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Insect-Symbiont Interactions

Indirect Effects of One Plant Pathogen on the Transmission of a Second Pathogen and the Behavior of its Potato Psyllid Vector

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ABSTRACT Plant pathogens can influence the behavior and performance of insect herbivores. Studies of these associations typically focus on tripartite interactions between a plant host, a plant pathogen, and its insect vector. An unrelated herbivore or pathogen might influence such interactions. This study used a model system consisting of *Tobacco mosaic virus* (TMV), the psyllid *Bactericera cockerelli* Sulc, and tomatoes to investigate multipartite interactions among a pathogen, a nonvector, and a plant host, and determine whether shifts in host physiology were behind potential interactions. Additionally, the ability of TMV to affect the success of another pathogen, '*Candidatus* Liberibacter solanacearum,' which is transmitted by the psyllid, was studied. In choice trials, psyllids preferred nearly fourfold noninfected plants to TMV-infected plants. No-choice bioassays demonstrated that there was no difference in psyllid development between TMV-infected and control plants; oviposition was twice as high on control plants. Following inoculation by psyllids, '*Candidatus* Liberibacter solanacearum' titers were lower in TMV-infected plants than control plants. TMV-infected plants had lower levels of amino acids and sugars but little differences in phenolics and terpenoids, relative to control plants. Possibly, these changes in sugars are associated with a reduction in psyllid attractiveness in TMV-infected tomatoes resulting in decreased infection of '*Candidatus* Liberibacter solanacearum.'

KEY WORDS *Bactericera cockerelli*, pathogen transmission, multipartite interaction, *Candidatus* Liberibacter solanacearum, pathogen competition

Organisms are frequently subject to concurrent infection with multiple pathogens (Lello et al. 2004, Booth 2006, Srinivasan and Alvarez 2007, Seabloom et al. 2014). These pathogens are transmitted via different mechanisms and vectors. In animal and human systems, the interactions between these pathogens and vectors have been investigated with a particular focus on medical scenarios such as mosquitoes and malaria (Lacroix et al. 2005, Muturi et al. 2008), while in plants, most attention has been on co-occurance of potyviruses with other viruses (Rochow and Ross 1955, Vance 1991). In both the plant and animal systems examined, research typically considers the effects of infection on behavior of the pathogen's vector (Rossignol et al. 1985, Nault 1997, Karasev 2000) or on the effects of coinfection within the host (Vance 1991, Korenromp et al. 2005, Graham et al. 2007, Seabloom et al. 2014). However, there is essentially no information on mechanisms demonstrating how presence of one pathogen in a plant affects vector behaviors that are critical to transmission of a second pathogen.

Plants serve as hosts for a variety of organisms, including herbivorous insects, viruses, fungi, and bacteria. Many of these organisms can cause infection or damage to their host plants, which have evolved defenses in response. These defenses include physiological responses that putatively influence behaviors of attacking organisms. Not all plant host physiological responses are the same, as certain attacking organisms warrant different defense responses. Observed host responses can become quite complicated. This is exemplified by the multiple examples of an insect-transmitted virus or bacteria manipulating the host plant physiology or vector's behavior to promote its transmission (Malmstrom et al. 2011, Mauck et al. 2014).

There is a small but increasing body of research on tripartite interactions involving plant pathogens, plants, and herbivorous insects. This is reviewed in Mauck et al. (2012) and Hatcher (1995) who note that fungal pathogens are known to affect herbivores, primarily through changes in the quality of the host plant. Kruess (2002) demonstrated that the beetle *Cassida rubiginosa* Müller, prefers healthy thistle to thistle infected with the necrotic fungi *Phoma destructiva* Plowr. Viral pathogens also influence herbivore life histories and behaviors. For example, Nachappa et al. (2013) observed how *Tomato spotted wilt virus* affects *Tetranychus urticae* Koch (two-spotted spider mite) host choice and fecundity. Higher densities of *T. urticae* occur on *Tomato spotted wilt virus*-infected pepper plants versus uninfected plants (Belliure et al. 2010). Shapiro et al. (2013) examined the interactions of *Zucchini yellow mosaic virus*, *Acalymma vittatum* (F.) (cucumber beetle), and wild gourds. They describe complicated effects on

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plants, a secondary pathogen, and the beetle, including reduced visits to infected plants. Finally, *Leptinotarsa decemlineata* Say (Colorado potato beetle) survives better on plants infected with *Tobacco mosaic virus* (TMV) than on uninfected plants (Hare and Dodds 1987).

Interactions in these multipartite studies are often attributed to defense-associated changes in host physiology, especially those controlled by pathways regulated by jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and to a lesser extent, those signaled by gibberellins, brassinosteroids, abscisic acid, and auxins (Verhage et al. 2010). The different regulators, in combination with "cross-talk" between pathways, provide plants with various expressions of host response to infection or infestation and, consequently, the ability to target their defense responses to specific types of feeding or damage. The JA and ET pathways are typically associated with herbivory in which plant cells are damaged. The pathway regulated by SA is frequently associated with microbial pathogens, but can also be involved with defense against phloem-feeding herbivores (Zarate et al. 2007, Walling 2008). There can exist negative cross-talk between SA and JA/ET pathways, resulting in both increased susceptibility to insects (Gao et al. 2007, Pieterse and Dicke 2007, Zarate et al. 2007, Howe and Jander 2008) and increased attractiveness to insects (Prado and Tjallingii 1994, Zhu-Salzman et al. 2004).

