Sublethal effects of a neonicotinoid insecticide on the sharpshooter vectors of *Xylella fastidiosa*

by

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Abstract

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The neonicotinoid insecticide imidacloprid, applied in soil to grape seedlings in a series of doses, was tested on two major vectors of the bacterium *Xylella fastidiosa* in California: *Homalodisca vitripennis* (glassy-winged sharpshooter (GWSS), Hemiptera: Cicadellidae) and *Graphocephala atropunctata* (blue-green sharpshooter (BGSS), Hemiptera, Cicadellidae) for effects on longevity, possible repellency, and flight ability. The insecticide greatly reduced feeding of both insects at doses where mortality was not significantly different from untreated control insects. Imidacloprid did not affect the ratio of insects on the plant to that of off the plant, and *G. atropunctata* were found on the leaves rather than on the stems, whereas *H. vitripennis* was more frequently found on the stems. However, insecticide treatment reduced the rate of both *G. atropunctata and H. vitripennis* present on the stems. Sublethal doses did not reduce flight ability markedly and did not measurably reduce transmission of *X. fastidiosa* to grape beyond reductions in transmission ascribable to vector mortality.

Dedicated to my mother Tokue, who has never ceased to believe in me.

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Chapter 1

Introduction

Recent epidemics of Pierce's disease (PD) of grapevines (*Vitis vinifera* L.) (Sisterson 2009) and oleander leaf scorch in southern California (Bethke et al. 2001) demonstrated how the invasion of a region by a newly introduced vector insect species can drastically change the outlook of the associated diseases. After invading southern California in the late 1980s (Sorensen and Gill, 1996), *Homalodisca vitripennis* (glassy-winged sharpshooter: GWSS) was linked to a subsequent epidemic of PD in the Temecula Valley (Blua et al., 1999). The disease itself has been present in California for over 100 years (Pierce, 1892), yet had never been a problem of this magnitude since it was first noticed in the 1880s in the Los Angeles basin. In some areas, the disease so severely affected the vineyards that growing grapes commercially became extremely difficult, just as in regions of some states around the Gulf of Mexico, where GWSS is native (Perring et al., 2001).

Pierce's disease is caused by the bacterium *Xylella fastidiosa* (Hopkins, 1989). In their host plants, the bacterium is limited to the xylem, and is transmitted by xylem fluid-feeding Hemiptera, such as sharpshooter leafhoppers, spittlebugs, and cicadas (Redak et al. 2004). The transmission efficiency of *X. fastidiosa* varies widely, depending on the vector species, source and recipient plants of the bacterium, and the titer of *X. fastidiosa* cells in the source (Redak et al. 2004). *H. vitripennis* is relatively inefficient in transmission, yet its ecological and behavioral characteristics such as large population sizes in certain habitats (Perring et al., 2001), dispersal ability (Blua and Morgan, 2003), and feeding behavior that can result in transmission of X. *fastidiosa* to a dormant vine (Almeida et al., 2005) make it a major threat to the grapevines and other plant species that are fed on by GWSS and can be affected by some strain of *X. fastidiosa* (Purcell and Hopkins, 1996).

A broad range of research projects have been initiated to improve management of the disease. In order to prevent the further spread of the vector and the epidemic, control of GWSS populations through the use of insecticides constitutes one of the major pillars of such management effort. A wide variety of chemicals have been tested on GWSS (Akey et al. 2001, Bethke 2001, Wood and McBride 2001, Grafton-Cardwell 2003, Prabhaker et al. 2006 a, b, Prabhaker and Toscano 2007, Lauziere and Elzen 2007), pyrethroids and neonicotinoids found to be particularly effective. Neonicotinoid insecticides have received much attention, as this relatively new group of chemicals (first introduced to the market in 1991) move systemically in plants (Elbert et al. 2000), and have been especially effective on the sap-sucking insects in the order Hemiptera (Nauen and Denholm 2005). Insecticide treatments against GWSS, with imidacloprid as the primary agent, have reduced the number of vectors immigrating into the vineyards in the early spring, and the disease incidence has declined (Grafton-Cardwell et al. 2008). However, they were not effective for very susceptible grape varieties or for vines less than 3 years old (UC IPM Pest Management Guidelines: Grape UC ANR Publication 3448). Therefore, a closer look at how insecticide treatments work is necessary.

Characteristics of fastidious bacterial plant pathogens

There are at least 60 bacterial plant diseases (excluding phytoplasmas) that have been reported to be transmitted by insects (Harrison et al., 1980). The vectors may provide for pathogen survival (such as overwintering) and dissemination to new host plant individuals and/or species (Sigee 1993). In many cases, vector-pathogen relationships are not highly specific, nor are the pathogens entirely dependent on the presence of the vectors. However, the exceptions include the fastidious, vascular-colonizing prokaryotes that depend on insect vector transmission to infect plants (Sigee 1993). They are divided into two groups: non-circulative prokaryotes and circulative prokaryotes.

Non-circulative prokaryotes, which include *X. fastidiosa*, are the gram-negative xylem-limited bacteria (Chikowski 1987, Almeida et al. 2005). All are transmitted by Hemipteran insects specializing on xylem-feeding, which are insects in the families cercopidae, cicadellidae, machaerotidae and cicadidae (Redak et al 2004). Their transmission can occur shortly after acquisition (without an apparent latent period) and adult vectors retain infectivity for long periods, if not for life. The loss of infectivity after molting and the failure to inject the pathogen into vectors to produce vector transmission are evidence that the transmissible bacteria are located in the foregut area and are not circulative (Purcell and Finney 1979).

Circulative prokaryotes are the mollicutes (MLOs and spiroplasmas) and phloem-limited bacteria (Chikowski 1987). These pathogens, after acquisition from source plants, are able to pass through the gut wall, survive in the insect hemocoele, enter into the salivary glands, and from there enter plant vascular tissues during vector feeding. The process requires some time (latent period) during which insects cannot transmit the pathogen (Sigee 1993).

Comparison with vector-transmitted viral plant pathogens

Pathogen-vector-plant host systems involving viral pathogens have been extensively studied, as many such diseases are economically important. Despite important biological differences between viruses and bacteria, viral disease studies may provide some useful principles applicable to vector-borne bacterial pathogens, as both require inoculation by a mobile insect vector.

Nault (1997) classified plant pathogenic viruses into four classes according to the mechanism and the persistence of transmission: nonpersistently transmitted, stylet-borne viruses (NSV or NP), semipersistently transmitted, foregut borne viruses (SFV or SP), persistently transmitted, circulative viruses (PCV or CP), and persistently transmitted, propagative viruses (PPV or PP). They are described in Table 1, along with the characteristics of the bacterial pathogens for comparison. The concept has been widely accepted, and some mathematical models incorporating the class differences of viral transmission characteristics have been developed in order to better understand the vector-mediated viral plant disease epidemiology and to explore possible management options.

Model epidemiology

Jeger et al. (1998) suggested that propagative viruses, due to their need for a latent period and with a longer acquisition period, require much larger vector populations or increased vector activity to sustain an epidemic, thus reducing vector-population density or activity would be effective against them. Chan and Jeger (1994) discussed the effect of rouging (removal of diseased plants) and concluded that it would only be effective for NPs at relatively low vector-population densities. Also, rouging is likely to be ineffective when there is a continual influx of virulent vectors and no epidemic thresholds. This may explain the ineffectiveness of the diseased vine removal in the case of Pierce's disease (Almeida et al. 2005), even though it is suggested for managing within vineyard source of inoculums (UC IPM 2008).

Madden et al. (2000) expanded on the work of Jeger et al. (1998) with modeling of a range of vector migration conditions, and found that the epidemic behavior and sensitivity to model parameter changes varied greatly in different virus transmission classes. Their model indicated that small changes in vector activity (plants visited per day by an insect) greatly affected disease incidence for the NPs, whereas with CPs and PPs, even large changes in the number of plants visited had only a small effect. They also pointed out that reducing vector density would not be effective for NPs if insect mobility (plants per day) is high.

With regard to the vector migration, results depended on how many of the vectors lost by death and emigration were replaced by immigrating insects, and the fraction of those immigrants that were infected. These factors most affected persistently transmitted (once vector begins to transmit, it continues to be infectious for at days to weeks up to indefinitely) viruses (Madden et al 2000).

Changing inoculation or acquisition rates had a direct effect on the disease incidence of CPs and PPs, but required a much greater rate reduction in order to affect rates with NPs and SPs. PPs were least affected by changes in vector activity. Persistently transmitted viruses, especially the PPs, were most affected by reducing the life time of vectors, but this manipulation had no effect on the NPs unless vector density was low (Madden et al 2000).

Grilli and Holt (2000) considered the importance of the mean feeding period in relation to the mean period required for one inoculation event to occur. They suggested that genetic changes in the host or vector that induced an increased variability of feeding period would give unpredictable changes in disease dynamics.

Effects of vector and/or disease aggregation have also been considered, as it is likely to affect the assumption of constant acquisition/inoculation parameters. In a study by Zhang et al. (2000), altering the model to account for vector aggregation gave a much better fit of model predictions to the field data. McElhany et al. (1995) examined the impact of vector preference for diseased plants on the dynamics of disease spread. They found that such an effect depends on the frequency of the diseased plants in the field and on how long the disease persists. The vectors that prefer the plant condition (diseased/not diseased) not dominant in the field produced higher rates of disease spread, and persistence increased the rate of spread for vectors that preferred healthy plants more than that of a diseased plant-preferring vector. They warned that a control strategy of disrupting vector preference should be given careful thought before

implementation. Another important factor was the scale of movement, where "global" vectors can move to any host in the field, and "local" vectors can only move to the proximity of current host. They showed that the rate of disease spread by a local vector with a limited distance of movement may be decreased if the spatial patchiness of the disease is increased. With the "global" vectors, the spatial autocorrelation of disease incidents became essentially zero, resulting in randomly distributed disease.

Insecticides and plant viral disease management

Perring et al (1998) reviewed the reports of insecticide use for the management of plant virus disease spread. A high number (94 of 119 cases, 80.0%) of successes were for persistently and semi-persistently transmitted viruses, whereas most of the reported failures (32 of 48 cases, 66.7%) were nonpersistently transmitted viruses. Their reasoning for these results was that persistently/semipersistently transmitted viruses take a longer time for vector inoculation/acquisition, thus exposing the insect to sufficient doses of insecticide to kill them or alter their behavior before transmission began. For nonpersistently transmitted viruses, in which acquisition and inoculation can occur in much shorter time, the source/host plants generally are not accepted as feeding hosts by the vector; vectors leave soon after alighting and test probing, thus there is not enough time for pesticide exposure before the acquisition/inoculation to occur. Moreover, they indicated that many insecticides can actually induce unsettling behavior of insects (at least temporarily), with vectors moving from plant to plant to escape the insecticide or find a suitable host.

Perring et al. (1998) also noted that the mode of transmission affects the efficacy of insecticide on disease management. When virus spread is mainly primary (with virus brought from outside of the field by vectors already infective), treating the target crop rarely is effective, especially if large number of infective insects enter the field. This was particularly true for NPs transmitted by non-colonizing, transient vectors. In such cases, control required applying pesticides to the plants that can host the vector or virus outside the target field. Application of insecticides directly to the target crop is more effective when spread is mainly secondary (from crop plant to crop plant within the same field) and/or vectors accept the plants in the field as their feeding hosts.

Overall, effects of transmission characteristics and changes on epidemic behavior seem to stem directly or indirectly from the length of the time needed for the pathogen acquisition/inoculation and transmission latent period, although exceptions certainly warrant more detailed study. In addition to vector transmission characteristics, sources of the pathogen (pathogen host plant species and their distribution around field), disease distribution patterns, and the manner of insect movement within and between fields can be crucial information when considering management tactics.

Concerns over the sublethal effects of insecticides

In the selection of insecticide for a specific target insect, experiments are carried out to estimate each chemical's lethal dosage (LD) or lethal concentration (LC) (the dose/concentration that induce a given mortality rate) for comparison of their effectiveness. Test doses are chosen to enable an accurate estimation of those values (Priesler 2007). However, even though the primary purpose of an insecticide is to kill the target insects, it is also important to observe the chemical's effect at dose/concentration ranges sufficiently wide around the level of intended field use. Insecticides can affect a variety of behaviors at lower than lethal doses (Hoy et al. 1998). In a rare case, Simons and Blaney (1996) found that azadirachtin worked as antifeedant at higher doses, and became toxic to insect at lower doses.

There are three scenarios under which target insects are exposed to lower-than-expected titer of insecticides in the field. Firstly, shortly after an insecticide application, depending on its method and/or the applicator's handling, uneven distribution of the chemical can occur. Secondly, after an insecticide application (or the peak of its uptake by the treated plant), chemical degradation occurs within/on/around the plant, and as a result, the target insect will be exposed to a continuum of decreasing doses at different localities within a plant. Lastly, if the target insect population widely varies in its tolerance to the insecticide, then the response of individuals among the population to a certain dose/concentration may also vary. If the target insects respond differently at higher or lower doses of an insecticide, then it should be noted and interpreted in the context of the pest management.

Implication for Pierce's disease system

So, what can be drawn from the findings on vector-transmitted plant viral diseases that can be applied to PD? The PD pathogen *X. fastidiosa* is a non-circulative prokaryote that shares some transmission characteristics of SP and PP (Table 1. shaded areas). *Xylella* can be transmitted in a relatively short period of time, and has virtually no latent period, even though it increases its abundance within the foregut of the vector (Almeida et al. 2005); vectors of *Xylella* remain infective for long time, perhaps for life in adults. The shorter acquisition/inoculation time required for a successful transmission, in conjunction with feeding/probing processes, can contribute to an increased rate of disease spread, and lessen the efficacy of insecticidal control. The lack of a required latent period increases the pool of an infective vector population. The persistence can prolong pathogen spread for a vector preferring healthy plants. The dispersal ability of GWSS could make the sequential spread of disease by this vector of special concern.

Zitter and Simons (1980) identified three important questions for using insecticides as a (viral) disease management measure through vector control: (1) Can the insecticide affect the vectors fast enough to prevent their acquisition/inoculation of the viruses, (2) are there insects that have become resistant to the insecticide, and (3) can the use of a weak insecticide encourage rather than discourage the rate of virus spread? Perring et al. (1998) mentioned fast action, long persistence, and good coverage as the characteristics of insecticides that influence chemical control.

Imidacloprid is a neonicotinoid insecticide, a group of relatively new insecticides that act on the insect central nervous system as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Bai et al. 1991). It depolarizes and blocks synaptic transmission, causing uncoordinated movement, paralysis, and tremor (Nauen 1995). Its effect on reducing the GWSS population is well recognized, and the chemical has been used in area-wide treatment programs (Hix et al., 2003). At a whole tree scale with citrus, the distribution of imidacloprid within the plant was found to be uniform (Castle et al., 2005), but it is also known that on smaller plants, concentration of the chemical in upper and lower leaves vary (Mendel et al. 2000, Laurent and Rathahao 2003, Olson et al., 2004). Uneven distribution of the chemical can also result from poor application techniques, insecticide metabolism, plant growth (Nauen and Denholm 2005), or condition of the plant (Cloyed 2002, Byrne and Toscano 2005). Because of the high chances that vectors can encounter lower, sublethal doses of imidacloprid in field, knowing the effect of such sublethal doses on the insects may be useful. In this study, I investigated some effects of sublethal doses of imidacloprid on the two major *X. fastidiosa* vectors in California, *Homalodisca vitripennis* (glassy-winged sharpshooter, Hemiptera: Cicadellidae) and *Graphocephala atropunctata* (bluegreen sharpshooter, Hemiptera, Cicadellidae), and the implications for disease management through vector control with this insecticide.

SUMMARY OF CHAPTERS

Most of the chapters and all details of the original research conducted for this dissertation are presented in the format of separate manuscripts to be submitted for publication. This approach unavoidably results in some repetition or redundancy. The final chapter (Chapter. 6) summarizes main points of the dissertation and integrates the findings in an overall discussion.

Chapter 2 describes a series of dosage-mortality experiments with imidacloprid tested on GWSS and BGSS, in order to observe the effects of the different level of chemicals on insect mortality and behavior. This established the standard doses used for later experiments that explore the details of the behavior of imidacloprid-affected insects.

Chapter 3 explains how, using the selected doses from the previous chapter, the effects of imidacloprid on insect feeding and flight were further investigated, and the host selection of the vectors was tested with or without the choice of imidacloprid treated/untreated plants.

Chapter 4 describes studies of GWSS flight using flight mills to quantify the effect of imidacloprid on individual insect. Also, the control insects in this study provide basic information on GWSS flight.

Chapter 5 summarizes the result of all experiments, and discusses the implications of experimental results with sublethal doses of insecticide for the spread of Pierce's disease.

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Transmission characteristic	Virus transmission group				Fastidious prokaryotes transmission group	
	NSV	SFV	PCV	PPV	Non-circulative	Circulative
Acquisition/inoculation time	Seconds, minutes	Minutes, hours	Hours, days	Hours, days	~Hours	
Retention time	Minutes	Hours	Days, weeks	Weeks, months	Until molt/months	
Transstadial passage	No	No	Yes	Yes	No	
Pathogen in vector hemolymph	No	No	Yes	Yes	No	Yes
Latent period	No	No	Hours, days	Weeks	No/short	2-6 weeks
Pathogen multiplied in vector	No	No	No	Yes	Yes	Yes
Transovarial transmission	No	No	No	Often	No	Only 2 pathogens reported
Insecticide control of epidemics	Lower success	Higher success	Higher success	Higher success		

Table 1. Comparison of transmission characteristics of virus and fastidious prokaryotes. Shaded area indicates the characteristics shared by X. fastidiosa, a non-circulative fastidious bacterium and viruses.

NSV: nonpersistently transmitted, stylet-borne viruses

SFV: semipersistently transmitted, foregut borne viruses

PCV: persistently transmitted, circulative viruses

PPV: persistently transmitted, propagative viruses

Chapter 2

Dose-dependent effect of imidacloprid on the mortality, feeding behavior, and flight of *Homalodisca vitripennis* and *Graphocephala atropunctata*

Abstract

The systemic insecticide imidacloprid was applied to potted grape seedlings in a series of doses to test its effects on mortality and behavior of two major sharpshooter vectors (Hemiptera: Cicadellidae, Cicadellinae) of *Xylella fastidiosa* in California, *Homalodisca vitripennis* (glassy-winged sharpshooter) and *Graphocephala atropunctata* (bluegreen sharpshooter). The insects were caged with grape seedlings treated with different doses of imidacloprid, and their location within the cage, feeding status, and mortality were recorded. The number of feeding insects showed significant differences among doses, but those non-feeding insects remained on the plants. This suggests that the insects are not repelled by imidacloprid under no-choice conditions. At 0.05 mg imidacloprid/380g soil, insect mortality was not significantly different from that of untreated controls but insect feeding activity was reduced on average by over 60%. However, most of these insects survived throughout the three to five-day test period; this is longer than the time that the insects are known to survive without feeding. In addition, most of the minimally feeding insects retained the ability to fly at the end of the experiments. By 9 hours after the insect introduction, the numbers of either BGSS or GWSS on stems was higher for control plants than for other doses, indicating a possible avoidance of imidacloprid-treated stems by the insects. Sublethal dosages of imidacloprid that markedly reduce feeding may also reduce their energy consumption, thus limiting their dispersal potential and other traits.

Introduction

The xylem-limited bacterium *Xylella fastidiosa* (Wells et al., 1987) infects a large number of host plant species, most of which show no symptoms in response to *Xf* infection (Freitag, 1951; Hopkins and Purcell 2002). Some strains of this bacterium, however, can cause serious diseases in agricultural and ornamental plants such as citrus, coffee, grape, peach, plum, and oleander (Hopkins, 2005, Hearon et al., 1980; Chang et al., 2009). The bacterium is transmitted from plant to plant by xylem feeding insect vectors in the order Hemiptera, namely sharpshooter leafhoppers (subfamily Cicadellinae in leafhopper family Cicadellidae), spittlebugs (family Cercopidae), and cicadas (family Cicadidae) (Almeida et al., 2005).

In California, Pierce's disease of grapevine (PD) has been recognized since the 1880s (Gardner and Hewitt 1974). Three regions of the state have been recognized as having different

key vector insect(s) for PD: the blue-green sharpshooter (BGSS) (*Graphocephala atropunctata* (Signoret)) on the North Coast, the green sharpshooter (*Draeculacephala minerva* Ball) and the red-headed sharpshooter (*Xyphon fulgida* (Nottingham)) in the Central Valley, and the glassy-winged sharpshooter (*Homalodisca vitripennis* (Germar)) in parts of Southern California (Sisterson, 2009). Among these, the glassy-winged sharpshooter (GWSS), a native of the southeastern United States and northern Mexico (Martensson, 2007), is a newly introduced species in Southern California detected in the late 1980s (Sorensen and Gill, 1996). This vector caused two PD epidemics in Southern California and the southern portion of the Central Valley (Sisterson, 2009). These new epidemics differed from the ones caused by the native sharpshooter species; although both native (Purcell, 1976) and invasive (Lauziere et al., 2008, Northfield et al., 2009) sharpshooters immigrated into vineyards from the surrounding vegetation, native vectors produced chronically-diseased vines only at the edges of vineyards (Purcell 1974) while the disease incidence caused by GWSS was higher and distributed further into vineyards (Blua and Morgan, 2003). These differences prompted new control strategies targeting multiple aspects of host-pathogen-vector-environment relationships (Almeida et al. 2005). The primary target of GWSS control was to reduce the spread and number of the vectors through chemical and biological control.

Chemicals from all of the major insecticide classes (e.g., insect growth regulators, repellents) have been screened against different life stages of GWSS (Akey et al. 2001, Byrne and Toscano 2007, Grafton-Cardwell et al. 2003, Lauziere and Elzen 2007, Prabhaker et al. 2006a, b, Prabhaker and Toscano 2007, Puterka et al. 2003, Tubajika et al. 2007). Pyrethroids and neonicotinoids were found to be the best in terms of their rapid killing and long persistence in the environment. Among these, a systemic neonicotinoid insecticide, imidacloprid, became the main agent for managing *Xylella* vectors in both commercial agriculture and urban landscapes (UC IPM website, accessed 12-08-2009, Glafton-Cardwell et al. 2008). So far, the management program in Southern California has successfully suppressed GWSS population and the spread of Pierce's disease. In addition to its insect mortality, imidacloprid has also been shown to affect feeding by GWSS (Bethke et al, 2001), and some impact on deterring *Xylella* transmission has been reported (Bethke et al. 2001, Krewer et al. 2002).

However, comparison of the efficiency of pathogen transmission by different vector insects (Redak et al. 2004, Daugherty and Almeida 2009) and by simple mechanical inoculation with needle puncture indicates that connections between insect feeding, pathogen transmission and disease establishment are not simple linear relationships (Bextine et al. 2004, Jackson et al. 2008, Daugherty and Almeida 2009). For example, GWSS transmission from grape to grape is about 5-15% per insect per day under lab conditions (Almeida and Purcell 2003), but GWSS delivery of bacteria during closely monitored feeding experiments was 100% (Backus 2006), indicating the complexity of these relationships. The genetics of *X. fastidiosa* are also critical to its vector transmission. Knock-out mutants of the bacterium with no functional cell-cell signaling system are not vector transmissible (Chatterjee et al. 2008) and strains of *X. fastidiosa* vary markedly in vector transmissibility and even in vector specificity (Lopes et al. 2009). Therefore, the impact of imidacloprid's anti-feeding effect on PD transmission is not yet clear.

Another aspect of imidacloprid's possible impact on the PD epidemics is through its effect on vector flight. Imidacloprid is an agonist of nicotinic acetyl chorine receptors that depolarizes and blocks synaptic transmission at the postsynaptic membrane of cholinergic

synapses (Bai et al. 1991). Typical symptoms for the insects administered with imidacloprid are uncoordinated movement, paralysis, and tremor (Nauen 1995). With BGSS and GWSS, intoxication can be observed as twitching of the hind legs. As leafhopper insects (Hemiptera, Auchenorrhyncha, Cicadellidae), they use their long hind legs to propel their jumps, which frequently launch flights (Burrows 2007). Therefore, if imidacloprid interferes with controlled movement of the hind legs, it may also interfere with flight ability. As flight is directly involved in the dispersal of the vector, and thus the pathogen, this may lead to affecting the PD epidemiology.

