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**Residual toxicity of insecticides used against the Asian citrus psyllid and resistance management strategies
with thiamethoxam and abamectin**

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Abstract

The Asian citrus psyllid (ACP) is the key pest of citrus due to its role as vector of huanglongbing. ACP control mostly relies on frequent insecticide applications therefore increasing selection for insecticide resistance. Optimization of field application rates and rotation of insecticide modes of action (MoA) would minimize this risk. Baseline toxicity responses of ACP to commonly used insecticides provide a basis for defining application field rates and are thus needed to track potential development of resistance.

Residual toxicities of 12 insecticides were evaluated through quantal response bioassays. The development of ACP resistance in response to monthly applications of two insecticides with different MoAs, abamectin and thiamethoxam, applied separately, in rotation or as mixtures was also tested.

Highest residual toxicities were found for the abamectin + thiamethoxam mixture. In general, nymphs were more sensitive than adults to residues. Nymphs were 10 and 28 times more sensitive than adults to residues of thiamethoxam and imidacloprid respectively. We found a wide range among ratios of maximum label field concentrations and estimated LC_{90} s. Highest label rates for malathion and dimethoate were more than 30 times greater than LC_{90} s, in contrast to abamectin, zeta-cypermethrin and fenpyroximate which were 4-7 times lower.

Abamectin appeared to synergize the mixture of it with thiamethoxam. We observed resistance to both active ingredients, especially abamectin, after eight months of sequential applications of both insecticides individually or in mixture. Rapid resistance development of the mixture relative to rotation was attributed to loss of the synergistic effect of abamectin on thiamethoxam.

Key words: neonicotinoids, avermectins, insecticide resistance management, susceptibility, huanglongbing, synergism, vector

Key message:

- Asian citrus psyllid (ACP) management mostly relies on insecticide control.
- Baseline toxicities to commonly used products are the basis for optimal insecticide management.
- Residual toxicities of 12 insecticides on ACP were evaluated.
- Laboratory residual toxicities strongly differed from recommended field concentrations.
- Rotation of abamectin, thiamethoxam and the mixture was preferred over sequential application of their mixture.
- Information presented will help to adjust application rates and provide a new tool to monitor and reduce resistance development in ACP.

Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is one of the two known vectors of huanglongbing (HLB) or citrus greening, probably the most destructive disease of citrus worldwide (Bove 2006; Grafton-Cardwell et al. 2013, Cocuzza et al. 2017). HLB is associated with α -proteobacterias in the genus *Candidatus Liberibacter* that grow in the phloem and alter nutrient transport within the plant (Bove 2006; Gottwald 2010). Citrus groves without intervention are rendered unproductive within 5-10 years (Bove 2006). Such intervention includes nutrient management to mitigate symptoms of HLB and ACP suppression (Stansly et al. 2014; Tansey et al. 2016).

Effective ACP management is considered key to both slowing advancement of HLB as well as decreasing impact of the disease on infected trees (Belasque et al. 2010; Boina and Bloomquist 2015; Chiyaka et al. 2012; Monzo and Stansly 2017; Stansly et al. 2014; Tansey et al. 2016). Nevertheless, resistance of ACP field populations to several key insecticides such as chlorpyrifos, fepropathrin, imidacloprid and thiamethoxam has already been reported (Tiwari et al. 2013; Tiwari et al. 2011). Aggressive insecticidal control also greatly compromises biological control, increasing the probability of ACP and secondary pest outbreaks (Hall and Nguyen 2010; Monzo et al. 2014; Qureshi and Stansly 2009). Direct and indirect costs of intensive insecticide applications may render this approach unprofitable in the near future. For this reason, special emphasis on designing insecticide strategies which efficiently control ACP populations and are compatible with integrated pest management principles is essential (Boina and Bloomquist 2015; Monzo and Stansly 2017; Qureshi et al. 2014; Qureshi and Stansly 2010). Among these strategies, the selection of suitable modes of action (MoA) (www.irac-online.org/modes-of-action/) and their appropriate rotation over time should constitute the basis of any ACP insecticide resistance management (IRM) program.

One important step towards effective IRM is determination of the baseline toxicity for commonly used active ingredients (a.i.s) to a susceptible ACP population. Baseline responses can then be used to assess the presence and prevalence of resistant field populations (Prabhaker et al. 2014; Tiwari et al. 2011). Laboratory quantal response bioassays provide the basis for such decisions (Robertson et al. 2007).

Previous works that comprehensively evaluated baseline toxicity of commonly used a.i.s against ACP studied contact toxicity by directly applying predetermined amounts and concentrations of a.i. to the insect body using micropipettes (Morse et al. 2016; Tiwari et al. 2011). Others ways of acquiring the toxicant such as ingestion or residual activity are not evaluated using this approach. Translaminar and/or systemic activity as well as metabolic activation in the plant are characteristic of some a.i.s (Jeschke 2016). Furthermore, adjuvants and other inert ingredients of commercial formulations may affect activity in the field. Hence, methodologies applying the formulated products to plant substrates may better simulate field applications.

Optimizing use of selected products is a rational goal of an insecticide application program. Field efficacy studies (Qureshi et al. 2014) and grower experience indicate differential efficacies of approved insecticides for ACP. Results from toxicity evaluations under laboratory conditions cannot be extrapolated directly to field conditions, but do provide fundamental information that can be verified in field trials aimed to optimize insecticide application rates.