Induced defense compounds, which may include phenolics and terpenoids, and the production of physical barriers such as thickened cells (Franceschi et al. 2005), can be metabolically costly to produce in terms of resources required for initial production (Eyles et al. 2010). Consequently, plants may take advantage of specificity of induction as a mechanism for conserving resources, resulting in the upregulation of compounds–proteins that may also be used as constitutive defenses. In addition to the production of defense-associated proteins and defensive metabolites following infection, plants may shift primary metabolism to become less suitable and deprive attackers of nutrition (Awmack and Leather 2002, Lin et al. 2008).

As a consequence of these physiological changes, the potential exists to influence behavior or performance of nontarget species that are using that plant as a host (Eyles et al. 2007, Wallis et al. 2008). Therefore, infection with a plant pathogen may affect insects even when the insects are unassociated with the pathogen (Wallis et al. 2008). Pathogen infection-induced changes in the host plant that could affect insect behavior include shifts in odor profile (Cardoza et al. 2002, McLeod et al. 2005), changes in visual cues (Mauck et al. 2010), production of antibiotics, or all (Eyles et al. 2010). In addition, another mechanism by which pathogens can affect nontarget insect herbivores is by changing plant quality for the herbivore (Stout et al. 1998, Traw and Dawson 2002, Zandt and Agrawal 2004).

TMV is a positive sense single-stranded RNA virus in the large genus *Tobamovirus*. TMV typically enters a plant through minor wounds, but it can also be transmitted in seeds, sap, and infective plant material in soil. In most plants, replication can occur within minutes of infection and symptoms can appear within days of infection. TMV has numerous hosts including many solanaceous plants and especially tomato. Symptoms of TMV vary and include distorted "fern-like" leaves, pointed leaves, necrosis, and the eponymous mosaic pattern (Sutic et al. 1999). Since its initial discovery >100 yr ago, TMV has become a model system and probably the most well-studied of all plant viruses (Scholthof 2004). As a result, its biology has been reviewed and discussed in numerous books and articles.

The tomato–potato psyllid *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) is a phloem-feeding insect with a geographic distribution that includes much of western North and Central America (Butler and Trumble 2012). *B. cockerelli* has a large reported host plant range and has been found on 20 plant families, with a particular affinity for plants in the family Solanaceae (Pletsch 1947, Wallis 1955, Butler and Trumble 2012). This affinity makes the psyllid a pest of vegetable crops including bell pepper (*Capsicum* spp.), eggplant (*Solanum melongena* L.), tomato (*Solanum lycopersicum* L.), and potato (*Solanum tuberosum* L.). Recently, *B. cockerelli* has gained attention as the vector of '*Candidatus* Liberibacter solanacearum' (Lso) (syn. '*Ca.* L. psyllaurous'), the bacteria associated with zebra chip disease (ZC) of potatoes and a similar disorder (vein greening disease) in tomatoes (Hansen et al. 2008, Brown et al. 2010, Crosslin et al. 2010). Symptoms of Lso infection in tomato can include: interveinal chlorosis, curling of leaves, stunting, bleached leaves, foliar purpling, necrosis, wilting, and eventually death (Brown et al. 2010, Crosslin et al. 2010). Transmission of Lso occurs quickly, and a single individual of *B. cockerelli* can infect a plant within 2 h of colonization (Munyaneza 2010), and tomatoes become symptomatic in ~3–4 wk postinoculation (Hansen et al. 2008).

Despite increasing research on effects of pathogens on herbivores vectors of plant pathogens, or on unassociated arthropod species, much less is known about how a particular pathogen affects herbivores that transmit a second pathogen. In this article, a combination of TMV, tomato plants, and the tomato–potato psyllid *B. cockerelli* the putative vector of Lso was used to examine this scenario. Three types of bioassays (choice, no-choice, and transmission) were combined to evaluate effects of TMV infection on *B. cockerelli* behavior, development, and Lso transmission. Additionally, key aspects of plant chemistry were compared to determine if infection with TMV caused similar results to infection with Lso, and whether observed host responses are associated with pathogen infection in general or specific to a given pathogen.

Materials and Methods

Plants and Insects. These studies were conducted using the heirloom tomato variety 'Yellow Pear' (*Solanum lycopersicum esculentum* L.,; Territorial Seed Company, Cottage Grove, OR). Yellow pear tomatoes

are suitable for the psyllid, and susceptible to infection by both TMV and Lso. This plant variety was used for all experiments, for maintaining psyllid colonies, and for propagating TMV. All plants were grown from seed in seedling trays and were then transplanted into 700-ml pots. Plants were grown in UC soil mix III (Matkin and Chandler. 1957) and fertilized weekly with Miracle Gro nutrient solution (Scotts Company, Marysville, OH) at label rate. All plants were watered ad libitum.

B. cockerelli used for these experiments came from colonies maintained at the University of California, Riverside. The insect material was originally collected in tomato and potato fields near Weslaco, TX. *B. cockerelli* were tested for genetic haplotype using the methods of Swisher et al. (2012) and confirmed to be the "western" haplotype. Colonies were maintained in multiple mesh tents (Bugdorm, BioQup, Rancho Dominguez, CA) at conditions of 21–26°C and 40–60% relative humidity, and held under long day light conditions (12–16 h light). All psyllids used in these studies were from colonies infected with Lso haplotype 'A', and the presence of Lso was confirmed periodically via a Taqman-based real-time polymerase chain reaction (PCR) assay with the methods of Butler et al. (2011).

