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Application of an Enzyme-linked Immunosorbent Assay for the Analysis of Imidacloprid in Wiliwili Tree, *Erythrina sandwicensis* O. Deg, for Control of the Wasp *Quadrastichus erythrinae*

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A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the neonicotinoid insecticide imidacloprid was evaluated for its reproducibility, accuracy, and comparability to results from a conventional high-performance liquid chromatography (HPLC) for the analysis of imidacloprid in the endemic wiliwili tree (*Erythrina sandwicensis* O. Deg) found in dryland forests and landscapes in Hawaii. Imidacloprid was applied to these wiliwili trees in an attempt to control the newly introduced erythrina gall wasp, *Quadrastichus erythrinae* Kim. Leaf samples were freeze-dried and extracted with acidic aqueous methanol followed by methylene chloride partitioning. After solvent removal, the extract residue was reconstituted in 1 mL of water/methanol (1:1, v/v) for ELISA; no significant matrix interference was observed at 10-fold or more dilution. The average recoveries of imidacloprid from fortified samples ranged from 78% to 100% by ELISA. The correlation between the ELISA and HPLC results was excellent ($r^2 = 0.98$). Imidacloprid was detected with the ELISA in all treated samples and its level varied in the samples among different treatments and in those from different parts of the trees. The infestation severity rating of leaf samples was inversely related to the concentration of imidacloprid. It is clear that imidacloprid effectively controls the wasps. The ELISA is a suitable method for quantitative and reliable determination of imidacloprid in wiliwili trees and the application provides information to understand how to control the wasps.

KEYWORDS: ELISA; imidacloprid; wiliwili trees; leaves; wasps

INTRODUCTION

Erythrina sandwicensis O. Deg. is an endemic deciduous tree that grows in dryland forest areas of leeward portions of the Hawaiian Islands up to elevations of about 1950 ft (*1*). It is also known as the wiliwili or Hawaiian coral tree and produces showy claw-shaped flowers that are commonly orange but other forms can produce red, salmon, peach, light green, yellow, or white flowers (*2*). In addition to growing in natural areas, *E. sandwicensis* can be found in resort landscape settings. One of the most recent threatening invasive species to wiliwili trees is the erythrina gall wasp (EGW), *Quadrastichus erythrinae* Kim (*3*−*4*). In addition to *E. sandwicensis*, EGW attacks *E. variegata* and *E. crista-galli* (*3*).

The EGW was described in 2004 as a new species by Kim et al. (*5*) from specimens from Singapore, Mauritius, and Reunion. The adult female wasp inserts eggs into the young leaves. Larvae develop in the leaf tissue, and the trees respond to its feeding by producing galls. After pupation, the wasp exits through a small hole in the gall. Heavily infested trees stop growing, lose vigor, and may die. Since its discovery on Oahu in April 2005, EGWs have spread rapidly to all the other major islands of Hawaii (*3*).

Presently, chemical and biological controls are being investigated. Chemical control is a short term measure which mainly focuses on effective use of insecticides. For long-term control, classical biological control, involving the importation of specific natural enemies, is the optimal choice because it is long lasting and friendly to the environment and biological diversity. Preliminary systemic insecticide trials suggest that imidacloprid may help in reducing damage to erythrina caused by the gall wasp (*3*).

Imidacloprid, 1-(6 chloronicotinyl)-2-(nitroimino) imidazolidine (**Figure 1**), is a neonicotinoid insecticide with high activity against sucking insects (*6*). It is the most widely used systemic insecticide in the world (around 70 crops in more than 100

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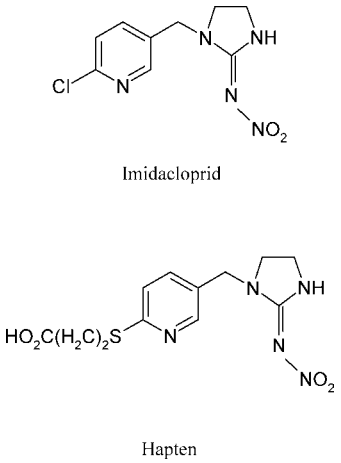

Figure 1. Structures of imidacloprid and hapten.

countries). Imidacloprid works by interfering with the transmission of stimuli in the insect's nervous system. It causes a blockage in at least one type of nicotinergic neuronal pathways that is more abundant in insects than in warm-blooded animals. This makes imidacloprid much more toxic to insects than to other animals. Furthermore, imidacloprid has a highly specific affinity to insect nicotinic acetylcholine receptors (nAChR) (*7*−*9*). Its binding leads to the accumulation of acetylcholine, resulting in the paralysis and death of insects (*10*).

