Postharvest Quarantine Treatments for *Diaphorina citri* on Infested Curry Leaves

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Abstract

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Studies were conducted to evaluate treatments that reduce survival and attachment of *Diaphorina citri* nymphs on infested curry leaves (*Bergera koenigii*). Decontamination of curry leaves infested with *D. citri* in relation to disinfectant (none or Pro-San), temperature (0, 40, and 50°C), and treatment duration (0, 5, 10, and 20 min) was examined using a split-split plot design. Experiments were performed three times. Treatment duration did not significantly affect *D. citri* nymph survival or removal ($P > 0.2$). Temperature and disinfectant each significantly affected *D. citri* nymph survival and removal ($P < 0.031$). The interaction of temperature and disinfectant was significant with respect to nymph survival ($P < 0.0001$) but

did not significantly affect removal ($P = 0.4589$). Tissue damage was significantly affected by temperature ($P = 0.0056$), duration ($P = 0.0023$), the interaction of temperature and duration ($P = 0.0320$), and the interaction of disinfectant, temperature, and duration ($P = 0.0410$). Of the treatments resulting in 100% *D. citri* nymph mortality on infested curry leaves, 40°C for 5 min with Pro-San was accompanied with the least proportion of curry leaf tissue damage (0.14 greater than untreated control, $P = 0.25$). Results from these studies may be useful in formulation of future regulatory policies regarding trade of citrus foliage, especially those used as condiments.

Huanglongbing (HLB) has been considered the most devastating disease of citrus (Bové 2006; Gottwald 2010; McClean and Schwarz 1970). Although promising progress is being made, there is currently no cure for this disease. The bacterium and vector associated with HLB in Florida are '*Candidatus Liberibacter asiaticus*' and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Asian citrus psyllid), respectively. Foliage of citrus and its relatives serves a variety of purposes, from decorations (e.g., *Citrus limon* leaf garlands and wreaths) to medicinal uses (Ferguson and Spann 2010). For example, *Bergera koenigii* (L.) Spreng. (syn. *Murraya koenigii*) has many important uses within traditional Ayurvedic medicine, including relief from vomiting, diarrhea, inflammation, and numerous other conditions (Jain et al. 2012). Additionally, leaves of *B. koenigii* have been widely used for centuries in Indian cooking (Jain et al. 2012), and *C. hystrix* leaves are commonly used in Thai cooking (Bastyra and Johnson 2007).

Citrus relatives' leaves are increasingly traded internationally, and accompanying this is the concern of pest (e.g., *D. citri*) and pathogen (e.g., '*Ca. L. asiaticus*') movement. At present, precautionary decontamination treatment options prior to international importation of *B. koenigii* or *C. hystrix* foliage into the United States consist of treatment with methyl bromide for 2 h at 48 g·m⁻³ (3 lb. per 1,000 ft³) at 10°C or irradiation at 400 Gy (USDA 2013). Although both of these current methods have many beneficial qualities for quarantine and preshipment applications (e.g., effective biocide, noncorrosive, and efficient area treatments; US EPA 1986, 2009), they simultaneously have numerous drawbacks. Irradiation requires a dedicated facility that is expensive to build, although operating costs can become economical compared with other treatment options (e.g., steam treatment) when a minimum capacity of samples are treated (Venugopal 2005). Methyl bromide causes stratospheric ozone depletion and is highly toxic and hazardous to work with (Butler and Rodriguez 1996; UNEP

2007; US EPA 2010). Although use of methyl bromide for quarantine (and preshipment) purposes is covered by an exemption from the Montreal Protocol, several countries have passed legislation to discontinue its use (Russian Federation) or have indicated that they will soon end such use (European Community and Brazil) (US EPA 2010). Furthermore, alternatives to methyl bromide for quarantine treatment have been sought, and it is the policy of the International Plant Protection Convention that, where possible, methyl bromide alternatives should be used (US EPA 2010). Previous studies have reported immersion in hot water to be applicable for quarantine-level disinfestation (Durgapal et al. 1969; Follett and Sanxter 2001; Gaur et al. 1984; Rao and Durgapal 1967; Shellie and Mangan 2002). The objective of the current studies was to examine effects of postharvest water-temperature treatments alone and in combination with a disinfectant on survival and removal of *D. citri* nymphs on infested curry (*B. koenigii*) leaves.