TMV used in these experiments originated from infected tobacco (*Nicotiana tabacum* L.) plants maintained in greenhouses at the University of California, Riverside. Tobacco source material was used to infect tomatoes, and TMV was subsequently maintained in tomato plants. Tomatoes were inoculated with TMV by grinding infected tomato plant material in a combination of phosphate buffer (K_2HPO_4; pH 7.5) and diatomaceous earth (mild abrasive celite from Sigma-Aldrich, St. Louis, MO) at ratio of 2:1 buffer: plant material. Young plants (~12 cm in height, 5 leaves) were infected by rubbing ~1 ml of the virus-laced buffer material into three leaflets of each plant. Control plants were treated with buffer and celite but no virus. Following infection, tomato plants were maintained in a climate-controlled rearing room until use. Because damaged plants could not be used for bioassays, subsets of plants were tested for TMV infection using polyclonal ImmunoStrips (Agdia, Eikhart, IN), according to manufacturer's instructions, and plants were tested again after use in bioassays.

Choice Bioassays. Choice bioassays were conducted to test whether *B. cockerelli* either distinguishes or demonstrates preferences between TMV-infected and uninfected plants. Bioassays were conducted in large rectangular screen cages (Bioquip 36" jumbo cages, Bioquip, Rancho Dominguez, CA). Each cage contained two tomato plants (one uninfected control and one TMV-infected, but nonsymptomatic), assigned randomly to opposite corners. To perform bioassays, 50 unsexed adults of *B. cockerelli* were released into the cage. The number of *B. cockerelli* on each plant was inspected daily for 5 d, after which plants were removed and the number of eggs was counted. It has been established that in similar bioassays, many *B. cockerelli* fail to settle prior to ~24 h postrelease (Prager et al. 2014a). This time period should encompass the period of time before plant defense transcripts (Casteel et al. 2012) or major changes in host defense from Lso are expected to occur (Rashed et al. 2013a, Wallis et al. 2013). Additionally, it has been previously established that host selection behavior of psyllids is similar between sexes, and also under light and dark conditions (Davis et al. 2012, Mann et al. 2012). Choice-experiments were replicated 10 times. Because the act of inoculating plants may also cause physiological changes to the plant, and because these may have influenced *B. cockerelli* behavior, choice bioassays were conducted by presenting either an uninfected plant or a plant that had been treated with buffer and cellite but no virus, and no significant differences were with respect to location (Wilcoxon test: $W = 79$, $P = 0.37$, $n = 10$) or oviposition (Wilcoxon test: $W = 31$, $P = 0.96$, $n = 10$). Therefore, it was assumed that the inoculation process alone did not influence behavior.

No-Choice Bioassays of Oviposition and Development. Since pathogen infection can influence plant physiology and host plant quality for insect development, a series of no-choice bioassays were conducted to compare *B. cockerelli* performance on TMV-infected and uninfected plants. No-choice bioassays were conducted on whole tomato plants 2 wk after manual inoculation with TMV (nonsymptomatic), or using uninfected control plants. In each bioassay, a set of two male:female pairs (four in total) of postteneral *B. cockerelli* were caged on a terminal leaflet in the top third of the plant using white 10.16 by 15.25 cm mesh sachet bags (JoAnn Fabric and Craft Stores, Hudson, OH). A single bag and set of psyllids was used per plant. *B. cockerelli* were caged onto plants for 48 h, removed, and the number of eggs on the caged leaflet was counted. After eggs were counted, plants were maintained in a climate-controlled insect rearing room at 21–26°C and 40–60% relative humidity and were inspected daily for the numbers of eggs, small (first or second instar) nymphs, large (third to fifth instar) nymphs, and adults until all potato psyllids were either removed as adults or died. Once no *B. cockerelli* remained on a plant, TMV infection was confirmed using ImmunoStrips. No-choice bioassays were replicated 10 times for each treatment.

Lso Transmission Experiments. To determine whether infection with TMV affects Lso transmission rate or severity of infection with Lso, Lso titers were compared between TMV-infected nonsymptomatic ($n = 11$) and uninfected tomato plants ($n = 14$) exposed to *B. cockerelli* from an Lso-infected colony. These experiments were conducted similarly to no-choice bioassays described above. However, following the 48-h exposure period, *B. cockerelli* were collected from plants and stored in 70% ethanol, eggs were removed without excising the leaflet, and plants were retained for 14 d in a rearing room at 27°C. Following the 14-d period, whole plants were collected and Lso titers in both plants and insects were evaluated via quantitative real-time PCR using the methods of Butler et al. (2011).

Biochemical Analyses. To analyze TMV infection effects on host biochemistry, six tomato plants were inoculated with TMV according to previously described

protocols, with an additional six tomato plants left non-inoculated as controls. Two weeks after inoculations, while infected plants were nonsymptomatic, leaf tissue was harvested, flash-frozen on dry ice, and was kept at −20°C until further analyses.