Pesticide mixtures are used in agriculture for multi-target pest control and may also improve efficacy against specific pests (LeBaron 1986). Active ingredients are thus synergistic (Bielza et al. 2007; Jones et al. 2012). The use of a.i. mixtures with different MoAs is also proposed as an IRM strategy to reduce selection pressures associated with individual a.i.s (Sparks and Nauen 2015). The theory is that selection against a specific MoA showing partial resistance may be hindered by mixing with a different MoA. On the other hand, simultaneous exposure to two different MoAs reduces the number of potential rotation partners. Agri-Flex® is a commercial mixture of two a.i.s, abamectin and thiamethoxam, with different MoAs. Abamectin (avermectin B1, IRAC group 6) is a macrocyclic lactone compound that affects membrane chloride conductance on a variety of ligand-gated chloride channels by binding to glutamate-gated, histamine-gated and γ -aminobutyric acid gated chloride channel receptors (Clark et al. 1995; McCavera et al. 2007). Thiamethoxam (IRAC group 4A) is a neonicotinoid insecticide that acts as an agonist to insect postsynaptic nicotinic acetylcholine receptors (Casida and Durkin 2013; Nauen and Denholm 2005; Tomizawa et al. 1995). Target-site based resistance mechanisms could be lower with the mixture of these two active ingredients compared to each individually, although the response over time to both could be less by exposure to a temporal rotation (Georghiou 1980).

The objectives of this research were to improve the current insecticide resistance strategies for ACP by 1) implementing a residual toxicity bioassay methodology that simulates field applications of insecticides against ACP, 2) determining baseline susceptibility of ACP adults and nymphs to formulated products used for ACP management based on this methodology, 3) comparing the obtained baselines to currently labeled field doses, 4) evaluating possible synergy between an avermetin and neonicotinoid insecticide in a typical commercial mixture against ACP, and 5) comparing selection for resistance by ACP to these same active ingredients applied as a mixture versus rotation.

Materials and Methods

Insecticides and dosages

Twelve commonly used insecticides registered in the USA for control of *D. citri* were selected to estimate concentration-response curves for ACP adults and nymphs based on UF IFAS recommendations (Rogers et al. 2016) (**Table 1**). Maximum dosages recommended by manufacturers for ground applications were converted to parts per million (ppm) to compare recommended concentrations with lethal concentrations obtained from laboratory bioassays. Conversions assumed ground insecticide applications at 1169 L/ha (125 gal/ac) (Qureshi et al. 2014).

Colonies and plants

Susceptible ACP colonies, reared without selection by pesticides, were established using insects collected from citrus groves at the SWFREC-IFAS University of Florida, Immokalee research farm in 2005. These insects were reared under greenhouse conditions on *Murraya paniculata* L. (Jack) (Sapindales: Rutaceae) (orange jasmine). Orange jasmine seedlings were transplanted into 3.92 L air-pots with a soil-less growth medium (40:60 mix of Canadian Sphagnum Peat and Fafard 4P mix Professional Growing Mix soil, Conrad Fafard Inc.). Plants were fertilized monthly with a woody ornamental fertilizer, 200132 - 12-6-8 Nursery Polyon (Harrell's LLC, Lakeland,

FL) and maintained under ambient conditions in a screen-house. M-pede soap (Dow AgroSciences LLC, Indianapolis IN) was applied monthly to plants to control whiteflies, ACP and occasional mealybugs. Aphids (*Aphis crassivora*) were controlled using a ladybeetle (*Diomus terminatus*) reared on the corn leaf aphid, *Rhopalosiphum maidis* maintained on sorghum (*Sorghum bicolor* (L.)) plants (Tiffet et al. 2006). Orange jasmine plants were pruned on a rotating basis every three weeks to induce flush. Plants with 2-5 approximately 4 cm flush shoots each were transferred to 60 cm x 60 cm base, 90 cm tall wood-framed, mesh cages (6 to 9 plants per cage) for ACP infestation. Twenty ACP adults were released into each cage for 24 h for oviposition. Infested plants were transferred to a similar cages maintained in an air-conditioned rearing room at 25-30 °C, 60-80% RH and 14:10 (L:D) photoperiod.

ACP bioassays

Concentration response curves

Stock suspensions of all insecticides tested (**Table 1**) were prepared of 1,000 ppm a.i. in 100 ml of distilled water. Serial dilutions (1000, 300, 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.02, 0.01, 0.005 ppm; between 6 to 11 serial dilutions per product) were prepared from each stock solution based on formulated concentrations as documented in specimen labels. A control treatment of distilled water was included with each product tested.

Cuttings of *M. paniculata* were collected from plants reared in an insecticide-free greenhouse and placed individually into 1.5 ml micro-centrifuge tubes (Fisherbrand™, Fisher Scientific, Pittsburgh, PA), containing distilled water. Cutting stems were sealed within micro-centrifuge tubes using modelling clay. Each cutting was sprayed to 100% coverage with its assigned insecticide concentration using a Crown Spray-Tool System (Woodstock, Illinois). Sprayed cuttings were air-dried before bioassay.

Recently emerged adult ACP from the susceptible colony were aspirated into 20 dram (74 ml) (Fisherbrand™ Polystyrene centrifuge tubes, Fisher Scientific, Pittsburgh, PA) and placed in a freezer at -7°C for 3 min to slow insect movement. Adults were then transferred to 50 ml plastic centrifuge tubes (Fisherbrand™, Fisher Scientific, Pittsburgh, PA) containing the treated cuttings (experimental unit). Twenty to 25 adult psyllids were introduced onto each treated cutting. For ACP nymphs, between 15 and 25, 3rd or 4th instars, as representatives of ACP immature stages, were transferred from the susceptible colony to treated cuttings using a fine camel-hair brush. Insects were confined to treated cuttings and ventilated by sealing tubes with cloth mesh fixed with elastic rubber bands.

