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2013媒介昆蟲與蟲媒病害國際研討會專刊

Proceedings of the 2013 International Symposium on Insect Vectors and Insect-Borne Diseases

主編　張宗仁 李啟陽 石憲宗
Edited by Chung-Jan Chang, Chi-Yang Lee, and Hsien-Tzung Shih



行政院農業委員會
Council of Agriculture, Executive Yuan
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中華民國一〇二年八月
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The 2013 International Symposium on Insect Vectors and Insect-Borne Diseases

2013 媒介昆蟲與蟲媒病害國際研討會

Agenda 議程表

Schedule	Program/ schedule	Speaker (Institution)	Moderator (Institution)
August 6th (Tue)			
08:30-09:20	Registration		
09:20-09:40	Opening Session: Opening Remarks: Welcome Address (Dr. Junne-Jih Chen, Director-General, TARI, COA)		
09:40-10:00	Tea Break and Group Photo		
10:00-11:00	Keynote speech: Invasive potential of *Xylella fastidiosa*	Dr. Alexander H. Purcell (UC Berkeley, USA)	葉 瑩處長 Dr. Ying Yeh, Director General (Department of Science and Technology, Council of Agriculture, Executive Yuan)
11:00-12:00	Keynote speech: Fastidious prokaryotes and plant health	Dr. Chung-Jan Chang (University of Georgia, USA)	
12:00-13:20	Lunch Break		
13:20-13:50	Matsumura's collection of froghoppers and sharpshooters (Hemiptera: Cicadomorpha) of Taiwan in the Hokkaido University Insect Collection	Dr. Kazunori Yoshizawa (University of Hokkaido, Japan)	何琦琛博士 Dr. Chyi-Chen Ho (Applied Zoology Division, TARI: Retired Senior Entomologist)
13:50-14:40	Overview of the phylogeny, taxonomy and diversity of the leafhopper (Hemiptera: Auchenorrhyncha: Cicadomorpha: Membracoidea:Cicadellidae) vectors of plant pathogens	Dr. Christopher H. Dietrich (INHS, Prairie Research Institute, USA)	
14:40-15:00	Tea Break		

15:00-15:50	How effective is sharpshooter control at limiting Pierce's disease spread in California vineyards?	Dr. Matthew P. Daugherty (UC Riverside, USA)	蔡志偉博士 Dr. Chi-Wei Tsai (Department of Entomology, National Taiwan University (NTU))
15:50-16:40	Habitat effects on population density and movement of insect vectors of *Xylella fastidiosa* in California, USA	Dr. Rodrigo Krugner (USDA-ARS in Parlier, USA)	
16:40-17:30	Panel Discussion		Dr. Chung-Jan Chang (University of Georgia, USA)
August 7th (Wed)			
08:30-09:10	Registration		
09:10-10:10	Keynote speech: *Xylella fastidiosa* diversity	Dr. Rodrigo P. P. Almeida (UC Berkeley, USA)	高靜華組長 Cing-Hua Kao, Director (Applied Zoology Division, TARI)
10:10-10:30	Tea Break		
10:30-11:10	*Xylella fastidiosa*-elicited leaf scorch diseases in Taiwan	Dr. Wen-Ling Deng (NCHU, Taiwan, ROC)	
11:10-12:00	Taxonomy and biology of egg parasitoids of Auchenorryncha of economic importance in Taiwan and adjacent countries, and of Proconiine sharpshooters in the New World	Dr. Serguei V. Triapitsyn (UC Riverside, USA)	
12:00-13:00	Lunch Break		
13:00-13:50	The occurrence of Pierce's disease of grapevines and its control strategies in Taiwan	Dr. Chiou-Chu Su (TACTRI, Taiwan, ROC)	詹富智主任 Fuh-Jyh Jan, Director (Department of Plant Pathology, National Chung Hsing University (NCHU))
13:50-14:40	Potential vectors of Pierce's disease in Taiwan: ecology and integrated management	Dr. Hsien-Tzung Shih (TARI, Taiwan, ROC)	
14:40-15:40	Understanding bacterial virulence genes and mechanisms of host response in insect-mediated citrus Huanglongbing	Dr. Hong Lin (USDA-ARS in Parlier, USA)	
15:40-16:00	Tea Break		
16:00-16:50	An integrated management of citrus Huanglongbing in Taiwan	Dr. Chia-Hsin Tsai (TARI, Taiwan, ROC)	安寶貞組長 Pao-Jen Ann, Director (Plant Pathology Division, TARI)
16:50-17:30	Panel Discussion		

August 8th (Thu)			
08:20-08:40	Registration		
08:40-09:40	Keynote speech: The new third-generation, AC-DC EPG monitor and its usefulness for IPM research on vectors of plant pathogens	Dr. Elaine A. Backus (USDA-ARS in Parlier, USA)	楊恩誠教授 Prof. En-Cheng Yang (Department of Entomology, NTU)
09:40-10:00	Tea Break		
10:00-10:50	Tospoviruses and thrips- is there an evolutionary relationship?	Dr. Laurence A. Mound (CSIRO Ecosystem Sciences, Australia)	王清玲博士 Dr. Chin-Ling Wang (Applied Zoology Division, TARI: : Retired Senior Entomologist and Director)
10:50-11:30	Tomato leaf curl disease in Taiwan and breeding for resistance against it	Dr. Wen-Shi Tsai (AVRDC- The World Vegetable Center)	路光暉主任 Kuang-Hui Lu, Director (Department of Entomology, NCHU)
11:30-12:10	Insect transmission of tomato yellow leaf curl viruses	Dr. Chi-Wei Tsai (NTU, Taiwan, ROC)	
12:10-13:10	Lunch Break		
13:30-14:10	Visit to the Insect Collection at TARI		李奇峰博士 Dr. Chi-Feng Lee (Applied Zoology Division, TARI)
14:30-16:30	Group Discussion for Invited Guests Only (1) Group 1: insect vectors and insect-borne diseases [At Seminar Room, Taiwan Soil Museum, Agricultural Chemistry Division, TARI]		張宗仁博士；林宏博士 Dr. Chung-Jan Chang & Dr. Hong Lin
	(2) Group 2: Taxonomy workshop [At Room 225, Applied Zoology Division, TARI]		楊正澤博士 Dr. Jeng-Tze Yang (Department of Entomology, NCHU; Department of Plant Medicine, NPUST)

Contents

Preface i

Invasive Potential of *Xylella fastidiosa* ..... A. Purcell 1

Fastidious Prokaryotes and Plant Health ..... C. J. Chang 17

Matsumura's Collection of Froghoppers and Sharpshooters (Hemiptera: Cicadomorpha) of Taiwan in the Hokkaido University Insect Collection ..... K. Yoshizawa 35

Overview of the Phylogeny, Taxonomy, and Diversity of the Leafhopper (Hemiptera: Auchenorrhyncha: Cicadomorpha: Membracoidea: Cicadellidae) vectors of plant pathogens..... C. H. Dietrich 47

How Effective is Sharpshooter Control at Limiting Pierce's Disease Spread in California Vineyards? ..... M. P. Daugherty 71

Habitat Effects on Population Density and Movement of Insect Vectors of *Xylella fastidiosa* in California, USA ..... R. Krugner 83

Xylella fastidiosa Diversity ..... R. P. P. Almeida 107

Xylella fastidiosa-Elicited Leaf Scorch Diseases in Taiwan..... W. L. Deng 117

Taxonomy and Biology of Egg Parasitoids of Auchenorryncha of Economic Importance in Taiwan and Adjacent Countries, and of Proconiine Sharpshooters in the New World ..... S. V. Triapitsyn 123

The Occurrence of Pierce's Disease of Grapevines and Its Control Strategies in Taiwan..... C. C. Su 145

Potential Vectors of Pierce's Disease in Taiwan: Ecology and Integrated Management..... H. T. Shih 163

Understanding Bacterial Virulence Genes and Mechanisms of Host Response in Insect-Mediated Citrus Huanglongbing..... H. Lin 177

An Integrated Management of Citrus Huanglongbing in Taiwan..... C. H. Tsai 193

The New, Third-generation, AC-DC Electrical Penetration Graph (EPG) Monitor and Its Usefulness for IPM Research on Vectors of Plant Pathogens ..... E. A. Backus 211

Tospoviruses and Thrips-is There an Evolutionary Relationship? ..... L. A. Mound 231

Tomato Leaf Curl Disease in Taiwan and Breeding for Resistance Against it ..... W. S. Tsai 239

Insect Transmission of Tomato Yellow Leaf Curl Viruses..... C. W. Tsai 255

Preface

Economic losses caused by insect-borne diseases on crops are much higher than those caused solely by insects or diseases. Insect-borne diseases may influence food security (production issues) and food safety (pesticide residues issues). So far, no simple ways to deal with these diseases. The integrated management strategy has been considered a good practice for disease prevention at present, including eradication of infected plants, insect control and breeding of disease-resistant crops. Unfortunately, early symptoms of insect-borne diseases are similar to those of physiological disorders caused by lack of nutrients or water deficiency. Misdiagnosis, therefore, occurs easily. Until the insect-borne diseases are confirmed, in most cases, it is too late to initiate appropriate and timely remedies. That is why the development of the techniques for the integrated management of insect-borne diseases has become an important and urgent issue worldwide.

The effective techniques of the integrated management for insect-borne diseases can benefit both producers and consumers. To maximize the performance of these techniques it requires the consideration of the characteristics of crop growth, manure management, and climatic conditions. Additionally these methods should be appropriated for and recognized by farmers. When applied at right time, the use of suitable control methods would achieve both economic benefits and environmental safety concerns.

For this purpose and under the International Agricultural Cooperation Act of 2013, the Council of Agriculture (COA) is interested in providing funds to support this international symposium. The symposium is co-organized by COA, Taiwan Agricultural Research Institute (TARI) and Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ). Topics discussed at the meetings include current state of important vector-borne diseases, innovation of new technologies, techniques of the integrated management and ecology and classification of insect vectors. Eleven experts from the United States, Australia and Japan and six scholars from local institutions have been invited as keynote speakers. The scientific findings and recommendation in the papers contained in this volume are contributions of all participating scientists and published as a special publication of TARI.

On behalf of TARI, I wholeheartedly welcome our distinguished speakers as well

as all participants. I look forward to witnessing this symposium becoming a communication platform for domestic and international scholars that undertake the study of insect-borne diseases and insect vectors. Papers published in this proceedings would be a good reference for agricultural authorities to develop a plan for the integrated researches of insect-borne diseases and insect vectors. Foreign scholars are in particular encouraged to keep contact with researchers in Taiwan. Together we can build up the fundamental knowledge of insect-borne diseases that leads to reduce the economic impact of diseases and insect vectors in agricultural industry.

Junne-Jih Chen, Ph.D.
Director General
Taiwan Agricultural Research Institute
Council of Agriculture, Taiwan ROC
August, 2013

序

蟲媒病害對農作物所造成的經濟損失，遠高於昆蟲或病害單獨造成的危害，其直接影響範圍包括糧食安全 (產量問題) 與食品安全 (農藥殘留問題) 兩大議題。由於這類病害皆不易防治，降低此類病害對產業造成經濟衝擊的最佳策略，在於剷除病株、防治媒介昆蟲與抗病育種等。惟蟲媒病害初期受害徵狀與植物缺乏養分之生理病徵或缺水極為相似，容易誤判，一旦確認為蟲媒病害再予以防治，多為時已晚。基此，蟲媒病害的整合防治技術成為全球重要的課題。

有效的蟲媒病害及其媒介昆蟲之整合管理技術，對產區與社會的經濟效益皆有所助益，其操作過程必需考量作物生育特性、肥培管理與氣候條件，於適當時機、運用適用防治方法，使防治過程兼具經濟效益與環境安全，才能建立適地適用且受農友認同的整合管理技術。

農委會在 2013 年的「國際農業合作」領域，以計畫經費支持辦理本次國際研討會，由農業試驗所和防檢局共同策劃，針對國內外重要蟲媒病害研究現況、創新研究技術、整合防治技術與媒介昆蟲之分類與生態研究等主題，邀請十一位來自美國、澳州與日本之國外專家和六位國內學者進行專題報告，並將專家之書面論文編印成專刊。

本人謹代表本所同仁，熱烈歡迎參與本次研討會的國內外專家學者。也期待本次研討會能夠作為從事蟲媒病害與媒介昆蟲國內外學者的交流平台，建立起蟲媒病害與媒介昆蟲整合研究之知識基礎，期以有助於未來降低此類病害與媒介昆蟲對產業的經濟衝擊。

行政院農業委員會農業試驗所

所長 陳駿季

中華民國一〇二 年八月

Invasive Potential of *Xylella fastidiosa*

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ABSTRACT

Evaluating the risks of invasion by the bacterium *Xylella fastidiosa* to geographic regions where this plant pathogen currently does not occur is an important challenge. Various strains of *X. fastidiosa*, differentiated by their plant host range, comprise a formidable variety of serious plant diseases throughout the tropical through subtropical Americas. These diseases do not seem present as significant threats outside the Western Hemisphere, except for Taiwan, which has recorded diseases in pear and grape caused by *X. fastidiosa*. Identifying the factors limiting or even prohibiting the spread of *X. fastidiosa* and their modes of action would be useful in attempts to estimate risks of new invasions by this bacterium and-more importantly-to identify the most effective phytosanitary strategies to prevent the bacterium from establishing in new regions. Although it is clear that cold severity of sub-freezing winter climates limit the geographic spread of *X. fastidiosa*, we lack an understanding of the underlying mechanisms of how freezing eliminates it from plants. Other aspects of climatic temperature regimes, such as limiting high temperatures or sustained cool but above freezing temperatures need to be addressed. For some regions, the lack of suitable insect vectors or suitable alternative hosts of *X. fastidiosa* may prevent introductions of *X. fastidiosa* in infected plant hosts from establishing a permanent presence. It is likely the permanent establishment of *X. fastidiosa* requires a suitable combination of vectors' distribution, abundance, plant preferences, phenology, transmission efficiency, and dispersal behavior in conjunction with the abundance and distribution of plant hosts of *X. fastidiosa* and the characteristics of the plant communities in which they are embedded. The intriguing possibilities of interactions with other bacteria and viruses have only begun to be explored as limiting factors.

Keywords: *Xylella*, Pierce's disease, citrus variegated chlorosis, sharpshooter, quarantine, phytosanitary

INTRODUCTION

Why be concerned about the invasiveness of *Xylella fastidiosa*?

Our practical concerns with the bacterium *Xylella fastidiosa* emphasize the prevention and control of the numerous plant diseases that this plant pathogen causes. Countries without these diseases want to prevent their invasion and establishment. The large diversity of serious diseases in numerous crop and forest species that are caused by *X. fastidiosa* have been reviewed recently [9, 27, 45]. These range from diseases affecting grape, almond, oleander and alfalfa in California and other southwestern parts of North America, to diseases of grape, peach, plum, pecan, blueberry and numerous forest tree species in southeastern North America, to diseased orange, coffee and plum crops in South America. Outside of the Americas, the only confirmed occurrences of diseases caused by *X. fastidiosa* are diseases of pear and grape in Taiwan [9, 30, 52]. .*X. fastidiosa* has also been reported in grape in Serbia (now Bosnia) [4] and a brief report in almond in Turkey [21], but it unclear if the diseases concerned are spreading in Europe.

The theoretically huge list of plant species that support the multiplication of *X. fastidiosa* (considerably more than 50% of those species tested so far) [16, 25, 45, 47] should facilitate the spread of the bacterium through the commercial movements of live plants. Yet there is no convincing evidence that this has contributed to transoceanic of *X. fastidiosa*. The introduction of grapevines unknowingly infected with *X. fastidiosa* may have introduced Pierce's disease (PD) into commercial vineyards in France, where new plantings with PD were removed and insecticides applied to prevent the establishment of PD [7]. Without untreated controls, the subsequent disappearance of PD could not prove the effectiveness of removing suspect plants in conjunction with insecticide applications. Classically, most introductions of exotic organisms fail to establish a permanent presence, but eventually many exotic invaders establish permanently [31]. Plant quarantines by practical necessity are based on logic instead of scientific proof.

Why hasn't *X. fastidiosa* invaded more regions outside of the Americas? Numerous fungal and viral pathogens of grape that are native to North America have

become widespread throughout much of the world, beginning in the late 1700s with of the importation of grape species to European botanical collections. Yet PD, another disease of grape indigenous in southern North America, never became established. This was despite the massive importation from the southern United States to Europe of wild grapevines for use as rootstocks against the grape phylloxera[18]. It is inconceivable that *X. fastidiosa* was not present in some of these vines. Species of wild grape native to the southern United States are tolerant of *X. fastidiosa* [22]. We can only speculate about possible explanations to answer the question of why any invasive organism has not established where it is continually introduced.

For *X. fastidiosa*, we have evidence for some limiting factors such as winter climate. For regions where freezing temperatures are rare, we must search for explanations, realizing that key limitations may be completely different from one location to another. Finding explanations of factors that limit invasions of *X. fastidiosa* into new locations may not only provide new ideas or improved quarantine measures or other phytosanitary strategies, but may also provide new ideas for control of *Xylella*-caused diseases in areas where this pathogen is native.

POSSIBLE LIMITING FACTORS

Climate

Winter climate is an important feature of the epidemiology of PD in northern California [27, 40] and probably elsewhere [27]. Subfreezing winter temperatures are important for recovery of grapevines with PD [29, 36, 40]. Almond leaf scorch disease has similar results in California [8]. Vector-borne new infections of *X. fastidiosa* in grapevines after the first two months of the growing season in northern California had recovered and were free of *X. fastidiosa* after the next winter [13, 41]. Northern California vines that did not recover overwinter had populations of *X. fastidiosa* that were sufficient for vector acquisition only after June [51]. The overwinter recovery phenomenon, in conjunction with the seasonal changes in *X. fastidiosa* populations, explains why the early growing season is critical for establishing chronic infections (no recovery) of *X. fastidiosa* [27, 43, 45]. They also offer a hypothesis to explain the failures of *X. fastidiosa* to permanently establish in Europe. Europe lacks known or potential vectors of *X. fastidiosa* that overwinter as adults, thus avoiding having flying vectors during the critical early growing season that is so important to establishing chronic

infections [42]. Chronically infected plants eventually die of disease, age or other factors. For *X. fastidiosa* to persist in a location indefinitely, the rate of spread of *X. fastidiosa* to new host plants must exceed the rate at which the bacterium disappears from colonized hosts (either diseased or symptomless).

Overwinter recovery has been modeled with regression-based mathematical models based on experimental data [33]. Despite some promising clues [36], an understanding of how freezing temperatures induce recovery is still a mystery. Understanding the mechanism of cold therapy might provide insights in how to develop climate-based models to predict the geographic range of *X. fastidiosa* or provide new ideas for disease control or therapies.

Another way that climatic temperature patterns can influence the potential for the spread of diseases caused by *X. fastidiosa* is the pattern of growing season temperatures. Some temperate regions may lack the subfreezing temperatures needed for environmental therapy of diseased vines, but not have sufficient degree-days to support population growth of *X. fastidiosa* to sustain the development of severe symptoms or to promote rapid plant-to-plant spread. Temperature also may affect vector transmission [11]. It is surprising that a pathogen that is most virulent in tropical climates has a maximum temperature for sustained growth under 34^{o}C [14] and is susceptible to temperatures below 15^{o}C (Fig. 1). Is it possible some climates are too hot to sustain PD? Both of thee possibilities are unexplored.

Diseased Plant Hosts

Hybrids of grapevines that are tolerant or resistant to *X. fastidiosa* nonetheless can harbor populations of *X. fastidiosa* [17] that are adequate for vector acquisition of the bacterium [24]. Illegal importations of new PD-resistant grape varieties could thus introduce plants with persistent populations of *X. fastidiosa* if the winter climate is suitable for survival of the bacterium. This is also true of resistant cultivars of other perennial plants that may harbor populations of *X. fastidiosa* but with mild or no symptoms. Nursery plants of citrus with CVC represent a proven threat for the movement of CVC to new locations, as occurred in Brazil [19]. The use of nursery plants free of *X. fastidiosa* is a fundamental part of the current control methods for CVC in Brazil [19].

The importance of having diseased crop plants as inoculum is quite clear for CVC in Brazil [19, 20, 29, 49] (reviewed in [43]), despite the CVC strains being able to infect

many weed species [35]. This is not so apparent for PD in California or Florida, where it has been most extensively studied. The genetic regulation of growth and movement of *X. fastidiosa* in European grape (*Vitis vinifera*) apparently is not adequate to prevent severe symptoms in grape. This may make commercial vineyards with PD especially important as acquisition sources in late summer for adult vectors that inoculate the bacterium into grapevines the following spring.

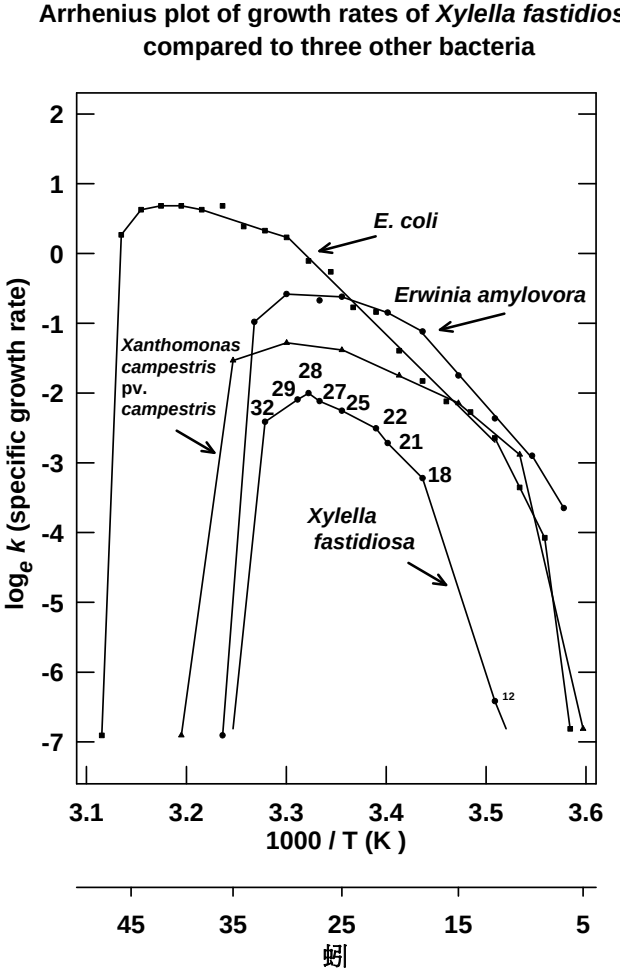


Fig. 1. The response of growth rates of *Xylella fastidiosa* and 3 other bacteria to temperature (Arrhenius plot). Note the narrower range of temperatures suitable for *X. fastidiosa* compared to two other plant pathogenic bacteria (*Erwinia* and *Xanthamonas*). Adapted from [14].

Symptomless Plant Hosts

Current evidence suggests that the cell signaling system of *X. fastidiosa* directs gene expression in ways that minimize host injury by slowing its population growth and increasing adhesion (immobility) to retard systemic movements and spread within the host plant [10, 38]. In the experiments [46] to identify the host status of plants that were favorable for an important vector for PD, recoveries of *X. fastidiosa* were attempted only after an incubation period of about 2 to 3 months. In later repetitions of these experiments for plants in which no growth of *X. fastidiosa* had been detected, populations of inoculated *X. fastidiosa* increased for several weeks, then rapidly decreased to undetectable levels [46]. It appears that xylem elements in which *X. fastidiosa* completely fills the vessel, most of the bacterial cells are dead [10]. This emphasizes the importance to *X. fastidiosa* of systemic movement within a host plant. The bacterium dies out in the plant if it completely packs the host cell and cannot move to new xylem cells [46].

The most common disease syndrome caused by infections of *X. fastidiosa* begins with the progressive decline and eventual death of foliar tissue, usually beginning at the leaf margins and progressing toward the leaf base before spreading further to woody tissues and fruit. PD of grapevine, which is the first described disease now attributed to *X. fastidiosa*, exemplifies this “leaf scorch” syndrome (Fig. 2). However, we can describe two other categories of syndromes. A second symptom type includes the dwarfing or stunting diseases such as alfalfa dwarf [54], phony peach [55], and citrus variegated chlorosis (CVC) [32]. These three diseases have a slower decline than the leaf scorch diseases and without leaf “burn” symptoms (Figs. 3-4). For example, the distinctive foliar lesions (Fig. 4) on citrus with CVC do not resemble the marginal decline and death of leaf tissues characteristic of PD. Like phony peach disease, the most damaging aspect of CVC is the dwarfing of fruits and new stem growth (Fig. 4). These diseases typically take longer to appear following inoculation via insect vector transmission. A third symptom group includes the “symptomless infections”, where *X. fastidiosa* multiplies within the host plant and may or not may not move systemically [16, 25, 46]. This third group may differ from the dwarfing syndrome group only in the rapidity and degree to which stunted growth results from colonization of the plant by *X. fastidiosa*. Most experimental evaluations of symptoms are made within a year of inoculation or even shorter, so chronic stunting that causes substantial reductions in

growth may not be seen for many months or years. The most difficult potential sources of *X. fastidiosa* to detect for plant quarantines are plants that have small and scattered populations of the bacterium and have a long incubation period before they develop symptoms, although these may be the least important for introducing *X. fastidiosa* because the bacterium is most likely to die out in such plants and they are poor sources for vector acquisition. The persistence of *X. fastidiosa* in and symptomless hosts varies with plant species [(3)] and probably with winter climate.



Fig. 2. Leaf scorch symptoms of Pierce's disease (PD) in grape in a Florida experimental vineyard. Note the young and missing vines in the background in this resistance screening planting – the result of high rates of infections with *X. fastidiosa* and favorable conditions for severe symptom development. Photo by A. H. Purcell.



Fig. 3. Citrus variegated chlorosis disease (CVC) symptoms in orange fruit (dwarfing) and leaves (stunting, chlorosis). Photo by A. H. Purcell.



Fig. 4. Symptoms of alfalfa dwarf (left)-stunting of leaves and stems, darker leaf color-after 9 months in greenhouse conditions. Photo by A. H. Purcell.

Vector abundance and distribution

Vector transmission is an essential part of the disease cycle for *X. fastidiosa*. It is clear that the types and numbers of vectors are important requirements to sustain the presence of PD. For example, the invasion of a new vector species can cause major changes in the incidence of PD. The successful invasion of southern California in the 1990s by the sharpshooter leafhopper *Homalodisca vitripennis* (formerly *coagulata*) caused major outbreaks of PD where PD previously had not been a major problem [(6)]. In California, PD occurs primarily near vector breeding habitats [(23)]. In Napa Valley, California, the sharpshooter *Graphocephala atropunctata* is the major vector [(23, 39)]. The spatial pattern of populations of *G. atropunctata* during the early growing season is very similar to spatial gradients of the incidence of PD in vineyards, with the proportion of vines with PD decreasing with distance from the sharpshooter's overwintering habitat.

Other vector characteristics are also influential. The following comments address characteristics of vectors that are known to be important for the spread of *Xylella* diseases.

Transmission efficiency

Not every individual vector that has acquired *X. fastidiosa* transmits it to all plants on which the insect feeds. Vector transmission efficiency is the proportion of plants infected per insect access period. Vector species vary greatly in transmission

efficiency, depending on the combination of vector species and plant species (reviewed in [48]). For example, the grass-feeding sharpshooter *Draeculacephala minerva* is more efficient in transmitting *X. fastidiosa* to alfalfa than *G. atropunctata*, which prefers woody plants, whereas the reverse is true for transmission to grape [50]. The differences in distribution and anatomy between grasses and dicotyledonous plants may require differences in feeding behavior to respond to the different cues found among various plant species.

Although it has been long hypothesized that all xylem sap-feeding, sucking insects are potentially vectors of *X. fastidiosa* [15], there are strains of this bacterium that differ in transmission efficiency depending on the vector and plant species [34]. This makes it difficult to predict how a certain strain of *X. fastidiosa* will be transmitted in a new location with different vectors and plant communities.

Plant and habitat preferences

Xylem feeders often have wide plant host ranges (reviewed in [48]), but all species demonstrate strong preferences when they have a choice of hosts. The condition of the plant can also be as important as the species in feeding preferences. Xylem feeders generally prefer more succulent, fast-growing plant tissues [37]. The most important native vectors (*Draeculacephala minerva* and *Xyphon fulgida*) in central California are two grass-feeding sharpshooters [23, 44]. Because they prefer succulent plants, they reach their highest populations during California's dry summers where grasses are irrigated [23, 44] Both of these two species are rarely found feeding on grape; their importance as vectors is deduced from their consistent association with PD outbreaks in vineyards next to habitats harboring these sharpshooters [23, 44].

In contrast, grape is a highly preferred host of the major vector (*G. atropunctata*) in coastal California vineyards [23, 44]. Surprisingly the incidence of PD in central California vineyards compared to Napa Valley vineyards can be very similar, despite only rarely observing vectors on grape in central California vineyards [45]. In addition, the central California vectors are much less efficient transmitters of *X. fastidiosa* to grape. The feeding of *D. minerva* and *X. fulgida* on grape is hypothesized to occur in the evenings, as these two species fly mostly in early evening, when they may drift or wander into nearby vineyards from their normal habitats [44].

Vector dispersal

Vector mobility can compensate for inefficient transmission, as exemplified by the sharpshooter *H. vitripennis.* The transmission efficiency of *X. fastidiosa* to grape by *H. vitripennis* is relatively low, about 5% to 15% per day per insect, [(1)], but the longer range dispersal and frequent daily movements or over longer time periods by *H. vitripennis* compared to more traditional vectors created epidemic spread of PD [(5, 6)].

It is the combination of vector traits that determines its overall effectiveness and importance as a vector, not just a single trait. This can be illustrated with a simple mathematical model. The probability of transmission by *n* vectors per plant per time interval (P_{nt}) depends on how many vectors (*n*) and the number of time intervals (*t*) they are present on a plant, as well as the fraction of vectors that are infective with the pathogen (*i*) and that transmit per time interval (transmission efficiency *E*). The relationship is

$$P_{nt} = 1\text{-}P^{-niEt} \qquad (41)$$

In this equation, all four determinants (*n, i, E, t*) are mathematically equivalent. Thus an abundant (high *n*) but inefficient (low *E*) vector can be very damaging as a vector if it has frequent plant to plant movements (high *t*) or a high rate of infectivity (high *i*). Host preferences can affect the frequency of vector movements. As already discussed, transmission efficiency (*E*) by a single vector species can vary with host plant. Thus the mix of plant host species can affect how important any vector species will be.

Phenology

Recall that in temperate climates with almost all grapevines inoculated with *X. fastidiosa* after the early growing season recover completely. Thus overwintering adult vectors that are infective with *X. fastidiosa* establish most of the infections destined to be chronically diseased. This is one possible explanation as to why PD never established in Europe, where most xylem sap-feeders found in vineyards overwinter in the egg stage[(42)]. Another explanation for the increase in PD accompanying the establishment of *H. vitripennis* in southern California is that this sharpshooter is able to transmit *X. fastidiosa* to dormant vines during winter [(2)]. *H. vitripennis* overwinters in California as an adult, so this characteristic could be important.

Antagonists of *Xylella fastidiosa*?

There is not yet much to be said about microbial antagonists of *X. fastidiosa* because of a lack of research in this area. Bacteriophages that attack *X. fastidiosa* have been identified [53], as well as a number of prophages in the genome of *X. fastidiosa* identified from genome sequencing [12] (reviewed in [43]).

Strains of *X. fastidiosa* that do not cause disease in grape but protect against PD strains of *X. fastidiosa* have been reported in Florida [26]. Other xylem inhabiting bacteria have shown some antagonism but are not yet been proven to be effective as protective agents against *X. fastidiosa* [28].

It would be unexpected to find that antagonistic interactions with other microbes would completely inhibit the invasion of *X. fastidiosa* into a new geographic region, but relatively few studies of microbial antagonism to *X. fastidiosa* have been made. This is an area that may have potential for control.

CONCLUSION

The persistence of *X. fastidiosa* in a given environment depends on a combination of suitable conditions at the proper times, not just the introduction of the bacterium in a plant or insect. Climate, host plants, suitable vectors and the vegetation and habitats needed to support them are all essential requirements to sustain the spread *X. fastidiosa* from plant to plant.

Xylella fastidiosa may be present in some geographic regions but unrecognized because it is causes no or very subtle symptoms or if any severely affected plants are not common. PD was not recognized in the southeastern USA until the 1950s, yet is one of the limiting factors for growing grapes there [22].

Taiwan is unique in being the only location outside the western hemisphere with documented established diseases caused by *X. fastidiosa*. If we can discover the explanation for the success of *X. fastidiosa* in Taiwan, it might provide new ideas on how to prevent invasions by *X. fastidiosa* from occurring in other countries.

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Fastidious Prokaryotes and Plant Health

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ABSTRACT

The prokaryotes are almost everywhere or we can phrase like this “prokaryotes are wherever there is life”. They were the earliest organisms on earth. Today, they still dominant the biosphere for the following two facts: 1) their collective biomass outweighs all eukaryotes combined at least tenfold, and 2) more prokaryotes inhabit a handful of fertile soil or the mouth or skin of a human than the total number of people who have ever lived. They thrive in habitats that are too cold, too hot, too salty, too acidic, or too alkaline for any eukaryote because they display diverse adaptations that allow them to inhabit many environments and they have great genetic diversity. Phytopathogenic fastidious prokaryotes are plant pathogens that either resist to grow in any available bacterial culture media or require specific or enriched media to grow. They include *Xylella fastidiosa*, *Leifsonia xyli* subsp. *xyli*, *L. xyli* subsp. *cynodontis* and *Clavibacter michiganensis* subsp. *sepedonicus* and *C. michiganensis* subsp. *michiganensis* that reside in xylem and spiroplasmas, phytoplasmas and *Candidatus* Liberibacter spp. that reside in phloem. *X. fastidiosa* is the causal agent of more than 19 diseases; among them Pierce’s disease of grape and citrus variegated chlorosis are two major maladies that cause serious economic loss on wine and citrus juice industry. *L. xyli* subsp. *xyli*, and *L. xyli* subsp. *cynodontis* are associated with ratoon stunting disease of sugarcane and Bermuda grass stunting respectively and *C. michiganensis* subsp. *sepedonicus* with bacterial ring rot in potato and *C. michiganensis* subsp.

michiganensis with bacterial tomato canker. Spiroplasmas are the causal agents of citrus stubborn, corn stunt and periwinkle diseases. Phytoplasmas are associated with more than 500 diseases worldwide. *Ca*. Liberibacter spp., are the causal agents of citrus Huanglongbing or citrus greening, zebra chip disease of potato and others. Pierce's disease is the limiting factor for the establishment of wine industry for the entire southeastern United States from Texas to the Carolinas along the gulf coast of Mexico. Recent introduction of the glassy-winged sharpshooter leafhoppers in California has threatened the winery industry of California. The successful isolation of *X. fastidiosa* from the tissues with citrus variegated chlorosis (CVC) symptoms followed by the identification of the major insect vectors provided crucial information for citrus growers and citrus juice industry to deal with the CVC crisis in Brazil. The successful isolation of *X. fastidiosa* from blueberry tissues with leaf scorch symptoms followed by the identification of the susceptibility/resistance of various blueberry cultivars provided significant information for the blueberry industry which has recently become the number one fruit commodity in Georgia. The biological characteristics of the three phloem-limited prokaryotes, namely spiroplasmas, phytoplasmas and *Ca*. Liberibacter spp., and the diseases they induce and their vectors will be discussed. Most plant pathogenic prokaryotes do not require an active insect vector to spread them from plants to plants, while *X. fastidiosa*, *Ralstonia syzygii*, *Ca*. Liberibacter spp., phytoplasmas, and spiroplasmas do. To date among all known vectors, the single most successful insects vectoring the diseases belong to the Order of Hemiptera.

Keywords: fastidious prokaryotes, *Xylella fastidiosa*, *Ca*. Liberibacter spp., spiroplasmas, phytoplasmas, Huanglongbing, Hemiptera, glassy-winged sharpshooter, Pierce's disease of grape, citrus variegated chlorosis, bacterial leaf scorch of blueberry

INTRODUCTION

In the Kingdom Prokaryotae, there are two domains, Archaea and Bacteria which differ in structure, physiology, and biochemistry[33]. Archaea like bacteria but are thought to be more closely related to eukaryotes than to bacteria. The prokaryotes are almost everywhere or we can phrase like this "prokaryotes are wherever there is life". They were the earliest organisms on earth. Today, they still dominant the biosphere for the following two facts: 1) their collective biomass outweighs all eukaryotes combined at least tenfold, and 2) more prokaryotes inhabit a handful of fertile soil or the mouth

or skin of a human than the total number of people who have ever lived. They thrive in habitats that are too cold, too hot, too salty, too acidic, or too alkaline for any eukaryote because they display diverse adaptations that allow them to inhabit many environments and they have great genetic diversity. [http://www.course-notes.org/Biology/Outlines/Chapter_27_Prokaryotes].

The prokaryotes are small and most are unicellular with the cell sizes ranging from 1 μm to 10 μm, but they can vary in size from 0.2μm to 750μm. Being so small, they have both harmful and beneficial impacts on humans and plants. Human life is only possible due to the action of prokaryotic microbes, both those in the environment and those species that call us home. Internally, they help us digest our food, produce crucial nutrients for us, protect us from pathogenic microbes, and help train our immune systems to function correctly. However on the harmful side, though pathogenic prokaryotes represent only a small fraction of prokaryotes species, yet they cause about half of human diseases. For example, there are between 2 and 3 million people a year die of the lung disease tuberculosis, caused by the bacillus *Mycobacterium tuberculosis*.

The prokaryotes that cause plant diseases belong in the Bacteria Domain. In the Division Gracilicutes, the Gram-negative bacteria, under the Class Proteobacteria, prokaryotes that cause plant diseases belong in three known Families and one unnamed Family. In Family Enterobacteriaceae, there are four Genera: *Erwinia, Pantoea*, *Serratia,* and *Sphingomonas*. In Family Pseudomonadaceae, there are seven Genera: *Acidovorax, Pseudomonas, Ralstonia, Rhizobacter, Rhizomons, Xanthomonas,* and *Xylophilus.* In Family Rhizobiaceae, there are two Genera: *Agrobacterium* and *Rhizobium*. In a still unnamed Family, there are two Genera: *Xylella* and *Candidatus* Liberibacter. In the Division Firmicutes, the Gram-positive bacteria, under the Class Firmibacteria, there are two Genera: *Bacillus* and *Clostridium* whereas under the Class Thallobacteria, there are six Genera: *Arthrobacter, Clavibacter, Curtobacterium, Leifsonia, Rhodococcus,* and *Streptomyces.* In the Division Tenericutes, under the Class Mollicutes, prokaryotes that cause plant diseases belong in two Families. In the Family Spiroplasmataceae, there is one Genus, *Spiroplasma* and in the Family Acholeplasmataceae, there is one Genus, *Candidatus* Phytoplasma [(1)].

Fastidious prokaryotes are those that either resist to grow in any available media, such as phytoplasmas, *Ca*. Liberibacter spp., and *Ca*. Phlomobacter fragariae or those that require specific and enriched media, such as spiroplasmas, *X. fastidiosa*, *Leifsonia xyli* subsp. *xyli, L. xyli* subsp. *cynodontis* and *Clavibacter michiganensis* subsp.

sepedonicus. Based on the inhabitant, *X. fastidiosa, Leifsonia* spp., and *C. michiganensis* subsp. *sepedonicus* are xylem-inhabiting while spiroplasmas, phytoplasmas, *Ca*. Liberibacter spp., and *Ca*. Phlomobacter fragariae are phloem- inhabiting prokaryotes.

Xylem-limited bacterial plant pathogens

According to Wells et al. [32], *X. fastidiosa* possesses the following characteristics: predominately single, straight rods with a cell size ranges from 0.25-0.35 μm in width and 0.9-3.5 μm in length; two types of colonies: convex to pulvinate smooth opalescent with entire margins or umbonate rough with finely undulated margins; Gram-negative, nonmotile, aflagellate, oxidase negative, catalase positive, and strict aerobic; nonfermentative, nonhalophilic, nonpigmented; and require a specific and enriched medium such as CS20, PD2, PW, or BCYE for growth. The optimal temperature for growth is 26-28 ℃, whereas the optimal pH is 6.5-6.9. The habitat is the xylem of plant tissue. The G+C content of the DNA is 51.0 to 52.5 mol% determined by thermal denaturation or 52.0 to 53.1 mol% determined by bouyant density.

Ever since Wells et al. [32] named then xylem-limited bacterium as *X. fastidiosa* in 1987, *X. fastidiosa* has been reclassified into five subspecies according to their differences in genetic makeup, host range, physiology, and biochemistry. They are *X. fastidiosa* subsp. *fastidiosa* for strains of grape, almond, alfalfa, and maple, *X. fastidiosa* subsp. *multiplex* for strains of peach, plum, almond, elm, sycamore, and pigeon grape, *X. fastidiosa* subsp. *pauca* for strains of citrus [25], *X. fastidiosa* subsp. *sandyi* for strains of oleander, daylily, jacaranda, and magnolia [26], and *X. fastidiosa* subsp. *tashke* for strains of *Chitalpa tashkentensis*, a common ornamental landscape plant [23]. However, the last two subspecies have not been officially recognized by the researchers in the community of systematic bacteriology.