Primary (amino acids and sugars) and defensive (phenolics and terpenoids) metabolite levels were analyzed according to procedures of Rashed et al. (2013a) and Wallis et al. (2008, 2012). In brief, frozen leaf tissue was pulverized in liquid nitrogen using a mortar and pestle, and three aliquots of 0.10 g were moved into separate 1.5-ml microcentrifuge tubes. Each of these aliquots were twice extracted overnight at 4°C in 0.5 ml of either phosphate-buffered saline (PBS) solution (pH 7.8) for use in amino acid and sugar analyses, methanol (Fisher Scientific, Pittsburgh, PA) for phenolic analyses, or methyl tert-butyl ether (Sigma-Aldrich, St. Louis, MO) for terpenoid analysis. After extractions, 1 ml of each extract was kept at −20°C until analysis by chromatography.

Amino acids were analyzed by using 100 µl of PBS buffer extract with the commercially available kit from Phenomenex (Torrance, CA) that derivatives and analyzes amino acids by gas chromatography. Derivatives were examined according to manufacturer's protocols, with a Shimadzu (Columbia, MD) GC-2010 gas chromatograph (GC) with a flame ionization detector (FID), and a kit-provided Zebron AAA column to identify and quantify amino acids.

Sugars (fructose and glucose) were analyzed by injecting 50 µl of PBS buffer extract into a Shimadzu high-performance liquid chromatograph equipped with 300 by 7.8 mm^2 Supelco C-611 carbohydrate column (Sigma-Aldrich) and a refractive index detector (RID-10 from Shimadzu; Rashed et al. 2013a). Fructose and glucose standards (both from Sigma) were used to identify the appropriate peaks and covert peak areas into gram amounts.

Phenolics were analyzed by injecting 50 µl of methanol extract into a Shimadzu high-performance liquid chromatograph system equipped with an XR-ODS C18 column (Shimadzu) and a Shimadzu photodiode array detector (set at 280 nm; Wallis et al. 2008, 2012). A binary solvent program was used for the separation and involved moving from 95% solvent A (water with 0.2% v/v acetic acid; Sigma) to 100% solvent B (methanol with 0.2% v/v acetic acid) and returning to initial conditions for subsequent samples, with runs lasting 40 min (Wallis et al. 2012). Phenolic compounds were identified by matching retention times with commercial standards (from Sigma), matching UV/Vis spectra of peaks, or determining molecular weights by running representative samples on a Shimadzu LCMS-2020 liquid chromatograph-mass spectrometer (Wallis et al. 2012). Representative compounds from each phenolic class, each obtained from Sigma, were used to convert peak areas to gram amounts, such as chlorogenic acid for all chlorogenic acid derivatives, quercetin-β-3-glucoside for flavonoid glycosides, catechin for catechin and derivatives, and ferulic acid for phenolic acids (Rashed et al. 2013a).

Terpenoids were analyzed by injecting 2 µl of methyl tert-butyl extract into a Shimadzu GC-2010 gas chromatograph equipped with a GCMS-2020S mass spectrometer and Shimadzu 30 m, 0.25 mm ID, 0.25 µm 5-MS column. A temperature gradient was used for separation by proceeding from 60 to 320°C with changes of 5°C per minute. Compounds were identified by matching retention times with known standards (from Sigma) or matching mass ion breakdowns with those reported in literature (Wallis et al. 2012). For monoterpenoids, peak areas were converted to gram amounts using a standard curve derived from +-α-pinene (Sigma), whereas sesquiterpenoids were converted using a standard curve derived from β-caryophellene (Sigma).

Statistical Analyses. Location data for the number of psyllids in two-choice bioassays were not normally distributed, but were a repeated measure as the same psyllids are examined every 24 h. Consequently, these data were analyzed using permutational multivariate analyses of variance (MANOVA) technique (PERMANOVA). PERMANOVA is a method for partitioning the sums of squares similar to that used for parametric MANOVA, but does not require normally distributed data (McArdle and Anderson 2001, Anderson 2006). PERMANOVA was conducted using a model with number of psyllids as a response variable and treatment (infected or control) as the sole fixed factor. Permutated MANOVA was implemented using the adonis function of the vegan package (Oksanen 2013, Oksanen et al. 2013) with 1,000 permutations. Results were further examined using a generalized linear model with a negative binomial probability distribution (chosen based on Akaike information criterion values) that included fixed effect terms for both days after release and treatment. This method made it possible to test for possible changes in preference over time as has been observed in response to Lso infection (Davis et al. 2012). The number of eggs was compared between TMV-infected and uninfected plants using Welch's *t*-test. Growth index is a measurement of developmental success which ranges from 0, which indicates no eggs developed to adults, to 1, which indicates that all individuals developed to adults (Zhang et al. 1993). Growth index was not normally distributed and was log-transformed prior to analysis. Growth index was then compared between infected and uninfected plants with a *t*-test. PCR on TMV-infected and uninfected plants were examined as both the number of positive and negative plants (positive was defined as a cycle threshold value <35). The numbers of positive and negative samples were compared using chi-square, while the Ct values were compared using a two-group Mann–Whitney *U* test.

In examining biochemical data, prior to analysis all variables were tested for assumptions of normality. MANOVA was utilized to determine differences in amounts of sugars, monoterpenoids, organic acids, and flavonoids owing to infection with TMV. As they are non-normally distributed, amino acids, chlorogenic acids, and sesquiterpinoids were examined using PERMANOVA. Individual compounds were examined for differences between TMV-infected and control plants using either Welch's or Wilcoxon signed-ranked tests,

as appropriate. *P*-values were adjusted for multiple comparisons using the Holm–Bonferroni method (Holm 2014). For all analyses, $N = 12$.