In order to develop a guideline for managing the wasps in wiliwili trees, it is clear that groundwork information on the activity profile of imidacloprid in wiliwili trees would be required. This information is often less apparent for a systemic insecticide such as imidacloprid than for a foliar contact insecticide, in part because of the longer period required for translocation throughout a plant compared with the immediate contact and exposure of a foliar-applied insecticide. Therefore, in this study, our goal is to apply a monoclonal-based enzyme-linked immunosorbent assay (ELISA) to monitor imidacloprid residues in wiliwili leaves. The assay should be a suitable tool for researchers to use to improve imidacloprid application for control of insect pests.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless specified otherwise. Analytical standard imidacloprid (96.9% purity) was obtained from Bayer Corp, Stillwell, KS. Goat anti-mouse IgG-horseradish peroxidase (IgG-HRP), phosphate−citrate buffer capsules with sodium perborate, carbonate−bicarbonate buffer capsules, and *o*-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO). Monoclonal antibodies against imidacloprid were previously prepared in our laboratory (*11*). The purified Mab IgG in phosphate-buffered saline (PBS, 5 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136 mM NaCl, and 2.7 mM KCl, pH 7.5) was stored at −20 °C until use.

Treatment Methods. Imidacloprid treatments were made using commercially available injection equipment and according to label recommendations of the formulations. Wedgle Direct-Inject (Arbor Systems, Omaha, NE) utilizes a 0.75 in. (19 mm) long needle-like tip which is inserted through a rubber "WedgeCheck" and is placed into the trunk acting as a stopper to prevent the chemical from escaping from the tree. One milliliter of 5% active ingredient (a.i.) imidacloprid (Pointer, Arbor Systems) was injected every 6 in. (15 cm) around the circumference of the trunk near the ground. Sidewinder precision tree injector (Noosaville, QLD, Australia) treatment was conducted by drilling a 6 mm hole into the trunk of the tree; an application nozzle was then screwed into the hole and 5 mL of imidacloprid was applied under pressure (<40 bar). Following the treatment application, a plug was screwed into the hole to seal the wound and prevent the chemical from bleeding out. Imicide HP formulation (10% imidacloprid; JJ Mauget Co, Arcadia, CA) was applied through the sidewinder equipment at 1.5 mL and 2 mL per inch diameter measured at breast height (DBH) for 10−36 in. (25−91 cm) and >36 in. (>91 cm) DBH trees, respectively. The third treatment was Mauget Imicide (10% imidacloprid; JJ Mauget Co, Arcadia, CA) packaged in ready-to-use 3-mL microinjection capsules. An 11/64 in. (4.4 mm) hole was drilled into the trunk of the tree, and a capsule fitted with a feeder tube was placed at a depth corresponding to the conductive xylem tissue. The number of capsules used was determined by dividing the diameter by 2. Unlike the other treatments that were applied to the trunk near the ground, the capsules were applied to the main limbs of the tree 4−6 ft from the ground. Treatments were applied to wiliwili trees in a native dryland forest at Pu'u Wa'awa'a and Waikoloa, Hawaii, and in an irrigated resort landscape at the Hualalai Resort, Hualalai, Hawaii, all located on the Island of Hawaii. Hualalai Resort treatment using Mauget capsules occurred March 13, 2006. At Pu'u Wa'awa'a, Wedgle and Sidewinder treatment occurred November 10 and December 2, 2005, respectively. Waikoloa Wedgle and Sidewinder treatment occurred December 7 and December 19, 2005, respectively.

Table 1. Infestation Severity Ratings

rating	description	approx gall weight/ 20 g leaf
1	very light infestation, only very slight galling	<3 g
2	moderate galling	3–8 g
3	heavy galling of leaves but minimal leaf deformity	8–14 g
4	heavy galling moderate leaf deformity	14–18 g
5	extreme galling and deformity with no expanded leaves	>18 g

Sampling. Leaf samples were obtained by cutting 15 cm long growing tips from the outer edge of the canopy. The samples were collected at the lower, middle, and upper canopy levels in at least four different locations at each level. The samples for imidacloprid analysis were stored at −20 °C until analyzed.