Imidacloprid formulations (Admire®, Bayer CropScience, NJ) can be applied to soil, from which it is taken up systemically and moved throughout plants. Residues of the chemical can remain in soil and provide prolonged efficacy (Oi, 1999, Elbert et al, 2008). However, as Almeida (2005) showed, the threat of pathogen transmission by GWSS can be year-round, even during winter, when the concentration of the chemical to which the insects are exposed would be much lower than initial concentrations (Byrne and Toscano 2007). Sublethal doses of imidacloprid may also result from its uneven distribution within a plant. Mendel et al. (2000) used a radioactive tracer to find that the active substance was preferentially supplied to new shoots. As the ecological safety of imidacloprid and other neonicotinoids comes into question (Department of Pesticide Regulation Notice, 2009), more detailed evaluation of its effects and the potential for minimal use of the chemical become more desirable.

The objectives of this study were to 1) evaluate mortalities of the BGSS and GWSS vectors at varying dosages of imidacloprid to determine how mortality changes with dosages, 2) evaluate the residual effects of sublethal doses of imidacloprid over several weeks, and 3) observe the responses of the insects in feeding, flying and insect location on plants at sublethal dosages. Throughout this work, we refer to the "dose" as an amount applied to the test plant unit, as the actual intake by insects could not be measured. We were particularly interested in the lower doses, where the mortality may be the sum of direct toxicity and weakening by food deprivation, which may not have been relevant at higher doses where insects were killed quickly. We expected imidacloprid to remain effective for an extended period, and the anti-feeding effect to starve the insects, reducing their flight ability and potentially make them abandon the plant as a feeding host.

Materials and Methods

Insects and plants

We collected BGSS from wild grapevines (*Vitis californica* Benth.) in an urban park in Berkeley, California. After capture, the animals were maintained in a greenhouse insectary on California mugwort [*Artimisia douglasiana* Bess. Ex Hook (Asteraceae)]. We collected GWSS from citrus groves and eucalyptus trees southeast of Edison, California, and maintained a breeding colony in an insectary on a mixture of grapes, mugwort and basil [*Ocimum basilicum* L.

(Lamiaceae)]. We germinated grape seedlings (*V. vinifera*, cv. Cabernet sauvignon) in a flat of vermiculite and transplanted the seedlings into UC soil mix (50% peat moss and 50% sand) after at least two true leaves emerged. Two weeks before insecticide treatments, we selected the seedlings of similar sizes (about 18 cm), trimmed the roots to 5 cm length from the base of the main stem, and transplanted each seedling into 380 g of soil (air-dried weight, 360 ml round plastic pot), collected from a single location in a Napa Valley vineyard. The soil type was loamy sand/sandy loam with a low organic matter content (%C = 2.3), and imidacloprid adsorption to the soil was expected to be low. We watered the plants just enough to not remove imidacloprid from the pot, and thus most of the applied chemical should have remained available to the roots.

Time-dosage-mortality tests on BGSS and GWSS

We made a stock solution of imidacloprid (Admire®2F, Bayer Co., Kansas City, MO, 21.4%AI) at 0.75 g Admire®2F per liter of water (0.16 mg imidacloprid/ml solution); subsequent dilutions of imidacloprid were prepared from this stock. We applied 20 ml imidacloprid solution of a designated concentration to the soil containing test plants; we were careful to avoid wetting the above-ground portion of plants since the chemical can also act as a contact insecticide. For the same reason, we covered the surface of the potted soil with a 5mm layer of coarse sand just before the experiments started in order to prevent the insects from having direct contact with the treated soil. We watered plants twice daily, just enough to wet the soil, without flushing water from the bottom of the pot. For the time-dose-mortality experiment, plants were used two weeks after the treatment to optimize systemic uptake.

Experiments typically began at about noon. At the start of the experiment, we transferred ten adult insects (five females and five males) to each test plant, which was then individually covered within a plastic tube cage inserted into the soil. The doses given to the plants were 3.2, 0.8, 0.2, 0.05, and 0 (control) mgAI/pot, and there were five replicates per dose. We observed insects hourly for up to six hours after the starting time, and then after 24, 48, 72, 96, and 120 hours in the greenhouse insectary (under natural lighting, 22 – 35 C˚). We did not remove the bodies of dead insects, as it would have disturbed the surviving insects too much and influence the later results. The lethal concentrations (LC) and lethal time (LT: time to achieve certain % of mortality) values were calculated using probit and complementary log-log (CLL) analysis (Robertson et al. 2007). In addition, at the time of the observation, we recorded the insects' location (ground - and whether dead or alive - cage, stem, petiole, leaf blade). Locations were compared with Fisher's exact test, first over all doses, and if the result was significant, the same test was conducted on each pair of doses.

Residual effect of sub-lethal dose imidacloprid

To calculate the change in toxicity of a dose (LT_{50}) over time following application, the plants were treated with 1.6, 0.8, 0.4, 0.2, 0.1, and 0 mg AI/pot with 3 replications per dose, and were used at 1, 3, 5, and 7 weeks after the treatment, with the same preparation and test procedures described above. The results were analyzed with CLL analysis.

Effect of sublethal doses of imidacloprid on GWSS Flight ability

We conducted three sets of the tests in which insects were exposed for different lengths of time to plants treated with different doses of imidacloprid. The specific treatment regimes used were as follows: 1) 3 weeks after imidacloprid application to the plant, 72 hour exposure, 2) 1 week after application, 120 hour exposure, 3) 3 weeks after application, 168 hour exposure. The doses used were 1.6, 0.8, 0.4, 0.2, 0.1, and 0 mg imidacloprid per 380 g (air-dried weight) soil. After the designated exposure time, we brought the cages into an enclosed room and performed the following procedures on a bench that was located in front of a south-facing window and that was enclosed on both sides and the ceiling by a smooth surface painted bright yellow, to which the insects are attracted to (Patt and Setamou 2007). We removed the plastic cage from each plant, and counted the number of insects that flew. Insects that did not fly spontaneously upon the cage removal were individually picked up from the plant by hand and thrown about 30 cm into the air to stimulate flight. We classified insects that did not fly after being tossed into the air three times as "non-fliers". The frequency of fliers was analyzed with Fisher's exact test, and if it was significant, then pairwise comparisons were used to distinguish where the differences occurred. This was a crude flight assay, and we did not quantify flight performance.

Feeding activity

During the dosage-mortality experiments on BGSS and GWSS, we used visible excretion as an indicator of feeding and recorded the number of "feeding" and "non-feeding" sharpshooters at each observation time. "Feeding" meant that we saw evidence of excretion in the form of visible droplets being expelled from the anus of the insect or of accumulated moisture behind or below the insect. Excretion is not a direct sign of active ingestion, but Dolezal et al. (2004) observed that intense ingestion was often preceded by excretion. They also found that stylet insertion is not necessarily the sign of ongoing feeding. Therefore, we assumed active excretion as an indication of feeding, realizing that some minor ingestion without visible excretion could have occurred. The counts of feeding and non-feeding individuals were analyzed with Fisher's exact test, and if it was significant, then pair-wise comparisons were used to detect where the differences occurred.

Statistical analyses

All statistical analyses were conducted using the statistical computing environment R (R Development Core Team, 2009).

Results

Time-dosage-mortality tests on BGSS and GWSS

Figures 1 and 2 show how the cumulative mortality rate of BGSS and GWSS increased over time at different doses, and the LD_{50} and LD_{90} values are shown on Table 1. The doses used ranged from 3.2 mgAI/pot that resulted in 100% mortality to 0.05 mgAI/pot that did not differ significantly (judging by the overlaps of LT_{50} 95% CI (Table 2) from mortality for insects on control (untreated) plants. For both insect species, we noted mortality as early as 2 hours after the insect entered the cage; some insects were observed on the ground as early as 1 hour after introduction to plants treated with the highest dose ($\geq$ 0.8 mg AI/pot) of imidacloprid. Insect mortality varied among the plants within dose group. For instance, at the highest dose, three out of five plants had 90-100% mortality for BGSS by 24 hour after insect introduction, but the other two plants at that dosage still had 4 or 5 insects alive at the end of that time interval. This gives an average $\pm$ SD mortality rate of 0.8 ± 0.235. In addition, one GWSS remained alive for at least 72 hours on the highest dose plant. Only the highest dose, 3.2 mg imidacloprid/pot, killed 100% of both BGSS and GWSS within the 120 hrs of observation. The next highest dose, 0.8 mg imidacloprid/pot killed all GWSS but not all BGSS. We also note the high control mortality of GWSS after 48 hour and BGSS after 72 hour may have reduced the sensitivity of statistical analyses (Fig. 1 and 2).

At 24 hr, LD_{50} (Table 1) was higher for BGSS, but at 48 hr, the value was similar for both insects. With GWSS, the slope of the probit line did not change for 24 and 48 hr, but BGSS had a steeper slope for the 48 hr probit line. From the comparison of the LT_{50} 95% confidence intervals (CI), (Table 2), the values at the highest dose were similar for both insects, but it was lower for the GWSS at the two smaller doses. For either vector species, 95% CI of mortalities at the control treatment and the 0.05 mg imidacloprid/pot dosage overlapped. Therefore, the "sublethal" dosages in a strict sense (i.e. not causing a detectable increase in insecticide-induced death) should lie somewhere below 0.2 mg AI/pot. The range of 0.2 – 0.8 mg imidacloprid/pot may also be considered sublethal in the sense of very low mortality. The LC_{50}, LC_{90}, and dose-mortality slopes calculated from standard probit analyses are shown on Table 1 for reference.

Residual effect of sub-lethal dose imidacloprid

The transition of LT_{50} value for each dose from 1 to 7 weeks after the treatment is displayed on Fig. 3. The LT_{50} decreased or remained about the same for 1.6 mg imidacloprid/pot over 7 weeks after application, but for all other lower doses the LT_{50} increased substantially after $\geq$ 5 weeks following application.

Effect of sublethal doses of imidacloprid on GWSS Flight ability

The number of survivors and the number of surviving insects that could fly are shown in Table 3. At imidacloprid doses resulting in larger numbers of survivors, most (> 75%) of individuals could fly. Even at the dose (≥ 0.4 mg AI/pot) resulting in the highest mortality, the remaining small number of individuals maintained their flight ability. The surviving insects were either able to fly or were nearly immobile. Therefore, there was no evidence that low-lethality doses of imidacloprid inhibited flight activity by itself, without affecting the overall condition of the insect

Feeding activity

At all doses, the effect of imidacloprid on feeding behavior was dramatic and immediate. With few exceptions (BGSS at 1 and 3 hr, GWSS at 1h after the insect introduction), significantly ($p << 0.001$) fewer BGSS and GWSS fed on treated plants at all doses (Figs 4 and 5). Even at the lowest dose of 0.05 mg imidacloprid/pot, where there was no difference in mortality compared to controls, feeding by both insect species decreased drastically. BGSS feeding on imidacloprid-treated plants mostly diminished after 3 hrs. With GWSS, the number of feeding insects at the lowest dose (0.05 mgAI/pot) decreased by about 50 % compared to the control, but feeding was observed throughout the observation time. At the highest dose, both GWSS and BGSS ceased all external signs of feeding even after one hour of being introduced onto treated plants.

Insect location on plant

For most of the post-introduction observation period, Fisher's exact tests did not detect significant differences ($p < 0.05$) in the ratio of insects on plants versus on the cage among the different doses, including untreated controls, for either BGSS or GWSS (Figs. 6, 7, and Table 4). With GWSS, significant differences between any two doses did not persist to the next observation (Table 4). For BGSS, significant differences occurred from 3 to 5.5 hr after insect introduction, where control and the lowest dose 0.05 mg AI/pot had a lower ratio of insects on the cage than the other doses, and at 48 hr, where 0.8 mg AI/pot had a higher ratio of insect than the other lower doses.

When we compared the ratio of GWSS on stems to those on leaves (in either leaf blade + petiole (Table 5) or leaf blade only (data not shown)), the control consistently had a significantly higher ratio of insects on the stem compared to other doses (Table 5, Fisher's exact test, $p < 0.05$); the only exception occurred at the first hour and at 72 hr post-introduction,. With BGSS, there were no significant differences for the initial 6 hours (Table 5, Fisher's exact test, $p < 0.05$), but the control had significantly higher ratio of insects on the stem for the observation period after that.

Discussion

Both BGSS and GWSS are vigorous feeders, excreting droplets of waste at rates of 0.2 ml/insect per hour (Mittler 1967) and 0.09-0.27 ml/insect per hour (Andersen et al. 1989), respectively. Although feeding by itself rarely causes damage to the plants, it is an entry point of the pathogen into the host and reducing feeding rates can impact PD spread. In this study, imidacloprid's anti-feeding effect was observed within a few hours of insect introduction, even at the lowest dose at which mortality didn't differ from that for untreated controls. This suggests that the insects detected the chemical at such sublethal levels, and possibly lower for BGSS, as the lowest dose still almost completely deterred them from feeding. Whether the insects halted feeding to avoid the chemical or from getting too intoxicated to continue feeding is not clear from this experiment. However, the doses used here were lower than the application rate in most of the previous studies (Akey et al. 2001, Byrne and Toscano 2007, Lauziere and Elzen 2007, Prabhaker et al. 2006a, b, Prabhaker and Toscano 2007, Puterka et al. 2003, Tubajika et al. 2007), and the speed of killing was much slower. At the lower doses, the mortality did not reach 100% within our observation period, which extended beyond the time these insects can survive without feeding. If the insects were too intoxicated to feed, their death might have occurred sooner, at the higher rates.

With a systemic insecticide with anti-feeding effects, distinguishing the cause of mortality is difficult. Unless the chemical is also a repellent, some amount of it will be ingested during the initial test feeding. However, as the titer in the plant decreases, or the insect acquires some physical (i.e. resistance by detoxification) or behavioral (i.e. avoidance) tolerance to the chemical, the relative contribution of direct toxicity versus starvation from anti-feeding effect becomes difficult to distinguish. In our study, at the highest dose, many insects were already on the ground at 1 hour after introduction, so the lack of feeding may simply have been a sign of intoxication. However, many of the insects survived beyond four days, the period for which GWSS normally can survive without feeding at this range of temperature (Scott and De Barro 2001). This suggests that the insects were either 1) feeding at a reduced rate (possibly on plants parts that were less toxic) or 2) not feeding, but were in a physiological state that required less food. The first scenario may be supported by the results of our insect location experiment, in which insects on the imidacloprid-treated plants tended to be found more often on leaf blades. The quantification of imidacloprid titer in citrus leaves by Byrne and Toscano (2007) showed that the concentration of imidacloprid in different leaves from the same tree varies. Therefore, if insects can find an acceptable and less-toxic feeding site before becoming intoxicated, they may be able to survive. The second scenario may be explained by the findings of Marshall et al. (2006). In their study, GWSS feeding diminished below 15 C˚, yet the time it took for 50% death was longer than 5 days, and up to 11 days at 6 C˚, possibly due to the reduced activity of the insect. If the detection of imidacloprid in the plant caused the insects to switch their physical condition to something similar to that in low temperature, then the insects may survive longer without feeding. Of course, these tactics may be occurring at the same time, therefore it is necessary to monitor the insects feeding more closely in order to discern the specific effect of sublethal imidacloprid.

Uptake and persistence of systemically applied imidacloprid in the field to the trees of different plant species showed similar trends among themselves, with peak titer appearing at 2 to 8 weeks post-treatment and effects sustained for up to 10 weeks post-treatment (Byrne and Toscano 2006 & 2007, Byrne et al. 2005, Castle et al. 2005). The uptake was most rapid at the highest rate of the application and with younger vines (Byrne and Toscano 2006). Byrne and Toscano (2005) also noted that soil and irrigation practices can affect imidacloprid uptake. In our study with potted seedlings, insect mortality increased up to 3 weeks after the treatment for all doses, but started to decline by the 5th week, except for the highest doses which sustained their efficacy through all 7 weeks of experimental period. This is somewhat in line with the previous studies on potted plants (Bethke et al. 2001, Grafton-Cardwell et al. 2003), where high mortality was already achieved at 1 week after treatment and was sustained for 4 to 8 weeks. A difference may be that at a very low application rate, the peak of uptake may occur sooner than at a higher rate. Whether this is due to the fundamental uptake potential at different doses or differences in imidacloprid metabolism in the plant or the soil is not yet known. In any case, these different factors should be considered when designing a bioassay system.

The high mortality on control plants observed in this study means that test plants of this size, with the designated number of insects per plant, may not be appropriate for measuring mortality over extended periods. This is understandable, as *H. vitripennis* is a highly polyphagous insect and selects and thrives on nutritionally preferred hosts (Brodbeck et al. 1990). The insects often reject unfavorable hosts even when there are no other hosts available, resulting in a lower survival on such plants (Brodbeck et al. 2007). Nevertheless, Hummel et al. (2005) have demonstrated that *H. vitripennis* can develop and reproduce when confined to a single host and bioassays of systemic insecticides with (possible) anti-feeding effects should run for longer terms, beyond the insect's starvation threshold. The use of larger test plants may ameliorate this issue, but then higher within-plant heterogeneity in insecticide distribution and any accompanying insects' responses to the insecticide may increase in importance.

Bioassay systems to evaluate systemic insecticides that have an anti-feeding effect require longer time intervals (several days) following insecticide application than most dosage-mortality assays and may not distinguish death due to direct toxicity from indirect effects of the toxins such as severely reduced feeding. Field tests and tests with laboratory plants would still constitute important parts of the insecticide study. However, in order to compare different populations on the same standard, variability arising from things other than the insects (e.g., different soils and individual plants, especially if they are not genetically identical) should be minimized. In addition, the relative contributions of intoxication versus anti-feeding effects on insect mortality should be measured. This is especially important if one tries to detect insecticide resistance among the populations, as insecticide tolerance resulting from behavioral changes can be greatly influenced by the test system and obscure the test results. For such purposes, a feeding system with sharpshooter food with known imidacloprid concentrations would be ideal. Unfortunately sharpshooter feeding on artificial diets is usually poor, with high mortality (>50%) in less than a day (Purcell, A. and Finlay 1979). If a monitoring and recording device such as electrical penetration graph can be added to the system, then the exact step by step procedure of feeding can be analyzed (Backus et al. 2009) in relation to the insect response to the insecticide and further, the pathogen transmission. Through such studies, the true effect of anti-feedant such as imidacloprid on disease transmission could be elucidated, and the chemical's potential can be better utilized.

In this study, insects were confined in cages, and did not have a choice to move to another host plant. Whether the insects would abandon the imidacloprid treated plants that they do not actively feed on, in the presence of alternative host plant nearby, would be the next question. Also, whether the insects would learn to avoid the plant they once fed on and abandoned is an important issue in terms of chemically managing vector population in vineyards. Finally, the most question remains as to whether this anti-feeding effect of imidacloprid, especially at the sublethal dose, can decrease transmission of *X. fastidiosa* to host plants. Using the sublethal dose range obtained in this study, these studies should be conducted.

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Table 1. Probit analyses for the vector mortality of systemic (soil-applied) imidacloprid on grape

Insect	Exposure time	n	Slope ± SE	LD_{50} (95%CL)	LD_{90} (95%CL)
BGSS	24 h	50	0.86 ± 0.09	2.15 (1.83 - 2.47)	3.64 (3.11 - 4.18)
	48 h	50	2.36 ± 0.34	0.73 (0.60 - 0.87)	1.28 (1.02 – 1.54)
GWSS	24 h	50	1.26 ± 0.17	1.32 (1.04 – 1.61)	2.34 (1.81 – 2.87)
	48 h	50	1.28 ± 0.25	0.79 (0.56 – 1.02)	1.79 (1.22 – 2.37)

LD50 values are expressed in units of mg imidacloprid per 380g soil.

Table 2. LT_{50} (SE) for the vector mortality at each dose of systemic (soil-applied) imidacloprid on grape

	Dose (mgAI/pot)				
Insect	3.2	0.8	0.2	0.05	0
BGSS	14.77 (0.84)	70.34 (2.61)	102.78 (3.11)	140.19 (8.76)	160.69 (16.12)
GWSS	13.85 (1.41)	45.00 (1.89)	76.16 (2.49)	106.19 (3.33)	116.99 (3.88)

LT_{50} values are expressed in units of hour.

Table 3. Flight ability of GWSS after exposure to grapes systemically treated with imidacloprid.

Dose (mg imidacloprid /380 g soil)	72 hr exposure, 3 wk after treatment		120 hr exposure, 1 wk after treatment		168 hr exposure, 3 wk after treatment	
	% survivor	% flier	% survivor	% flier	% survivor	% flier
1.6	0	-	0	-	0	-
0.8	13.3	10.0a	20.0	3.3b	0	-
0.4	30.0	20.0b	10.0	3.3b	13.3	6.7
0.2	13.3	6.7b	66.7	60.0a	20.0	16.7
0.1	100	86.7a	76.7	76.7a	43.3	43.3
control	96.7	93.3a	60.0	60.0a	60.0	53.3

The flier numbers followed by the same letters or have no letters are not significantly different ($\alpha = 0.05$). The analyses were done on the counts, in terms of the ratio of flier and non-flier, with Fisher's exact test.

Table 4. Significant Fisher's exact test results on insect location: plant v. cage

Insect	Time after insect introduction (hr)	Dose 1 (mg AI/pot)	Dose 2 (mg AI/pot)	p-value
BGSS	3	0.2	Control	0.026
			0.05	0.002
			0.8	0.029
			3.2	0.020
	4	Control	3.2	0.007
			0.8	0.010
			0.2	0.026
		0.05	3.2	0.007
			0.8	0.010
			0.2	0.026
	5	0.05	3.2	0.011
			0.8	0.040
	48	0.8	Control	0.017
			0.2	0.019
GWSS	6	3.2	Control	0.003
			0.05	0.009
			0.2	0.023
	12	3.2	0.05	0.027
		0.8	Control	0.026
			0.05	< 0.001
			0.2	0.007
	96	0.8	Control	0.034
			0.05	0.031

Table 5. Significant Fisher's exact test results on insect location: stem v. leaf + petiole

Insect	Time after insect introduction (hr)	Dose 1 (mg AI/380g soil)	Dose 2 (mg AI/380g soil)	*P*-value
BGSS	9	control	0.2	0.026
	24	control	0.8 0.2 0.05	0.005 < 0.001 < 0.001
	48	control	0.2 0.05	< 0.001 < 0.001
	72	control	0.2 0.05	0.003 0.022
	96	control	0.2 0.05	0.002 < 0.001
GWSS	2	control	3.2 0.8	0.008 0.004
	3	control	3.2 0.8	< 0.001 < 0.001
	4	control	3.2 0.8 0.2 0.05	< 0.001 < 0.001 0.011 0.027
	5	control	0.8 0.2	< 0.001 0.009
		0.05	0.8	< 0.001
		0.2	0.8	0.027
	6.5	control	3.2 0.8	0.018 < 0.001

			0.2	0.012
		0.05	0.8	0.003
		0.2	0.8	0.022
	9	control	0.8 0.2 0.05	< 0.001 < 0.001 0.002
	12	control	0.8 0.2 0.05	0.010 < 0.001 0.005
	24	control	0.8 0.2	0.004 0.001
	48	control	0.2 0.05	0.011 0.040
	96	control	0.05	0.003

Table 5. Continued

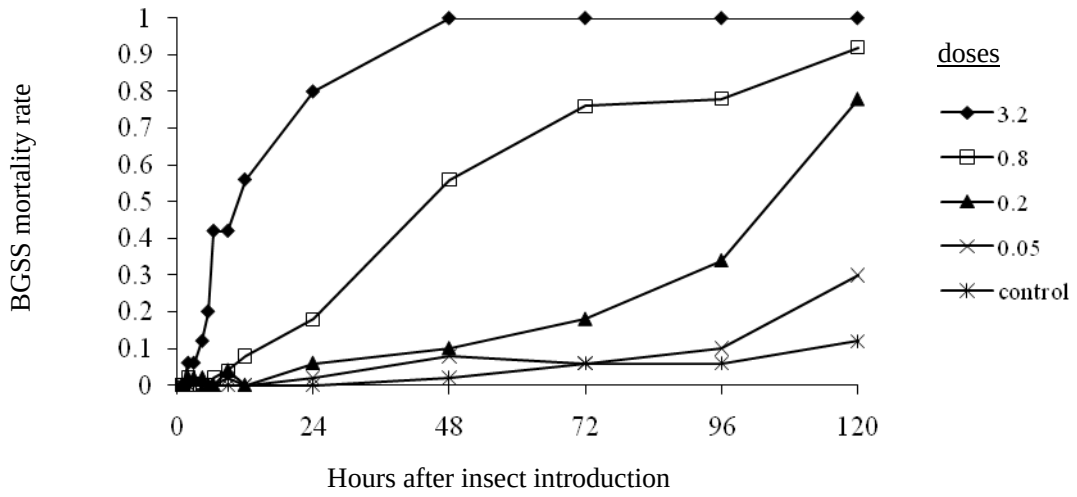


Fig. 1. Cumulative mortality rate of BGSS exposed to different amounts of imidacloprid. Unit of doses are expressed in mg imidacloprid/380 g soil.