All statistical analyses were conducted using R 3.1.1 (R core team 2014).

Results

Choice Bioassays. In two-choice bioassays, nearly four times as many psyllids settled on the control plants than on those infected with TMV (PERMANOVA: $F = 5.14$; df = 1,18; $P < 0.05$, $R^2 = 0.22$; Fig. 1), and nearly twice as many laid eggs on the uninfected plants (1,256.5 ± 461.8) than on the TMV-infected plants (517.5 ± 343.6; TTEST: $t = 4.06$, $P < 0.001$, df = 16.6). Further examination of settling via generalize model confirmed a significant effect of treatment (GLM: $\chi^2 = 18.8$; df = 3; $P < 0.0001$) but revealed no effect of day (GLM: $\chi^2 = 0.45$; df = 3; $P = 0.9$), or day by treatment interaction (GLM: $\chi^2 = 0.8$; df = 3; $P = 0.84$). These results suggest that *B. cockerelli* chooses to settle on control plants versus those infected with TMV, and this preference does not change over time.

No-choice and Development Bioassays. Similar to the choice bioassays, in no-choice bioassays psyllids laid significantly more, nearly twice as many, eggs on control plants (69.2 ± 28.5) than on TMV-infected plants (37.7 ± 18.6; TTEST: $t = 3.24$; $P < 0.001$; df = 18.75). On TMV-infected plants the mean growth index was 0.89 ± 1.06, while on control plants it was 0.52 ± 0.65, which were not significantly different (TTEST: $t = -1.11$; $P = 0.29$; df = 12.6).

Lso Transmission Assays. Infection with TMV significantly reduced the inoculation rate of Lso (chi-square: $\chi^2 = 4.233$; $P < 0.05$; df = 1). Specifically, 1 of the 14 control plants tested negative for Lso, while 7 of the 11 TMV-infected plants tested negative. Similarly, the median Ct (cycle threshold, a non-normalized relative measure of the concentration of target in the PCR) value in TMV-infected plants (34.23) was significantly higher than that of control plants (21.59; Mann–Whitney *U* test: W = 8; $P < 0.001$; $n = 24$), which is indicative of relatively lower Lso titers in TMV-infected plants.

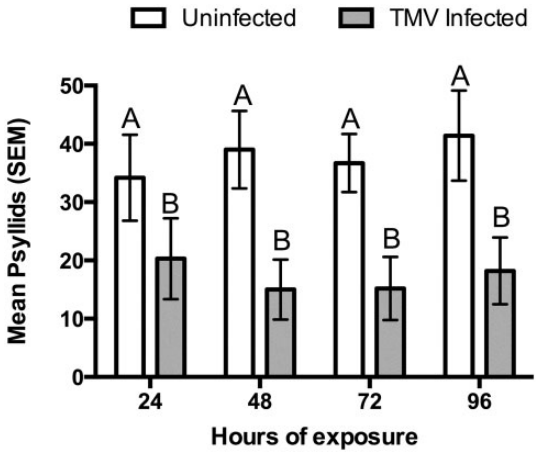

Fig. 1. Mean (±SEM) psyllids on TMV-infected plants and uninfected control plants at 24, 48, 72, and 96 h of exposure.

Effects of TMV Infection on Primary Metabolites. In total, 15 amino acids were analyzed including alanine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, proline, serine, threonine, tryptophan, tyrosine, and valine. Overall, amino acid levels differed significantly with respect to TMV infection (PERMANOVA: $F = 6.28$; df = 1, 10; $R^2 = 0.39$; $P < 0.05$). Follow-up analyses (Table 1) demonstrated significant effects of infection on alanine and glutamine, but no other amino acids differed individually. In general, there is a pattern of reduced levels of amino acids in TMV-infected plants.

Overall, sugar levels were also significantly affected by TMV infection (MANOVA: $\Delta = 0.74$; $F = 12.9$; $P < 0.001$). Infection status had significant effects on levels of both fructose ($t = 4.52$; df = 4.52; $P < 0.001$) and glucose ($t = 5.28$; df = 7.58; $P < 0.001$; Fig. 2). Both fructose and glucose levels were reduced in TMV-infected plants relative to controls.

Effects of TMV Infection on Defense-associated Metabolites. In total, 19 phenolic compounds were analyzed including chlorogenic acid, chlorogenic acid derivative, cryptochlorogenic acid, two dichlorogenic acids, ascorbic acid, caftaric acid, citric acid, protocatechulic acid, quinic acid, catechin, a procyanidin B isomer, genistin, isoquercetin, kaempferol apioxil-rutinoside, kaempferol rutinoside, quercetin apioxil-rutinoside, quercetin hyperoside, and quercetin rutinoside. Overall, the levels of chlorogenic acids differed in response to TMV infection (PERMANOVA: $F = 6.288$; $R^2 = 0.39$; $P < 001$). Both dichlorogenic acid 2 and quinic acid differed between the treatments, but in opposing directions (Table 2). There were no significant differences in organic acids (MANOVA: $\Delta = 0.76$; $F = 3.7$; $P = 0.7$) or flavonoids (MANOVA: $\Delta = 0.76$;

