23) a	6.24 (1.77) ab	6.07 (3.37) ab	3.87 (0.39) b
Isoleucine	2.97 (0.33) abc	3.37 (0.38) abc	4.96 (1.32) ab	5.45 (1.51) a	2.26 (2.92) bc	1.19 (0.47) c
Leucine	2.26 (0.39) b	2.55 (0.60) b	3.67 (0.49) a	4.41 (1.00) a	1.50 (0.34) bc	1.11 (0.36) c
Lysine	11.9 (2.3) a	12.4 (1.6) a	12.4 (1.6) a	10.5 (2.99) a	8.12 (8.23) ab	4.42 (0.87) b
Methionine	6.35 (1.32) a	6.35 (0.54) a	6.03 (1.00) a	6.12 (0.80) a	3.78 (1.31) b	2.63 (0.33) b
Phenylalanine	4.46 (0.71) a	4.37 (0.79) a	4.58 (0.68) a	3.51 (0.87) a	1.87 (0.85) b	1.78 (0.32) b
Proline	12.8 (3.4) a	11.0 (2.5) abc	12.1 (2.7) ab	20.7 (8.4) d	4.91 (3.87) bc	3.90 (0.66) c
Serine	35.1 (8.7) a	39.7 (11.3) a	40.0 (7.5) a	38.8 (15.2) a	35.5 (22.8) a	20.6 (6.0) a
Tryptophan	7.43 (1.05) a	6.09 (0.66) a	5.95 (0.65) ab	5.13 (0.75) ab	4.77 (4.06) ab	3.20 (0.12) b
Tyrosine	1.90 (0.26) a	1.92 (0.19) a	1.83 (0.16) a	1.79 (0.16) ab	1.73 (0.06) ab	1.61 (0.05) b

^z Letters represent differences due to Tukey's honestly significant difference means separation tests.

3.19, $P < 0.001$). Similar to the basal ends, infection duration had a significant effect on overall amino acid levels at the apical ends ($\Lambda = 2.91$, $F_{70,150} = 2.98$, $P < 0.001$). Methionine, phenylalanine, and proline were elevated in infected tubers (at least in those infected for 9 weeks) over uninfected tubers in both basal (Table 1) and apical (Table 2) tuber ends. Across different infestation dates, amino acid levels averaged $\approx 705 \pm 31$ $\mu\text{mol/g}$ fresh weight (FW) (mean $\pm$ SE) at the basal ends. Amino acid levels at the apical ends averaged at 427 ± 26 $\mu\text{mol/g}$ FW. A similar pattern existed between basal (568 ± 44 $\mu\text{mol/g}$ FW) and apical (372 ± 51 $\mu\text{mol/g}$ FW) ends of uninfected control tubers. Leucine, isoleucine, serine, proline, aspartic acid, methionine, glutamic acid, phenylalanine, lysine, histidine, tyrosine, and tryptophan levels were all significantly positively correlated between basal and apical tuber ends (Pearson correlations, $P < 0.028$). At basal ends of the tubers, 9 of 14 of amino acids were positively correlated with disease severity ($P < 0.05$). However, tyrosine, glutamine, aspartic acid, and serine were not associated with disease severity ($P > 0.05$). Glutamic acid was negatively correlated with symptom score ($\rho = -0.375$, $P = 0.019$).

Variations in phenolic levels. Phenolic concentrations were significantly affected by the infection duration (MANCOVA: $\Lambda = 2.339$, $F_{56,92} = 2.45$, $P < 0.001$). However, phenolic concentrations also co-varied with '*Ca. L. solanacearum*' titer ($\Lambda = 0.773$, $F_{14,20} = 4.81$, $P = 0.001$). Phenolic concentrations did not differ among plant replicates ($\Lambda = 1.41$, $F_{42,57} = 1.19$, $P = 0.262$) and, therefore, this factor was not included in the final model. Separate MANOVAs, which included the noninfected controls, were utilized for both basal and apical tuber ends. In both cases, there was a significant effect of infection duration on phenolic concentrations (basal end: $\Lambda = 2.29$, $F_{70,155} = 1.87$, $P = 0.004$, Table 3; apical end: $\Lambda = 2.39$, $F_{70,160} = 2.09$, $P < 0.001$, Table 4). At all infestation dates, basal tuber ends (mean $\pm$ SE: 115 ± 16 $\mu\text{g/g}$ FW) of the infected tubers possessed relatively higher phenolic levels compared with apical ends (58.9 ± 9.6 $\mu\text{g/g}$ FW). This pattern did not exist in the uninfected controls (basal end: 7.80 ± 0.84 and apical end: 9.33 ± 1.95 $\mu\text{g/g}$ FW). Basal levels of all individual phenolics were significantly positively correlated with apical levels of individual phenolics (Pearson correlations, $P < 0.005$).