Materials and Methods

Effect of postharvest treatments on *D. citri* survival on and removal from infested curry leaves. Curry trees were grown in a greenhouse in single-plant cups (16 cm in diameter by 16 cm in height). Three trees each were placed within six insect-rearing tents (mesh aperture = 0.16 mm; BugDorm-2120F; MegaView Science Co., Ltd., Taichung, Taiwan). Four weeks prior to each infestation, plants were pruned to induce a new flush of leaves. Plants were infested by placing cuttings of curry leaves with *D. citri* adults (approximately 120 adults per tent) within each tent. Adult *D. citri* were propagated from approximately 20 individuals from a ('*Ca. L. asiaticus*'-negative) colony maintained at the U.S. Horticultural Research Laboratory, Fort Pierce, FL. Approximately 3 weeks after initiation of infestation, leaves with *D. citri* nymphs (minimum number of nymphs per leaf = 4 and average number of nymphs per leaf = approximately 14) were arbitrarily detached, placed within resealable plastic bags, and stored at 10°C overnight. The next day, treatments were applied by submerging leaves held between T-304 stainless-steel bolting cloth (mesh aperture = approximately 0.32 by 0.32 mm and wire diameter = approximately 0.10 mm) in a temperature-controlled recirculating water bath similar to that used by Anco et al. (2014). A nymph-proof gasket at mating interfaces of screen perimeters was formed using polyvinylchloride foam tape (0.635 cm thick by 1.27 cm wide; Foamtapes.net, West Deptford, NY). The water bath was constructed around a 160-liter cooler (Tropical Ice Boxes cooler; Braid Products, Palmdale, CA). Water was heated and cooled via an 11-kW CAST-X 3000 water heater

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(Cast Aluminum Solutions, Batavia, IL) and an Arctica DA-1000B chiller (JBJ Lighting, Inglewood, CA), respectively. A Flojet 4100-500-115 pump (Xylem Flojet, Irvine, CA) provided turbulence and circulation within the water bath. Bath temperature was monitored in 2-s intervals using DASYLab V11 (Measurement Computing, Norton, MA) as the average of four T-type 20-gauge thermocouples (Omega, Stamford, CT). One thermocouple was positioned in each of the four corners of the water bath at half the height of the water level. Actual temperatures (standard error) maintained in the temperature-controlled recirculating water bath corresponding to target treatment temperatures of 0, 40, and 50°C were 0.5 (0.007), 40.2 (0.009), and 49.9°C (0.009), respectively. Screens were arbitrarily removed from the water bath after 5, 10, and 20 min of treatment, after which leaves were examined for live and dead *D. citri* nymphs, as well as those that were removed from leaves and trapped by screens that were alive or dead. For treatments with Pro-San (Pro-San LC; active ingredients: lactic acid, phosphoric acid, citric acid, sodium acid pyrophosphate, sodium dodecylbenzene sulfonate, and sodium benzoate; Microcide, Inc., Detroit), the effective pH range (2.2 to 2.8) was reached by adding approximately 710 ml of Pro-San to the water bath (working water volume = 140 liters). The average pH of the untreated tap water was 5.5. Water bath pH was determined via colorpHast pH test strips (EMD Chemicals Inc., Darmstadt, Germany). Sample size for each treatment was 12 leaves, because preliminary experimentation indicated that sample sizes ≥ 12 leaves (up to 20) resulted in similarly minimized estimated mean variances of numbers of nymphs per leaf compared with smaller sample sizes. Experimental design was a split-split plot, with disinfectant assigned to whole-plots, temperature assigned to subplots, and treatment duration assigned to sub-subplots. The experiment was performed three times.

Effect of postharvest treatments on leaf appearance. Fourteen days posttreatment (storage at 10°C), proportion of tissue damage was estimated from five representative curry leaves per treatment (or untreated control). Digital images were taken with a Canon EOS 10D digital camera (Canon USA, Inc., Melville, New York) at a standardized distance of 32 cm. Pixels were classified as healthy or damaged using Photoshop CS4 (Adobe Systems, Inc., San Jose, CA), from which proportion of tissue damage was calculated. Pixels were converted to square millimeters via multiplying by $29^2/440^2$ (determined empirically). This experiment was performed three times.