Table 1. Mean (±SE) concentrations (μmol/g FW) of amino acids in tissues from noninfected controls and TMV-infected plants for each trial

Compound	Control	TMV	Test statistic	*P*-value
Alanine	**2.63 ± 0.76**	**0.46 ± 0.06**	**36**	**0.03**
Asparagine	2.43 ± 0.74	3.62 ± 2.34	25	1.0
Aspartic acid	0.59 ± 0.03	0.53 ± 0.06	22	1.0
Glutamic acid	6.30 ± 1.78	0.55 ± 0.37	36	0.28
Glutamine	**3.13 ± 0.73**	**1.38 ± 1.12**	**35**	**0.04**
Glycine	1.47 ± 0.60	0.07 ± 0.04	35	0.07
Isoleucine	0.21 ± 0.05	0.15 ± 0.02	22	1.0
Leucine	0.34 ± 0.08	0.24 ± 0.03	25	1.0
Lysine	0.04 ± 0.02	0.05 ± 0.03	25	0.3
Proline	2.45 ± 1.61	0.23 ± 0.04	36	0.28
Serine	3.04 ± 0.92	1.71 ± 0.31	23	1.0
Threonine	0.34 ± 0.05	0.22 ± 0.03	2.2[a]	0.054
Tryptophan	0.01 ± 0.01	0.03 ± 0.01	8	0.9
Tyrosine	0.04 ± 0.02	0.04 ± 0.01	16	1.0
Valine	0.34 ± 0.08	0.24 ± 0.03	25	1.0

Pairwise comparisons are made with Wilcoxon sign-rank test except normally distributed variables that are tested with Welch's t-test. All *P*-values are adjusted using the Holm–Bonferroni method. $N = 12$ throughout. Significant effects are in bold.

[a] T-Test.

Table 2. Mean (±SE) concentrations (μg/g FW) of phenolic compounds in tissues from noninfected controls and TMV-infected plants

Compound class	Compound	Control	TMV	Test statistic	*P*-value
Chlorogenic acids	Chlorogenic acid	3.91 ± 1.30	0.98 ± 0.16	34.50	0.24
	Chlorogenic acid derivative	4.30 ± 0.49	4.21 ± 1.24	25	0.24
	Cryptochlorogenic acid	4.74 ± 0.74	2.23 ± 0.21	35	0.36
	Dichlorogenic acid 1	6.63 ± 0.92	5.01 ± 1.05	1.16	0.36
	Dichlorogenic acid 2	**1.18 ± 0.42**	**1.71 ± 0.72**	**0.13**[a]	**0.01**
Organic acids	Ascorbic acid	2.13 ± 0.43	1.09 ± 0.20	2.19[a]	0.24
	Caftaric acid	0.22 ± 0.02	0.12 ± 0.05	2.08[a]	0.24
	Citric acid	1.05 ± 0.18	0.79 ± 0.13	1.16[a]	0.36
	Protocatechulic acid	0.05 ± 0.03	0.00 ± 0.00	1.54[a]	0.36
	Quinic acid	4.27 ± 0.45	2.04 ± 0.25	4.29[a]	0.01
Flavanoids	Catechin	1.44 ± 0.13	1.66 ± 0.33	0.60[a]	1.0
	Procyanidin B isomer	11.70 ± 2.80	26.80 ± 8.10	1.78[a]	1.0
	Genistin	0.41 ± 0.09	0.36 ± 0.10	0.29[a]	1.0
	Isoquercetin	0.22 ± 0.08	0.26 ± 0.05	0.45[a]	1.0
	Kaempferol apioxil-rutinoside	1.05 ± 0.16	0.88 ± 0.35	0.44[a]	1.0
	Kaempferol rutinoside	0.31 ± 0.14	0.27 ± 0.06	0.24[a]	1.0
	Quercetin apioxil-rutinoside	5.02 ± 0.64	7.07 ± 1.92	15	1.0
	Quercetin hyperoside	0.75 ± 0.11	1.02 ± 0.20	17	1.0
	Quercetin rutinoside	1.66 ± 0.22	2.36 ± 0.74	17	1.0

Pairwise comparisons are made with Wilcoxon sign-rank test except normally distributed variables that are tested with Welch's t-test. All *P*-values are adjusted using the Holm–Bonferroni method. $N = 12$ throughout. Bold indicates a significant effect when the overall class is also significant.

[a] T-Test.

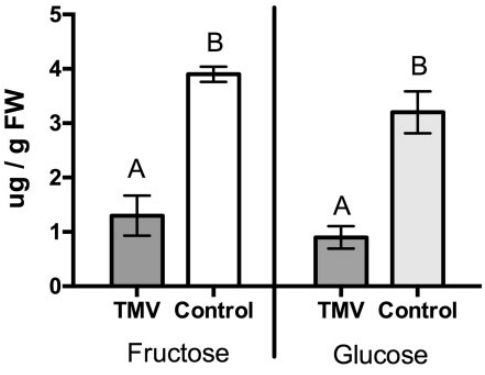

Fig. 2. Mean (±SEM) concentrations (μg/g FW) of sugars in tissues from noninfected controls and TMV-infected plants.

$F = 3.7$; $P = 0.07$) between TMV-infected and control plants.