The sealed insects on cuttings were placed undisturbed in a climate-controlled chamber (HOBO® RH/Temp/Light/External Data Logger H08- 004-02, Onset Computer Corp., Bourne, Massachusetts) for 48 h at 26.8 ± 2°C, 70% RH, and a photoperiod of 14:10 (L:D). Mortality was then assessed by touching psyllids with a blunt probe to check for movement. Insects were considered to be alive if able to move appendages or head when prodded. Insects that remained immobile or only twitched when prodded were considered dead.

Each bioassay was replicated four times; each replicate run was conducted on different days. Results of trials in which control mortality was > 20% were discarded and trials repeated.

Resistance management strategies

Twenty screened cages (61 x 61 x 91 cm) with four pesticide-free *M. paniculata* plants per cage were placed in a greenhouse under controlled temperature ($26 \pm 2^{\circ}\text{C}$). Approximately two thousand ACP, collected from the SWFREC susceptible colony, were introduced to each cage and allowed to feed and reproduce for three months. Two old plants were removed every month from each cage and replaced with new flushing plants to continuously provide the colonies with fresh oviposition sites. The following three insecticides were applied monthly: 1) thiamethoxam (Actara®), 2) abamectin (Agri-Mek® 0.15 EC) and 3) abamectin + thiamethoxam (Agri-Flex®), and 4) a rotation of these products. The rotation treatment consisted of alternating each month between these insecticides. These four treatments plus a control were replicated four times in randomly assigned cages.

Tolerance of the susceptible colony to each formulated insecticide was assessed as the lethal response to residues applied at the concentration of active ingredient that killed 25% (LC_{25}) and 80% (LC_{80}) of the population in concentration response bioassays. Treatment-specific estimated LC_{25} concentrations (18.26 ppm for abamectin and 1.67 ppm for thiamethoxam insecticides, and 0.35 ppm for the commercial mixture (i.e. 0.29 ppm of abamectin and 0.06 ppm of thiamethoxam) were applied to ACP colonies every 4 weeks, using the same spray system as above. LC_{25} was chosen to balance induction of resistance with survival in the treated population. Sublethal concentrations in residual toxicity studies simulate field degradation of insecticide residues and do not jeopardize the survival of the treated colonies (Smirle et al 2017). Forty of these adults per cage (160 per treatment) were then collected at random and subjected to the LC_{80} of their corresponding treatment. The LC_{80} was chosen as diagnostic concentration to maintain precision (Robertson et al. 2007). Two temporal replicates per treatment of twenty adults per cage were tested, including a control application of distilled water. Treated cuttings were placed undisturbed in a climatic chamber for 48 h at $26.8 \pm 2^{\circ}\text{C}$, 70% RH, photoperiod 14:10 (L:D) until assessment as explained above. LC_{25} treatments to cages were terminated after 5 months if ACP population mortality in response to the original LC_{80} concentrations did not exceed 50%. In that case, a new concentration response curve of that resistant population was then estimated to assess differences in susceptibility between the putative resistant and original, susceptible colony.

Data analysis

Concentration-mortality regressions for the susceptible ACP colony and each insecticide tested were estimated assuming a normal distribution of the binomial variable ‘probability of response’ based on the probit model (PoloPlus statistical software, LeOra Software, Petaluma, CA). Chi-square values of each regression divided by the degrees of freedom of the model near 1, and t-ratios of estimated slopes over 1.96 were considered indicative of a good-fitting model and thus a normal distribution (Robertson et al. 2007). Plots of standardized residuals were examined for the presence of outliers and/or non-linear responses. Outliers were removed from subsequent evaluations. Intercepts, slopes and lethal concentrations 50 and 90 (LC_{50} and LC_{90}) and their corresponding confidence limits were estimated for each insecticide. Ratios of maximum field-recommended rates (concentrations) of each product tested and their estimated LC_{90} for adults and nymphs were calculated.

To test whether the two a.i.s that make up Agri-Flex® mixture (abamectin and thiamethoxam) had an independent, uncorrelated joint action or they had antagonist or synergist action, observed ACP mortality at different concentrations of the mixture was compared to the expected mortality under the hypothesis that the three potential

causes of mortality (natural mortality, mortality caused by abamectin and mortality caused by thiamethoxam) were independent. A Chi-square statistic was used through PoloMix (PoloPlus) software. Chi-square values significantly greater than the tabular value for the corresponding degree of freedoms would mean a rejection of the null hypothesis of independent, uncorrelated joint action. In that case, observed mortality values above predicted values would indicate synergist action, whereas observed mortality values below predicted values would be indicative of antagonist action (Robertson et al. 2007).

Mortality rates of ACP colonies at the original LC₈₀ were calculated each month by correcting mortality in the insecticide treatments with that observed at the controls using Abbott's formula (Abbott 1925). Changes in resistance throughout the experiment to specific insecticides measured as mortality rates were evaluated using the General Linear Model Procedure (SAS Version 9.3, SAS Institute, Inc. 2010, Cary, North Caroline, USA). 'Mortality' rate was the dependent variable of the quantitative variable 'month'. To test whether the strategies of using a binary mixture of a.i.s or monthly rotating them reduced the risk of developing resistance, for each insecticide, changes in ACP mortality at LC₈₀ throughout the study in ACP colonies under monthly applications of the same product were compared to those in colonies sprayed monthly by the binary mixture or by rotation through General Linear Model procedure. For each comparison, a categorical variable 'strategy' with two levels (mixture or rotation) and its interaction with 'month' were included in the model. A significant 'month' effect ($P < 0.05$) was indicative of overall slopes different from zero; a significant 'strategy' effect was indicative of intercept differences among the three regression lines, and a significant interaction effect was indicative of different slopes what would mean different resistance attributable to strategy over the course of the study.