X. fastidiosa requires specific and enriched media to grow as compared to other bacteria [8]. There are seven complex components that are used in the listed four media: soy peptone (Scott Laboratories), Bacto tryptone (Difco), phytone peptone (BBL), trypticase peptone (BBL), soytone (Difco) or phytone (BBL), and yeast extract; either one or two complex components for each medium; two iron sources for the medium either hemin chloride (Sigma) or soluble ferric pyrophosphate; four inorganic salts: ammonium phosphate, potassium phosphate (monobasic or dibasic) or magnesium sulfate; three amino acids and two Krebs cycle intermediates: citrate or succinate; and three detoxifying components: potato starch (J. T. Baker), activated charcoal (Norit

SG), or bovine serum albumin (Sigma). Rippled cell walls seemed to be unique for all *X. fastidiosa* cells regardless of the origin of its host plants. That was one of the reasons why they were first described as "rickettsia-like bacteria". However, a thorough study of Pierce's disease (PD) strain by Huang et al. [19] disclosed that in addition to the predominated rippled cell walls there are intermediate cell walls and smooth cell walls.

Based on the diseases reported around the world, *X. fastidiosa* causes diseases in the America Continent including North and South America. In the US, they occur in the whole southeastern States along the Gulf coast of Mexico, and California. In the southern hemisphere, the diseases occur in Brazil, Argentina, and Paraguay. In Asia, the pear leaf scorch [21] and PD of grapes [29] were reported in Taiwan. In Europe there was a report describing PD of grapes in Kosovo [3], former Yugoslavia which sits in southern Europe. The *X. fastidiosa*-induced diseases seemed to occur in the region between 15-45 degrees latitude of both north and south of Equator. It is interesting to note that Taiwan sits at the Tropic of Cancer where the pear leaf scorch disease and PD occur and that Sao Paulo in Brazil sits at the Tropic of Capricorn where the severe citrus variegated chlorosis (CVC) [10,16] and coffee leaf scorch occur. Kosovo sits at about 45 degree North of Equator.

There are 19 diseases that were confirmed to be caused by *X. fastidiosa.* They are Pierce's disease of grape, alfalfa dwarf, phony peach (PP), plum leaf scald, CVC, periwinkle wilt, ragweed stunt, and leaf scorch of almond, elm, mulberry, oak, sycamore, pecan, maple, oleander, blueberry, coffee, pear, and Chitalpa [8,10,16,17,21, 23,25,26,27]. The common symptoms induced by *X. fastidiosa* include marginal leaf necrosis, scorching or scalding of leaves, early leaf fall, dieback of branches, and wilting to death. The specific symptoms vary among different hosts. Symptoms of Pierce's disease of grapes usually start with marginal leaf necrosis to chlorosis; normally a yellow band would form between the green and necrotic tissues for white wine grapes and a purple band for red wine grapes. The following unique symptoms will follow: petioles remain attached to the canes, green island formation due to irregular maturing process of barks, dried up raisins, and eventual dying and dead vines occurs in 2-4 years after initial infection in GA (Fig. 1). The specific symptoms on peach of phony disease include darker green leaves and extremely shortened terminal growth which resulted in a shape of an umbrella canopy. In the Order Hemiptera, four main sharpshooters in the Family Cicadellidae, e. g., glassy-winged

sharpshooter (GWSS), blue-green sharpshooter, red-headed sharpshooter, and green sharpshooter were the important vectors for PD *X. fastidiosa* and GWSS vectoring phony peach and plum leaf scald diseases as well.

CVC was first observed in 1987 on sweet orange trees in the southwestern part of Minas Gerais, Brazil. Since then, the disease has been observed in the neighboring State of San Paulo and other citrus producing states [10]. Rossetti et al. [24] were the first to show by electron microscopy that a xylem-limited bacterium, probably a strain of *X. fastidiosa*, was present in all symptomatic leaves and fruits tested but not in similar tissues from symptomless trees.

CVC causes severe leaf chlorosis between veins when young trees are infected. Symptomatic leaves exhibit brown gummy lesions on the lower side in corresponding to the chlorotic yellow areas on the upper leaf surface. Reduced growth vigor and abnormal flowering and fruit set occur in infected trees. Fruits from affected trees are often small and hard with high acidity which is not fitting for juice making and no fresh market value [10,16]. A bacterium was consistently cultured from plant tissues from CVC twigs of sweet orange trees but not from tissues of healthy trees on several cell-free media known to support the growth of *X. fastidiosa*. Bacterial colonies typical of *X. fastidiosa* became visible on PW (Fig. 2), CS20 and PD2 agar media after 5 and 7-10 days of incubation, respectively. The cells of the CVC bacterium were rod-shaped, 1.4-3 µm in length, and 0.2-0.4 µm in diameter, with rippled walls. An antiserum against an isolate (8.1.b) of the bacterium gave strong positive reactions to double-antibody-sandwich (DAS), enzyme-linked immunosorbent assay (ELISA) with other cultured isolates from CVC citrus, as well as with several type strains of *X. fastidiosa* (Table 1) [15]. Sweet orange seedlings inoculated with a pure culture of the CVC bacterium supported multiplication of the bacterium, which became systemic within 6 months after inoculation and could be re-isolated from the inoculated seedlings. Symptoms characteristic of CVC developed 9 months post inoculation. *X. fastidiosa* can infect most of the citrus cultivars, species and hybrids, yet the severity of symptoms varies. Sweet oranges are the most susceptible. Grapefruit, mandarins, mandarin hybrids, lemons, limes, kumquat and trifoliate orange are moderately susceptible, showing less severe symptoms. Rangpur lime, citron, and pummelo are less susceptible. The major vectors for citrus variegated chlorosis in Brazil are *Acrogonia terminalis, Dilobopterus costalimai, Oncometopia fascialis,* and *Oncometopia nigricans*.

Relative to total sales, blueberries are the number one fruit commodity in the state of Georgia, surpassing even peaches. Production is concentrated in the southern coastal flatwoods. Rabbiteye blueberry (*Vaccinium virgatum* Aiton), a native species, has long been the predominant blueberry species cultivated in Georgia. More recently, however, growers have increased the production of the southern highbush cultivars (*V. corymbosum* interspecific hybrids) as a result of a very favorable market window. Growers and scientists started to observe a new disorder affecting the southern highbush selection FL 86-19 in the Georgia blueberry production region. An initial symptom was marginal leaf scorch (burn) of the older leaves which is very distinct and is surrounded by a dark line of demarcation between green and dead tissue (Fig. 3A), similar to that observed with extreme drought or fertilizer salt burn. New developing shoots were usually abnormally thin with a reduced number of flower buds. Leaf drop eventually occurred with young twigs or stems of the southern highbush selection FL 86-19 developing a yellow, "skeleton-like" appearance (Fig. 3B) which was why "yellow stem" or "yellow twig" was often used to describe the disorder. At this stage, the root system still appeared healthy, except for the possible loss of fine new roots. Whole plants or individual canes showed symptoms. The plant eventually died after leaf drop, typically during the second year of observation [(11)].

This prompted the enzyme-linked immunosorbent assay (ELISA) tests and isolations of *X. fastidiosa*. A single diseased blueberry bush of the selection FL 89-16 was excavated from a blueberry farm in South Georgia on 2 Feb. 2006. The bush was subsequently stored under cold room conditions (5 °C)-in a plastic trash bag to prevent moisture loss—until attempted detection of *X. fastidiosa* using direct isolation and ELISA tests (Agdia, Inc., Elkhart, IN). From this initial plant, two leaf and two root tissue samples were collected for isolation and ELISA testing on 2 Mar. 2006. The diseased blueberry bush was then moved to a greenhouse and planted in a 30.5-cm diameter pot. This original diseased plant was used to monitor the survival of the bacterium and symptom development on new growth after being stored for 48 d at 5 °C. ELISA results indicated all four tissues tested positive for the bacterial pathogen, *X. fastidiosa*, whereas only the two root tissues provided positive isolations. One leaf and one root tissue sample were later collected from each of five additional diseased plants for isolation and ELISA testing. Both isolation and ELISA testing methods obtained positive results. Cultures were multiplied to inoculate seedlings of three cultivars: 'Southern Belle' (eight plants), 'Premier' (six), and 'Powderblue' (six) on 23 May 2006

and one selection, FL 86-19 (eight), on 31 May 2006. Two FL 86-19 plants started to show symptoms of marginal necrosis 54 days postinoculation , whereas one plant each of ‘Southern Belle’ and ‘Powderblue’ started to show symptoms of marginal necrosis 63 days postinoculation and ‘Premier’ stayed symptomless. All eight culture-inoculated FL 86-19 plants (100%) showed symptoms 72 days postinoculation, but no symptoms were observed on the control plants. One hundred twenty-six days postinoculation, two ‘Powderblue’ and four ‘Southern Belle’ plants showed mild symptoms, whereas all ‘Premier’ plants were asymptomatic. Positive reisolations of the bacteria from the inoculated symptomatic plants, not from asymptomatic plants, fulfilled Koch's postulates, which confirmed *X. fastidiosa* was the causal bacterium of the new blueberry disorder, the bacterial leaf scorch of blueberry.

This original blueberry bush provided valuable information on the survivability of the *X. fastidiosa* blueberry strain. The bacterium was able to survive at 5 °C for 48 d when the bush was kept in a plastic bag before being planted in a large pot and kept in the greenhouse. On 10 July 2006, tissues from this bush were collected for isolation and ELISA and the results were positive for both methods. The blueberry industry-particularly growers-in the southeastern United States will find this information especially important, because the research suggests that the bacteria is able to survive in the aboveground tissues through the south Georgia winter because it is unlikely for the temperature to remain at 5 °C 24 h a day for a consecutive 48 d in the winter. Furthermore, the source of inoculum for transmission would likely be available year-round [(11)].

By 3 months after initial inoculation, all eight *X. fastidiosa*-injected FL 86-19 plants showed symptoms, whereas all four PW medium-only-injected plants remained asymptomatic. For the other three cultivars, only two of six ‘Powderblue’ and four of eight ‘Southern Belle’ showed mild symptoms, whereas zero of six ‘Premier’ plants were symptomatic even at 4 months postinoculation. Both ELISA and direct isolations confirmed the presence of *X. fastidiosa* in symptomatic plants. Yellow stems or twigs were a strong symptomatic indicator of *X. fastidiosa* infection. There seemed to be a different degree of susceptibility among the three cultivars and one selection with selection FL 86-19 clearly being the most susceptible consistent with what had been observed in the field [(11)].

Further studies indicated that there is varietal resistance in some southern highbush blueberries. The FL 86-16 variety is particularly susceptible to infection. When compared with other southern highbush or rabbiteye varieties, the “V5” variety

showed resistance to the bacterium (Fig. 4). This is encouraging, since it indicates that breeding can be utilized to develop varieties that are highly resistant to Xylella. Likewise, surveys have shown that there are other varieties that either do not develop symptoms or that slow epidemic spread of the disease (Fig. 5). 'FL 86-19' is highly susceptible, as is the 'O'Neal' cultivar. 'Star' is susceptible, but it is representative of desirable cultivars that will develop the disease but still likely be economically viable; field epidemics observed in 'Star' and similar cultivars do not develop as rapidly, allowing adequate time to recoup investments [(6)].

Insect vectors for the blueberry bacterial leaf scorch disease are under investigation in Georgia and the glassy-winged sharpshooter leafhopper, *Homalodisca vitripennis* (formerly *H. coagulata*), is likely an important suspect.

Phloem-limited plant pathogenic prokaryotes

In Mollicutes, the cell wall-less and phloem-limited prokaryotes, there are two major plant pathogens: spiroplasmas and phytoplasmas. Spiroplasmas are cells with helical forms during log phase growth. Most spriroplasmas are cultivable in enriched media that contain supplemented sterols and other ingredients [(9)]. They are facultative anaerobic or microaerophilic. They are associated with three plant diseases: citrus stubborn and horseradish brittle root disease by *Spiroplasma citri*, corn stunt disease by *S. kunkelii*, and periwinkle disease by *S. phoeniceum*. Phytoplasmas have been associated with more than 500 plant diseases worldwide [(22)] ever since the historical discovery by Doi et al. [(14)] of then referred as mycoplasma-like organisms (MLO) found in the pholem elements of plants infected with mulberry dwarf, potato witches'-broom, aster yellows, or paulownia witches'-broom. Phytoplasmas are still noncultivable even though they have been classified into 30 group-subgroups and four undetermined entities based on the 16S rDNA RFLP grouping (http: //plantpathology. ba. ars.usda.gov/pclass/pclass_taxonomy.html).

There were unintentional fans of phytoplasmas for as early as 1000 years ago in Song Dynasty and as recent as nowadays. When phytoplasmas infect peonies, the plants produced flowers not in the typical red or yellow colors, but in a delicate green we call virescence. The green flower was considered so attractive and valuable about 1000 years ago in China that the Song Dynasty's imperial court received a special annual tribute composed of the blossoms. More recently, phytoplasmas have helped brighten winter holidays by transforming otherwise lanky poinsettias, with their eye-catching red

leaves, into bushy ornamentals for their fans, lovers of Christmas decorations (28).

But most effects of phytoplasmas on plants are not that appealing. They were actually naively quite destructive and malicious. For example, they wither grapes in Europe and Australia; dwarf corn growth in South America; devastate pears and apples in the United States and Europe; destroy peanuts, sesame, and soybean in Asia; and sicken elms, coconuts, asters, and hydrangeas on multiple continents. Just one 2001 phytoplasma outbreak in apple trees caused a loss of about 25 million Euros in Germany and about 100 million Euros in Italy [28].

For all the destruction they inflict, you might expect that lots of big agricultural companies and respected academic labs have garnered ample amount of information about them. Well to the contrary, to this day, the inability to grow these bacteria outside plants or insects hinders efforts to get a handle on their biology and genomes despite the fact that plant pathologists had spent over half a century thinking that phytoplasmas were viruses.

Walnut witches'-broom disease was reported by Chang et al. [7] after the MLO particles were observed in the sieve cells of the symptomatic tissues collected from Griffin, GA. Abnormal proliferation of numerous small shoots with lighter green color which resembled the shape of a broom became evident in mid-July. The insect vector for this disease is still unknown even though DNA fragments were isolated and cloned from diseased walnut and later DNA probes were developed to monitor the seasonal occurrence of walnut witches'-broom MLO [12, 13]. There are other economically important phytoplasma diseases, such as lethal yellowing of coconuts in Jamaica and lime witches'-broom in Oman and many others in Taiwan.

Recent finding of secreted AY-WB protein 11 (SAP11) by Bai et al [2] and secreted protein TENGU by Hoshi et al [18] seemed to suggest that phytoplasmas are finally giving up some of their secrets of how they used the proteins to modify the activity of plant genes that participate in the disease development. Some scientists are already contemplating whether they can create plants with only the positive attributes of an infection. Perhaps adding a single phytoplasma gene to a plant's DNA could create bushy poinsettias or green peonies that don't carry the annoying pathogens [28].

Spiroplasma citri causes stubborn disease on citrus and brittle root disease on horseradish. *S. citri* is transmitted in a propagative, circulative manner by several leafhoppers including *Circulifer tenellus* and *Scaphytopius nitridus* in citrus-growing regions of California and Arizona and *C. haematoceps (syn. Neoaliturus haematoceps)*

in the Mediterranean region. The pathogen multiplies in the vector but no transovarial transmission occurs. Spatial and temporal analysis of CSD incidence indicate only primary spread occurring and no or very limited secondary spread (citrus to citrus). *C. tenellus and N. haematoceps* have a wide host range which includes many natural hosts of *S. citri* but citrus is a non host of these leafhoppers. Citrus becomes infected when inoculative *Circulifer* vectors feed temporarily on citrus during migratory flights [(5)].

Spiroplasma kunkelii causes characteristic small chlorotic stripes at the leaf bases of new leaves 25-30 days after initial inoculation. The chlorotic stripes fused together and extended toward the leaf tips with green spots and stripes exhibited on a chlorotic background. The infected plants are stunted due to much shorter internodes and a proliferation of secondary shoots in leaf axils; thus it is named corn stunt disease. Corn stunt disease is transmitted by *Dalbulus maidis* (DeLong and Wolcott) and *D. elimatus* (Ball) in nature whereas it can be transmitted experimentally by *Graminella nigrifrons* (Forbes), *G. sonora* (Ball), *Stirellus bicolor* (Van Duzee), *Exitianus exitiosus* (Uhler), and *Euscelidius variegatus* (Kirsch.) [(31)].

The other phloem-limited bacteria are the causal agent of Huanglongbing (HLB) and other diseases, *Ca*. Liberibacter spp. Striking symptoms of “yellow shoots” were often seen in sweet orange of young and high density orchard (1,000 trees per hectare). Two most characteristic symptoms of HLB are leaves with blotchy mottle and fruits with small size and color inversion [(4)]. HLB are transmitted by psyllid vectors. In Asia, Southeast Asia, and Oceania, *Diaphorina citri* is the vector, *Ca*. L. asiaticus is the HLB agent, and both are heat tolerant (Asian form of HLB). In Africa and Madagascar island, *Trioza erytreae* is the vector, *Ca*. L. africanus is the HLB agent, and both are heat-sensitive (African form of HLB) [(4)]. Another HLB agent, *Ca*. L. americanus, was found in 2004 in Sao Paulo State, Brazil [(30)] and 2005 in Florida, USA [(20)] and its vector is *D. citri.*

The phloem-limited plant pathogenic prokaryotes, phytoplasmas, spiroplasmas, and the pathogens (*Cadidatus* Liberibacter spp.) of Huanglongbing (HLB), are transmitted to plants by phloem-feeding insects belonging to the Order of Hemiptera.

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Table 1. Serological relatedness between the CVC-bacterium and strains of *Xylella fastidiosa*

Antigens Tested	ATCC Number	OD[a] at 405 nm 15 min	OD[a] at 405 nm 30 min
CVC-bacterium, isolate 8.1.b	--	0.860	>2
Xylella fastidiosa from			
Mulberry leaf scorch	35868	0.022	0.057
Mulberry leaf scorch	35869	0	0.032
Oak leaf scorch	35874	0.014	0.045
Ragweed stunt	35876	0.071	>2
Periwinkle wilt	35878	0	0.07
Almond leaf scorch	35870	0.743	>2
Pierce's disease of grape	35879	0.852	>2
Pierce's disease of grape	35881	0.815	>2
Pierce's disease of grape			
Georgia isolate Chateau 3C	--	0.613	>2
Georgia isolate 112.V1	--	0.814	>2
Georgia isolate 116.V6	--	0.737	>2
Georgia isolate 116.V11	--	0.601	>2
Georgia isolate MS7	--	0.654	>2
E. coli	--	0.059	0.083

[a] ELISA conducted using antiserum prepared against isolate 8.1.b of the CVC bacterium.



Fig. 1. Symptoms of Pierce's disease of grapes: A close-up view of marginal leaf necrosis (A), petioles remained attached to the canes after leaves fall (B), green island (C) formed due to irregular maturing process of barks, and dried up raisins (D), and eventual dying and dead vines in 2-4 years after initial infection in GA, USA. (Photo by Chung-Jan Chang)

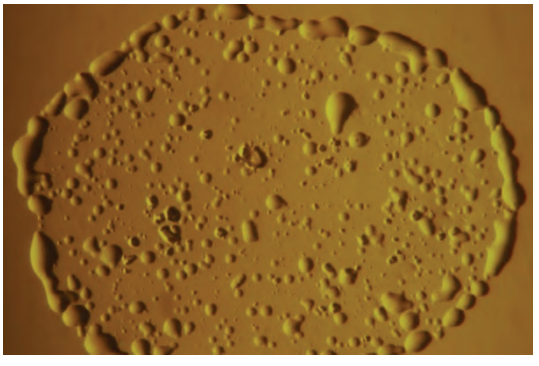


Fig. 2. Colonies of the CVC-bacterium obtained from a drop of tissue homogenate on PW 9 days after inoculation (x 16). (photo by Chung-Jan Chang).



Fig. 3. Symptoms of bacterial leaf scorch of blueberry. Marginal leaf necrosis or burn (A) which is very distinct and is surrounded by a dark line of demarcation between green and dead tissue. Prior to complete plant death, all leaves fall off, and the remaining stems display a yellow "skeletal" appearance (B) which was why "yellow stem" or "yellow twig" was often used to describe the disorder before "bacterial leaf scorch" was designated for this disease. (Photo by P. M. Brannen, University of Georgia)



Fig. 4. Resistance. In this planting, a single row of 'V5' plants was alternately planted after 10 rows of 'FL86-19' plants (repeated numerous times). The surrounding 'FL86-19' plants were all infected, with significant mortality, and they have been removed at this point. The 'V5' plants consistently showed no symptoms of disease or mortality after five years at this site. This indicates field resistance in the 'V5' line. (Brannen *et al*., 2011. UGA Cooperative Extension Circular 922:1-6).

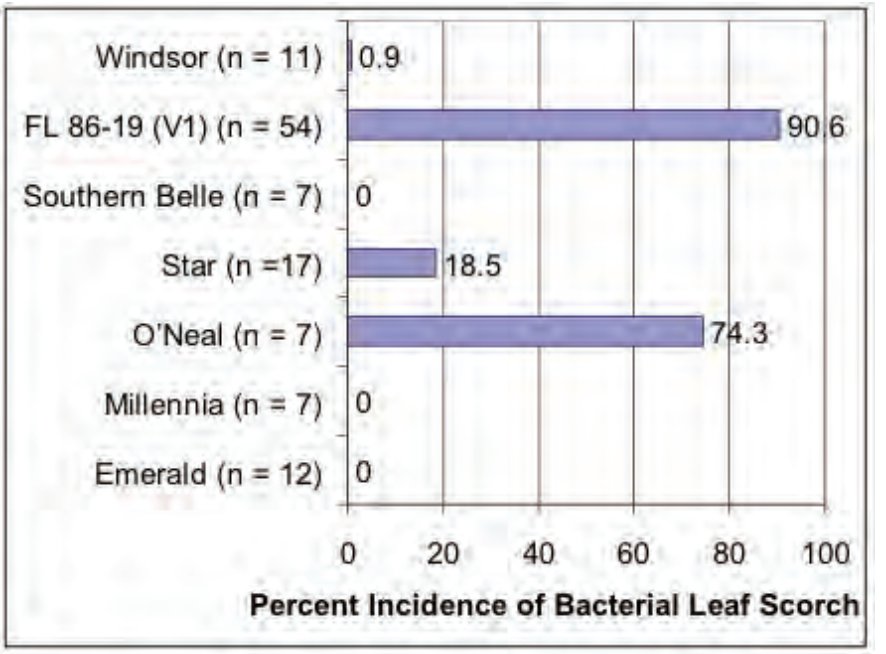


Fig. 5. Incidence (percentage of symptomatic plants) of bacterial leaf scorch by cultivar at one site. The number of rows surveyed (n) is shown in parentheses next to the cultivar name. 'FL 86-19' is highly susceptible, as is the 'O'Neal' cultivar. 'Star' is susceptible, but it is representative of desirable cultivars that will develop the disease but still likely be economically viable; field epidemics observed in 'Star' and similar cultivars do not develop as rapidly, allowing adequate time to recoup investments. (Brannen *et al*., 2011. UGA Cooperative Extension Circular 922:1-6).

Matsumura's Collection of Froghoppers and Sharpshooters (Hemiptera: Cicadomorpha) of Taiwan in the Hokkaido University Insect Collection

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ABSTRACT

The Laboratory of Systematic Entomology, Faculty of Agriculture, Hokkaido University, Sapporo, Japan (SEHU), former Entomological Institute of Sapporo Agricultural College, was founded by Professor Shonen Matsumura. SEHU has preserved the insect specimens named by Professor Matsumura for a long time that includes insect specimens collected from Taiwan. Some of them are xylem-feeders, such as froghoppers and sharpshooters which can transmit xylem-limited bacteria (XLB). This article provides the checklist of Taiwan froghoppers and sharpshooters named by Professor Matsumura preserved in SEHU. The checklist contains 44 froghopper species and 4 sharpshooter species. This checklist could be a reference for studying the taxonomy of xylem-feeders in Taiwan.

Keywords: Taiwan, froghopper, sharpshooter, Hokkaido University, Shonen Matsumura

INTRODUCTION

Xylem feeders belonging to Hemiptera are capable to transmit xylem-limited bacteria (XLB). Therefore, these xylem feeders were considered as potential vectors of XLB [(34)]. Some of the xylem feeders confirmed as vectors of XLB belong to Cercopoidea (commonly known as froghoppers or spittlebugs) and Cicadellinae

(commonly known as sharpshooters) [1, 3, 9, 33, 34, 35, 38].

The known species of xylem-limited bacteria in Asia are *Ralstonia syzygii* and *Xylella fastidiosa* [2, 7, 15, 45]. The vector of transmitting *Ralstonia syzygii* is *Hindola striata* (Machaerotidae of Cercopoidea). In order to identify the vectors that transmit Pierce's disease of grapevines occurred in Taiwan, the survey of xylem feeders which harvest *Xylella fastidiosa* was performed. The results showed that the DNA fragments of *X. fastidiosa* could be detected in three cicadelline species (*e.g. Kolla paulula* (Walker, 1858), *Bothrogonia ferruginea* (Fabricius, 1787), and *Anatkina horishana* (Matsunura, 1912)), and one aphrophorid species (*Poophilus costalis* (Walker, 1851)). Therefore, these four insects were considered as candidate vectors (Su *et al*., 2011; Su *et al*., 2013). Among them, *Kolla paulula* and *Bothrogonia ferruginea* were confirmed as insect vectors through Koch's postulates (Su and Shih, unpublished data).

There were 100 cercopoid species and 18 cicadelline species recorded in Taiwan [21, 30, 36, 37, 39, 40, 41, 43, 47, 48]. Among them, 48 species (40.7% of known species), including 44 froghoppers and 4 sharshooters, were named by a Japanese insect taxonomists, Professor Shonen Matsumura (1872-1960) (Fig. 1). Professor Matsumura was the father of Japan entomology. He was born in Akashi, Hyogo Prefecture, Japan. Professor Matsumura published 240 reports and 35 books between 1895 and 1945 (Liang and Suwa, 1998). Insect specimens Professor Matsumura worked on were mainly from East Asia, such as Japan, Korea, China, Taiwan, and Sakhalin (Sakhalin) [13, 17]. Most of the specimens are still stored at the laboratory of Systematic Entomology, Faculty of Agriculture, Hokkaido University, Sapporo, Japan (SEHU) [13].

Professor Matsumura described the froghopper and leafhopper species of East Asia based on body color and external morphology (e.g. the structures and sharps of antennal ledge, tylus, frons, and clypeus). However, the difference of male genital structures was not included. Therefore, examinations of the type specimens are crucial in proper identification of these species. Based on the subsequent examinations of the type specimens, some of the diagnostic features used by Matsumura have been identified as the differences between individuals or genders. Therefore, lots of species he described were now treated as synonyms [10, 11, 12, 14, 16, 31].

So far, four species were identified as PD candidate insect vectors, which are only 3.4% of the total froghoppers and leafhoppers known in Taiwan. Therefore, it is still possible to find other candidate insect vectors in the future. The correct information of classification and identification of froghoppers and leafhoppers are

required for further studies on ecology and prevention of these candidate insect vectors. For this purpose, this article provides a checklist of Cercopoidea and Cicadellinae of Taiwan from the Matsumura's collection at SEHU.



Fig. 1. Professor Shonen Matsumura.

A checklist and remarks of Taiwan froghoppers and sharpshooters named by Shonen Matsumura

Type specimens of Cercopoidea [13] and Cicadellinae of Taiwan described by Professor Matsumura were preserved at SEHU. The type localities of froghoppers and sharpshooters in the checklist refer to Matsumura's original descriptions [18, 19, 20, 21, 22, 23, 24, 25, 26]. The synonymies follow Metcalf [27, 28, 29, 30] and Shih and Yang [39, 40].

Family Aphrophoridae

1. *Aphrophora arisana* Matsumura, 1940

Distribution: Taiwan.

2. *Aphrophora habonis* (Matsumura, 1940)

Distribution: Taiwan.

3. *Aphrophora horishana* Matsumura, 1940

Distribution: Taiwan.

4. *Aphrophora kikuchii* (Matsumura, 1940)

Distribution: China, Taiwan.

5. *Aphrophora maritima* Matsumura, 1903 New Record

Distribution: China, Japan, Korean Peninsula, Taiwan (Matsu Is.)

Specimens examined: CHINA: 1♂, Fujian, Chongan, 580-650 m, 21. V. 1960, C. L. Ma; 1♀, Fujian, Chongan, 580-650 m, 19. VI. 1960, Y. R. Zhang; TAIWAN**:** 1♀, Machu, Hsijiu Island, Tienau, 14. V. 2002, J. A. Pan; 1♂, Machu, Dungyin Island, Chungliu, 4. VI. 2002, H. T. Shih; 2♂, 1♀, Machu, Hsijiu Island, Tienau, 3. IX. 2002, W. H. Chen & T. Y. Chang.

Remarks: In 2002, HTS found that nymphs of this species feed on the stems or roots of *Angelica* sp. (Umbelliferae) at Hsijiu Island, Machu, Fujian.

6. *Aphrophora mushana* (Matsumura, 1940)

Distribution: Taiwan.

7. *Aphrophora nagasawae* Matsumura, 1907

Distribution: Taiwan.

8. *Aphrophora nomurella* (Matsumura, 1942a)

Distribution: Taiwan.

9. *Aphrophora arisanella* (Matsumura, 1940)

Distribution: Taiwan.

10. *Aphrophora tsuruana* Matsumura, 1907

Distribution: China; Taiwan.

11. *Jembrophora sawadai* Matsumura, 1942a

Distribution: Taiwan.

12. *Jembra inouyei* (Matsumura, 1942a)

Distribution: Taiwan.

13. *Jembrana centralis* Matsumura, 1940

Distribution: Taiwan.

14. *Jembrana daitoensis* Matsumura, 1940

Distribution: Taiwan.

15. *Jembrana kankonis* Matsumura, 1942a

Distribution: Taiwan.

16. *Jembrana kanoniella* Matsumura, 1940

Distribution: Taiwan.

17. *Nokophora nokoensis* Matsumura, 1940

Distribution: Taiwan.

18. *Ariptyelus arisanus* (Matsumura, 1942b)

Distribution: Taiwan.

19. *Ariptyelus auropilosus* (Matsumura, 1907)

Distribution: Taiwan.

20. *Peuceptyelus excavatus* (Matsumura, 1940)

Distribution: Taiwan.

21. *Peuceptyelus kanmonis* (Matsumura, 1940)

Distribution: Taiwan.

22. *Peuceptyelus nigriceps* (Matsumura, 1940)

Distribution: Taiwan.

23. *Peuceptyelus nitobei* (Matsumura, 1940)

Distribution: Taiwan.

24. *Peuceptyelus rokurinzana* (Matsumura, 1940)

Distribution: Taiwan.

25. *Peuceptyelus takaosanus* Matsumura, 1934

Distribution: Taiwan.

26. *Nagaclovia formosana* Matsumura, 1940

Distribution: China, Taiwan, Japan (Okinawa).

27. *Lepyronia okadae* (Matsumura, 1903)

Distribution: China, Japan, Korean Peninsula, Taiwan.

28. *Philaenus arisanus* Matsumura, 1940

Distribution: Taiwan.

29. *Philaenus mushanus* Matsumura, 1940

Distribution: Taiwan.

30. *Philaenus nigripectus* (Matsumura, 1903)

Distribution: Taiwan.

31. *Mesoptyelus arisanus* Matsumura, 1940

Distribution: China, Taiwan.

32. *Mesoptyelus karenkonis* Matsumura, 1940

Distribution: Taiwan.

33. *Ptyelus tamahonis* Matsumura, 1940

Distribution: Taiwan.

34. *Philagra kanoi* Matsumura, 1940

Distribution: Taiwan.

35. *Philagra kuskusuana* Matsumura, 1942b

Distribution: Taiwan.

36. *Kotophora botelensis* (Matsumura, 1938)

Distribution: Taiwan.

Family Cercopidae

37. *Tadascarta rubripennis* Matsumura, 1940

Distribution: Taiwan.

38. *Eoscarta zonalis* (Matsumura, 1907)

Distribution: Taiwan, Japan, China.

39. *Baibarana uchidai* Matsumura, 1940

Distribution: Taiwan.

40. *Kuscarta koshunella* Matsumura, 1940

Distribution: Taiwan.

41. *Kanozata arisana* Matsumura, 1940

Distribution: Taiwan.

42. *Cosmoscarta uchidae* Matsumura 1906

Distribution: Daito Island, Ryukyu Islands, Taiwan.

43. *Kanoscarta kanoniella* Matsumura, 1940

Distribution: Taiwan.

44. *Eoscarta bimaculata* (Matsumura, 1907)

Distribution: China, Japan, Taiwan.

Family Cicadellidae

Subfamily Cicadellinae

45. *Anatkina hopponis* (Matsumura, 1912)

Tettigonia hopponis Matsumura, 1912, Annot. Zool. Jap. 8: 37-38.

Anatkina hopponis (Matsumura) [transferred by Young 1986: 46].

Distribution: Taiwan.

46. *Anatkina horishana* (Matsunura, 1912)

Tettigoniella horishana Matsumura 1912: 36-37.

Anatkina horishana (Matsumura) [transferred by Young 1986: 46].

Distribution: Taiwan.

47. *Atkinsoniella rinkihonis* (Matsumura, 1912)

Tettigoniella rinkihonis Matsumura 1912: 36.

Atkinsoniella rinkihonis Young 1986: 97. [transferred by Young 1986: 46].

Distribution: Taiwan.

48. *Bothrogonia formosana* (Matsumura, 1912)

Tettigonia formosana Matsumura 1912: 34.

Remarks: Ishihara [8] concluded that *B. formosana* (Mats.) should be identified as *B. ferruginea*.

Future Prospects

In addition to Prof. Matsumura, the other key scholar of froghoppers of Taiwan was Masayo Kato. However, the depository of Masayo Kato's collection is still unknown. Furthermore, neither Matsumura nor Kato has studied the male genital structures of froghoppers and leafhoppers, and many species were described based on the superficial differences, or sometimes difference in coloration, only. Numerous studies on color polymorphism of *Philaenus* (Cercopoidea) have been performed [4, 32, 46, 49, 50, 51]. Through these studies, it has been confirmed that intraspecific color polymorphism exists in some species of Cercopoidea. For example, chromosomal alleles, eographic and climatic factors were suspected to influence intraspecific color polymorphism of *P. spumarius* (L.) [5, 6]. Therefore, inspection of Mstsumura's collection stored at SEHU is especially crucial in solving some taxonomic problems regarding the Taiwanese species possibly described on the bases of intraspecific color polymorphism. These will greatly contribute for establishing the base for classification of potential insect vectors that transmit xylem-limited bacteria in Taiwan.

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Overview of the Phylogeny, Taxonomy and Diversity of the Leafhopper (Hemiptera: Auchenorrhyncha: Cicadomorpha: Membracoidea:Cicadellidae) Vectors of Plant Pathogens

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ABSTRACT

Comprising~20,000 described species, leafhoppers (Cicadellidae) are the largest family of sap-sucking herbivores and comprise the largest number of known vectors of plant pathogens of any insect family. Although recent studies of tropical faunas indicate that the vast majority of extant species remain to be discovered, availability of new cybertaxonomic tools is enabling newly trained taxonomists to increase the rate of species discovery. Phylogenetic relationships among leafhoppers remain largely unexplored, but recent published phylogenies based on morphological and molecular data have begun to elucidate the phylogenetic status and relationships of previously recognized tribes and subfamilies. As a result of such studies, several changes to the higher classification have been proposed, including changes to the concepts of subfamilies Cicadellinae, Delocephalinae and Megophthalminae, the groups comprising the majority of known vector species. Taking newly available phylogenetic information into account may help focus the ongoing search for competent vectors on the groups most closely related to known vector species. New molecular phylogenetic and functional genomic tools and techniques may facilitate more rapid and extensive surveys of the microbiota associated with non-pest leafhoppers and promote more comprehensive approaches to the study of the evolution of leafhopper-pathogen-plant associations.

Keywords: Homoptera, phylogeny, taxonomy, evolution, endosymbiont, bacteria, Mollicutes, virus, *Xylella*

INTRODUCTION

Leafhoppers (Cicadellidae) are the largest family of the insect order Hemiptera and

the most diverse family of sap-sucking herbivores. They are distributed worldwide, from tropical rainforests to arctic tundra and from sea level to >4,000 meters elevation and may be found feeding on nearly all major groups of vascular plants. Beginning with their first appearance during the lower Cretaceous [35], the evolution of leafhoppers has been closely tied with that of their vascular plant hosts and, as is becoming increasingly clear, various endosymbiotic and pathogenic microbes [9, 43, 55, 64]. Unfortunately, the vast majority of leafhopper species are known only from a few museum specimens, often from a single locality. Little is known of the ecology of most species, including their feeding preferences and competence as potential vectors of plant pathogens. Host plant data are available for less than 10% of known species and many of the available records represent incidental collections that have not been confirmed by detailed study of feeding behavior. Although basic knowledge of leafhopper taxonomy and ecology is increasing steadily, ecological data for most species will remain scarce for the foreseeable future. Thus, strategies are needed that allow predictions about ecological characteristics to be made based on data available for the few species that have been studied in detail and their inferred relationships to other, less well studied species. Only about 1% of known leafhopper species have been shown to be capable of transmitting plant pathogens but this probably reflects the still very poor state of knowledge of leafhopper-host plant-pathogen associations. The number of species that are actual or potential vectors is likely to be much larger than the ~200 currently documented. Indeed, vectors have not yet been identified for the great majority of plant pathogens thought to require an insect vector. Nevertheless, the fact that competent vectors are clustered among a few taxonomic groups suggests that at least some of the traits associated with vector competence are phylogenetically conservative. Phylogenetic analysis therefore represents a tool for discovery of new or potential leafhopper vectors. Some evolutionarily conservative traits, such as preferential feeding on particular vascular fluids (phloem vs. xylem) and associated morphological modifications allow predictions to be made regarding the competence of various leafhopper species as vectors for particular kinds of pathogens. An improved understanding of leafhopper phylogeny may facilitate the development of models that predict disease outbreaks based on the presence and abundance of particular leafhopper species and higher taxa in agroecosystems. In this paper, I summarize current knowledge of the phylogeny and biodiversity of leafhoppers, review recent changes to the higher classification, examine the distribution of known vectors and the pathogens

vectored among leafhopper lineages, and discuss the implications of recent phylogenetic results for the study of pathogen-vector associations. Detailed lists of leafhopper species known to be vectors and their associated pathogens have been published by Nielson [57], Maramorosch and Harris [51], Harris [39], Redak *et al.* [59], Weintraub and Beanland[69],Weintraub[68] and Ammar *et al.* [2], but new vector species continue to be discovered [1].

Known diversity

The number of valid, described species of Cicadellidae presently stands at ~20,000. These species are included in ~2,400 currently valid genera. Over the past 60 years, cicadellid taxonomists have described an average of 201 new species per year (Fig. 1). Discovery and description of new species increased steadily in the post-WWII period and reached its peak in the 1970s and early 80s but fell precipitously thereafter due to the retirement of nearly all of the most prolific leafhopper taxonomists, mostly in the USA and Europe (Blocker, DeLong, Freytag, Knight, Kramer, Linnavuori, Nielson, Oman and Young). Unfortunately, these individuals were not replaced by new leafhopper experts and, although a few of them trained graduate students, almost none of the students succeeded in obtaining employment as taxonomists. Following a lull during the 1990s, leafhopper species discovery has increased slowly but steadily over the past decade and again exceeded the 60-year average for species described per year in 2010 and 2011, the most recent years for which complete data are available. Three major factors account for this recent trend and suggest that leafhopper species discovery will continue to increase dramatically in the coming decades.

First, thanks to recent increases in support for basic science in several countries, especially Brazil and China, newly invigorated cicadellid systematics research programs have emerged and many new students are being trained in leafhopper taxonomy. Indeed, two large laboratories in China have accounted for 133 (54%) of the 247 papers published on leafhopper taxonomy over the past 5 years. If such training programs can be sustained and if even a few of the trainees are able to obtain full-time employment as systematists, then we may be on the verge of a new golden age of leafhopper systematics.

Second, recent bioinventory projects in previously undersampled biodiversity hotspots worldwide have yielded enormous numbers of new specimens, many representing new species and higher taxa. Some such projects have employed

previously underutilized sampling methods such as insecticidal fogging of forest canopies and vacuuming in grasslands. Study of such samples indicates that 90% or more of the tropical species of Cicadellidae remain unnamed [20, 41]. Recent taxonomic revisionary studies based on this newly collected material [11, 12, 15, 17, 18, 19, 49, 50] have increased numbers of species in previously known tropical genera by 50-94% in addition to erecting many new genera. Because large backlogs of samples from the tropics remain unstudied and vast areas remain unsampled, the species represented in these recent studies probably represent only a small fraction of global leafhopper biodiversity.

Finally, increased adoption of cybertaxonomic methods that streamline the process of describing species and publishing revisionary studies will likely accelerate species discovery as well as facilitate efficient storage and retrieval of large amounts of taxonomic information via relational databases and web applications [17, 26, 71]. The availability of such labor saving tools and a well-trained workforce of early-career scientists should provide the momentum needed to complete the task of documenting the world leafhopper fauna. Indeed, the tremendous diversity of the world fauna demands that new more efficient approaches to species discovery and synthesis be applied by leafhopper systematists. If taxonomists continue to describe species at present rates, barring a dramatic increase in the number of active leafhopper taxonomists, several more centuries of work will be required to completely document the extant world fauna. Given present rates of habitat fragmentation and destruction, many leafhopper species are undoubtedly being driven to extinction each year, most without our even being aware of their existence.

Leafhopper phylogeny

Knowledge of phylogenetic relationships of leafhoppers has improved substantially over the past 20 years but remains highly incomplete. The vast majority of leafhopper taxa have never been included in an explicit phylogenetic analysis. Most published phylogenetic studies have focused on relationships among major lineages [12, 23, 24, 25, 29, 34, 47, 62, 76, 77] rather than among species within a single genus [10, 21, 22, 66] and some of the latter have been based on intuitive assessments of character evolution rather than explicit cladistic analysis [33, 36, 70]. DNA sequence data, representing only a handful of gene regions, have been incorporated into only a few species-level phylogenetic analyses of Cicadellidae [21, 22, 29]. Such molecular data are crucial for species-level

phylogenetics of leafhoppers because the morphological differences among species within a single genus are often subtle, shape-based, and difficult to encode in a character matrix.