Fig. 1. Example of the zebra chip (ZC) severity scale used in this study ('FL 1867'), with 0 representing no disease and 3 representing severe ZC symptoms.

Fig. 2. Relationship between symptom severity and pathogen quantity in infestations conducted 3, 5, 7, and 9 weeks before harvest. Tuber of the plants infested 1 week before harvest were asymptomatic and tested negative for '*Candidatus Liberibacter solanacearum*'. Error bars represent ± 1 standard error.

All of the phenolic compounds were elevated at early infestation dates compared with the later infestations, or at least the ones infested 1 week before harvest (Tables 3 and 4), and all were significantly positively associated with symptom severity ($p > 0.571$, $P < 0.001$, $N = 40$).

Variations in carbohydrate levels. Overall, the effects of plant replicate ($\Lambda = 0.327$, $F_{9,90} = 1.22$, $P = 0.290$), '*Ca. L. solanacearum*' titer ($\Lambda = 0.100$, $F_{3,27} = 1.00$, $P = 0.408$), and time of infestation ($\Lambda = 0.528$, $F_{12,99} = 1.76$, $P = 0.065$) on overall

carbohydrate concentrations were nonsignificant. However, out of three evaluated carbohydrates, glucose was significantly influenced by infection duration ($F_{4,33} = 2.68$, $P = 0.049$). This significant effect was primarily due to the observed difference between glucose levels of tubers infested 7 weeks before harvest and tubers which were infested only a week prior to harvest (Fig. 3A). Likewise, glucose was the only carbohydrate that was positively correlated with symptom severity ($p > 0.571$, $P_s < 0.001$, $N = 40$).

TABLE 2. Mean ($\pm 95\%$ confidence interval) amino acid concentrations ($\mu\text{mol/g}$ FW) at apical tuber ends from plants infested in different dates throughout the season^z

Compound	' <i>Candidatus Liberibacter solanacearum</i> ' infection duration (weeks before harvest)					Noninfected
	9	7	5	3	1	
Aspartic acid	40.7 (13.8) a	33.9 (7.7) a	37.0 (33.4) a	31.1 (9.9) a	30.6 (15.1) a	32.7 (10.0) a
Asparagine	354 (132) a	251 (35) a	185 (108) a	198 (24) a	243 (127) a	209 (102) a
Glutamic acid	9.60 (8.98) a	6.35 (3.58) a	7.44 (5.26) a	7.51 (3.53) a	13.6 (5.4) a	15.4 (5.0) a
Glutamine	84.1 (36.6) a	54.4 (17.1) ab	52.6 (22.9) ab	50.3 (17.8) ab	41.8 (24.5) ab	36.0 (20.2) b
Histidine	7.64 (1.43) a	5.29 (1.10) ab	5.42 (1.66) ab	5.10 (1.26) b	5.48 (2.22) ab	3.76 (1.14) b
Isoleucine	3.94 (1.05) a	2.28 (0.54) a	2.77 (0.83) a	2.79 (0.57) a	2.72 (2.82) a	2.06 (1.44) a
Leucine	3.07 (0.74) a	2.48 (0.66) ab	2.54 (0.44) ab	2.97 (0.76) a	1.61 (0.45) bc	1.36 (0.61) c
Lysine	10.3 (2.5) a	8.18 (1.18) ab	8.81 (2.74) a	7.66 (1.61) ab	6.30 (3.86) ab	4.52 (1.48) b
Methionine	6.68 (2.00) a	4.88 (0.67) ab	4.48 (0.57) b	4.20 (0.70) b	3.40 (0.71) b	3.28 (0.76) b
Phenylalanine	5.26 (1.46) a	3.32 (0.40) bc	3.73 (0.80) b	2.82 (0.50) bcd	1.82 (0.43) d	1.86 (0.54) cd
Proline	16.1 (5.9) a	7.95 (1.60) bc	7.01 (1.61) bc	11.0 (5.1) ab	4.96 (4.06) bc	3.04 (0.94) c
Serine	46.4 (10.0) a	35.3 (7.8) ab	26.4 (4.2) b	29.4 (8.5) b	31.8 (7.4) ab	20.1 (8.8) b
Tryptophan	7.10 (1.08) a	5.03 (0.37) ab	5.39 (1.12) ab	4.69 (0.82) b	4.50 (2.64) b	3.40 (0.41) b
Tyrosine	1.77 (0.09) a	1.75 (0.14) a	1.72 (0.08) a	1.66 (0.08) a	1.70 (0.14) a	1.65 (0.06) a