Evaluation of Severity of Infestation. Samples were evaluated for severity of infestation by a five-point numerical rating system (**Table 1**). A rating of 1 signified very light infestation levels with only very slight galling. A rating of 3 represented samples with heavy galling of leaves but minimal leaf deformity. Samples with ratings of 5 exhibited extreme galling and deformity with no expanded leaves. Wasp emergence was quantified by excising galls that lacked emergence holes and holding them in many 473 mL waxed paper bowls (Georgia Pacific, Atlanta, GA) covered with silkscreen to prevent escape. Galls were weighed at the time of excision so that the number of wasps per gram of gall tissue could be calculated. Three weeks after collection, wasps were counted with the aid of a dissecting microscope.

Extraction Procedures. Leaf samples without peduncle were freeze-dried and ground to powder. One gram of leaf powder was weighed in a 100 mL beaker. Imidacloprid was extracted ultrasonically with 50 mL of methanol/H_2SO_4 0.04% (4:1, v/v) at 60 °C for 20 min. The mixture was vacuum filtered through Whatman No. 4 filter paper (ID 9.0 cm, pore size 2.5 μm) with 1 g of Celite 545 on it. The filtrate was concentrated to 10−15 mL of water by evaporating with a rotary evaporator, at 55 ± 2 °C. The residue was centrifuged (6000 rpm) for 10 min, and the aqueous supernatant was transferred to a 60 mL separation funnel.

For ELISA determination, the supernatant was extracted with methylene chloride (20 mL × 3). The methylene chloride layer was collected and concentrated to 1−2 mL with a rotary evaporator. The organic remainder was transferred to a tube and dried under nitrogen. The residue was dissolved in 1 mL of water/methanol (1:1, v/v) which was diluted at least 10-fold with water for ELISA.

For HPLC determination, the supernatant was washed with 20 mL of hexane and the aqueous layer was collected. The hexane layer was extracted once again with 20 mL of 0.04% H_2SO_4. The aqueous phases were combined and transferred to a 125 mL separate funnel followed by extraction with methylene chloride (30 mL × 3). The combined methylene chloride extract was concentrated to 2 mL with a rotary evaporator. The organic remainder was passed through a C18 cartridge (Analtech, Inc., Newark, DE) that was preactivated with 5 mL of

methanol followed by 5 mL of water. The cartridge was eluted with 5 mL of methylene chloride/acetonitrile (85:15, v/v). The eluate was collected and dried under a gentle nitrogen stream. The residue was reconstituted in 2 mL of acetonitrile/water (1:1, v/v) and filtered through a 0.45-μm syringe filter (Gelman Sciences, Ann Arbor, MI) before HPLC analysis.

ELISA Determination. The ELISAs were carried out in 96-well polystyrene microplates (MaxiSorp F96; Nalge Nunc International, Copenhagen, Denmark) as previously described (*11*). Briefly, microplate wells were coated with conjugates (4 ng in 100 μL per well in 0.05 M carbonate−bicarbonate buffer, pH 9.6) of hapten (**Figure 1**) and BSA overnight at 4 °C. The following day, the plates were washed four times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 1% BSA in PBS (150 μL per well) by incubation for 1 h at room temperature. The plates were washed again 4 times; a solution of 50 μL per well of samples or standard diluted in PBST and 50 μL per well (0.2 μg of antibody per well) of imidacloprid MAb was added and incubated at room temperature for 1 h. Peroxidase-labeled goat anti-mouse IgG (1:5000 in PBST; 100 μL per well) was then added, and the plates were incubated at room temperature for 1 h. The plates were again washed 4 times as above, and then substrate solution (100 μL per well of 0.05 M citrate−phosphate buffer, pH 5.0, containing 0.03% sodium perborate and 1.0 mg/mL of OPD) was added. After 10−15 min at room temperature, the reaction was stopped with sulfuric acid (4 N, 50 μL per well), and absorbance at 490 nm was read with a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Samples and standards were analyzed in four replicate wells. Inhibition curves were fitted with the four-parameter logistic equation using Softmax version 2.35 software (Molecular Devices).

HPLC Determinations. A Dionex BioLC system (Dionex Corp., Sunnyvale, CA) consisted of a PDA-100 photodiode array detector, AS50 autosampler, GP50 gradient pump, and column oven, which were controlled by Chromeleon software. The HPLC was operated at the following conditions: mobile phase, acetonitrile/5 mM ammonium acetate (20:80, v/v); injection volume, 30 μL; flow rate, 1.5 mL/min; column, Inertsil ODS-3V, 5 μm, 4.6 × 250 mm; column temperature, 30 °C; wavelength, 270 nm.