Phylogenetic analyses based on morphological and molecular data strongly support the monophyly of superfamily Membracoidea[6, 23, 34], the group comprising leafhoppers and treehoppers. However, current best estimates of the phylogeny of this group (Fig. 2a) indicate that the family Cicadellidae is paraphyletic with respect to a lineage comprising the three recognized families of treehoppers (Aetalionidae, Melizoderidae and Membracidae). The treehopper lineage is derived from within a lineage of leafhoppers mostly comprising species with short crowns and facial ocelli, including Idiocerinae,Macropsinae and Megophthalminae (sensulato, including Agalliini), which include many vectors of phloem-borne plant pathogenic viruses and phytoplasmas.

Another major lineage recovered with strong branch support by combined analysis of morphological and molecular data includes Deltocephalinae (sensulato), the concept of which was recently expanded [77, 78] to include ten other groups treated as separate subfamilies in the most recent published world catalogue (Table 1), plus a few tribes previously included in other subfamilies [58].

The same analysis supported Young's [72] restricted definition of the sharpshooter subfamily Cicadellinae, but indicated a close relationship between this group and three other subfamilies, Evacanthinae, Mileewinae, and Typhlocybinae. A more detailed analysis of relationships within Cicadellinae based on morphological and molecular data and including nearly all genera of Proconiini and representatives of all genus groups of Cicadellini recognized by Young [62, 72, 73, 74] indicates that neither of the two tribes recognized by Young is monophyletic. The *Oncometopia* genus group, included by Young in Proconiini, was recovered as a distinct lineage separate from other proconiines and Cicadellini was paraphyletic with respect to both groups [62].

Recent and ongoing analyses have begun to explore finer-scale relationships within a few other cicadellid lineages (subfamilies) identified by the broader prior analyses [7, 12, 76, 77] but, because of the high diversity of the group, many aspects of membracoid phylogeny remain poorly studied. Comprehensive morphology-based phylogenetic analyses of relationships among genera within tribes have so far been performed for only five of the 130 tribes of leafhoppers [47, 52, 62, 75]. Other genus-level analyses of leafhoppers have sampled primarily within the faunas of particular regions and/or are not comprehensive [7, 12, 16, 29, 32, 44, 46, 48]. Species-level phylogenetic relationships have

been explored within only a few genera [5, 10, 18, 21, 22, 36, 49, 63, 66].

Recent changes in the higher classification of Cicadellidae

Although many aspects of the phylogeny of leafhoppers and related membracoids remain poorly resolved, strong support for certain groupings of genera in published phylogenies has prompted several recent changes in the higher classification of leafhoppers. In the most recent comprehensive world leafhopper checklist, Oman *et al.* [58] proposed a provisional higher classification that recognized 40 subfamilies and 121 tribes of Cicadellidae (Table 1). This classification retained many of the elements proposed by Evans [28] in his comprehensive review of the world fauna, but also included nine subfamilies and 47 tribes described more recently as a result of improved knowledge of tropical faunas. Subsequent to the 1985 cut-off date for Oman *et al.* [58], an additional new subfamily and 11 new tribes have been proposed [31, 78]. To better reflect hypothesized phylogenetic relationships, substantial reductions in the number of subfamilies have been proposed recently [13, 14, 77], so only 25 cicadellid subfamilies are recognized currently (Table 1).

Among the major changes to the classification proposed recently are the synonymy of ten groups recognized as separate subfamilies by Oman *et al.* [58] with Deltocephalinae and the transfer of some tribes previously placed in other subfamilies (Cicadellinae, Nirvaninae, Nioniinae) to Deltocephalinae [77]. Other proposed changes include the treatment of Agalliini as a tribe of Megophthalminae rather than as a separate subfamily, and placement of Makilingiini and Tinteromini as tribes of Mileewinae rather than as separate subfamilies. Additional subfamily synonymies are likely to be proposed in the near future, given ongoing phylogenetic studies. For example, recent analyses [62]suggest that Phereurhininae and Signoretiinae are derived from within Cicadellidae (sensu Young) and that Idiocerinaeand Eurymelinaeare closely related with the former having apparently given rise to the latter [23, 48].

One change possibly warranted by the phylogenetic results that has, nevertheless, not been proposed is the treatment of Cicadellidae and Membracidae as synonyms (yielding "Membracidae, sensulato" because Membracidae is the older name), despite strong evidence that the former is paraphyletic with respect to the latter. Cicadellidae has been retained as a paraphyletic taxon because it is well defined morphologically, is a well known group among entomologists, and a strategy for reclassifying the families of Membracoidea so that each represents a monophyetic group and is not unnecessarily

confusing (e.g., resulting in a proliferation of small families or a lumping of well known groups) is not yet apparent.

Implications for the study of vector-pathogen associations

Biologists studying vector-pathogen associations have long recognized the need for phylogenetic information to inform research aimed at identifying the most likely vectors among the numerous leafhopper species usually present in agroecosystems. Until very recently, however, absence of explicit phylogenetic estimates has required that taxonomy serve as a surrogate. Published phylogenetic studies have shown that many traditionally recognized higher taxa (genera, tribes, subfamilies) are monophyletic groups (Table 1). Nevertheless, such studies have also shown that some previously recognized groups are para-or polyphyletic and this has important implications for the study of vector biology.

Examination of the current best estimate of the phylogeny of Cicadomorpha (Fig. 2a) indicates that plant pathogen vectors are distributed among several distantly related lineages of leafhoppers and treehoppers. This probably reflects the fact that searches for competent vectors have focused almost entirely on the small minority of species that occur in agroecosystems and feed on economically important plants. The widespread occurrence of known vectors among most of the major lineages of leafhoppers strongly suggests that more systematic surveys would reveal that many other members of these lineages, as well as members of related groups that have not yet been reported to include vectors, are capable of transmitting plant pathogens. Alternatively, the physiological traits associated with vector competence may be extremely labile evolutionarily, but this seems unlikely, given the complexity of such associations [2, 69].

The phylogenetic results (Fig. 2a) reveal that feeding preference (inferred for most groups based on mouthpart morphology) is conservative, but not without homoplasy. The outgroup Cicadoidea and Cercopoidea feed preferentially on xylem and some have been shown to be competent vectors of the xylem borne pathogen *Xylella fastidiosa* [59]. These two superfamilies comprise the sister group to Membracoidea. Myerslopiidae, a relict group of flightless, soil-dwelling membracoids, may also be xylem feeders, given their inflated faces, but feeding has never been observed in this group [37]. The branching sequence of the earliestdivergences in the lineage that includes Cicadellidae and the three treehopper families have not yet been resolved satisfactorily (Fig. 2a), but a most parsimonious reconstruction of feeding preference indicates that the derivation

of this lineage likely involved a shift from xylem to phloem feeding. Most major lineages of leafhoppers and treehoppers are thought to prefer to feed on phloem, the main exceptions being found in the lineage comprising Cicadellinae and Typhlocybinae. Cicadellinae sensustricto (sharpshooters) feed preferentially on xylem and Typhlocybinae (microleafhoppers) feed preferentially on mesophyll. The phylogeny revealed a close relationship between Cicadellinae and Evacanthinae. Several species of Cicadellinae are competent vectors of *Xylella fastidiosa* [59]and some members of the related Evacanthinae (tribe Pagaroniini: *Friscanus* and *Pagaronia*) have also been shown to be capable of transmitting this pathogen (Nielson 1968). Mileewinae[14]are relatively rare inhabitants of tropical forests not known to feed on economically important plants but, given their close relationship with Cicadellinae, they should be tested for competence as vectors of *Xylella*.

Detailed phylogenetic studies of Deltocephalinae, the group comprising the largest number of confirmed vector species, show that the subfamily as previously defined [58] is polyphyletic (Table 1). A substantial expansion of the concept of this subfamily was proposed recently and Deltocephalinae, in its present sense[78], is a well-supported monophyletic group. Known vectors of plant pathogenic mollicutes (phytoplasmas and spiroplasmas) and viruses are distributed among several major deltocephaline lineages including two early-diverging lineages (Acinopterini, Fieberiellini) and several more recently derived groups (e.g., Chiasmini, Deltocephalini, Macrostelini, Opsiini). The largest deltocephaline tribe, Athysanini, which includes numerous identified vectors, is highly polyphyletic, with various genera grouped together in distantly related lineages and several of these lineages include known vectors (Fig. 2b). The scattered distribution of known vectors across multiple lineages of deltocephalines suggests that many undiscovered vectors exist within this group. None of the groups currently included in Deltocephalinae that were previously placed in other subfamilies is known to include competent vector species, but failure to find vectors in these groups may have resulted in part from their not having been targeted in surveys of potential vectors because they were previously not included in one of the subfamilies known to include vector species.

Other groups of Membracoidea known to include smaller numbers of vectors of plant pathogens are Aphrodinae, Coelidiinae, Iassinae, Idiocerinae, Macropsinae, and Typhlocybinae. Aphrodinaeis sister to the lineage comprising Neocoelidiinae and Deltocephalinae. Given that both Aphrodinae and Deltocephalinae include vectors of

mollicutes and viruses, Neocoelidiinae should also be tested as potential vectors. The vast majority of neocoelidiines are restricted to the Neotropics but a few species occurring in temperate North America feed on Asteraceae and *Pinus*. Coelidiinae, a pantropical group most common in rainforests and only rarely present in agroecosystems, includes only one known vector species. Iassinae (sensulato) includes tribes Gyponini (=Scarinae) and Iassini, each of which includes one known phytoplasma vector species. These arboreal phloem feeders primarily inhabit tropical forests and savannas, and are uncommon in agroecosystems. Idiocerinae, Macropsinae, and Megophthalminae (=Agalliinae) are closely related members of a large lineage that also includes Membracidae and other treehoppers. Idiocerinae and Macropsinae are restricted to woody hosts, but many megophthalmines feed on herbaceous plants. Several members of these leafhopper subfamilies are vectors of mollicutes and/or viruses, but only one treehopper has, so far, been shown to transmit a plant pathogen (*Micrutalis malleifera*, a vector of pseudo-curly top virus [61]). Oak-feeding treehoppers have more recently been implicated via PCR screening as potential vectors of bacterial leaf scorch in oaks (*Quercus* spp.), caused by *Xylella fastidiosa*, but transmission tests have not been performed [79]. Like other members of the lineage including Idiocerinae and Megophthalminae, treehoppers are thought to feed preferentially on phloem sap, but these results indicate that they also, at least occasionally, ingest sufficient quantities of xylem to become infected with *Xylella*.

Although they are derived from within a large lineage that mostly includes the xylem-feeding sharpshooters and related groups, Typhlocybinae feed preferentially on mesophyll. However, a few species have been shown to be competent vectors of phloem borne pathogens, and this is consistent with observations of occasional phloem feeding in some species [30, 45].

Future research

Relatively few leafhopper species have been shown to be vectors of plant pathogens. These species are distributed among most of the major lineages of leafhoppers (Fig. 2), but known vectors are over-represented in the faunas of Europe, North America and Australia compared to other regions [53]. This undoubtedly reflects greater efforts to document pathogen/vector associations in the more economically developed parts of the world rather than any tendency of these regional faunas to be more efficient vectors. Greater efforts are needed in non-anthropogenic ecosystems, especially in the tropics, to

identify and characterize leafhopper-pathogen associations because some of these will ultimately spread to human-dominated landscapes asa result of anthropogenic habitat alterations and climate change. The vast majority of leafhopper species either do not feed on economically important plants and/or do not occur in large enough numbers to be considered pests. Previous work on leafhopper vectors and associated pathogens has focused primarily on species associated with crops and, to a much lesser extent, on weeds associated with crops [69].Nevertheless, vectors remain unknown for the vast majority of plant pathogens thought to require an insect vector[69]. More thorough and systematic screening would undoubtedly reveal that many, if not most, species of Cicadellidae harbor plant pathogens. Recent PCR screening has often shown this to be the case [1, 40] although only a minority of species that tested positive for pathogens in such PCR-based studies have been shown to be competent vectors. Available evidence also suggests that related pathogens tend to be associated with particular lineages of leafhopper vectors [42].

Recent molecular genomic and phylogenetic studies of leafhopper-associated bacteria have revealed that pathogens and endosymbionts are often closely related and indicate that the physiological and evolutionary mechanisms that facilitate associations between such microbes and leafhoppers are essentially the same [8, 9, 43, 55, 60]. Indeed infection of the leafhopper vector by a plant pathogen has often been shown to benefit the vector in various ways [33, 54] although negative and neutral effects have also been reported [3, 4]. In most well-studied plant pathogen- leafhopper vector associations, the leafhopper-associated pathogenic organisms require an insect vector to facilitate their spread and inhabit and multiply within cells of the insect vector, indicating a substantial degree of co-adaptation between pathogen and vector. In the most common circular, propagative associations, the pathogen must first travel from the gut to the salivary gland and traverse at least three intercellular barriers before it can be injected into the host plant by the feeding insect [69]. The complexity of such physiological co-adaptations suggests that, although the number of potential vectors is large, the number of actual vectors is much smaller.

A few well-studied model systems [56, 59] have provided tantalizing clues regarding the co-evolutionary processes involved in vector-pathogen-plant associations. More comprehensive studies are needed to elucidate the distributions of specific plant pathogens and vector competence across entire lineages of leafhoppers. Such knowledge will facilitate the development of more robust models for predicting the

vector potential of various leafhopper groups. Ultimately, it should also improve our understanding of the roles pathogenic and mutualistic microbes have played in the evolutionary diversification of leafhoppers, and vice versa.

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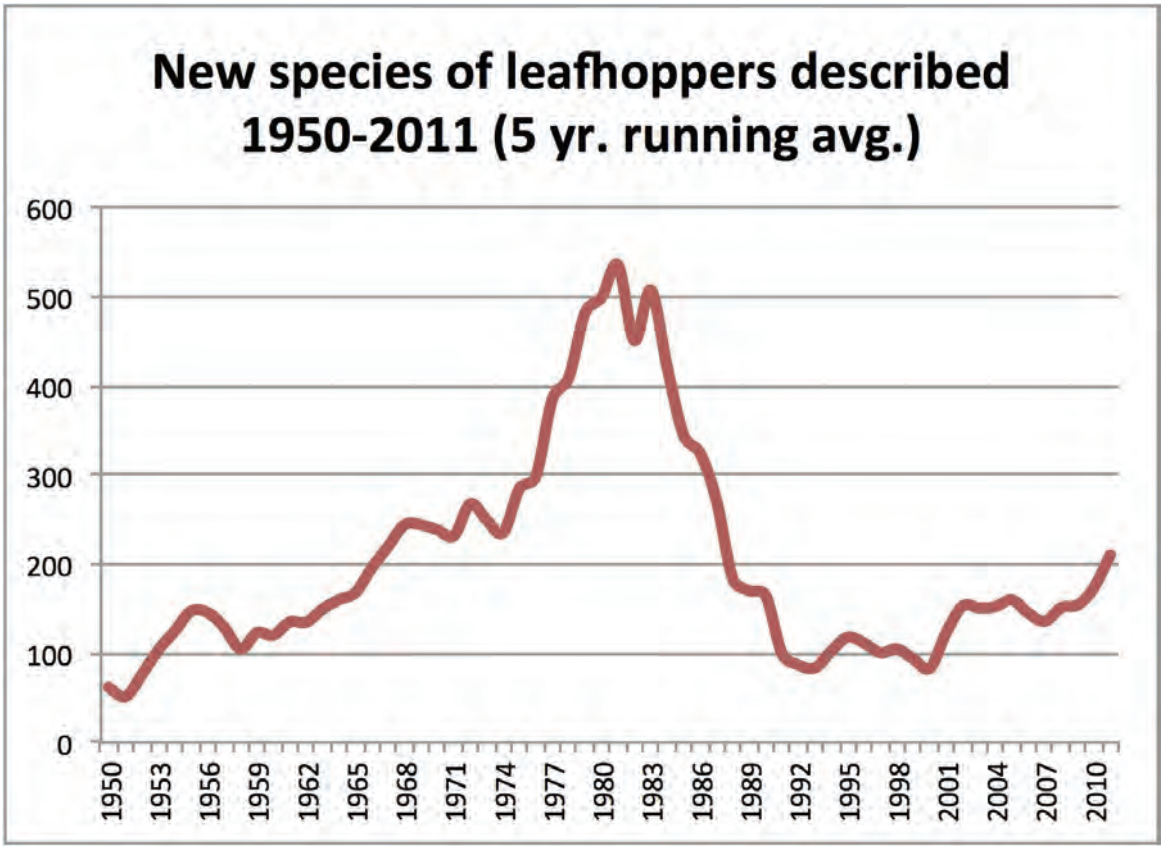


Fig. 1. Numbers of new leafhopper species described per year, 1950-2011, plotted as five-year running averages.

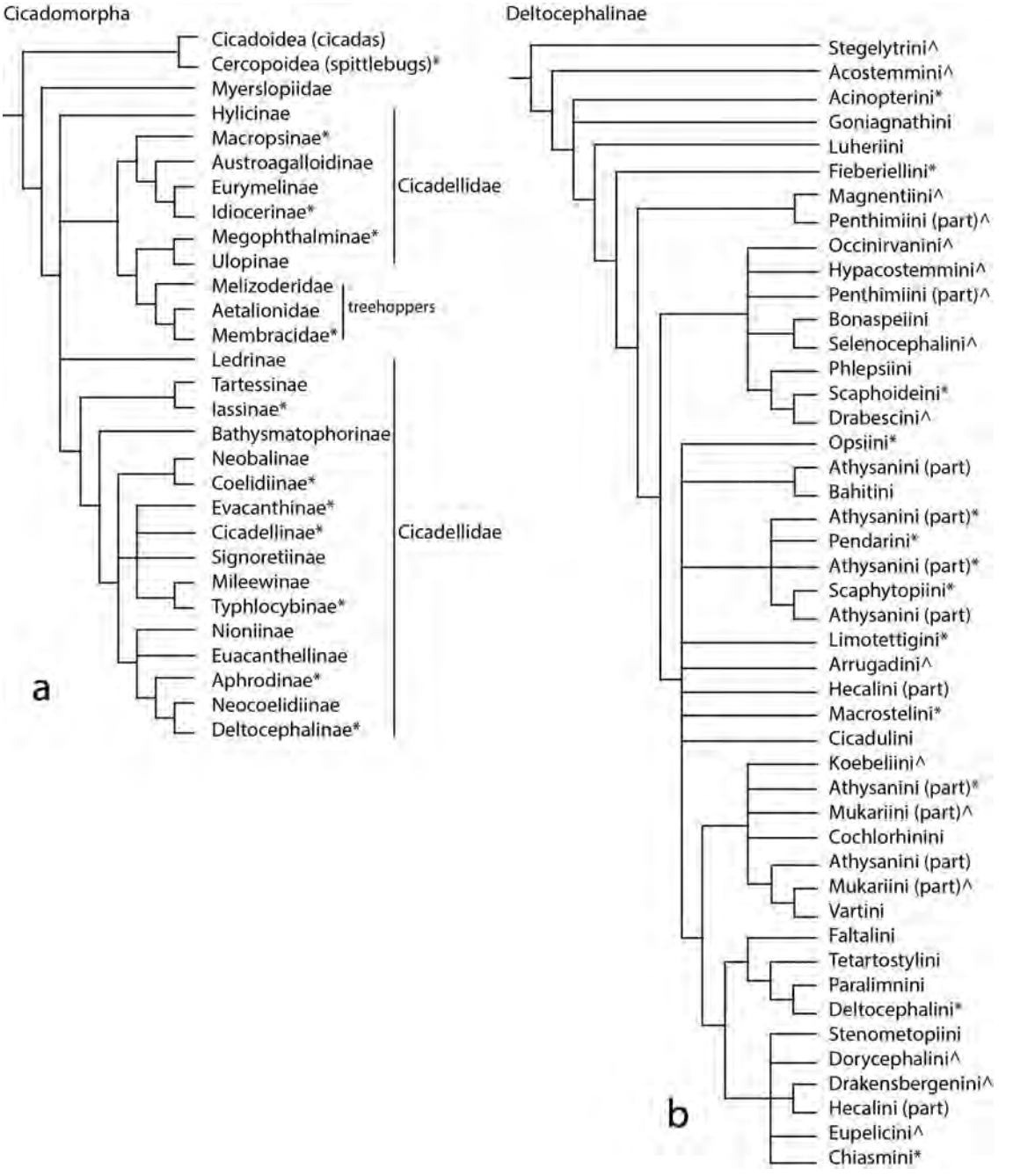


Fig. 2. Current best estimates of the phylogeny of Membracoidea (a) and Deltocephalinae (b) based on analyses of morphological and molecular data [24, 25,78]. Poorly supported branches are collapsed into polytomies. Groups previously included in other subfamilies but added to Deltocephalinae recently are marked with ^. Groups that include one or more confirmed vector species are marked with *.

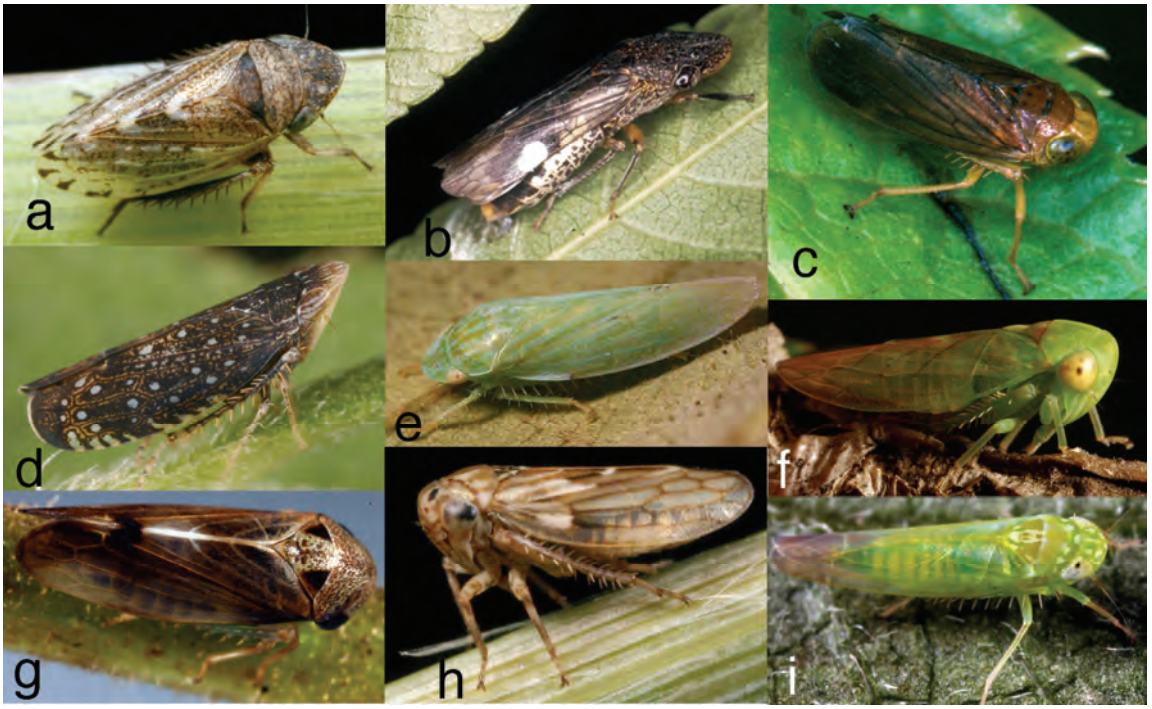


Fig.3. Representatives of the nine currently recognized leafhopper subfamilies known to include vectors of plant pathogens: a, *Anoscopusserratulae* (Aphrodinae); b, *Homalodisca vitripennis* (Cicadellinae); c, *Jikradia olitoria* (Coelidiinae); d, *Scaphytopius frontalis* (Deltocephalinae); e, *Gyponana* sp. (Iassinae); f, *Idiocerus* sp. (Idiocerinae); g, *Pediopsoides distinctus* (Macropsinae); h, *Ceratagallia agricola* (Megophthalminae); i, *Empoasca* sp. (Typhlocybinae).

Table 1. Subfamilies recognized by Oman *et al.* (1990), their current taxonomic and phylogenetic status. Feeding preferences are for most groups are inferred based on head morphology. Under vector status, presence in the group of confirmed vectors of *Xylella,*Mollicutes (phytoplasmas and spiroplasmas), and viruses are indicated by X, M, and V, respectively.

type genus	family-group sensu Oman *et al.* (1990)	current status	reference	feeding preference	vector status	phylogenetic status
Acostemma	Acostemminae	tribe of Deltocephalinae	77	phloem	?	monophyletic but relationships among genera unknown
Adelungia	Adelungiinae	tribe of Megophthalminae	13	phloem	?	phylogenetic status and relationships among genera unknown
Agallia	Agalliinae	tribe of Megophthalminae	37	phloem	M, V	probably paraphyletic ([32])
Aphrodes	Aphrodinae	subfamily (concept expanded)	38	phloem	M, V	monophyletic or paraphyletic with respect to Deltocephalinae
Arrugada	Arrugadinae	tribe of Deltocephalinae	77	phloem	?	monophyletic (monobasic)
Austroagalloides	Austroagalloidinae	subfamily	13	phloem	?	monophyletic (monobasic)
Bathysmatophorus	not included	subfamily	67	xylem	?	monophyletic but relationships among genera unknown
Bythonia	Bythoniinae	tribe of Iassinae	13	phloem	?	monophyletic (monobasic)
Cicadella	Cicadellinae	subfamily	13	xylem	X, M, V	paraphyletic with respect to Phereurhininae ([62])
Coelidia	Coelidiinae	subfamily	13	phloem	M	monophyletic but relationships among genera unknown
Deltocephalus	Deltocephalinae	subfamily (concept expanded)	77	phloem	M, V	monophyletic but many tribe and genus relationships unresolved
Drakensbergena	Drakensbergeninae	tribe of Deltocephalinae	77	phloem	?	monophyletic (monobasic)
Euacanthella	Euacanthellinae	subfamily (concept expanded)	37	phloem	?	monophyletic but relationships among genera unknown
Eupelix	Eupelicinae	tribe of Deltocephalinae	77	phloem	?	monophyletic
Eurymela	Eurymelinae	subfamily	13	phloem	?	monophyletic but relationships among genera unknown
Evacanthus	Evacanthini (Cicadellinae)	subfamily (concept expanded)	12	xylem	X	subfamily monophyletic but some tribes paraphyletic
Evansiola	Evansiolinae	tribe of Megophthalminae	37	phloem	?	monophyletic (monobasic)
Gypona	Gyponinae	tribe of Iassinae	13	phloem	M	monophyletic but relationships among genera unknown
Hylica	Hylicinae	subfamily	13	phloem	?	monophyletic but relationships among genera unknown
Iassus	Iassinae	subfamily (concept expanded)	13	phloem	M	monophyletic but relationships among genera unknown
Idiocerus	Idiocerinae	subfamily	13	phloem	M	paraphyletic with respect to Eurymelinae ([48])
Koebelia	Koebeliinae	tribe of Deltocephalinae	77	phloem	?	monophyletic but relationships among genera unknown
Ledra	Ledrinae	subfamily (concept narrowed)	44	phloem	?	monophyletic
Macropsis	Macropsinae	subfamily	13	phloem	M, V	monophyletic but relationships among genera unknown
Makilingia	Makilingiinae	tribe of Mileewinae	14	xylem	?	monophyletic (monobasic)
Megophthalmus	Megophthalminae	subfamily	13	phloem	M	possibly paraphyletic with respect to Ulopinae
Mileewa	Mileewini (Cicadellinae)	subfamily (concept expanded)	14	xylem	?	paraphyletic with respect to Typhlocybinae
Mukaria	Mukariinae	tribe of Deltocephalinae	77	phloem	?	monophyletic but relationships among genera unknown
Neobala	Neobalinae	subfamily	13	phloem	?	monophyletic but relationships among genera unknown
Neocoelidia	Neocoelidiinae	subfamily	52	phloem	?	monophyletic
Nionia	Nioniinae	subfamily (concept narrowed)	13	phloem	?	monophyletic but relationships among genera unknown
Nirvana	Nirvaninae	tribe of Evacanthinae	12	xylem	?	monophyletic but relationships among genera unknown

Paraboloponа	Paraboloponinae	tribe of Deltocephalinae	77	phloem	?	monophyletic but relationships among genera unknown
Penthimia	Penthimiinae	tribe of Deltocephalinae	77	phloem	?	polyphyletic
Phereurhinus	Phereurhininae	subfamily	13	xylem	?	monophyletic but relationships among genera unknown
Phlogis	Phlogisinae	tribe of Signoretiinae	65	xylem	?	monophyletic but relationships among genera unknown
Selenocephalus	Selenocephalinae	tribe of Deltocephalinae	77	phloem	?	polyphyletic
Signoretia	Signoretiinae	subfamily (concept expanded)	65	xylem	?	monophyletic but relationships among genera unknown
Stegelytra	Stegelytrinae	tribe of Deltocephalinae	77	phloem	?	monophyletic but relationships among genera unknown
Tartessus	Tartessinae	subfamily (concept expanded)	44	phloem	?	monophyletic but relationships among genera unknown
Tinteromus	not included	tribe of Mileewinae	14	xylem	?	newly described by Godoy & Webb [31]
Typhlocyba	Typhlocybinae	subfamily	13	mesophyll	M	monophyletic but relationships among genera unknown
Ulopa	Ulopinae	subfamily	13	phloem	?	monophyletic but relationships among genera unknown
Xestocephalus	Xestocephalinae	tribe of Aphrodinae	13	phloem	?	monophyletic (monobasic)

How Effective is Sharpshooter Control at Limiting Pierce's Disease Spread in California Vineyards?

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ABSTRACT

Pierce's disease management in southern California vineyards hinges on chemical control of populations of the vector, the invasive glassy-winged sharpshooter (*Homalodisca vitripennis*), residing in citrus. Systemic insecticides (imidacloprid) are regularly applied to citrus, which is a preferred plant type for the sharpshooter, to reduce insect abundance before they move into vineyards. These treatment programs have been successful, reducing regional sharpshooter populations to a fraction of what they once were. Grape growers also frequently apply systemic insecticides in vineyards, but the efficacy of these treatments for disease management is not known. Over the last three years we conducted a series of surveys in treated and untreated vineyards in Temecula Valley to determine the relative economic value of within-vineyard chemical control for Pierces disease management. In each of the past three seasons we surveyed 34 vineyards in the Temecula Valley that differ in their use of systemic insecticides, and monitored regularly populations of sharpshooters and beneficial insects. Among the years overall Pierce's disease prevalence was low; averaging approximately 1% based on visual symptoms. Prevalence differed slightly among fields of different treatment categories with the lowest infection rates in those vineyards that were either consistently or intermittently treated with imidacloprid. Based on sticky trap monitoring, consistently or intermittently treated vineyards also had lower catches of sharpshooters than untreated fields, but natural enemy catch did not differ appreciably among the three treatment categories. Finally, tap sampling results showed slightly lower natural enemy abundance in consistently treated sites, but the abundance of non-predatory arthropods was also substantially lower in those sites. Collectively, these results suggest that imidacloprid treatments may reduce slightly disease spread, at least in part due to reductions in vector pressure, but without any clear non-target

effects on natural enemies that may lead to secondary pest outbreaks. However, it may not be critical to treat vineyards every year; at least not as long as regional vector populations continue to be reduced through areawide control programs.

Keywords: *Xylella fastidiosa*, vector-borne pathogen, vector control, transmission efficiency, disease prevalence, disease incidence

INTRODUCTION

Chemical control of insect vectors plays a crucial role in many disease mitigation programs. This is true not only for the management of mosquito-borne diseases of humans, such as malaria and dengue fever [8, 9, 26], but also for limiting disease epidemics in a wide range of agricultural crops [12, 22]. In southern California vineyards chemical control at both the areawide and local scales may affect the severity of Pierce's disease, caused by the pathogenic bacterium *Xylella fastidiosa*, by reducing the density or activity of the primary vector, the invasive glassy-winged sharpshooter (*Homalodisca vitripennis* [7, 14]).

Xylella fastidiosa is endemic to the Americas, and is widespread throughout the western and southeastern U.S. This xylem-limited bacterium is pathogenic to a wide variety of plants, including several important crop, native, ornamental, and weedy species [15, 20]. In the Western U.S. the most economically significant host is grapevine, in which *X. fastidiosa* causes Pierce's disease. Multiplication of the bacterium in vines plugs xylem vessels, which precipitates leaf scorch symptoms and typically kills susceptible vines within a few years [20].

X. fastidiosa can be spread by several species of xylem sap-feeding insects, the most important being the sharpshooter leafhoppers [21]. Historically Pierce's disease prevalence has been moderate, with a pattern that is consistent with primary spread into vineyards from adjacent riparian habitats by the native blue-green sharpshooter (*Graphocephala atropunctata* [19]). However, beginning in the late 1990s severe outbreaks occurred in southern California and the southern San Joaquin Valley that are attributable to the establishment and proliferation of the glassy-winged sharpshooter. This invasive sharpshooter is not inherently more efficient at transmitting the pathogen than are native sharpshooters [1, 10]. Instead its threat as a vector appears to stem from a combination of ability to achieve extremely high densities [3] and promote vine-to-vine (i.e. secondary) disease spread [2].

Citrus trees themselves are not susceptible to the strains of *X. fastidiosa* found in

the U.S. (though strains found in Brazil have caused significant economic losses to their citrus industry – [20]). None-the-less citrus plantings figure prominently in the epidemiology of *Xylella* diseases in California. Many portions of southern California and the southern San Joaquin Valley have vineyards in close proximity to citrus groves [23]. This is important because citrus is a preferred habitat for the glassy-winged sharpshooter at key times of the year, allowing this vector to achieve very high densities [4, 16, 17]. High vector populations then disperse seasonally out of citrus into nearby vineyards, resulting in clear gradients of Pierce's disease prevalence (i.e. proportion of infected plants) as a function of proximity to citrus [18].

Given the importance of citrus in Pierce's disease epidemiology, citrus groves have been the focus of areawide chemical control programs, initiated in the Temecula and Coachella Valleys in the early 2000s and shortly afterward in Kern and Tulare Counties [14, 23]. The southern California programs use targeted application of systemic insecticides, such as imidacloprid, to limit *H. vitripennis* populations residing within citrus. Census data in citrus show substantial year to year variation in sharpshooter abundance that may stem from incomplete application, the use of less effect organically-derived insecticides, or inadequate irrigation to facilitate uptake - which makes the consistent management of sharpshooter populations a challenge [24, 25]. None-the-less trap counts have been, overall, much reduced compared to pre-areawide counts. The effect of chemical control can be seen clearly in early insect surveys which found significantly fewer sharpshooters in treated relative to untreated citrus and in vineyards bordering treated versus untreated groves (R. Redak and N. Toscano, unpublished data). Thus, these areawide control programs have been considered successful in southern California [16, 24, 25], and the swift implementation of an areawide management program in Kern County has been credited with limiting the severity of Pierce's disease outbreaks [23].

Research into imidacloprid uptake by grape also has been initiated, and target concentrations high enough to suppress glassy-winged sharpshooter activity (approx. 10 μg/L of xylem sap) can be achieved and will endure for several weeks in mature vines [6]. This information coupled with the success of areawide programs in citrus appears to have led to relatively widespread adoption by grape growers of imidacloprid application in vineyards to reduce further exposure to *X. fastidiosa*. In Temecula Valley, for example, it is estimated that 70% of vineyards use imidacloprid, at an approximate cost of $150-200 per acre (N. Toscano, personal communication). Yet consistent treatment of

vineyards with systemic insecticides is neither universal, nor have there been any measures of how effective these costly treatments are at reducing Pierce's disease.

We studied the epidemiological significance of chemical control in vineyards, via a multi-year series of field surveys in Temecula Valley. This work addresses gaps in empirically-derived observations regarding the cascading effects of vineyard imidacloprid applications on glassy-winged sharpshooter abundance and, ultimately, Pierce's disease severity. The overall goal of this project was to understand **does within-vineyard sharpshooter chemical control reduce vector pressure and Pierce's disease spread?** As part of this overall objective we have been evaluating the following set of research questions:

1. Do vineyards from different treatment categories (untreated, intermittent treatments, or consistently treated) differ in insecticide concentration?

2. Do imidacloprid applications reduce vector abundance or activity in vineyards?

3. Do treatments reduce disease spread in vineyards?

4. Are treatments disrupting biological control and contributing to secondary pest outbreaks?

MATERIALS AND METHODS

In the summer of 2010 we interviewed several vineyard owners and vineyard managers in the Temecula region to identify vineyards with a range of imidacloprid treatment histories. Of the 88 distinct properties for which we acquired information 66 were treated regularly with imidacloprid, 14 were treated intermittently (one to three years of the last 5), and 8 properties were not treated with imidacloprid for at least the last 4 years. 34 of these properties (8 to 14 replicate fields from each of the three treatment categories) were then used for disease surveys and arthropod monitoring over the next three seasons (Fig. 1).

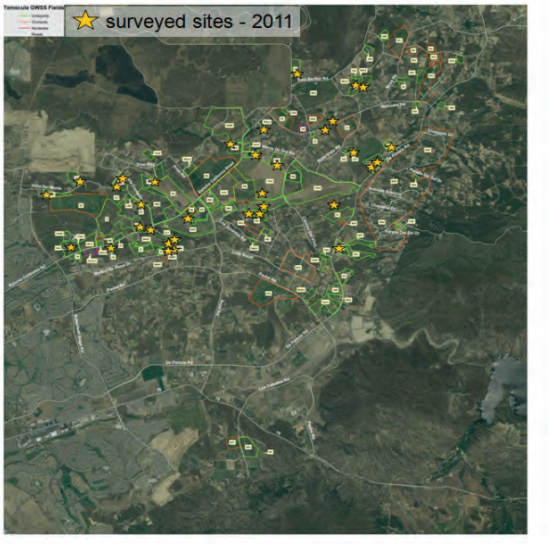


Fig. 1. Location of 34 vineyard sites throughout Temecula Valley (approximately 90 miles southeast of Los Angeles, CA) for use in Pierce's disease and arthropod surveys.

First, to verify that field sites were appropriately categorized based on recent insecticide use history, in late summer we collected leaf samples from 10 vines at each site. For each site 2 vines were sampled in 5 different rows spread throughout the vineyard block. For each vine sampled we collected 2 healthy, fully expanded leaves from mid-cane. These samples were then subjected to an ELISA analysis to calculate imidacloprid concentration, using a slight modification to established methods [6, 7]. Briefly, from each leaf we used a #6 cork borer to punch a disc of leaf tissue, weighed that disc, and then ground it in 1% methanol. After incubation dilutions were made and this material was added to Envirologix Imidacloprid Quantiplate Kit.

In the second and third years of the study we surveyed monthly populations of *H. vitripennis*, the native smoke-tree sharpshooter, *Homalodisca liturata*, and generalist natural enemies. We placed 4-8 yellow sticky-card traps at 0.5-1 m above the vines at each site. The number of traps depended on the area of vines planted. Traps were replaced monthly. For each trap, we counted the number of *H. vitripennis, H. liturata,* and four groups of generalist predators: minute pirate bugs (*Orius* spp.), assassin bugs (Reduviidae), big-eyed bugs (*Geocoris* spp.), and spiders (Aranea). These four groups appear to be the most important generalist predators of *H. vitripennis* [11].

In addition to sticky traps, over the summer of 2012 we conducted tap sampling from each of the sites to estimate the abundance of generalist predators and pest insects. At each site, 6 rows approximately evenly spaced throughout the vineyard block were chosen for sampling, avoiding edge rows. Within each row, 5 vines approximately spaced throughout the row were "tap-sampled," avoiding vines at the end of rows, with no neighbors, or which were obviously diseased/dying. Tap sampling was conducted by tapping the vine 40 times to dislodge arthropods from the upper canes, foliage and branches into a shallow beat net. Arthropods collected within a single row were pooled and aspirated into plastic vials and placed on ice during transport. Upon return to the lab, vials were partially filled with 70% ethanol and placed in the freezer until identification.

In the fall of each year we surveyed each vineyard for Pierce's disease prevalence. This time of year was chosen because disease symptoms are strongest late in the year. At each size all vines were visually inspected for obvious leaf scorch-type Pierce's disease symptoms to estimate a percent of vines showing disease. These visual estimates of disease prevalence were adjusted for false positives using plate culturing of bacteria and for false negatives using ELISA. For false positive testing, we collected

petioles from 50 randomly selected vines that were visually categorized as symptomatic for Pierce's disease. We then plate cultured isolates from these petioles for detection of *X. fastidiosa* [13]. To estimate the frequency of false negative, we collected petioles from 100 randomly selected asymptomatic vines found in the vicinity of symptomatic vines. These 100 samples were then subjected to ELISA tests for the presence of *X. fastidiosa*. Estimated false positives and false negatives were used to adjust the disease prevalence based on the visual surveys.

RESULTS AND DISCUSSION

Estimates of imidacloprid concentrations *in planta* match the classes for management practices. All but one of the untreated sites had no detectable imidacloprid in leaf tissue samples. Conversely, both the regularly treated and those treated intermittently (i.e. "mixed" treatment history) had markedly higher insecticide concentrations (Fig. 2A). These results support the classification of most sites into the three treatment categories. Notably, however, several of the mixed treatment sites tended to have equivalent (or even higher) average imidacloprid concentrations to those of consistently treated sites in some years, indicating recent treatments occurred at those sites.