Concentration-mortality regressions for ACP treatment colonies were estimated following the same procedure as in the concentration response experiments. Test hypotheses of equality of slopes and intercepts of the probit model were used to study differences in concentration response between treated and original colonies. Lethal concentration ratios (LCR) (relative toxicity ratios) and 95% confidence limits were calculated for LC₅₀ and LC₈₀ to compare changes in susceptibility at predetermined lethal concentrations between resistant and susceptible colonies. Lethal concentrations were considered significantly different when LCR confidence limits did not include 1 (Robertson et al. 2007).

Results

Concentration response curves

The probit model explained the relationship between residual concentration of insecticide a.i.s and ACP adult mortality for 11 of the 12 insecticides tested (**Table 2**). The exception was spirotetramat (Movento) for which there was insufficient adult toxicity to fit a curve. Product ranked by toxicity to adults based on LC₅₀, (the most reliable point of comparison for concentration-response regressions (Robertson et al. 2007)), was 1st the abamectin + thiamethoxam mixture (0.536 ppm of the mixture $\Leftrightarrow$ 0.095 ppm of abamectin + 0.441 ppm of thiamethoxam), followed by 2nd thiamethoxam (1.93 ppm) > 3rd beta-cyfluthrin (2.77 ppm) > 4th imidacloprid (3.35 ppm) > 5th spinetoram (5.86 ppm) > 6th dimethoate (6.23 ppm) > 7th zeta-cypermethrin (6.90 ppm) > 8th fenprothrin (14.88

ppm) > 9th malathion (29.34 ppm) > 10th abamectin (30.05 ppm) > 11th fenpyroximate (70.15 ppm). At LC₉₀, the decreasing order of toxicity for ACP adults showed similar trends (**Table 2**).

The probit model also explained relationship between exposure to insecticide by application concentration and mortality to ACP nymphs for the 10 insecticides tested (**Table 2**). Concentration at LC₅₀ of the insecticides tested were ranked as follows: 1st abamectin + thiamethoxam (0.05 ppm of the -mixture $\Leftrightarrow$ 0.009 ppm of abamectin + 0.041 ppm of thiamethoxam) > 2nd imidacloprid (0.12 ppm) > 3rd thiamethoxam (0.18 ppm) > 4th abamectin (0.57 ppm) > 5th beta-cyfluthrin (0.76 ppm) > 6th zeta-cypermethrin (1.13 ppm) > 7th dimethoate (2.22 ppm) > 8th spinoteram (2.91 ppm) > 9th fenpyroximate (10.05 ppm) > 10th spirotetramat (45.42 ppm). Adults/nymphs ratios of their LC-50s ranked highest for agrimek (52.7) followed by admirePro (27.9), agriFlex (10.8) and actara (10.7), then by portal 6.98), mustang (6.11), baythroid (3.64), dimethoate (2.81), and delegate (2.01) (**Table 2**).

Significantly greater mortality than predicted by the model for independent, uncorrelated joint action of agri-Flex® (abamectin + thiamethoxam) was observed for both adults ($\chi^2 = 55.981$, $df = 7$, $P < 0.0001$) and nymphs ($\chi^2 = 15.785$, $df = 7$, $P = 0.0271$) indicating synergist toxicity for the combination.

Maximum labeled field rates for most of the insecticides tested were markedly higher than estimated LC₉₀ for both ACP adults and nymphs (**Table 3**). The greatest discrepancies for adults were observed with malathion and dimethoate for which maximum label application rates (599 and 5,392 ppm) were 57 and 36 times greater respectively than the estimated LC₉₀. In contrast, maximum recommended field rates for abamectin, zeta-cypermethrin and fenpyroximate were well below (0.24-0.13 times) estimated adult LC₉₀ (**Table 3**). The highest discrepancy for ACP nymphs was observed for abamectin + thiamethoxam where the maximum field concentration was 136 times greater than the estimated LC₉₀. In contrast, maximum field rates were well below to estimated LC₉₀ for spirotetramat (**Table 3**).

Resistance management strategies

Monthly applications of the two a.i.s tested at the estimated LC₂₅ resulted in a progressive decrease in sensitivity within populations (abamectin: $F = 77.29$; $df = 1, 5$; $P = 0.0009$; $r^2 = 0.95$ | thiamethoxam: $F = 18.72$; $df = 1, 8$; $P = 0.0035$; $r^2 = 0.73$). ACP adult mortality at the LC₈₀ estimated for the susceptible colony was reduced from 80% to 51% in 6 months in colonies subjected to monthly applications of abamectin. After this period, estimated probit lines for the new abamectin-resistant and original susceptible ACP populations were neither equal ($\chi^2 = 203$, $df = 2$, $P < 0.0001$) nor parallel ($\chi^2 = 9.23$, $df = 1$, $P = 0.002$). According to the estimated 95% confidence limits for the LCR₈₀ from susceptible and treated colonies, LCs₈₀ were significantly different (**Table 4**). Repeated thiamethoxam treatment nevertheless only reduced ACP mortality associated with this insecticide at LC₈₀ to 67% after 8 months of repeated applications. Monthly application of the commercial mixture of abamectin and thiamethoxam (Agriflex®) also resulted in a progressive decrease in sensitivity to this product ($F = 28.60$; $df = 1, 6$; $P = 0.0031$; $r^2 = 0.85$). Colonies treated with monthly applications of the mixture reduced LC₈₀ from 80% to 50% after seven months. However, the hypotheses of parallelism and equality between the estimated probit lines of the original susceptible population and the selected resistant population were both rejected, although in the former, marginal differences were found (Equality: $\chi^2 = 3.31$, $df = 2$, $P = 0.191$; Parallelism: $\chi^2 = 3.30$, $df = 1$, $P = 0.069$).