Data analysis. The GLIMMIX procedure of SAS (ver. 9.3; SAS, Cary, NC) was used to examine fits of generalized linear mixed models to the *D. citri* nymph mortality and removal data. The best fitting models were linear models fit to mean proportion of *D. citri* nymph death and removal. Fixed-effect factors in the final models were temperature, disinfectant, and their interaction term. All fixed effects involving duration were not significant ($P > 0.2$); thus, duration was removed from the models. Random effects corresponding to whole-plot (replication–disinfectant), subplot (replication–disinfectant–temperature), and sub-subplot error

terms were estimated to be zero and were excluded from the model (Littell et al. 2007). Laplace approximation (Pinheiro and Chao 2006; SAS Institute Inc. 2011; Stroup 2013) was utilized to improve standard error estimation. One-sided contrasts of least squares means were used to determine which treatment combinations resulted in significantly less *D. citri* nymph survival or more removal compared with untreated controls. The Monte Carlo simulation method (Edwards and Berry 1987) was used to adjust contrast *P* values for multiple comparisons.

Fixed effects from the model best fitting the tissue damage data included disinfectant, temperature, duration, and all corresponding interactions. Random effects (in addition to the residual) consisted of a subplot error term (replication–disinfectant–temperature) and an error term corresponding to the leaf area of the three terminal-most nodes per leaf (terminal leaf area [TLA]). This error term (TLA class–temperature) was incorporated into the model after observation and subsequent determination of a significant ($P < 0.0001$) negative exponential relationship of the form

$$y_{uz} = \alpha e^{-\beta TLA_u} + T_z + \varepsilon_{uz}$$

incorporating temperature (T_z) as a random effect, with mean 0 and variance s_T^2 between TLA of leaf *u* (an inverse relative proxy for leaf age) and proportion of tissue damage (*y*) 14 days posttreatment of leaf *u* treated at temperature *z*. Parameter ε_{uz} represents experimental error associated with leaf *u* treated at temperature *z* (mean 0 and variance s_e^2), while both α and β are parameters representing abstract predicted proportion of tissue damage at TLA 0 and the rate of decrease in predicted proportion of tissue damage as TLA increases, respectively. Modeling of TLA data was performed in the NLMIXED procedure of SAS. Fit statistics (Madden et al. 2007), parameter, and covariate estimates are shown in Table 1. According to the fitted model, predicted proportion of tissue damage corresponding to $TLA \geq 325.80 \text{ mm}^2$ was no longer significantly greater than 0.01 ($P \geq 0.06$). This yielded an empirically derived threshold beyond which TLA class was subsequently undifferentiated. Classes of TLA were as follows: $1 < 45.01 \leq 2 < 77.32 \leq 3 < 116.25 \leq 4 < 165.28 \leq 5 < 231.78 \leq 6 < 325.80 \text{ mm}^2 \leq 7$. As a note, none of the sampled leaves possessed TLA equal to class boundaries. TLA class boundaries were calculated as the midpoint of adjacent TLA class midpoints, which were determined by inputting midpoints of intervals (six classes, each approximately 0.038 wide) of proportion of tissue damage from the range of observed values (approximately 0.057 to 0.28) preceding the previously mentioned threshold of 325.80 mm^2 into the fitted negative exponential model. Categorizing TLA into seven classes produced the best fitting model among the range examined (two through eight classes). Because inclusion of the whole-plot error term (replication–disinfectant) and the effect of replication did not improve fit of the model to the data, these terms were excluded from the final model. Laplace approximation was used. One-sided contrasts of least squares means were used to determine which treatment combinations resulted in significantly more curry leaf tissue damage compared with untreated controls. Here, contrast *P* values were not adjusted for multiple comparisons to more stringently scrutinize amounts of posttreatment tissue damage.