Over the two years of sticky trap monitoring more than 150 sharpshooters were caught in vineyards, the vast majority of which were the invasive glassy-winged sharpshooter. For example, we collected more than 75 sharpshooters, both *H. vitripennis* and *H. liturata*, among all sites between November 2011 and May 2012. In both years, mean sharpshooter catch depended on treatment history grouping. Untreated sites had the highest sharpshooter catches, consistently treated sites were intermediate, and mixed sites had the lowest catches over this period (Fig. 2C). It plausible that regularly treated sites had relatively higher catches than mixed treatment sites because their location exposes them to inherently more sharpshooters (i.e. proximity to citrus); hence encouraging more frequent applications by growers. Regardless, these results overall support the idea that imidacloprid application reduces vector pressure, through mortality or by having anti-feedant effects[5], which may underlie any differences in disease spread among the treatment categories.

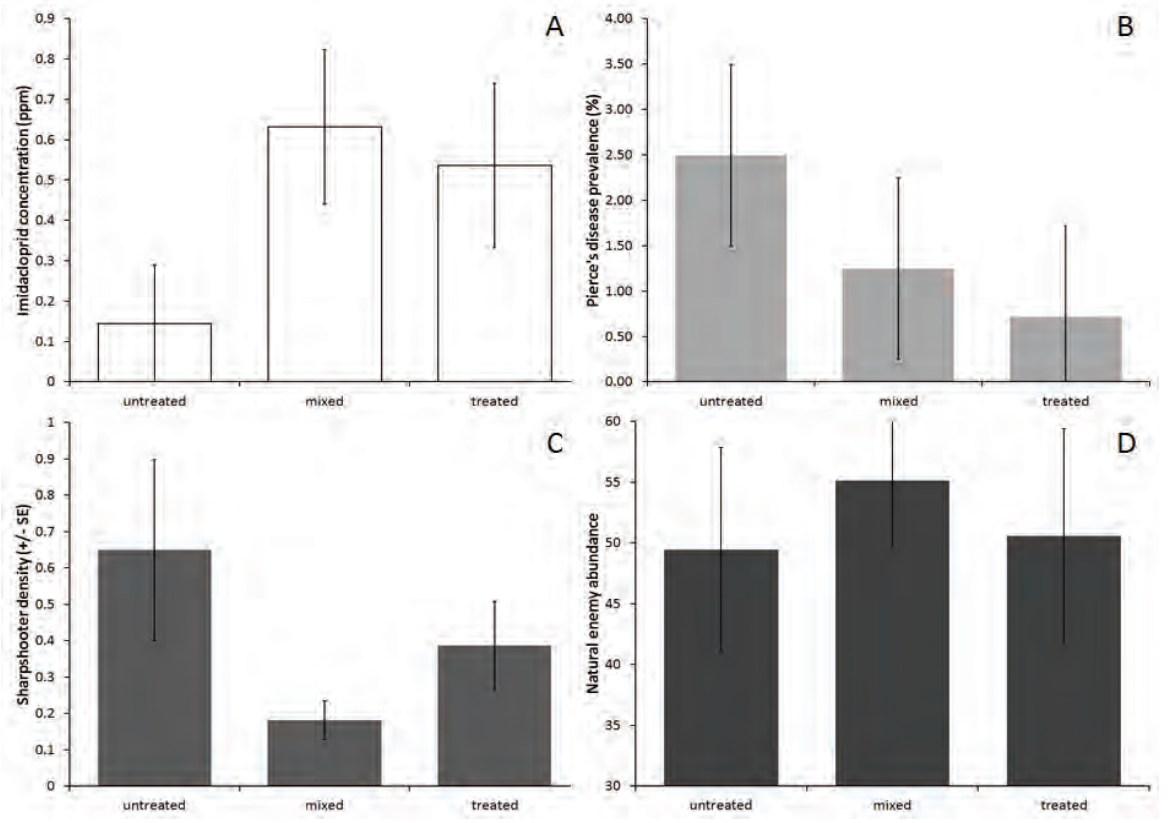


Fig. 2. A) Mean imidacloprid concentration, B) mean estimated Pierce's disease prevalence, C) mean sharpshooter catch on sticky traps, and D) mean natural enemy catch on sticky traps among 34 Temecula vineyards based on treatment category: untreated, intermittently treated (i.e. "mixed") or consistently treated with imidacloprid. Data shown for only 2012.

Estimated Pierce's disease prevalence varied substantially among fields from several sites that had no detectable cases of disease to a single site with on the order of 10% infection. Yet, overall prevalence among the three years of disease surveys was consistently low, with an overall mean of approximately 1.3%. The results suggest that at least part of the variability in prevalence is attributable to systemic insecticide treatment history (Fig. 2B). Specifically, Pierce's disease prevalence tended to be higher in untreated sites, was lowest in the consistently treated sites, and was intermediate in intermittently treated sites. Though not significant, this trend is at least consistent with the expectation of beneficial, albeit only slightly, effects of imidacloprid application on reducing vector pressure and pathogen spread.

Based on sticky trap catches there were little differences among the treatment categories with respect to natural enemy abundance in vineyards. We found similarly high catches of several natural enemy taxa, especially spiders, at all three types of sites (Fig. 2D). Tap sampling results showed a slightly different pattern. Generalist natural enemies were more common in untreated sites than mixed or consistently treated sites (Fig. 3A). However, this result does not appear to stem from disruption of natural enemy activity at treated sites. Rather, the abundance of all non-predatory arthropods (including several pest species such as grape leafhopper) were up to 7-fold higher on average at untreated sites compared to treated sites (Fig. 3B). Collectively these two sources of data indicate that imidacloprid applications do not dramatically upset natural enemy activity in vineyards. Rather, if anything, natural enemy populations may track pest populations, which are strongly affected by whether fields were treated with imidacloprid.

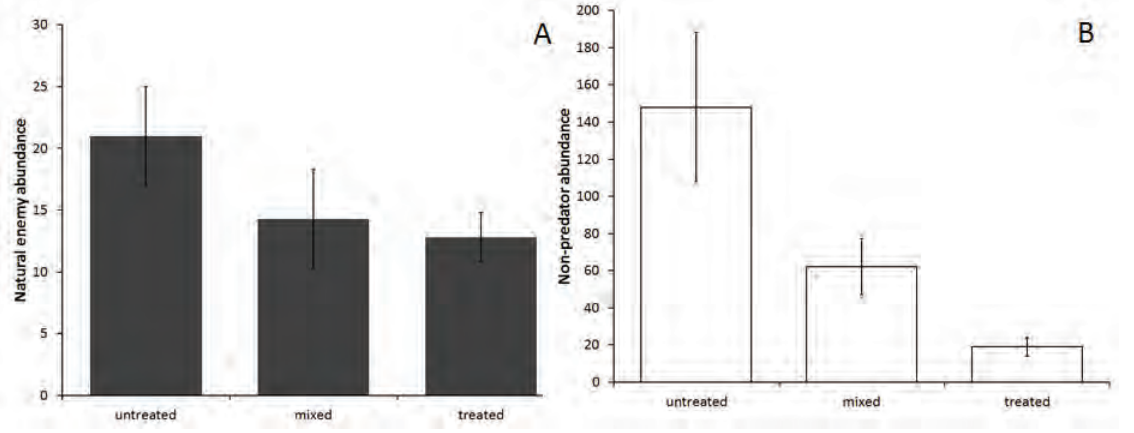


Fig. 3. A) Mean natural enemy abundance, and B) mean non-predatory arthropod abundance (+/- SE) based on tap sampling in Temecula Valley vineyards in 2012.

CONCLUSIONS

For many Southern California grape growers vector control is seen as a critical component to managing Pierce's disease within vineyards. Yet epidemiological theory suggests that disease management via vector control is only practical for pathosystems in which vectors are of limited efficiency. In such situations, as seems to be the case for *H. vitripennis*, the expectation is that reducing vector populations should limit the potential for pathogen spread.

Broadly, the work presented here suggests that within-vineyard chemical control

has the potential to reduce vector pressure and curb pathogen spread. Moreover, at least in this system, there do not appear to be strong non-target effects of the preferred systemic insecticide on natural enemies that would contribute to secondary pest outbreaks. However, three aspects of these results are worth exploring further. First, the observed differences in prevalence among years appear to be based on chemical control strategies, with untreated vineyards having the highest average prevalence, but whether those differences are due to recent management, historical artifacts, or differences in vector pressure at individual vineyards remains unclear. Ultimately analyses that grapple with year-to-year changes in prevalence are needed (i.e. disease incidence), which are ongoing. Second, it is interesting to note that there were little to no differences in Pierce's prevalence between the intermittently and consistently treated sites – especially in 2010 and 2011 surveys (MP Daugherty, unpublished data). This suggests that it may not be necessary to treat vineyards every year to effectively manage sharpshooter populations. Rather, in systems such as this one where there is substantial interannual variability in vector populations, targeting only the "outlier" years may be sufficient; assuming such years can be identified prior to treatment decisions. Finally, it is worth noting that such modest levels of observed disease are likely attributable to very low vector populations that exist currently relative to conditions during the peak of the *H. vitripennis* outbreak in the Temecula Valley region 15 years ago. The apparently slight differences in disease incidence among treatment categories might be expected to be substantially greater should the effective areawide control of sharpshooters be discontinued.

ACKNOWLEDGEMENTS

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Habitat Effects on Population Density and Movement of Insect Vectors of *Xylellafastidiosa* in California, USA

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ABSTRACT

Xylella fastidiosa is a xylem-limited bacterium that causes disease in grapevines, almonds, citrus, pear, alfalfa, and many other economically important plants. In California, USA, the bacteria are transmitted by several species of leafhoppers including the cicadellids *Draeculacephala minerva* Ball and *Homalodisca vitripennis* (Germar), the glassy-winged sharpshooter (GWSS). The pathogen and vectors have a wide host range including natural vegetation, cultivated crops, and ornamental plants in urban areas. Management of the diseases caused by *X. fastidiosa* requires knowledge of all possible infection pathways and biotic and abiotic factors that affect primary and secondary spread of the pathogen into and within agricultural landscapes. Two field studies were conducted to (*i*) determine patterns of insect vector population dynamics and temporal distribution of *X. fastidiosa*-infected plants relative to host plant assemblages in natural and cultivated habitats, and (*ii*) quantify movement and net dispersal rates of insect vectors in a manipulated experimental area. The first study investigated the role of *D. minerva* on movement of *X. fastidiosa* from different habitats into commercial almond nurseries, whereas the second study investigated the effects of deficit irrigated citrus trees on the spatiotemporal distribution and net dispersal rates of GWSS within the orchard. Surveys near commercial nurseries revealed that only habitats with permanent grass cover sustained *D. minerva* populations throughout the

season. A total of 87 plant samples tested positive for *X. fastidiosa* (6.3%), with a higher number of *X. fastidiosa*-infected plants found in weedy alfalfa fields than in other habitat types. Among plant species infected by *X. fastidiosa*, 33% were winter annuals, 45% were biennials or perennials, and 22% were summer annuals. Collectively, these findings identified a potential pathway for *X. fastidiosa* infection of almonds in nursery situations. Sex-specific net dispersal rates showed that GWSS males and females moved consistently and contributed equally to the level of population change within the citrus orchard. Trees under severe water stress were the least preferred by GWSS and yet, ca. 80% of the population were inflow individuals. Movement towards less preferable plants indicates that in agricultural landscapes dominated by perennial monocultures, there is a random component to GWSS movement, which may result from the inability of GWSS to use plant visual and/or olfactory cues to make well-informed long-range decisions.

Keywords: *Homalodisca vitripennis*, *Draeculocephala minerva*, plant water stress

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae), is an invasive insect pest native to the southeastern United States and northeastern Mexico [53] that was first discovered in California in the late 1980's [51]. The establishment of GWSS in California represents a serious threat due to its ability to vector *Xylella fastiodosa* Wells et al., a xylem-limited bacterium that causes Pierce's Disease in grapes [8], almond leaf scorch disease (ALSD) [9, 37], and many other diseases in economically important woody crops.

Since its initial detection, GWSS has expanded its range in Southern California and can also be found in southern portions of the San Joaquin Valley [3] and Pacific islands such as French Polynesia, Hawai'i, and Easter Island [17]. Pierce's disease affects grapevine (*Vitis vinifera* L.) production in the western and southeastern USA, whereas ALSD is found throughout almond production areas of California. Strains of *X. fastidiosa* are transmitted by several other species of xylem sap-feeding insects [13, 22, 42, 46, 47], but *Draeculacephala minerva* Ball (Hemiptera: Cicadellidae) is perhaps the only species that plays a role in pathogen spread to almond in California, as the distribution of *D. minerva* overlaps with almond production regions. GWSS is a polyphagous leafhopper with over 100 known hosts [25, 54]. GWSS populations are strongly associated with citrus plantings in California. Infested citrus orchards can

act as a source of vectors to adjacent vineyards as a result of the movement of GWSS between these two crops [4], which affects Pierce's disease incidence [39].

Effective management of a disease requires knowledge of all infection pathways. Proximity of susceptible crops to insect vector habitats is known to affect incidence of Pierce's disease in vineyards [16]. In almond orchards, in contrast, the random distribution of symptomatic trees and the absence of distinct disease gradients associated with adjacent vector habitat [18, 42] demonstrate that the relationships among proximity to vector habitat, the distribution of vectors in the orchard, and disease incidence are not as clear. Nonetheless, it is known that *D. minerva* moves between almond orchards and adjacent pastures and alfalfa fields [32], and the pathogen is present in *D. minerva* and in vegetation both in and around almond orchards [9]. Clearly, this species is an important vector of *X. fastidiosa* strains causing ALSD, and it may be responsible for some level of primary pathogen spread into orchards. Alternatively, another route of primary pathogen spread could be from infected nursery stock at the time of orchard establishment. Infection may occur in nurseries, either by the use of infected bud wood or transmission of the pathogen by insect vectors from surrounding vegetation into the nursery. Work by Hutchins et al. [27], Mircetich et al. [37], and Boyhan et al. [5] showed that *X. fastidiosa* can be transmitted by grafting in peach, almond, and plum, respectively. To our knowledge, primary spread of *X. fastidiosa* through the planting of infected almond nursery stock has not been considered.

Irrigation is the most significant input in agrosystems in arid and semi-arid regions worldwide. In California, future climate projection models predict reduced water reservoir carryover storage, reduced water availability to farmland in the Western San Joaquin Valley, and increased groundwater pumping [10]. Consequently, studies have developed water-saving strategies such as regulated deficit irrigation to improve water-use efficiency and sustainability in numerous perennial crop systems such as almonds [14, 52], citrus [15], and grapevines [55]. Regulated deficit irrigation is a strategy to maximize water use efficiency by reducing irrigation during drought-tolerant growth stages of a plant.

A significant amount of research has been generated to characterize the impact of plant stress on insect outbreaks and regulation of insect population dynamics. In general, resulting responses often appear to be insect feeding-guild dependent [33].

In this manuscript, results from two field studies, conducted separately, are

presented to illustrate the effects of habitat characteristics on population density and movement of insect vectors of *X. fastidiosa* in California, USA. In the first study with *D. minerva*[30], the objective was to evaluate the risk of infection of almond nursery stock from outside sources by quantifying vector populations and pathogen infection in host plant assemblages in habitats surrounding commercial almond nursery growing grounds. The hypothesiswas that natural vegetation in and around nursery plots included hosts for both *X. fastidiosa* and insect vectors. In the second study with GWSS [29, 31], the objective was to investigate the effects of deficit irrigation regimes in citrus trees on the population dynamics of GWSS. Results from the latter study demonstrated a relationship between GWSS population density and host plant quality, as measured by degree of water stress. However, differences in insect density among irrigation treatments may be a result of several mechanisms that act independently or in concert including differences in insect performance (e.g., fecundity and longevity) among irrigation treatments and differences in rates of movement based on treatment. Therefore, another goal of this study was to assess the extent to which movement affected GWSS population density and structure among irrigation treatments. Quantification of movement of GWSS was achieved through the combination of a mark-capture technique using multiple immunomarkers[19] and manipulation of irrigation levels in the orchard thereby inducing movement of marked GWSS individuals within the spatially heterogeneous habitat.

MATERIALS AND METHODS

Draeculocephala minerva in habitats surrounding almond nurseries.

Monitoring of *D. minerva* population dynamics. The population dynamics of *D. minerva* in vegetation located in and around commercial almond nurseries was monitored for one year. During this period, sharpshooter activity was monitored using yellow sticky traps placed around the perimeter of five almond nursery blocks. Seven common vegetation types (habitats) located adjacent to nursery blocks (10 to 15 m) were selected for sampling: irrigated pasture, drainage ditch, alfalfa field, weedy alfalfa field, non-cultivated perimeter, orchard floor, and cover crop. Throughout the trapping period, insect population densities in surrounding vegetation were monitored by collecting sweep net samples every six weeks.

Incidence of *X. fastidiosa* and plant species composition in *D. minerva* habitats. At each site, the relative cover and abundance of plant species were measured in 10 transects in the habitats described above. Linear transects were placed paralleled to the nursery blocks at ~ 14 m from the edge of the crop. Plant species composition within linear transects (10 m long × 0.3 m wide) was measured every six weeks for one year by recording species richness and species diversity (Simpson's Index of Diversity) [(49)]. At each sampling date, leaf samples were collected from plant species present within each transect and tested for presence of *X. fastidiosa* using an ELISA kit.

GWSS population density and dispersal in a water-stressed citrus orchard block

Experimental site and irrigation treatments. A two year study was conducted on the campus of the University of California, Riverside, in 5.4 ha of a citrus orchard [*Citrus sinensis* cv. 'Valencia'] maintained under micro-sprinkler irrigation. The experiment was designed as a 3 x 3 Latin square with three irrigation treatments: 1) trees irrigated at 100% of the crop evapotranspiration rate (ET_c), 2) a continuous deficit-irrigated treatment maintained at 80% ET_c, and 3) a continuous deficit-irrigated treatment maintained at 60% of ET_c throughout the two years of the experiment. Each of the nine plots consisted of 120 trees (23.6 m^2 canopy cover).

Plant conditions. The severity of water stress was characterized weekly by measurements of stem water potential using a pressure chamber. To monitor the water potential, the fourth leaf from the tip of two mature branches per tree was covered with a bag made of foil-laminate material for 30 min before being excised from the branch. Leaves were excised and immediately processed.

Fruit quality and yield. All oranges were harvested and taken to a local commercial packing house where oranges were mechanically counted, sized, and color graded. Fresh market oranges were categorized as "first" (higher quality) or "second" (lower quality) grade.

GWSS populations. Populations of GWSS within experimental plots were sampled weekly for two years. A 3-min visual inspection of leaves and branches around sample trees was conducted to monitor for GWSS egg masses, nymphs, and adults. The same trees were sampled for GWSS adults and nymphs by collecting a beat net sample from each tree. Yellow sticky traps were used to monitor insect

activity. Six traps were placed on the south side of three rows per plot (two traps per row placed five trees apart). Traps were replaced weekly and placed into a freezer until inspection.

Mark and capture of GWSS. Three unique proteins were used in the study including cow's milk (casein), chicken egg white (egg albumin), and soy milk (soy trypsin) to mark GWSS in the 60, 80, and 100% ET_c treatments, respectively. Homogenized whole milk, chicken egg white, and soy milk were purchased from local wholesale distributors and stored at 4°C until use. On the application date, each of the marking materials were diluted in water to a 5% solution and applied to trees in the respective treatment plots at a rate of 1870.6 l / ha using a tractor PTO-driven, airblast sprayer. Applications were repeated on three different dates in 30-day intervals starting in late-June and ending late-August in each year of the study. Yellow sticky traps deployed as described above were used tomonitor insect activity. GWSS adults were removed from the traps and placed into individual 1.5-mlvials for ELISA analysis.

ELISA for marker detection. A bovine casein, egg albumin, and soy trypsin indirect ELISA was performed on field-captured GWSS as described in detail by Jones et al. [28] to determine the captured individual area of origin.

Net dispersal rate of GWSS. A weekly, sex-specific net dispersal rate (NDR) of GWSS for each irrigation treatment was calculated as the ratio of the difference between the number of inflow and outflow individuals to the number of residents, as follows:

$$NDR = (i - o)/r \quad [1]$$

where the number of residents (*r*) was the number of insects caught in the reference irrigation treatment that were ELISA-positive only for the protein marker applied to the reference irrigation treatment. The number of inflow individuals (*i*) was the number of insects caught in the reference irrigation treatment that were ELISA-positive for one or both of the protein markers applied to the other irrigation treatments. Finally, the number of outflow individuals (*o*) was the number of insects caught outside the reference irrigation treatment that were ELISA-positive for the protein marker applied to the reference irrigation treatment. Positive values for NDR indicate that more GWSS entered an irrigation treatment than left, whereas negative values for NDR indicate that more GWSS left an irrigation treatment than entered. The impact of such movement on population composition was measured relative to the size of the resident population. Individuals that were ELISA-positive for two or three markers were not included in the number of outflow individuals because their origin was unknown.

RESULTS

Draeculocephala minerva in habitats surrounding almond nurseries.

Monitoring of insect vector population dynamics. A total of 22 species of Cicadomorphs were collected in sweep net samples. Of these, *D. minerva* was the only known vector of *X. fastidiosa* captured. The numbers of *D. minerva* adults captured were highest on the edges of irrigated pastures, followed by drainage ditches and edges of weedy alfalfa fields (Fig. 1A). Insect population densities in weedy alfalfa fields were about three-fold higher than in weed-free alfalfa fields.

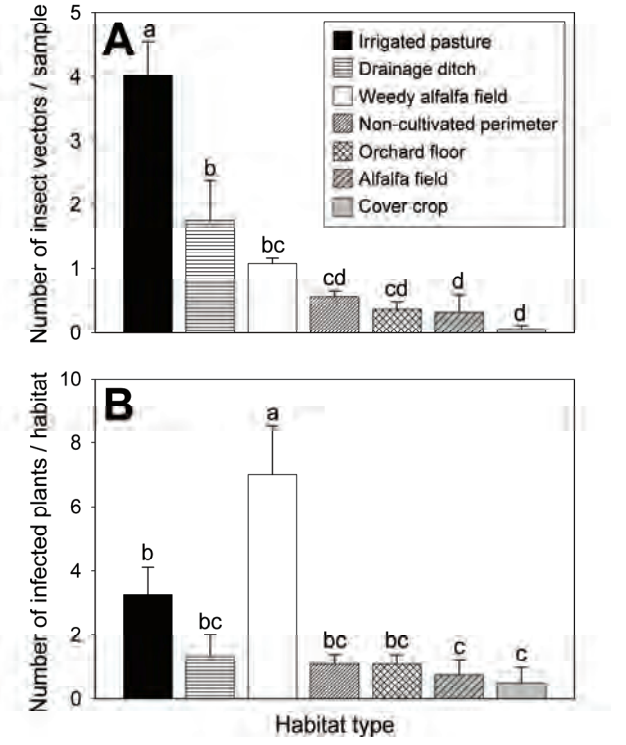


Fig. 1. Mean (± SEM) numbers of *Draeculacephala minerva* adults in sweep net samples collected from vegetation in habitats surrounding almond nursery grounds, **A**, and mean number of *Xylella fastidiosa*-infected plants per habitat, **B**. Bars representative of habitat type having the same letter above them do not differ significantly ($P< 0.05$) according to a Tukey's HSD test.

There was a curvilinear relationship between the numbers of *D. minerva* and the percentage of cover by grass species in sampled habitats (Fig. 2), such that the numbers of

insects caught in the samples increased with increasing grass cover. Although some habitats referred to here as non-cultivated perimeter, orchard floor, and cover crop had a high percentage of grass cover during winter and spring months, only habitats with permanent grass cover (i.e., irrigated pastures and drainage ditches) were shown to sustain robust *D. minerva* populations throughout the season.

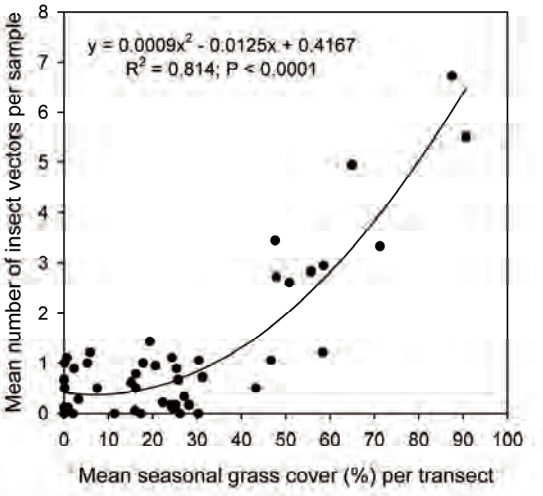


Fig. 2. Relationship between the mean (± SEM) numbers of *Draeculacephala minerva* adults captured in sweep net sampling and the mean seasonal grass cover on habitats surrounding almond nursery grounds.

Insect catch data from yellow sticky traps, when pooled across all habitats, showed five peaks of *D. minerva* adult activity throughout the sampling period (Fig. 3). Traps located on the edge of surrounding habitats consistently captured more *D. minerva* adults than traps located on the edge of nursery stock growing grounds. Despite the reduced insect activity from mid- March to early May, trap catches within nursery stock grounds indicated that *D. minerva* adults were actively moving between the surrounding vegetation and the nursery crop.

Incidence of *X. fastidiosa* and plant species composition in vector habitats. A total of 102 plant species were identified and 1387 samples were collected. A total of 87 samples tested positive for *X. fastidiosa* (6.3%) with a higher number of infected plants found inweedy alfalfa fields than in the other habitat types (Fig. 1B).

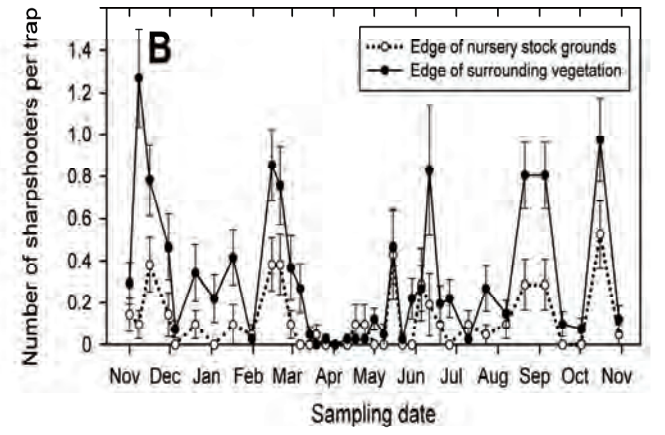


Fig. 3. Mean (± SEM) numbers of *Draeculacephala minerva* adults captured in yellow sticky traps placed between nursery growing grounds and surrounding vegetation. Bars representative of sampling dates having the same letter above them do not differ significantly ($P< 0.05$) according to a Tukey's HSD test.

Measurements of plant species richness and species diversity showed that alfalfa fields and drainage ditches were the least and the most rich and diverse habitats, respectively. The mean (± SEM) plant species richness in alfalfa fields and drainage ditches was 1.133 ± 0.133 and 5.778 ± 1.806 species per transect per sampling period, respectively. Species diversity (Simpson's Index of Diversity) in alfalfa fields and drainage ditches was 0.004 ± 0.004 and 0.569 ± 0.161 per transect per sampling period, respectively. Values of species richness and diversity for irrigated pastures and weedy alfalfa fields were intermediate among the habitats. On average (± SEM), plant species richness in irrigated pastures and weedy alfalfa fields was 3.62 ± 0.73 and 4.22 ± 1.13 species per linear transect per sampling period, respectively. Species diversity values in irrigated pastures and weedy alfalfa fields were 0.225 ± 0.081 and 0.287 ± 0.082 per linear transect per sampling period, respectively.

Although measurements of plant species richness and diversity within habitats did not markedly vary throughout the sampling period, plant species composition in habitats changed according to plant species' life cycle (e.g., annual vs. perennial) and seasonality (e.g., winter vs. summer). Among the 40 plant species that tested positive for *X. fastidiosa*, about one third were winter annuals, one third were biennials or perennials, and one third were summer annuals that accounted for about 33.3, 44.8, and 21.8% of all *X. fastidiosa*-positive plants, respectively (Table 1). Although the majority of the *X. fastidiosa*-positive plant species reported here had been reported as

hosts in previous surveys, a total of 19 new plant species are reported here as potential hosts of *X. fastidiosa*. Among the sampling dates, *X. fastidiosa* detection was highest during the month of February, followed by July. There were no significant differences in proportion of infected plants among the other sampling dates.

GWSS population density and dispersal in a water-stressed citrus orchard

Plant conditions. Stemwater potential was consistently lower in the 60% ET_c treatment than in the 80 or 100% ET_c treatments. There were no differences in stem water potential between the 80 and 100% ET_c treatments.

Fruit quality and yield. In 2006, there were no differences in total numbers of harvested fruit or in the number of fruit per grade category among irrigation treatments. However, the percentage of first grade fruit was higher in the 80% ET_c treatment. Moreover, the percentage of first grade fruit was significantly lower in the 60% than in the 100% ET_c treatment. There were no differences in the percentages of low quality, non-juice (second grade) fruit among treatments. In 2007, the total number of harvested fruit in the 60% ET_c treatment was significantly lower than in the 80 and 100% ET_c treatments. The numbers of fruit across all fruit grade categories were lower in the 60% ET_c treatment than in the 80 and 100% ET_c irrigation treatments. There were no differences in total number of fruit and number of fruit per grade category between the 80 and 100% ET_c irrigation treatments.

GWSS populations. Visual counts in 2005 revealed an increase in adult GWSS levels from late June to a peak in mid July. During this period, about 50% fewer adults were counted on trees irrigated with 60% of the ET_c than with 80 and 100% ET_c. There was no difference in the number of GWSS adults observed per tree between the 80 and 100% ET_c treatments. In 2006, there was an increase in the overall number of adult GWSS observed per tree in early July to the population peak in late July. Up to the peak of GWSS numbers in late July, fewer adults were found on trees irrigated at 60% ET_c than at 80 and 100% ET_c. There was no difference in the number of GWSS adults observed per tree between 80 and 100% ET_c treatments. Averaging over the early July to early October interval, fewer adult GWSS were found in trees irrigated at 60% ET_c than at 80% ET_c. The number of adult GWSS counted in the 100% ET_c treatment was not different from those observed in the 60% or 80% ET_c irrigation treatments.

During the 2005 sampling period, there were two peaks of GWSS oviposition (mid-May and mid-July). However, there were no differences in the mean number of GWSS egg masses observed among the irrigation treatments throughout either the

mid-April to mid-May interval or during the second, and highest, egg mass peak lasting from mid-June to early August. In 2006, there appeared to be four discrete periods of GWSS oviposition. The first period, resulting from oviposition by overwintering adults, occurred from late February to early March. A second peak occurred from late April to early June, and the third and largest peak occurred from early July to early September. The fourth discrete oviposition period occurred from late September to late October. In only one of the four periods were any differences in GWSS egg masses observed as a result of deficit irrigation treatment. Specifically, fewer egg masses were found in the 60% than in the 80 and 100% ET_c treatments during the second peak ovipositional period of 2006.

Sex-specific net dispersal rate of GWSS. Male and female GWSS NDRs were similar and followed the same trend during the 2005 and 2006 seasons (Fig. 4). Weekly NDRs calculated for each irrigation treatment showed that inflow movement (i.e., individuals moving into a block) was consistently higher than outflow movement (i.e., individuals moving out of a block) in the 60% ET_c (2005 and 2006) and 100% ET_c treatments (2005 only). NDRs were generally neutral in the 80% ET_c treatment in both years (Figs. 4C and 4D) and neutral in the100% ET_c treatments in 2006 only (Fig. 4F). In the 100% ET_c treatment, inflow and outflow movements were the same except for the period of 24 July to 7 August 2006 when the number of inflow individuals exceeded the number of outflow individuals (Fig. 4F).

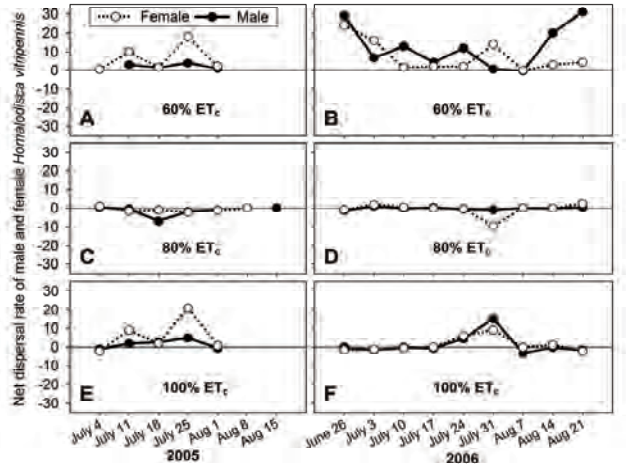


Fig. 4. Net dispersal rates (NDRs) of male and female *Homalodisca vitripennis* in irrigation treatments during the 2005 and 2006 sampling seasons obtained from data on number of dispersing individuals captured on traps. NDRs were calculated using equation 1. Positive and negative NDRs in the 60% (Fig. A and B) and 80% ET_c irrigation treatments (Fig. C and D) show higher inflow and outflow movement, respectively.

Ignoring gender, more individuals moved into the 60% ET_c treatment than moved out of the 60% ET_c treatment in 2006. Conversely, more individuals moved out of the 80% ET_c and 100% ET_c treatment areas than moved into the 80% ET_c and 100% ET_c treatments. Resident populations peaked on 24 July in the 80% ET_c treatment and were overall higher than resident populations in the other irrigation treatments.

Composition of GWSS populations. The composition of GWSS populations within irrigation treatments was similar during the 2005 and 2006 seasons. In 2005, inflow individuals that originated from the 80% ET_c irrigation treatment were more abundant in the 60% ET_c (~51% of ELISA-positive insects) and 100% ET_c treatments (~65% of ELISA-positive insects) than residents and other inflow individuals (Table 2). In 2006, individuals that originated from the 80% ET_c treatment were also more abundant than the resident populations in the 60% ET_c (~27 vs. 12% of ELISA-positive insects) and 100% ET_c treatments (~55 vs. 37% of ELISA-positive insects, respectively) (Table 2). Resident populations in the 80% ET_c treatment were higher than the inflow populations (Table 2). Resident populations in the 60 and 100% ET_c treatments were in the minority (<50%) in both 2005 and 2006.

DISCUSSION

One of the goals of the study on *D. minerva* in almond nurseries was to investigate the potential role of infected nursery stock in contributing to ALSD occurrence in commercial almond orchards. Surveys conducted in vegetation found near commercial nursery growing grounds revealed that vector population densities and incidence of *X. fastidiosa* are highly dependent on vegetation type. As both vector and pathogen were found in close proximity to almond nurseries, spread of *X. fastidiosa* into nurseries is considered plausible.

In the past 60+ years, numerous studies have demonstrated the importance of non-crop plants species as potential sources of *X. fastidiosa*[2, 6, 12, 20, 21, 23, 24, 26, 35, 36, 43, 44, 45, 48, 50, 56]. Although most *X. fastidiosa* host plant species documented here have been reported as hosts in other surveys, 19 new plant hosts were identified.

Draeculacephala minerva is well known to be abundant in irrigated pastures, stream banks, and weedy alfalfa fields with perennial grass cover [7, 41, 50]; proximity of such habitats near almond orchards with high incidence of ALSD has been documented [40]. Results from surveys reported here, such as high vector abundance and presence of *X. fastidiosa* in host plants located adjacent to the crop, are in agreement with

findings from previous investigations. However, this study is the first to establish presence of vectors and *X. fastidiosa* specifically with almond nurseries.

Proximity of *X. fastidiosa* and insect vectors to commercial almond nurseries in California was demonstrated, providing evidence for *X. fastidiosa* infection of nursery stock. However, as ALSD incidence in California is typically low in almond orchards, primary spread via infected nursery stock also must be low under the current conditions. Nursery plants may not show symptoms of ALSD while in the nursery, which makes it impossible to use symptom expression for roguing prior to commercialization. Moreover, screening the large numbers of plants cultivated by commercial nurseries (12,000 plants/ ha) for presence of *X. fastidiosa* using assays such as culturing, PCR, or ELISA is impractical, laborious, and could result in the addition of unnecessary production costs. Therefore, removal and replacement of diseased plants soon after orchard establishment may be the most cost effective practice for both almond growers and almond nursery stock producers.

The study on the effects of citrus deficit irrigation on GWSS showed that the two irrigation deficit regimes, 60 and 80% ET_c, differentially affected the population dynamics of GWSS in the experimental citrus plots. GWSS populations were negatively affected by severe host plant water stress, but GWSS population density was not linearly correlated with decreasing water availability in plants. Trees irrigated at 60% ET_c were host to fewer GWSS eggs, nymphs, and adults than trees irrigated at 80% ET_c. Interestingly, the 100% ET_c treatment hosted similar numbers of GWSS eggs, nymphs, and adults as the 60% ET_c treatment in some periods of the study and lower numbers of GWSS nymphs than the 80% ET_c. Moderate water stress in trees (e.g., 80% ET_c) may increase solute concentrations used for osmotic adjustment (i.e., carbohydrates, amino acids, and organic acids) that may also serve as feeding stimulants and nutritional substrates [(34)]. However, reduced water potential beyond a certain threshold in more severely water-stress irrigation treatments (60% ET_c) might impede GWSS feeding because more energy would be needed to extract xylem fluid [(1)]. Conversely, well-watered plants (100% ET_c) with higher mean water potentials may facilitate extraction of xylem fluid. However, as the energy required for extracting xylem fluid was reduced in well-watered trees, more fluid would have to be ingested and filtered to compensate for a more dilute xylem food source. Thus, citrus trees irrigated at 80% ET_c may combine two important plant characteristics for GWSS: 1) a nutrient-concentrated food source and 2) a water potential above

acceptable thresholds for GWSS xylem fluid extraction.

Our data demonstrated that a water saving of 20% (i.e., irrigation at 80% ET_c) over two years did not induce significant reduction in yield and fruit quality compared to full irrigation (100% ET_c). The 20% water saving practice improved water use efficiency (yield per unit water) and thus, seems a viable option for commercial practice to maintain productivity and reduce irrigation costs in areas with scarce water resources. However, long term effects of this deficit irrigation regime on plant vegetative growth needs further investigation.

Findings from this study have generated significant new information regarding the host selection behavior of GWSS in California. Trees under severe water stress had lower water potential and consequently hosted fewer GWSS than trees maintained under moderate water stress. Although the adult GWSS population was reduced, on average, by 50 to 65% in citrus plots maintained under continuous severe water stress, the negative economic impacts to citrus growers reflected by lower yield and fruit quality (50% overall reduction), especially after two consecutives years of severe water stress, impedes the adoption of this management strategy to reduce GWSS populations in Valencia orchards in southern California. Nevertheless, regulated deficit irrigation (RDI) regimes are widely practiced over millions of hectares worldwide to reduce usage of irrigation water and increase farmer's profits [(11)]. A more complete understanding of the effect of RDI applied during less vulnerable phenological stages of citrus fruit development and the operative host-plant cues that influence GWSS host selection behavior may result in the deployment of strategies to improve control efforts.

Irrigation levels established in the experiment induced movement revealing the mobility of both male and female GWSS. The mark-capture method used in the current study aided our ability to document and track insect movement in the experimental area demonstrating the effects of movement on GWSS population density and structure among irrigation treatments. Sex-specific net dispersal rates showed that males and females moved consistently within the habitat and contributed equally to the overall level of population change within and among irrigation treatments. Resident GWSS represented the minority in the 60 and 100% ET_c irrigation treatments during the 2005 and 2006 seasons, whereas resident GWSS were in the majority in the 80% ET_c irrigation treatment in both seasons. Insects originating from the 80% ET_c treatment plots were also in the majority in the other irrigation treatments; except in 2006 when there was no difference in the percentage of inflow individuals in the 60%

ET_c treatment. A total of 5,970 and 10,478 individuals in 2005 and 2006, respectively, were tested for the presence of each protein mark. Of these, about 10 and 27% tested positive for at least one marker in 2005 and 2006, respectively. Despite the fact that the percentages of double- (or triple-) marked individuals were generally low compared to single-marked individuals, the presence of multi-marked individuals showed that movement of GWSS within the discrete habitat was not unidirectional. That is, between 0.4 and 1% of all ELISA-positive individuals had visited the three different marked areas before being captured.

Differential inflow and outflow movement within and among experimental treatments were the major factors contributing to the observed unbalanced distribution and composition of the population within the experimental area. Among all protein-marked individuals trapped in the 60% ET_c irrigation treatment, about 75 and 88% of them in 2005 and 2006, respectively, were inflow individuals. Movement towards less preferable host plants indicates that GWSS were unable to make well-informed decisions based on visual or olfactory cues in selecting suitable host plants and habitats during movement. These findings are in agreement with those of Northfield et al. [38] who reported movement of GWSS individuals from a high to low quality patch. Data also support their hypotheses that: 1) GWSS move into low quality patches from a distance, but leave the patch after assessing host plant quality such as xylem fluid quality, and 2) GWSS host selection behavior occurs on the plant rather than from plant visual or physical cues available prior to landing. Collectively, these studies demonstrate that there is a random component to GWSS movement and dispersal in agricultural landscapes dominated by perennial monocultures.

The overall goal of this study was to improve our understanding of the factors that: 1) influence GWSS movement in a managed ecosystem, 2) control fluctuating population densities within a manipulated habitat, and 3) reduce GWSS impact on affected crops. In addition to quantifying net dispersal rates and describing the composition of GWSS populations, this study is an important step towards predicting seasonal movement of GWSS according to habitat conditions.

