Glutathione Transferase and Cytochrome P_{450} (General Oxidase) Activity Levels in *Candidatus* Liberibacter Asiaticus-Infected and Uninfected Asian Citrus Psyllid (Hemiptera: Psyllidae)

SIDDHARTH TIWARI,[1] KIRSTEN PELZ-STELINSKI,
RAJINDER S. MANN, AND LUKASZ L. STELINSKI

Department of Entomology and Nematology, Citrus Research and Education Center,
University of Florida, Lake Alfred, FL 33850

Ann. Entomol. Soc. Am. 104(2): 297–305 (2011); DOI: 10.1603/AN10128

ABSTRACT *Candidatus* Liberibacter asiaticus (Las) has been reported to increase the susceptibility of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), to selected insecticides. Reduced general esterase activity in Las-infected, compared with uninfected, *D. citri* has been proposed as a possible explanation for this difference in insecticide susceptibility. The current study was conducted to quantify glutathione transferase (GST) and cytochrome P_{450} (general oxidase) activities in Las-infected *D. citri* to further explain the possible mechanisms for altered susceptibility to insecticides due to Las infection. GST and cytochrome P_{450} activities (indirectly through general oxidase levels) were quantified in Las-infected and uninfected *D. citri* nymphs and adults. Mean (±SEM) GST activity was significantly lower in Las-infected (468.23 ± 26.87 μmol/min/mg protein) than uninfected (757.63 ± 59.46 μmol/min/mg protein) *D. citri* adults. Likewise, mean cytochrome P_{450} activity was significantly lower in Las-infected (0.23 ± 0.02 equivalent units [EU] cytochrome P_{450}/mg protein) than uninfected (0.49 ± 0.05 EU cytochrome P_{450}/mg protein) *D. citri* adults. Immature stages (second and fifth instars) were characterized by significantly lower GST activity than adults for uninfected *D. citri*. However, cytochrome P_{450} activity was significantly higher in second instar nymphs than adults and fifth-instar nymphs for uninfected *D. citri*. Lower activities of GST and general oxidase in Las-infected *D. citri* indicate that infection with Las alters *D. citri* physiology in a manner that could increase insecticide susceptibility. The reduced activities of these detoxifying enzymes due to Las infection may be explained by examining expression levels of associated genes in Las-infected and uninfected *D. citri*.

KEY WORDS Asian citrus psyllid, *Candidatus* Liberibacter asiaticus, citrus greening, cytochrome P_{450}, glutathione transferase

Glutathione transferase (GST) and cytochrome P_{450} (general oxidase) are two important groups of multifunctional detoxifying enzymes responsible for metabolizing an array of xenobiotic compounds as well as endogenous compounds (Booth et al. 1961, Feyereisen 1999, Scott and Wen 2001, Yang et al. 2007, Gui et al. 2009). GST acts by catalyzing the nucleophilic addition of reduced glutathione (GSH) to electrophilic substrates, leading to the formation of water-soluble conjugates (Booth et al. 1961). In insects, GSTs are primarily associated with the metabolism of insecticides from organophosphate, organochlorine, and pyrethroid groups (Clark and Shamaan 1984, Grant and Matsumura 1989, Ranson et al. 1997). Insects resistant to the above-mentioned groups of insecticides have been found to possess higher GST activity. Quantification of GST activity in major insect pests is used to monitor resistance against organophosphate, organochlorine, and other plant-based xenobiotic compounds (Tang and Tu 1994; Huang et al. 1998; Vontas et al. 2000, 2002). Cytochrome P_{450} is known to have a wide range of substrates and therefore has a greater role in the metabolism and detoxification of various compounds (Scott 1999). The cytochrome P_{450} enzyme complex is often noted as the most important group of enzymes responsible for causing metabolism-based insecticide resistance (Scott 1999).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is one of the most destructive pests of citrus, primarily because it vectors the presumed causal pathogens of huanglongbing (HLB) and also causes direct injury losses. HLB is one of the most destructive and economically important diseases of citrus throughout the world (Halbert and Manjunath 2004, Manjunath et al. 2008). HLB is associated with either of three species of fastidious phloem-inhabiting gram-negative bacteria: *Candidatus* Liberibacter asi-

[1] Corresponding author, e-mail: stiwari@ufl.edu.

aticus (Las), *Candidatus* L. americanus (Lam), or *Candidatus* L. africanus (Garnier et al. 1984, Jagoueix et al. 1996). *D. citri* vectors both Las and Lam in Asia and the Americas. HLB reduces yield by causing premature fruit drop and increases fruit bitterness. HLB-infected trees typically die within 5–10 yr of infection, and currently there is no available cure to treat diseased trees. The overall HLB infection rate in Florida is estimated to be 1.6%, reaching up to 100% in the southern and eastern parts of the state and 1.4–3.6% in central Florida (Morris et al. 2009, Tiwari et al. 2010a). Lack of a specific cure for HLB disease has led to several efforts to identify vulnerabilities in the insect–pathogen interaction that would improve pest management programs for *D. citri.*

The presence of bacteria and yeast is known to alter the susceptibility of host insects to insecticides or toxins (Shen and Dowd 1991, Lauzon et al. 2003, Duron et al. 2006, Kontsedalov et al. 2008, Ghanim and Kontsedalov 2009, Tiwari et al. 2010b). Infection of *Culex pipiens* L. (Diptera: Culicidae) by *Wolbachia* bacteria is hypothesized to increase susceptibility to insecticides (Duron et al. 2006). Likewise, infection of whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) with *Rickettsia* species is found to increase the host's susceptibility to acetamiprid, thiamethoxam, spiromesifen, and pyriproxyfen (Kontsedalov et al. 2008). In our previous study, we found that Las-infection renders *D. citri* more susceptible to carbaryl, chlorpyriphos, fenpropathrin, imidacloprid, and spinetoram (Tiwari et al. 2010b). In addition, total protein content and general esterase activity were significantly reduced in Las-infected *D. citri* compared with uninfected counterparts, suggesting that presence of bacteria alters the physiology of the host insect. In the current investigation, GST and cytochrome P_{450} (general oxidase) activities were compared between Las-infected and uninfected *D. citri* to test the hypothesis that Las infection reduces GST and cytochrome P_{450} (general oxidase) activities. Reduced GST and cytochrome P_{450} activities (general oxidase levels) in Las-infected *D. citri* may be additional possible mechanisms responsible for increased susceptibility of *D. citri* to insecticides due to Las infection.