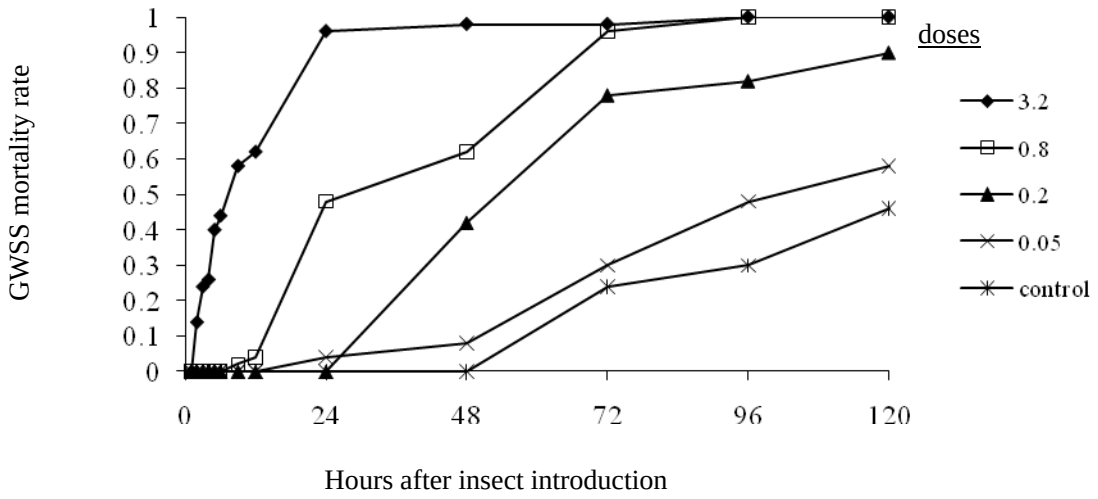


Fig. 2. Cumulative mortality rate of GWSS exposed to different amounts of imidacloprid through systemically-treated grape seedlings at different doses.

Units of doses are expressed in mg imidacloprid/380 g soil.

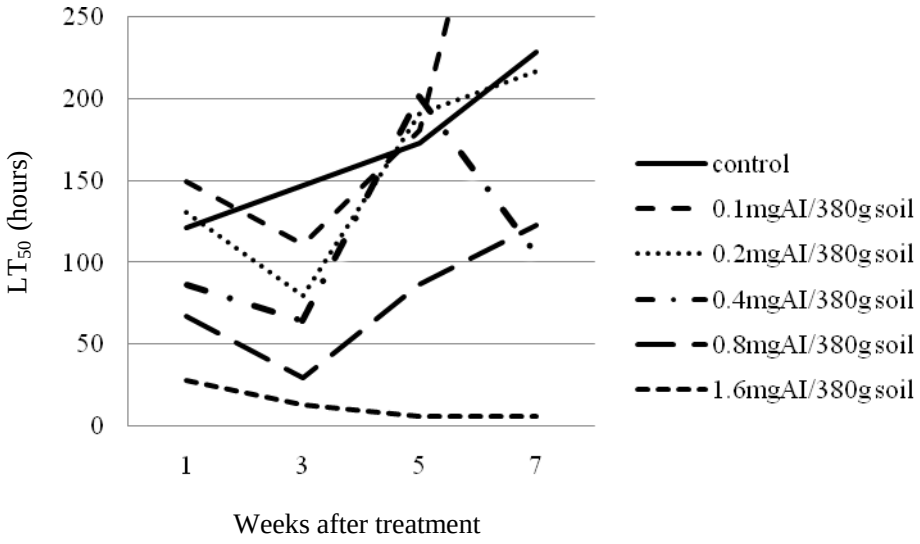


Fig. 3. Transition of LT_{50} for GWSS at different doses, from 1 to 7 weeks after the systemic imidacloprid application to the grape seedlings.

Units of LT_{50} are expressed in hours after insect introduction.

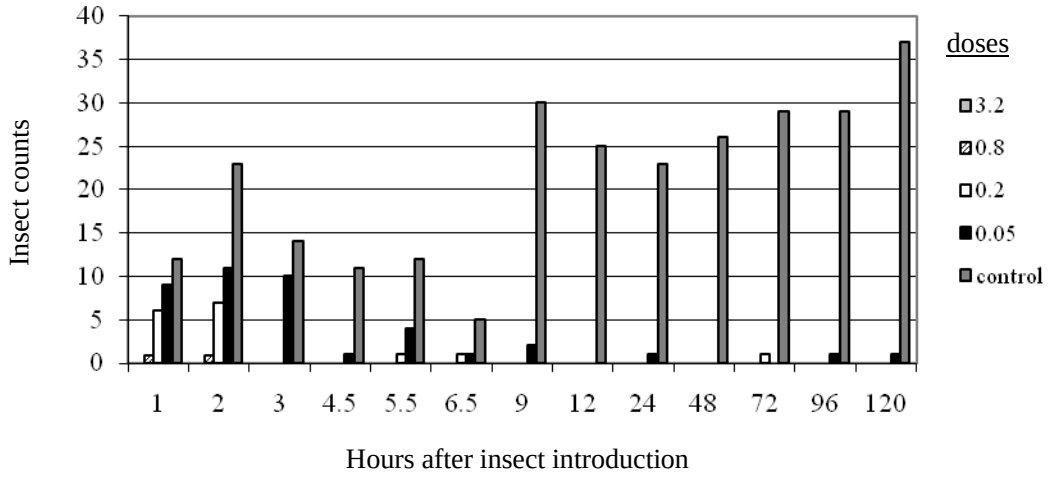


Fig. 4. Number of excreting BGSS exposed to different amounts of imidacloprid through grape seedlings systemically-treated with different doses.

n = 50 test insects per dose.

Units of doses are expressed in mg imidacloprid/380g soil.

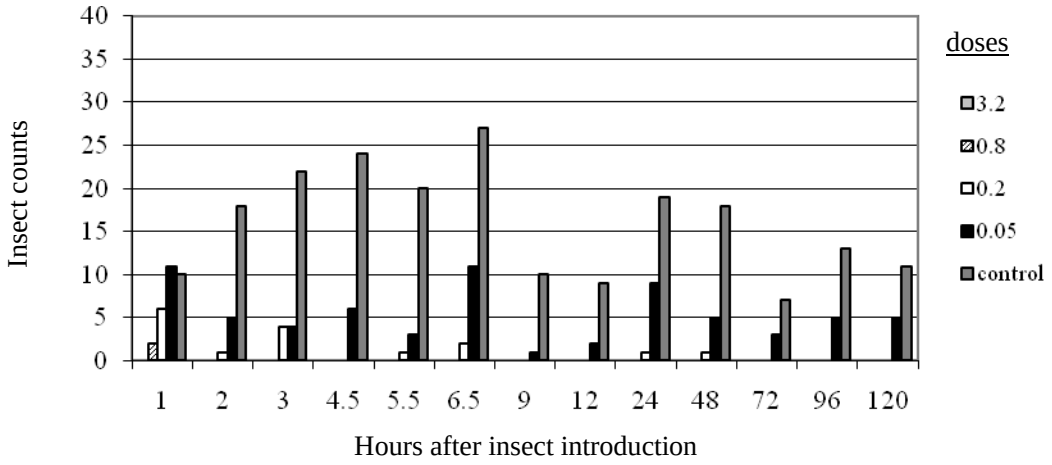
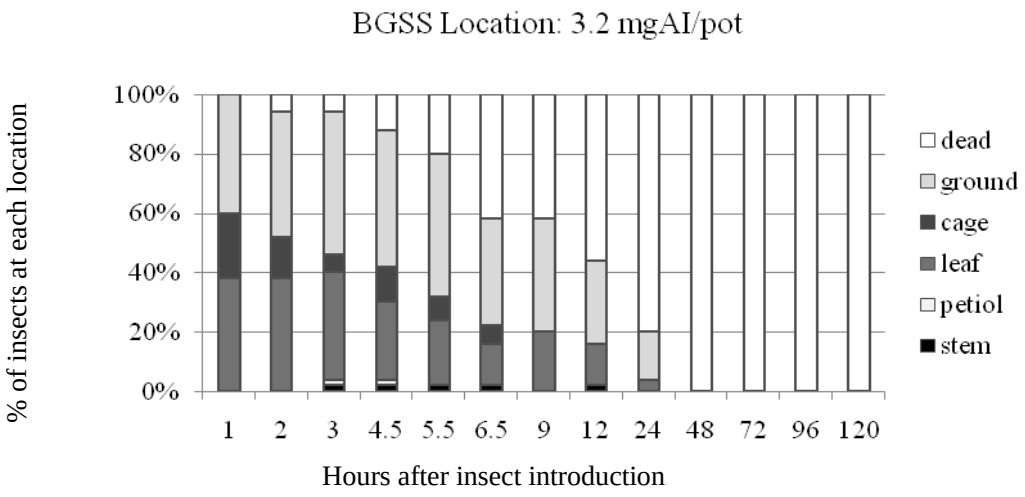
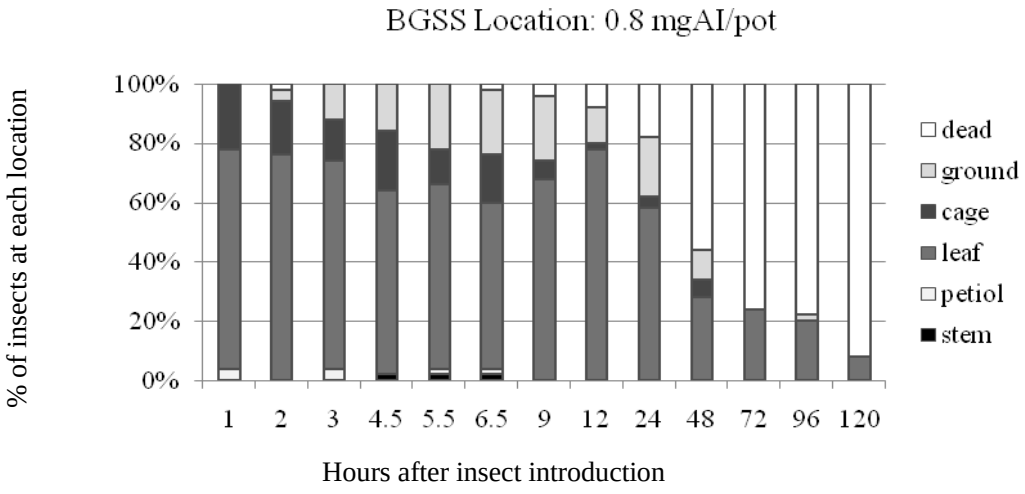


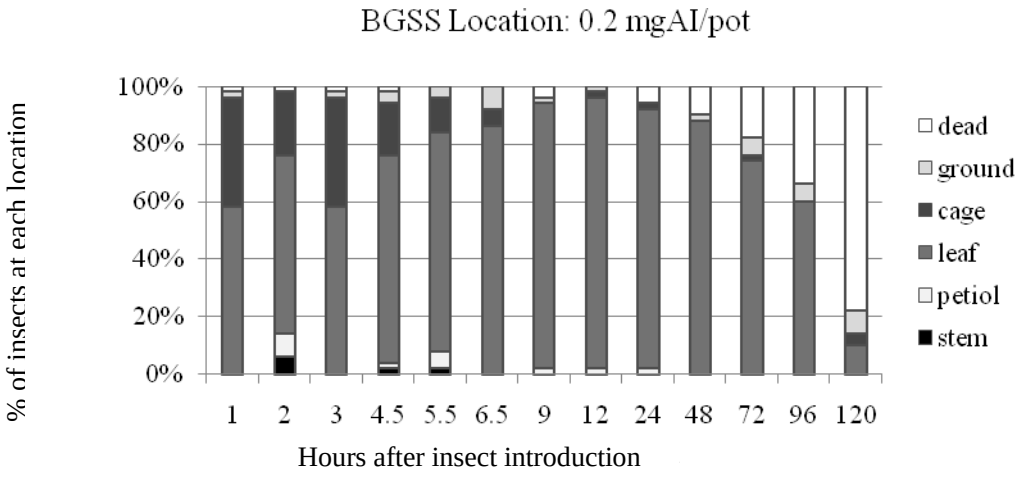
Fig. 5. Number of excreting GWSS exposed to different amounts of imidacloprid through grape seedlings systemically-treated with different doses.

n = 50 test insects per dose.

Units of dose are expressed in mg imidacloprid/380g soil.


BGSS Location: 3.2 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem


BGSS Location: 0.8 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem


BGSS Location: 0.2 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem

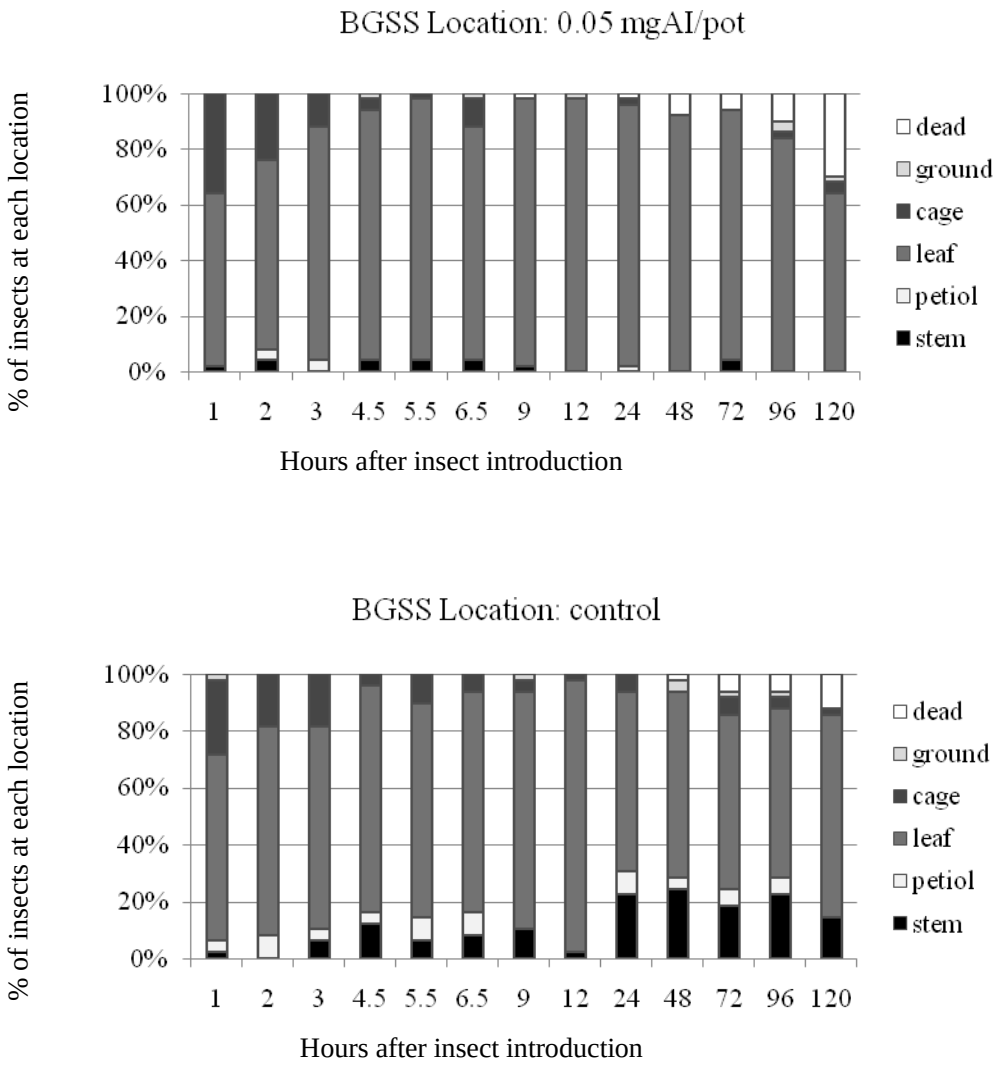
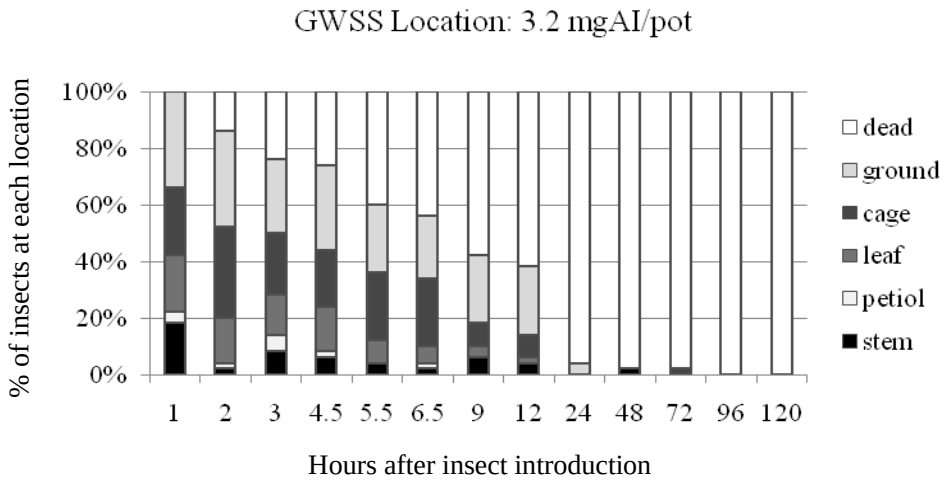
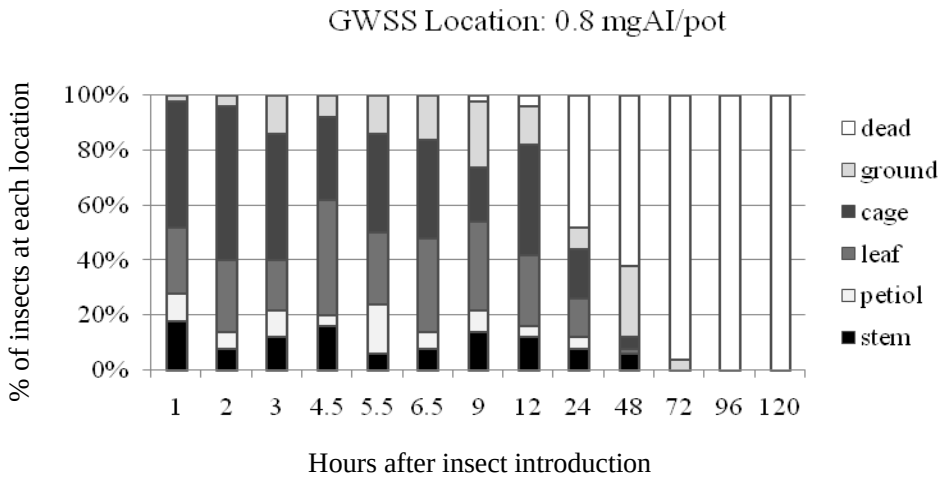


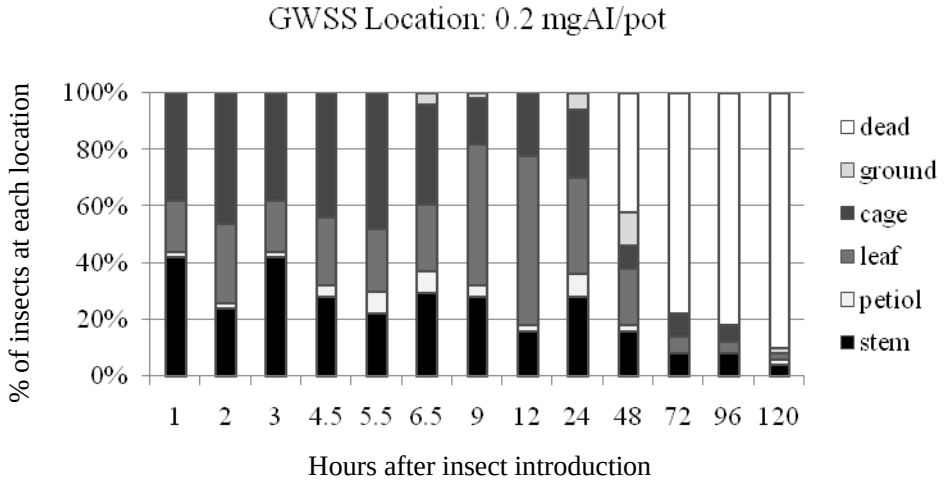
Fig. 6. Location of BGSS exposed to grape seedlings that were systemically-treated with different doses of imidaclorpid.

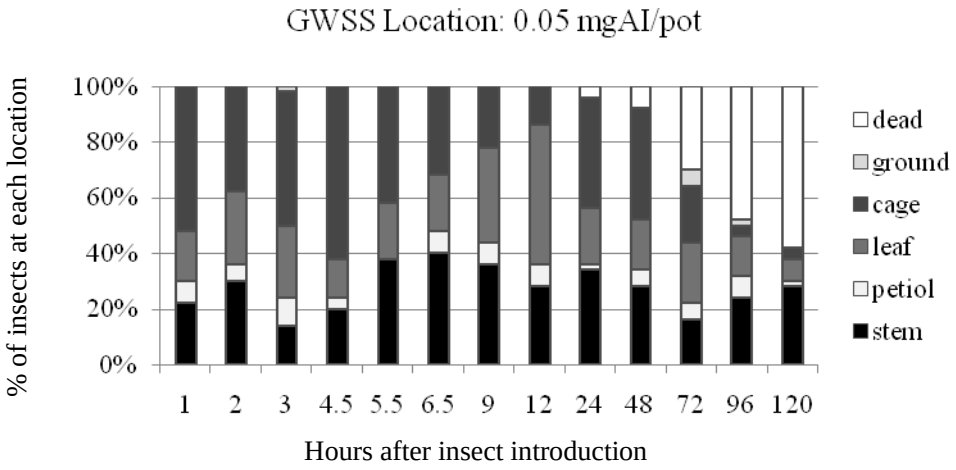
n = 50 test insects per dose.

Units of doses are expressed in mg imidacloprid per 380g of soil.


GWSS Location: 3.2 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem


GWSS Location: 0.8 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem


GWSS Location: 0.2 mgAI/pot
% of insects at each location
100%
80%
60%
40%
20%
0%
1 2 3 4.5 5.5 6.5 9 12 24 48 72 96 120
Hours after insect introduction
dead
ground
cage
leaf
petiol
stem



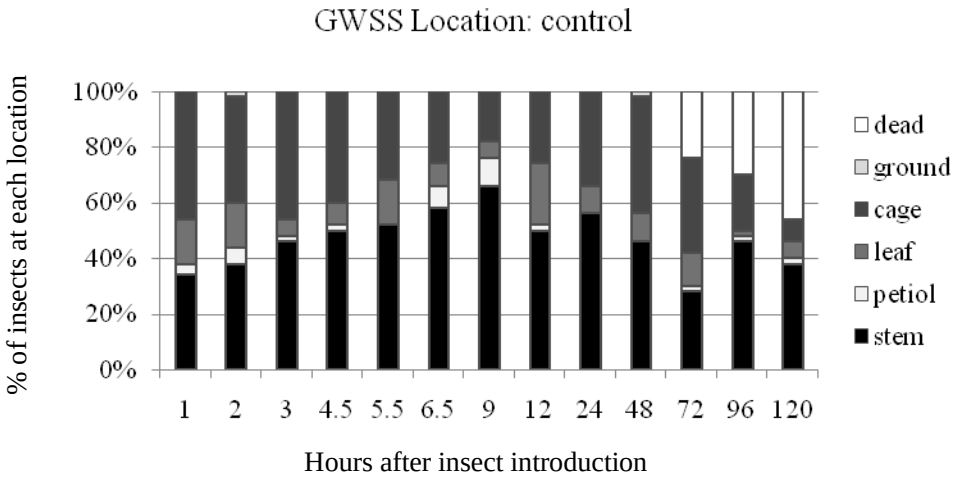


Fig. 7. Location of GSS exposed to grape seedlings that were systemically-treated with different doses of imidaclorpid.

n = 50 test insects per dose.

Units of doses are expressed in mg imidacloprid per 380g of soil.