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Table 1. Total number of infected plants over the number of sampled individuals on each collection date and their temporal distribution in habitats surrounding five nursery stock blocks

Plant species	Plant family	Sampling date					
		2/6/08	3/21/08	5/23/08	7/24/08	9/11/08	10/23/08
Bromus diandrus Roth[a]	Poaceae	1/1	d	d	d	d	d
Avena fatua L.	Poaceae	4/4	d	d	d	d	d
Hordeum murinum L. ssp. *murinum*	Poaceae	2/5	0/3	d	d	d	d
Erodium botrys (Cav.) Bertol.[a]	Geraniaceae	1/4	0/4	d	d	d	d
Lolium perenne L.[a]	Poaceae	5/5	c	d	d	d	d
Capsella bursa-pastoris (L.) Medik.[b]	Brassicaceae	1/4	0/3	0/2	d	d	d
Poa annua L.	Poaceae	3/6	c	c	d	d	d
Stellaria media (L.) Vill.	Caryophyllaceae	1/10	0/2	c	d	d	d
Senecio vulgaris L.	Asteraceae	2/5	1/8	0/1	d	d	d
Ranunculus repens L.[b]	Ranunculaceae	1/2	0/2	c	c	d	d
Cyperus eragrostis Lam.	Cyperaceae	1/1	0/1	c	c	d	d
Geranium dissectum L.[b]	Geraniaceae	0/3	0/3	c	1/1	d	d
Medicago polymorpha L.	Fabaceae	2/5	0/2	c	c	c	d
Lactuca serriola L.	Asteraceae	c	c	0/6	2/6	0/3	d
Verbena litoralis Kunth[b]	Verbenaceae	0/3	1/2	0/3	c	0/3	d
Silybum marianum (L.) Gaertn.[b]	Asteraceae	1/6	0/3	0/1	0/1	c	0/1
Erodium moschatum (L.)	Geraniaceae	1/22	0/13	0/1	c	0/1	c
Ludwigia grandiflora (Michx.)[b]	Onagraceae	0/3	1/12	c	c	0/6	c
Marrubium vulgare L.[b]	Lamiaceae	3/6	0/4	0/1	0/2	0/1	0/2
Medicago sativa L.	Fabaceae	7/26	0/23	1/30	0/26	0/31	0/32
Cynodon dactylon (L.) Pers.	Poaceae	c	c	2/9	1/2	1/7	0/4
Sonchus oleraceus L.	Asteraceae	1/2	0/12	0/8	1/5	0/1	0/2
Malva parviflora L.	Malvanaceae	7/29	0/17	0/10	2/6	0/11	0/16
Conyza canadensis (L.) Cronquist	Asteraceae	c	c	0/10	1/9	0/8	0/6
Rumex crispus L.	Polygonaceae	3/14	0/7	0/9	c	0/1	3/5
Coronopus didymus (L.) Smith[b]	Brassicaceae	1/10	c	0/1	0/2	c	1/1

Plantago lanceolata L.[b]	Plantaginaceae	0/2	0/1	0/2	0/3	0/3	1/3
Datura wrightii Regel	Solanaceae	d	0/8	0/25	4/18	0/24	0/34
Prunus dulcis (Mill.) D. A. Webb	Rosaceae	d	0/12	0/14	2/20	0/33	0/35
Vitis spp.	Vitaceae	d	0/2	0/2	1/2	0/2	0/2
Convolvulus arvensis L.	Convolvulaceae	d	d	0/6	2/10	0/3	0/2
Salsola tragus L.[b]	Chenopodiaceae	d	d	0/2	1/2	0/1	0/2
Eriochloa contracta Hitchc.[a]	Poaceae	d	d	d	1/1	c	d
Echinochloa crus-galli (L.) P. Beauv.	Poaceae	d	d	d	1/4	0/2	0/2
Polygonum arenastrum Jord.[a]	Polygonaceae	d	d	d	1/5	0/1	0/3
Polygonum lapathifolium L.[a]	Polygonaceae	d	d	d	1/5	c	0/3
Agrostis gigantea Roth[b]	Poaceae	d	d	d	0/1	c	1/5
Carex L.[b]	Cyperaceae	d	d	d	c	0/1	1/1
Xanthium spinosum L.[a]	Asteraceae	d	d	d	0/1	0/13	1/5
Portulaca oleracea L.	Portulaceae	d	d	d	2/8	d	d

[a] Congener species previously reported as a host.

[b] First report of *Xylella fastidiosa* detection in plant genus and species.

c No plant samples collected.

d Plant species not present in or near line transects.

Table 2. Accumulated percentage distribution of protein-marked GWSS in irrigation treatments during the 2005 and 2006 summer seasons

Irrigation treatment	Sex	Insects tested	Insects marked	Mean (± SE) percentage of protein-marked individuals					
				Casein[a] (60% ET_c)	Egg albumin (80% ET_c)	Soy trypsin (100% ET_c)	Casein + egg albumin[b]	Casein + soy trypsin	Egg albumin + soy trypsin
2005									
60% ET_c	♂	406	28	30.90 ± 9.25	52.12 ± 11.91	12.12 ± 9.29	-	-	4.84 ± 3.37
	♀	510	72	21.00 ± 7.66	49.17 ± 8.84	14.34 ± 4.64	-	-	15.47 ± 5.60
	Total	**916**	**100**	**25.19 ± 5.87 Ab**	**50.42 ± 7.02 Aa**	**13.4 ± 4.65 Ab**	**-**	**-**	**10.97 ± 3.63 Ab**
80% ET_c	♂	472	36	7.69 ± 7.69	58.49 ± 12.33	33.81 ± 11.68	-	0	-
	♀	595	60	8.29 ± 4.48	71.40 ± 7.62	20.30 ± 6.19	-	0	-
	Total	**1067**	**96**	**8.03 ± 4.11 Ac**	**65.81 ± 6.84 Aa**	**26.15 ± 6.16 Ab**	**-**	**0**	**-**
100% ET_c	♂	479	32	20.45 ± 12.06	63.63 ± 13.63	11.36 ± 6.18	4.54 ± 4.54	-	-
	♀	523	66	19.04 ± 9.86	65.67 ± 10.53	12.20 ± 7.34	3.07 ± 2.09	-	-
	Total	**1002**	**98**	**19.66 ± 7.49 Ab**	**64.77 ± 8.23 Aa**	**11.83 ± 4.83 Ab**	**3.72 ± 0.97 Ab**	**-**	**-**
				$F_{2, 71}$ = 2.426	$F_{2, 71}$ = 0.981	$F_{2, 71}$ = 2.311		$F_{1, 43}$ =2.933	
				P = 0.096	P = 0.380	P = 0.107		P = 0.094	
2006									
60% ET_c	♂	1011	205	10.20 ± 3.31	26.42 ± 3.43	38.58 ± 4.38	-	-	24.79 ± 4.43
	♀	654	124	13.21 ± 2.81	27.07 ± 3.87	25.23 ± 4.68	-	-	34.47 ± 3.46
	Total	**1665**	**329**	**11.70 ± 2.15 Ab**	**26.75 ± 2.55 Ba**	**31.91 ± 3.35 Aa**	**-**	**-**	**29.63 ± 2.89 Aa**
80% ET_c	♂	1208	422	1.69 ± 0.75	62.11 ± 2.76	35.35 ± 2.85	-	0.83 ± 0.48	-
	♀	722	268	5.91 ± 1.51	52.12 ± 2.22	41.09 ± 2.92	-	0.85 ± 0.50	-
	Total	**1930**	**690**	**3.80 ± 0.91 Bc**	**57.12 ± 1.94 Aa**	**38.22 ± 2.07 Ab**	**-**	**0.84 ± 0.34 Bc**	**-**
100% ET_c	♂	1023	235	5.58 ± 1.51	52.45 ± 4.28	39.73 ± 4.24	2.22 ± 1.86	-	-
	♀	621	176	6.76 ± 2.57	57.64 ± 5.64	34.79 ± 5.25	0.80 ± 0.61	-	-
	Total	**1644**	**411**	**6.17 ± 1.47 Abc**	**55.05 ± 3.52 Aa**	**37.26 ± 3.35 Ab**	**1.51 ± 0.97 Bd**	**-**	**-**
				$F_{2, 98}$ = 4.575	$F_{2, 98}$ = 39.359	$F_{2, 98}$ = 2.623		$F_{2, 98}$ = 126.998	
				P = 0.013	P< 0.001	P = 0.078		P< 0.001	

[a] Casein, egg albumin, and soy trypsin, which were applied to irrigation treatments 60, 80, and 100% ET_c, respectively.
[b]GWSS ELISA-positive for two protein markers.
The same upper case letters in columns and lower case letters in rows indicate irrigation treatments and protein markers, respectively, did not significantly affect the number of captured and marked individuals (α = 0.05).

Xylella fastidiosa Diversity

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ABSTRACT

The bacterial plant pathogen *Xylella fastidiosa* causes disease in a wide range of host plant species. However, the species is very diverse phylogenetically and phenotypically. There are four broadly recognized subspecies of *X. fastidiosa*, which primarily cause disease in a host specific manner. Furthermore, clusters within subspecies may also be host specific. Despite the fact that host specificity is an important characteristic of *X. fastidiosa* phylogenetic clusters, the determinants of specificity are unknown; this bacterium lacks a type III secretion system and effectors, often associated with specificity in other bacterial plant pathogens. Furthermore, field populations have been shown to have large genetic diversity. Because horizontal gene transfer, rearrangements, and other mutational processes appear to be frequent in *X. fastidiosa*, understanding how it evolves is of importance, especially in face of the fact that new diseases caused by this bacterium continue to emerge.

Keywords: *Xylella fastidiosa*, vector-borne, Pierce's disease, Pear leaf scorch, xylem.

INTRODUCTION

The first disease caused by the bacterium *Xylella fastidiosa*, Pierce's disease of grapevines, was described in 1892 by Newton Pierce. An epidemic in California, USA, in the 1930-40s led to a large number of advancements in our knowledge of this and other *X. fastidiosa* diseases, including the identification of insect vectors [28, 29]. Nevertheless, only in the 1970s was the bacterial etiology of these diseases demonstrated [11], as prior to that they were thought to be caused by viruses. This breakthrough resulted in the *in vitro* cultivation of the bacterium [9], which was a decade later named *Xylella fastidiosa* [38]. More recently, it became the first bacterial plant pathogen to be fully sequenced [32], and epidemics in citrus and grapevines in

Brazil and California, USA, respectively, led to an increase in interest and research on this bacterium. The history of important phases of *X. fastidiosa* research has been elegantly summarized in a recent review [25].

There are several reviews that have addressed various aspects of the biology and ecology of *X. fastidiosa* [2, 4, 12, 25, 27]. The specific goal of this article is to summarize current information available on the diversity of *X. fastidiosa*. Furthermore, because it has been shown that this bacterium has a dynamic genome, and that recombination among subspecies occurs in the field, a robust knowledge about its evolution may assist in the development of strategies to reduce the impact of emerging diseases. This is relevant because, in the last decade, several new *X. fastidiosa* diseases have been described in North and Central America, in addition to Taiwan [34].

The species *Xylella fastidiosa*

Xylella fastidiosa is a gamma-proteobacterium in the family Xanthomonadaceae [38]. The main sister clade to *Xylella* is the genus *Xanthomonas* [23]. There are important and significant differences among these bacteria; for example, *Xanthomonas* spp. do not require insect vectors for dispersal, while *X. fastidiosa* does. In addition, the genome of *X. fastidiosa* is much smaller than that of *Xanthomonas* spp., which may be a consequence of a lifestyle fully dependent of plant and insect hosts [23]. However, there are several similarities between these two genera at the genome level, resulting in substantial degree of gene conservation. For example, both produce exopolysaccharides via a highly conserved operon [8, 15], and cell-cell signaling is mediated by another conserved set of genes [17]. A comparison between *Xanthomonas* spp. and *X. fastidiosa* is available elsewhere [4, 23].

Although generally thought of as a plant pathogen, *X. fastidiosa* needs to colonize both its plant and insect hosts. Plant colonization is a process that has received more attention, and is therefore better understood from both biological and mechanistic perspectives (see 4 for a review). Bacterial colonization of plants is based on multiplication within xylem vessels and movement through the xylem network via pit membranes. Vectors of *X. fastidiosa* are xylem sap-sucking insects such as sharpshooter leafhoppers (Hemiptera, Cicadellidae) and spittlebugs (Hemiptera, Cercopidae) [2]. Insect colonization is more poorly studied, but the current hypothesis proposes that *X. fastidiosa* forms a biofilm on the cuticular surface of the foregut of vectors, much like other biofilm-forming bacteria [13]. In addition, there are two other

essential steps to the biology of *X. fastidiosa*. First, the bacterium must be able to initiate an insect colonization event when acquired from an infected plant, a process that is mediated by cell-surface adhesins [14]. Second, once inhabiting a vector it must be dislodged from the cuticle and be inoculated into a susceptible host plant. The processes involved in this step are yet to be determined.

Xylella fastidiosa subspecies

The species *X. fastidiosa* has a very broad host range, one study estimated that species in 29 plant families are susceptible to colonization [10]. However, colonization does not equal disease development. In fact, most plant species that are capable of sustaining a *X. fastidiosa* infection are asymptomatic hosts [24]. In addition, *X. fastidiosa* phylogenetic clusters are largely host specific, meaning they cause disease in one or few host plants, while being able to colonize many asymptomatically [e.g. 1]. Therefore, although at the species level this bacterium has a very large number of hosts, at finer levels of phylogenetic resolution its range is very limited. It should be mentioned that, up to now, even the most divergent *X. fastidiosa* isolates share more than 97% 16S rRNA gene homology, suggesting that they belong to the same bacterial species.

The species is subdivided into four subspecies based on DNA:DNA hybridization [31] and multi-locus sequence typing [MLST, 30]. The subspecies are *fastidiosa*, *multiplex*, *pauca*, and *sandyi*. A fifth subspecies has been proposed [26], but its placement needs to be accurately determined with a larger number of loci or other approaches [21]. Lastly, the cluster that causes pear leaf scorch in Taiwan, representing the most divergent *X. fastidiosa* genotype known so far, will eventually be classified as another subspecies [33]. The subdivision at the subspecies level is not only of taxonomical importance. These groups represent phylogenetically robust clusters that are also phenotypically similar to each other. Similarities include observations *in vitro*, but more important are host associations. Isolates belonging to ssp. *fastidiosa* are primarily associated with grapevines, ssp. *multiplex* with almonds, oaks, and other trees, ssp. *pauca* with citrus and coffee, and ssp. *sandyi* with oleander.

Currently, the best method to determine the phylogenetic placement of novel isolates is MLST [21]. This method uses seven house-keeping loci spread through the genome of *X. fastidiosa*, which are all incorporated into the analysis. The main benefit of this approach is that all sequence data for type isolates is freely available at a database (http://pubmlst.org/xfastidiosa/), which currently contains 250 isolates (as of

June 2013). Most of the known *X. fastidiosa* genetic diversity is already represented in this database, so that phylogenetic coverage is adequate in most instances. Furthermore, there is no need to develop new typing tools, or request isolates or DNA for comparison purposes. In other words, once seven loci are sequenced (~500 bp each), one uses a database to infer its placement within the species. In addition to practical advantages, the use of a multi-locus approach is of relevance because *X. fastidiosa* is naturally competent for transformation [16], meaning that the sequence of a single gene does not necessarily reflect the phylogeny of the genome. Homologous recombination events have been detected between subspecies and may lead to incorrect inferences if loci used for tree-building have recombined [3, 20, 22].

The four subspecies likely evolved in geographic isolation, with ssp. *fastidiosa* having its center of origin in Central America, ssp. *multiplex* and ssp. *pauca* in North and South America respectively. Subspecies *sandyi* has only been found in the USA infecting oleander (*Nerium oleander*). However, evidence indicates recent anthropogenic movement of this bacterium, probably via contaminated plant material. Pierce's disease of grapevines in North America is caused by ssp. *fastidiosa* isolates, but those appear to have originated in Central America [19]. A very similar case has recently been reported from Taiwan [34], in which Pierce's disease isolates group within ssp. *fastidiosa*. Isolates of plum leaf scald in Brazil belong to ssp. *multiplex* [18]; because it is the only case of ssp. *multiplex* in South America, the assumption is that it was introduced. In the case of ssp. *pauca* and *multiplex*, there is evidence of between-subspecies recombination, raising the interesting possibility that horizontal gene transfer of novel alleles may lead to the emergence of new *X. fastidiosa* diseases [20].

The level of genetic diversity within subspecies is variable. For example, ssp. *sandyi* has little diversity and has only been found in oleander, while ssp. *multiplex* is subdivided into several clusters that colonize a wide range of tree species. The other two subspecies are also capable of causing disease in multiple host plant species, but in the case of ssp. *pauca* there are at least two genetic clusters, one which infects citrus and another coffee [3]. In general there is concordance between phylogenetic clustering and host plant association when MLST is used as a diagnostic tool.

Diversity at the population level

The expectation that different genetic markers would be useful at different levels of resolution has also been supported for *X. fastidiosa* [3, 19]. DNA sequence-based

markers are useful at the species and subspecies levels, while fast-evolving markers such as tandem repeat regions are more useful at the population level. Although there is substantial genetic diversity within phylogenetic clusters of host-specific *X. fastidiosa*, few studies have addressed ecological questions other than estimating the degree of genetic diversity with unstructured populations. Therefore, little is known about *X. fastidiosa* populations.

A series of studies analyzed the spatial structure of citrus variegated chlorosis-causing *X. fastidiosa* in the state of São Paulo, Brazil. It was found that host plant variety did not affect genetic structure, and that populations were geographically isolated [5, 6]. In addition, a significant degree of clonality was observed in populations. Another study considered the structure of populations at a smaller spatial scale, by analyzing the ecology of *X. fastidiosa* isolates causing Pierce's disease of grapevines in Napa Valley, USA [7]. In this case, vineyards within ~10 km of each other were found to be infected with a highly diverse pathogen population. In addition to, essentially, a lack of clonality and high genetic diversity, isolates could not be genetically assigned to vineyards where they were collected, suggesting high rates of among-vineyard dispersal of genotypes.

Horizontal gene transfer

Similarly to other bacteria, a range of processes leads to genetic diversity in *X. fastidiosa*. Horizontal gene transfer via transduction and transformation appears to be very important in *X. fastidiosa*. There is high diversity among prophage regions in *X. fastidiosa*, suggesting that bacteriophage infections are common in this pathogen [36], even though so far only one phage has been described for this bacterium [35]. MLST studies also suggested that homologous recombination was responsible for allele diversity, potentially at higher rates than point mutations [30]. Because homologous recombination rates have been reported to be larger than mutation rates for *X. fastidiosa in vitro* [16], it is possible recombination events are common in field populations. However, because recombination between identical sequences are undetectable, it is possible that its rate has been underestimated.

SUMMARY

The bacterium *Xylella fastidiosa* is a genetically and phenotypically diverse plant pathogen. Although considered to have broad host range, it is becoming increasingly

evident that there is a high degree of phylogenetic cluster host specificity, and that *X. fastidiosa* infections of most plant species do not lead to disease symptoms. Therefore, it is important to understand the diversity of this pathogen, so that inferences of applied relevance such as host range and accurate detection protocols can be made in a robust manner. Recent advances in our knowledge about the genetic diversity of *X. fastidiosa* have not been followed by efforts to understand its phenotypically diversity, representing and important gap in information about the biology of this bacterium. Integrating all aspects of *X. fastidiosa* diversity is also relevant because it may provide insights into its evolutionary biology, and consequently processes leading to new and emerging diseases.

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Xylella fastidiosa-Elicited Leaf Scorch Diseases in Taiwan

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ABSTRACT

Xylella fastdiosa is a gram negative, nonflagellate, nutritionally fastidious bacterium that resides only in xylem tissues and requires specific and enriched media for *in vitro* growth. Two *X. fastidiosa*-elicited leaf scorch diseases, pear leaf scorch (PLS) and Pierce's disease (PD), were found in Taiwan in 1991 and 2002. Phylogenetic analyses using 16S rRNA gene and 16S-23S internal transcribed spacer sequence (ITS) revealed that the Taiwan PLS and PD strains may have been independently introduced into and evolved in the host plants. The completion of PLS genome sequences will provide valuable insights into the genome organization and genetic components that diversify PLS bacteria from the other *X. fastidiosa* strains.

Keywords: Pierce's disease, pear leaf scorch disease, shotgun sequencing, genome comparison

The world-wide occurrence of *X. fastidiosa*-induced plant diseases

Xylella fastidiosa is a gram-negative, xylem-limited fastidious bacterium [(13)] that causes numerous scorching, scalding, and stunting diseases worldwide [(4)], and its diverse host range makes the bacterium a serious threat to agricultural activities. Economically important diseases caused by the bacterium include citrus variegated chlorosis (CVC), Pierce's disease of grape (PD), phony peach disease, alfalfa dwarf, periwinkle wilt, and bacterial leaf scorch of almond, coffee, plum, pear, mulberry, elm, oak, sycamore, maple, oleander, pecan, and landscape plants [(4, 8)]. In nature, the bacterium is acquired and transmitted by sharpshooter leafhoppers (*Hemiptera*: Cicadellidae) and spittlebugs (*Hemiptera*: Cercopidae) [(1)] by forming a biofilm of polar-attached cells inside the foreguts of vectors [(6)]. The bacterium can multiply in the

foreguts and be persistently transmitted by adult vectors, and very few live bacteria are required for transmission (for a review, see (1) and references therein).

X. fastidiosa-induced plant diseases are generally found in the region between 15-45 degrees latitude of both north and south of Equator, predominately in North and South Americas. In the US, they occur in the whole southeastern States along the Gulf coast of Mexico, and California. In the southern hemisphere, the diseases occur in Brazil, Argentina, and Paraguay. There is only one report of Pierce's disease of grapevine in Kosovo, the former Yugoslavia, in southern Europe. In 1991, *X. fastidiosa*-induced pear leaf scorch (PLS) disease was found in low altitude areas (below 800 m) in Taiwan where the low-chilling pear cultivar Hengshan (*Pyrus pyrifolia*) was grown, which was the first *X. fastidiosa*-induced plant diseases in Asian Continent [(5)]. Leaf scorch symptoms were observed in early July, 6 months after the sprouting of dormant buds. Accompanying with the scorching symptoms on the leaf margin, early defoliation, declining of tree vigor, dieback and wilting, and significant yield losses in severe cases, pear leaf scorch disease has been a major limiting factor of the pear industry in Taiwan [(9)]. In 2002, another leaf scorch disorder, Pierce's disease (PD) of grapevines, was found in the major grape production areas (Taichung City, Miaoli and Nantou counties) in central Taiwan [(12)]. Scorch symptoms on grapevine leaves were observed at the onset of berry ripening (veraison phase) in late May to early June. Necrotic tissue with yellow or burgundy margins was developed at the edge of the symptomatic leaves and became coalesced in later stage, followed by the systematic development of scorch symptoms in upper and lower leaves. Severely affected leaves became fully necrotic and dropped early, showing matchstick-like petioles attached to the cane. Affected twigs and branches of the grapevines declined and dieback in 1 to 5 years post infection.

The relatedness of Taiwan PD and PLS bacteria with the other *X. fastidiosa* strains

To characterize the genetic relatedness of the *X. fastidiosa* strains isolated in Taiwan, PCR-based DNA amplification and phylogenetic analyses were applied. DNA fingerprinting patterns amplified by arbitrary primers show that PLS strains are genetically distinct from *X. fastidiosa* strains isolated from other host plants [(10)]. Phylogenetic analyses using 16S rRNA gene and 16S-23S internal transcribed spacer sequence (ITS) reveal that the Taiwan PD strains are grouped together with the other

PD strains collected from North and South Americas that belonged to *X. fastidiosa* subsp. *fastidiosa* [8], whereas the PLS strains are distantly related to the PD strains and the other *X. fastidiosa* strains collected from different plant species [11, 12], suggesting the Taiwan *X. fastidiosa* bacteria that infects grapevines and Asian pear might have different origins.

Genome-wide comparison of *X. fastidiosa*

Microarray-based comparisons using strain 9a5c (CVC strain) as a reference genome to be hybridized with 11 *X. fastidiosa* strains reveal that these bacteria display a large set of flexible genes, with several horizontally transferred elements contributing up to 18% of the total genome [7]. Whole genome sequences of *X. fastidiosa* strains, including 5 complete genomic sequences of 9a5c (Citrus variegated chlorosis), Temecula 1 and GB514 (Pierce's disease of grapevine), M12 and M23 (almond leaf scorch), and 2 draft sequences of Dixon (almond leaf scorch disease) and Ann1 (oleander leaf scorch disease), are available (http://www. ncbi.nlm.nih.gov/genome/genomes/173). Comparative analyses of these genome sequences show that 76.2% of the genome sequences are conserved among *X. fastidiosa* strains and identified significant variations among elements coding for additional functions that are not essential for bacterial growth [3]. The overall genomic diversity observed among these *X. fastidiosa* strains provides evidence that different *X. fastidiosa* strains might carry unique genetic factors for adaptability and host specificity.

Analyses of the draft sequences of PLS bacteria

Genomic DNAs of *X. fastidiosa* strains PLS235 and PLS244 were extracted from pure culture in PD2 medium. The random shotgun method was used for genome sequencing, and sequences were de-novo assembled with MIRA assembler and annotated in the web-based RAST server [2]. Nucleotide comparison showed that PLSB whole-genome sequences share approximate 78% similarity with the other *X. fastidiosa* bacteria, suggesting that PLS strains might be a new subspecies of *X. fastidiosa*. Assembly and annotation of PLS235 draft sequences revealed an estimate genome size of 2.99 Mb putatively coding for 3,196 genes. Among the predicted genes, 1,714 genes involved in metabolism, protein synthesis, cell cycle and other house-keeping activities are conserved between PLS235 and *X. fastidiosa* strain 9a5c. INDELs and strain specific genes identified in PLS235 genome are the main source of

variations to differentiate the genetic compositions of *X. fastidiosa* strains, which is similar to the conclusion derived from the genome comparison of *X. fastidiosa* strains Temecula 1, Ann1, Dixon, and 9a5c [(3)]. Sequence analysis of the PLS235 genomic contigs also identified the association of repeat sequences with hypothetical and phage related functions, and many of them are unique to the PLS genome, suggesting horizontally transferred genes may drive the evolution of *X. fastidiosa* genomes toward metabolically compromised and host-specific strains.

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Taxonomy and Biology of Egg Parasitoids of Auchenorrhyncha of Economic Importance in Taiwan and Adjacent Countries, and of Proconiine Sharpshooters in the New World

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ABSTRACT

An overview of the taxonomic and biological studies on the egg parasitoids (Hymenoptera: Mymaridae and Trichogrammatidae) of Auchenorrhyncha (Hemiptera) of economic importance in Taiwan (Republic of China) and adjacent countries is given, and their current status is discussed. Also provided is a summary of taxonomy and biology of the hymenopteran egg parasitoids of various Proconiini (Hemiptera: Cicadellidae: Cicadellinae), particularly the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar), in the New World, with references to its biological control in the non-native range.

Keywords: Cicadellidae, Delphacidae, Proconiini, *Homalodisca vitripennis*, vector, Aphelinidae, Mymaridae, Trichogrammatidae, egg parasitoid, biological control.

INTRODUCTION

Most common egg parasitoids of Auchenorrhyncha (Hemiptera) belong to two families of Hymenoptera, Mymaridae and Trichogrammatidae (superfamily Chalcidoidea). Worldwide, they are largely responsible for the natural control of leafhopper (Cicadellidae), planthopper (Delphacidae), and treehopper (Membracidae) species, including economically important pests. Therefore knowledge of their taxonomy (for identification) and biology is very important for biological control, ecological, and biodiversity studies. Provided herein is an overview of the history and current status of the taxonomy and biology of these two groups of egg parasitoids in Taiwan (Republic of

China) and the adjacent countries. Due to the recent identification of the phytopathogenic bacterium *Xylella fastidiosa* in the vineyards of Taiwan [84], which causes Pierce's disease of grapevines (the first such record in Asia), a summary of the known egg parasitoids of the proconiine sharpshooter leafhoppers (its vectors) in the New World is also given. Information on egg parasitoids of leafhoppers from the tribe Cicadellini, also from the subfamily Cicadellinae, is scarce globally. Cicadellini contain several known vector species for *X. fastidiosa* in the USA, where their eggs are parasitized by a few *Gonatocerus* spp. and also a *Polynema* sp. [5, 6, 125]; *Kolla paulula* (Walker) is a potential vector of *X. fastidiosa* [82] and a common cicadellid species in Taiwan [84]. In Japan and elsewhere, *Gonatocerus longicornis* Nees ab Esenbeck and several *Anagrus* spp. are known as egg parasitoids of *Cicadella viridis* (Linnaeus) [21, 107].

Taxonomy and biology of egg parasitoids of Auchenorrhyncha of economic importance in Taiwan and adjacent countries

Biogeographically, the fauna of Taiwan is primarily Oriental, although at high altitudes it arguably fits more in the Palaearctic ecozone, with some Himalayan elements. The faunas of the adjacent countries are either within the Oriental (the Philippines) or Palaearctic (Republic of Korea), or both (People's Republic of China and Japan) ecozones, although most of the latter country, except for the southern Ryukyu Islands, has a Palaearctic fauna as does the more remote Russian Far East. The entire region also has a number of cosmopolitan or Old World taxa, some transpacific elements (genera and species) that occur from Australasia (Queensland) to the eastern Palaearctic (Russian Far East and northern Japan), endemic, relict, and possibly a few unintentionally introduced species. Historically, dealing with such diversity has been challenging for taxonomists of parasitic Hymenoptera, particularly due to the fact that many species of egg parasitoids of Auchenorrhyncha, including economically important ones, are often widespread in distribution. A "single country" focal approach (rather than regional or hemisphere, or global) to parasitoid taxonomy thus has been particularly problematic and detrimental for these groups of insects: to be able to correctly identify a parasitoid from one country in east and southeast Asia a taxonomist needs to have knowledge, at a minimum, of the congeneric taxa from the northern Australasian, Oriental, and Palaearctic regions. That includes availability of comparative, identified material and access to type specimens, scientific literature, etc. While generic identifications of most Mymaridae and Trichogrammatidae in the region

are generally relatively easily available, species identifications are still a major problem [96]. Expensive and time-consuming special preservation and mounting techniques, such as microscopic slides in Canada balsam, are usually required to be able to identify any mymarid or trichogrammatid to species based on morphology. Overall, taxonomic studies on the diversity of egg parasitoids lag significantly compared to those of leafhoppers and other Auchenorrhyncha; the latter have seen considerable progress recently, especially in the People's Republic of China. However, unlike their taxonomy, the natural history of most leafhoppers and other groups is still poorly known, besides a few species that are agricultural pests, primarily of rice. Despite numerous publications on mymarid and trichogrammatid egg parasitoids of the rice leafhoppers and planthoppers in Asia (reviewed by Gurr *et al.* [28] for the planthopper hosts), their identifications, particularly those in the fairyfly genera *Anagrus* Haliday and *Gonatocerus* Nees ab Esenbeck and trichogrammatid genera *Oligosita* Walker and *Paracentrobia* Howard, are generally unreliable and many, particularly pre-2000s, are incorrect or outdated. *Aprostocetus* (*Ootetrastichus*) spp. (Eulophidae) are not covered in this review because they act more as egg predators than parasitoids of various Auchenorrhyncha.

Keys and taxonomic revisions of regional importance.

These are unfortunately few, but can be helpful to recognize genera: Subba Rao and Hayat [85] and Triapitsyn and Huber [116] for Mymaridae, and Doutt and Viggiani [22] for Trichogrammatidae. For relevant records and recognition of species in some genera of Mymaridae: Chiappini *et al.* [19], Triapitsyn [93], Triapitsyn and Beardsley [103], and Triapitsyn and Berezovskiy [107] for *Anagrus*; Triapitsyn [100] for *Ooctonus* Haliday; Triapitsyn [102] for *Gonatocerus*; Triapitsyn and Berezovskiy [104] for *Mymar* Curtis; Triapitsyn and Berezovskiy [109] for *Acmopolynema* Ogloblin, with the description of *A. orchidea* Triapitsyn and Berezovskiy from Orchid Island (Lan Yü), Taiwan; Huber [34] for *Chaetomymar* Ogloblin (a synonym of *Palaeoneura* Waterhouse); Huber and Fidalgo [35] for *Stephanodes* Enock. Owen [72] revised the world species of *Ufens* Girault (Trichogrammatidae).

Taxonomic and biological studies on egg parasitoids of Auchenorrhyncha, listed by country. TAIWAN (REPUBLIC OF CHINA): Taguchi [87, 88, 89, 90] published on taxonomy of *Himopolynema* Taguchi, *Mymar*, *Palaeoneura*, and *Stephanodes*,

which contain descriptions of several new species. Unfortunately, the entire Hidenari Taguchi collection (originally in Ehime University, Matsuyama, Japan) of Mymaridae is lost, including the types. Lin [43], Miura *et al.* [64], and Chen [16] reported on egg parasitoids of the rice leafhoppers and planthoppers; Chen and Yu [17] studied *Anagrus* egg parasitoids of brown rice planthopper, *Nilaparvata lugens* (Stål) (Delphacidae); Chu and Hirashima [20] summarized the earlier Taiwanese literature on the natural enemies of rice leafhoppers and planthoppers. PEOPLE'S REPUBLIC OF CHINA: Lin and Xu [48] keyed the genera of Mymaridae; Lin [44, 46] and Guo *et al.* [27] reported on the classification of Mymaridae and Trichogrammatidae and their use in biological control; Pang and Wang [73] and Chiappini and Lin [18] reviewed the Chinese species of *Anagrus*; Xu and Lin [128] published on *Acmopolynema*; Zhang *et al.* [130] reported on *Stethynium* Enock. A taxonomic revision of the Chinese Trichogrammatidae by Lin [45] contains keys to the species of the genera that are known as egg parasitoids of Auchenorrhyncha; also important are studies by Hu and Lin [30, 31, 32] and Hu *et al.* [33] on several trichogrammatid genera. Publications on egg parasitoids of the rice leafhoppers and planthoppers, mainly on *Anagrus* spp., in mainland China are so numerous that it is impossible to list them here; those on egg parasitoids of Delphacidae were reviewed by Gurr *et al.* [28]. Mao *et al.* [54] reported on egg parasitoids of *Empoasca vitis* (Goethe); *Hispidophila sophoniae* Lin and Lin and *Ufens rimatus* Lin (Trichogrammatidae) were among the complex of 11 species of mymarid, trichogrammatid, and aphelinid egg parasitoids of *Sophonia* spp. (Cicadellidae) in southern China [47, 55]. JAPAN: Doutt [21] published on some egg parasitoids of leafhoppers; Taguchi [86, 87, 88, 89, 90] on *Acmopolynema*, *Himopolynema*, *Mymar*, *Palaeoneura*, *Polynema* Haliday, and *Stephanodes*; Sahad [79] and Sahad and Hirashima [81] on *Anagrus* and *Gonatocerus*. *Himopolynema hishimonus* Taguchi is a parasitoid of *Hishimonus sellatus* (Uhler) (Cicadellidae) [89]. *Mymar taprobanicum* Ward was reported from an egg of *Delphacodes striatella* (Fallén) (Delphacidae) [87]. Chantarasa-ard *et al.* [13, 14, 15], Miura [56, 57, 58, 59], Miura and Miura [60, 61], Miura and Yano [62, 63], Otake [67, 68, 69, 70, 71], Sahad [77, 78, 80], and Sahad and Hirashima [81] studied biology of *Anagrus* spp. and *Gonatocerus* spp. as well as that of *Paracentrobia andoi* (Ishii) (Trichogrammatidae). REPUBLIC OF KOREA: Chang [11], Kim [39], Kim *et al.* [40], Yeo *et al.* [129], and Chang *et al.* [12] reported on *Anagrus* spp. and *P. andoi* egg parasitoids of the rice leafhoppers and planthoppers. PHILIPPINES: Chandra [8, 9], Chandra and Dyck [10], and Tran [92] studied *Anagrus* parasitoids of the rice leafhoppers

and planthoppers. RUSSIA: in the Russian Far East, taxonomic studies on Mymaridae were conducted by Berezovskiy and Triapitsyn [2], Triapitsyn [97], and Triapitsyn and Berezovskiy [104, 105, 106, 108], and on Trichogrammatidae by Fursov [23].

Looking ahead. There are several taxonomic challenges that can be identified as priority projects in the region for future studies, as follows: 1) Figuring out the true taxonomic identity of *Anagrus nilaparvatae* Pang and Wang, a common and economically important egg parasitoid of *N. lugens* and other rice pests in the Oriental and eastern Palaearctic regions. As noted by Triapitsyn and Berezovskiy [107], *A. nilaparvatae* is morphologically practically indistinguishable from the common, widespread, polyphagous Palaearctic species *A. incarnatus* Haliday. Collections of reared specimens of both taxa should be made from various hosts and locations throughout their ranges, including the type localities, and a combination of morphometric and molecular studies, and also of cross-breeding experiments, should be considered to solve this problem. 2) Taxonomy of other egg parasitoids (Mymaridae and Trichogrammatidae) of the rice leafhoppers and planthoppers in Asia needs to be revised including the use of molecular methods to distinguish cryptic species. 3) Taxonomic revisions of the most important genera which contain known egg parasitoids of various Auchenorrhyncha, for the entire Oriental and adjacent regions: *Anagrus*, *Gonatocerus* (Oriental and Australasian regions), *Himopolynema*, and *Polynema* (Mymaridae), as well as *Aphelinoidea* Girault, *Oligosita*, *Paracentrobia*, and *Pseudoligosita* Girault (Trichogrammatidae). Such revisions would be particularly useful for identifications of the natural enemies of leafhopper, planthopper, and other agricultural pests. 4) Establishing host-parasitoid associations for the most common and economically important species of leafhoppers (particularly from the tribe Cicadellini), planthoppers, and other Auchenorrhyncha, based on thorough rearings (including using sentinel eggs) and taxonomic identifications of both the host and parasitoid(s). The recent, notable advances in leafhopper taxonomy (but not biology, knowledge of which is often lacking), particularly in People's Republic of China, would facilitate such studies.

Taxonomy and biology of egg parasitoids of Proconiini in the New World and beyond

Proconiine sharpshooters belong to the New World cicadelline leafhopper tribe Proconiini (Hemiptera: Cicadellidae: Cicadellinae). The most notorious is the

glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar), a vector of plant diseases caused by the phytopathogenic bacterium *Xylella fastidiosa* [3]. The glassy-winged sharpshooter is a self-introduced pest in California from the southeastern USA. Turner and Pollard [125] provided a brief overview of proconiine sharpshooter egg parasitoids (Mymaridae and Trichogrammatidae) in Georgia. For a long time this was the only available publication on this group of natural enemies, which more recently has been shown to be amazingly diverse; almost every sharpshooter species has been found, upon closer examination, to have a complex of associated egg parasitoids. Establishment of *H. vitripennis* in California in the 1990s, later in Hawaii, USA and French Polynesia, and even more recently in Easter Island (Chile) [76] prompted interest in proconiine sharpshooter investigations, including studies of their egg parasitoids in North America [111, 115, 120, 122, 123], mainly for classical biological control purposes [65, 66, 75, 76, 113, 114]. An overview of the genera in several families of Chalcidoidea (Hymenoptera) containing known egg parasitoids of Proconiini follows.

APHELINIDAE

Centrodora Förster: Species of this genus appear to be more polyphagous egg parasitoids of some Hemiptera and are very difficult to identify. The two species reared from eggs of Proconiini remain undetermined: one *Centrodora* sp. from Tahiti and Moorea Islands, French Polynesia is a parasitoid of the non-native *H. vitripennis* [26]; the other *Centrodora* sp. was reared in Argentina from eggs of *Tapajosa rubromarginata* (Signoret) and *Tretogonia notatifrons* Melichar (G. A. Logarzo and S. V. Triapitsyn, unpublished).

MYMARIDAE

Acmopolynema: One species of this genus, *A. sema* Schauff, was reared in Florida and Georgia, USA, from eggs of *Homalodisca insolita* (Walker) [115].

Anagrus: Four species of this genus have been recorded as egg parasitoids of Proconiini: *A. epos* Girault in Minnesota, USA, a gregarious parasitoid of *Cuerna fenestella* Hamilton [29, 99], which was mass-reared and released without much success in California, USA against *H. vitripennis* [7, 42], where its host specificity and other biological traits were studied by Krugner *et al.* [41]; *A. stethynioides* Triapitsyn, an occasional and poorly known parasitoid of *H. vitripennis* in Texas, USA [99], which is also known from several countries in Central and South America [95]; *A. breviphragma*

Soyka from *Dechacona missionum* (Berg) in Argentina [51]; and an *Anagrus* sp., which was reared in Tahiti Island, French Polynesia, from an egg mass of the invasive *H. vitripennis* [26].

Gonatocerus: This large and common genus contains many species that are known as egg parasitoids of various Proconiini, including those used in the biological control program against *H. vitripennis* in California [7], as summarized by Triapitsyn [99] for the Nearctic region and Triapitsyn *et al.* [117] for the Neotropical region. Details on their diversity, taxonomy, host associations and other biological traits are therefore omitted for brevity: these are readily available and too numerous to fit in this communication. All are solitary egg parasitoids, producing one wasp per host egg, except for *G. fasciatus* Girault which is a gregarious parasitoid [121]. Biology of several species of *Gonatocerus* was thoroughly studied [36, 37, 38, 83, 126]. They do not appear to be too host specific but rather are usually able to attack eggs of at least several genera and species of Proconiini [4, 49, 53]. All of them belong to the subgenus *G.* (*Cosmocomoidea* Howard), which also contains a large number of undescribed species from Central and South America which are likely egg parasitoids of Proconiini [94, 117], thus suggesting apparent co-evolution. Triapitsyn [101] reported the following species of *Gonatocerus* from eggs of *H. vitripennis* in California: *G. ashmeadi* Girault, *G. fasciatus* (intentionally introduced), *G. incomptus* Huber, *G. morgani* Triapitsyn, *G. morrilli* (Howard) (intentionally introduced), *G. novifasciatus* Girault, *G. triguttatus* Girault (intentionally introduced), and *G. walkerjonesi* Triapitsyn. The southern and southeastern USA strains of *G. ashmeadi* were released in California against *H. vitripennis* [65, 76]. *Gonatocerus ashmeadi* was found self-introduced in Oahu Island, Hawaii (USA), where it provides a good control of *H. vitripennis* [99]; it was intentionally and successfully introduced into French Polynesia for very effective biological control against *H. vitripennis* [24, 25]. It was also found self-introduced in Easter Island [99]. Paradell *et al.* [74] summarized the known egg parasitoids of Proconiini in Argentina, the majority of which are *Gonatocerus* species described or revised taxonomically by Triapitsyn *et al.* [117, 118, 119, 124]; *T. rubromarginata* is the most thoroughly studied host of many of these parasitoids.