Results

Effect of postharvest treatments on *D. citri* survival on and removal from infested curry leaves. Proportion of *D. citri* nymph mortality without Pro-San increased with increasing temperature, and treatments with Pro-San yielded 100% (40 or 50°C) or 99.49% (0°C) nymph mortality. All fixed effects in the model describing proportion of nymph mortality were significant (Table 2). Mortality ranged from 35% (0°C without Pro-San) to 100% (treatments including Pro-San or 50°C) (Fig. 1A). All temperature and disinfectant treatment combinations resulted in significantly (or, in the case of 0°C with no disinfectant, nearly significantly) greater *D. citri* nymph mortality compared with the untreated control (Table 3). Average proportion of nymph mortality on untreated controls was 0.24.

Across all temperatures, a significantly greater proportion of nymphs (fold difference of approximately 1.6, proportion difference

Table 1. Terms regarding the negative exponential relationship between proportion of tissue damage of curry leaves 14 days posttreatment and terminal leaf area

Term ^a	Value (SE) ^b	<i>t</i> Value	<i>P</i>
Robust pseudo- <i>R</i> ²	0.3936 (...)	...	...
Concordance correlation coefficient	0.8798 (...)	...	...
α	0.3085 (0.06388)	4.83	<0.0001
β	0.5202 (0.1540)	3.38	0.0026
s_e^2	0.02321 (0.001974)	11.75	<0.0001
s_T^2	0.08708 (0.02564)	3.40	0.0025

^a Terms are fit statistics (robust pseudo-*R*² and the concordance correlation coefficient; both have an upper limit of 1, which would represent perfect agreement), parameters (α and β), and variances of covariates (s_e^2 and s_T^2).

^b Standard errors (SE) are not applicable for the fit statistics.

of approximately 0.07) were removed from infested curry leaves when treatments included Pro-San ($P = 0.0013$; Fig. 1B). Within the model describing proportion of nymph removal, simple effects (but not the interaction) of disinfectant and temperature were significant (Table 2). All temperature and disinfectant combinations resulted in a significantly greater proportion of nymph removal compared with the untreated control ($P < 0.021$), with the exception of treatment at 0°C without Pro-San ($P = 0.219$). The most removal of nymphs from curry leaves occurred following treatment at 40°C with Pro-San (approximately 24%). Average proportion of nymph removal on untreated controls was 0.022.

Effect of postharvest treatments on leaf appearance. Symptoms of tissue damage (proportions of which are shown in Fig. 2) on curry leaves 14 days posttreatment ranged from isolated water-soaked cells (more common after treatment with Pro-San) to total necrosis (more common after treatment for 20 min at 50°C) (Fig. 3). For treatments containing Pro-San, the proportion of leaf area with water-soaked cells was exacerbated by increased treatment temperature and duration (e.g., treatment at 40°C for 5 min resulted in a proportion of tissue damage of 0.17, whereas 20 min at 40°C resulted in a proportion of tissue damage of 0.35). Significance of fixed effects and covariance parameter estimates are shown in Table 4. Average proportion of tissue damage on untreated control curry leaves after

14 days storage at 10°C was 0.034. Treatments resulting in significantly more tissue damage than untreated controls are shown in Table 5. Although specific examples shown (Fig. 3E) were treated at 0°C with Pro-San, increased tissue damage in relation to small TLA was not unique to specific treatments and was also observed on untreated controls after 14 days of storage at 10°C.

Discussion

The current studies have contributed knowledge of efficacy of alternative postharvest treatments combining water-mediated temperature transfer, treatment duration, and an acidic disinfectant for curry leaves infested with *D. citri* nymphs. In the absence of disinfectant, *D. citri* nymph mortality increased with increasing temperature to 100% mortality at 50°C. With the disinfectant, mortality was 100% after treatment at $\geq 40^\circ\text{C}$, and nearly 100% after treatment at 0°C. Overall, more removal occurred when treatments included the disinfectant, and the most removal occurred at 40°C. Posttreatment tissue damage generally increased with increasing temperature and, for treatments with Pro-San, it further generally increased with increasing treatment duration. Although the nymph stage of *D. citri* was the focus of the current studies (due to their being much more difficult to remove from plant material than their adult counterparts), it was consistently observed that treatments resulting in 100% mortality of nymphs concurrently resulted in 100% mortality of *D. citri* adults.