Materials and Methods

Asian Citrus Psyllid Cultures. Uninfected and Las-infected *D. citri* used for quantifying GST and cytochrome P_{450} activities were drawn from cultures continuously reared at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, FL. The uninfected culture was established in 2000 by using field populations collected in Polk Co., FL (28.0′ N, 81.9′ W) and maintained on sour orange (*Citrus aurantium* L.) seedlings without exposure to insecticides in a greenhouse at 27–28°C, 60–65% RH, and a photoperiod of 14:10 (L:D) h. The Las-infected *D. citri* culture was established in 2009 from the uninfected laboratory population by rearing *D. citri* on Las-infected sour orange seedlings in a separate greenhouse approved for rearing Las-infected citrus plants and *D. citri* under the environmental conditions described above.

Enzyme Preparations. Enzyme preparations for GST and cytochrome P_{450} (general oxidase) were performed in following batches: head + thorax section of uninfected male, head + thorax section of uninfected female, head + thorax section of Las-infected male, and head + thorax section of Las-infected female. Likewise, all of the above-mentioned enzyme preparations were repeated using the abdomen section of *D. citri* adults. Enzyme preparations for GST also were conducted on the two sections of Las-infected fifth-instar nymphs. In addition, GST and cytochrome P_{450} (general oxidase) activities were measured using whole bodies of uninfected second instar, fifth instar, and adult *D. citri.* Second-instar Las-infected *D. citri* nymphs were not used for GST quantification because of low Las titers. Likewise, cytochrome P_{450} activity was not quantified for nymphal sections of Las-infected *D. citri* because of the fragility of *D. citri* nymphs.

Although *D. citri* adults and nymphs reared on Las-infected host plants and presumed to be infected with Las were used in the study, Las infection was confirmed with quantitative real-time polymerase chain reaction (qPCR). Sections of *D. citri* found positive for Las were retained and matched with their corresponding sections to be used for GST and cytochrome P_{450} quantifications. Initially, abdomens were used for qPCR and corresponding head + thorax sections of Las-positive *D. citri* were used to quantify GST and cytochrome P_{450} (general oxidase) activities. This was repeated a second time, where head + thorax sections were used for qPCR and the corresponding abdomen sections of Las-positive head + thorax sections were used to quantify GST and cytochrome P_{450} (general oxidase) activities. Sectioning of uninfected *D. citri* adults and nymphs was performed to allow direct comparisons with equivalent sections of Las-infected *D. citri* adults.

Each half section of a *D. citri* adult used in GST and cytochrome P_{450} (general oxidase) assays was homogenized using a hand held homogenizer with a plastic pestle (Thermo Fisher Scientific, Waltham, MA) in ice-cold phosphate buffer (0.1 M; pH 7.5; 200 μl) containing 0.3% Triton X-100 (vol:vol) (Sigma Aldrich, St. Louis, MO) in a 1.5-ml microcentrifuge tube. Sections of adults and nymphs were homogenized in batches of 10 and 25, respectively. Microcentrifuge tubes were centrifuged at 12,600 rpm (Eppendorf, Centrifuge 5415R, Thermo Fisher Scientific) for 15 min at 4°C. After centrifugation, 100 μl of the supernatant was transferred into a clean microcentrifuge tube and mixed with phosphate buffer (0.1 M; pH 7.5; 120 μl) and placed on ice until use in assays.

GST Assay. GST activity was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) (Sigma Aldrich) as the substrate (Habig et al. 1974). Three aliquots of 10 μl of the enzyme solution were pipetted into separate wells of the 96-well microplate (NUNC PolySorp, Thermo Fisher Scientific). The total reac-

tion volume per well of a 96-well microplate was 200 μl, consisting of 10 μl of enzyme solution, 2 μl of 200 mM CDNB [containing 0.1% (vol:vol) ethanol)], and 188 μl of 10.35 mM GSH in phosphate buffer (0.1 M; pH 7.5; pH 7.5). GST activity was determined by the change in absorbance as measured continuously for 5 min at 340 nm and 25°C using a kinetic microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA). Control wells consisted of 2 μl of CDNB, 188 μl of GSH, and 10 μl of phosphate buffer (0.1 M; pH 7.5; pH 7.5). Changes in absorbance per minute were converted into micromoles of CDNB conjugated per min per milligram of protein by using the extinction coefficient of the resulting 5-(2, 4-dinitrophenyl)-glutathione: $\varepsilon_{340\ nm} = 9.6\ nM^{-1}\ cm^{-1}$ (Habig et al. 1974).

Cytochrome P_{450} (General Oxidase) Assay. Cytochrome P_{450} activity was quantified and expressed in terms of general oxidase level, which is an indirect measure of cytochrome P_{450} by using heme peroxidation (Brogdon et al. 1997, Penilla et al. 2007). The heme peroxidation method has been considered a reliable tool for comparing differences in general oxidase levels based on hemoprotein levels (Penilla et al. 1998, 2007; Casimiro et al. 2006). Because heme constitutes the majority of cytochrome P_{450} in nonblood-fed insects, quantification of heme activity has been used to compare the levels of cytochrome P_{450} on the basis of general oxidase levels (Brogdon et al. 1997).