Palaeoneura: One undetermined species of this genus was reared in Tahiti Island from eggs of *H. vitripennis* [26].

Polynema: An undescribed species of *P.* (*Doriclytus*) sp. was reared in California, USA from eggs of *H. vitripennis*; it is only an occasional parasitoid of this host [101].

One or two other *P.* (*Doriclytus*) spp. were reared in Argentina from sentinel eggs of *T. rubromarginata* (G. A. Logarzo and S. V. Triapitsyn, unpublished).

TRICHOGRAMMATIDAE

Members of several genera are gregarious egg parasitoids of various Proconiini, all native to the New World [98]; trichogrammatids are generally relatively more common on grassy vegetation and in dry habitats.

Burksiella De Santis: *Burksiella spirita* (Girault) is a common egg parasitoid of *H. vitripennis* and also of *Oncometopia orbona* (Fabricius) in the southeastern USA [98, 101]; a related form, which may or may not belong to this species, was reared in Montana, USA, from eggs of *Cuerna sayi* Nielson [112]. One or two different, undetermined species of *Burksiella* spp. were reported from eggs of *Homalodisca liturata* Ball in Mexico [110]. *Burksiella platensis* (De Santis) is known from eggs of *T. rubromarginata* in Argentina [98].

Ittys Girault: One undescribed species of this genus was reported from eggs of *H. liturata* in Mexico [110].

Oligosita: An *Oligosita* sp., possibly *O. americana* Girault, was reared in Georgia, USA from eggs of *Homalodisca insolita* (Walker) [98, 125]. Undetermined *Oligosita* spp. were also reared from eggs of two species of Proconiini in Argentina [74]; however, at least one of these is a member of *Pseudoligosita* [110].

Paracentrobia: *Paracentrobia acuminata* (Ashmead) was reared in Florida and Georgia, USA from eggs of *Cuerna costalis* (Fabricius), *H. insolita,* and *H. vitripennis* [91, 98, 125]. Also *P. americana* (Girault) is a parasitoid of *H. insolita* in Florida [91]. In Argentina, *P. tapajosae* Viggiani is known from eggs of *T. rubromarginata* [127], who studied biology of the parasitoid. Undetermined *Paracentrobia* spp. were also reared from eggs of three other species of Proconiini in Argentina [74].

Pseudoligosita: Two species of this genus were reared from eggs of Proconiini: *P. plebeia* (Perkins) from *H. liturata* in Mexico [101, 110] and also from eggs of *T. rubromarginata* in Argentina [110]; and an undetermined and apparently undescribed *Pseudoligosita* sp. from eggs of *H. vitripennis* in California [101]. Biological traits of *P. plebeia*, which was reared under quarantine conditions in California on eggs of *H. vitripennis*, were reported by Triapitsyn and Bernal [110] and Lytle *et al.* [52].

Ufens: *Ufens ceratus* Owen and *U. principalis* Owen are common egg parasitoids of *H. liturata* and *H. vitripennis* in Mexico and USA [1, 101, 110], and *U.*

ceratus is also known from *Oncometopia clarior* (Walker) in Mexico [111] (as *Ufens* sp.). *Ufens niger* (Ashmead) was reported from eggs of *C. costalis* and *Homalodisca* sp. [98, 125].

Zagella Girault: The sharpshooter *T. rubromarginata* is a known host of *Z. delicata* De Santis, the biology of which was reported by Logarzo *et al.* [50]. Undetermined *Zagella* spp. were also reared from eggs of three other species of Proconiini in Argentina [74].

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The occurrence of Pierce's disease of grapevines and its control strategies in Taiwan

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ABSTRACT

PD of grapes caused by *Xylella fastidiosa,* a xylem-limited bacterium, has been listed as one of the international quarantine plant diseases. From 2002, the Bureau of Animal and Plant Health Inspection and Quarantine launched a survey project to detect PD in Taiwan. So far, a total of 13,666 grapevines were confirmed to be infected by *X. fastidiosa* via direct isolations and PCR protocols. Other than grapes, the following four plants were confirmed to be the alternative hosts of PD in Taiwan: *Diplocyclos palmatus* (L.) C. Jeffrey, *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder., *Humulus scandens* (Lour.) Merr., and *Mallotus paniculatus* (Lam.) Muell.-Arg. The 16S rRNA sequences of PD strains isolated from tissues of grapevines exhibiting PD symptoms collected from 5 counties and 4 alternative hosts were compared with *X. fastidiosa* from other hosts. The phylogenetic trees constructed with neighbour-joining method revealed that *X. fastidiosa* strains from different hosts could be divided into five subgroups and the PD strains from Taiwan were grouped with grapevine and mulberry strains from the Americas. The control strategies recommended for limiting the spread of PD are as follows: (1) to plant the healthy seedlings and eradicate the PD-like grapevines immediately (2) to eradicate the alternative hosts and the weeds that insect vectors favor (3) to control the population of indigenous insect vectors and avoid the invasion of foreign insect vectors.

Keywords: *Xylella fastidiosa*, Pierce's disease of grape, alternative hosts, phylogenetic analysis

INTRODUCTION

Pierce's disease (PD) of grapes, caused by *Xylella fastidiosa*, a xylem-limited bacterium, was described in California in 1892 [14] as Anaheim disease of gapes. The bacterium inhabits only in xylem tissues and requires specific and enriched media for *in vitro* growth [40]. All strains of *X. fastidiosa* are classified as one species but infect different host range. Besides PD, *X. fastidiosa* also causing diseases on citrus [3, 16], pear [19], elm [11], almond [22], oak [4], plum [27], peach [39] and mulberry [18]. Ever since Wells et al.[40] named then xylem-limited bacterium as *X. fastidiosa* in 1987, *X. fastidiosa* has been reclassified into five subspecies according to their differences in genetic makeup, host range, physiology, and biochemistry. They are *X. fastidiosa* subsp. *fastidiosa* for strains of grape, almond, alfalfa, and maple, *X. fastidiosa* subsp. *multiplex* for strains of peach, plum, almond, elm, sycamore, and pigeon grape, *X. fastidiosa* subsp. *pauca* for strains of citrus [31], *X. fastidiosa* subsp. *sandyi* for strains of oleander, daylily, jacaranda, and magnolia [32], and *X. fastidiosa* subsp. *tashke* for strains of *Chitalpa tashkentensis*, a common ornamental landscape plant [26]. However, the last two subspecies have not been officially recognized by the researchers in the community of systematic bacteriology.

Distribution of PD

PD was reported from countries in the Americas including Mexico and the United States in North America, Costa Rica [1] and Venezuela [15] in Central America and Caribbean, and Argentina and Peru [24] in South America. In 1998, Kosovo, former Yugoslavia [2], was the only country that reported a PD incidence in European Continent. In Asian Continent, description of PD-like disorder from China was reported in 2001[7], and PD incidence was confirmed in Taiwan in 2002 [36]. In the southeastern United States, PD is endemic and plays an important role in limiting the development of winery industry. During the period from1994 to 2000, PD destroyed more than 1000 acres of vineyards and caused losses of $30 million in northern California.

The pathogen of PD is *X. fastidiosa*, a gram-negative, non-flagellate, nutritionally fastidious, rod-shaped with rippled cell walls. The cell size was measured 0.2–0.4 μm

in width and 1–3 μm in length (Fig. 1). *X. fastidiosa* only resided in the xylem tissue and the optimal temperature for growth was 26-28℃ and the optimal pH was 6.5-6.9. *X. fastidiosa* grows slowly even in specific and enriched media. At 12-day post-subculture, the opalescent circular colonies reached 1 mm in diameter and were convex with smooth margins in morphology.

Diseased grapevines usually begin with scorch symptoms, necrotic tissue with yellow margins, and followed by the systemic development of leaf scorch symptoms in upper and lower leaves (Fig. 2) [12]. Severely affected grapevines drop leaves early, leaving petioles remain attached to the canes, decline in vigor, followed by stunting, dieback and eventual death in 2-4 years after initial infection [34, 38].

The primary goal of this report was to describe the occurrence of PD and the potential alternative hosts in Taiwan. Moreover, the epidemiological information of PD would be drawn to develop control strategies to reduce the risk of PD in Taiwan.

The occurrence of PD in Taiwan

Table grapes and winemaking grapes were both cultivated in central Taiwan, including Miaoli County, Changhua County, Nantou County, Taichung City. Table grapes including Kyoho, Italia, Honey red and Himrod seedless are competitive to other imported fruits, and gradually become one of the high economic valued fruits in Taiwan; Golden Muscat, Black queen and Muscat Bailey A are for winemaking. In 2002, the Bureau of Animal and Plant Health Inspection and Quarantine launched a survey project to investigate the occurrence of PD in central Taiwan. From 2003 to 2012, a total of 399 vineyards and 13,666 grapevines, including table grapes and winemaking grapes, were confirmed to be infected by *X. fastidiosa* by performing direct isolation and cultivation of the bacterium and PCR [21, 30]; and hence eradicated except those in Changhua County (Table 1).

The survey result showed the morbidity of vineyards conducted from 2002 to 2012 is 19% in Caoton Township, 57% in Zhushan Township, 69% in Waipu District, 93% in Houli District, and 87% in Tonxiao Township (Fig. 3). The winemaking grapes mainly cultivated in Waipu District, Houli District, and Tonxiao Township showed higher morbidity than the table grapes cultivated in areas where they were managed accurately and effectively. In the case of the vineyards in Zhushan, the abandoned vineyards might conserve the pathogen of PD in the diseased plant and the complex vegetation. Once, the alternative hosts were eradicated around the vineyard the insect vectors were forced

to migrate to the adjacent vineyards and spread PD.

The geographic distribution of diseased vineyards in Taiwan.

According to the survey results obtained from central Taiwan, the geographic distribution of diseased vineyards can be summarized as two characteristics: (1) The vineyards in Tonxiao Township, Waipu District and Houli District were typically located in hilly terrain; (2) The vineyards adjacent to the river or valley were found in Zhusan Township, Caoton Township, Zhuolan Township and Xinshe District. These mentioned counties were considered as high risk areas for PD, and the diseased vineyards were almost found in the margin of these counties where they were surrounded by undeveloped mixed forest which favors the survival of the pathogenic bacteria and the insect vectors.

The vineyards in Xinyi Township, Shuili Township and Shigang District have been free of PD incidence. However, the geographical characteristics of vineyards in these counties were similar to those mentioned above; hence they were classified as moderate risk area of PD that need constant survey of PD incidence.

On the other hand, Changhua County, where about 1800 hectares of table grapes were cultivated, has been free of PD according to the detection survey and report of Taichung District Agricultural Research and Extension Station, were classified as low risk area. Based on the following assumptions, we think those in Changhua County might avoid PD explosion: (1) the cultivating area were located in plains, and dense planting cultivation were performed; (2) grapevines are fully replaced approximately every seven years; and (3) immediate eradication of PD-like plants to keep the vegetation clean and simple.

Alternative hosts of PD in Taiwan

X. fastidiosa has a broad host range of native plant species, and many of them appear to be symptomless. In California more than 94 species host plants in 28 families are identified as alternative hosts, including *Acacia longifolia*, *Artemesia vulgaris*, *Avena fatua*, *Chenopodium ambrodioides*, *Fuchsia magellanica*, *Hydrangea paniculota*, *Lolium multiflorum*, *Marjorana hortensis*, *Poa annua*, *Rosa California*, *Rosemary officinalis*, *Rubus vitifolius*, *Salix* spp., *Veronica* spp. and *Vitis califonica* [9,28].

From 2003 to 2012, about 5,404 plant samples (Table 2) were collected from the proximity of PD-confirmed vineyards in central Taiwan and they were classified as 251 species in 72 families. So far, the result showed 5 *Diplocyclos palmatus* (L.) C. Jeffrey samples in Taichung City and Miaoli county, 8 *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder. in Taichung City, Nantou county and Miaoli county, 10 *Humulus scandens* (Lour.) Merr. in Taichung City and Miaoli county, and just 1 *Mallotus paniculatus* (Lam.) Muell.-Arg in Taichung City were confirmed to be the alternative hosts of PD strains based on the direct isolation and PCR detection (Fig. 4). The pathogenic bacteria isolated from four alternative hosts were used for artificial inoculation to healthy grapevines by xylem-infiltration method [8, 13]. The symptomatic leaves appeared from the base near the inoculation sites and moved upwards 1 month post-inoculation.

Upon further analysis, it was revealed that there was a geographic correlation between the distribution of alternative hosts and the diseased grapevines in the vineyard which were indirectly confirmed that the indigenous insects were the vectors in Taiwan. In previous reports, 39 species of Cicadellinae and 5 species of Cercopoidea were confirmed as the vectors of different strains of *X. fastidiosa* in the United States and Brazil [29]. Results of extensive surveys revealed that one *Kolla paulula* (Walker), a xylem-feeding leafhopper, was identified as a candidate insect that may transmit *X. fastidiosa* in central Taiwan [33]. Other than *Kolla paulula* (Walker), we have got positive PCR detection from *Bothrogonia ferruginea* (Fabricius) and *Anatkina horishana* (Matsumura). Nevertheless, the transmission protocol by *B. ferruginea* (Fabricius) and *A. horishana* (Matsunura) has not been tested, and it awaits further investigations for the fulfillment of the Koch's postulates.

Phylogenetic analysis of the PD strains from Taiwan and from the Americas and *X. fastidiosa* from other hosts

The 16S rRNA gene [6,16] sequences of PD strains from Taiwan have been deposited in GenBank under the accession numbers indicated in Table 3 and were used as queries for the similarity search. Twenty sequences of Pierce’s disease strains (Table 4) from grapevines and other alternative hosts found in Taiwan were compared with strains from other host plants via multiple sequences alignment by Clustal X program

[17] using *Xanthomonas axonopodis* pv. *citri* strain XCW as an outgroup [5, 10, 36].

The phylogenetic tree was constructed with neighbour-joining method and evaluated by bootstrap analysis for 1000 replicates using MEGA 4 program [37] with the orthologous sequences of XCW as outgroups for phylogenetic analyses [20]. The neighbour-joining trees showed two distinct monophyletic groups of the 31 strains: Group 1 contained 2 PLS strains [35] and group 2 contained the other 29 strains. The strains in group 1 and group 2 were closely related to each other with bootstrap probabilities of 88% and 99% for the 16S rRNA phylogenetic tree. Group 2 can be subdivided into 4 subgroups: GM (grape and mulberry), C (coffee and citrus), PS (peach, pecan, plum and sycamore) and O (oleander) (Fig. 5). The PD strains from grapevines and confirmed alternative hosts in Taiwan were grouped with American PD and mulberry strains that belonged to the subspecies *fastidiosa* [31] which demonstrated that the pathogens might exist in indigenous alternative hosts and from which the indigenous insect vectors spread the disease.

DISCUSSION

Even though the PD of grapes in Taiwan was not reported in a respected journal until this year, it still represents the very first PD in Taiwan as well as in the entire Asian Continent [36]. We did take immediate action from 2002 onward to survey the vineyards using direct isolation and PCR detection and to eradicate diseased grapevines, PD however has not been successfully controlled. In Taiwan, the diseased vineyards were usually in hilly terrains, and therefore it is possible that the vegetation in the vineyards serves as the natural bacterial reservoirs. More than 94 species of plants in 28 families were reported as hosts of PD bacterium in California, and most of them were symptomless hosts. This study represents the first report confirming that *D. palmatus*, *A. brevipedunculata*, *H. scandens* and *M. paniculatus* serve as alternative hosts of *X. fastidiosa* in Taiwan. Because of the availability of the alternative hosts that reserve *X. fastidiosa* in its vegetation, the insect vectors could acquire *X. fastidiosa* at any time and inoculate grapevines. Understanding the stages of *X. fastidiosa* transmission by insect vectors will provide information for PD control in Taiwan.

At last, we should understand thoroughly the epidemiology of PD in order to develop successful control measures. The control strategies include the follows:

(1) Enter Quarantine: The pathogen of PD may be brought in with diseased branches or insect vectors. Therefore, the government must enhance the quarantine of grapevines imported for use as planting material originated from counties where PD occurs and must strongly restrict smuggling.

(2) We should always survey the grapevines for suspicious PD-like symptoms which should be marked up immediately and experts should be contacted for further identification in the high risk area.

(3) Integrated control:

(a) Plant healthy seedlings to prevent the pathogens being brought into the vineyard.

(b) Eradicate the abnormal grapevines immediately.

(c) Monitor the indigenous insect vector population.

(d) Eradicate the confirmed alternative hosts.

(e) Eradicate the weeds that the insect vectors favor.

(f) Pesticides should be applied to the weeds and insects in the vineyards on weekly basis or as recommended. Pesticides with different inhibition mechanisms should be used and changes of pesticides should be done after one type is being used consecutively for 2-3 times.

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Table 1. Survey results of Pierce's disease incidence: total numbers of diseased plants and vineyards in various counties conducted from 2002 to 2012

Country		Diseased grapevine / Diseased vineyard																					
		91 S	91 W	92 S	92 W	93 S	93 W	94 S	94 W	95 S	95 W	96 S	96 W	97 S	97 W	98 S	98 W	99 S	99 W	100 S	100 W	101 S	101 W	Total
Nantou County	Caoton	1/1	9/3	7/5	17/6	16/4	2/2	7/3	8/6	11/3	5/3	6/5	0	19/3	3/2	25/9	15/3	13/7	3/3	13/7	6/2	6/2	2/1	194/29
	Zhushan	0	1/1	5/1	1/1	4/1	0	3/1	0	0	0	30/4	5/2	52/4	1/1	693 /29	60/9	221 /20	40/4	161 /17	18/4	184/17	2/1	1,481/59
	Jiji	0	0	0	0	0	0	0	0	0	0	0	0	0	0	65/1	15/1	0	0	0	0	0	0	80/1
Taichung City	Fengyuan					1/1	0	1/1	0	0	0	0	0	1/1	0	0	0	0	0	1/1	0	0	0	4/3
	Dongshi			7/1	2/1	1/1	0	5/2	0	0	1/1	0	0	1/1	0	0	0	4/1	0	5/1	0	1/1	0	27/4
	Xinshe		2/1	5/2	9/3	0	0	2/2	11 /1	25/4	1/1	7/3	0	53/7	11/3	119 /13	11/4	106 /14	6/2	55/13	1/1	0	2/2	426/26
	Waipu					128 /13		84/8	3/1	102 /7	28/3	41/7		301 /12		160 /12		145 /16		129 /19		7/3		1,128/47
	Houli					187 /21		1,341 /42		576 /38	71/7	501/43		1,649/68		1,732 /96		863 /57		560 /42		166/32		7,313/184
Miaoli County	Zhuolan				4/3	5/2		167/2	121/3	93/2	67/2	20/1	10/2	231/6	22/4	100/6	0	32/3	4/2	39/5	6/2	4/1	2/1	927/15
	Tongxiao					246/9		599 /18		107 /12		113/9		131 /12		150 /14		81/9	53/11	268/9		13/3		1,761/31
Total		1/1	12/5	24/9	33 /14	588/52	2/2	2,209/79	143/11	914 /66	173 /17	718/72	15/4	2,438/114	37/10	3,044/ 180	101/17	1,465 /127	106 /22	1,231 /114	31/9	381 /59	8/5	13,674 /399

Table 2. Survey results of other possible host plants for *Xylella fastidiosa* Pierce's disease (PD) strains: total number of various plant species collected from the proximity of PD-confirmed vineyards and sample sizes conducted from 2003 to 2012

Country	Vineyard numbers	Plant species / Sample number										
		2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Total
Nantou County												
Caoton	4	44/76	72/210	39/52	11/12	—	11/82	13/78	13/62	12/71	15/81	77/643
Zhushan	3	21/27	56/85	—	—	7/23	10/56	11/89	12/81	9/78	10/67	65/439
Taichung City												
Dongshi	2	69/131	106/290	41/132	—	29/112	—	—	—	—	—	106/665
Houli	13	—	30/73	31/109	70/245	45/142	36/146	38/155	37/172	28/128	26/135	93/1,170
Waipu	5	—	44/85	20/22	44/75	21/59	20/112	22/124	21/131	16/101	17/100	60/709
Xinshe	5	62/112	94/284	—	54/138	35/91	11/78	12/75	13/77	11/69	16/75	72/924
Miaoli County												
Zhuolan	3	—	76/151	39/52	41/149	23/33	10/54	13/55	13/23	10/85	—	68/602
Tongxiao	2	—	35/56	36/83	42/79	17/34	—	—	—	—	—	42/252
Total	37	131/346	205/1234	92/450	127/698	98/494	80/528	80/576	80/546	71/532	67/458	251/5,404

Table 3. Detection of *Xylella fastidiosa* Pierce's disease (PD) strains in four confirmed alternative hosts collected from the proximity of PD-confirmed vineyards conducted from 2003 to 2012

Country	Vineyard numbers	Host plant (positive*/attempted)			
		H. scandens	*A. brevipedunculata*	*D. palmatus*	*M. paniculatus*
Nantou County					
Caoton	4	0/5	0/5	0/11	0/3
Zhushan	3	0/5	3/52	ND	ND
Taichung City					
Dongshi	2	0/8	ND	1/2	ND
Xinshe	5	0/1	0/32	1/27	1/26
Houli	13	1/21	1/19	0/25	0/13
Waipu	5	7/26	4/34	2/14	ND
Miaoli County					
Zhuolan	3	2/4	1/26	1/13	0/8
Tongxiao	2	0/1	ND	ND	0/2
Total		10/71	8/168	5/92	1/52

*: Positive isolation and PCR detection.

ND: Non-detection

Table 4. Source of *Xylella fastidiosa*: Pierce's disease strains from grapevines and other alternative hosts in Taiwan

Species/host or vector	Strains	GenBank accession no.	Source of reference
Xylella fastidiosa			
Grape			
Nantou, Caotun	GV26	JN820134	Su *et al.*, 2013
	GV61	JN990271	Su *et al.*, 2013
Nantou, Zhushan	GV14	JN990274	Su *et al.*, 2013
	GV102	JN990276	Su *et al.*, 2013
Taichung, Houli	GV78	JN831760	Su *et al.*, 2013
	GV84	JN831759	Su *et al.*, 2013
Taichung, Xinshe	GV18	JN990273	Su *et al.*, 2013
	GV20	JN990272	Su *et al.*, 2013
	GV93	JN831758	Su *et al.*, 2013
Miaoli, Zhuolan	GV63	JN935275	Su *et al.*, 2013
	GV103	JN990277	Su *et al.*, 2013
	ATCC35876	DQ991182	Su *et al.*, 2011
	ATCC35879	DQ987477	Su *et al.*, 2011
D. palmatus	DP10	JN990280	This Study
A. brevipedunculata	AB3	JN990278	This Study
	AB4	JN990279	This Study
H. scandens	HS2	JN831755	This Study
	HS3	JN831756	This Study
	HS4	JN831757	This Study
M. paniculatus	MP1	JN831752	This Study
Pear	PLS2	DQ987473	Su *et al.*, 2011
	PE.PLS	AF203392	Mehta and Rosato, 2001
Citrus	CI.52	AF203389	Mehta and Rosato, 2001
Coffee	CO.01	AF203390	Mehta and Rosato, 2001
Mulberry	GHS505	DQ991183	Su *et al.*, 2011
Oleander	GH-9	DQ991185	Su *et al.*, 2011
	Ol	DQ991186	Su *et al.*, 2011
Peach	4-5	DQ991187	Su *et al.*, 2011
Plum	2-4	DQ991188	Su *et al.*, 2011
Pecan	4BD2	DQ991190	Su *et al.*, 2011
Sycamore	SLS55	DQ991193	Su *et al.*, 2011
Xanthomonas axonopodis* pv. *citri			
Citrus	XCW	DQ991194	Su *et al.*, 2011

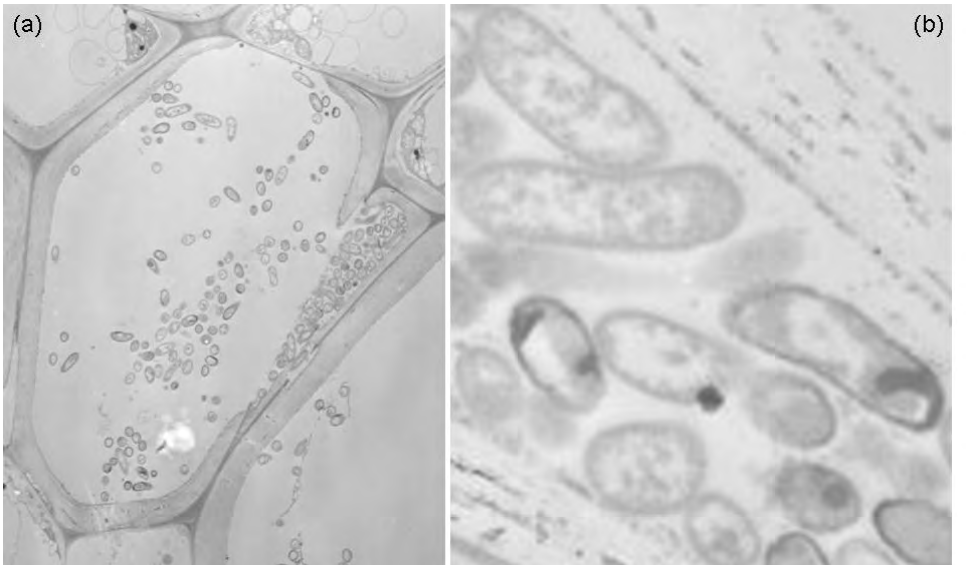


Fig. 1. Cell morphology of Pierce's disease (PD) strains in Taiwan. (a) Transmission electron micrograph of *X. fastidiosa* in the xylem vessel of *D. palmatus* in the vineyard. (b) Enlarged transmission electron micrograph showing cells of *X. fastidiosa* with rippled cell walls.



Fig. 2. (a) Symptoms of PD begins with leaf margin necrosis. (b) Systemic development of leaf scorch (c) Petioles remain attached to the cane (d) Symptoms on the one-year-old grapevine cuttings.

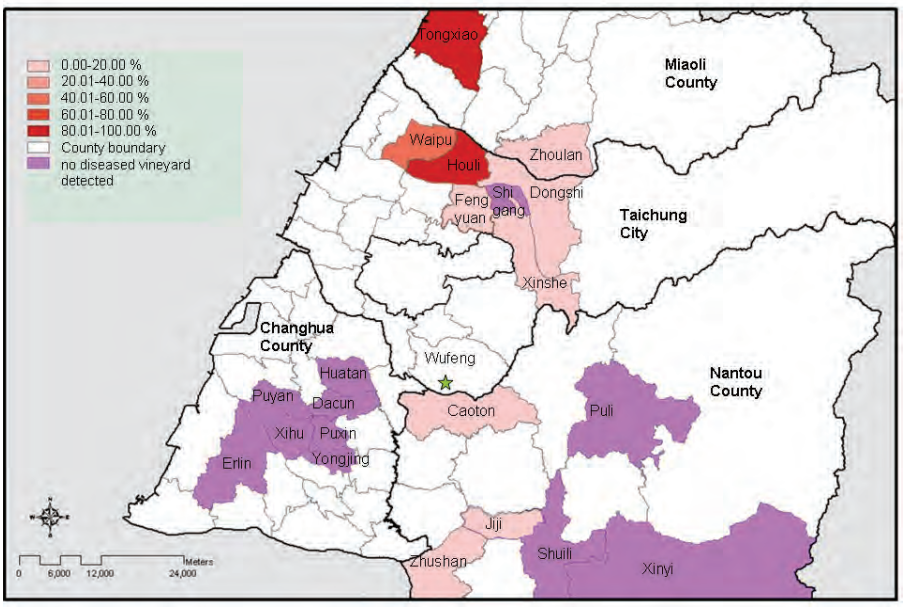


Fig. 3. Survey results of Pierce's disease incident: morbidity of orchards in various counties in central Taiwan conducted from 2002 to 2011.

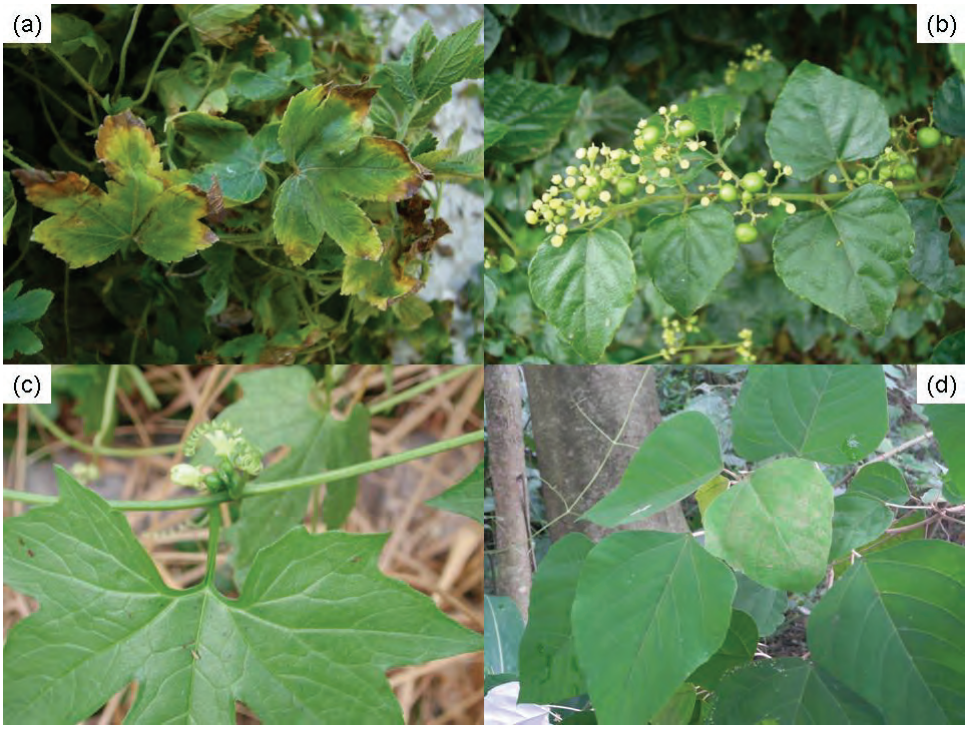


Fig. 4. Four confirmed alternative hosts in Taiwan. (a) *Humulus scandens* (Lour.) Merr.、(b) *Ampelopsis brevipedunculata* (Maxim.) Trautv var. *hancei* (Planch.) Rehder.、(c) *Diplocyclos palmatus* (L.) C. Jeffrey and (d) *Mallotus paniculatus*（Lamm.）Mull. –Arg.

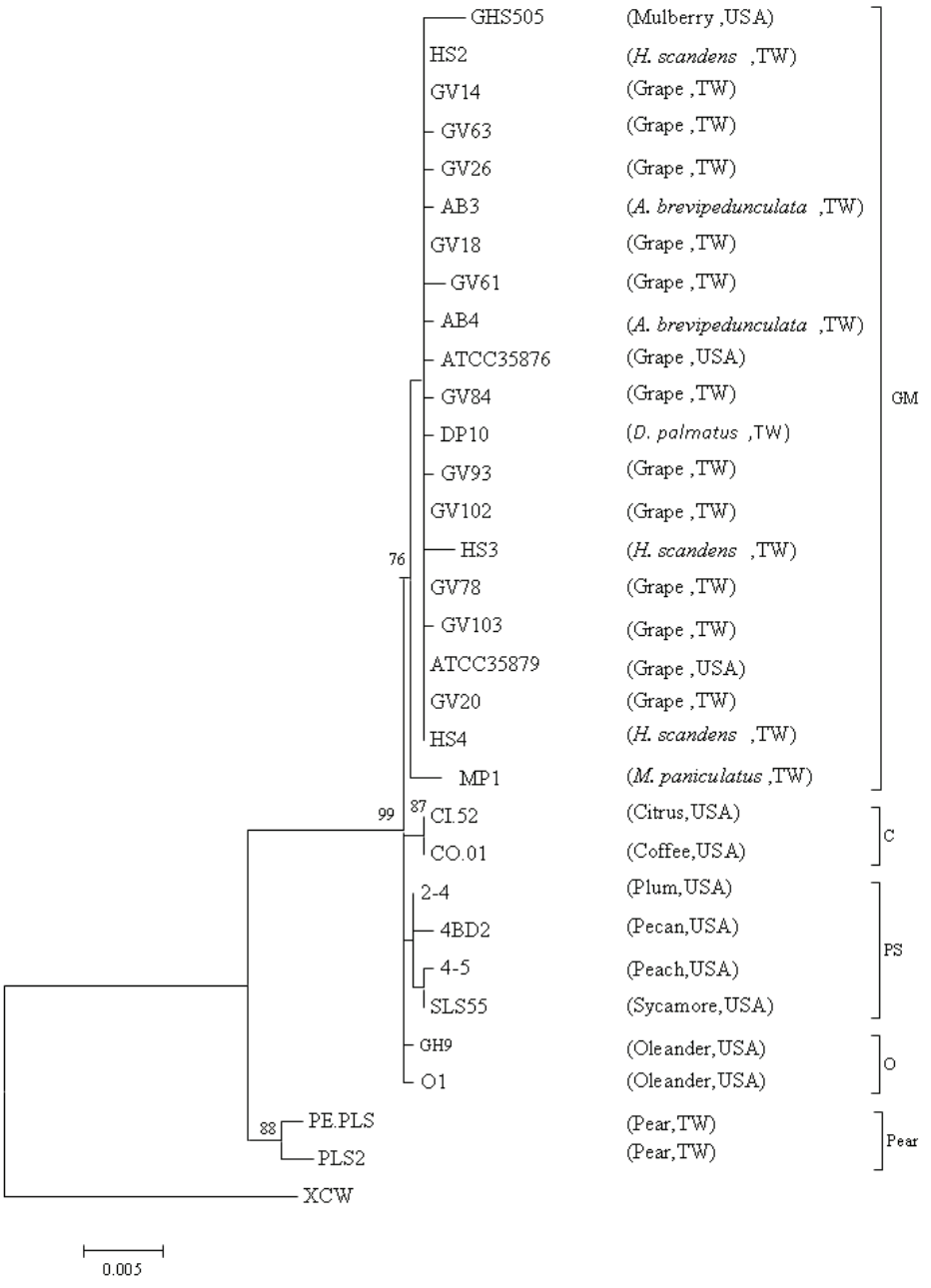


Fig. 5. A neighbour-joining (NJ) tree expressing the relationship of Pierce's disease strains from Taiwan with the other *Xylella fastidiosa* strains based on the 16S rRNA sequence. The tree was rooted using the 16S rRNA gene of *Xanthomonas axonopodis* pv. *citri* as an outgroup. The scale bar is equivalent to 0.05 nucleotide substitution per site. The probabilities of bootstrap analyses (in percentage) for 1000 resamplings that are greater than 70% are shown above the internal branches. The characteristics of the bacterial strains used in this experiment are listed in Table 4.

Potential Vectors of Pierce's Disease in Taiwan: Ecology and Integrated Management

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ABSTRACT

Since Pierce's disease (PD) of grape was reported in Taiwan in 2002, *Kolla paulula* has been a common leafhopper found at PD infected areas. The study showed that *K. paulula* is a potential insect vector for both PD and pear leaf scorch. In order to establish the integrated management techniques for the environment of vineyards in Taiwan, the authors describe how to apply the data on the integrated management of the potential insect vector. Data represent the study results including life history of *K. paulula*, population dynamics, habitats and host ranges conducted from 2009 to 2012. In addition, this article also describes the effects of temperature and rainfall on the changes of the population of *K. paulula*.

Keywords: Taiwan, Pierce's disease, potential insect vector, *Kolla paulula*, host plant, population dynamics, integrated management.

INTRODUCTION

Pierce's disease (PD) of grapevines is an infectious disease caused by *Xylella*

fastidiosa, a xylem-limited bacterium. PD was found in all commercial grape cultivars, and has been an important limiting factor for the wine industry. PD is one of the typical insect-borne diseases. So far, it is known that crops including grapes, pears, almonds, peach, citrus, coffee, alfalfa and many others are infected with various *X. fastidiosa* species or subspecies. In addition to crops, more than 150 plant species are also infected [6, 7, 8, 9, 13]. PD was first discovered in California in 1892 [15]. After that, PD was mainly reported from the American continent, such as U.S., Costa Rica, Mexico and Peru [8, 9, 15].

Since 1998, PD has been found in other areas such as Kosovo [2], China [4, 5] and Taiwan [22]. In 2002, suspected PD-like symptoms were reported at Nantou County in Taiwan. In 2013, the PD-like disorder was confirmed as the PD caused by *X. fastidiosa*. However, between 2002 and 2013, 12023 infected grapevines at Miaoli County, Taichung County and Nantou County were eradicated [21, 22]. The vineyards with infected grapevines are located in the hilly land (*e.g.*, Tunghsiao Township in Miaoli County, Houli District and Waipu District at Taichung City), or near the ravines or rivers (*e.g.*, Cholan Township in Miaoli County, Hsinshe District and Tungshih District in Taichung City, and Tsaotun and Chushan areas in Nantou County). These vineyards are surrounded by shrubs or weeds. Some of the plant species are the host plants for *X. fastidiosa* or its potential vector- *Kolla paulula* (Walker, 1858) [19, 20, 21]. On the contrary, no PD was yet to be found in the vineyards at Changhua County. One possible reason was because these vineyards at Changhua County are relatively large, and the lands are generally flat. Moreover, the high level of underground water underneath the vineyards could damage grapevines after a long period of time. So the grapevines have to be replaced approximately every 7 years. The replacement could be the reason why PD-infected grapevines are not found in these vineyards [21].

Grapevines in Taiwan are considered as high valued fruit trees. The average fruit price per kilogram is only lower than loquat, lychee, persimmon and Irwin mango. According to the AG Statistics Yearbook 2010, Agriculture and Food Agency, COA, Executive Yuan, Taiwan (http://agrstat.coa.gov.tw/sdweb/public/book/Book.aspx), the grape total harvest area was 3,054 hectares, and the output value of grapes was $ 6 billion 730 million New Taiwan dollars. The main production regions for vineyards are located at Changhua County (1330 ha), Taichung (679 ha), Nantou County (518 ha) and Miaoli County (503 ha). The amounts of grapes produced in these four areas accounted for 99.54% of total production in Taiwan. The data shown above indicates

that the grapevines in Miaoli County, Taichung City and Nantou County are highly susceptible to PD infection. These vineyards produce 55.66% of total production. Therefore, if the integrated management of PD and insect vectors are not effectively established in these high-risk areas, PD would become a threat to other vineyards in Taiwan.

It has been understood that only phytophagous hemipteran insects are the potential vectors for the transmission of prokaryotic plant pathogens. Among these insects, the potential vectors capable of transmitting xylem-limited bacteria, *e.g.*, *X. fastidiosa*, only belong to the Aphrophoridae, Clastopteridae, Machaerotidae, and Cicadellinae (Hemiptera: Cicadomorpha) [1, 3, 9, 12, 14, 16, 17,]. These vectors are known as xylem feeders or xylem-feeding insects [12]. Su *et al*. [21] reported that among the xylem feeders, *Kolla paulula* is a dominate species found in the PD infection areas. Moreover, the DNA fragment of *X. fastidiosa* was detected in the *K. paulula* individuals, suggesting that *K. paulula* is a candidate vector for transmitting PD in Taiwan.

Currently, there are no chemicals for practical treatment of PD. Prevention of PD would focus on vector control, eradication of infected plants and alternative host plants of PD, breeding disease resistant vines, and cultivation of healthy seedlings. For vector control, the insecticides are used for emergency prevention or rapid suppression of population density of insect vectors. Other methods such as biological control, cultural control and physical control are also used to further reduce the density of vector insects. The goal of vector control is to effectively reduce the speed of the spread of PD. In fact, the development of integrated management for insect-borne diseases would consider the relationships between the pathogens, insect vectors, host plants and environmental factors. Multiple approaches should be used to prevent these insect-borne diseases and hence to reduce economic damages. The data in this context includes field surveys, research and a report regarding the promotion of practical prevention and treatment of insect vectors. The potential vectors of PD in Taiwan are briefly introduced. The ecology of the potential vectors such as host range, feeding habits, population dynamics and fly capacity, as well as the relationship between the potential vectors and environmental factors are included. This report could be used for the development of the integrated management strategy for prevention of the vectors transmitting PD in Taiwan.