Chapter 3

Effects of imidacloprid and kaolin particle film on host preference by *Homalodisca vitripennis* and *Graphocephala atropunctata* and transmission of *Xylella fastidiosa*

Abstract

Imidacloprid is a necotinoid insecticide that is widely used in California for control of the glassy-winged sharpshooter (*Homalodisca vitripennis* Germar. Hemiptera: Cicadellidae), an important vector for Pierce's disease (PD) of grapevines. This insecticide is well known for its effectiveness against piercing-sucking insects and has been employed against a large variety of pest insects and of diseases spread by insect vectors. In order to better understand how this pesticide works against PD, we compared the relative effects of imidacloprid's sublethal effects on *H. vitripennis* and another native vector *Graphocephala* atropunctata (Signoret) with respect to their host acceptance and transmission of *Xylella fastidiosa* (*Xf*), the causal bacterium of PD. For comparison, a known repellent of *Xf* vectors, kaolin particle film (Surround®, Engelhard Corp. NJ), was also tested. In laboratory trials, the kaolin film repelled insects from landing on plants. The incidence of PD among kaolin-covered plants was significantly lower than that for controls. Even when given a choice between imidacloprid-treated and untreated plants, vector mortality increased with time, suggesting that the insects were not discriminating between treated and untreated plants. Transmission experiments provided no evidence that sublethal doses of imidacloprid substantially reduced the transmission efficiency of *H. vitripennis*. The observed efficacy of imidacloprid against PD appears to be most likely due to its lethality to vectors.

Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) is an invasive insect in California (Sorensen and Gill 1996) that is an important vector of Pierce's disease (PD), a lethal disease of grapevines that is caused by the bacterium *Xylella fastidiosa* (*Xf*). As part of efforts to control PD, the insecticide imidacloprid has been applied to soils for systemic uptake by plants in GWSS breeding areas and vineyards. Because of the low caloric value of xylem sap and the high energy outlays required to ingest it, xylem sap-feeders that are vectors for PD must ingest huge volumes of xylem sap (Mittler 1967; Raven 1984). Thus xylem sap-feeders such as GWSS should be especially sensitive to systemic insecticides applied to the soil for uptake through roots.

Historically, insecticidal control of PD vectors has been of little or no use in reducing PD spread. Application of DDT to vineyards during the late 1940s was not effective in reducing the

spread of PD (Hewitt et al. 1949). Experiments in Napa Valley during the 1970s showed that insecticidal reduction of populations of the blue-green sharpshooter (BGSS) vector *Grapholacephala atropunctata* (Signoret) outside vineyards during early spring (i.e. before or as the vector moved into vineyards from adjacent riparian habitats) reduced the subsequent spread of PD in some but not all grape varieties (Purcell 1979). In sum, insecticidal control of PD vectors has been erratic and of limited success in PD prevention.

Fortunately, the imidacloprid applications and careful monitoring in response to the newly-introduced GWSS have so far successfully suppressed the vector population and subsequent spread of PD (CDFA, 2009). Still, the threat of pathogen transmission by GWSS can be year-round, even during winter (Almeida 2005), when the concentration of the chemical to which the insects are exposed would be much lower than initial concentrations (Byrne and Toscano 2007). In addition, there are concerns of insecticide resistance buildup and ecological safety of neonicotinoid insecticides (CDPR 2009); therefore, it is important to study this chemical's effect at a wider range of application rates.

Sublethal doses of imidacloprid sharply reduced feeding activity by GWSS (Bethke et al. 2001 and Ch 2 of this dissertation). This raises a number of questions. First, does this reduction in feeding directly lead to reduced transmission rates (transmission efficiency) by the vector? Second, does the anti-feeding effect make the vectors avoid the plant as a feeding host, thus making imidacloprid work as a repellent? Third, if it does, would it lead to increased plant to plant movements of the vector searching for acceptable hosts within vineyards? Increased vector movement among vines may increase the number of vector visit per vine and increase the chance of pathogen acquisition/inoculation. In such a case, imidacloprid may counteract PD management, if it fails to kill the insects quickly enough before they could acquire the causal bacterium or inoculate it into plants.

However, a repellent doesn't necessarily increase pathogen transmission. A sprayable suspension of kaolin clay particles (Surround®, Engelhard Corp. NJ), which has proven to be highly repellent to some insect pests of tree crops (Glenn et al. 1999, Puterka et al. 2000), proved to be effective against GWSS in field trials (Puterka et al. 2003, Tubajika et al. 2007) and was claimed to reduce the spread of PD (Tubajika et al. 2007). By coating the plant surfaces with a thin layer of mineral barrier, kaolin particle film deters insect activities, such as feeding and oviposition (Puterka et al. 2003). When the insects were given no choice but kaolin-coated plants to feed on, they were not able to settle on the plants and died faster (Puterka et al. 2003). Although there was no description of whether the vectors actually engaged in any feeding activity on the treated plants, or the coating simply eliminated vector landing, the reduction of PD incidence observed in the study suggests that the repellency and reduced feeding caused by kaolin treatments led to decreased spread of PD. Therefore, it would be worthwhile to test whether imidacloprid's anti-feeding effect at sublethal doses is comparable to that of this known repellent.

In our previous study, the test insects were confined in small tube cages together with the test plant, thus the insects were to some extent forced to be on the plant. The objectives of this study were to 1) investigate whether the anti-feeding effect of imidacloprid makes the insects abandon or avoid the plant when either an increased space and/or a choice of untreated plants are available 2) compare the behavior of insects exposed to imidacloprid-treated plants to that of

insects exposed to a repellent-treated plants, and 3) determine whether the anti-feeding effect of imidacloprid can reduce *Xf* transmission by the vectors at sublethal doses. For these purposes we observed imidacloprid's effects on host acceptance by GWSS and BGSS of grapevines systemically treated with imidacloprid. To address the effects of sublethal doses of imidacloprid on vector efficiency in transmitting *Xf* to grape, we evaluated transmission rates at several dosages of the insecticide, both in choice (with both treated and non-treated plants) and no-choice (with either one of treated and non-treated plants only) conditions. We tested GWSS and BGSS, the latter of which is less threatening than GWSS but more efficient in *Xf* transmission. Because the insects quickly stop feeding on treated plants yet retain their flight ability after such exposures (Ch. 2), we expected that with the presence of alternative host plants, the insects would move to other, untreated plants. In comparison with the kaolin-coated plants, the major difference between the two is whether they can repel insects from landing on the plant or not. Depending on how well the kaolin prevents landing, and how the combined effect of reduced feeding and potential changes in plant visit frequency, some difference in their ability to prevent pathogen transmission was expected. We were particularly interested in how imidacloprid might induce behavioral changes and how these might affect PD transmission.

Materials and Methods

Insects and plants

We collected BGSS from wild grapevines (*Vitis californica* Benth.) growing in an urban park in Berkeley, California, after which the insects were maintained in a greenhouse insectary on California mugwort [*Artimisia douglasiana* Bess. Ex Hook (Asteraceae)]. We collected GWSS from citrus groves and eucalyptus trees southeast of Edison, California. Captive, breeding colonies of this species were maintained in the same insectary on a mixture of grapes, mugwort and basil [*Ocimum basilicum* L. (Lamiaceae)]. We germinated grape seedlings (*V. vinifera*, cv. Cabernet sauvignon) for experimental trials in a flat of vermiculite; seedlings were transplanted into UC soil mix (50% peat moss and 50% sand) after at least two true leaves emerged. Two weeks before insecticide treatments, we selected seedlings of similar sizes (about 18 cm), trimmed their roots in the shape of 5 cm diameter half-sphere and transplanted each of them into 380 g of test soil (air-dried weight; collected from a single location in Napa Valley vineyard) in a 10 cm diameter (360 ml) round plastic pot. Because the soil type was loamy sand/sandy loam with low organic matter content (2.3% C) imidacloprid adsorption to the soil organic matter was expected to be small; because water was not added to excess, most of the applied chemical should have been available to the roots.

Prior to the experiments, the insects for transmission tests were put in cages with PD-symptomatic grapes for 4 days to let them acquire *Xf* from the plant. This duration has been shown to be sufficient to ensure the insects' pathogen acquisition, and about twice the number of insects actually needed for the experiments was placed on the symptomatic plant, to account for the mortality that occurred during the acquisition period. The PD plants were shaken daily to

encourage the insects to change feeding locations within the diseased plant to increase the chances of *Xf* acquisition.

Comparison of effects of imidacloprid and kaolin coat on host acceptance and *Xf* transmission by GWSS and BGSS.

We set 8 arenas (60 x 60 x 45 cm) constructed of thin white mesh cloth, each containing 2 plants, inside in a greenhouse insectary. There were 3 different combinations of grape seedlings (*Vitis vinifera*, Pinot Noir) available in the enclosures: 2 arenas each of No-choice (+/+, 2 treated plants per arena) and No-choice (-/-, 2 untreated plants per arena), and 4 arenas of Choice (+/-, one treated and one untreated per arena). The treatments applied here were either imidacloprid (Admire 2F®, Bayer CropScience LP. NC), 0.2mgAI/pot applied to the soil 7 days prior to the experiments) or Surround WP (6% kaolin, sprayed for full coverage the day before the experiments).

We released 4 insects (GWSS or BGSS) in the center of each arena for the imidacloprid experiments, and 4 GWSS or 2 BGSS per arena for the kaolin-coat experiment. For the latter experiment, it was difficult to locate the BGSS in arenas due to the coating and the mesh cover, so the number of insects per arena was reduced. The insects were marked with a spot of color so that each individual could be distinguished. Observations of the insect location (on either plant, or on arena) and mortality occurred at 10 min, 30min, and 1, 2, 3, 6, 9, 12, and 23 hrs after the insects were released. The experiment was replicated 7 times for the kaolin-coat tests, and 4 times each for the imidacloprid tests for each vector species.

. For 4 of the replications on each insect, detailed data on their location on the plant were also recorded. The data from all replications were compiled for each insect species. We compared the number of insects on treated plants and that on untreated plants in two ways: 1) Between the treated/untreated plants from the two no-choice (+/+ and -/-) tests and the choice (+/-) test, and 2) Between the treated/untreated plants within the choice (+/-) test, using t-test. The insect mortality counts were compared at the end by Fisher's exact test. After each experiment, the test plants were maintained in the greenhouse for subsequent assessment of PD by culturing, following the dilution plating method of Hill and Purcell (1995). We used Fisher's exact test to compare the numbers of diseased/healthy plants for each treatment. This test was chosen instead of the chi-square test, as the incidences of PD was very low for the latter test.

Effect of imidacloprid treatement on the transmission of *Xf* by GWSS

For tests of the effects of imidacloprid applications on *Xf* transmission by GWSS, we treated grape seedlings with 0.8, 0.1 and 0 (control) mgAI/pot of Admire 2F®, as described previously. A week after the application, each plant was covered with a plastic cage and 4 GWSS were introduced to the enclosure. The insects had been previously fed on grapes infected with PD for two days to let them acquire *X. fastidiosa*. At 1, 2, 3, 4, 5, 6, 9, 12, and 24 hours after introduction, the insect locations and the presence/absence of excretion (emission of the

small droplets from the tip of the abdomen) by each insect were recorded. After 24 hrs, the insects were removed and the plants were set aside for later diagnosis of PD.

Statistical analyses

All statistical analyses were conducted using the statistical computing environment R (R Development Core Team, 2009).

Results

Comparison of effects of imidacloprid and kaolin coat on host acceptance and *Xf* transmission by GWSS and BGSS

Effect of kaolin coat on the host acceptance and mortality of BGSS

The mortality of the BGSS after 23 hr was markedly higher in the no-choice arena with kaolin-coated plants being significantly higher than in no-choice untreated control arenas (FET, $P = 0.02$) or choice arenas ($P = 0.01$) (Fig. 1). The choice arena mortality was not significantly different from those of no-choice, untreated only arenas (FET, $P = 0.65$) (Fig. 1). In the comparison of the number of BGSS on plants (regardless of treatment) in each arena category, no-choice, kaolin-treated plants only arena was significantly lower than the untreated plants only arenas at 1, 12 and 23 hr after insect introduction (t-test, $P = 0.019$, 0.007, and 0.02, respectively) (Table 1). The numbers at 6 hr and in arena category combinations other than the above did not differ significantly (Table 1). Within the choice arenas, comparison between the number of insects on kaolin-treated and that on untreated plants revealed that there was a strong tendency for the insects to land and remain on the control plants throughout the observations (t-test, $P = 0.007$ at 1 hr, and $P << 0.001$ thereafter) (Fig. 2). However, the number of insects per kaolin-treated plant did not differ between choice and no choice arenas (Table 2).

On arenas containing at least one kaolin-treated plant, there were 5 or 6 insects that were never observed landing on a plant, whereas all of the insects in no-choice, untreated plants only arenas landed on a plant at some point (Table 3). However, such difference was statistically significant only between the two no-choice arenas (FET, $P = 0.02$). Once landing on a plant, either kaolin-treated or untreated, the insects did not actively move elsewhere. When the BGSS on no-choice, kaolin-treated plants only arenas landed on a treated plant, only 2 out of 11 insects moved away from it (Table 3). In the choice arenas, 3 insects out of 26 that ever landed on a plant were observed on kaolin-treated plants: one remained on the plant, another one moved to an untreated plant, and one moved from an untreated to a treated plant (data not shown). Statistical significance was not tested for in these results, as the number of these events was very low.

Effect of kaolin coat on the host acceptance and GWSS mortality

Mortality of the GWSS at 23hr in the no-choice arena with kaolin-coated plants was significantly higher than in the no-choice untreated control arenas (FET, $P = 0.001$) and choice arenas ($P = 0.0001$)(Fig. 3). The mortality rate was similar, at about 40% of that of BGSS, adjusting for the mortality in the no-choice, untreated only arenas. The choice arena mortality was not significantly different from those of no-choice, untreated only arenas (FET, $P = 1$) (Fig. 3). In the comparison of the number of GWSS on plants (regardless of treatment) in each arena category, no-choice with kaolin-treated plants only arena was significantly lower than the untreated plants only and choice arenas at 12 and 23 hr after insect introduction (t-test, $P = 0.0002$ and 0.002 at 12 hr, and $P = 0.013$ and 0.04 at 23 hr, respectively) (Table 4). There were no significant differences at 1 and 6 hr after insect introduction and between no-choice, untreated only arenas and choice arenas (Table 4). Within the choice arenas, comparison between the number of insects on kaolin-treated plants and that on untreated plants revealed that there was a strong tendency for the insects to land on and remain on the control plants throughout the observations (t-test, $P = 0.02$ at 1 hr, and $P << 0.001$ thereafter)(Fig. 4). Similar to the results from BGSS experiment, the number of insects per kaolin treated plant did not differ between choice and no choice arenas (Table 5). More than half of the insects released in the no choice, kaolin-treated plants only arenas were not observed to be on a plant throughout the experiment (Table 6). There were no significant differences among the arenas on the number of insects that landed on a plant then moved away, or the number of insects that switched between the two plants within an arena (Table 6).

Effect of imidacloprid treatment on the host acceptance and mortality of BGSS

Mortality data among the arenas with three different combinations of treated and untreated plants were not consistent, and a Fisher's exact test of 23 hr data was not significant (Fig. 5, $P = 0.93$). In two of the four experiments, no insect died in any arenas. The numbers of BGSS on plants (regardless of treatment) remained nearly constant throughout the post-introduction sampling period, and there was no significant difference among the arena categories (ANOVA, $P = 0.87$, 0.70, 0.6, and 0.66 at 1, 6, 12, 23 hr, respectively) (Table 7). Within the choice arenas, there was no difference between the number of BGSS on the imidacloprid-treated and untreated plants ($P = 0.33$, 0.85, 0.68, and 0.68 at each 1, 6, 12, and 23 hr, respectively) (Fig. 6). There were only a few insects that were never observed on a plant (Table 8). The number of location changes or plant switching by the insects did not differ among the arena categories ($P = 0.54$ and 0.81, respectively) (Table 8).

Effect of imidacloprid treatment on the host acceptance and mortality of GWSS

Mortality of GWSS in no choice, imidacloprid-treated plant arenas and choice arenas were similar and higher than that in no choice, untreated plants only arenas (Fig. 7). This result was reflected in the per arena number of insects on plant data, as the number became lower toward the end of the observation period for the former two arenas because more insects died in them (Table 9). Consequently, at 23hr, per arena number of insects was significantly higher for

the no choice, untreated plants arenas than the no choice, treated only arenas (t-test, $P = 0.01$) and choice arenas ($P = 0.01$). Within the choice arenas, initially there were no significant differences between the number of GWSS on the treated plant and that on the untreated ones (t-tests at 1 and 6 hr, $P = 0.22$ and 0.21, respectively), but at the end of the observation, the number of insects was higher for the untreated plants ($P = 0.03$) (Fig. 8). After landing on a plant, GWSS moved away to somewhere else in the arena or to the other plant more actively than other insect-treatment combinations, especially in the no choice, imidacloprid-treated plants only or choice arenas (Table 10). Among the 26 plant switches by the insects in choice arenas, 9 moved from an untreated plant to an imidacloprid-treated plant, and 17 moved the other way around. Three insects switched location (at least) twice, each of which was counted as one switching, and those all landed on an untreated plant first, moved to a treated plant, then back to the untreated plant again (data not shown).

Effect of kaolin coat and imidacloprid on *Xf* transmission

GWSS did not transmit PD to any of the 112 kaolin treated/un-treated plants ($n = 56$ plants for each of them). With BGSS, which had half the number of insects per arena compared to the GWSS experiment, 13 plants tested positive for PD, among the total of 64 plants tested. Two of the infected plants were kaolin-coated plants in the no choice, kaolin-coated plants only arenas, and none of the kaolin-coated plants in the choice arenas was diseased. Among the untreated plants, 4 from the no choice, untreated plants only arenas ($n = 28$ plants), and 7 from the choice arenas ($n = 28$ plants) became infected. Therefore, the largest difference was between the treated and untreated plants within the choice arenas, and this difference was the only one that was statistically significant ($P = 0.01$).

There was no significant difference between the number of PD plant among the imidacloprid-treated plants and that among the untreated plants, for either vector species. With BGSS, 5 treated plants and 3 untreated plants were infected, out of 32 plants each (Fisher's exact test, $P = 0.67$) (the information on whether the infected plant came from choice or no-choice arenas were lost). With GWSS, 6 imidacloprid-treated plants were infected, half of which were from no-choice arenas (with treated plants only) ($n = 16$ plants), and the other half were from choice arenas ($n = 16$ plants). There were 8 untreated-plants infected, 3 of which were from no-choice, untreated only arenas, and 5 were from choice arenas. There was no significant difference among these combinations of treatment and arena category (Fisher's exact test, $P = 0.87$)

Effect of imidacloprid treatment on the transmission of *Xf* by GWSS

In each of the three experiments, the higher doses achieved over 50 % mortality by 24 hr after insect introduction, and the lower doses either had low or comparable to control plants (Fig. 9 - B, E, and H). Except for the lowest dose (0.1 mg AI/pot) tested, GWSS feeding on imidacloprid-treated plants diminished within 3 hr (Fig. 9 – C, F, and I). The PD infection rate relationships among the treatment groups were not consistent, but in general, *Xf* transmission

rates of imidacloprid-treated plants were either lower than that of untreated control plants or not significantly different from it (Fig. 9 – A, D, and G).

Discussion

In accordance with the previous studies (Puterka et al. 2003, Tubajika et al. 2007), kaolin particle films on the grape seedlings reduced the number of BGSS and GWSS landing on the plants. Although the significant differences were found only after > 12 hrs post insect introduction, this may be due to the time it took for the insects to settle after the disturbance of handling and the small sample numbers. However, we should note that the number of insects on the treated plants in the no-choice, treated only arenas did not decrease even after the insect mortality of over 40% occurred. When the insects were given no choice but kaolin treated plants, they did land on these plants, and did not move away from the treated plants. In the case of BGSS, transmission of *Xf* did occur, though only in few cases, indicating that the insects actually fed on the kaolin-treated plants. The difference between the per plant number of insect on the kaolin-treated plants from choice and no choice arenas was not significant; however, none of the kaolin-treated plants from choice arenas were infected, whereas 2 treated plants out of 16 were infected in the no-choice arenas. In fact, all of the *Xf* transmission in the choice arenas occurred on untreated plants, which was statistically significant. This may suggest that kaolin coat can be more effective in preventing PD in vineyards where alternative host plants are nearby.

In contrast, BGSS landed on the imidacloprid treated and untreated plants similarly. Their mortality was low, and the number of diseased plants among the treated and untreated plants was not significantly different. The number of GWSS on imidacloprid treated plants eventually become significantly lower than that on untreated plants, but this seems due to the mortality from the insecticide reducing the number on the treated plants. Even with the high mortality of GWSS, *Xf* transmission rate did not differ between treated and untreated plants. Therefore, the sub-lethal dose of imidacloprid does not repel the insects from landing on the treated plants. Also, it seems that reduction of total feeding does not seem to be enough for reducing the transmission. One notable thing was that both sharpshooter species, especially GWSS, moved around on or off plant or switched between the plants more often in arenas with imidacloprid treated plant(s) than the insects in kaolin-treated plant(s). As GWSS did not infect any of the plants in kaolin coat experiment, and our sample numbers are yet small, we may not be able to draw conclusion on the effect of insect relocation on PD transmission, but such action does increase the insect visit per plant and potentially increase the chance of *Xf* transmission.

This is the first report of Surround repelling BGSS. Such repellency may become useful where BGSS is the main vector. Puterka et al. (2003) found that white was one of the least attractive color to GWSS. Therefore, with the color and barrier effects together, kaolin treatment at the border of vineyards facing BGSS overwintering areas may reduce visits of BGSS as they move into vineyards during spring months (Purcell, 1975). Because the vector inoculation of vines in coastal California establish chronic PD (vines do not recover overwinter) only during the first 70 days of the growing season (Feel et al. 2003), the period that vines must be protected from infectious vectors is relatively short. Puterka et al. (2003) reported that GWSS did not settle on test plants well under caged condition and remained on the cage until their death. In our test study, GWSS took more time to settle on the test plants compared to BGSS; therefore the

above mentioned tendency may be present. However, BGSS quickly landed on plants within the arena, so they may not be prone to such issues and present more direct result from the interaction with the kaolin film.

In the results of our three experiments, effects of imidacloprid treatment on *Xf* transmission by GWSS were not consistent. Transmission rates to treated plants were not always lower than to control plants, nor did the imidacloprid dosage show a positive relationship with the transmission. The treated plants at least never showed significantly higher PD infection than the control plants, so imidacloprid applications did not increase transmission rates.

The transmission rate of *Xf* by GWSS was low and inconsistent (about 5-15%), as previously reported (Almeida and Purcell, 2003). Information on the exact mechanism of vector feeding and its relation to pathogen transmission, and subsequent disease manifestation seems to be crucial to fully understand the potential of an insecticide like imidacloprid. In the mean time, field insecticidal management program should be closely monitored and evaluated for any new insight or change in the reaction of vectors to this chemical.

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Table 1. Number of BGSS (mean ± sd) on the plants in choice/no choice arenas with kaolin treated/untreated plants.

	Time after insect introduction (hr)			
arena	1	6	12	23
No choice (+/+)	0.5 ± 0.85^{a}	1 ± 0.82^{a}	1 ± 0.82^{a}	1 ± 0.82^{a}
No choice (-/-)	1.4 ± 0.70^{b}	1.5 ± 0.71^{a}	1.9 ± 0.32^{b}	1.8 ± 0.63^{b}
Choice (+/-)	1 ± 0.65^{ab}	1.55 ± 0.60^{a}	1.5 ± 0.61^{ab}	1.55 ± 0.60^{ab}

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant within an arena. Number of insects on plant data were accumulated for each arena category, and ANOVA test on the per arena number of insect on plant was performed at each hour. If the result was significant, each pair of arena categories was compared using t-test. Values followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 2. Number of BGSS (mean ± sd) on kaolin treated plants in choice/no choice arenas.

	Time after insect introduction (hr)			
Arena	1	6	12	23
Choice (+/-)	0.25 ± 0.44	0.2 ± 0.41	0.15 ± 0.37	0.25 ± 0.69
No choice (+/+)	0.25 ± 0.55	0.5 ± 0.69	0.5 ± 0.76	0.5 ± 0.69
P-value	1	0.1041	0.07463	0.1817

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant within an arena. Number of insects on plant data were accumulated for each arena category, and t- test on the number of insect on plant per plant was performed at each hour.