In total, six monoterpenoids (α-phellandrene, α-terpinene, β-linalool, eucalyptol, para-cymene, and γ-terpinene) were quantified, but did not differ with infection status (MANOVA: $\Delta = 0.76$; $F = 3.7$; $P = 0.07$). Conversely, six sesquiterpenoids (α-humulene, β-caryophellene, β-farnesene, citronellyl isobutyrate, germacrene D, and nerolidol) were also quantified and these did differ with respect to infection (PERMANOVA: $F = 6.05$; $R^2 = 0.37$, $P < 0.05$). Individually, none of the sesquiterpenoids were affected by TMV infection. While, multiple compounds had marginal results that would be significant if not corrected for multiple comparisons but there is no obvious overall trend (Table 3).

Discussion

The role of insect preference with pathogen infection is the subject of some theoretical consideration (Zhang et al. 2000). Modeling studies by McElhany et al. (1995), for instance, demonstrate that vector preferences can have dramatic effects on pathogen spread. These models are expanded on by Sisterson et al. (Sisterson 2008) who determine that preferences based on feeding (gustatory cues) will have different implications for disease spread than those based on orientation (volatiles and olfactory cues). Feeding cues lead to changes in the rate of pathogen spread, but not in the association of the percentage of plants infected and the rate of pathogen spread. Other models (McElhany et al. 1995) indicate that when the vector of a persistent pathogen (such as Lso) prefers a healthy host, then pathogen spread is increased. Unfortunately, there are few empirical studies available, and thus evaluating these models is difficult. Here, we provide an empirical study of vector preference in a multipartite system. While it is difficult to predict how these models would function specifically for the system examined in this study, they suggest that infection with viral pathogens such as TMV may result in decreased spread of Lso through crop fields. If these models are accurate, our results also have important implications for applied management decisions regarding *B. cockerelli*.

Multiple studies show that infection with a vector-borne microbial pathogen will alter plant phenotype in a manner that influences the interaction between the host and the pathogen, or the host and the vector (Roy and Raguso 1997, Ebbert and Nault 2001, Eigenbrode et al. 2002, Belliure et al. 2005, Lefèvre et al. 2006, Mann et al. 2012, Mauck et al. 2014). In this study, *B. cockerelli* (a vector) demonstrate a preference for noninfected hosts to those infected with TMV. This preference is exhibited both as a settling behavior in

Table 3: Mean (± SE) concentrations (μg/g FW) of terpenoids in tissues from noninfected controls and TMV-infected plants

Compound class	Compound	Control	TMV	Test statistic	*P*-value
Monoterpenoids	α-phellandrene	1.93 ± 0.20	1.48 ± 0.08	2.12[a]	0.28
	α-terpinene	299.00 ± 57.00	375.00 ± 72.00	0.83[a]	0.78
	β-linalool	2.63 ± 0.35	1.83 ± 0.33	1.68[a]	0.36
	Eucalyptol	1.66 ± 0.10	1.31 ± 0.05	3.13[a]	0.08
	Para-cymene	**8.56 ± 2.62**	**12.50 ± 4.20**	**4.59[a]**	**0.006**
	γ-terpinene	2.18 ± 0.09	1.61 ± 0.08	12	0.78
Sesquiterpenoids	α-humulene	0.87 ± 0.16	1.01 ± 0.21	30	0.20
	β-caryophellene	1.89 ± 0.79	5.01 ± 1.38	−2.96[a]	0.06
	β-farnesene	53.60 ± 6.50	40.00 ± 2.90	1.28[a]	0.23
	Citronellyl isobutyrate	1810.00 ± 340.00	3210.00 ± 330.00	−2.96[a]	0.07
	Germacrene D	234.00 ± 21.00	193.00 ± 24.00	2.99[a]	0.07
	Nerolidol	279.00 ± 51.00	490.00 ± 50.00	−1.96[a]	0.21

Pairwise comparisons are made with Wilcoxon sign-rank test except normally distributed variables that are tested with Welch's t-test. All *P*-values are adjusted using the Holm–Bonferroni method. $N = 12$ throughout. Bold indicates a significant effect when the overall class is also significant.

[a] T-Test.

choice bioassays, and as oviposition preference in both no-choice and choice bioassays. Further there is no difference in growth index (performance) between vectors reared on TMV-infected and uninfected hosts. This suggests that while the psyllid vectors respond variably to infected and uninfected plants, this effect is not realized as differential performance.

Two previous studies test the affect of Lso infection on *B. cockerelli* host choice. Davis et al. (2012) show that Lso-negative *B. cockerelli* initially prefers hosts infected with Lso, with preference changing to uninfected plants over time. Mas et al. (2014) also found that Lso-infected *B. cockerelli* prefer to settle on uninfected plants, while the inverse applied to Lso-uninfected *B. cockerelli* that prefer Lso-infected plants. In each instance, volatile organic compounds differed between Lso-infected and uninfected plants, and this is considered a mechanism for these behaviors. These studies can be compared with the results of this study that demonstrated a preference of Lso-infected *B. cockerelli* for plants that were not infected with TMV. These similar findings may indicate a general trend in which Lso-infected *B. cockerelli* prefer pathogen free plants. However, it may also be a confounding effect of Lso. It is an inherent property of this study that plants will be exposed to feeding by Lso-infected vectors. As such, it is possible that both control and TMV-infected plants also were infected with Lso, and some aspect of the trends results from this. However, there was no change in preference over time, and the first time point of 24 h is likely before any effect of Lso infection would occur. Similar to the results presented here, Davis et al. (2012) report no effect of infection on performance (as measured by population growth). Interestingly, this corresponds to other studies in which performance is not associated with preference in *B. cockerelli* (Prager et al. 2014b) and raises some interesting questions about how *B. cockerelli* host plant preferences have evolved.