Heme peroxidase activity was measured using 3,3′5,5′-tetra-methylbenzidine (TMBZ) (Sigma Aldrich) as the substrate. Four aliquots of 20 μl of enzyme preparation were pipetted into separate wells of the 96-well microplate. The total reaction volume per well of a 96-well microplate was 325 μl, consisting of 20 μl of enzyme solution, 80 μl of 0.625 M potassium phosphate buffer (pH 7.2), 200 μl of TMBZ solution, and 25 μl of hydrogen peroxide (3%). TMBZ solution was made by dissolving 0.01 g of TMBZ in 5 ml of methanol and 15 ml of 0.25 M sodium acetate buffer (pH 5.0). Plates were incubated at room temperature (25–27°C) for 2 h before reading at 450 nm as the endpoint in the microplate reader at 25°C. Control wells consisted of 20 μl of distilled water, 80 μl of 0.625 M potassium phosphate buffer, 200 μl of TMBZ solution, and 25 μl of hydrogen peroxide (3%). A standard curve for heme peroxidase activity was prepared using different concentrations of cytochrome C from horse heart (Sigma Aldrich). Cytochrome P_{450} (general oxidase) activity obtained from plate reading was expressed as equivalent units (EU) of cytochrome P_{450} per milligram of protein by using the standard curve of cytochrome C.

Total Protein Estimation. Total protein content in the enzyme preparations was estimated using bovine serum albumin (BSA, Sigma Aldrich) (Smith et al. 1985). Three aliquots of 20 μl of enzyme preparation were pipetted into separate wells of the 96-well microplate. Bicinchoninic acid (180 μl) in 4% cupric sulfate solution (Sigma Aldrich) was added to each well. The plate was covered with aluminum foil and incubated for 30 min at 37°C. After incubation, the plate was set aside at room temperature for 5 min to develop color and read at 562 nm by using a microplate reader at 25°C. Total protein content in the enzyme extraction was estimated based on the standard curve generated from serial dilutions of BSA.

DNA Extraction. Samples used for DNA extractions from the GST assays consisted of *D. citri* half sections (head + thorax or abdomen, as described above). Sections were homogenized in a buffer solution (QIAGEN, Valencia, CA) by using a sterile mortar and lysed overnight at 56°C in a hybridization oven (model 136400, Boekel Scientific, Feasterville, PA) before extraction of total DNA. DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's protocol with modifications for extraction of bacterial DNA from arthropods.

qPCR. All qPCR assays were performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) by using a multiplex TaqMan qPCR assay developed for detection of *Ca.* Liberibacter asiaticus (Li et al. 2006). qPCR was performed in 25.5-μl reaction volumes by using 96-well MicroAmp reaction plates (Applied Biosystems). Reactions, conducted in duplicate, contained the following: 1 μl of template DNA, 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 235 nM each of target primers (LasF, 5′-TCGAGCGCGTATGCAATACG-3′; LasR, 5′-GCGTTATCCCGTAGAAAAAGGTAG-3′) (GenBank accession L22532) (Li et al. 2006), internal control primers specific to the *wingless* (*wg*) gene (GenBank accession AF231365) (WgF, 5′-GCTCTCAAAGATCGGTTTGACGG-3′; WgR, 5′-GCTGCCACGAACGTTACCTTC-3′) (Thao et al. 2000), and 118 nM of each probe (WGp, JOE-5′TTACTGACCATCACTCTGGACGC3′-BHQ2) (Duan et al. 2009); HLBp, FAM-5′AGACGGGTGAGTAACGCG-BHQ1) (Li et al. 2006) (Integrated DNA Technologies, Inc., Coralville, IA). qPCR reactions consisted of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each 96-well plate contained a no template control (containing no DNA sample), a positive control (containing Las DNA in DNA extractions from *D. citri*), and a negative control (no Las DNA in DNA extractions from *D. citri*). Samples were considered positive for *wg* gene or Las if the cycle quantification (C_q) value determined by the ABI 7500 Real-Time software (version 1.4, Applied Biosystems), was ≤35 (Tiwari et al. 2010b).

Statistical Analysis. Three-way analysis of variance (ANOVA) followed by Fisher protected least significant difference (LSD) mean separation tests were performed to determine differences in GST and cytochrome P_{450} (general oxidase) activity between uninfected and Las-infected body sections of *D. citri* adults, where body section (head + thorax or abdomen), infection type (uninfected or Las-infected), and gender (male or female) served as main effects (PROC GLM) (SAS Institute 2005). Separate ANOVAs followed by Fisher protected LSD mean separation tests were performed to compare GST activities among various growth stages of uninfected *D. citri* by using data for the entire *D. citri* body (PROC GLM) (SAS Institute 2005). Likewise, sep-

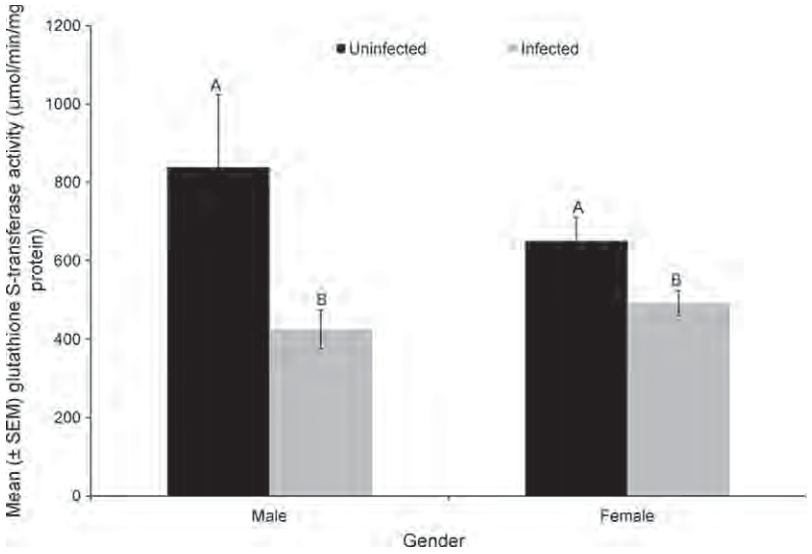

Fig. 1. GST activity in *Candidatus* Liberibacter asiaticus-infected and uninfected *D. citri* adults. Columns marked with different letters within a gender are significantly different ($P \leq 0.05$; Fisher protected LSD test). Although GST activities were measured using head + thorax and abdomen sections separately, herein means for both sections are represented.

arate ANOVAs were performed to compare GST activities among various growth stages by using abdomen and head + thorax sections for uninfected and Las-infected *D. citri* (PROC GLM) (SAS Institute 2005). Based on a significant interaction between main effects, one-way ANOVA was performed to determine differences within simple effects. Likewise, all above-mentioned analyses were analogously performed for cytochrome P_{450} (general oxidase) activity.