RESULTS AND DISCUSSION

Matrix Interference. Several instrument methods (*12*−*14*) and immunoassays (*15*−*16*) have been reported for the analysis of imidacloprid in environmental matrices and agricultural products. As it is well-known, immunochemical methods for residual pesticides have many advantages. On the other hand, although these methods are susceptible to matrix interference from samples, especially biological samples, they can be overcome by simple dilution with water or appropriate buffer without troublesome cleanup steps (*17*). The ELISA for residual imidacloprid monitoring was highly specific and sensitive (*11*). No significant matrix interference from the water and cucumber samples was observed after simple dilution of the extracts before analysis in our previous studies (*11*). The results indicated that the ELISA method could be suitable to perform residual analysis for imidacloprid in the environmental and biological matrixes. So, in this study, we applied the ELISA method to analyze imidacloprid in wiliwili tree leaves.

An ultrasonic extraction with a mixture of methanol and 0.04% H_2SO_4 (4:1, v/v) was applied to wiliwili leaf samples (*13*, *18*). The extracts may contain numerous constituents such as chlorophyll, carotenoids, and wax, and therefore, it is essential to assess the influence of interference on the ELISA performance. The optimal dilution factor with water was investigated for the extract (**Figure 2**). Although the IC_{50} value shifts slightly, the curve of the 5-fold dilution sample is apart from the standard curve, which is apparently due to the matrix interference. Little position shift of the curves of 10-fold or more dilutions relative to the standard curve indicates no significant matrix interference

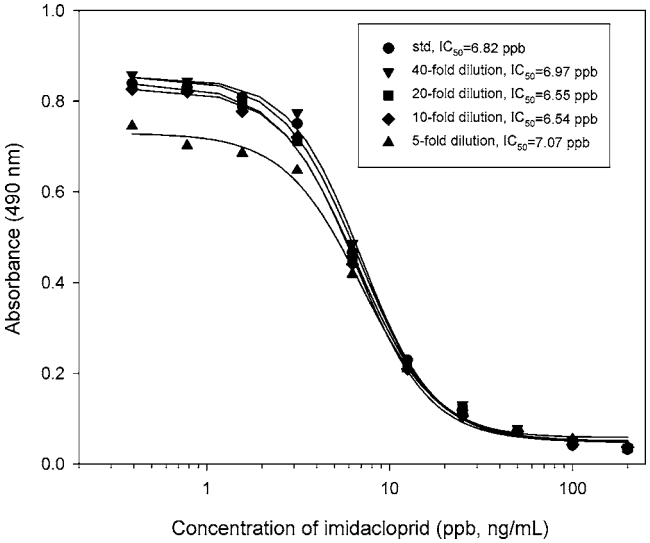

Figure 2. Inhibition curves of imidacloprid in wiliwili leaf extracts that were diluted in different folds. The data are an average of three replicates.

on the assay. Therefore, it is necessary to dilute the extracts at least 10-fold for ELISA to minimize the matrix effects.

The extracts of the plant leaves were too complicated for direct analysis by HPLC. Thus, a further cleanup procedure was necessary after extraction. Several methods such as liquid−liquid partition (LLP) (*18*), supercritical fluid extraction (SFE) (*19*), and solid-phase extraction (SPE) (*20*) have been successfully applied to clean up the extract of imidacloprid residues from environmental samples. In this study, LLP and SPE were used to clean up the extracts. Methylene chloride was used to eliminate polar compounds followed by a C18 column cleanup to remove nonpolar interference such as lipids from the matrices. Elution of imidacloprid was carried out with a different solvent and its proportions to establish the best elution procedure. Elution with 100% of methanol or acetonitrile provided good recoveries of imidachloprid, but the eluates obtained were dirty because of waxes and pigments. In contrast, elution with 100% of methylene chloride gave low recoveries of imidacloprid and required more solvent. In this study, different ratios of methylene chloride and acetonitrile were tested. Elution with a mixture of methylene chloride/acetonitrile at 85:15 (v/v) had a minimal amount of co-extractives and gave satisfactory recoveries. The eluates did not interfere with the accurate determination of imidacloprid by HPLC (**Figure 3**).