Potential vectors of PD in Taiwan

During the fruit seasons, both summer and winter, between 2002-2012, the two corresponding authors preformed surveys of potential vectors using sweeping net,

yellow sticky paper, and D-vac at PD infected areas, including Cholan Township and Tunghsiao Township in Miaoli County, Tungshih District, Houli District, Waipu District and Hsinshe District in Taichung City, and Tsaotun and Chushan areas in Nantou County. A total of 4 froghopper and 32 leafhopper species were captured [18, 21]. Among these insects, 6 cicadelline leafhoppers, 3 aphrophorid insects and 1 cercopid insect were identified as xylem feeders (potential vectors), including *Cicadella viridis* (Linnaeus, 1758), *Cofana spectra* (Distant, 1908), *Kolla paulula* (Walker, 1858), *Bothrogonia ferruginea* (Fabricius, 1787), *Anatkina horishana* (Matsunura, 1912), *Poophilus costalis* (Walker, 1851), *Clovia puncta* (Walker, 1851), *Aripty*elus *auropilosus* (Matsumura, 1907) [21], *Eoscarta zonalis* (Matsumura, 1907) and *Xyphon* sp. Among these xylem feeders, the DNA fragments of *X. fastidiosa* were detected in *K. paulula* [21, 22], *B. ferruginea* [21], *A. horishana* [21] and *P. costalis* [21]. These four insects are the candidate vectors for transmitting PD in Taiwan. Moreover, *K. paulula* is a common insect inhabiting in weeds or shrubs outside the vineyards in Taiwan [19].

In order to understand whether foreign xylem feeders had been introduced to Taiwan, since 2002, the two corresponding authors not only surveyed insect vectors in fields every year, but also checked insect specimens perpetually persevered at several agencies and schools (such as the Taiwan Agricultural Research Institute, Insect and Mite Collection, Wufeng, Taichung, Taiwan (TARI); Taiwan Forestry Research Institute, Insect Collection, Taipei, Taiwan (TFRI); National Museum of Natural Science, Taichung, Taiwan (NMNS); National Taiwan University, Department of Entomology, Taipei, Taiwan (NTU); National Chung-Hsing University, Insect Collection, Department of Entomology, Taichung, Taiwan (NCHU)). The results showed that five confirmed vectors of Pierce's disease in United States of America *i.e.*, *Homalodisca vitripennis* (Germar) (GWSS), *Graphocephala atropunctata* (Signoret), *Draeculacephala minerva* (Ball), *Xyphon fulgida* (Nottingham) and *Philaenus spumarius* (Linnaeus), were not found in Taiwan [21].

The ecology and habitats of leafhoppers at PD-infected areas- using *Kolla paulula* as an example

Excluding leafhoppers belonging to *Mileewa*, there are 18 cicadelline leafhoppers and sharpshooter leafhoppers) [10, 23, 24]. Seven of them are commonly found in field, including *Anatkina horishana* (Matsunura, 1912), *Cicadella viridis* (Linnaeus, 1758), *Cofana spectra* (Distant, 1908), *Kolla atramentaria* (Motschulsky, 1859), *Kolla*

insignis (Distant, 1908), *Kolla paulula* (Walker, 1858), and *Bothrogonia ferruginea* (Fabricius, 1787). In addition to *Kolla atramentaria* (Motschulsky, 1859) and *Kolla insignis* (Distant, 1908), the other 5 species can be found in vineyards located at mountainsides.

In this study, *K. paulula* was used as a model insect to investigate the relationship between the ecology of potential insect vectors (xylem feeders) and their inhabitations at the PD-infected areas in Taiwan. Since 2009, four vineyards with different environments were chosen as studying sites. The first vineyard locates at Bai-mao-tai area of Hsinshe District in Taichung City. This vineyard was also assigned as a long-term studying site for the integrated management of potential insect vectors. The second vineyard was at Tsaotun area in Nantou County. No insect vectors control has been performed at this vineyard. The other two vineyards were at Houli District in Taichung City and Chushan area in Nantou County, respectively. The grapevines at these two vineyards were eradicated due to PD infestation in 2008. But the weeds outside the vineyards are still growing without any treatments. Therefore, the areas outside these two vineyards were chosen for monitoring the occurrences of potential insect vectors. In this study, yellow sticky papers were used for monitoring the species and occurrences of potential insect vectors year round. The survey and data collection was performed every two weeks. The data shown below are our recent studies on *K. paulula* and the research results of PD from foreign countries. Scholars who participate in the international symposium would discuss how to promote PD researches in Taiwan based on this information, as well as through the international cooperation in the future.

The geographic distribution and habitat of *Kolla paulula* in Taiwan

Kolla paulula distributes in Palearctic region and Oriental region, including the Indian subcontinent, Indochina, China, Taiwan, Malaya Peninsula and Indonesia [(10)]. Shih *et al*. [(19)] indicated that *K. paulula* is a common xylem-feeder founded in the medium and low altitude areas in Taiwan. To understand the changes in the geographical distribution of *K. paulula* in Taiwan, since 2002, the first author not only performed field surveys, but also examined about 1,500 specimens of *K. paulula* (both dried and alcohol soaked specimens) stored at the TARI, NMNS, and NTU. The results indicated that most specimens of *K. paulula* were recorded at altitude of 500-1,300 meters mountainous area before 1990. However, in between 1990 and 2012, surveyed

records have included those collected from ground level to 800 meters mountains. In the meantime, two of *K. paulula*'s favorable host plants, mile-a-minute, *Mikania micrantha* Kunth, and spanish needles, *Bidens pilosa* L. var. *radiata* Sch. Bip, have been widely distributed in plains and in low to medium altitude mountains. These results suggest that *K. paulula* might follow their host plants to move down from the mountains. In addition, during the process of checking the specimens, the first author also found various marking and colors on the head, pronotum, and scutellum of *K. paulula* individuals captured from the same or different areas. The result of indoor subculture showed that leafhoppers feed on the same host plants also developed the same variance. Whether these phenotypes occurred resulted from chromosomal mutations warrants further study.

The results of field survey and collection conducted between 2002 and 2012 showed a few *K. paulula* individuals inhabiting inside the dense woods area. The characters of the habitat could be summarized as (1) before the sun rises and after 5 to 6:00 pm, *K. paulula* inhabits on wood and weeds at the edge areas of the orchards, and feeds on those host plants nearby and (2) most of the habitats are in cool and dry areas.

Identified host plants of *Kolla paulula*

The tests of host plants of *K. paulula* were performed according to the definitions made by Oman [(11)]: "Host plants" refers to that adults and nymphs can finish their life cycles on this plant; otherwise, "Food plant" refers to the adults or nymphs only feed on the plant. Based on this definition, the plants identified as the host plants of *K. paulula* mainly belong to the families of Compositae (*e.g*., *Mikania micrantha* Kunth, *Bidens pilosa* L. var. *radiata* Sch. Bip., and *Ageratum houstonianum* Mill.), Commelinaceae (*e.g*., *Commelina diffusa* Burm f.) [(19)], Moraceae, and Convolvulaceae (Shih, unpublished data).

The possible factors affecting the population dynamics of *Kolla paulula* throughout the year

In the period from 2009 to 2012, four PD-infected vineyards were selected as the studying sites, including Bai-mao-tai 60 (Bai-mao-tai, Hsinshe District, Taichung City), Houli-80 (Houli District, Taichung City), Pinglin-117 (Tsaotun, Nantou County), and Chushan-66 (Chushan, Nantou County). Monitoring of the population dynamics of *K. paulula* and other xylem feeders at the studying sites was performed using yellow

sticky papers throughout the year. The survey was conducted every two weeks. The results of four-year-survey demonstrated that the population of *K. paulula* was affected by several environmental factors, such as abundance of weed, temperature, rainfall and geographic location.

Integrated management of potential insect vectors of PD-using Bai-mao-tai 60 studying site (Hsinshe District, Taichung City) as an example

The Bai-mao-tai area (Hsinshe District, Taichung City) is located in the hillside tableland near Ta-Chia River. The altitude of Bai-mao-tai area is about 600 to 700 meters. Large temperature difference between day and night is recorded at Hsinshe District in Taichung City. It is an important area where high-quality grapes (Kyoho grapes, *Vitis vinifera* L. X *Vitis labruscana* B.) are produced. Annual production was unable to meet the domestic demand. Export markets include Japan, Singapore and Hong Kong. From 2002 to 2010, 368 grapevines (5 to 15 years old) at more than 20 vineyards at Bai-mao-tai area were infected with PD, then be eradicated [(21)], resulting in huge economic losses. The vineyards at Bai-mao-tai area are located on sloping valleys covered with weeds and shrubs. Most of these plants are *K. paulula*'s host plants (such as *Mikania micrantha* Kunth, *Bidens pilosa* L. var. *radiata* Sch. Bip., and *Commelina diffusa* Burm f.). The environment and species of potential vectors are different from those identified in the United States. Therefore, it is necessary to establish the integrated management of potential insect vectors in Taiwan for reducing farmers' economic losses.

To do so, from 2009-2012, one of the infected vineyards, Bai-mao-tai 60, at Bai-mao-tai area was selected by Shih and Su to be a long-term studying site for investigation of the population density and the integrated prevention of *K. paulula* adults. In addition, the first author cultured *K. paulula* in a laboratory condition to establish the timing of chemical control of *K. paulula*. The results showed that the mean generation time (from eggs to adults) of *K. paulula* was about 62-94 days. The mean generation time in autumn and winter (October to February the following year) was 1.2 to 1.5 times longer than that in summer.

Preliminary results described below are based on the results of the integrated insect vector control at Bai-mao-tai 60 from 2009 to 2010 as well as the modified control strategies from 2011 to 2012.

1. Chemical control

Kolla paulula is one of the potential insect vectors of PD in Taiwan [22].The tests showed that in a laboratory condition, grapevines are only the food plants of *K. paulula*. Moreover, the life cycle of *K. paulula* is about 2 to 3 months. In order to reduce the population of *K. paulula*, the chemical control was tested in this study. Chemicals such as Imidacloprid (6,000 time dilutions of the 28.8% stock solution), Carbosulfan (2,500 time dilutions of the 48.34% stock solution), lambda-Cyhalothrin (1,000 time dilutions of the 2.8% stock solution) were applied at weeds outside the vineyard once every two weeks. Each chemical was administered three consecutive times. This study was conducted between March and August, two months before and after a period of germination to bursting of summer fruit and winter fruit from 2009 to 2010.

The results showed that from 2009 to 2011, the ratios of the populations of the first peak of *K. paulula* in each year were 1.7: 1: 1.5. It suggests that chemical control could not significantly reduce the population of *K. paulula*. Even though, before the confirmation of habitats and primary host plants of potential insect vectors, chemical control is still the best practical method to suppress the population density.

2. Weed control- prevention of the *K. paulula's* host plants

Due to ineffective results of chemical control preformed at Bai-mao-tai 60 studying site, since March, 2011, Shih and Su changed the strategy and focused on weed control, especially against spanish needles, within and outside the vineyard. Prevention was conducted by the farmers. The spanish needles on roadsides and within the vineyard were removed by mower once every 1.5 months. The results showed that in 2011, the maximum population of *K. paulula* was only 6. In 2012, Three peaks of population of *K. paulula* were 7 (in mid-March), 6 (in mid-July) and 3 (late December). There is a correlation between the trends of disease and presence of large amount of *K. paulula* and its host plants (such as *Bidens pilosa* L. var. *radiata* Sch. Bip., *Ageratum houstonianum* Mill., and *Commelina diffusa* Burm f.) in other PD infected vineyards in Taiwan (such as Cholan Township and Tunghsiao Township in Miaoli County, Tungshih District, Houli District, Waipu District and Hsinshe District in Taichung City, and Tsaotun and Chushan areas in Nantou County). Therefore, the key to potential insect vector control at PD high risk areas is the eradication of *K. paulula*'s host plants.

In Taiwan, grapevines have high economic value. Although, using mower to remove weeds requires manpower and high costs, it can significantly reduce the

number of potential insect vectors. And it is also very safe for agricultural environments. Therefore, it is necessary to use mower to remove weeds for weed control.

3. Educational promotion

In order to increase farmers' awareness of the knowledge regarding PD symptoms and the favorable environment for potential insect vectors, Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ) invites Su and Shih to hold informational meetings about the integrated management of PD and potential insect vector every year. So farmers can observe and cut down their infected grapevines, as well as prevent the host plants of potential insect vectors. At Bai-mao-tai 60 studying site, for example, after weed control by the farmers, the population of potential insect vectors was significantly reduced, and the numbers of PD-infected grapevines also continued to decline each year.

Future Prospects

PD was first reported in Taiwan in 2002. Since then, the researchers under the suggestion and support of the government performed immediate eradication of infected grapevines. Later, a cooperation between Taiwan Agricultural Chemicals and Toxic Substance Research Institute and Taiwan Agricultural Research Institute was established to study PD and the ecology of the leafhopper vectors, set up the management of potential insect vectors at different agriculture environments, investigate the hosts of pathogens and potential insect vectors, and reduce the population densities of pathogens and potential insect vectors in fields, thereby reducing economic losses.

Unfortunately, over the past decade, the researches on insect-mediated diseases and insect vectors received limited governmental funding. The studies of the potential insect vectors of PD in Taiwan, for example, require research works on technologies about monitoring migration of insect vectors and their feeding behavior, test of disease transmission, statistical population ecology, biological control, microbial control and new non-chemical materials. Therefore, it is urgent to incorporate domestic or international interdisciplinary collaborative researches, and to train research teams in this area which is one of the main purposes of this international symposium. With the publication of the papers presented at this symposium and the records of group discussions, it is expected to highlight the main scheme and bottlenecks of researches

on insect-mediated pathogens and insect vectors which can be a vital reference for the authorities in agriculture sector and researchers to develop related research plans. The scholars who participate in this international academic symposium are welcome to maintain cooperation with researchers in Taiwan. Together, the fundamental knowledge of insect-mediated pathogens and insect vectors would be built, and the technology that can fulfill the needs of the industry will be created in the future.

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Table 1. From January, 2009 to August, 2011, the monthly average temperature and total rainfall, and the populations of *Kolla paulula* at Bai-mao-tai area of Hsinshe District in Taichung City

		1	2	3	4	5	6	7	8	9	10	11	12
2009	The period of peak occurrence (every ten days)		※	※				○	○				□ □
	Monthly average temperature	12.16 ℃	18.59 ℃	17.71 ℃	19.99 ℃	23.25 ℃	25.48 ℃	26.14 ℃	26.32 ℃	25.62 ℃	23.75℃	19.82 ℃	15.23 ℃
	Monthly total rainfall	0 mm	15 mm	189.7 mm	385.6 mm	22 mm	93.8 mm	111 mm	996.5 mm	27.5 mm	13.5 mm	23.5 mm	22.5 mm
2010	The period of peak occurrence (every ten days)		※ ※						○				□
	Monthly average temperature	14.95 ℃	16.86 ℃	19.34 ℃	20.73 ℃	24.68 ℃	25.22 ℃	26.84 ℃	26.28 ℃	25.68 ℃	23.43 ℃	18.66 ℃	15.46 ℃
	Monthly total rainfall	47.9 mm	155.8 mm	32.5 mm	171.5 mm	26 mm	795 mm	392 mm	376.5 mm	228.8 mm	6.5 mm	22.9 mm	37.3 mm
2011	The period of peak occurrence (every ten days)	※ ※ ※			○								□
	Monthly average temperature	-	15.04 ℃	15.54 ℃	20.71 ℃	23.98 ℃	27 ℃	26.56 ℃	27.01 ℃	-	-	-	-
	Monthly total rainfall	-	33.5 mm	52.5 mm	2.5 mm	157.5 mm	185 mm	354.2 mm	250 mm	-	-	-	-

Remarks: ※ represents the first peak of the population occurred every year; □ represents the second peak of the population occurred every year; ○ represents the third peak of the population occurred every year.

Understanding Bacterial Virulence Genes and Mechanisms of Host Response to Insect-Mediated Citrus Huanglongbing

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ABSTRACT

A summary is provided for research progress in studying genomics and proteomics of molecular pathogen-host interactions for citrus huanglongbing (HLB), a destructive disease of citrus that presents a major threat to the citrus industries in US as well as other citrus production regions in the world. The disease is associated with a gram-negative, phloem-limited, insect-vectored, unculturable prokaryote: '*Candidatus* Liberibacter spp', that belong to the *Rhizobiaceae* family of α -Proteobacteria. Despite the fact that Koch's postulates have not been fulfilled, a considerable progress has been made in understanding molecular basis of HLB disease since the publication of HLB-associated Liberibacter genome. Annotation of the Liberibacter genome sequence has provided insights into the genetic basis of the virulence, physiological and metabolic capabilities of this organism. Functional determination of key virulence genes will permit researchers to design and develop a novel gene-based therapeutic treatment to control the disease. Since most commercial citrus cultivars are susceptible to HLB, understanding the molecular mechanisms of host response is crucial for the development and deployment of HLB-resistant citrus varieties. A long term and sustainable management of HLB is likely to be based on integrated strategies including removal or reduction of vectors or inocula, and the improvement of host resistance to HLB-associated Liberibacters and psyllid vectors.

Keywords: huanglongbing, *Candidatus* Liberibacter asiaticus, proteomics, virulence genes, host response

INTRODUCTION

Huanglongbing (HLB), also known as greening, is a destructive disease of citrus

worldwide that severely reduces productivity resulting in catastrophic economic losses [8, 20]. Generally, almost all commercial citrus cultivars are susceptible to HLB. There are some citrus cultivars or hybrids appear to be tolerance to HLB [43]. HLB-affected trees begin to decline within a few years of infection, produce reduced yields, poor quality fruit, and may die or become otherwise unproductive. HLB was first reported in Asian countries in the 1870s [30]. While Koch's postulates are yet to be determined, the etiology of the disease has been associated with '*Candidatus* Liberibacter spp', a group of Gram-negative, fastidious, phloem-limited α-proteobacteria. Taxonomically, there are three HLB-associated species, namely: '*Candidatus* Liberibacter asiaticus' (Las), '*Ca.* L. africanus' and '*Ca.* L. americanus', which are based on their presumptive origins from the Asian, African and American continents, respectively [8]. Among these three Liberibacter species, Las-associated HLB is the most prevalent and has been associated with increasing economic losses to citrus production worldwide. Since the first report of HLB in Florida in 2005, the disease has been observed in most citrus growing counties in Florida as well as in other citrus growing states in U.S. including recent occurrences in Texas and California [27, 28]. Las is transmitted and disseminated naturally by the Asian citrus psyllid (*Diaphorina citri*). The disease can also be transmitted by grafting with HLB-affected citrus plants or by dodder plants [8]. There is a latency period between times of infection and symptom development, which greatly complicates control strategies [20] making it crucial to develop fast, reliable and efficient methods for the early detection of infected plants.

Due to the fastidious nature of the Liberibacter, standard microbiological methods could not be directly applied for the causative agent study, thus, the details of etiology of the disease are limited. '*Candidatus* Liberibacter' associated diseases are usually present in very low titers and uneven distribution in plant hosts [17, 31], therefore attempts to obtain the complete Liberibacter genome directly using HLB-affected plants tissue failed. This difficulty was overcome by using a high through-put next generation deep sequencing technology in combination with the whole genome amplification approach [17]. The availability of Las genome has provided insight into the genetic basis of the virulence, physiological and metabolic capability of this organism. Annotation of the Las genome facilitates the identification and functional determination of the virulence genes responsible for HLB.

Since almost all citrus cultivars are susceptible or highly susceptible to HLB, research efforts have also been emphasized on understanding the molecular basis of host

responses to HLB. An important aspect of disease-associated plant-microbe interactions are the host responses during the disease development [36]. Identification of the host responses, especially at the infection or pre-symptomatic stage can be critical towards understanding the initial processes involved in disease development and could be exploited in the formulation of efficient disease management practices [12, 16]. At least three separate but complementary transcriptomics studies using microarray technology have been performed to elucidate the effects of Las infection on the total mRNA expression levels in tissues of sweet orange (*Citrus sinensis*) plants [2, 3, 25]. However, differential gene expression at the transcriptional (mRNA) level do not always correlate with differential gene expression at the translational (protein) level as posttranscriptional translational and/or posttranslational modifications; alternative splicing of mRNA transcripts and mRNA stability and interference factors play important roles in regulating gene expression [6, 22, 40, 46]. Proteins are the final products of gene expression and their expression levels directly correlate with cellular functions. In order to fully understand the molecular mechanisms involved in the response of citrus plants to Las-infection, it is therefore imperative to inquire beyond the transcriptional level and into the proteomic level of gene expression. To better understand Las associated HLB, we have developed research strategies focusing on: (1) genome-based analysis and functional determination of virulence genes of Las. (2) proteomic profile analysis of citrus in response to HLB.

Genome analysis and identification of putative virulence genes of Las

Motility is an important virulence factor in many pathogenic species. Several lines of evidence indicate that the activity of the flagellum may have an impact on virulence gene regulation [37]. Vascular disease pathogens require motility to establish a systemic infection and spread beyond the initially infected tissue. A complete set of genes involved in flagella biosynthesis were identified in the Las genome. In addition, genes involved in the biosynthesis and in the function of type IV pili are also present in Las genome. Sequence-based function analysis indicated that the functional domain of *pilG* is highly conserved in some members of proteobacteria included in *Xf* [45]. In *Xf*, *pilG* is a chemotaxis homologous gene in a Pil-Chp operon that regulates type IV pili. Interestingly, *Xf pilG* homologue gene was identified in the Las genome. Further gene structure analysis reveals that both share a functional REC superfamily domain. We hypothesize that the substitution of homologous Las *pilG* gene could restore a wild-type

phenotype of *Xf pilG* mutant. As Las is not yet culturable, conventional prokaryotic gene manipulation technique cannot be applied directly to this study. To circumvent this limitation, an *in vitro* heterologous gene expression system was employed.

Here we report a novel assay for characterization and functional determination of Las *pilG* gene. A site-directed deletion method was employed to generate a mutant *Xf pilG* [41]. To determine the function of Las *pilG* gene, a mutant *Xf pilG* was replaced with homologous gene of Las *pilG* (*XfΔpilG –R*-Las). In addition, the complementation of *Xf pilG* with native *Xf pilG* gene was also made (*XfΔpilG* –C). The function of twitching motility in *Xf pilG* and Las *pilG* were observed using microfluidic chambers. Microfluidic devices were fabricated using photo-lithographic procedures similar to that described previously [15, 33]. Specifically, *Xf* cells were collected from 4-6 day old grown on PD3 agar. Cell density was adjusted to an OD_{600nm} of 0.05 in PD3 broth. Cells were introduced through a separate inlet with 1 ml gas-tight syringes, and growth medium flow was maintained at 0.2 µL min^{-1} for 10 min to stabilize the system. Cell behavior was assessed microscopically using 40X phase-contrast optics from time-lapse image recordings using a SPOT-RT digital camera (Diagnostic Instruments, Inc., MI) controlled by MetaMorph Image software, NX version 2.0 (Universal Imaging Corp., PA). The number of cells exhibiting twitching motility was quantified using MetaMorph software. All experiments were conducted at room temperature.

Functional determination of Las *pilG*

In vitro growth curves showed that the biofilm formation by *XfΔpilG* was six-fold less than that of WT *Xf* after ten days of static incubation, measured by a crystal violet assay while genotype *XfΔpilG-C, ΔpilG–R*-Las had significantly a higher biofilm formation as compared with the mutant *Xf, XfΔpilG* (Fig.1), indicating that *XfΔpilG* has a reduced surface attachment ability, resulting in a reduced biofilm formation. On PD3 culture medium, colonies of the wild type, *XfΔpilG-C* and *ΔpilG–R*-Las exhibited a peripheral fringe, which is indicative of type IV pilus-mediated twitching motility by the bacteria, whereas *XfΔpilG* mutant exhibited a smooth and non-peripheral fringe phenotype (Fig. 2), implying that the *pilG* mutant phenotype results in.

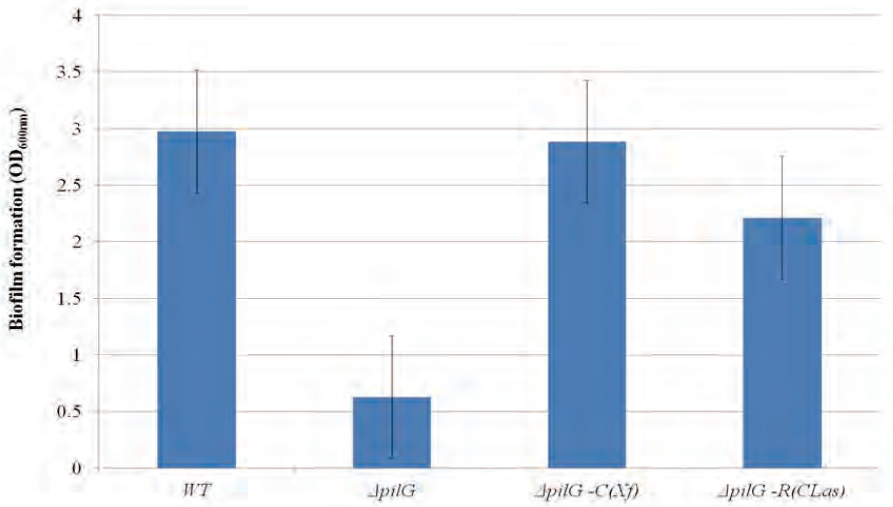


Figure 1. Quantitative assessment of biofilm formation of *X. fastidiosa* *XfΔpilG* (WT), mutant (*XfΔpilG*), complementation (*XfΔpilG-C)* and replacement (*XfΔpilG-R-Las)*. Means and standard deviations of three independent experiments.

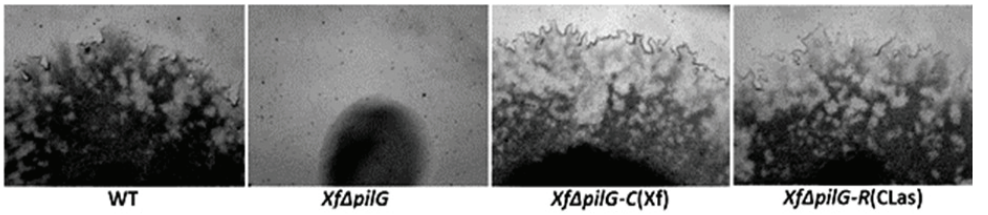


Figure 2. Colony characteristics of *X. fastidiosa* genotypes grown on PD3 agar. A peripheral fringe of colony was found in all genotypes except *XfΔpilG* mutant.

To confirm our results, *Xf* cells were assessed for motility in microfluidic flow chambers. Most *Xf* wild type cells exhibited twitching motility and developed cell aggregates in PD3 broth. In contrast, *Xf pilG* mutant cells had completely impaired twitching motility. However, twitching motility and the aggregation of cells were observed in both *XfΔpilG*-C and *XfΔpilG-R*-Las, suggesting *PilG* is required for cell twitching motility. Furthermore, this result indicates that pilus is highly conserved. *In vitro* heterologous gene expression experiments support the hypothesis that expression of type IV pili appears to be a requisite determinant of pathogenicity in Liberibacter associated disease. This study demonstrates the utility of heterologous gene expression

approach for functional characterization in unculturable Liberibacter.

Proteomic analysis of citrus host response to HLB

In this study, a comprehensive proteomic profile was constructed for proteomic analysis of citrus in response to HLB at asymptomatic and symptomatic stages. Two-year old grapefruit (*Citrus paradisi* cv. 'Duncan') plants were grown in an environment controlled, insect-proof greenhouse. Plants were either uninoculated or inoculated by side-grafting with 3-4 cm long bud sticks from PCR-confirmed HLB-affected (showing blotchy mottle and yellow shoots) lemon plants. Three months post-inoculation, 10-15 fully expanded leaves were collected from three individual plants each from the uninoculated or inoculated group. At this stage the infected plants were pre-symptomatic (no blotchy mottle, yellow shoots or symptoms of nutrient deficiency) but were PCR-positive for Las. Leaf samples from uninoculated or inoculated plants were grouped, respectively, as uninfected control for pre-symptomatic (UP) plants or infected pre-symptomatic (IP) plants. Six months post-inoculation, another set of 10-15 fully expanded leaves was collected from three individual plants each from the uninoculated or inoculated group. Successful inoculation was confirmed by PCR [(29)]. At this stage all of the inoculated plants were symptomatic for HLB and PCR-positive for Las. Leaf samples from uninoculated or inoculated plants were grouped, respectively, as uninfected control for symptomatic (US) plants or infected symptomatic (IS) plants.

Sample Preparation

The total leaf protein was extracted according to the early report [(35)]. Total protein extraction and quantification process were repeated three times generating three analytical replicates per plant. Total protein samples were then subjected two dimension electrophoresis separation and image analysis**.** Gel images were analyzed using the PDQuest software package (version 8.0, Bio-Rad, USA). A total of 36 gels were analyzed representing three analytical replicates per plant and three replicate plants per treatment. The gels were sorted into four groups namely: uninfected control for pre-symptomatic (UP) plants, infected pre-symptomatic (IP) plants, uninfected control for symptomatic (US) plants, or infected symptomatic (IS) plants. Gel spots were detected and matched so that a given spot had the same number across all gels. A master gel image containing matched spots across all gels was auto-generated.

Extensive analysis using the "Landmark" tool was used to resolve missed matches and spot volumes were normalized according to the total gel image density as suggested by the PDQuest software package. Only spots that had ≥10-fold increase over background and present in at least six of the nine gels per treatment as well as showed < 1.5 fold change ($P < 0.05$) compared to at least one other treatment group were considered to be differentially produced and further analyzed.

Protein mass spectrometry analysis

Prior to Protein mass spectrometry analysis, protein spots were manually excised (OneTouch Plus Spotpicker, The Gel company, USA) and digested with mass spectrometry grade trypsin in the presence of ProteaseMAX™ Surfactant according to the manufacturer's protocol (Promega, USA). For MALDI-TOF-MS/MS analysis (QSTAR XL Hybrid Quadrupole TOF LC/MS/MS System, Applied Biosystems, USA), the MASCOT search engine (Matrix Science, London, UK) was used to find matches of the PMF and MS/MS fragmentation spectra against a custom database containing entries for citrus (Citrus sinensis and Citrus clementina) available at http://www.citrusgenomedb.org/ and entries for grape (*Vitis vinifera*) available in the NCBI nonreduntant database. LC-MS/MS spectra were also searched via MASCOT against a custom citrus database. To gain functional information on identified proteins from MALDI-TOF and LC-MS/MS analysis, homology searches using $BLAST_P$ (http:www.ncbi.nlm.nih.gov/BLAST) was employed.

Effects of Las-infection on the leaf protein profile of pre-symptomatic and symptomatic grapefruit plants

There was no visible difference in leaf morphology between the uninfected control for pre-symptomatic stage plants and the infected pre-symptomatic stage plants but the uninfected control for symptomatic stage (US) plants was visibly different from the infected symptomatic stage (IS) plants. A total protein yield of over 10 mg g^{-1} was extracted from leaves and there was no significant difference in the total protein yield across treatments. A high resolution 2-DE separation of total leaf proteins from grapefruit plants was visualized in a p*I* range of 4-7 and M_r range of 10,000-150,000 (Fig. S1). Using PDQuest analysis software, over 700 spots per gel and over 440 reproducible spots within replicate gels were detected. Out of 191 differentially produced spots detected by PDQuest analysis, mass spectrometry analysis via

MALDI-TOF- or LC-MS was identified and summarized. An example of magnified view of the profiles of identified spots in representative gels from each treatment group is shown in Fig 3. Differentially expressed proteins were identified and categorized according to functional groups (Fig 4).

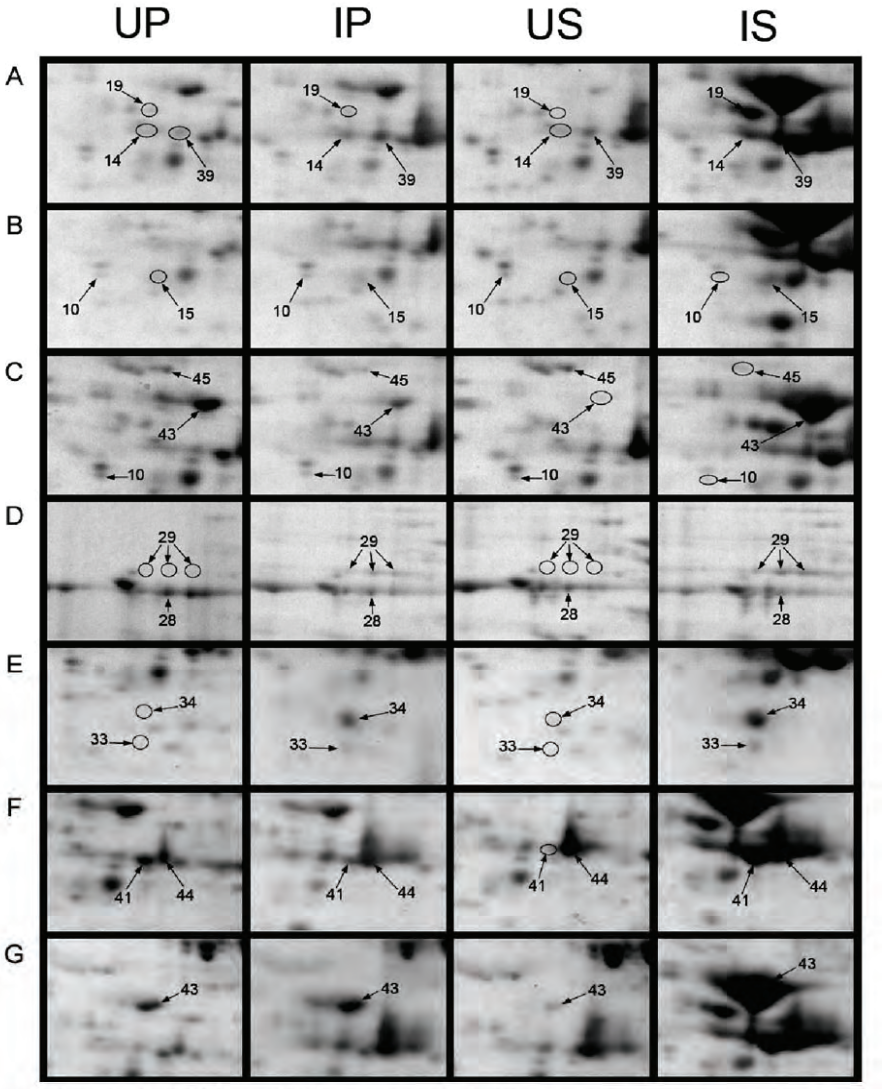


Figure 3. Differentially expressed protein spots were separated by 2D-electrophoresis and visualized by staining with Coomassie Brilliant Blue in UP (uninfected control for pre-symptomatic stage plants), IP (infected pre-symptomatic plants) US (uninfected control for symptomatic stage plants) and IS (infected symptomatic stage plants).

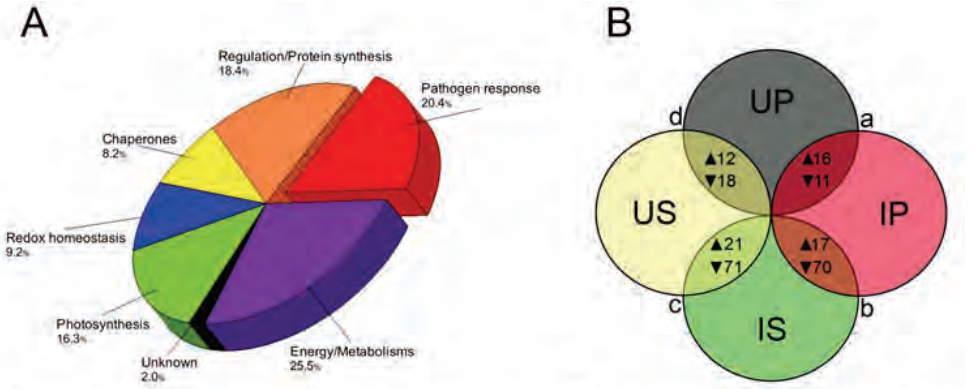


Figure 4. (**A**) Functional categories of identified differentially expressed protein spots from grapefruit plants that were Las-infected or uninfected and symptomatic or pre-symptomatic for HLB. (**B**) Venn diagram with intersections a, b, c and d, showing the number of identified protein spots that were significantly up- (▲) or down- (▼) regulated in comparison among four groups; IP, UP, IS and IP.

Pathogen response: Pathogenesis-related (PR) proteins are plant proteins that are induced in response to pathogen attack. However, several studies suggest that these proteins can also be induced by a variety of abiotic stresses, such as wounding and exposure to chemicals or heavy metals [(9, 13, 35)]. The PR-4 family of PR proteins consists of class I and class II chitinases, which differ by the presence (class I) or absence (class II) of a conserved N-terminal cysteine-rich domain corresponding to the mature hevein, a small antifungal protein isolated from rubber tree (*Hevea brasiliensis*) latex [(10)].

Lectin-like proteins are involved in vascular tissue differentiation [(14)] and are associated with the plugging of phloem sieve plates in response to wounding and defense against pathogens and insects [(39)]. Accumulation of Phloem protein 2 (PP2), a lectin-like protein, at the sieve plates together with phloem necrosis and blockage of the translocation stream was demonstrated by Kim [(25)] and Achor [(1)] in HLB-affected citrus plants. Furthermore, the deposition of PP2 with callose at the sieve plates played a role in the recovery of apple trees from apple proliferation disease caused by the phloem-limited pathogen '*Candidatus* Phytoplasma mali' [(34)]. Las, a phloem-limited bacterium, might induce the production of lectin-related proteins in host plants in order to inhibit phloem flow and accumulate photosynthates to nourish further bacterial growth as previously suggested. On the other hand host plants might induce the

production of lectin-like proteins as a defensive attempt to prevent the spread of Las by sealing off the sieve tubes. Additionally studies have demonstrated that lectin-like proteins are able to interact with RNA molecules, and are involved in the long-distance trafficking of macromolecules and may play a role in long-distance signaling in response to infection by plant pathogens [38].

In agreement with our results, a proteomics study by Fan et al. [18] also showed a Las-mediated up-regulation of miraculin-like proteins and gene transcripts in sweet orange plants. Recently, two distinct miraculin-like proteins, RlemMLP1 and RlemMLP2, were characterized in rough lemon (*Citrus jambhiri* Lush), and shown to have protease inhibitor activities as well as being involved in defense against pathogens [44]. During the development of citrus sudden death (CSD) disease, a miraculin-like protein was suppressed in susceptible plants but not in tolerant plants [11] . Increased levels of PR proteins as well as miraculin-like proteins was observed in leaves of *C. clementina* plants after infestation by the spider mite *Tetranychus urticae* or exposure to methyl jasmonate [32].

Redox homeostasis: Redox-homeostasis-related proteins are usually involved in the prevention of oxidative stress, which is induced by reactive oxygen species (ROS). ROS are by-products of electron transport and redox reactions from metabolic processes such as photosynthesis and respiration. The production of ROS is markedly increased under conditions of biotic or abiotic stress. We observed that Las-infection up-regulated the production of peroxiredoxins and Cu/Zn superoxide dismutase in IP and IS plants compared to their respective control plants. Additionally, we observed a Las-mediated up-regulation of a 2Fe-2S ferredoxin-like protein particularly in IP plants compared to UP plants. Antioxidants, such as superoxide dismutase (SOD), are among the most potent in nature in protecting living systems against oxidative stress. While the role of Cu/Zn SOD in HLB disease development in citrus plants has been previously demonstrated [4], this study provides novel evidence for the potential involvement of two other redox homeostasis-related proteins: peroxiredoxins and an uncharacterized 2Fe-2S ferredoxin-like protein. ROS are produced sequentially: superoxide (O_2^-) is the first reduction product of ground state oxygen and it can undergo spontaneous or SOD-catalyzed dismutation to H_2O_2, which is the second reactive product. Although, H_2O_2 is less reactive than superoxide, it is very diffusible and directly inactivates key cellular processes.

Regulation/Protein synthesis: Considering the general physiological decline that

accompanies HLB development, it is not surprising that proteins associated with regulation/protein synthesis including such as a 31 kDa ribonucleoprotein, EF-Tu, glutamine synthetase, an ATP-dependent zinc metalloprotease, a serine-type peptidase, nucleoside diphosphate kinase 1, and alanine aminotransferase 2, were markedly repressed by Las especially in IS plants compared to US plants. Interestingly, we observed a significant Las-mediated down-regulation of a transcription factor homolog (Btf3-like) protein and S-adenolsyl-L-methionine synthetase in IP and IS plants compared to control plants, suggesting that these proteins might be early targets of Las pathogenesis or an early susceptibility response by grapefruit during HLB development.

The RNA polymerase B transcriptional factor 3 (Btf3) was demonstrated to be associated with apoptosis in mammalian cells [7, 47] but its actual function in plants is not well understood. However, recently, Huh et al. [23] showed that silencing of Btf3 protein expression in *Capsicum annuum* and *Nicotiana benthamiana* plants led to reduced hypersensitive response (HR) cell death and decreased expression of some HR-associated genes. HR cell death upon pathogen infection has been described as a strategy devised by plants for inhibiting pathogen spread and obtaining systemic acquired resistance against further infection [24, 42]. Thus, an early Las-mediated reduction in the production of a Btf3-like protein in grapefruit plants would have facilitated the spread of the bacterium within the host.

Chaperones: Molecular chaperones [e.g. heat shock proteins (HSPs), chaperonins and peptidyl-prolyl cis-trans isomerases] are proteins involved in protein folding, refolding, assembly, re-assembly, degradation and translocation [2, 5, 19, 26]. It is, therefore, not surprising that the broad Las-mediated down-regulation of proteins associated with regulation/protein synthesis was accompanied by a corresponding down-regulation in the expression levels of chaperones including peptidyl-prolyl cis-trans isomerase, heat shock proteins and chaperonin-60 especially in IS plants compared to US plants.