Previous studies have demonstrated that high-temperature treatments provide quarantine-level disinfestation of pests. Follett and Sanxter (2001) reported 100% mortality of *Cryptophlebia* spp. (Lepidoptera) on lychee and longan fruit submerged in 49°C water for 16 or 20 min. Shellie and Mangan (2002) found that immersion of large mango fruit with Mexican fruit fly (*Anastrepha ludens* Loew) (Diptera) in 46.1°C water for 110 min provided quarantine security. Hot-air treatment combinations between 43 and 47°C for durations from 140 to 10 min have resulted in 100% mortality of *Conotrachelus nenuphar* (Herbst) (plum curculio) (Coleoptera) and *A. ludens* (Hallman 1998). After a longer treatment time of 240 min at 46°C, 100% mortality of *Rhagoletis pomonella* (Walsh) (Diptera) was observed on infested apple fruit (Blessington and Hallman 1999). Although similar temperature treatments (and duration ≤ 20 min) were examined in the current study, the presence of significant posttreatment tissue damage on curry leaves for treatments (without Pro-San) yielding 100% mortality rendered those treatments undesirable. Although not examined in the current study, it is possible that quarantine-level decontamination of infested curry leaves could have been reached after extended (>100 min) treatment duration at 40°C while also yielding statistically acceptable amounts of posttreatment tissue damage.

Although the current studies demonstrated that (visual) tissue damage observed on curry leaves treated for 5 min at 40°C with Pro-San was quite low and not significantly different from untreated controls, formal measurements regarding quantity or quality of flavor volatiles in treated leaves was absent. This information would be useful, because curry leaves are largely traded for their use in culinary applications (Jain et al. 2012), and previous research has reported how flavor volatiles can be affected independently of visual characteristics (Buttery et al. 1989; McDonald et al. 1996). Nevertheless, subjective observations did indicate that the aroma quality and intensity of leaves exposed to treatments resulting in insignificant amounts of visual tissue damage were analogous to those of untreated controls. Conversely, treatments at the significantly damaging end of the spectrum (e.g., 50°C treatments) produced leaves with noticeably weaker aromas less characteristic of curry.

The overall best treatment from the current study, 5 min at 40°C with Pro-San, has advantages and disadvantages compared with the currently prescribed quarantine treatments of irradiation and methyl bromide treatment. With regard to irradiation, it would be anticipated that the infrastructure necessary to conduct this study's recommended treatment would be considerably less expensive, because (depending on capacity and size) building an irradiation facility ranges from U.S.\$3 to 5 million (International Consultative Group on Food Irradiation 1999). On that note, it would have been beneficial if the current studies included treatments at room temperature

Table 2. Type III tests of fixed effects for models describing *Diaphorina citri* nymph data

Response	Effect	F value	P value
Proportion of nymph mortality	Disinfectant	123.62	<0.0001
	Temperature	113.15	<0.0001
	Disinfectant $\times$ temperature	48.70	<0.0001
Proportion of nymph removal	Disinfectant	4.97	0.0303
	Temperature	8.71	<0.0001
	Disinfectant $\times$ temperature	0.88	0.4589

Fig. 1. Effect of temperature and disinfectant on proportion of **A**, mortality and **B**, removal of *Diaphorina citri* nymphs from infested curry leaves. Error bars are standard errors of the mean of three replications.

because, based on the mortality and tissue damage data, it is quite plausible that 100% *D. citri* nymph mortality would have occurred after a room-temperature treatment with Pro-San for 5 min, with potentially even less associated tissue damage. If, indeed, this was the case, resulting infrastructure requirements would be further simplified and even more economical. The current studies also made no attempt to dry treated leaves before storing them in resealable plastic bags. Under an importation for subsequent trade scenario, it would perhaps be desirable to dry treated leaves. An easy way to do this would be a conveyor belt coupled with forced air. A great advantage of irradiation and methyl bromide is their versatility in treating multiple target organisms, as well as irradiation's applicability toward industrial use (e.g., sterilization of medical or scientific equipment). In the case of irradiation, facility versatility serves to offset, to an extent, costs of construction and operation. Although it is plausible that effective treatments from the current study would be effective against other pests of quarantine concern, experimental data would need to be established before expanded use of these treatments could be confidently executed.