Table 3. Summary of BGSS location record on kaolin treated/untreated plants

	Insects never observed on any plants	Insects that changed location after landing on a plant	Insects that moved between the plants
No choice (-/-) (n = 16)	0^{a}	7^{a}	3^{a}
No choice (+/+) (n = 16)	5^{b}	2^{a}	1^{a}
Choice (+/-) (n = 32)	6^{ab}	5^{a}	2^{a}

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant. There were 2 BGSS per arena. Values were compiled for the 4 replications of 2 no choice (-/-), 2 no choice (+/+), and 4 choice arenas, and n is the total number of insects released into each arena category. Those within a column followed by the same letter are not significantly different by Fisher's exact test ($\alpha = 0.05$)

Table 4. Number of GWSS (mean ± sd) on the plants in choice/no choice arenas with kaolin treated/untreated plants.

	Time after insect introduction (hr)								
arena	10 min	30 min	1	2	3	6	9	12	23
No choice (+/+)	0	0.13 ± 0.35	0.25 ± 0.70[a]	0.25 ± 0.70	0.25 ± 0.46	1 ± 0.93[a]	1.25 ± 1.04	1.13 ± 0.83[a]	1.63 ± 1.30[a]
No choice (-/-)	0.63 ± 0.47	0.63 ± 0.48	0.88 ± 0.5[a]	0.88 ± 0.62	0.88 ± 0.99	2.25 ± 0.82[a]	2.63 ± 0.68	3.25 ±0.89[b]	3.25± 0.89[b]
Choice (+/-)	0.5 ± 0.52	0.56 ± 0.63	0.88 ± 0.81[a]	0.81 ± 0.83	1.13 ± 1.15	1.75 ± 1.18[a]	2.44 ± 1.21	2.63 ± 1.26[b]	2.88 ± 1.15[b]

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant. Per arena number of insects on plant data were accumulated for each arena category. ANOVA tests were performed at 1, 6, 12, and 23 hr, and if the result was significant, each pair was compared using t-test. Values followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 5. Number of GWSS (mean ± sd) on kaolin treated plants in choice/no choice arenas.

	Time after insect introduction (hr)			
Arena	1	6	12	23
Choice (+/-)	0.19 ± 0.40	0.44 ± 0.51	0.56 ± 0.51	0.5 ± 0.52
No choice (+/+)	0.12 ± 0.34	0.5 ± 0.63	0.56 ± 0.63	0.82 ± 1.05
P-value	0.6396	0.761	1	0.4002

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant within an arena. Number of insects on plant data were accumulated for each arena category, and t- test on the number of insect on plant per plant was performed at each hour. Each arena category had total of 16 kaolin treated plants tested.

Table 6. Summary of GWSS location record

	Insects never observed on any plants	Insects that changed location after landing on a plant	Insects that moved between the plants
No choice (-/-) (*n* = 32)	6[a]	11[a]	3[a]
No choice (+/+) (*n* = 32)	18[b]	4[a]	1[a]
Choice (+/-) (*n* = 64)	9[a]	18[a]	2[a]

A "+" denotes kaolin-treated plant and a "-" denotes untreated plant. There were 4 GWSS per arena. Values were compiled for the 4 replications of 2 no choice (-/-), 2 no choice (+/+), and 4 choice arenas, and *n* is the total number of insects released into each arena category. Those within a column followed by the same letter are not significantly different by Fisher's exact test ($\alpha = 0.05$)

Table 7. Number of BGSS (mean ± sd) on the plants in choice/no choice arenas with imidacloprid treated/untreated plants.

	Time after insect introduction (hr)								
arena	10 min	30 min	1*	2	3	6*	9	12*	23*
No choice (+/+)	1.63 ± 1.51	1.75 ± 0.89	2 ± 1.31	2.88 ± 1.25	3.13 ± 0.83	3.5 ± 0.53	3.5 ± 0.53	3.5 ± 0.53	3.5 ± 0.53
No choice (-/-)	1.88 ± 0.99	2.5 ± 0.93	2.25 ± 1.28	2.38 ± 1.19	2.75 ± 1.39	3.13 ± 1.26	3.5 ± 0.93	3.38 ± 0.92	3.38 ± 1.19
Choice (+/-)	1.06 ± 1.00	1.63 ± 0.86	2 ± 0.97	2.63 ± 0.89	2.88 ± 0.72	3.25 ± 0.93	3. 81 ± 1.38	3.81 ± 1.38	3.81 ± 1.38

A "+" denotes imidacloprid-treated plant and a "-" denotes untreated plant within an arena. Per arena number of insects on plant data were accumulated for each arena category.

* For these observations, ANOVA tests were performed to compare plant treatment combinations, but none were significant (P = 0.87, 0.707, 0.67, and 0.66 at 1, 6, 12, 23 hr, respectively).

Table 8. Summary of BGSS location record on imidacloprid treated/untreated plants

	Insects never observed on any plants	Insects that changed location after landing on a plant	Insects that moved between the plants
No choice (-/-) (*n* = 32)	1	16	7
No choice (+/+) (*n* = 32)	0	14	6
Choice (+/-) (*n* = 64)	3	24	13

A "+" denotes imidacloprid-treated plant and a "-" denotes untreated plant. There were 4 BGSS per arena. Values were compiled for the 4 replications of 2 no choice (-/-), 2 no choice (+/+), and 4 choice arenas, and *n* is the total number of insects released into each arena category. Fisher's exact test did not detect differences among the arenas with different plant treatment combinations (P = 0.81, 0.55, and 0.81 for the categories above, from left to right)

Table 9. Number of GWSS (mean ± sd) on the plants in choice/no choice arenas with imidacloprid treated/untreated plants.

	Time after insect introduction (hr)						
arena	30 min	1*	2	3	6*	9	23*
No choice (+/+)	1.38 ± 0.96	1.5 ± 0.96[a]	1.75 ± 1.63	1.88 ± 2.06	1.75 ± 0.58[a]	2.13 ± 1.29	1.63 ± 1.73[a]
No choice (-/-)	1 ± 1.29	1.63 ± 0.96[a]	2.63 ± 0.96	3 ± 0.58	2.38 ± 1.73[a]	3.25 ± 0.5	3.38 ± 0.5[b]
Choice (+/-)	0.63 ± 1.20	0.88 ± 0.92[a]	1.13 ± 0.92	1.88 ± 1.31	1.94 ± 1.19[a]	1.94 ± 1.28	1.94 ± 0.83[a]

A "+" denotes imidacloprid-treated plant and a "-" denotes untreated plant within an arena. Per arena number of insects on plant data were accumulated for each arena category.

* For these observations, ANOVA tests were performed to compare plant treatment combinations. At 1 ($P = 0.19$) and 6 hr ($P = 0.64$), there was no significant difference in the per arena number of insects on plants among the arena categories.

Table 10. Summary of GWSS location record on imidacloprid treated/untreated plants

	Insects never observed on any plants	Insects that changed location after landing on a plant*	Insects that moved between the plants
No choice (-/-) (*n* = 32)	2	20[a]	10
No choice (+/+) (*n* = 32)	5	35[b]	17
Choice (+/-) (*n* = 64)	13	45[ab]	26

A "+" denotes imidacloprid-treated plant and a "-" denotes untreated plant. There were 4 GWSS per arena. Values were compiled for the 4 replications of 2 no choice (-/-), 2 no choice (+/+), and 4 choice arenas, and *n* is the total number of insects released into each arena category. Fisher's exact test on the accumulated data did not detect differences among the arenas with different plant treatment combinations (P = 0.20, 0.21, 0.09 for the above insect location categories, from left to right). However, t-test on the insect movement per arena from the four experiment dates found significance between the two no choice arenas, imidacloprid-treated plants only and untreated plants only (*P* = 0.35)

* Some insects changed location twice, and both were counted.

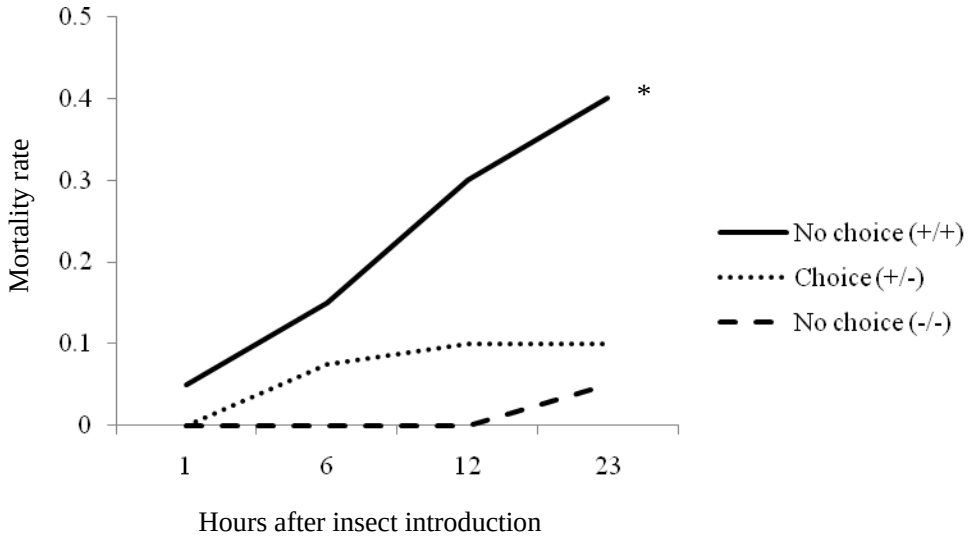


Fig. 1 Mortality rate of BGSS in the arenas with kaolin treated/untreated plants. A + means a kaolin-treated plant, and a – means an untreated plant, indicating which plants were present in each arena category of choice/no choice. The data from all replications were accumulated for each arena category. A * by the line denotes that the mortality count at 23 hr in the category is different from the unmarked ones ($P = 0.02$).

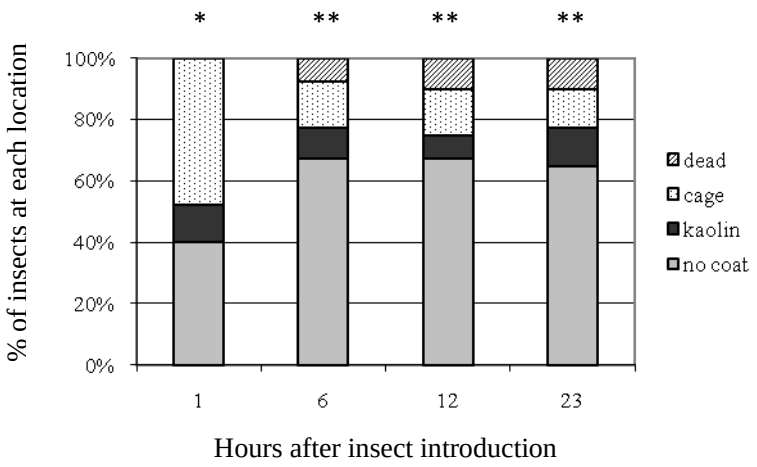


Fig. 2. Distribution of BGSS within choice arenas with kaolin-treated and un-treated plants. Insects are either on one of the plants, dead, or on other places (e.g. on the floor or the net of the arena). T-test was performed at each hour, and a "*" denotes that the difference between the number of insects on kaolin-treated plants is higher than that on untreated plants, at $P < 0.01$ level. A "**" denotes the same difference at $P << 0.001$ % level.

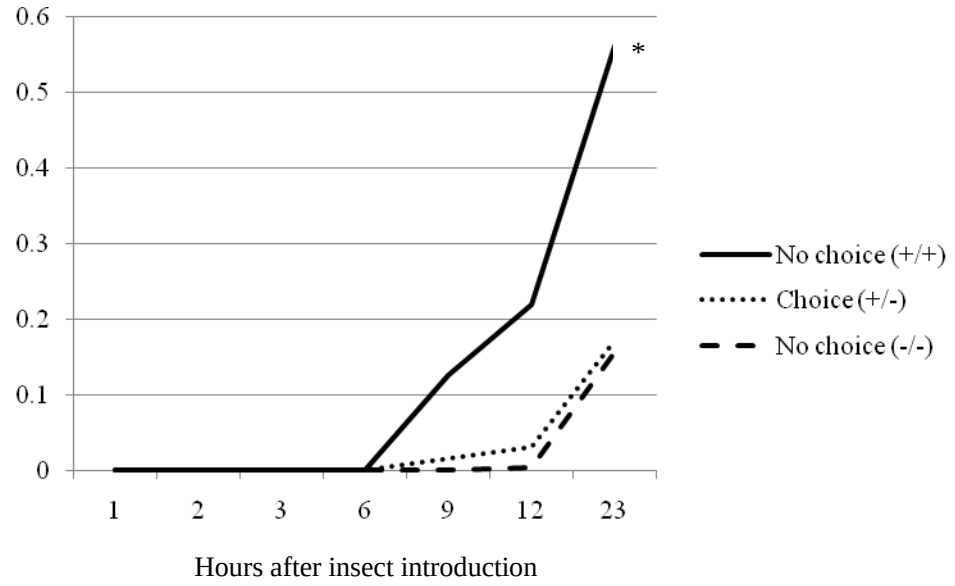


Fig. 3. Mortality rate of GWSS in the arenas with kaolin treated/untreated plants. A + means a kaolin-treated plant, and a – means an untreated plant, indicating which plants were present in each arena category of choice/no choice. The data from all replications were accumulated for each arena category. A "*" by the line denotes that the mortality count at 23 hr in the category is different from the unmarked ones.

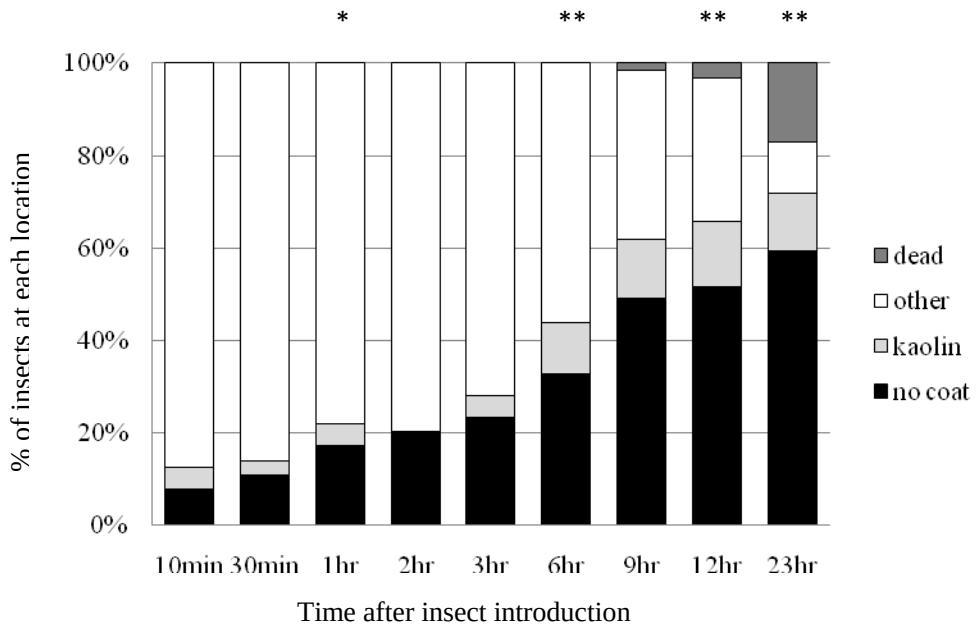


Fig. 4. Distribution of GWSS within choice arenas with kaolin-treated and un-treated plants. Insects are either on one of the plants, dead, or on other places (e.g. on the floor or the net of the arena). T-test was performed at 1, 6, 12, and 23 hr, and a "*" denotes that the difference between the number of insects on kaolin-treated plants is higher than that on untreated plants, at $P < 0.01$ level. A "**" denotes the same difference at $P << 0.001$ % level.

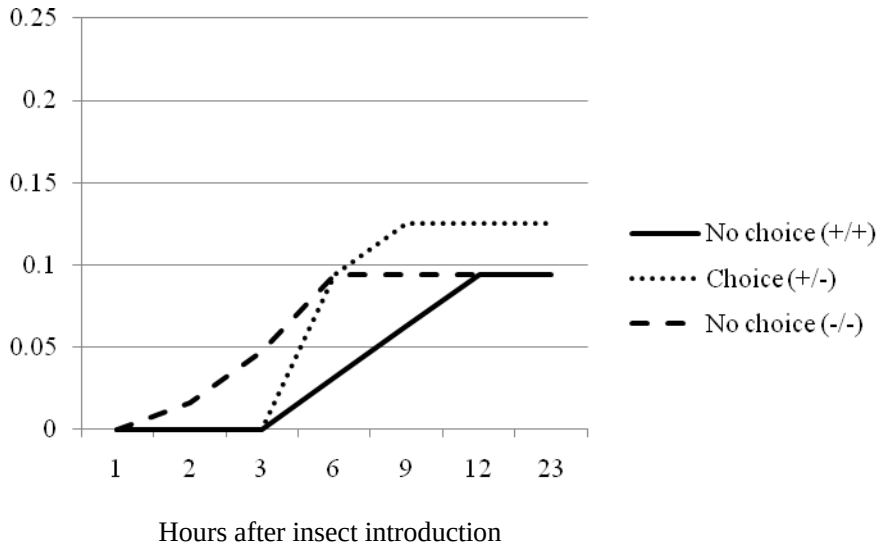


Fig. 5 Mortality rate of BGSS in the arenas with imidacloprid treated/untreated plants. A + means a treated plant, and a – means an untreated plant, indicating which plants were present in each arena category of choice/no choice. The data from all replications were accumulated for each arena category. The mortality count at 23 hr in the category was not significantly different (Fisher's exact test, $P = 0.93$).

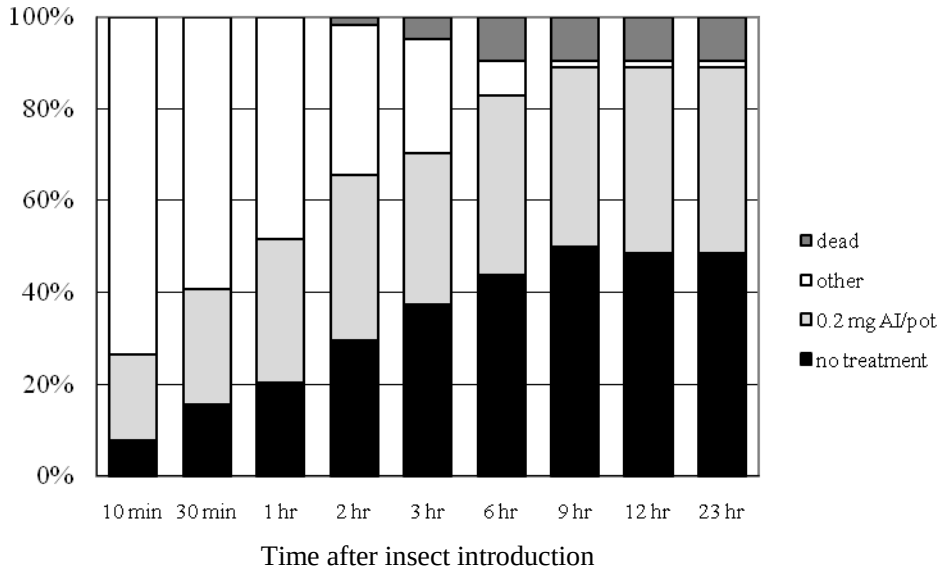


Fig. 6. Distribution of BGSS in the choice arenas with imidacloprid-treated (with 0.2 mg AI/pot) and untreated plants. Insects are either on one of the plants, dead, or on other places (e.g. on the floor or the net of the arena). T-tests were performed at 1, 6, 12, and 23 hr, to compare the number of insects on imidacloprid-treated plants and that on untreated plants, but there was no significant difference (P = 0.33, 0.85, 0.68, and 0.68 at each hour above, respectively).

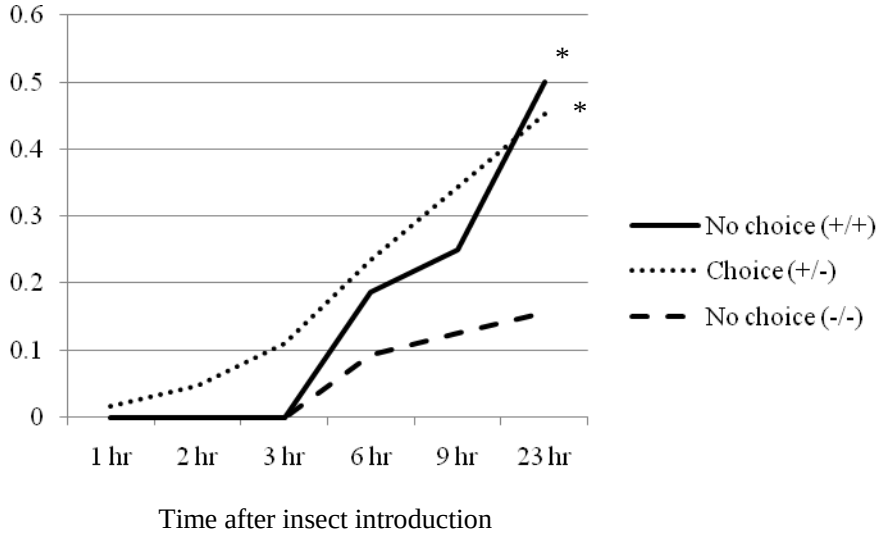


Fig. 7. Mortality rate of GWSS in the arenas with imidacloprid treated/untreated plants. A "+" means a treated plant, and a "–" means an untreated plant, indicating which plants were present in each arena category of choice/no choice. The data from all replications were accumulated for each arena category. A "*" by the line indicates that the mortality count at 23 hr in the category is different from the unmarked one ($\alpha = 0.01$).