^z Letters represent differences due to Tukey's honestly significant difference means separation tests.

TABLE 3. Mean ($\pm 95\%$ confidence interval) phenolic concentrations ($\mu\text{g/g}$ FW) at basal ends of tubers from plants infested in different dates throughout the season^y

Compound	' <i>Candidatus Liberibacter solanacearum</i> ' infection duration (weeks before harvest)					Noninfected
	9	7	5	3	1	
Chlorogenic acid peak 1 ^z	58.5 (36) a	33.7 (21.6) ab	12.5 (9.7) b	13.9 (8.9) b	0.33 (0.2) b	0.78 (0.8) b
Chlorogenic acid peak 2 ^z	8.32 (2.7) a	7.94 (2.8) a	6.02 (2.5) a	7.02 (3.4) a	1.14 (0.7) b	0.61 (0.3) b
Coumaric acid	0.03 (0.02) a	0.015 (0.01) ab	0.004 (0.001) b	0.005 (0.005) b	0.001 (0.0001) b	0.001 (0.0001) b
Cryptochlorogenic acid	21.5 (6.9) ab	26.3 (6.8) a	22.9 (7.6) a	42.0 (25.8) a	1.05 (0.9) b	0.64 (0.2) b
Chlorogenic acid derivative	59.2 (23.3) a	32.3 (13.7) b	12.5 (3.8) bc	8.86 (5.4) c	1.49 (0.7) c	1.53 (0.6) c
Flavanol glucoside 1	13.8 (6.5) a	9.46 (4.9) ab	5.8 (3.2) bc	7.62 (4.9) abc	1.08 (0.4) c	1.15 (0.3) c
Flavanol glucoside 2	19.8 (11.7) a	19.7 (5.8) a	17.8 (6.8) a	18.4 (8.5) a	0.490 (0.4) b	0.13 (0.03) b
Flavanol-3-o-methyl ester	25 (9.3) a	15.9 (5.7) b	7.91 (2.6) bc	6.54 (3.6) c	0.33 (0.3) c	0.31 (0.2) c
Epicatechin gallate	10.4 (5.5) a	6.84 (4.5) ab	3.66 (2.7) ab	7.93 (7.1) ab	0.96 (0.8) b	0.20 (0.05) b
Neochlorogenic acid	43.9 (22.8) a	26.9 (19.6) ab	12.4 (7.5) b	23.1 (13.3) ab	4.20 (6.8) b	1.09 (0.4) b
Quinic acid	0.31 (0.09) a	0.258 (0.06) ab	0.215 (0.07) ab	0.248 (0.09) ab	0.128 (0.05) b	0.131 (0.06) b
Protocatechuic acid hexoside	0.37 (0.08) a	0.276 (0.07) ab	0.206 (0.04) abc	0.204 (0.08) abc	0.12 (0.2) bc	0.014 (0.005) c
Salicylic acid glucoside	0.060 (0.02) a	0.040 (0.02) ab	0.021 (0.01) bc	0.020 (0.01) bc	0.003 (0.001) c	0.002 (0.001) c
Sinapic acid	0.070 (0.03) a	0.034 (0.01) b	0.014 (0.004) bc	0.010 (0.005) c	0.002 (0.001) c	0.002 (0.0005) c

^y Letters represent differences due to Tukey's honestly significant difference means separation tests.