Continued use of methyl bromide for quarantine decontamination stems from multiple considerations. Many importation situations leave methyl bromide as the only economical and practical treatment option (US EPA 2010). Often, ports of entry do not have applicable infrastructure to accommodate alternative treatments, and removal of imported materials from packaging containers costs additional time and labor (US EPA 2010). Methyl bromide has the corresponding advantage of potentially requiring minimal equipment to conduct treatments, because fumigation can be performed with as little as a sealed tarp, though more elaborate setups would be required to allow for fume recapture (US EPA 2010), due to the hazardous nature of the chemical, as described earlier. Conversely, Pro-San is nontoxic, free of volatile organic compounds, benign to work with, and environmentally friendly (Microcide 2002). In addition, and in contrast to irradiation (McGuire 2000), treatment with quarantine levels of methyl bromide has been shown to result in significant adverse effects on treated plant tissues: reduced quality, marketability (MBTOC 2007), and shelf-life (US EPA 2010). With explicit reference to curry leaves, quarantine-level treatment with methyl bromide resulted in leaves more than 50% decayed 6 days posttreatment (McGuire 2000), which is considerably more than the approximately 17% proportion of tissue damage observed on curry leaves 14 days posttreatment with Pro-San at 40°C for 5 min (which is also 115 min shorter than currently prescribed methyl bromide treatment) from the current studies. With respect to potential obstacles in creating infrastructure at ports of entry to conduct this alternative treatment, an option would be for exporting countries to conduct this as a preshipment treatment, which could

further reduce treatment costs compared with postentry treatment (US EPA 2010).

One potentially perceivable weakness of the current curry studies is that the results are based on experiments using an essentially clonal population of *D. citri* nymphs. This resulted in a negligible amount of genetic diversity of the sample of insect pests tested and would certainly raise critical speculation as to the relevancy of resulting "effective treatments" if the crux of those "effective treatments" was based on variables (e.g., upper or lower lethal temperatures) in response to which phenotypic plasticity of tolerable conditions could be influenced based on the geographical (e.g., latitudinal) origin or diversity of pest populations (Addo-Bediako et al. 2000). However, the ultimate result of the current studies (determination of 5 min at 40°C with Pro-San as the best evaluated treatment producing

Fig. 2. Effect of treatment duration at different temperatures in the **A**, absence and **B**, presence of the disinfectant Pro-San on proportion of tissue damage on curry leaves 14 days posttreatment. Error bars are standard errors of the mean of three replications.

Table 3. Proportion of *Diaphorina citri* nymph mortality and removal posttreatment and contrasts with untreated controls

Response	Treatment	Estimate (SE)	Contrast with untreated control ^a		
			Estimate (SE)	t Value	P value ^b
Mortality	0°C	0.3515 (0.02829)	0.1426 (0.06634)	2.15	0.0501
	40°C	0.5507 (0.02829)	0.3417 (0.06634)	5.15	<0.0001
	50°C	1.0000 (0.02829)	0.7911 (0.06634)	11.92	<0.0001
	0°C, Pro-San	0.9949 (0.03000)	0.7860 (0.06709)	11.72	<0.0001
	40°C, Pro-San	1.0000 (0.02829)	0.7911 (0.06634)	11.92	<0.0001
	50°C, Pro-San	1.0000 (0.02829)	0.7911 (0.06634)	11.92	<0.0001
	Control	0.2089 (0.06001)	...	...	...
	Control, Pro-San	0.2770 (0.04900)	...	...	...
Removal	0°C	0.09452 (0.02509)	0.07540 (0.05885)	1.28	0.2192
	40°C	0.1836 (0.02509)	0.1645 (0.05885)	2.79	0.0121
	50°C	0.1699 (0.02509)	0.1507 (0.05885)	2.56	0.0202
	0°C, Pro-San	0.2019 (0.02661)	0.1828 (0.05951)	3.07	0.0067
	40°C, Pro-San	0.2355 (0.02509)	0.2164 (0.05885)	3.68	0.0013
	50°C, Pro-San	0.2105 (0.02509)	0.1914 (0.05885)	3.25	0.0041
	Control	0.01913 (0.05323)	...	...	...
	Control, Pro-San	0.02563 (0.04346)	...	...	...