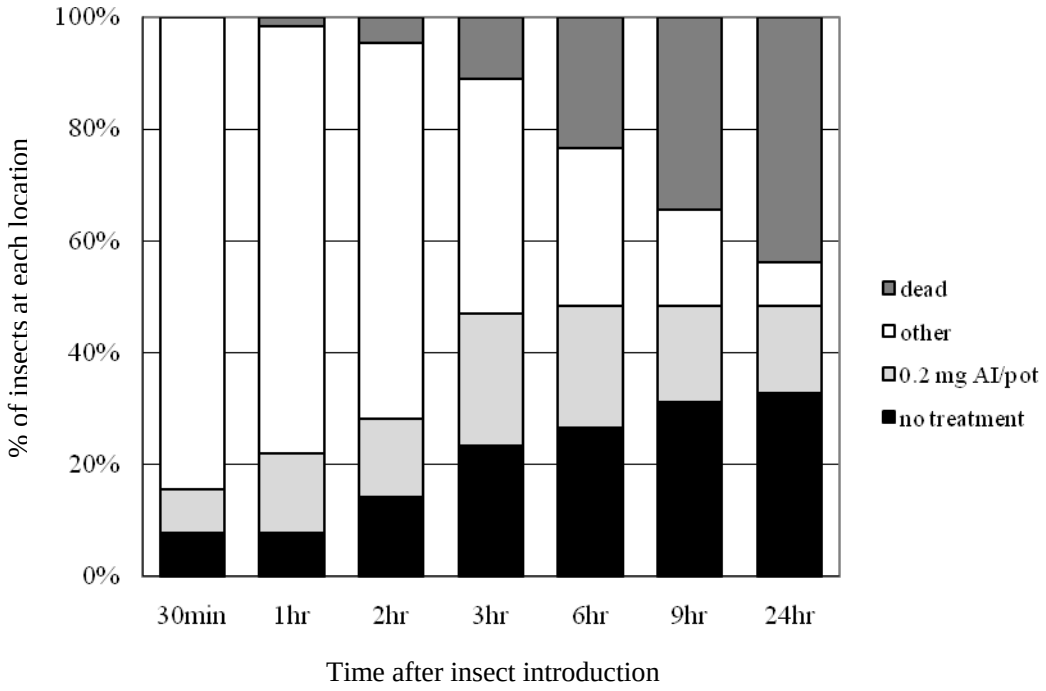
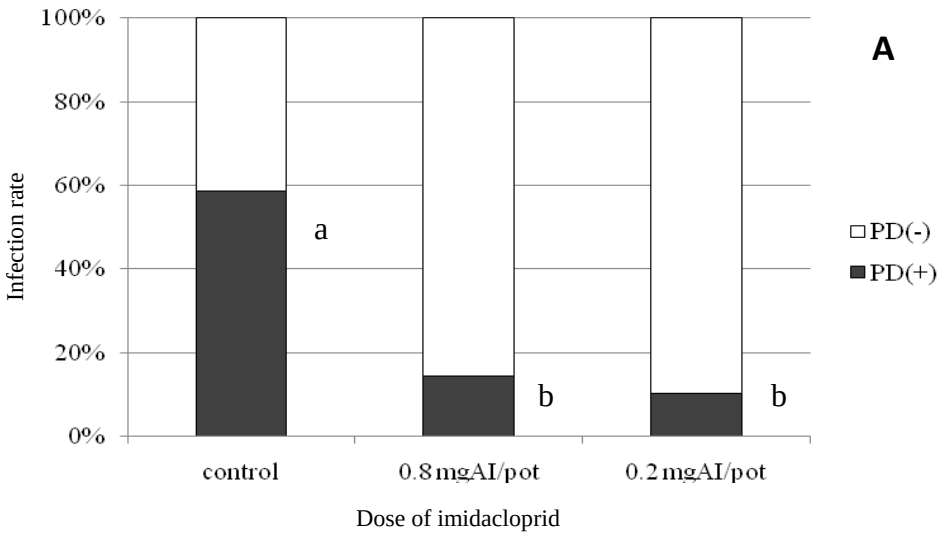
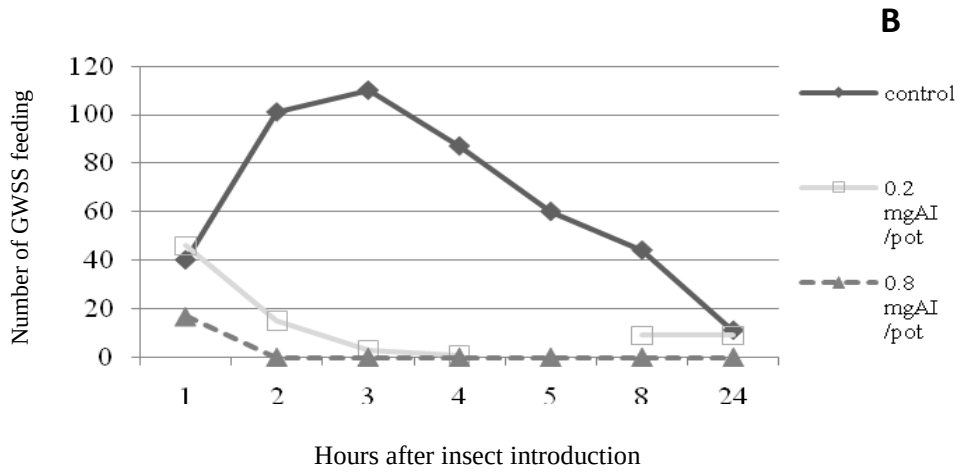
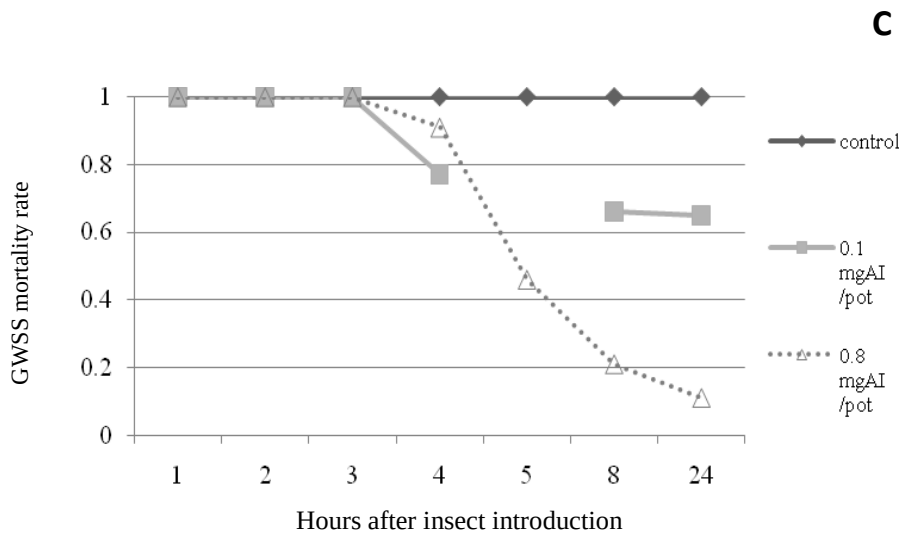
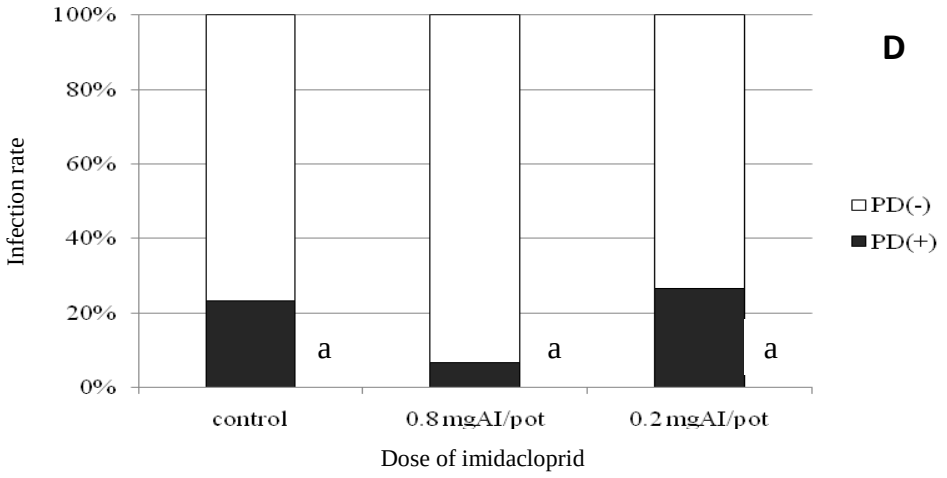


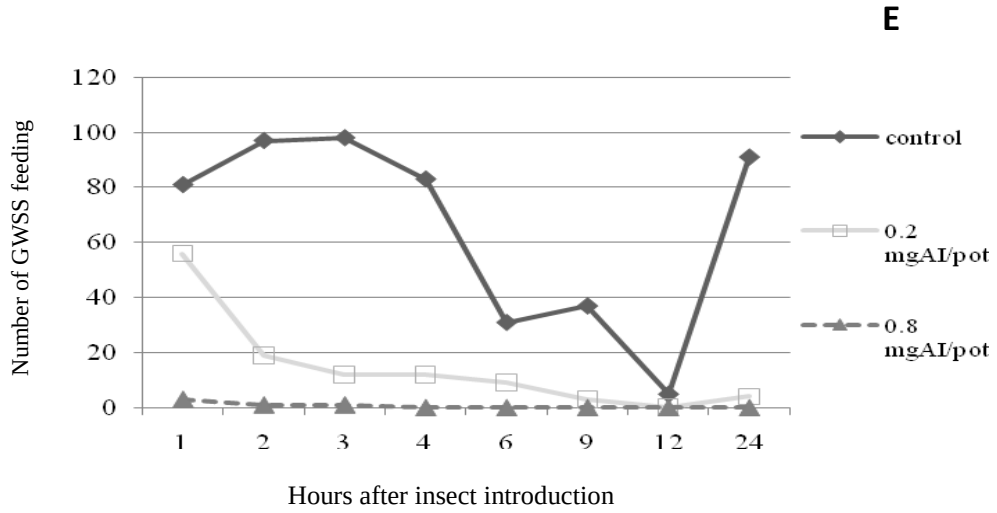
Fig. 8. Distribution of GWSS in the choice arenas with imidacloprid-treated (with 0.2 mg AI/pot) and untreated plants. Insects are either on one of the plants, dead, or on other places (e.g. on the floor or the net of the arena). T-test was performed at 1, 6, and 23 hr, to compare the number of insects on imidacloprid-treated plants and that on untreated plants. There was no significant difference for 1 and 6 hr (P = 0.22 and 0.21), and the number of insects on the untreated plants was significantly higher at 23 hr (P = 0.03).

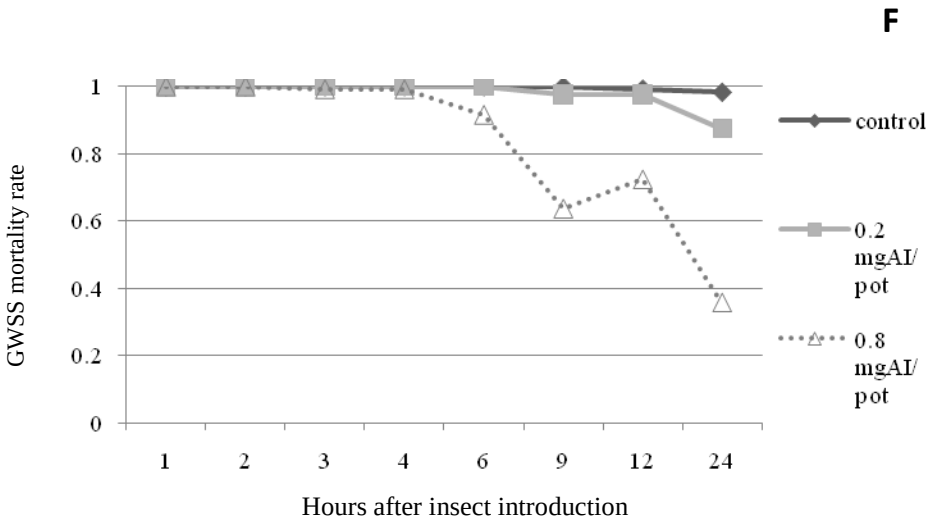

A
100%
80%
60%
40%
20%
0%
Infection rate
a
b
b
PD(-)
PD(+)
control
0.8 mgAI/pot
0.2 mgAI/pot
Dose of imidacloprid

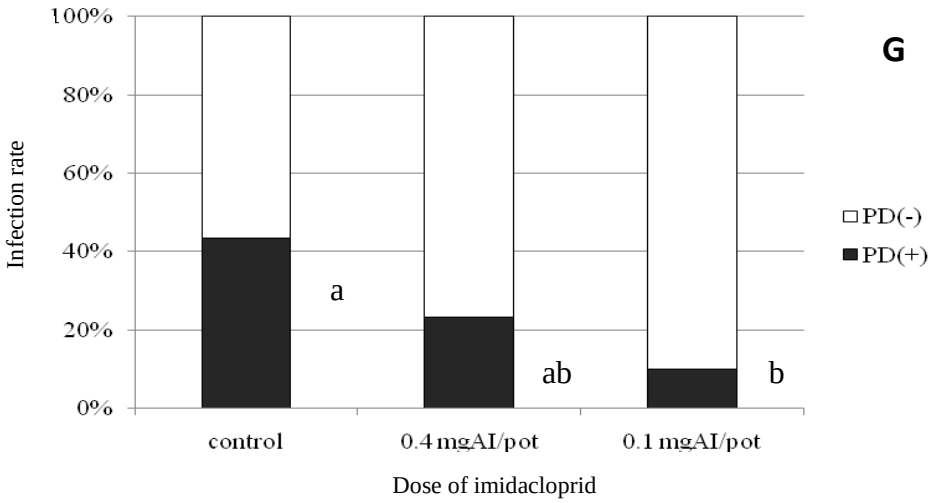

B
120
100
80
60
40
20
0
Number of GWSS feeding
control
0.2 mgAI /pot
0.8 mgAI /pot
1
2
3
4
5
8
24
Hours after insect introduction

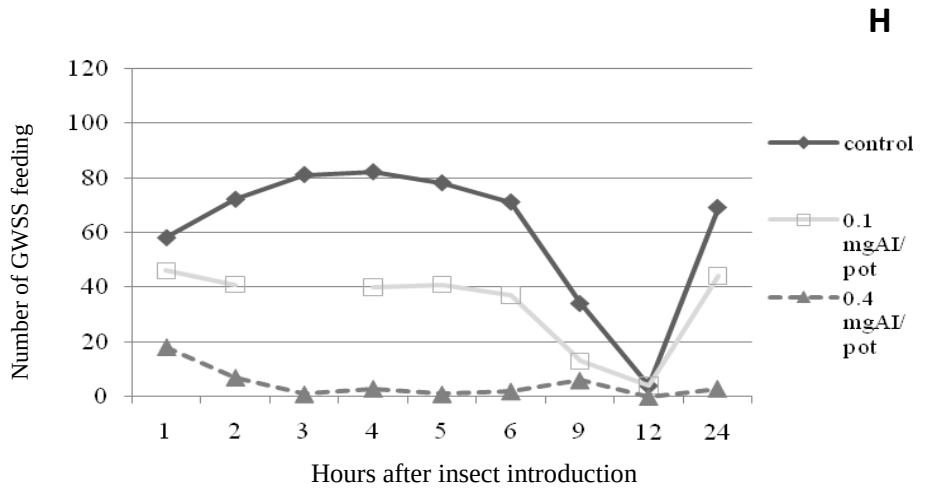

C
1
0.8
0.6
0.4
0.2
0
GWSS mortality rate
control
0.1 mgAI /pot
0.8 mgAI /pot
1
2
3
4
5
8
24
Hours after insect introduction


D
100%
80%
60%
40%
20%
0%
Infection rate
a
a
a
control
0.8 mgAI/pot
0.2 mgAI/pot
Dose of imidacloprid
PD(-)
PD(+)


E
120
100
80
60
40
20
0
Number of GWSS feeding
1
2
3
4
6
9
12
24
Hours after insect introduction
control
0.2 mgAI/pot
0.8 mgAI/pot


F
1
0.8
0.6
0.4
0.2
0
GWSS mortality rate
1
2
3
4
6
9
12
24
Hours after insect introduction
control
0.2 mgAI/ pot
0.8 mgAI/ pot


G
100%
80%
60%
40%
20%
0%
Infection rate
a
ab
b
control
0.4 mgAI/pot
0.1 mgAI/pot
Dose of imidacloprid
PD(-)
PD(+)


H
120
100
80
60
40
20
0
Number of GWSS feeding
1
2
3
4
5
6
9
12
24
Hours after insect introduction
control
0.1 mgAI/pot
0.4 mgAI/pot

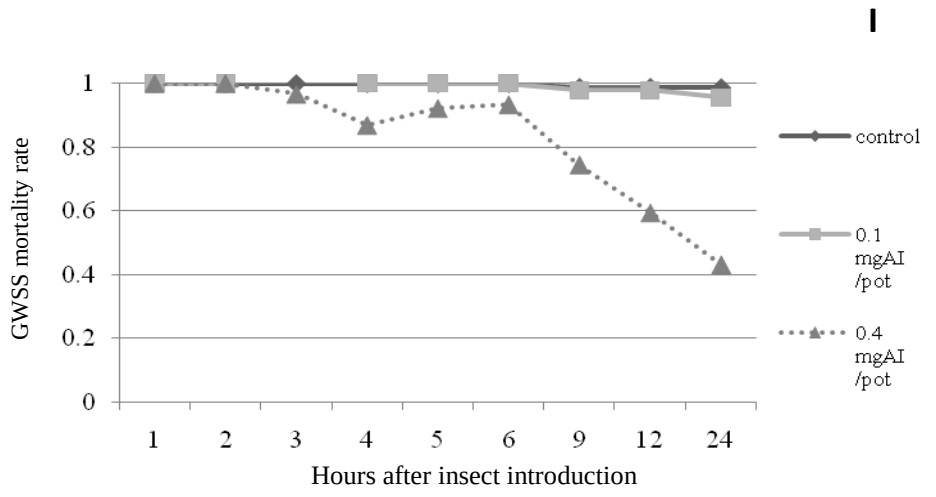

I
1
0.8
0.6
0.4
0.2
0
GWSS mortality rate
1
2
3
4
5
6
9
12
24
Hours after insect introduction
control
0.1 mgAI /pot
0.4 mgAI /pot

Fig. 9. Transmission rates of *Xylella fastidiosa* by *Homalodisca vitripennis* to plants with or without soil-applied imidacloprid. PD = test grapevines with (+) or without (-) Pierce's disease in three trials (A, D, G). Columns followed by the same letter are not significantly different (Fisher's exact test; $\alpha = 0.05$). (B, E, H) – Number of excreting insects observed at each time, and (C, F, I) – Survival rate of the test insects.

Chapter 4

Flight Performance of Glassy Winged Sharpshooter (Hemiptera: Cicadellidae) exposed to Imidacloprid-treated Grapevines

Abstract

Adults of field-collected glassy-winged sharpshooter (GWSS) were tethered on a computer-linked flight mill system, and their flight performances were monitored to determine their flight characteristics. Flight varied substantially among the individuals, but most of their flight activity occurred in the first 4-5 hours. The average maximum distances flown by males and females were 14.92 km and 9.56 km, respectively. There were no significant differences between males and females in the flight parameters we observed. In subsequent experiments, GWSS were exposed to grape seedlings treated with different doses of imidacloprid, and their tethered flights were then monitored. The insects that survived on the imidacloprid-treated plants showed flight activities comparable to those of the control insects, indicating that if the initial imidacloprid exposure failed to kill the insect, it did not hinder its further dispersal.

Introduction

The Pierce's disease vector (Adlerz and Hopkins 1979), *Homalodisca vitripennis* (Germar) [formerly, *H. coagulata* (Say)] (glassy-winged sharpshooter, GWSS) (Takiya et al. 2006) (Hemiptera: Cicadellidae) was first detected in California in the late 1980's (Sorensen and Gill 1996). A new epidemic of Pierce's disease on grapevines in Temecula Valley in southern California (Blua et al. 1999) in the fall of 1997 was associated with this newly introduced insect. Another epidemic followed in the southern part of the Central Valley, and these epidemics resulted in a magnitude of losses typically not experienced with native vectors (Sisterson 2009).

Pierce's disease has been in California since the 1880s (Gardner and Hewitt 1974). Until the arrival of GWSS, native sharpshooter (Cicadellidae: Cicadellinae) vectors of its pathogen, *Xylella fastidiosa* (Wells et al., 1987) were considered the most important vectors for the spread of PD. This includes *Draeculacephala minerva* Ball (green sharpshooter) and *Xyphon fulgida* Nott (red-headed sharpshooter) in the Central Valley and *Graphaecephala (Hordnia) atropunctata* (Sigmoret) (blue-green sharpshotter) in coastal region (Hewitt et al. 1949).

However, this new epidemic by GWSS showed some different characteristics, such as the vector moving further into the vineyards (Blua et al. 1999), effects of surrounding environment, such as proximity to citrus groves (Blua et al. 1999, Perring et al. 2001, Tubajika et al. 2004) and the spatial pattern of disease incidence within vineyards (Tubajika et al. 2004). For example, citrus proved to be a major breeding habitat for GWSS and was associated with PD in adjacent vineyards, but gradients of diseased vines were not as pronounced (Perring et al. 2001) as were epidemics involving native vectors (Purcell 1974, Hewitt et al. 1949).

As such, movement of GWSS into and within vineyards is considered a key factor in the PD epidemics caused by this new vector, and there have been studies on their flight and dispersal (Blua, M. J., et al. 2001, Blua and Morgan 2003, Blua et al. 2005, Blackmer et al. 2004, Petit et al. 2008). Petit et al. (2008) studied invasion dynamics of GWSS in French Polynesia and concluded that long-distance dispersion (e.g. between islands) was via human transport rather than long-distance flight by the insects. They also found that shorter-distance dispersal (e.g. within islands) is affected by the availability of host plants in favorable (e.g. well irrigated) conditions. Blua et al. (2001) monitored the seasonality of GWSS flight activities, and Blua and Morgan (2003) found that the peak GWSS dispersion into vineyards occurred in summer. A comparative study of GWSS and smoke-tree sharpshooter (STSS, *Homalodisca liturata* (Ball)) dispersal by Blackmer et al. (2004) showed STSS could move further and /or faster in the field than GWSS, suggesting that factors other than flight capacity, such as an expanded host range, higher densities, and possibly a greater tendency to move short distances between oviposition and feeding sites may be contributing to the faster spread of PD by GWSS.

The first aim of our study was to test the feasibility of a flight mill system to provide basic information on the GWSS's flight performance. Flight parameters such as total flight distance, longest single flight distance, speed, and frequency of flight initiation should provide insight about the flight potential of this insect. Although our GWSS sample was field collected and mixed in age and likely in mating status, influence of sex on flight is also a factor to consider when dealing with an invasive species. Flight mill studies on lepidopteran species found females flew longer in total and per single flight (Shirai and Kosugi 2000, Hughes and Dorn 2002, Elliott and Evenden 2009) and had more flight events (Sarvary et al. 2008). Other studies on lepidopteran (Ishiguri and Shirai 2004) and coleopteran flight found few or no significant difference between sexes, and Van Dam et al. (2000) found varied results among lepidopteran species. A study on leafhopper (*Nephotettix virescens* (Distant)) flight by Cooter et al. (2000) showed no effect of sex on flight duration. Our work examined if GWSS male and female flight characteristics differed.

Laboratory experimentation using a flight mill differs from natural flight conditions, as the insects are tethered and forced to fly (Yamanaka et al. 2001). Absence of something to land on (Yamanaka et al. 2001), or other flight-related cues, might greatly affect the initiation and/or termination of flights (Wilson 1961, Edwards 2006). Riley et al. (1997) pointed out that insects don't need to generate as much lift to support their flight on the mills, yet have to overcome both pivot friction and the aerodynamic drag on the mill arm. In their experiment with corn leafhopper *Dalbulus maidis* (DeLong & Wolcott), Riley et al. (1997) estimated that the insect's energy expenditure on the mills is only 20-30% of that needed for free flight. When the tethered and untethered flight of *Lygus* spp. were compared, their longest flights on flight mills were 17 – 18 times longer than the longest flight in the flight chamber.(Blackmer et al. 2004). On the other

hand, Tsunoda and Moriya (2008) compared the bean bug flight speed observed with flight mill and with hand-held speed sensor and found that the former speed is lower than the latter, Nevertheless, Taylor et al. (1992) suggest that flight mills may be used in comparative studies, where relative estimates of flight parameters are obtained. Stebbing et al. (2005) conducted such comparative studies on the sublethal effect of methyl-parathion in reducing flight ability of western corn rootworm. Alyokhin and Ferro (1999), examined the effect of Bt toxin ingestion on the flight of susceptible and resistant Colorado potato beetles and found that Bt resistant beetles significantly increased flight activity when fed on transgenic plant expressing Cry3A (Bt toxin), compared to non-transgenic plants.

Such a comparison is our second objective. We examined how exposure to sublethal doses (here, "dose" is not the amount the insects receive, but the amount applied per plant) of an insecticide imidacloprid affects the flight of GWSS. This neonicotinoid insecticide is often used for GWSS control in citrus and grape for prevention of PD (Byrne et al., 2005). The plants the insects were exposed to were systemically treated with a series of doses of imidacloprid, which are known to have an anti-feeding effect. Flight distance and duration of olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) decreased when its pre-flight supply of food and water were limited (Wang et al. 2009). Therefore, either through the chemical's direct toxic effect or the food deprivation through an anti-feeding effect, we expected changes in the insect's flight activity. If the flight mill system can differentiate the flights of GWSS with or without insecticide exposure, the data should provide information on how the chemical affects the dispersal of this insect.

Materials and Methods

Study insects and plants

We collected *H. vitripennis* adults from citrus groves and eucalyptus trees southeast of Edison, California, and maintained breeding colonies in an insectary on a mixture of grapes, mugwort and basil (*Ocimum basilicum*), kept under natural light conditions. We transplanted bare-root grape seedlings into 10 cm diameter pots when the plants were about 20 cm high. Pots contained 380 g clay-loam soil from just north of Napa, CA. Two weeks later and one week prior to the test, the plants were treated with randomly assigned dosages of imidacloprid (Admire 2F, Bayer Co). We covered the soil surface with coarse sand after insecticide applications to avoid insects' direct contact with the soil, and watered the plants twice daily, just enough to thoroughly moisten the soil and not wash the chemical from the soil.

Flight mills

To measure flight performance, we constructed a flight mill system, modified from a previously described flight mill design (Gorder 1990; Schumacher et al. 1997). There were nine channels of the flight mills connected to a computer through the printer port. Therefore, a maximum of nine insects per day were tested, and the tests were repeated to obtain sufficient numbers of samples. GWSS adults were glued at the highest arch of their thoraxes to the heads of insect pins using Beacon Hold the Foam! Glue (Beacon Adhesives, Mt. Vernon, NY), so that the glue did not touch any part of wings during their movement. The pins were inserted into the end (5 mm from the edge) of a plastic straw that served as the mill arm and rotated on a jewel bearing. At the other end of the straw was a balancing piece of aluminum foil-covered piece of thick paper, which cut the flow of infra-red beam into an electronic detector during the insect flight-driven rotations and were recorded on a computer as the time of completion of each revolution. Revolutions were counted on a per minute basis, and a bout (single continual flight event) was counted where there was more than one rotation per minute.