HPLC Separation. The chromatographic separation of imidacloprid using different mobile phases was investigated in detail according to the method of Liu et al. (*18*). The imidacloprid peak was relatively wide and tailed using aqueous acetonitrile (20%) as a mobile phase. This problem was overcome by adding ammonium acetate to the mobile phase. Further investigation showed that a reasonable retention time for imidacloprid could be obtained at about 11.2 min by adjusting the ratio of acetonitrile/5 mM ammonium acetate solution at 20:80 (v/v). With this mobile phase, imidacloprid could be completely separated from the matrix interferences (**Figure 3**). The concentrations of imidacloprid were calculated by calibration with the peak areas of external imidacloprid standard.

Comparison of Recoveries Determined on HPLC and ELISA. Recovery experiments were performed in control samples at four fortification levels (**Table 2**). The average recoveries of imidacloprid from the leaf samples were in a range of 78−100% for ELISA and 76−114% for HPLC, respectively.

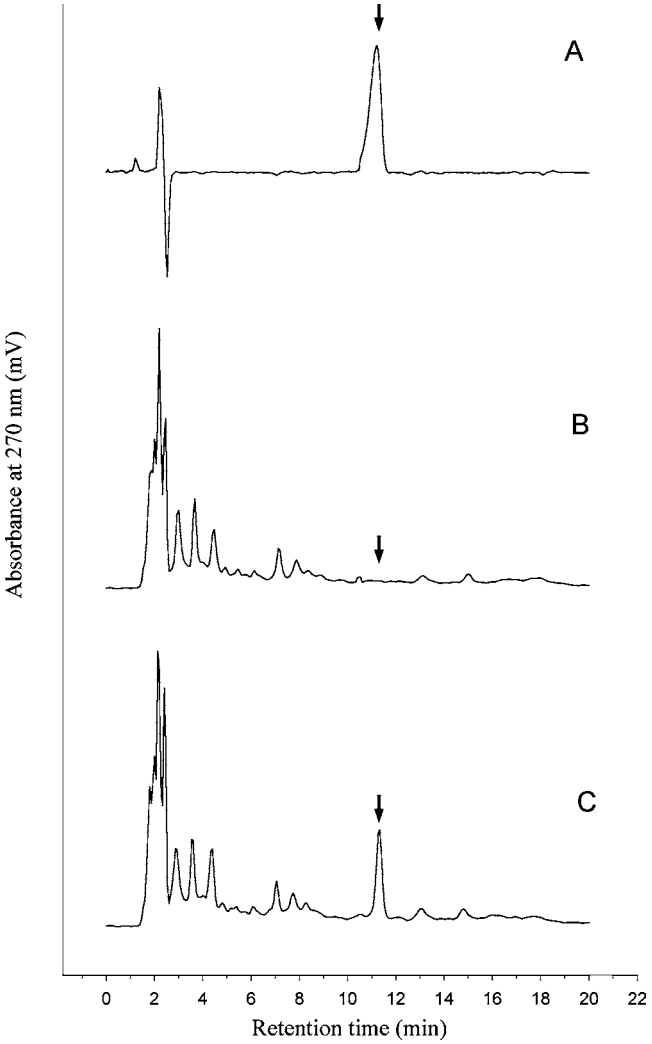

Figure 3. HPLC chromatograms of imidacloprid standard (2 μg/mL) in acetonitrile (**A**), extract of imidacloprid-free plant leaves (**B**), and extract of plant leaves fortified with imidaclopride standard (**C**).

Table 2. Recovery of Imidacloprid from Fortified Samples Determined by ELISA and HPLC

fortified concentration (μg/g)	concentration ± standard deviation (μg/g)		recovery (%, $n = 3$)	
	ELISA	HPLC	ELISA	HPLC
0	ND[a]	ND		
0.1	0.09 ± 0.01	0.08 ± 0.02	91	84
0.5	0.39 ± 0.02	0.38 ± 0.04	78	76
2	1.77 ± 0.1	2.28 ± 0.22	89	114
10	9.97 ± 0.06	10.54 ± 0.19	100	105

[a] ND, not detected.

Both the ELISA and HPLC procedures are sensitive enough to detect 0.1 ppm of imidacloprid in the leaf samples.

To validate the ELISA, correlation studies were performed. **Figure 4** shows an excellent correlation ($r^2 = 0.98$) between the results obtained by ELISA and those by HPLC analyses of samples which contained different levels of imidacloprid.