^z In methanol extracts, chlorogenic acid breaks down into two products with two peaks in high-performance liquid chromatography (25).

TABLE 4. Mean ($\pm 95\%$ confidence interval) phenolic concentrations ($\mu\text{g/g}$ FW) at apical tuber ends from plants infested in different dates throughout the season^y

Compound	' <i>Candidatus Liberibacter solanacearum</i> ' infection duration (weeks before harvest)					Noninfected
	9	7	5	3	1	
Chlorogenic acid peak 1 ^z	45.6 (46.5) a	14.3 (13) ab	14.4 (8.9) ab	3.03 (1.7) b	0.74 (0.5) b	0.62 (0.47) b
Chlorogenic acid peak 2 ^z	8.33 (4.1) a	5.76 (1.7) ab	5.45 (2.1) abc	3.90 (1.2) bc	2.06 (0.9) bc	1.35 (0.70) c
Coumaric acid	0.015 (0.01) a	0.0086 (0.005) ab	0.003 (0.001) ab	0.0013 (0.0004) b	0.002 (0.0008) b	0.0013 (0.0003) b
Cryptochlorogenic acid	6.46 (5.3) a	3.77 (1.8) ab	6.23 (4) a	3.77 (1.9) ab	0.304 (0.14) b	0.277 (0.10) b
Chlorogenic acid derivative	31.3 (14.5) a	11.3 (6.2) b	6.74 (2.5) b	2.54 (1.1) b	1.82 (1.1) b	1.28 (0.35) b
Flavanol glucoside 1	7.83 (4.5) ab	9.76 (4.2) a	8.62 (8.0) a	3.16 (1.2) ab	1.43 (0.6) b	0.99 (0.21) b
Flavanol glucoside 2	4.64 (1.9) ab	5.64 (4.2) a	5.65 (4.3) a	0.74 (0.3) b	0.55 (0.8) b	0.230 (0.17) b
Flavanol-3-o-methyl ester	21.3 (8.9) a	18.8 (6.1) a	14.2 (5.6) ab	14.37 (19.6) ab	0.545 (0.34) b	0.40 (0.3) b
Epicatechin gallate	7.18 (4.9) a	3.24 (1.3) ab	2.47 (1.1) ab	2.04 (2.1) b	0.443 (0.20) b	0.36 (0.34) b
Neochlorogenic acid	12.1 (2.7) ab	13.9 (10.3) a	8.5 (7.3) abc	3.41 (1.4) bc	3.57 (3.2) bc	1.44 (0.4) c
Quinic acid	0.253 (0.06) a	0.216 (0.05) ab	0.179 (0.03) ab	0.180 (0.03) ab	0.151 (0.02) b	0.142 (0.06) b
Protocatechuic acid hexoside	0.289 (0.06) a	0.181 (0.07) ab	0.164 (0.05) abc	0.093 (0.02) bc	0.10 (0.2) bc	0.028 (0.02) c
Salicylic acid glucoside	0.044 (0.03) a	0.050 (0.05) a	0.033 (0.02) a	0.010 (0.005) a	0.010 (0.005) a	0.007 (0.006) a
Sinapic acid	0.0320 (0.009) a	0.013 (0.005) b	0.008 (0.003) bc	0.004 (0.002) bc	0.002 (0.001) c	0.002 (0.0003) c

^y Letters represent differences due to Tukey's honestly significant difference means separation tests.

^z In methanol extracts, chlorogenic acid breaks down into two products with two peaks in high-performance liquid chromatography (25).

MANOVAs (including the uninfected controls) were performed and detected significant effects of infection duration on carbohydrate levels in basal ($\Lambda = 0.649$, $F_{15,117} = 2.15$, $P = 0.012$; Fig. 3A) but not apical ($\Lambda = 0.470$, $F_{15,126} = 1.56$, $P = 0.094$; Fig. 3B) tuber ends. Reducing sugar levels (total fructose and glucose) were similar at the basal (9.10 ± 0.78 mg/g FW) and apical (9.42 ± 0.64 mg/g FW) ends of infected tubers. Sucrose level, however, was greater in basal tuber ends (7.88 ± 0.50 mg/g FW) than apical ends (4.38 ± 0.54 mg/g FW). Levels of fructose ($R = 0.753$, $P < 0.001$, $N = 51$), glucose ($R = 0.587$, $P < 0.001$, $N = 51$), and sucrose ($R = 0.433$, $P = 0.002$, $N = 49$) in basal tuber ends were positively associated with levels in apical ends.