Flight measurements: male/female

For the male/female comparison, the insects were randomly drawn from the above mentioned colonies. Each experiment started around 10 am, and the flight events (number of rounds in a bout and length of time of the bout) were recorded in a computer. The insects were left on the system for overnight (~ 22 hours). A total of 31 males and 25 females were tested. The time to flight cessation (time since the start until when the last flight terminated), total flight duration, and total number of flight bouts were recorded, and total flight distance, maximum and median (as the distribution is positively skewed) single flight distance, and maximum and median flight speed were calculated from the number of rotations data.

Flight measurements: effect of exposure to imidacloprid-treated plants

For the imidacloprid effect study, we conducted 2 series of experiments. For the first experiment, about 30 males (numbers varied due to availability of the insects) were drawn from the colonies 24 hr prior to the start of each flight mill test, and were placed in one of the three smaller cages which contained two grape seedlings (*V. vinifera*, cv. Cabernet sauvignon) that were either 1) untreated or, 2) treated with a low dose of imidacloprid (0.1 mg AI /380g soil), or 3) treated with a high dose of imidacloprid (0.8 mg AI/380g soil). The low dose was previously determined (chapter 2) to drastically reduce feeding in both GWSS and another sharpshooter, *Graphocephala atropunctata*. After 24 hrs, we record mortality and then screened the percentage of non-fliers among the survivors by tossing them about 50 cm vertically into the air. From the individuals from each cage that attempted to fly, we selected 3 animals which were then tethered onto the flight mills and their flight behavior recorded as in the male/female experiments. For the second experiment, we followed the same procedure but with different doses of imidacloprid (0.2 mg AI for lower dose, 0.4 mg AI for higher dose, and untreated control). The total number

of insects tested on the flight mill for each dose, including the number of non-flyers were 18 GWSS per dose for the first experiment, and 15 GWSS per dose for the second experiment.

Statistical analyses

Flight performance data were compared using one-way analysis of variance (ANOVA) to test for the differences between sexes or exposure to imidacloprid-treated plants, unless it is noted otherwise individually. Statistical analyses were performed using R (R Development Core Team 2009). All data, except for that of non-flyers, are included in the statistical analysis.

Results

General characteristics of flight and sex differences

There was a great variability in flight characteristics of both female and male GWSS (Table. 1) Among the insects that were not weak fliers (i.e. flights per minute of less than 40 or with only a few of >40 rpm bouts) there were three general patterns of flight distribution (Fig. 1): 1) continual flight at the beginning, without many pauses, 2) pulses of flights with short, somewhat regular pauses, with the flight activity extending longer, and 3) irregular pulses of flights with longer pauses over longer periods (>5 hrs). On average, about 95 % of total flight by individual insect occurred in the first 5 hr, as exemplified by Fig. 1A and B. The distributions of flight parameter data, except for the flight velocity, are positively skewed, with a few outliers flying for an exceptionally long time (and thus long distance). The longest individual flight distance during a single bout of uninterrupted flight was 3.11 km, and the longest cumulative flight distance was 14.93 km for males and 9.55 km for females (Table 1). ANOVA tests did not detect statistically significant differences between males and females in any flight parameter (Table 1). Log frequency distributions of number of rounds in a single bout (i.e. single uninterrupted flight event) show that most of the flights occurred in short bursts (Fig. 2). Approximately 75 % of female and 60% of male flight bouts had 20 or fewer revolutions per minute ($\leq$ 12.6 m distance). Total distances and times flown were well correlated, as would be expected. The very close correlation of total distance and total flight time indicates that one could be a reasonable proxy for the other. However, the duration of an insect's sustained flight capability (the "last flight" data) was not significantly correlated to any other flight parameter. Figure 3 shows the scatter diagrams for correlations among flight parameters for males (Fig 3A) and females (Fig. 3B). There were no significant correlations between total number of bouts and median single flight distance (Pearson's product moment correlation coefficient r = -0.33, P = 0.10) or total flight time (r = 0.24, P = 0.24). Therefore, the insects that flew more often did not fly longer total durations, yet the distance flown per single flight did not decrease significantly.

Average speed and total flight time were weakly negatively correlated (Fig. 3 and Table 2), which makes sense energetically

Effect of exposure to imidacloprid-treated plants

In the first series of experiments, the high dose (0.8 mg/pot) of soil-applied imidacloprid killed about a third of the GWSS fed on these plants. Among the survivors (after 24 hrs) in the high dose group, about 60 % did not pass the simple screening test for "fliers." This was significantly different from the control and the low dose (0.1 mg AI/pot), and the latter two groups were not significantly different (at $P < 0.05$, Table 3). In the second series of experiments, although the higher dose did yield a higher average mortality rate, the value varied day to day, and as a result, significant differences were not detected among the doses (Table 3).

The number of GWSS that did not fly on the flight mill were: 1) In the first experiment series ($n = 18$ for each dose) 4, 0, 0 for each of 0.8, 0.1, and 0 (control) mg/pot groups ($P = 0.029$ with Fisher's exact test (FET)), and 2) In the second series ($n = 15$ for each dose). 4, 1, 0 for 0.4, 0.2, and 0 mg AI/pot groups ($P = 0.069$ with FET). There were small differences in tethered flights among the surviving insect groups exposed to grapes treated with different doses of imidacloprid and none of these were significant (Table 4) except for the total flight time and the total flight distance, for which the GWSS exposed to 0.4 mg AI/pot flew longer in time ($F_{2, 37} = 5.39$; $P < 0.01$) and distance ($F_{2, 37} = 8.12$; $P < 0.01$)(Fig. 4-c, d).

Discussion

In general, the flight parameters that we recorded varied greatly among individuals, with a few exceptionally strong fliers flying for long time and distance in every group, including the ones exposed to higher doses of imidacloprid. Although such flight performance heterogeneity with a small number of high-end outliers does not seem to be rare (Sarvary et al. 2008), the high variability in our results may be, at least partially, due to the heterogeneity in the life stages of insect population we used, whose influence has been noted in other insect species (Lopes et al. 1995, Sarvary et al. 2008, Lopes et al. 1995, Ishiguri and Shirai 2004, Alyokhin and Ferro 1999). In any case, in the field, insect populations will vary by age and mating status over time. With that fact in mind, future studies should control for distinct insect age groups, their known mating status, time of year, body size, female egg load/body fat content, nutritional status (such as feeding on different hosts), and *Xf* infectivity in order to estimate how much effect these factors have on the insect flight. Other environmental factors, such as temperature, light: darkness regime, visual/olfactory cues around the flight mills, could also be incorporated into the system. Refining the mill and its program should also be considered, as with the current system, we could

not detect a brief (< 1 min) break between the flights. Recording the flight with video may help to document the details of GWSS flight behavior.

The three patterns of flight observed in our study (continual, pulses with short breaks, and pulses with longer, varying length of breaks) are similar to those described by Bruzzone et al. (2009). Using a time-series analysis method called "wavelet analysis," which was first applied to tethered flight data series in their study, they numerically analyzed the flight patterns of woodwasps, correlated these patterns to individual wasp body mass, and suggested its implication to the insect's local and regional dispersal. Such analysis can be useful in further understanding GWSS flight characteristics, and may provide insight into the seasonal spread pattern of this vector insect. Detecting flight patterns, finding whether individuals can switch between the patterns and if there is any cue for such switching, and most importantly, connecting such pattern to the actual movement of GWSS in the field, we may be able to construct efficient strategies for preventing vectors from entering the vineyards, or simulate the PD spread within vineyard and consider effective method for its prevention or care

Most GWSS that fed on either treated or untreated plants completed their flights within a few hours. Exhaustion of their food reserves for flight is a plausible explanation for this limited time span. However, it doesn't fully explain the absence of differences between the GWSS on untreated and untreated plants. Even given the variability of flight ability among the individuals, failure to detect differences between imidacloprid-exposed and unexposed surviving insects was somewhat unexpected, especially with the higher mortality rates and greater percentage of non-fliers among the insecticide-exposed animals. Imidacloprid has anti-feeding effect (Nauen et al. 1998, Bethke et al. 2001) and can deter feeding of GWSS at sublethal doses (chapter 2). Therefore, the GWSS placed on imidacloprid-treated grapes are expected to be food-deprived. Moreover, once tethered, insects did not have access to food. The GWSS on untreated plants had been feeding until just before the flight and those on treated plants whose feeding had been deterred for nearly 24 hours on top of the food deprivation during the flight.

The fact that imidacloprid-exposed insects displayed comparable flight to the control insects suggests that those insects exposed to imidacloprid treated grapes either: 1) avoided feeding on xylem fluids with high titer of imidacloprid, and were able to obtain sufficient energy, or 2) conserved energy well even when their feeding was deterred, and/or 3) displayed the result as combinations of flight stimulation by imidacloprid and adaptation to energy intake limitation. Taking the observed individual variability into account, much higher number of insects should be tested to confirm our results.

Although tethered flight data tend to overestimate the flight distance and time (Riley et al. 1997, Bruzzone et al. 2009), our study confirmed the strong flight capacity of GWSS, which can be sustained for several hours in time and cover up to several kilometers without feeding. These data document maximum flight potential, not actual flight, as environmental factors should greatly affect their performances in the field. However, the short duration of most frequent bouts seems very similar to the flights of disturbed GWSS observed in vineyards and orchards, suggesting the usefulness of a flight mill system in monitoring some aspects of their flight behavior.

Imidacloprid appears to have little potential for disrupting disease spread by altering vector flight behavior, either through its toxic or anti-feeding effects. Rather, the main effect on disease spread seems to be its effect on vector mortality. Although there were some non-fliers among the survivors exposed to imidacloprid-treated plants, many of them flew well enough to disperse the pathogen within a vineyard or orchard or escaped to the untreated area. Therefore, imidacloprid's killing efficacy in the field should be carefully monitored in order to maintain the successful insecticidal management of the Pierce's disease.

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Table 1. Basic flight parameter results of GWSS males and females.

GWSS tested	Total bouts		Total flight (m)		Total flight (min)		Time to flight cessation (min)		Max. single flight (m)		Med. Single flight (m)		Max. flight speed		Ave. flight speed (m/s)	
	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
male n = 31	2 - 200	61.52 ± 7.20	85.95 - 14932	3930 ± 569	1.15 – 237.5	70.34 ± 11.07	124.3 - 1247	574.5 ± 48.10	10.74 - 3111	603± 142	4.78 - 116	25.84 ± 4.31	1.34 – 2.74	1.74 ± 0.058	0.91 – 1.66	1.20 ± 0.037
female n = 25	13 - 207	81.92 ± 10.20	96.70 - 9555	3048 ± 487	1.3 - 198	56.77 ± 10.24	106 - 1059	578.0 ± 53.94	7.16 - 2662	390± 105	4.78 – 124	20.15 ± 5.13	1.19 – 2.39	1.75 ± 0.061	0.88 – 1.73	1.20 ± 0.041

Table 2. Correlations among flight parameters

Male (n = 31)

Parameter	Other parameters with significant correlation	Correlation coefficient *r*	p-value
Total number of bouts	none	-	-
Total flight distance	Total flight time Max. single flight distance	0.97 0.77	$2.2e^{-16}$ $5.0e^{-07}$
Total flight time	Ave. speed Max. single flight distance	-0.53 0.79	0.0021 $1.4e^{-07}$
Ave. speed	Max. speed	0.68	$2.5e^{-05}$

Female (n = 25)

Parameter	Other parameters with significant correlation	Correlation coefficient *r*	p-value
Total number of bouts	none	-	-
Total flight distance	Total flight time Max. single flight distance	0.97 0.65	1.8e-15 0.00041
Total flight time	Ave. speed Max. single flight distance	-0.49 0.73	0.012 2.9e-05
Ave. speed	Max. speed	0.73	3.2e-05

The paired parameters with correlation coefficient near and above 0.5 are displayed with their statistical significance level.

Table 3. GWSS survival and the survivors' flight ability after 24 hr exposure to the test plants.

Experiment series	Imidacloprid dose per plant	GWSS Sample size n	Average mortality rate ±SD	Average rate of survivors that did not fly ±SD
1	0.8 mg/pot	57	0.33 ± 0.34 a	0.59 ± 0.38 a
	0.1 mg/pot	48	0.09 ± 0.09 b	0.07 ± 0.09 b
	control	48	0.03 ± 0.08 b	0.03 ± 0.08 b
2	0.4 mg/pot	59	0.43 ± 0.42 a	0.20 ± 0.40 a
	0.2 mg/pot	58	0.32 ± 0.46 a	0.03 ± 0.08 a
	control	59	0.12 ± 0.14 a	0.02 ± 0.04 a

Within the same experiment series, the values followed by the same letter are not significantly different at $\alpha = 0.05$ level.

Table 4. ANOVA results of flight parameters of GWSS with/without imidacloprid exposure.

Experiment series		Ave. speed	Max. speed	Total bout	Total distance	Total flight time	Time to flight cessation
1 (df = 2)	F-value	1.9173	1.5366	1.573	0.1587	0.0308	1.452
	P (> F)	0.1583	0.2257	0.2181	0.8537	0.9697	0.2444
2 (df = 2)	F-value	1.4207	0.5508	0.4209	8.1243	5.3927	2.8292
	P (> F)	0.25	0.58	0.66	0.0012 *	0.0088 *	0.072

Experiment series 1 tested the doses 0.8, 0.1, and 0 (control) mg imidacloprid/380g soil, and the series 2 tested the doses 0.4, 0.2, and 0 mg imidacloprid/380g soil. A "*" by the P-value indicates that there was significant difference within the series.

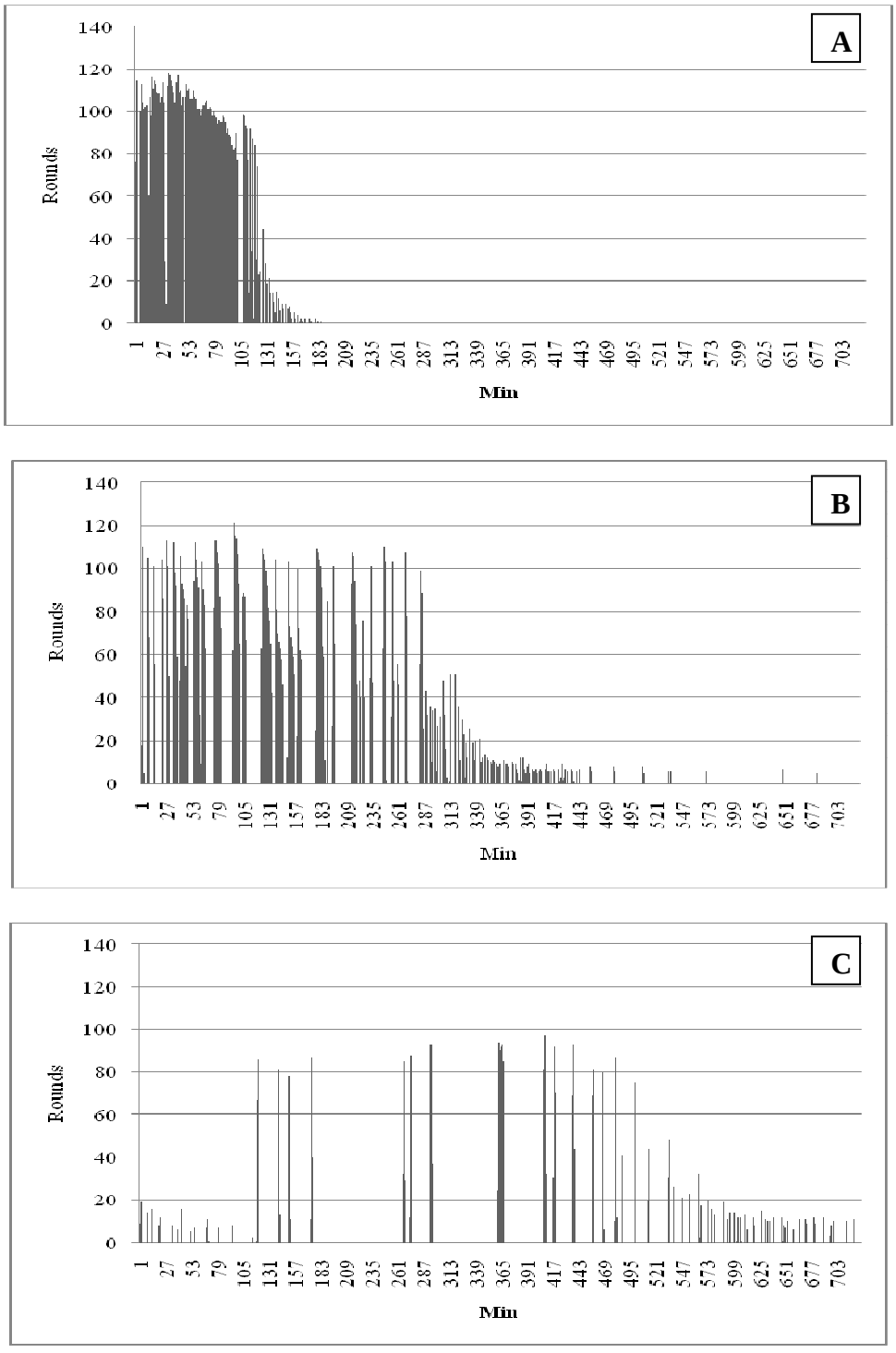
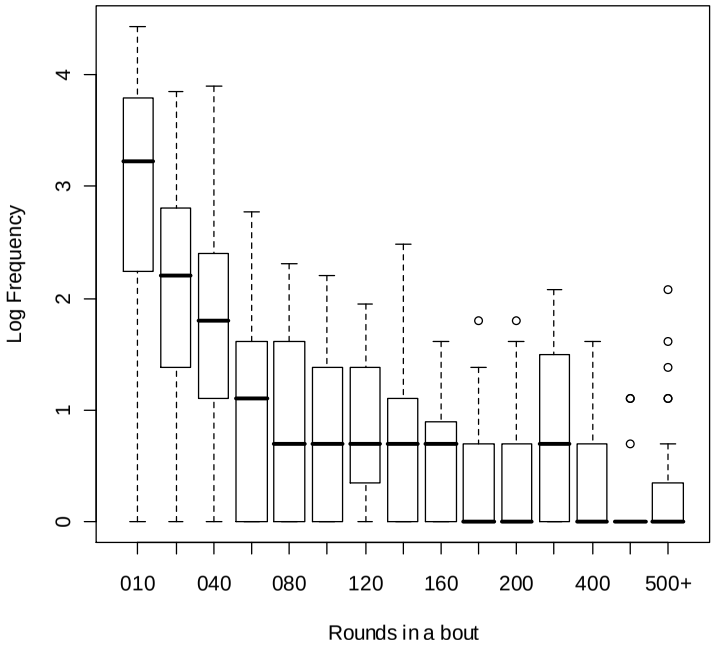
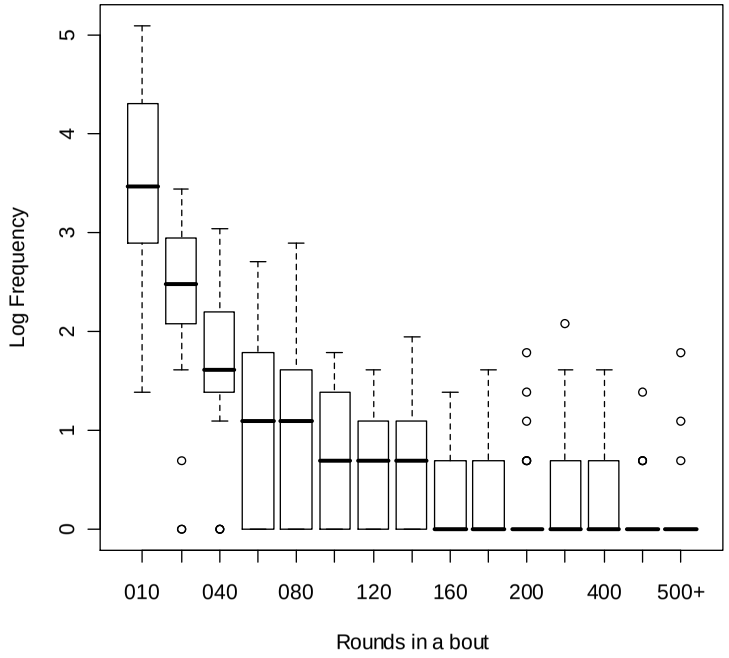


Fig. 1. Typical patterns of flight distribution: (A) continual flight, (B) pulse flights with short pauses, (C) pulse flights with varying pauses. Each vertical line represents the number of rounds observed within a minute interval.



Log frequency distribution of number of rounds in a single bout: GWSS male



Log frequency distribution of number of rounds in a single bout: GWSS female

Fig. 2. Log frequency distribution of rotations in a single bout. All bouts of males (n = 31) and females (n = 25) are accumulated and the number of bouts that fall within each scale of rounds are counted. In the box plot, the heavy center line is the median, the box boundaries are the 25- and 75-percentiles, and the extremes of the vertical bars are the 10- and 90-percentiles. The open circles above or below indicates outliers.

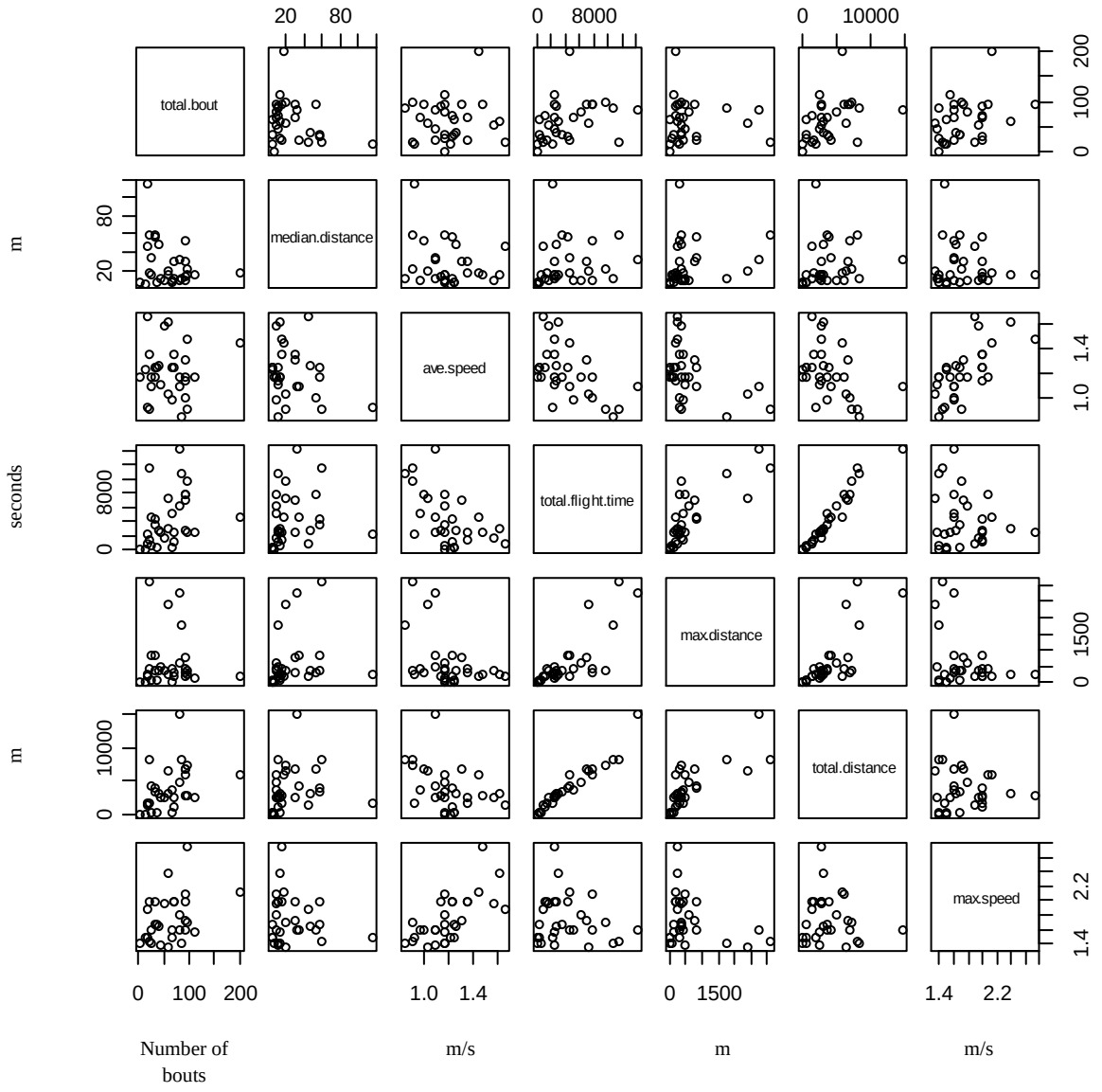


Fig. 3a. Correlations among male flight parameters in paired display. The specific analyses of high and significant correlations are provided in the table 2.