Similarly, based on the estimated LCR_{80} 95% confident limits, the estimated LC_{80} for treated ACP populations was not yet significantly higher than that of the original, susceptible population (**Table 4**).

Susceptibility reduction throughout the experimental period in colonies subjected to monthly applications of the commercial abamectin + thiamethoxam mixture was not statistically different from results obtained for colonies under the monthly abamectin treatment (treatment: $F = 0.37$; $df = 1$; $P = 0.557$ |month x treatment: $F = 0.00$; $df = 1$; $P = 0.954$) (**Fig. 1A**). Nevertheless, marginally greater susceptibility reduction was observed in response to monthly application of abamectin compared to monthly rotation of all 3 products (treatment: $F = 0.16$; $df = 1$; $P = 0.699$ |month x treatment: $F = 4.42$; $df = 1$; $P = 0.080$) (**Fig. 1A**). In contrast, marginally greater reduction of susceptibility was seen with monthly treatment with the mixture compared to thiamethoxam (treatment: $F = 1.45$; $df = 1$; $P = 0.252$ |month x treatment: $F = 4.06$; $df = 1$; $P = 0.067$) (**Fig. 1B**). No differences were found in susceptibility reduction between colonies treated with thiamethoxam compared to monthly rotation of all 3 products (treatment: $F = 0.04$; $df = 1$; $P = 0.839$ |month x treatment: $F = 2.46$; $df = 1$; $P = 0.151$) (**Fig. 1B**).

Discussion

We evaluated 12 insecticides (four never tested for ACP adults and eight for nymphs) belonging to eight MoAs commonly used for ACP management in Florida against ACP, against an insecticide-naïve colony using a residual methodology on plant cuttings that simulates field applications. Many of these products are translaminar for which the toxicant is also acquired by feeding. Residual toxicity evaluations would therefore better explain their field performance than contact bioassay studies for which toxicity depends only on cuticular penetration of a concentrated solution to reach the target site.

Residual toxicity to ACP adults of six insecticides (abamectin, dimethoate, imidacloprid, malathion, thiamethoxam and zeta-cypermethrin) was previously studied (Boina et al. 2009, Naeem et al. 2016, Pardo et al. 2018). All these studies used the leaf disc dipping technique and experiments had significantly smaller sample sizes than in our studies. Estimated LC_{50} for these a.i.s were in general 5 to 10 times lower than in our study, whereas these differences were less marked for estimated LC_{90s} . The use of different insecticide application methodologies (spraying vs dipping) may be behind these discrepancies. Future studies may therefore be required to define the most appropriate methodology for ACP residual toxicity studies.

Direct toxicity to ACP adults of six insecticides (abamectin, dimethoate, fenpropathrin, malathion, spinetoran and thiamethoxam) and two against nymphs (fenpropathrin and imidacloprid) were also previously tested by Tiwari et al. (2011) and Morse et al. (2016). Our results showed similar trends to those direct toxicity studies with most of the products tested. However, toxicity of neonicotinoids was more than ten times greater when applied directly to the adult by Tiwari et al (2011) than when applied to plants in the current study, although Morse et al. (2016) obtained results more similar to ours. In contrast, we found nymphs to be 10 and 28 times more sensitive than adults to thiamethoxam and imidacloprid respectively. These products are recommended primarily to protect young trees from HLB by systemic application in the soil (Qureshi et al. 2014; Qureshi and Stansly 2007). Our results confirm that this is an effective way to control nymphs which are primarily responsible for acquisition of *Candidatus Liberibacter asiaticus* (Lee et al. 2015; Pelz-Stelinski et al. 2010). Nevertheless, long continuous

exposure of ACP populations to systemically applied neonicotinoids provides ample opportunity to select for resistance. Tolerance to neonicotinoids has already been noted in Florida (Tiwari et al. 2011) and may be responsible for the relative underperformance of imidacloprid in field studies (Qureshi et al. 2014). Therefore, rotations with sprays of different MoA and constant monitoring of ACP susceptibility to neonicotinoids is important. Overall, our results reveal that the proposed methodology to evaluate residual toxicity of insecticides to ACP adults and nymphs are in most cases comparable to, and possibly in the case of neonicotinoids, more germane to use patterns than direct toxicity bioassays.

Lethal concentrations obtained through our methodology can be easily translated into field concentrations assuming spray volumes commonly used for ACP management. When LC_{S90s} were compared to maximum recommended field doses at the labels, important discrepancies, both positive and negative, were observed. For instance, the maximum label rate for the organo-phosphate (OP), dimethoate, is more than 30 times greater than our LC_{S90} for adults. Not surprisingly, this product has proven very effective in field studies (Qureshi et al. 2014). Lower application rates may therefore benefit growers by reducing material application costs, impacts on natural enemies, residues on fruit and in the environment. The same is true for the mixture of abamectin and thiamethoxam. In contrast, control in the field was only moderate with abamectin and fenpyroximate (Qureshi et al. 2014) with maximum label rates below the estimated LC_{50} . Likewise, our estimated LC_{90} for zeta-cypermethrin against adults was 5 times higher than the maximum label rate, in contrast to the other two pyrethroids tested, beta-cyfluthrin and fenpropathrin, again corresponding to field results for these three products (Qureshi et al. 2014). Lack of effectiveness of zeta-cypermethrin makes it a less favorable choice than the other two pyrethroids tested for controlling overwintering adults (Qureshi and Stansly 2010).