Variations in defense-associated enzyme activity. The activity levels of the evaluated defense proteins did not co-vary with '*Ca. L. solanacearum*' levels ($\Lambda = 0.105$, $F_{3,28} = 1.09$, $P = 0.368$) but were significantly influenced by the time of infestation ($\Lambda = 0.851$, $F_{12,102} = 3.36$, $P < 0.001$). Defense-associated enzyme

activity levels did not vary among plant replicates at each infestation time ($\Lambda = 0.371$, $F_{9,93} = 1.45$, $P = 0.175$), and this non-significant effect was removed from the final model. Peroxidase ($F_{4,34} = 8.41$, $P < 0.001$) and chitinase ($F_{4,34} = 5.45$, $P < 0.001$) activity levels were significantly affected by the time of infestation. This effect was nonsignificant for polyphenol oxidase enzyme activity ($F_{4,34} = 2.58$, $P < 0.054$). Peroxidase ($\rho = 0.800$, $P < 0.001$, $N = 40$), polyphenol oxidase ($\rho = 0.320$, $P = 0.044$, $N = 40$), and chitinase ($\rho = 0.730$, $P < 0.001$, $N = 40$) were all positively correlated with symptom severity.

MANOVA detected a significant effect of infection duration defense-associated enzymes at both basal ($\Lambda = 0.985$, $F_{15,126} = 4.106$, $P < 0.001$; Fig. 4A) and apical ($\Lambda = 0.949$, $F_{15,123} = 3.79$, $P < 0.001$; Fig. 4B) tuber ends. Levels of peroxidase ($R = 0.785$, $P < 0.001$, $N = 49$), polyphenol oxidase ($R = 0.490$, $P < 0.001$, $N = 51$), and chitinase ($R = 0.772$, $P < 0.001$, $N = 51$) in basal tuber ends were positively correlated with levels in apical ends.

Fig. 3. Mean carbohydrate levels quantified in **A**, basal and **B**, apical ends of potato tubers infected at different times. Different letters indicate statistical differences detected by Tukey's honestly significant difference. Error bars represent 95% confidence interval.

Fig. 4. Mean defense enzyme relative activities quantified in **A**, basal and **B**, apical ends of potato tubers infected at different times. Peroxidase and polyphenol oxidase are measured as $\Delta 450$ nm/5 min. Chitinase activity level unit of measure is $\Delta 405$ nm/6 h. Different letters indicate statistical differences due to Tukey's honestly significant difference. Error bars represent 95% confidence interval.

DISCUSSION

Phenolic, amino acid, glucose, and defense-associated enzyme levels were affected by '*Ca. L. solanacearum*' infection duration. Although these putatively induced shifts in metabolism were primarily associated with tuber symptom severity, with the exception from phenolics, none was associated with '*Ca. L. solanacearum*' titers.

Although '*Ca. L. solanacearum*'-positive psyllids were used to infest experimental plants, the observed variations in physiological responses in this study are primarily attributed to differences in infection durations with the pathogen. This is because, in all infestation dates, psyllids were removed after 1 week. Moreover, the lack of correlation between physiological responses and '*Ca. L. solanacearum*' titer is unlikely to be due to variations in psyllid titer levels or their inoculation success. Potato psyllids are highly efficient vectors of '*Ca. L. solanacearum*' because their inoculation success is >80% and as high as 100%, for multiple psyllids, within 48 h (17). In addition, no relationship has been detected between potato psyllid titer level and the amount of pathogen injected into the plant tissue (17). Disease incubation time was uniform across all infestation dates (22 to 25 days) (18), which further supports the homogeneity of infestations across treatments.