Results

GST. The GST activity level was compared between head + thorax and abdomen sections of uninfected and Las-infected adults and nymphs. Las infection ($F = 19.15$; df = 1, 193; $P < 0.001$) and the interaction between Las infection and *D. citri* gender ($F = 4.08$; df = 1, 193; $P = 0.0448$) significantly affected GST activity (as micromoles per minute per milligram of protein) when comparisons were conducted between uninfected and Las-infected body sections of psyllid adults. Body section ($F = 0.15$; df = 1, 193; $P = 0.6985$) did not significantly affect GST activity. Overall mean (±SEM) GST activity was significantly higher for uninfected (757.63 ± 59.46; $n = 98$) than Las-infected (468.23 ± 26.87; $n = 103$) adults. Because of the significant interaction between Las infection and adult gender, one-way ANOVAs were performed to differentiate between GST activity in uninfected and Las-infected adults of each gender. Infection with Las significantly affected GST activity in both males ($F = 11.43$; df = 1, 90; $P = 0.0011$) and females ($F = 6.45$; df = 1, 107; $P = 0.0125$) (Fig. 1). Mean GST activity was significantly higher in both uninfected male and female adults than Las-infected counterparts.

For uninfected *D. citri*, growth stage ($F = 5.45$; df = 3, 44; $P = 0.0028$) had a significant effect on GST activity; however, body section ($F = 0.35$; df = 1, 44; $P = 0.5547$) and the interaction between body section and growth stage ($F = 0.41$; df = 3, 44; $P = 0.7448$) did not significantly affect GST activity. Uninfected adults had significantly higher GST activity than second and fifth instar nymphs (Fig. 2A). For Las-infected *D. citri*, growth stage ($F = 8.96$; df = 2, 95; $P = 0.0003$), body section ($F = 14.36$; df = 1, 95; $P = 0.0003$) and the interaction between body section and growth stage ($F = 24.07$; df = 2, 95; $P < 0.0001$) significantly affected GST activity. Among Las-infected *D. citri*, female adults had significantly higher GST activity than males and fifth-instar nymphs (Fig. 2B).

Cytochrome P_{450} (General Oxidase). The standard curve generated in a linear peroxidation reaction using 0.0003–3.4 µg of cytochrome C was used to calculate general oxidase activity, as an indirect measure of possible changes in cytochrome P_{450} levels among various treatments (Fig. 3). Las infection ($F = 10.47$; df = 1, 86; $P = 0.0017$) and the interaction among infection, *D. citri* gender, and body section ($F = 5.12$; df = 1, 86; $P = 0.0261$) significantly affected cytochrome P_{450} activity. Overall mean (±SEM) cytochrome P_{450} activity was significantly higher for uninfected (0.49 ± 0.05; $n = 53$) than Las-infected (0.23 ± 0.02; $n = 41$) *D. citri* adults. Separate ANOVAs performed to compare cytochrome P_{450} activity among Las-infected and uninfected adults revealed that there was a significant effect of Las infection for female ($F = 6.79$; df = 1, 43; $P = 0.0125$) and a near significant effect for male ($F = 3.93$; df = 1, 43; $P = 0.0538$) adults (Fig. 4). *D. citri* growth stage ($F = 8.68$; df = 3, 64; $P < 0.0001$) had a significant effect on cytochrome P_{450} activity. Uninfected adults and fifth-instar nymphs had significantly lower cytochrome P_{450} activity than second-instar nymphs (Fig. 5).

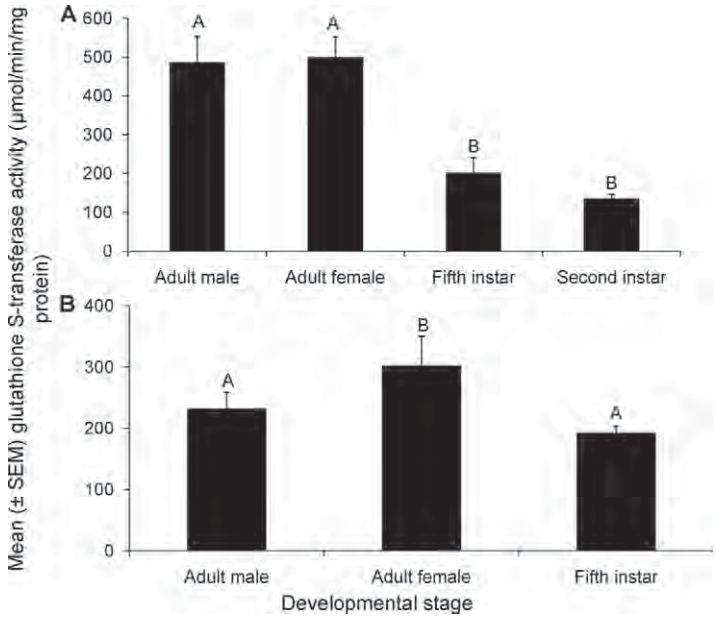

Fig. 2. GST activity at various growth stages of uninfected (A) and *Candidatus* Liberibacter asiaticus-infected *Diaphorina citri* (B). Columns marked with different letters are significantly different ($P \leq 0.05$; Fisher protected LSD test). Although GST activities were measured using head + thorax and abdomen sections separately, herein means for both sections are represented.