The toxicity of spirotetramat against adults was negligible and indeed, it is recommended for control of nymphs for which it has proved efficacious in various field studies (Qureshi et al. 2014). Nevertheless, our results indicated an LC_{90} for nymphs that was almost 15-fold above the maximum field rate (**Table 4**). Many field studies included additions of horticultural spray oil or other surfactants, whereas we did not use an adjuvant. Furthermore, spirotetramat is systemic and has to be metabolized into an active molecule in the plant (Jeschke 2016). Possibly, this transformation did not occur efficiently during the 48 h incubation time in the *M. paniculata* cuttings used in our bioassay.

Eight months of repeated exposure of our ACP susceptible colony to abamectin and thiamethoxam at a discriminatory concentration (LC_{25}) were sufficient to select for ACP resistance to these products. Resistance alleles in any laboratory strain may be in a low frequency compared to wild-type populations. This could result in a slower resistance induction compared to what might actually occur in the field. Consequently, our results may underestimate the potential resistance induction risks of the current insecticide management programs for ACP. In fact, a recent field study found a reduction of susceptibility to an organophosphate (Dimethoate) in a field ACP population just after 5 monthly sequential insecticide applications with this a.i. (Chen and Stelinski 2017). These and our results therefore demonstrate the rapid and high risks of resistance development associated to intensive ACP insecticide management and stresses the importance of developing IRM strategies for this pest. The strongest

response was to abamectin for which 6 monthly applications (approximately 6 generations) were sufficient to increase resistance 3-fold. Our toxicity laboratory studies with abamectin showed that currently maximum field recommended doses for this insecticide are below the obtained LC_{90} for both ACP nymphs and adults. Field applications at sublethal doses may therefore be favoring the resistance development to this a.i.. Resistance to abamectin is considered polyfactorial involving both general and specific mechanisms (Kwon et al. 2010; Stumpf and Nauen 2002; Wang and Wu 2007). Rapid selection for resistance to abamectin is likely associated with unspecific detoxifying enzymes such as cytochrome P450 dependent monooxygenases and Glutathione S-transferases (Wang and Wu 2007) and it would be specially favoured in laboratory populations with limited genotypic variability (Ffrench-Constant, 2013). Development of resistance to thiamethoxam has been linked to detoxifying cytochrome P450 dependent monooxygenases to imidacloprid-tolerant ACP field populations in Florida (Tiwari et al. 2013) although more specific resistance mechanisms to neonicotinoids such as target-site mutations have also been identified in other insects (Casida and Durkin 2013; Liu et al. 2005). Owing to the much less frequent use of thiamethoxam than imidacloprid in Florida citrus, cross-resistance mechanisms to the latter may underlie the resistance to thiamethoxam (Alyokhin et al. 2007; Zewen et al. 2003).

Synergistic toxicity was found between the two components of the commercial mixture of abamectin and thiamethoxam against both adults and nymphs. This could indicate that one of these two a.i.s. interferes with the metabolic detoxification of the other one. Monthly applications of this mixture at a discriminatory concentration also resulted in tolerance. In fact, tolerance to abamectin was similar, and to thiamethoxam greater, when applied monthly with the commercial mixture at the discriminatory LC_{25} (abamectin 0.29 ppm, thiamethoxam 0.06 ppm) than when each was applied alone at their respective LC_{25} s (abamectin 18.26 ppm, thiamethoxam 1.67 ppm). This result may be due to more rapid selection for resistance development to abamectin reducing its synergistic effect in the insecticide mixture.

The literature is divided on which strategy (rotation vs mixture) is the better approach for resistance management (Immaraju et al. 1990; Prabhaker et al. 1998). In our study, the rotation resulted in a better strategy even though more a.i. was applied by rotation (almost half as much as applied with each alone). Recent field surveys in Florida for ACP resistance development to several MoAs revealed a decrease of tolerance to a.i.s such as thiamethoxam (Coy et al. 2016). The authors attributed the decrease to the implementation of effective rotation strategies. Further research on the mechanisms involved in ACP resistance development to thiamethoxam and abamectin would be needed to understand why rotation appears to be a better IRM strategy with these a.i.s against ACP.

In conclusion, we have found reasonable agreement between published direct and our estimates of residual toxicities for numerous a.i.s with different modes of action. Therefore the susceptibility baselines provided in this study complement the information obtained in previous studies (Boina et al. 2009, Morse et al. 2016, Naeem et al. 2016, Pardo et al. 2018, Tiwari et al. 2011). The methodology we propose can therefore be similarly used to monitor resistance development in ACP field populations. We present susceptibility baselines for four a.i.s not previously tested for adult ACP and eight not previously tested for nymphs. Our methodology also permits easy translation of

laboratory concentrations to field rates. Although differences between laboratory concentrations and field rates were expected, we have observed discrepancies between ACP responses to concentrations provided at the labeled rates and toxicity data obtained in the laboratory. While some products have labeled rates well above the obtained LC₉₀, field concentrations for other products are insufficient to produce desired mortality. In order to optimize ACP insecticide management programs, further field studies with those products exhibiting the highest discrepancies could provide be used to adjust to more efficient field concentrations. We have observed that 8 months of repeated treatments to a naïve ACP colony with a discriminatory concentration of two insecticides with different modes of action were sufficient to lessen response to both a.i.s. We have found that although the binary mixture of the two a.i.s improves insecticide efficacy against ACP and reduces the development of resistance to these a.i.s., the temporal rotation of the two a.i.s seems to be a better IRM strategy. Information generated in this work will contribute to develop effective IRM strategies that circumvent risks of rapid development of resistance in ACP to the presently available insecticides.

Author contribution statement

PS and MJ conceived and designed the research. PV, MJ, JT, CM and XC participated in data collection and analyses. PV, CM and PS wrote the manuscript. All authors read and approved the manuscript.

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Table 1. Characteristics of tested insecticides for their residual activity on Asian citrus psyllid nymphs and adults.