The satisfactory recovery and correlation suggested that both ELISA and HPLC methods were suitable for the analysis of imidacloprid in the leaves. However, there are some differences of pretreatment between these two methods. Compared with ELISA, sample cleanup procedures are required for HPLC analysis. In addition, HPLC requires more organic solvents and

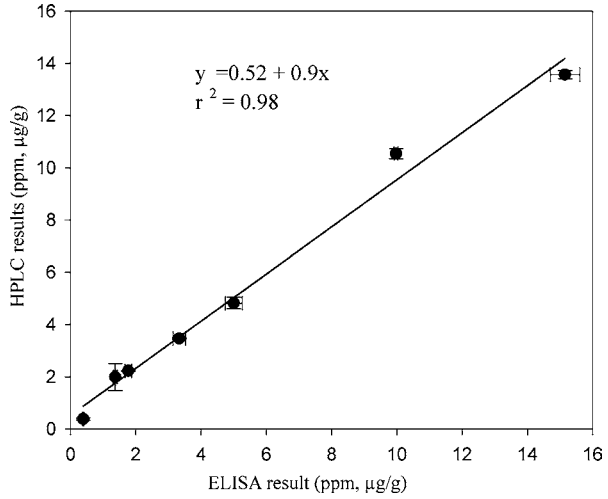

Figure 4. Correlation between ELISA and HPLC results of imidacloprid concentrations in leave samples. The error bars are standard deviations.

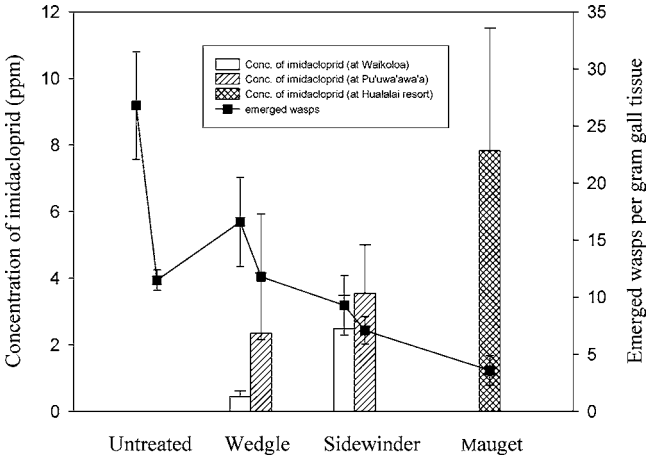

Figure 5. Correlation between treatments of wiliwili trees at different locations and concentrations of imidacloprid or emergence of the wasps. All leaf samples were collected from the middle canopy of trees. The error bars are standard deviations.

generates solvent wastes, which need proper disposal. Since ELISA has by far higher sample throughput than HPLC analysis and can fulfill the requirements for monitoring imidacloprid in the leaves, it was used to analyze the real samples.

Application to Real Samples. There had been very limited experience with imidacloprid against wasps in wiliwili trees (*21*). Expectations were highly based on knowledge of the superior performance of imidacloprid against sucking insects in various crop settings (*22*−*25*). Decision-making in pest management has traditionally relied upon field efficacy data related to a particular activity profile for any given insecticide. Thus, measuring insecticide concentrations within a plant may provide information on effective doses and help us improve wasp management. In the present study, imidacloprid was injected into trees in three different ways including Wedgle, Sidewinder, and Mauget. Imidacloprid was detected in all the samples collected from treated trees and low emergence of the wasps was observed for treated trees compared with untreated trees (**Figure 5**). Actually, no imidacloprid was detected in untreated trees. More wasps emerged from untreated trees at Waikoloa than those at Pu'u Wa'awa'a as shown in **Figure 5**. It is clear the infestation of wasps was different at two locations. Maybe that is a reason why a more significant decrease of wasps was observed at Waikoloa than at Pu'u Wa'awa'a under the same