^a SE = standard error.

^b Adjusted for multiple comparisons via Monte Carlo simulation.

100% nymph mortality while resulting in the least amount of [non-significant] tissue damage among treatments yielding 100% nymph mortality) was a treatment whose effectiveness resulted primarily from those examined nymphs being immersed in a relatively intolerably acidic environment (pH approximately 2.5). Thus, the question now becomes whether or not insect (more specifically, *D. citri*) pH tolerance varies by (geographically distinct) population of individuals or, more broadly speaking, whether or not sufficient evidence exists to support the plausibility of (relatively) currently known environmental stimuli (e.g., acidic deposition) contributing toward acidophilic adaptation by *D. citri* to such (currently) relatively extreme conditions of effective treatments proposed from the current studies. These questions bring the discussion into the realm of extremophiles and, more specifically, acidophiles. At the time of this writing, literature revealing relationships regarding variation in pH susceptibility or tolerance of populations of individual insect species with geographical population origin was not found. Nevertheless, the effect of this relationship would be anticipated to be negligible, as *D. citri* is a terrestrial organism whose known geographical range of existence does not typically exhibit a range of tropospheric-air pH, since the diatomic tendency of hydrogen within Earth's troposphere precludes the presence of gaseous hydrons for any appreciable amount of time. Because of decreased pressure and increased electromagnetic excitation energy available, gaseous ions, including hydrons, are present within the ionosphere, at altitudes of more than 60 km (Banks 1968; Shawhan and Gurnett 1966); however, *D. citri* is not found there (or at least not living for any appreciable amount of time). Regardless, the presence of free electrons within the ionosphere could cancel out a theoretically acidic pH of the air there (via proton donation) resulting from prevalence of gaseous hydrons. Examples of acidophile environments include acidic or hot springs, geysers, hydrothermal vents, and sulfide ore or coal deposits (Brock 1978; Kennish 1994; Prieur 2011; Rothschild and Mancinelli 2001) (all places currently unknown to harbor living *D. citri*). At typical atmospheric conditions, normal rain has a slightly acidic pH of approximately 5.6 (US EPA 2012). This is due to the formation of carbonic acid upon combination of carbon dioxide and water, which dissociates in water to form hydronium (Joesten et al. 2006). Since the industrial revolution, anthropogenic combustion of fossil fuels has increased deposition of carbon dioxide into the atmosphere (Solomon et al. 2009). Although this has its own effects, with regard to the possibility of increased tolerance of *D. citri* to lower pH values of rain potentially resulting from an increased tendency of the previously mentioned reactions toward formation of hydronium, the increased presence (now or future) of carbon dioxide by itself in the atmosphere is not likely to be the dominant factor

in influencing this relationship. Water saturated with carbon dioxide (at approximately 35°C, 0.38 MPa [approximately 3.75 times the pressure at sea level]) yielded a relatively measly pH of 3.71 (Peng et al. 2013), which has approximately 16 times less hydronium than available in treatments involving Pro-San in the current study (within the study of Peng et al. [2013], pH of water saturated with carbon dioxide at approximately 35°C reached 2.97, but only under 15.38 MPa [>151 times the pressure at sea level]). This demonstrates the relatively slow rate of increase in acidity of water exposed to increasing concentrations of carbon dioxide, and it can quickly be predicted that atmospheric levels of carbon dioxide

Table 4. Type III tests of fixed effects and covariance parameter estimates for the model describing curry leaf tissue damage 14 days posttreatment

Effect	F value	P value	Estimate (SE) ^a
Temperature	7.31	0.0056	...
Duration	6.24	0.0023	...
Temperature × duration	2.69	0.0320	...
Disinfectant	2.97	0.1040	...
Disinfectant × temperature	0.74	0.4927	...
Disinfectant × duration	2.74	0.0664	...
Disinfectant × temperature × duration	2.53	0.0410	...
Covariance parameter			
Subplot (replication × disinfectant × temperature)	...	...	0.02840 (0.008875)
Terminal leaf area (terminal leaf area class × temperature)	...	...	0.03127 (0.01397)
Residual	...	...	0.01679 (0.001504)

^a SE = standard error.