Discussion

Infection of *D. citri* with *Candidatus* Liberibacter asiaticus reduced GST and general oxidase activities compared with uninfected controls. This reduction occurred in adults of both genders and was roughly equivalent in head + thorax and abdominal body sections. These results are congruent with a previous investigation documenting reduced general esterase activity in *D. citri* as a result of Las infection (Tiwari et al. 2010b). Greater susceptibility of Las-infected *D. citri* adults to chlorpyriphos and spinetoram (Tiwari et al. 2010b) could be correlated with reduced general esterase, GST, and general oxidase activities in Las-infected *D. citri* adults compared with uninfected counterparts. Results from the current study and that by Tiwari et al. (2010b) indicate changes in the level of three major detoxifying enzymes in *D. citri* due to infection with the Las bacterium. These results are congruent with previous investigations indicating that microbial infection increases arthropod susceptibility

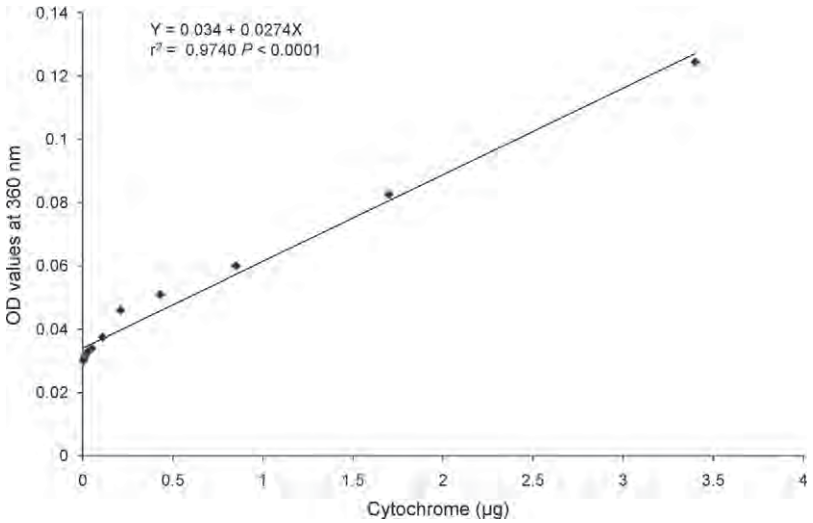

Fig. 3. Relationship between the peroxidation activity and concentrations of TMBZ substrate cytochrome C.

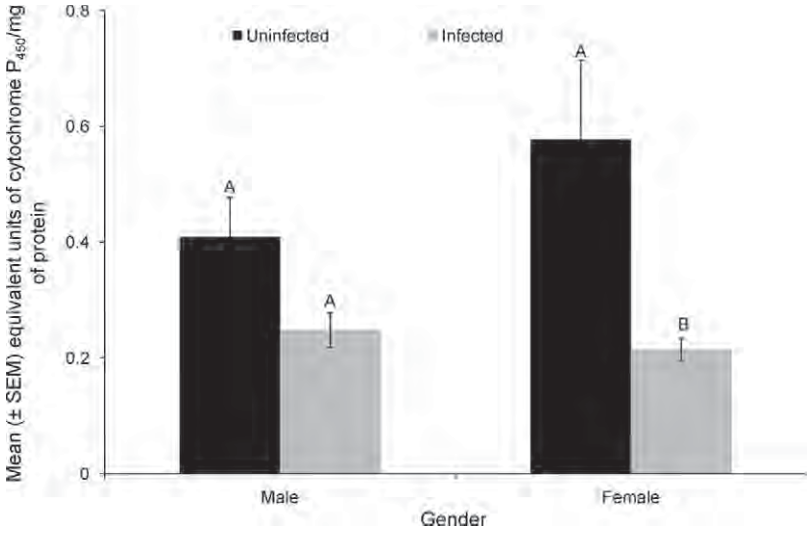

Fig. 4. Cytochrome P_{450} (general oxidase) activity in *Candidatus* Liberibacter asiaticus-infected and uninfected *D. citri*. Columns marked with different letters within a gender are significantly different ($P \leq 0.05$; Fisher protected LSD test). Although general oxidase activities were measured using head + thorax and abdomen sections separately, herein means for both sections are represented.

to insecticides and to other exogenous toxins (Shen and Dowd 1991, Lauzon et al. 2003, Duron et al. 2006, Kontsedalov et al. 2008).

Several studies have reported a positive correlation between levels of detoxifying enzymes and insecticide resistance. Resistant populations exhibit higher levels of metabolic detoxification than susceptible counterparts (Wang et al. 2009, Yang et al. 2009, Matambo et al. 2010). For example, the imidacloprid resistant 'NJ-Imi strain' of B-type *B. tabaci* exhibited higher GST and cytochrome P_{450} activities than the susceptible NJ strain (Wang et al. 2009). Also, an abamectin resistant strain of *B. tabaci* was characterized by higher GST and cytochrome P_{450} activities than a susceptible strain (Wang and Wu 2007). The presence of lower levels of GST and cytochrome P_{450} (indirect measurement through general oxidase)activities in Las-infected *D. citri*, compared with uninfected adults, might delay the development of resistance among Las-infected *D. citri* populations. Our current results indicate that the presence of the biological agent *Candidatus* Liberibacter asiaticus may increase the effi-

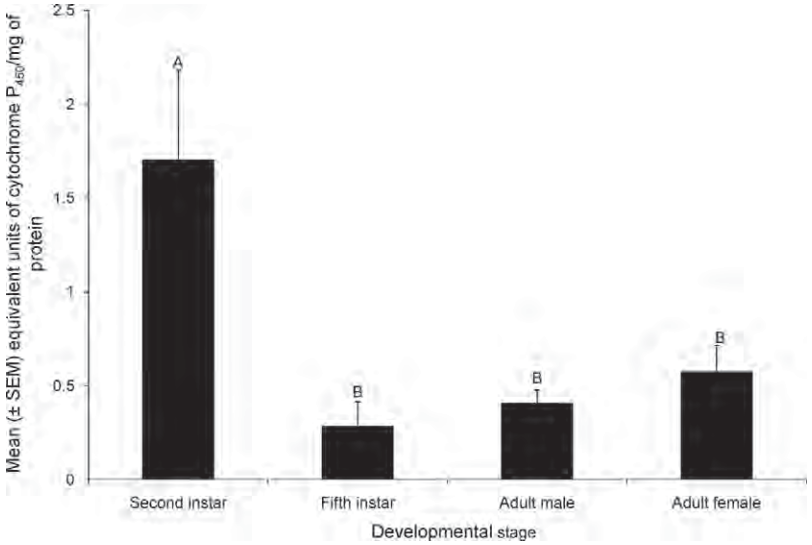

Fig. 5. Cytochrome P_{450} (general oxidase) activity at various growth stages of uninfected *D. citri*. Columns marked with different letters are significantly different ($P \leq 0.05$; Fisher protected LSD test). Although general oxidase activities were measured using head + thorax and abdomen sections separately, herein means for both sections are represented.