Brand Name	Active Ingredient	% a. i.	Company	IRAC Group	Chemical Sub-group or active ingredients	MoA
Actara®	Thiamethoxam	25	Syngenta Crop Protection, LLC	4A	Neonicotinoids	Nicotinic acetylcholine receptor (nAChR) competitive modulators
Admire® Pro	Imidacloprid	42.8	Bayer CropScience	4A	Neonicotinoids	Nicotinic acetylcholine receptor (nAChR) competitive modulators
Agri-Flex®	Abamectin + Thiamethoxam	3 + 13.9	Syngenta Crop Protection, LLC	6 + 4A	Abamectin + Neonicotinoids	Glutamate-gated chloride channel (GluCl) allosteric modulators + Nicotinic acetylcholine receptor (nAChR) competitive modulators
Agri-Mek® 0.15 EC	Abamectin	2	Syngenta Crop Protection, LLC	6	Abamectin	Glutamate-gated chloride channel (GluCl) allosteric modulators
Baythroid® XL	Beta-Cyfluthrin	12.7	Bayer CropScience	3	Pyrethroids, Pyrethrins	Sodium channel modulators
Danitol® 2.4 EC	Fenpropathrin	30.9	Valent U.S.A. Corporation Agricultural Products	3	Pyrethroids, Pyrethrins	Sodium channel modulators
Delegate® WG	Spinetoram	25	Syngenta Crop Protection, LLC	5	Spinosyns	Nicotinic acetylcholine receptor (nAChR) allosteric modulators
Dimethoate 4EC	Dimethoate	43.5	Drexel Chemical Company	1B	Organophosphates	Acetylcholinesterase (AChE) inhibitor
Malathion 57 EC	Malathion	57	Loveland Products, INC	1B	Organophosphates	Acetylcholinesterase (AChE) inhibitor
Movento® MPC	Spirotetramat	14.5	Bayer CropScience	23	Tetronic and Tetramic acid derivatives	Inhibitors of Acetyl CoA carboxylase
Mustang®	Zeta-Cypermethrin	17.1	FMC Corporation Agricultural Products Group	3	Pyrethroids, Pyrethrins	Sodium channel modulators
Portal® XLO	Fenpyroximate	5	Nichino America, Inc.	21 A	Meti acaricides and insecticides	Mitochondrial complex I electron transport inhibitors

Table 2. Concentration response of ACP adults and 3rd and 4th instar nymphs from a susceptible colony maintained at SWFREC to dried residues of several insecticides labeled for control of this pest.

	Chemical (active ingredient)	n total	Slope $\pm$ S.E.	t-ratio	χ^2	df	LC ₅₀ (ppm)	Rank*	95 % Limits	LC ₉₀ (ppm)	Rank	95 % Limits
Adults	Actara® (Thiamethoxam)	666	1.109 $\pm$ 0.12	8.77	3.29	4	1.93	2	1.16-2.87	28.28	3	17.79-53.67
	Admire® Pro (Imidacloprid)	340	1.25 $\pm$ 0.14	9.03	4.643	4	3.35	4	1.95-5.88	35.70	5	16.64-140.08
	Agri-Flex® (Abamectin + Thiamethoxam)	835	0.97 $\pm$ 0.08	12.29	4.42	5	0.54	1	0.34-0.78	11.27	1	7.69-17.93
	Agri-Mek® 0.15 EC (Abamectin)	897	2.60 $\pm$ 0.28	9.37	5.29	4	30.05	10	18.34-39.67	93.30	7	75.65-124.84
	Baythroid® XL (Beta-Cyfluthrin)	965	1.19 $\pm$ 0.14	8.68	7.70	6	2.77	3	1.26-4.56	33.24	4	20.33-71.60
	Danitol® 2.4 EC (Fenpropathrin)	400	1.40 $\pm$ 0.26	5.32	2.55	5	14.88	8	7.18-23.51	122.62	9	73.52-303.02
	Delegate® WG (Spinoteram)	1770	1.43 $\pm$ 0.10	14.83	9.40	5	5.86	5	3.90-8.04	46.40	6	32.04-78.50
	Dimethoate 4EC (Dimethoate)	1091	2.96 $\pm$ 0.27	10.92	7.68	5	6.23	6	5.02-7.33	16.86	2	13.61-23.83
	Malathion (Malathion)	880	2.51 $\pm$ 0.21	11.82	1.14	5	29.34	9	25.72-33.32	95.07	8	78.13-123.07
	Movento® MPC (Spirotetramat)	1292	-	-	-	-	-	-	-	-	-	-
	Mustang® (Zeta-Cypermethrin)	1551	0.86 $\pm$ 0.08	10.89	7.79	5	6.90	7	4.18-11.32	211.66	10	87.76-973.71
	Portal® XLO (Fenpyroximate)	1344	0.98 $\pm$ 0.08	12.18	2.55	5	70.15	11	51.98-92.22	1425.83	11	925.40-2512.36
Nymphs	Actara® (Thiamethoxam)	749	1.05 $\pm$ 0.09	12.01	5.671	4	0.18	2	0.090-0.32	2.97	2	1.43-8.94
	Admire® Pro (Imidacloprid)	450	0.91 $\pm$ 0.11	7.99	2.97	3	0.12	3	0.39-1.02	17.53	5	10.22-39.47
	Agri-Flex® (Abamectin + Thiamethoxam)	288	1.12 $\pm$ 0.15	7.62	4.78	5	0.05	1	0.03-0.08	0.73	1	0.41-1.70
	Agri-Mek® 0.15 EC (Abamectin)	317	0.78 $\pm$ 0.14	5.67	7.57	6	0.57	4	0.11-1.49	24.31	7	7.34-14.83
	Baythroid® XL (Beta-Cyfluthrin)	297	1.58 $\pm$ 0.27	5.95	3.51	3	0.76	5	0.27-1.48	4.90	3	2.27-48.03
	Delegate® WG (Spinoteram)	865	1.24 $\pm$ 0.12	10.50	3.08	4	2.91	8	2.02-3.97	31.24	8	21.37-51.33
	Dimethoate 4EC (Dimethoate)	849	1.74 $\pm$ 0.18	9.93	5.56	4	2.22	7	1.34-3.22	12.07	6	7.81-24.69
	Movento® MPC (Spirotetramat)	499	0.76 $\pm$ 0.11	7.21	1.35	4	45.42	10	25.32-74.28	2186.14	10	652.3-8495.6
	Mustang® (Zeta-Cypermethrin)	584	1.39 $\pm$ 0.14	9.73	5.23	3	1.13	6	0.41-2.07	9.44	4	4.94-31.95
	Portal® XLO (Fenpyroximate)	875	1.99 $\pm$ 0.22	9.28	6.45	3	10.05	9	5.50-18.03	43.98	9	22.94-225.03