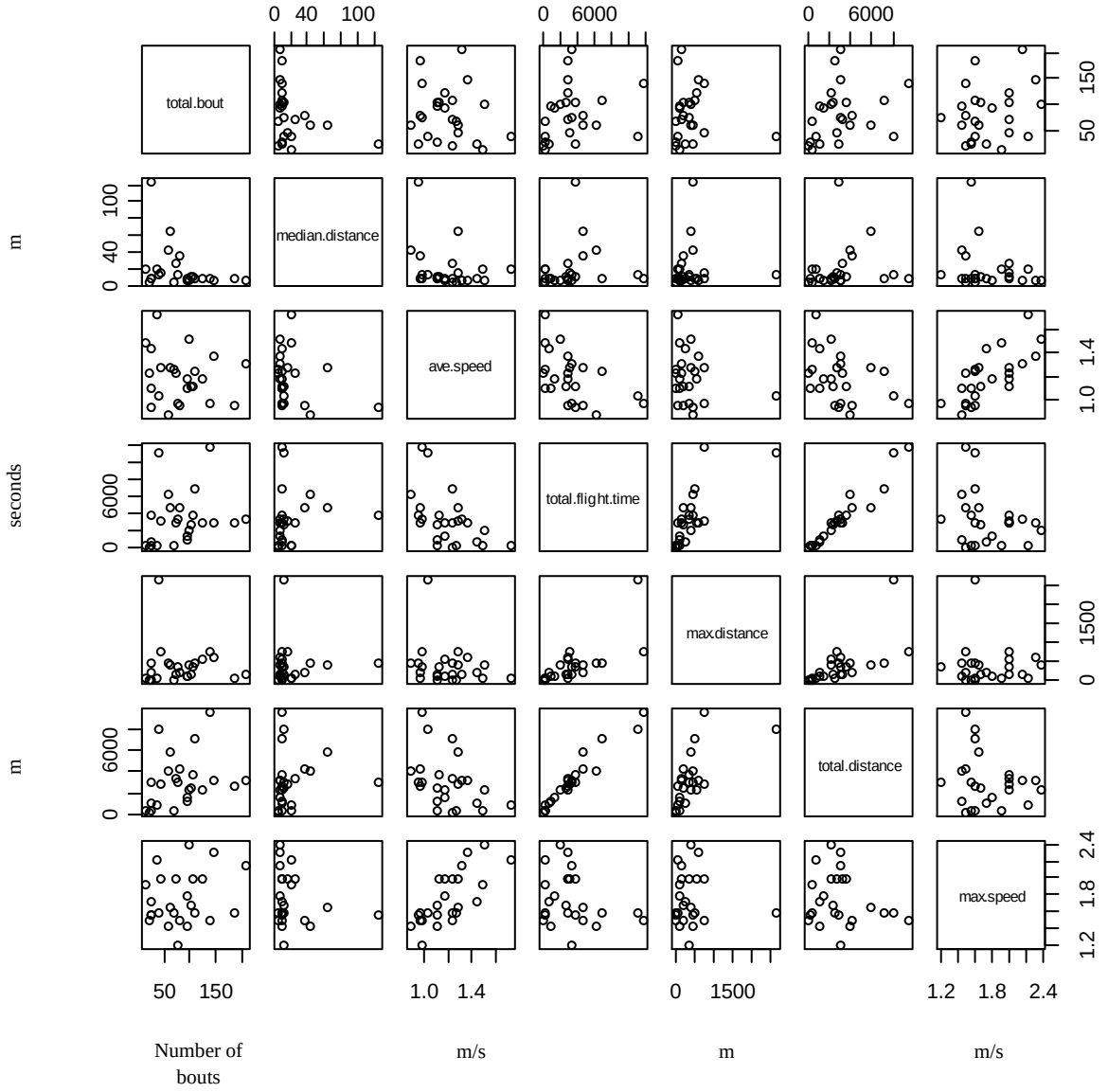
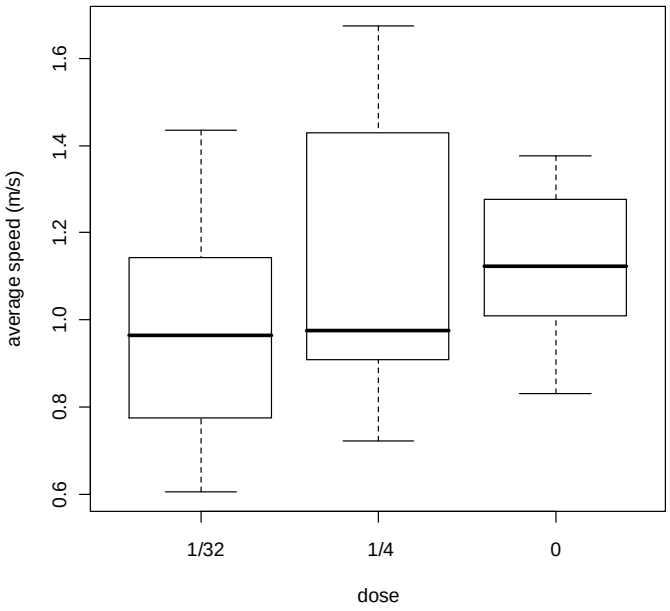


Fig. 3b. Correlations among female flight parameters in paired display. The specific analyses of high and significant correlations are provided in the table 2.



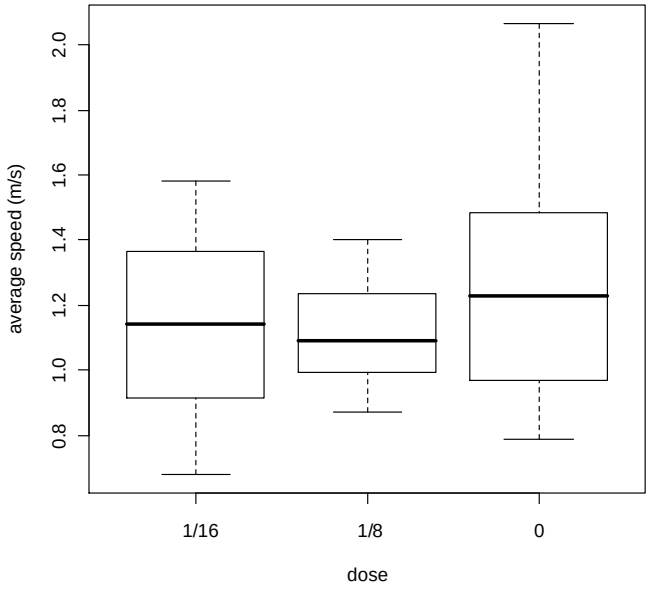


Fig. 4-a. Distribution of average flight speed by GWSS individuals exposed to grape seedlings treated with different doses.

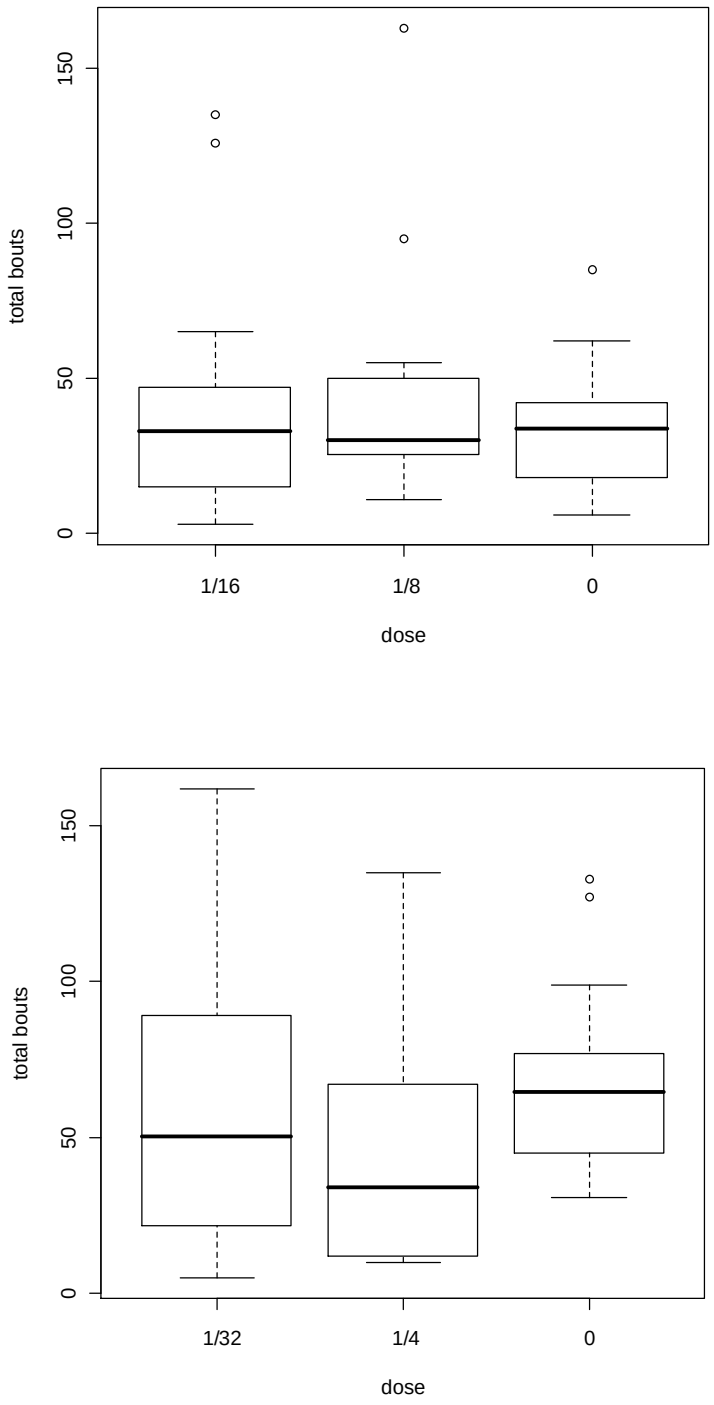
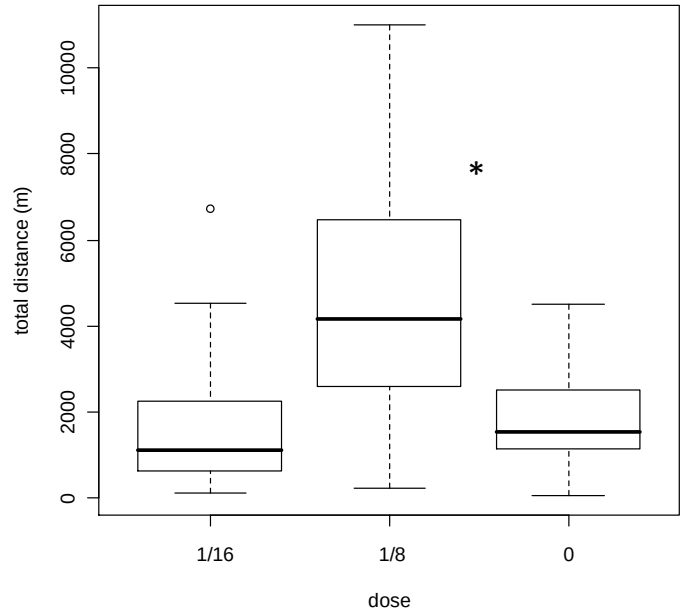


Fig. 4-b. Distribution of total number of bouts by GWSS individuals exposed to grape seedlings treated with different doses.



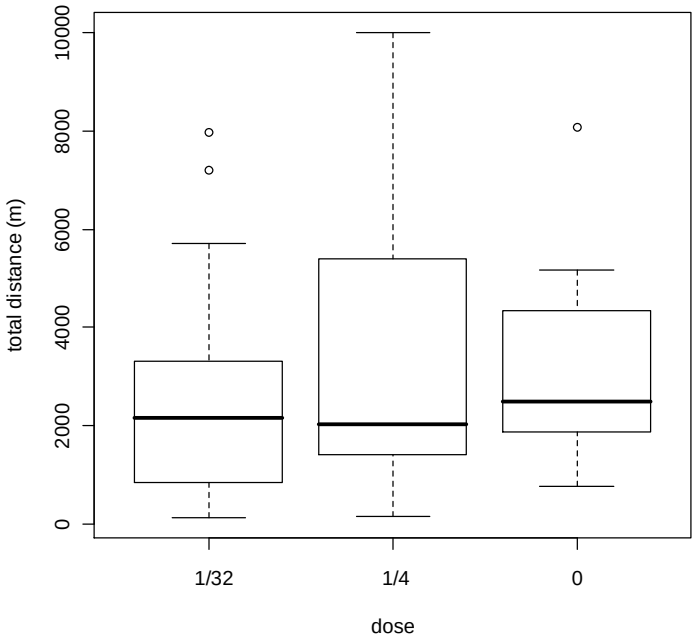


Fig. 4 – c. Distribution of total distance flown by GWSS individuals exposed to grape seedlings treated with different doses. A "*" indicates statistically significant difference with the ones without the mark at $\alpha = 0.01$ level.

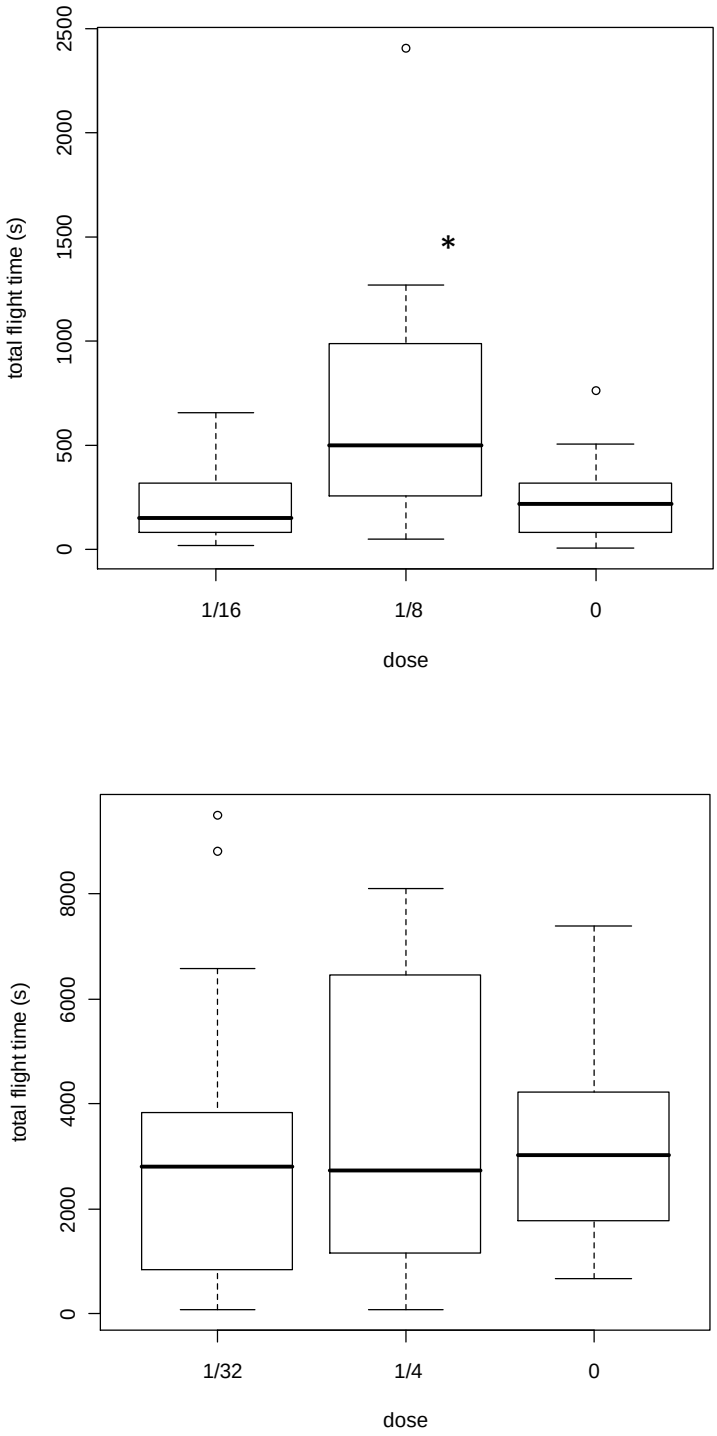


Fig. 4- d. Distribution of total time by GWSS individuals exposed to grape seedlings treated with different doses.

Chapter 5

Overview of the studies, in relation to the Pierce's disease epidemics

In the course of events resulting in the transmission of *Xylella fastidiosa* (Wells) (*Xf*) to grapevine (*Vitis vinifera* L.), an adult vector insect must: 1) acquire *Xf* from its *Xf*-hosting plant(s) through feeding, 2) initiate a flight, 3) locate a new host (grape) and land on it, 4) assess the new host and accept it, 5) probe superficial tissues and make a gustatory decision to accept it as a feeding host, 6) locate and insert stylets at the appropriate feeding site 7) salivate and initiate committed sap ingestion (Backus and McLean 1985, Perring et al. 1999, Fereres and Moreno 2009). As it is generally recognized that disease incidence is a function of vector abundance (Purcell 1981), insecticides are primarily used to directly reduce the vector population in attempts to control plant diseases caused by vector-borne pathogens. However, sub-lethal or even non-lethal effect of insecticide can potentially interfere with the 1) pathogen acquisition through anti-feeding effect, 2) flight through paralysis or loss of muscle coordination, 3) host location and 4) host acceptance through disrupting the cues for the vector, making the host plant unacceptable or repellent for it, and 5-7) by anti-feeding effects or modifications of feeding processes, possibly affecting the crucial step(s) involved in the actual pathogen deposition step.

In order to numerically express the relationship between the vector abundance and Pierce's disease (PD) infection, Purcell (1981) formulated a model

$$P_{\mathrm{nt}} = 1 - e^{-niEt}$$

Where P_{nt} is the probability of infection by n vectors in t unit of time, i is the probability that the insect has acquired *Xf*, and E is the vector's *Xf* transmission efficiency per unit time: the probability of transmission if the vector is infective. According to this model, reducing each term, n, i, E, t, would mean suppressing the disease incidence. Mortality by insecticides mostly directly reduce n, and interference with flight and repellency can also reduce the number of vectors arriving at or staying on the plant. The infectivity i may be reduced through decreasing *Xf* acquisition by mortality or anti-feeding effects. The transmission efficiency E may be reduced through interference with feeding processes. The contribution of duration of feeding t, however, to the *Xf* transmission probability is not clear. In Daugherty and Almeida's (2009) meta-analysis of *Xf* transmission experiments, effects of inoculation access period, i.e. t, was not significant. Depending on at which stage of feeding process the passage of bacterial cells from the vector precibarium and/or cibarium to the grape xylem tissue occurs and how often that happens in a single feeding bout, the relative contribution of feeding time and frequency of feeding bouts will change. An antifeedant will certainly reduce feeding time per bout, but how it affects the feeding process may depend on the chemical. If it is the frequency of the feeding bout that increases the chance of transmission, then an antifeedant may disrupt a feeding bout and make the vector choose another feeding site/host, in which case the chance of transmission could increase. If the chemical disturb the feeding process itself, it may interfere or facilitate the inoculation process, and possibly acquisition process, too. For example, when Shanks and Chapman (1965) treated tobacco with parathion and DDT, aphids fed longer on parathion-treated

foliage than on un-treated leaves while DDT had the opposite effect. However, neither insecticide affected acquisition of potato virus Y (PVY) by aphids, yet parathion tended to decrease and DDT increase the virus transmissions to treated plants. Therefore, it is crucial to find the details of transmission mechanisms, and test each chemical on how it affects at different points.

With glassy-winged sharpshooters, *Homalodisca vitripennis* (Germar) (GWSS), Backus et al. (2009) monitored its feeding with electrical penetration graph (EPG) and identified a distinctive pattern of activity designated as X waves. This phenomenon's fine structure was previously associated with the transmission of maize chlorotic dwarf virus in leafhopper probing behavior by Wayadande and Nault (1993). Further study is under way to associate the details of the X wave with specifics of the feeding process, and until such studies are confirmed, we can only construct hypotheses with circumstantial evidence.

In our study, a neonicotinoid insecticide imidacloprid (Bai, 1991) suppressed the feeding of both of the two vector insect species, GWSS and *Graphocephala* atropunctata (Signoret) (BGSS) at sub-lethal levels, where the mortality effect was not significantly different from untreated controls (Chapter 2). Such anti-feeding effects became significant within a few hours of insect exposure to the treated plants. As the insects survived longer than they would normally without feeding (Backus et al. 2006), imidacloprid seems to affect more than the simple act of xylem fluid ingestion, e.g. GWSS and BGSS physiology related to their energy expenditure.

Imidacloprid also affected the location of vector feeding sites within a plant. Although the differences were smaller for BGSS which already preferred to feed on leaves, the vectors exposed to treated plants tended to feed on the leaves rather than stems. This may be due to differential distribution of imidacloprid titer within the plant, and may affect the transmission efficiency *E* of the vectors.

How such changes affect *Xf* transmission by the vectors, the *E* term in the equation, was not clear from our transmission experiments (Chapter 3). As observed in some other works (Bextine et al. 2004, Jackson et al. 2008), the effects on transmission were variable. At least in our study, however, the transmission rate of imidacloprid-treated plants, in different doses from sub-lethal to moderately (below current field rate mortality) lethal, did not become significantly higher than that of untreated controls. This suggests that the effects of imidacloprid on the leafhoppers are unlike those of DDT on aphids with PVY (Shanks and Chapman 1965) or of pymetrozine on GWSS (Bextine et al. 2004).

The results of experiments to determine if imidacloprid-treated plants repelled sharpshooters (Chapter 3) also did not detect any substantial repellency effect of imidacloprid for sharpshooters. This was somewhat unexpected, as the treated plants were apparently unacceptable as a feeding host, and not all treated plants had an imidacloprid titer high enough to intoxicate the vectors quickly, giving them a chance to move to a better host. BGSS (Purcell 1981) and GWSS (Felix et al. 2007, Brodbeck et al. 2007) have been observed to display host preference among different varieties of grapes. However, vectors landed on imidacloprid treated and untreated plants indiscriminatingly, and even after the initial contact with the treated plants, the insects did not fly away from the treated plants. Such results were reflected in the rate of PD incidence among the plants used for the test. Diseased plants occurred at the same rate among the imidacloprid treated and untreated grapes, regardless of the vector insect. In contrast, a

known repellent, kaolin particle film, significantly reduced the landing of vectors on the treated plants, feeding, and *Xf* transmission, although once a vector settled on a treated plant, movement from treated to untreated plant was not significantly higher than the movement between any other combinations of plants. Therefore, reducing *n* term of the transmission equation via repellency is unlikely with imidacloprid.

Another unexpected result was the flight of GWSS exposed to the imidacloprid-treated plants. Above the sub-lethal doses, imidacloprid killed GWSS and reduced the flight of toxicated insects. However, flight ability of the surviving insects was not significantly different from those on untreated control plants. In one case, at a dose above the sub-lethal level, GWSS flew longer than the control insects. Even though flight mill system tends to overestimate insect flight potential (Riley et al. 1997), considering that the flight was performed without food supply, and further food deprivation due to the anti-feeding effect of imidacloprid, the flight capability of imidacloprid-exposed GWSS was substantial. If the effects of mortality and toxication were to be accounted for, the overall vector visitation rate would certainly decrease. However, the impact of surviving (and escaping, as the insects may eventually toxicated by neighbor treated plants if they don't fly outside of treated area) insects on spreading PD in later seasons should not be ignored.

In conclusion, the disease management through imidacloprid application mostly stems from its mortality effect, especially at the current rates of application. Imidacloprid cannot be expected to have a repellency effect before or after the vector lands on the plants. Its anti-feeding effect does not seem to have an adverse effect on PD epidemics. The relative contribution of the anti-feeding effect on disrupting transmission, and whether it can be effective against different levels of GWSS infestation should be better known once the details of their feeding processes are deciphered.

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