cacy of insecticides against its host *D. citri* by reducing the levels of detoxifying enzymes. Increased efficacy of insecticides in the presence of a biological agent in host insects has been reported previously. Infection by *Beauveria bassiana* and *Metarhizium anisopliae* was found to increase the toxicity of insecticides by 72-fold in *Spilarctia obliqua* (Walker) compared with the toxicity observed in the absence of such pathogens (Purwar and Sachan 2006). Likewise, infection of *Leptinotarsa decemlineata* (Say) with *B. bassiana* increases susceptibility to insecticides as compared with uninfected counterparts (Anderson et al. 1989).

Increased activity of detoxifying enzymes is correlated with overexpression of associated genes in insecticide resistant populations of insects (Gui et al. 2009; Matambo et al. 2010; Ranson et al. 2001; Daborn et al. 2002, 2007; Wondji et al. 2009). DDT resistant *Anopheles gambiae* Giles are characterized by higher mRNA levels of *aggst3-2*, a class III GST gene, than susceptible counterparts (Ranson et al. 2001). Another GST gene found in *Bombyx mori* (L.), *BmGSTS2*, was found to be overexpressed in the larval midgut after 6–12 h of exposure to permethrin and glyphosate (Gui et al. 2009). Likewise, the cytochrome P_{450} genes CYP6P9 and CYP6P4 were overexpressed 25 and 51 times, respectively, in pyrethroid-resistant *Anopheles funestus* Giles (Wondji et al. 2009). In *Drosophila melanogaster* (Meigen), overtranscription of *Cyp6g1* alone is responsible for DDT resistance and overexpression of eight cytochrome P_{450} genes was correlated with resistance to DDT, nitenpyram, dicylanil, and diazinon (Daborn et al. 2002, 2007). Collectively, the above-mentioned findings suggest that reduced activity of detoxifying enzymes in Las-infected *D. citri* may be due to reduced gene expression, which requires further investigation.

GST and general oxidase activity levels varied among different growth stages of both uninfected and Las-infected *D. citri.* In general, GST activity was higher in adults than nymphs. In contrast, general oxidase activity was higher in second-instar nymphs than adults. GST activity is known to vary among insect developmental stages (Hazelton and Lang 1983, Wood et al. 1986, Kotze and Rose 1987, Kostaropoulus et al. 1996). In *Aedes aegypti* (L.) (Hazelton and Lang 1983) and *Tenebrio molitor* L. (Kostaropoulus et al. 1996), GST activity is greatest in the early pupal stage. In contrast, GST activity was highest in newly emerged *Lucilia cuprina* (Wiedemann) adults (Kotze and Rose 1987). Our results are similar to those observed with *Triatoma infestans* Klug, where GST activity was almost 50 fold higher in adults than nymphs (Wood et al. 1986). Peak GST activity, such as that observed in *D. citri* adults, could be associated with high expression of metabolic pathways (Hazelton and Lang 1983). In addition, the mobile adult may encounter a relatively greater array of exogenous toxic compounds than the predominantly sessile nymphal stages and therefore may be characterized by higher metabolic pathways. Alternatively, sessile nymphs may be more susceptible to insecticide applications than mobile adults and therefore benefitted by higher levels of general oxidase. It seems that there is no general trend among the different detoxifying enzyme groups among various developmental stages, given that GST activity is relatively greater in adults, whereas cytochrome P_{450} (general oxidase) activity is greater in early nymphal instars.

Greater cytochrome P_{450} (general oxidase) activity in *D. citri* nymphs than adults is congruent with results obtained with *Helicoverpa armigera* (Hübner) where expression of two homologous cytochrome P_{450} mRNAs peaked during the larval stage (Ranasinghe et al. 1997). Likewise, midgut CYP4 mRNA levels were higher during the active feeding stage of *Manduca sexta* (L.) than during prepupal and pupal stages (Snyder et al. 1995). In addition, it has been reported that specific cytochrome P_{450}s are expressed at each developmental stage, such as CYP6B2 during larval stage and CYP6D1 during the adult stage (Scott et al. 1996, Ranasinghe et al. 1997). The quantity of molting hormone is also known to influence the expression of cytochrome P_{450} enzymes at various growth stages. Expression of cytochrome P_{450} was elevated in the presence of an ecdysone, 20-hydroxyecdysone, in *D. melanogaster* (Spiegelman et al. 1997). Collectively, the above studies suggest that higher cytochrome P_{450} levels in nymphs than in adults could be related to active feeding and molting during nymphal stages.

The current study indicates that *Candidatus* Liberibacter asiaticus infection reduces levels of two important detoxifying enzymes in *D. citri*, thus potentially rendering them more susceptible to toxins. Our results also indicate that levels of two detoxifying enzyme groups vary depending on *D. citri* developmental stage, which may allow the formulation of developmental stage-specific insecticide recommendations, such as timing of sprays during peak activities of adults or nymphs. Insecticides specifically targeted by GST enzymes such as pyrethroids and organochlorines might be more effective during peak nymph activity, whereas those targeted by cytochrome P_{450} such as organophosphates and carbamates may be more effective when adults are most abundant. Based on the differential levels of enzymes between Las-infected and uninfected adults, we propose that further investigations are needed to compare expression levels of associated genes between uninfected and Las-infected *D. citri* during various growth stages. This will help elucidate the genetic mechanisms underlying the variations in levels of detoxification enzymes due to Las infection.

Acknowledgments

We thank W. Dawson for the access to the Las-infected *D. citri* culture. We also thank J. Burns for access to real-time PCR equipment. We acknowledge A. Hoyte, I. Jackson, R. Campos-Herrerra and M. Flores for technical assistance. This project was supported by a grant from the Florida Citrus Production Research Advisory Council (to L.L.S.).