It has been suggested that Lso manipulates plants, vectors, or both to its own benefit. Such effects are known for other vectors of plant pathogens (Kahn and Saxena 1985, Stout et al. 2006). In fact, Lso is known to manipulate plant defense in tomatoes, with differences in plant defense gene expression between Lso-infected plants, uninfected plants with insect feeding, and plants with no insect feeding (Casteel et al. 2012). We found that Lso-infected *B. cockerelli* response to Lso and TMV is similar. This is interesting because it suggests that they may interpret a general signal associated with "sick plants," rather than to a specific pathogen. However, it is unclear how this results in attraction of uninfected *B. cockerelli* to infected plants. In particular, we did not examine volatile compounds that may reflect long-distance attraction, and thus cannot say if TMV-induced responses resemble those of Lso-infected plants.

Biochemical analyses reveal significant differences between TMV-infected and uninfected plants with respect to groups of primary and defensive metabolites. Overall, the trends are for less sugars and amino acids in the infected plants. This is important, as the psyllids consistently prefer the "healthy" control plants. Therefore, it is likely that *B. cockerelli* have a reduced preference for TMV-infected plants that have reduced nutritional value. Psyllids are phloem-feeding insects, and it has been proposed that host plant selection is chemo-gustatory (Hodkinson 1974). Additionally, like many other phloem-feeding sucking insects, psyllids must consume large amounts of sap to overcome the limited essential amino acids available in sap (Dolling 1991; Douglas 1993, 2006). Consequently, there is a substantial benefit for selecting host plants with higher levels of sugars, and especially amino acids. These findings may explain why psyllids demonstrated a behavioral response despite no apparent shifts in defensive compound levels. However, we cannot rule out some response to volatiles. TMV infection is known to cause increases in methyl salicylate, a known signaling compound in plant defense (Deng et al. 2004) in tomatoes. Further, it has been demonstrated that *Diaphorina citri* Kuwayama, will respond to plant defense volatiles (Mann et al. 2012), and *B. cockerelli* has been shown to respond to olfactory cues (Diaz-Montano and Trumble

2012). Although, volatile compounds are likely to influence "long-range" attraction, while bacterial transmission is a function of feeding and thus requires contact with a plant.

The results of this study suggest that reduced transmission of Lso results from behavioral changes in the vector. It has been demonstrated that transmission can occur with an access period of ~2 h (Sandanayaka et al. 2014), and feeding interruption can prevent Lso transmission (Butler et al. 2012). Additionally, both Lso titer and probability of infection is associated with psyllid density (Rashed et al. 2013b). Thus, we suggest that the observed differences in Lso titer and proportions of infected plants result from less insects feeding, and perhaps shorter durations of feeding. However, we cannot rule out the possibility that lower Lso titers and incidence of infection are because of competition within the plant host. We did not examine potential interactions of TMV and Lso, which may have occurred once the vectors fed on plants. Such competitive interactions are known for some plant systems (Rochow and Ross 1955, Vance 1991, Seabloom et al. 2014), and often result increased success of one pathogen relative to the second. However, these studies typically consider multiple viral pathogens rather than a combination of bacteria and viral pathogens, as in our study.

The findings of this article have important applied implications for developing management techniques for *B. cockerelli*. Recently, it has been proposed that TMV can be used to deliver RNAi effectors for control of potato psyllids (Wuriyanghan et al. 2011, Wuriyanghan and Falk 2013). However, the results presented here indicate that the very fact a plant is infected with TMV, as would occur in such a scenario, will alter the psyllid's behavior such that the psyllid avoids infected plants. This avoidance will limit the psyllid's exposure to the RNAi. Additionally, it suggests that when examining these technologies, one must ascertain if the effects being observed, such as reduced feeding or reproduction, stem from the effect of RNAi or from psyllid's response to infected plants. Finally, because TMV is typically a less damaging infection than Lso, these results open the door for potential prophylactic infection with attenuated TMV as a management strategy for Lso.

This study was designed to evaluate the influence of TMV infection on transmission of Lso. As vector transmission of a pathogen requires infected vectors, only Lso-positive psyllids were used throughout the experiments. Consequently, one question that remains to be examined is whether Lso-negative psyllids would exhibit similar behavioral responses to those that are infected with Lso. Regardless, plants infected with TMV exhibited lower Lso titers and were infected less frequently, suggesting that a pathogen can influence the infection success of a second pathogen via changes in host physiology, vector behavior, or both. A probable mechanism for this is reduced feeding (in duration, instances, or number of individuals) by the psyllid vector(s). It is likely that fewer *B. cockerelli* individuals feeding on TMV-infected plants result in reduced transmission of Lso. However, this study did not evaluate feeding, proxies of feeding, or feeding-like behaviors, and these need to be explicitly examined. Furthermore, direct interactions between the pathogens *in planta* were not examined, and it remains to be tested whether or not viral presence could impact the bacterial infection processes or induction of plant defense. Future studies of feeding responses, gene expression studies in Lso- and TMV-infected plants, and further biochemical studies focusing on volatile compounds are all necessary to elucidate the specifics mechanism of these results.

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