Table 5. Treatments resulting in significantly more tissue damage than untreated controls 14 days posttreatment

Treatment	Estimate of difference (SE) ^a	t Value	P value
50°C, 5 min	0.3705 (0.1916)	1.93	0.0272
50°C, 10 min	0.5600 (0.1905)	2.94	0.0018
50°C, 20 min	0.4671 (0.1914)	2.44	0.0077
50°C, 5 min, Pro-San	0.6184 (0.1891)	3.27	0.0006
50°C, 10 min, Pro-San	0.6584 (0.1902)	3.46	0.0003
50°C, 20 min, Pro-San	0.7461 (0.1899)	3.93	<0.0001

^a SE = standard error.

Fig. 3. Examples of tissue damage on curry leaves 14 days posttreatment. Leaves were A, untreated or treated at B and F; C and G, 40; or D and H, 50°C F to H, with or B to D, without the disinfectant Pro-San. E, increased tissue damage in relation to small terminal leaf area. Where applicable, leaflets were detached to facilitate imaging.

approaching saturation would render the importance of potentially increased *D. citri* pH tolerance from a possible adaptation to subsequently (carbon dioxide concentration-dependent) increasingly acidic rain inconsequential.

In the absence of neutralization reactions (e.g., resulting from aerosolic mingling of alkali species [e.g., ammonium] or dust with aqueously dissociated acidic moieties [Spengler et al. 1990]), acidic deposition (e.g., acid rain) resulting from chemical reactions involving compounds other than carbon dioxide (sulfur dioxide, nitroxides, or organic acids) typically ranges in pH from 3.5 to 5 (Menz and Seip 2004). Globally, major citrus-producing areas (Liu et al. 2012) generally exhibit an annual pH of wet deposition between 4.2 and 4.5 (Rodhe et al. 2002). Even if *D. citri* were to be constantly exposed to this acidic stimulus and subsequently successfully adapt to it (not considering effects on its host or ecosystem, for that matter), it would need to do so in a manner that would allow it to tolerate approximately 66 times more extracellular hydronium in order to render the current study's effective treatments ineffective. Consideration now shifts to how acidophiles tolerate relatively acidic conditions and how closely related *D. citri* is to known acidophiles.

Mechanisms of organismal tolerance of acidic environments are essentially based on maintaining intracellular homeostasis, because adenosine triphosphate, nucleic acid, and nicotinamide adenine dinucleotide are all acid sensitive and vital to cell function (Brock 1978). More specifically, acidophiles may expel intracellular hydronium via highly active membrane-associated antiporters (proton-pumps), which may be further aided by greater than average expression of proton export enzymes, or they may prevent net proton influx via alternative permeability properties, cationic polymers on cell walls, or having robust internal pH buffering (Krulwich et al. 1998; Pick 1999; Rothschild and Mancinelli 2001). Current knowledge indicates that the most acidophilic insects can only tolerate pH between 2 and 3 (Rothschild and Mancinelli 2001). Exemplifying this are certain members of the Ephydriidae family (e.g., *Ephydra thermophila* and *Scatella paludum*; Brock 1978). Although *D. citri* and Ephydrid flies are both members of the Neoptera infraorder, taxonomic divergence of the two begins at the level of superorder, with *D. citri* belonging to Paraneoptera (order Hemiptera) and Ephydrid flies to Holometabola (order Diptera) (ITIS 2014a,b). Thus, because *D. citri*'s environment is neither continually nor nearly as acidic as treatments involving Pro-San, and *D. citri* is only relatively distantly related to acidophilic insects, it is unlikely (given present knowledge) that geographically distinct populations of *D. citri* would exhibit increased pH tolerance and, thus, be capable of surviving treatment with Pro-San, though this would have to be verified experimentally for maximal certainty. Collectively, this information supports quarantine-level effectiveness of treatment of *D. citri*-infested curry leaves for 5 min at 40°C with Pro-San.

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