References Cited

Anderson, T. E., A. E. Hajek, D. W. Roberts, H. K. Preisler, and J. L. Robertson. 1989. Colorado potato beetle (Coleoptera: Chrysomelidae): effects of combinations of *Beauveria bassiana* with insecticides. J. Econ. Entomol. 82: 83–89.

Booth, I., E. Boyland, and P. Sins. 1961. An enzyme from rat liver catalyzing conjugation with glutathione. Biochem. J. 79: 516–524.

Brogdon, W. G., J. C. McAllister, and J. Vulule. 1997. Heme peroxidase activity measured in single mosquitoes identifies individuals expressing an elevated oxidase for insecticide resistance. J. Am. Mosq. Control 13: 233–237.

Casimiro, S., M. Coleman, J. Hemingway, and B. Sharp. 2006. Insecticide resistance in *Anopheles arabiensis* and *Anopheles gambiae* from Mozambique. J. Med. Entomol. 43: 276–282.

Clark, A. G., and N. A. Shamaan. 1984. Evidence that DDT-dehydrochlorinase from the house fly is a glutathione S-transferase. Pestic. Biochem. Physiol. 22: 249–261.

Daborn, P. J., C. Lumb, A. Boey, W. Wong, R. H. ffrench-Constant, and P. Batterham. 2007. Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic overexpression. Insect Biochem. Mol. Biol. 37: 512–519.

Daborn, P. J., J. L. Yen, M. R. Bogwitz, G. Le Goff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D. Heckel, P. Batterham, et al. 2002. A single P450 allele associated with insecticide resistance in *Drosophila.* Science 297: 2253–2256.

Duan, Y., L. Zhou, D. G. Hall, W. Li, H. Doddapaneni, H. Lin, L. Liu, C. M. Vahling, D. W. Gabriel, K. P. Williams, et al. 2009. Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus* liberibacter asiaticus' obtained through metagenomics. Mol. Plant Microbe Interact. 22: 1011–1020.

Duron, O., P. Labbe, C. Berticat, F. Rousset, S. Guillot, M. Raymond, and M. Weill. 2006. High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. Evolution 60: 303–314.

Feyereisen, R. 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44: 507–533.

Garnier, M., N. Danel, and J. M. Bové. 1984. The organism is a gram-negative bacterium, pp. 115–124. *In* S. M. Garnsey, L. W. Timmer, and J. A. Dodds [eds.], Proceedings of 9th Conference of the International Organization of Citrus Virologist, 9–13 May 1983, Riverside, CA. University of California, Riverside.

Ghanim, M., and S. Kontsedalov. 2009. Susceptibility to insecticides in the Q biotype of *Bemisia tabaci* is correlated with bacterial symbiont densities. Pest Manag. Sci. 65: 939–942.

Grant, D. F., and F. Matsumura. 1989. Glutathione S-transferase 1 and 2 in susceptible and insecticide resistant *Aedes aegypti.* Pestic. Biochem. Physiol. 33: 132–143.

Gui, Z., C. Hou, T. Liu, G. Qin, M. Li, and B. Jin. 2009. Effects of insect viruses and pesticides on glutathione *s*-transferase activity and gene expression in *Bombyx mori.* J. Econ. Entomol. 102: 1591–1598.

Habig, W. H., M. J. Pabst, and W. B. Jakoby. 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130–7139.

Halbert, S. E., and K. L. Manjunath. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. Fla. Entomol. 87: 330–353.

Hazelton, G. A., and C. A. Lang. 1983. Glutathione S-transferase activities in the yellow-fever mosquito [*Aedes aegypti* (Louisville)] during growth and aging. Biochem. J. 210: 281–287.

Huang, H. S., N. T. Hu, Y. E. Yao, C. Y. Wu, S. W. Chiang, and C. N. Sun. 1998. Molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella.* Insect Biochem. Mol. Biol. 28: 651–658.

Jagoueix, S., J. M. Bové, and M. Garnier. 1996. PCR detection of the *Candidatus liberibacter* species associated with greening disease of citrus. Mol. Cell Probes 10: 43–50.

Kontsedalov, S., E. Zchori-Fein, E. Chiel, Y. Gottlieb, M. Inbar, and M. Ghanim. 2008. The presence of *Rickettsia* is associated with increased susceptibility of *Bemisia tabaci* (Homoptera: Aleyrodidae) to insecticides. Pest Manag. Sci. 64: 789–792.

Kostaropoulus, I., A. E. Mantzari, and A. I. Papadopoulos. 1996. Alterations of some glutathione S-transferase characteristics during the development of *Tenebrio molitor.* Insect Biochem. Mol. Biol. 26: 963–969.

Kotze, A. C., and H. A. Rose. 1987. Glutathione S-transferase in the Australian sheep blowfly, *Lucilia cuprina* (Wiedeman). Pestic. Biochem. Physiol. 29: 77–86.

Lauzon, C. R., S. E. Potter, and R. J. Prokopy. 2003. Degradation and detoxification of the dihydrochalcone phloridzin by *Enterobacter agglomerans*, a bacterium associated with the apple pest, *Rhagoletis pomonella* (Walsh) (Diptera: Tephritidae). Environ. Entomol. 32: 953–962.

Li, W. B., J. S. Hartung, and L. Levy. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. J. Microbiol. Methods 66: 104–115.

Manjunath, K. L., S. E. Halbert, C. Ramadugu, S. Webb, and R. F. Lee. 2008. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus Huanglongbing in Florida. Phytopathology 98: 387–396.

Matambo, T. S., M.J.I. Paine, M. Coetzee, and L. L. Koekemoer. 2010. Sequence characterization of cytochrome P450 CYP6P9 in pyrethroid resistant and susceptible *Anopheles funestus* (Diptera: Culicidae). Genet. Mol. Res. 9: 554–564.

Morris, R. A., C. Erick, and M. Estes. 2009. Greening infection at 1.6%, survey to estimate the rate of greening and canker infection in Florida citrus groves. Citrus Ind. 90: 16–18.