The amino acids phenylalanine, methionine, and proline were increased in potato infected for at least 3 weeks prior to harvest, compared with the healthy controls. Leucine, isoleucine, and phenylalanine were previously observed to be associated with ZC-symptomatic tubers (28). Although previous studies documented that higher levels of tyrosine were associated with ZC disease symptoms (25,28), the level of this amino acid was not found at significantly greater levels in infected tubers compared with uninfected controls in this study.

The significance of greater amino acid levels in infected tubers is magnified because relatively greater glucose and, potentially fructose levels were also observed in infected tubers (Fig. 3A and B). Gao et al. (2) also observed that '*Ca. L. solanacearum*' infection increased tuber glucose levels. Greater amino acids and reducing sugars contribute to the excessive browning of ZC-affected tubers during the frying process (16,25). Furthermore, the brown-colored product acrylamide is linked to the Maillard-driven reaction of free amino acids and reducing sugars during thermal processing (22).

Although Navarre et al. (16) observed no differences in chlorogenic acid and neochlorogenic acid quantities between ZC-positive and -negative tubers, this study revealed the presence of both of these phenolic compounds in significantly greater concentrations in tubers infected earlier with '*Ca. L. solanacearum*' than those inoculated just 1 week prior to harvest, as well as the uninfected controls. Such phenolic compounds are putatively toxic to pathogens and contribute to the enzymatic browning of freshly cut tubers, because polyphenol oxidase converts phenolic compounds to brown-colored products upon exposure to air (16,25).

Activities of the host defense-associated enzymes peroxidase, polyphenol oxidase, and chitinase were higher in '*Ca. L. solanacearum*'-infected tubers than in uninfected tubers and in tubers infected for longer durations than shorter durations. Greater activities of these enzymes suggest a host defense response to infection (13,21,24). Although polyphenol oxidizing enzyme levels were correlated with symptom severity and time of inoculation, they were not associated with '*Ca. L. solanacearum*' titers in infected tubers, an observation consistent with that of Wallis et al. (25).

In addition to its effect on yield and quality, one important impact of ZC on potato production is reduced germination of seed potato (7), a phenomenon thought to be influenced by ZC-associated metabolic shifts. Greater concentrations of phenolics, amino acids, and peroxidase in basal tuber ends could result in reduced germination from those ends, albeit increased levels were found

throughout the tuber compared with noninfected tubers. Some of these compounds, such as chlorogenic acid and coumarin, are known to be associated with reduced seed germination (27). Total amino acid concentration also differed between apical and basal ends of noninfected tubers, even though greater amino acid levels were hypothesized to be present in the apical ends (budding end) of tubers, because that is where sprouting is more likely to occur (3).

Glucose and fructose levels did not differ between tuber ends, although sucrose was observed at greater levels in basal ends. Regardless of any potential effect of carbohydrates on seed germination, an increase in sucrose may not be economically as significant as the increase in reducing sugars. However, at high concentrations, this nonreducing sugar may contribute to an undesirable sweet taste.

In conclusion, this study showed that '*Ca. L. solanacearum*' infections, initiated 3 to 5 weeks before harvest, can cause significant increases in several amino acids, phenolics, reducing sugars, and defense enzymes; the four biochemical classes which were associated with ZC symptom severity. In the cases of glucose, peroxidases, and chitinases, as well as several phenolics and amino acids, the shifts appeared to start as early as 1 week post infection. Although the observed changes were statistically nonsignificant, this conclusion is supported by the fact that a considerable percentage of seed potato produced from the plants which were infected 1 to 2 weeks before harvest failed to germinate during field trials (C. M. Rush, A. Rashed, and F. Workneh, *unpublished data*). Because of the early shifts in host-plant metabolites following '*Ca. L. solanacearum*' inoculation, it is important that control measures for the psyllid vectors continue until at least 1 week before harvest.

The lack of associations between plant biochemistry and '*Ca. L. solanacearum*' titers suggests that, although defense mechanisms might be activated very soon after inoculation, accumulation of compounds continues independently of pathogen presence within tubers. Such a response may have implications in seed potato germination success, and studies are underway to quantify the relationship between infection duration and the probability of successful germination events. These results can facilitate the development of potato cultivars resistant to ZC, suggesting that, perhaps by silencing genes responsible for physiological responses which are associated with disease symptoms, ZC would not develop even in potato infected with '*Ca. L. solanacearum*'.

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