* Toxicity ranking of each insecticide for ACP adults and nymphs according to their estimated LC₅₀

Table 3. Maximum labeled field concentrations (ppm assuming 935 L/ha), estimated lethal concentration 90 [LC₉₀] (ppm) in ACP susceptible colonies for adults and nymphs, maximum field concentration / LC₉₀ ratios for adults and nymphs and discrepancy ranking between field recommendation and LC₉₀, for 12 commercial products used for ACP control in citrus groves in Florida.

Brand Name	Active Ingredient	MoA	Max field concentration (ppm)	LC ₉₀ (ppm) Adults	LC ₉₀ (ppm) Nymphs	Field concentration/LC ₉₀ (ppm) Adults	Rank	Field concentration/LC ₉₀ (ppm) Nymphs	Rank
Actara®	Thiamethoxam	4A	82.38	28.29	2.98	2.91	6	27.69	3
Admire® Pro	Imidacloprid	4A	241.15	35.70	17.53	6.76	4	13.76	4
Agri-Flex® ¹	Abamectin + Thiamethoxam	6 + 4A	98.53	11.27	0.73	8.74	3	135.53	1
Agri-Mek® 0.15 EC	Abamectin	6	22.47	93.30	24.31	0.24	9	0.92	9
Baythroid® XL	Beta-Cyfluthrin	3	47.93	33.25	4.90	1.44	8	9.79	5
Danitol® 2.4 EC	Fenpropathrin	3	383.38	122.62	-	3.13	5	-	
Delegate® WG	Spinetoram	5	89.87	46.40	31.24	1.94	7	2.88	8
Dimethoate 4EC	Dimethoate	1B	599.13	16.86	12.07	35.54	2	49.63	2
Malathion 57 EC	Malathion	1B	5392.19	95.07	-	56.72	1	-	
Movento® MPC	Spirotetramat	23	149.78	-	2186.14	-		0.07	10
Mustang®	Zeta-Cypermethrin	3	48.31	211.66	9.44	0.23	10	5.12	6
Portal® XLO	Fenpyroximate	21 A	191.72	1425.83	43.98	0.13	11	4.36	7

¹ Agri-Flex® ppm indicate the total amount of the two a.i.s of the mixture, presented in ratio abamectin:thiamethoxam (0.178:0.822)

Table 4. Concentration response comparisons predicted by the probit model, of the induced ACP adult resistant populations to two commercial insecticides commonly used for ACP control: Agri-Mek® 0.15 EC (Abamectin) and Agri-Flex® (Abamectin + Thiamethoxam), and the original susceptible population, once mortality rates at the original LC₈₀ in the resistance populations were under 50%. LCR confident limits not including 1 are indicative of significant differences on LC between the induced resistant population and the original susceptible population.

Chemical (active ingredient)	ACP ¹	n total	Slope ± S.E.	t-ratio	χ^2	df	LC ₂₅ (ppm)	95 % CL	LC ₈₀ (ppm)	95 % CL	LCR ₂₅ ²	95 % CI	LCR ₈₀ ³	95 % CL
Agri-Flex® (Abamectin + Thiamethoxam)	S	666	1.10 ± 0.12	8.77	3.29	4	0.47	0.21 - 0.82	11.25	7.65 – 17.91	1.69	0.62 – 4.65	0.73	0.39 – 1.35
	R	403	1.50 ± 0.26	5.77	3.53	5	0.80	0.28 - 1.43	8.17	5.40 - 14.17				
Agri-Mek® 0.15 EC (Abamectin)	S	897	2.60 ± 0.2	9.37	5.29	4	16.55	7.62– 24.77	63.23	50.13 – 78.14	5.91	4.08 – 8.55	3.02	2.47 – 3.68
	R	554	4.26 ± 0.50	8.51	7.12	3	97.84	40.14 - 130.10	221.98	179.03 - 339.17				

¹ACP: Asian citrus psyllid colony; S: Susceptible; R: Resistance; ² Lethal Concentration 80 ratio; ³ Lethal Cncentration 25 ratio

Fig. 1. Mortality responses over 8 months to the originally estimated LC_{80} rates of 3 insecticides of susceptible ACP populations subjected to monthly applications at LC_{25} of: **A)** abamectin (Agri-Mek®), the thiametoxam + abamectin commercial mixture (Agri-Flex®) and a rotation of Agri-Flex®, Agri-Mek® and Actara®; **B)** thiamethoxam (Actara®), the thiametoxam + abamectin commercial mixture (Agri-Flex®) and a rotation of Agri-Flex®, Agri-Mek® and Actara®.

B)