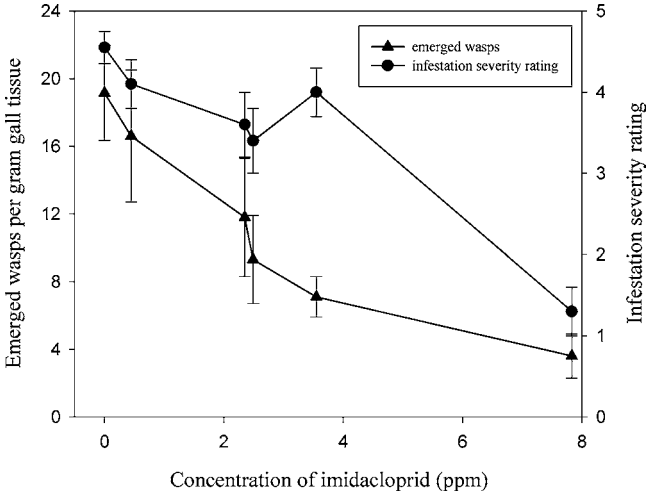

Figure 6. Correlation between concentration of imidacloprid and emergence of the wasps and infestation severity rating of samples. The error bars are standard deviations.

treatment. The concentrations of imidacloprid in the leaf samples correlated inversely with the emergence of the wasps and infestation severity rating (**Figure 6**). Trees that were treated with the Mauget Imicide microinjection capsules at 0.15 mL a.i./inch diameter were sampled approximately 3 weeks after treatment and contained the highest concentration of imidacloprid in the leaves (**Figure 5**) and consequently had the best control efficacy among the three treatment methods (**Figure 6**). Wedgle (applied at 0.026 mL a.i./inch diameter) and Sidewinder (applied at 0.15−0.2 mL a.i./inch diameter) treatments were applied during approximately the same period and were sampled between 4 and 5 months after treatment. The Wedgle system is purported by the manufacturer to provide greater efficiency of imidacloprid utilization due to the targeted nature of the injection method. The results of this study may indicate greater utilization despite lower concentration values for the Wedgle treatment. The Wedgle treatment had 1.5 and 5.6 times less imidacloprid than those treated by Sidewinder which applied 5.8−7.7 times more imidacloprid. This study focused on imidacloprid extraction and measurement for *Erythrina*. The analysis results indicate that the analytical method could be used to determine efficiency differences among injection equipment and method, efficacy thresholds, and control periods. Imidacloprid distribution in wiliwili trees was obtained to relate to injection techniques and control efficacy. The tests were carried out by analyzing the leaves collected from lower, middle, and upper canopies of the trees treated via the Sidewinder technique and the galling or non-galling leaves collected from the middle canopy. It is interesting that the concentration of imidacloprid in the leaves decreased gradually from the low canopy to the top canopy. The imidacloprid levels in the non-galling leaves from two of the three trees were much higher than those in the galling leaves (**Table 3**). The imidacloprid level in the non-galling leaves from tree-2 was slightly lower than that in the galling leaves. The data suggest field control variations. After imidacloprid was injected into trunks or main limbs, it was slowly taken up into different parts of trees.

ELISA is an effective method to quantify and monitor imidacloprid in wiliwili trees. We will continue to use this assay in our further work on gathering more basic knowledge of imidacloprid in wiliwili trees such as the nature of the exposure to wasps, its spatial and temporal dynamics, and the intrinsic susceptibility of the wasps to imidacloprid.

Table 3. Spatial Distribution of Imidacloprid in Trees with Sidewinder Treatment

source of leave samples	average concentration of imidacloprid ± standard deviation determined by ELISA (μg/g, $n = 3$)		
	Tree 1	Tree 2	Tree 3
upper canopy	1.15 ± 0.05	0.78 ± 0.05	0.59 ± 0.02
middle canopy	1.32 ± 0.13	1.34 ± 0.06	1.1 ± 0.14
lower canopy	2.18 ± 0.29	2.15 ± 0.05	1.31 ± 0.05
galling absent	1.50 ± 0.06	0.72 ± 0.01	1.03 ± 0.03
galling present	0.73 ± 0.03	1.03 ± 0.03	0.59 ± 0.03

Conclusion. A monoclonal antibody-based ELISA was used to measure concentrations of imidacloprid in wiliwili leaf samples for control of the gall wasp, *Quadrastichus erythrinae*. The satisfactory recovery of imidacloprid by ELISA and the good correlation between ELISA and HPLC results suggest that ELISA is a highly sensitive and relatively simple method to quantify imidacloprid in wiliwili tree leaves. Imidacloprid was distributed into different parts of the trees after treatment. The inverse relationship between the imidacloprid concentration and the infestation severity rating suggests imidacloprid work effectively against the wasps. The ELISA is a useful tool to measure imidacloprid for management and control of the wasps.

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