Penilla, R. P., A. D. Rodríguez, J. Hemingway, J. L. Torres, J. I. Arredondo-Jiménez, and M. H. Rodríguez. 1998. Resistance management strategies in malaria vector mosquito control. Baseline data for a large-scale field trial against *Anopheles albimanus* in Mexico. Med. Vet. Entomol. 12: 217–233.

Penilla, R. P., A. D. Rodríguez, J. Hemingway, A. Trejo, A. D. López, and M. H. Rodríguez. 2007. Cytochrome P^{450}-based resistance mechanism and pyrethroid resistance in the field *Anopheles albimanus* resistance management trial. Pestic. Biochem. Physiol. 89: 111–117.

Purwar, J. P., and G. C. Sachan. 2006. Synergistic effect of entomogenous fungi on some insecticides against Bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). Microbiol. Res. 161: 38–42.

Ranasinghe, C., M. Headlam, and A. A. Hobbs. 1997. Induction of the mRNA for CYP6B2, a pyrethroid inducible cytochrome P450, in *Helicoverpa armigera* (Hubner) by dietary monoterpenes. Arch. Insect Biochem. Physiol. 34: 99–109.

Ranson, H., L. Prapanthadara, and J. Hemingway. 1997. Cloning and characterization of two glutathione S-trans-

ferases from a DDT resistant strain of *Anopheles gambiae.* Biochem. J. 324: 97–102.

Ranson, H., L. Rossiter, F. Ortelli, B. Jensen, X. Wang, C. W. Roth, F. H. Collins, and J. Hemingway. 2001. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae.* Biochem. J. 359: 259–304.

SAS Institute. 2005. SAS, users guide. SAS Institute, Cary, NC.

Scott, J. G. 1999. Cytochromes P450 and insecticide resistance. Insect Biochem. Mol. Biol. 29: 757–777.

Scott, J. G., and Z. Wen. 2001. Cytochromes P450 of insects: the tip of the iceberg. Pest Manag. Sci. 57: 958–967.

Scott, J. G., P. Sridhar, and N. Liu. 1996. Adult specific expression and induction of cytochrome P450lpr in house flies. Arch. Insect Biochem. Physiol. 31: 313–23.

Shen, S. K., and P. F. Dowd. 1991. Detoxification spectrum of the cigarette beetle symbiont *Symbiotaphrina kochii* in culture. Entomol. Exp. Appl. 60: 51–59.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fugimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Ann. Biochem. 150: 76–85.

Snyder, M. J., J. L. Stevens, J. F. Andersen, and R. Feyereisen. 1995. Expression of cytochrome P450 genes of the CYP4 family in midgut and fat body of the tobacco hornworm, *Manduca sexta.* Arch. Biochem. Biophys. 321: 13–20.

Spiegelman, V. S., S. Y. Fuchs, and G. A. Belitsky. 1997. The expression of insecticide resistance-related cytochrome P450 forms is regulated by molting hormone in *Drosophila melanogaster.* Biochem. Biophys. Res. Commun. 232: 304–307.

Tang, A. H., and C. D. Tu. 1994. Biochemical characterization of *Drosophila* glutathione S-transferases D1 and D21. J. Biol. Chem. 269: 27876–27884.

Thao, M. L., N. A. Moran, P. Abbot, E. B. Brennan, D. H. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. Appl. Environ. Microbiol. 75: 7097–7106.

Tiwari, S., H. Lewis-Rosenblum, K. Pelz-Stelinski, and L. L. Stelinski. 2010a. Incidence of *Candidatus* Liberibacter asiaticus infection in abandoned citrus occurring in proximity to commercially managed groves. J. Econ. Entomol. 103: 1972–1978.

Tiwari, S., K. Pelz-Stelinski, and L. L. Stelinski. 2010b. Effect of *Candidatus* Liberibacter asiaticus infection on susceptibility of Asian citrus psyllid, *Diaphorina citri,* to selected insecticides. Pest Manag. Sci. 67: 94–99.

Vontas, J. G., G. J. Small, and J. Hemingway. 2000. Comparison of insecticide resistance gene copy number, expression levels and esterase activity in insecticide susceptible and resistant *Nilaparvata lugens* (Stal). Ins. Mol. Biol. 9: 655–660.

Vontas, J. G., G. J. Small, D. C. Nikou, H. Ranson, and J. Hemingway. 2002. Purification, molecular cloning and heterologous expression of a glutathione S-transferase involved in insecticide resistance from the rice brown planthopper, *Nilaparvata lugens.* Biochem. J. 362: 329–337.

Wang, L., and Y. Wu. 2007. Cross-resistance and biochemical mechanisms of abamectin resistance in the B-type *Bemisia tabaci.* J. Appl. Entomol. 131: 98–103.

Wang, Z., M. Yao, and Y. Wu. 2009. Cross-resistance, inheritance and biochemical mechanisms of imidacloprid resistance in B-biotype *Bemisia tabaci.* Pest Manag. Sci. 65: 1189–1194.

Wondji, C. S., H. Irving, J. Morgan, N. F. Lobo, F. H. Collins, R. H. Hunt, M. Coetzee, J. Hemingway, and H. Ranson. 2009. Two duplicated P450 genes are associated with pyrethroid resistance in *Anopheles funestus,* a major malaria vector. Genome Res. 19: 452–459.

Wood, E., N. Casabe, F. Melgar, and E. Zerba. 1986. Distribution and properties of glutathione S-transferase from *T. infestans.* Comp. Biochem. Physiol. 84B: 607–617.

Yang, M. L., J. Z. Zhang, K. Y. Zhu, T. Xuan, X. J. Liu, Y. P. Guo, and E. B. Ma. 2009. Mechanisms of organophosphate resistance in a field population of oriental migratory locust, *Locusta migratoria manilensis* (Meyen). Arch. Insect Biochem. Physiol. 71: 3–15.

Yang, Z., H. Yang, and G. He. 2007. Cloning and characterization of two cytochrome P450 CYP6AX1 and CYP6AY1 cDNAs from *Nilaparvata lugens* Stal (Homoptera: Delphacidae). Arch. Insect Biochem. Physiol. 64: 88–99.

Received 20 August 2010; accepted 30 November 2010.