CONCLUSION

HLB is currently one of the most destructive diseases of citrus and Las has been responsible for increasing economic losses in citrus production worldwide. Management of HLB remains elusive largely because the physiological and molecular processes involved in HLB-disease development are unresolved. In addition, almost all citrus cultivars tested so far are susceptible to HLB. Nevertheless, a great deal of research progress has been made in our understanding of the HLB since the publication of the

Las genome. Functional determination of key virulence genes will permit researchers to design and develop novel gene-based therapeutic treatment to control the disease. In addition, dissecting molecular mechanisms of host responses will advance our knowledge in understanding the genetic basis of HLB. A long term and sustainable management of HLB requires integrated strategies including the removal or reduction of vectors or inocula, and the improvement of host resistance to HLB-associated Liberibacters and psyllid vectors.

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An Integrated Management of Citrus Huanglongbing in Taiwan

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ABSTRACT

Citrus Huanglongbing (HLB) was first found in China in 1943. This psyllid-borne disease caused by *Candidatus* Liberibacter asiaticus has been devastating the citrus industry in Taiwan since 1951. In order to formulate adequate control measures of HLB, etiological and epidemiological studies on HLB have been made thenceforth. So far four strains of HLB were identified in Taiwan. The HLB disease, commonly mix-infected with citrus tristeza and/or tatter leaf viruses, causes severe yellow mottling and decline. These systemic diseases are generally controlled by the integrated management measures including cultivation of pathogen-free seedlings, elimination of inoculum sources, and management of secondary spread by vector insects. The pathogen-free nursery system has been established since 1983 in Taiwan, which is primarily important for preventing HLB and virus diseases. The pathogen-free nursery has been properly managed through improved techniques of shoot-tip micrografting for obtaining HLB/virus-free citrus budwoods. The molecular diagnostic probes and polymerase chain reaction (PCR） were developed for indexing HLB trees and vectors. The ELISA, rapid diagnostic strips, RT-PCR techniques were also made for citrus virus diseases. The transmission of psyllid was studied for management of HLB spread. In chemotherapy, three applications of 1000 ppm tetracycline could reduce disease index of HLB in field trails.

Keywords: Huanglongbing, Greening, HLB management, Pathogen-free citrus seedling

INTRODUCTION

Citrus greening was first reported in 1947 from South Africa, while a similar disease known as Huanglongbing（HLB）was already found in 1943 from South China. HLB disease, locally called Likubin, was first found in Taiwan in 1951. This destructive

virus-like disease was spread throughout Southeast Asia including Philippines, Indonesia, and India, during 1960s. Eventually this disease spread to tropical and subtropical climate region such as Japan[13,15], Brazil[10], and Florida [1]. While the South African HLB organism（*Candidatus* Liberibacter africanus）is heat sensitive form and only causes severe symptoms in cool temperature climates (22-24℃）, the Asian HLB organism (*Candidatus* Liberibacter asiaticus） can cause serious symptoms in warm climates (27-32℃） and has been identified as a heat-tolerant form. The Asian form has caused greater devastation by shortening tree lifespan to less than 10 years and lowering fruit yield and quality in recent decades in tropical and subtropical Asian regions. For developing control measures, etiological and epidemiological studies on HLB have been conducted in Taiwan since 1956 up to present decade[4,6,7,8,9,11,18,19,21].

Moreover, HLB disease commonly co-infected citrus with citrus tristeza and/or tatter leaf viruses, and exocortis viroid, resulted in severe yellow mottling and tree decline, and ultimately death of citrus trees. This systemic infection is generally controlled by integrated control measures including production and cultivation of pathogen-free seedlings, elimination of inoculum sources, prevention of secondary spread by vector insects[2,3,16,17,19]. Establishment of a pathogen-free nursery system is primary measures for preventing prevalence of these diseases. The precise and rapid indexing techniques are indispensible for management of pathogen-free nursery system and health management of citrus trees in the field. Development and application of molecular diagnostic probes and polymerase chain reaction (PCR） for HLB include the followings; ELISA, rapid diagnostic strips and RT-PCR for CTV and CTLV [6,7,8,20]. In order to decrease the inoculum source and keep the old citrus trees continuously producing good quality fruit, chemotherapy was also studied to treat HLB-infected trees.

Symptoms

HLB symptoms in most susceptible mandarin cultivars are yellow mottling i.e., yellowing of the veins and adjacent tissue, followed by yellowing with pale-green mottling of entire leaf. With age, diseased leaves become harden, curling outwards, and occasionally develop vein corking. Leaf symptoms are followed by premature defoliation, dieback of branches, loss of fibrous roots and decline in vigor. In later stage, the diseased trees produce small and slender leaves along with symptoms of mineral deficiency. HLB-affected trees may become stunted, bear multiple off-season flowers, most of which fall off, and produce small irregularly shaped fruits with thick

peel of poor color-changing patterns (Fig. 1)[12].

Etiological studies

1. Causal organism

Electron microscope of HLB

The HLB bacteria causing Likubin was found in sieve tubes. Electron microscopy, using serial sections and three-dimensional assessments, confirmed the presence of various forms of HLB pathogen. The HLB bacteria were pleomorphic. The matured form of the pathogen was a rigid rod with dense cytoplasm, measuring 350-500 x 660-1500 nm in size, and surrounded by a two-layered envelop, 20-25 nm thick consisting of a cell wall and an inner cytoplasmic membrane (Fig. 2).

2. HLB strains

HLB first occurred in northern Taiwan in 1951. Previously the HLB pathogen infected main citrus cultivars in Taiwan such as Ponkan mandarins, Tankan tangors and Liucheng sweet oranges whereas it did not infect pummelos. However, HLB pathogen began to infect pummelos in 1970s implying that the evolution of pathogenicity occurred in recent decades. Four different HLB bacteria (HLBB） were identified based on the results of their pathogenicity and virulence on indicator plants. Strain I was only pathogenic on mandarins and sweet oranges by inducing typical HLB symptoms. Strain Ⅱ was pathogenic with high virulence on all differential cultivars with fast multiplication rates. Strain Ⅲ only caused intermediate symptoms on mandarins and sweet oranges, and mild symptoms on pummelos. Strain Ⅳ was a mild strain which could only infect but cause no symptoms on mandarin and sweet oranges (Fig. 3). Strain Ⅱ was prevalent in the field.

3. Diagnosis and indexing

HLB is tentatively diagnosed in the field by characteristic foliage and fruit symptoms described above. Further diagnosis requires indexing on susceptible mandarin or tangor, e.g. Ponkan and Tankan seedlings by graft-inoculation. Because of the low population and uneven distribution of HLBB within the plant, bioassay with an indicator plant requires numerous test plants and inoculum buds at least 2 buds/indicator plants, and is time-consuming[11]. HLB can be confirmed in the indicator plant by examining HLBB bodies in sieve tube of phloem section by electron microscopy[4].

The HLBB unevenly colonize the sieve tube of host plants at a low concentration. A highly sensitive and specific DNA probe, developed with DNA cloning methods has been used to detect HLBB in infected citrus hosts[6]. One of the clones containing a 0.24-kb HLBB-specific DNA fragment was labeled with biotinylated nucleotides by a PCR-labeling technique. A dot hybridization assay with the biotin-labeled DNA probe was successfully used for detecting HLBB in various citrus hosts including mandarins, tangors, sweet oranges and pummelos. This probe could specifically react with all HLBB strains from several Asian countries including Malaysia, China, Thailand, Philippines and Okinawa, Japan, but not with those from South Africa (Fig. 4). The probe developed was specific and sensitive enough to detect low levels of HLBB infection. Highly specific primer pairs for HLBB detection have been developed through HLBB-DNA cloning and sequencing. The sensitivity of HLB detection has been enhanced by the development of polymerase chain reaction (PCR) with appropriate primer pairs followed by electrophoresis analysis. The PCR detection also reacted with the foreign HLBB isolates of the Asian form, including Okinawa, Japan, China, Malaysia, Vietnam, Thailand and Saudi Arabia (Fig. 5). The technique was successfully applied for detection and ecological study of HLB pathogen. PCR has been developed and used for the detection of HLBB in host plants and vector insects[6,9]. This technology is routinely applied to indexing citrus foundation stocks and pathogen-free seedlings[9,17]. DNA primer pair used for HLBB detection via PCR of HLBB is: Forward: CAC CGA AGA TAT GGA CAA CA; Reverse: GAG GTT CTT GTG GTT TTT CTG.

Epidemiology

1. Transmission

The systemic HLB disease is transmitted mainly via vegetative propagation of citrus seedlings, and spreads by Asian citrus psyllid (*Diaphorina citri*) in persistent manner[5]. The Taiwanese biotype of the psyllids is less efficient in pathogen transmission of HLB. Less than 5% of adults acquired HLB pathogen after acquisition feeding on diseased citrus plants over a one day period, while the nymphs acquired the pathogen at a much higher rate. Transovarial passage of the pathogen by psyllid was not demonstrated. Transmission of HLB via graft propagation with infected budwood, and marcotted (layering) seedling from infected trees also plays an important role in spreading HLB.

2. Alternative hosts

Four suitable hosts of the Asian psyllid, to be considered as possible alternative hosts of HLBB, were investigated by graft-inoculation test and psyllid transmission. The multiplication of HLBB in plants was monitored by PCR. The results demonstrated that HLBB can replicate and persist in Chinese box orange (*Severinia buxifolia*) and wild lime (*Atalantia citroides*) (Fig. 6.) from Cambodia, but not in common jasmine orange (*Murraya paniculata* var. *paniculata* and *M. exocortica*) and curry leaf (*Murraya koenigii*) in Malaysia. HLBB failed to replicate in common jasmine orange and curry leaf with negative detection by PCR. HLBB could be detected in woody apple (*Limonia acidissine*) from Thailand at the sixth to tenth month after graft-inoculation. Inoculated woody apple produced yellows symptom about 6 months after graft inoculation, but plants recovered to a healthy state after one year. Accordingly, woody apple is considered a transient host of HLBB. Chinese box orange is a good host of HLBB because HLBB replicates in it as well as in citrus. Yellow-mottling symptoms developed in Chinese box oranges which can be served as donor plants of psyllid transmission as well as Valencia plants can. However, HLBB has been detected in diseased samples of jasmine orange in Brazil[(10)].

3. Ecology

For controlling the spread of the disease, production and cultivation of pathogen-free citrus seedlings have been implemented since 1983. Once the healthy trees are established in the field, the control of HLBB infection of the orchards is accomplished by controlling the psyllids. Transmission of HLBB by citrus psyllid is related to disease prevalence[(11)]. Epidemics occur when vector population is high and a reservoir of the inoculum is present. Natural spread of the disease is greatest during periods of bud-sprouting that promote the greatest psyllid feeding, reproduction and transmission[(5)] (Fig. 7.). The transmission of HLBB by citrus psyllid is related to high vector population, incidence of HLBB infection of psyllids and dispersal of the psyllid. The epidemic infection of healthy citrus trees in an orchard near HLB-affected groves showed a sigmoid progress curve of disease incidence. With the latent period set at 1.5-month after infection, the highest infection rate occurred in late February to mid-March, when HLBB infection incidence of viruliferous psyllids and the dispersal of psyllids in the field reached its peak. This time period was critical for psyllid control with insecticide sprays. The disease incidence increased up to 70% within two years

without spray and rouging of diseased trees. A survey of HLB disease progress of infection rate (%) was conducted over a 5-year period from 1999 to 2004 in southern Taiwan. The cumulative rate of HLB infection reached 17% in the psyllid control plot and 57% in the unsprayed plot[5] (Fig. 8.). Adequate insecticides such as Dimethoate and Confidor have been applied.

Disease management

1. Pathogen-free citrus nursery system

HLB disease was documented as a major threat to economic viability of citrus production in the subtropics and tropics. Since the systemic infection of HLB and viruses diseases are transmitted primarily by vegetative propagation, and then rapidly spread by vector insects in the field, integrated disease management is highly recommended for its control. Integrated disease management includes propagation of pathogen-free citrus seedling, elimination of inoculum sources, and prevention of secondary infection by insect vectors. Establishment of a pathogen-free citrus nursery system (PFNS) is fundamental to prevention of the disease epidemic. In Taiwan, a technique of modified shoot-tip micrografting in combination with heat therapy is conducted for preparing the citrus pathogen-free foundation stock. Precise and rapid indexing techniques are indispensable for management of a pathogen-free nursery system. The PFNS in Taiwan consists of the following four steps; 1) shoot-tip micrografting (STG), 2) establishment of pathogen-free citrus foundation blocks, 3) pathogen-free nurseries and 4) issue of health certificate *via* indexing. The current scheme for the PFNS and bud-wood certification program in Taiwan was initiated in 1983 and has been operated under a joint program of National Taiwan University (NTU), Agricultural Research Institute (ARI), and Council of Agriculture (COA) of Taiwan.

(1) Shoot-tip micrografting (STG) for obtaining virus-free citrus foundation stocks

STG is the most reliable method to recover pathogen-free (PF) citrus propagation material from infected parental sources. The shoot tip or meristem of auxiliary buds of infected plants is generally free of virus and HLB, and plants regenerated from the shoot tips are usually free of the systemic pathogens. The common STG method[14] was greatly improved by exchanging the inverted T cut with newly developed triangle-hole cut method [19]. The entire procedure of STG is carried out in a laminar-flow hood under sterile conditions (Fig. 9). The procedure of modified STG is described in detail

in recent publication[17]. A double grafting technique has been developed to enhance the growth of STG plants. The procedure of this technique is briefly summarized as follows : 1) The well-developed micro-grafted plants about l and half months old are removed from the test tubes. The upper part of the plant serves as the scion tissue. 2) The scion is side-grafted to a healthy and well-grown rootstock seedling and the bud was wrapped with parafilm. The grafted plant is covered by a plastic bag, with zip unsealed, and the plant placed in a greenhouse. 3) The plastic bag is removed 3 to 4 weeks after grafting. After about three months, the new shoot will grow from the shoot-tip and the new shoot is ready for use as scion-wood for further propagation. 4) Indexing for HLB and viruses is done before the STG plant is used for further propagation. For increase multiplication of pathogen-free seedlings, scion wood is harvested from double grafted STG-plants at 3 to 4 month intervals.

(2) Pathogen-free（PF） citrus foundation block

The STG-seedlings that are indexed to be free of the citrus viruses and HLB can be used as the certified pathogen-free foundation stocks. The stocks are kept in the citrus foundation block repository (Fig. 10 A) which is an insect-proof screen house constructed with a double-door entrance and surrounded by a water canal to prevent entry of ants and mites (Fig. 10 B&C). The screen house is installed with an air curtain over the first door. Concrete benches are set on gravel floor for maintaining the PF foundation trees in containers 30 cm above the floor surface (Fig. 10 D). The foundation trees are generally propagated on Troyer or Carrizo citrange from the clean STG plants. Two to four plants are maintained per cultivar, and they are pruned every year to produce a few fruits for verification of horticultural characteristics and removal of off-types. The plants are indexed periodically and inspected for fruit abnormality. The protected foundation blocks are maintained by public agencies. The national repository of pathogen-free foundation blocks is located in Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute (CAES/ TARI). The citrus foundation repository is located within a rice-paddy field of CAEA/TARI.

(3) Production of pathogen-free citrus seedling

A healthy citrus orchard planted with pathogen-free seedling may outlive the grower. Healthy citrus trees have a great potential for sustainable high yield over many decades, provided appropriate horticultural and disease management practices are

followed. Accordingly, production and cultivation of pathogen-free and high-quality nursery trees are fundamentally important component of HLB management. In a screen-house nursery, effective preventive measures to control diseases caused by *Phytophthora* spp., nematodes, and bacterial canker disease have to be practiced. Budwood-propagating blocks are established ahead of the production of PF citrus seedlings. The blocks contain certified parent plants propagated by using budwood from foundation trees and maintained in screen houses. Only a limited number of foundation trees are used for producing the budwood-propagating trees because of the stringent indexing for vector-transmitted pathogen and inspection of off-type mutation. These trees must be reindexed periodically, and used for the bud-supply up to three for avoiding re-infection and mutations during the propagated saplings. New budwood-propagating blocks must be periodically established with clean buds from foundation trees. The PF citrus rootstock seedlings are produced in screen houses well-constructed with 30 mesh screen and double doors. Rootstock seedlings are grown from seeds of selected cultivars in seedling tubes (5 cm in diameter and 18 cm in height) or in a seedbed tank of soil containing sterile potmixture. The rootstock seedlings are transplanted to perforated plastic containers (10 cm in diameter and 30 cm in height) for further cultivation when they grow to more than 40 cm high. A rootstock cultivar should be selected in terms of the compatibility with targeted scion-cultivar, i.e., Sunki and Cleopatra mandarin for mandarin cultivars; Troyer and Carrizo citrange for sweet orange and mandarin; Swingle citrumelo and pummelo for pummelo; and Volkamer lemon for mandarin and sweet orange. The PF seedling are propagated by bud- or side grafting with wood curving knife and parafilm using bud/scions from PF budwood--propagating trees. The production of PF seedlings is accelerated by rapid propagation technique described in detail in a recent publication [(17)].

2. Health management of pathogen-free trees in the orchard

Rehabilitation of citrus orchards planted with PF seedlings has been showing vigorous growth and good fruit-setting. The PF citrus trees may begin fruiting as early as 2 years after orchard establishment provided appropriate health management and cultural practices such as watering, fertilization, and pruning are followed. Health management of pathogen-free citrus seedlings in orchards needs to be properly performed using the following strategies: 1) Prompt elimination of HLB-diseased citrus trees and alternative host plants serving as inoculum sources to prevent spread of

HLB to adjacent healthy citrus trees, 2) Protection of pathogen-free trees from vector transmission by effectively spraying insecticides at critical sprouting periods, and with biological control of the vector using natural enemies including *Tamarixia radiate* introduced from Reunion by Dr. Bernard Aubert. 3) Protecting the orchards with physical barriers such as wind breaks or distance barriers. 4) Chemotherapy of HLB-infected citrus trees: Tetracycline (Achromycin) injection has been utilized by some citrus growers using the method formulated by Su and Chang in 1976[(18)]. Recently, the efficacy of the antibiotic injection has been improved by using an air-pressured injector (Fig. 11). Three applications (2 autumn, 1 spring) of 1,000 ppm Achromycin (2~4 L per tree) by air-pressured injector with 80-lbs pressure which improved the injection speed and had good recovery efficacy of diseased trees. The repeatedly injected trees recover well and produce normal fruits. Pruning dead branches in the upper canopy improves the therapeutic efficacy. The antibiotic injection is frequently associated with temporary phytotoxicity such as mild vein necrosis, slender leaves and defoliation, but the trees quickly recover to normal growth. The tree injection method is recommended to treat the diseased trees with diseased twigs less than half of canopy in early stage of disease development.

CONCLUSION

The Asian form of HLB has been evolving into strains with different virulence and consequently spreading over citrus-growing areas worldwide, and devastating citrus industry. The systemic disease can be well controlled by the integrated management including cultivation of PF seedlings, elimination of inoculums sources and prevention of secondary spread by vector psyllid. Establishment of pathogen-free nursery system for production and cultivation of PF seedlings is primary important for citrus rehabilitation from HLB and virus invasion. The HLB has been under control in large acreage of 200,000 hectares through prompt removal of diseased trees and adequate insecticide application in region-wide orchards in Brazil by March 2012. So far, the complete HLB management has not been well established in Asia. However, the management strategy and techniques are expected to be improved through further international collaboration in epidemiological study and disease management.

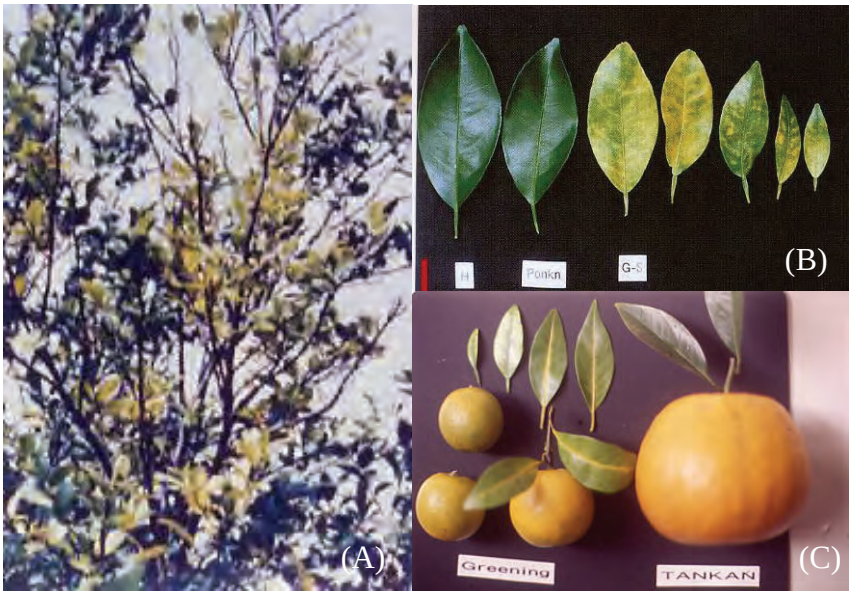


Fig. 1. Symptom expression of HLB in mandarin trees. **(A)** The diseased branch and twigs of ponkan mandarin showing yellow mottling leaves and die back. **(B)** Leaf symptoms of Ponkan leaves, showing yellow mottling of mature leaves and zinc deficiency of younger leaves（five leaves from the right）. Two leaves from the left were healthy. **(C)** HLB-disease Tankan fruits showing atrophy with greening (L) and H-ck, normal large fruit (R).

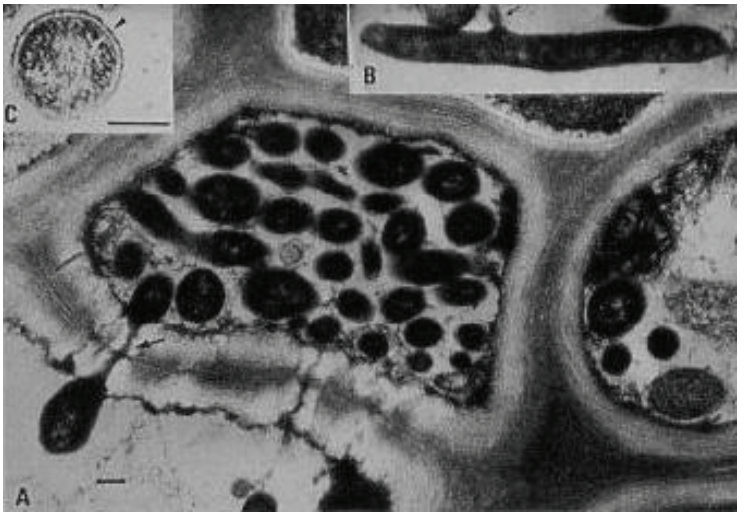


Fig. 2. Electron micrograph of citrus Huanglongbing bacteria (HLBB). A. HLBB packed in sieve tube of Ponkan mandarin. B. The rod HLBB with side-budding. C. HLBB consisted of a cell wall and inner cytoplasmic membrane from a cross-sectioned cell [(5)].

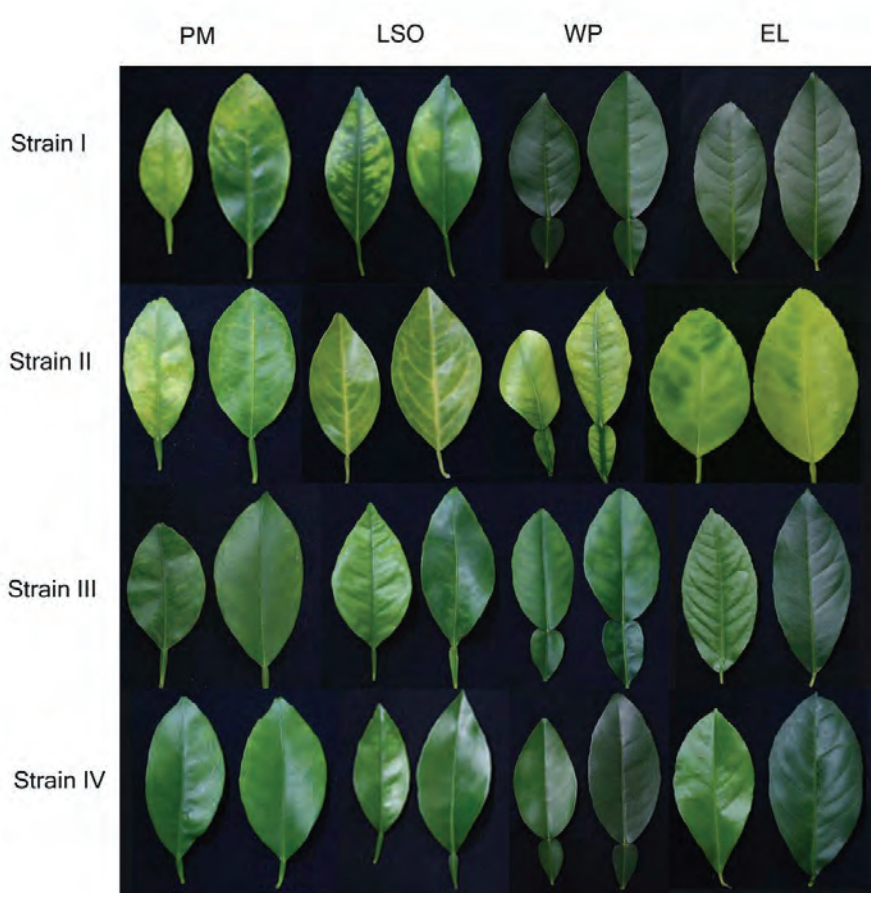


Fig. 3. Different types and severities of the symptoms induced by the 4 HLBB strains (Ⅰ~Ⅳ) in 4 differential citrus cultivars including Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL) under greenhouse conditions from 2005-2006.

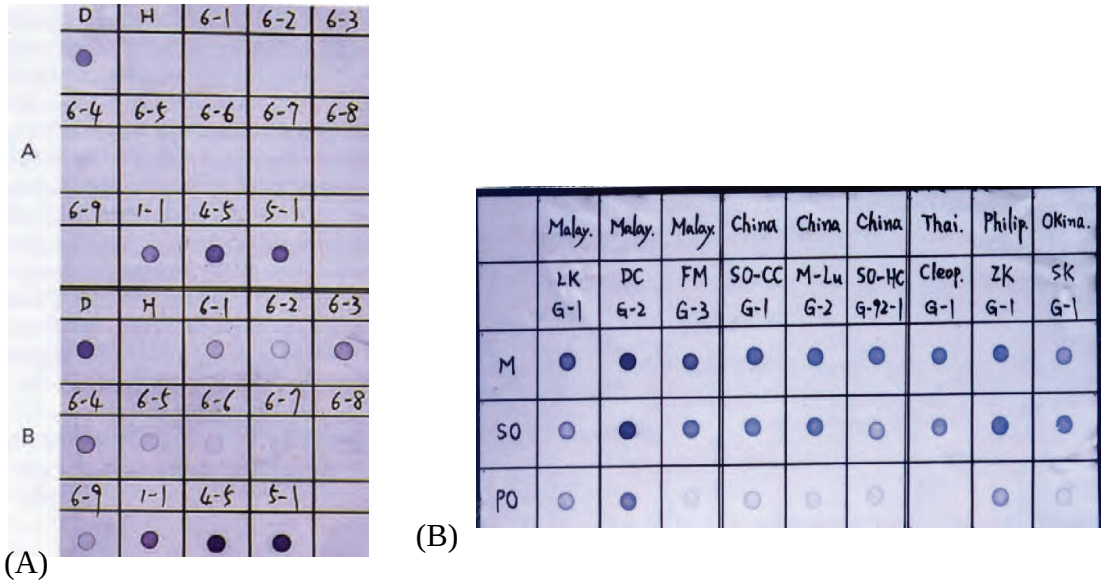


Fig. 4. Detection of HLBB by dot hybridization tests, with HLBB DNA extract (A-A), and by PCR amplification of HLBB DNA followed by DH with PCR products (A-B). D, diseased sample; H, healthy sample from shoot-tip-grafted ponkan used as negative control ; 6-1~6-9, healthy-looking citrus samples collected from field; 1-1, 4-5, 5-1, citrus samples with HLB symptoms.(B) Detection of HLBB by dot hybridization including Malaysia (Duncy and Fremont mandarin), China (Chanychou, Lukan mandarin,Honchian sweet orange), Thailand (Cleopatra), Philippine (zinkom mandarin), and Okinawa Japan (Shikuashia) isolates.

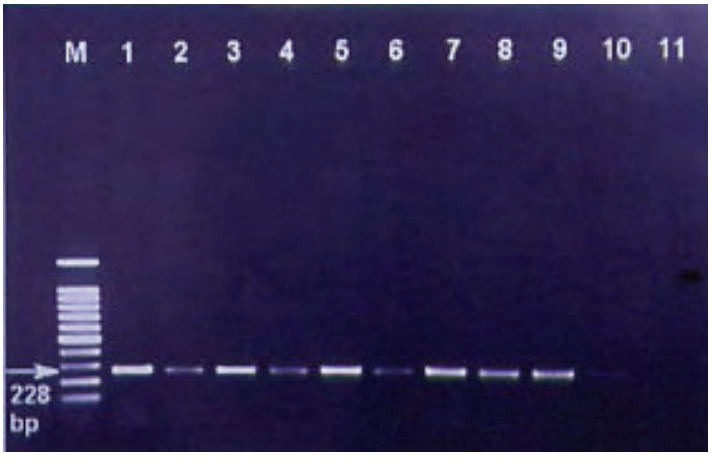


Fig. 5. Detection of HLB pathogen by polymerase chain reaction (PCR) followed with electrophoresis analysis: 1~4, Taiwan HLBB isolates; 5, Okinawa isolate, Japan; 6, Chain isolate; 7, Malaysia isolate; 8, Vietnam isolate; 9, Thailand isolate; 10, Saudi Arabia isolate; 11, Healthy CK.

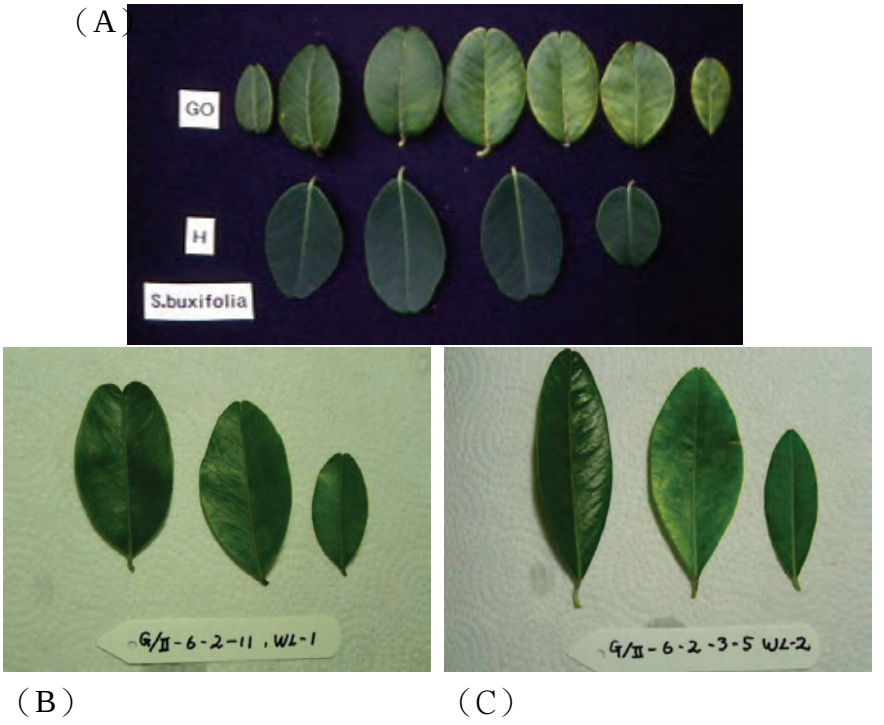


Fig. 6. **(A)** Leaves of Chinese box orange (*Severinia buxifolia*), alternative host of HLB. GO: HLB-diseased leaves showing yellow-mottling symptoms; H: healthy leaves (lower). **(B & C)** Leaf symptom on wild lime (*Atalantia* (=*Severinia*) *citroides*) infected by HLBB.

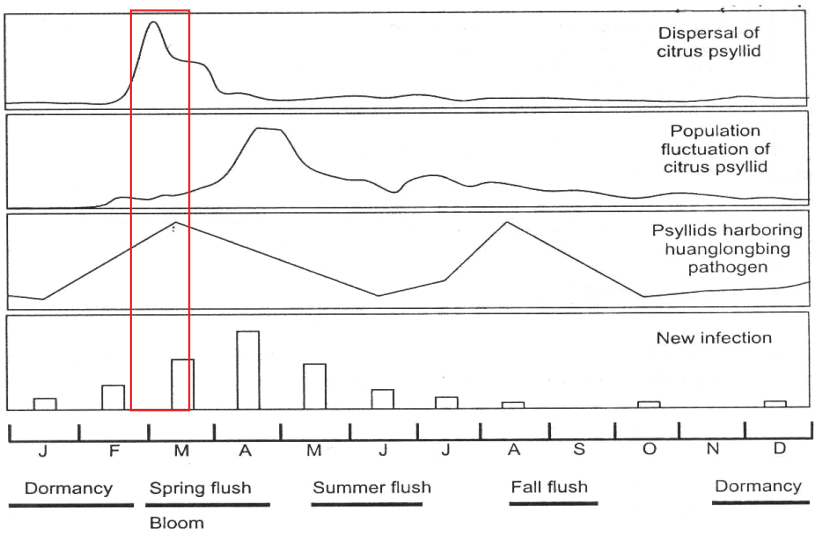


Fig. 7. Phenological patterns, population dynamic and HLBB infection of citrus psyllid in orange orchards of Southern Taiwan [6].

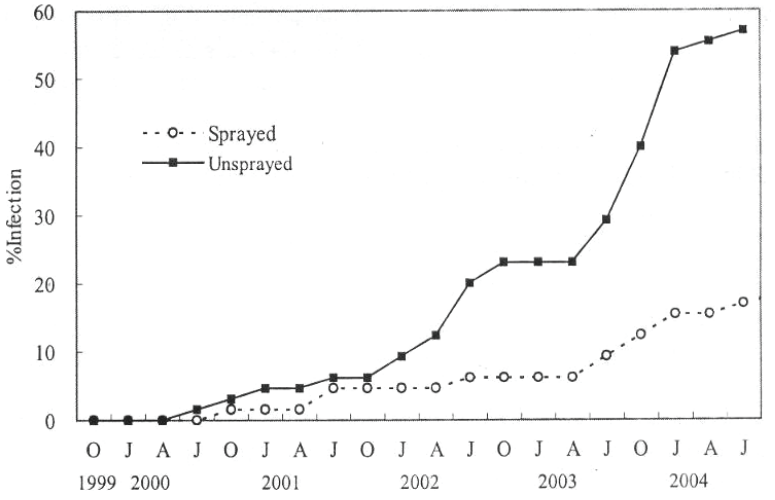


Fig. 8. HLB progresses of infection rate (%) in sprayed and unsprayed plots during a 5year period. The infection cumulative rate of HLBB infection reached 17% in sprayed plot and 57% in unsprayed plots [6].

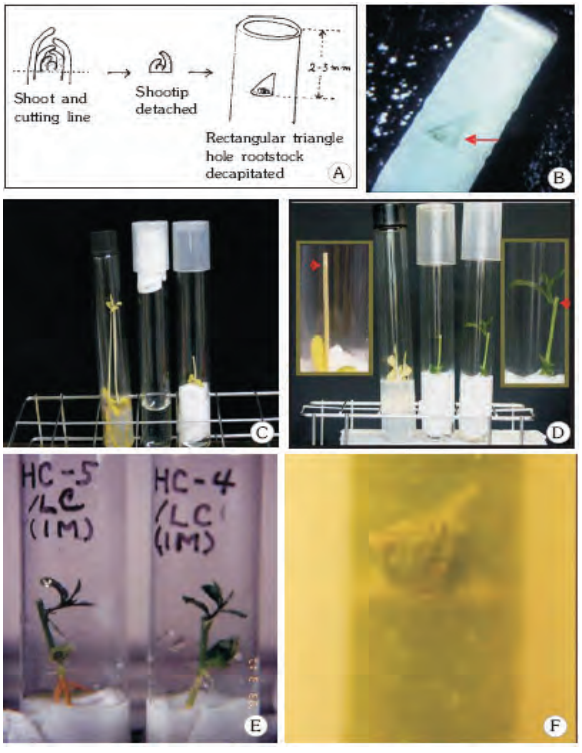


Fig. 9. Procedure for shoot tip (ST) micrografting. (A) Diagram showing excision of shoot tip with 2-leaf primodia from top of a shoot and making a rectangular triangle hole (0.3~ 0.5 mm) on a decapitated rootstock seedling by removing cortex layer with cutting edge of STG knife. (B) A shoot tip piece placed within the triangular hole of a decapitated rootstock seedling. (C) Two-week old rootstock seedlings on solid medium (left), a sterile test tube with a center-perforated filter-paper platform, containing liquid medium (center), and a test tube containing the micrografted rootstock seedling supported by filterpaper platform on liquid medium (right). (D) Different stages of STG rootstock seedlings, also showing a new sprout regenerated from the grafted shoot-tip (right). (E) Two new shoots of Hongjian sweet orange regenerated from ST in triangular hole (left) and V-shaped incision (right) of rootstock seedlings one month after micrografting. (F) A new sprout from ST in triangular hole on rootstock.

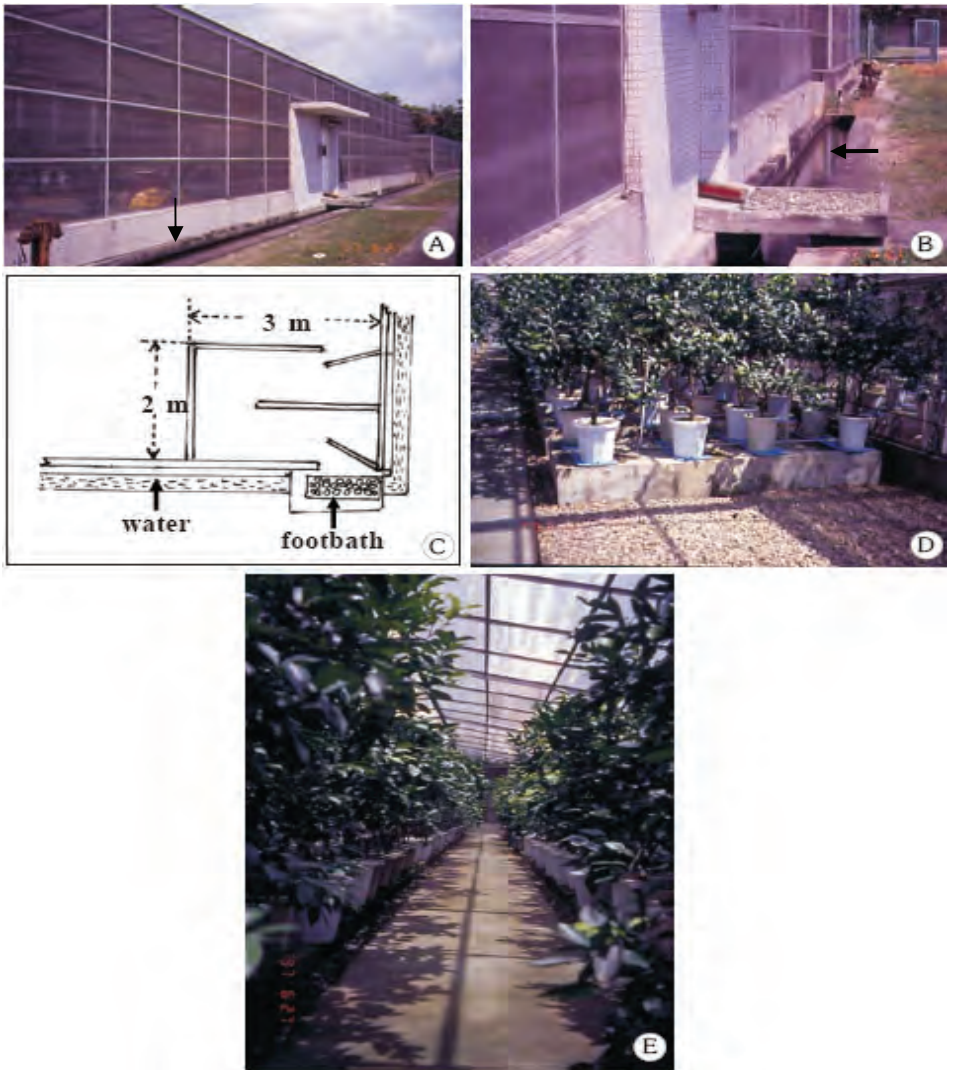


Fig.10. National repository for maintaining the certified pathogen-free citrus foundation blocks. (A) Insect-proof stainless screen-house surrounded by water canal (arrow head) and with double door entrance. (B) Close-up picture of double doors. (C) Drawing of double door, and footbath containing gravels mixed with copper sulfate solution. (D) Inside view of the screen house showing healthy foundation trees on concrete benches in gravel floor. (E) Certified PF budwood increase trees grown in a screen house attached to the foundation depository.



Fig. 11. Injection of antibiotic solution by an improved air-pressured plastic injector in the field.

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The New, Third-generation, AC-DC Electrical Penetration Graph (EPG) Monitor and Its Usefulness for IPM Research on Vectors of Plant Pathogens

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ABSTRACT

The most rigorous method to identify feeding behaviors of hemipteran vectors of plant pathogens is electrical penetration graph (EPG) monitoring. The purpose of this talk was to review: 1) principals of EPG as a tool for developing novel integrated pest management tools against vectors, and 2) application of EPG to identify feeding behaviors leading to inoculation of *Xylella fastidiosa*. *X. fastidiosa* is a xylem-limited bacterium that causes several scorch diseases in important crops, such as Pierce's disease of grape. Bacteria form a dense biofilm on the foregut cuticle of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) and other xylem-feeding vectors. Bacteria are inoculated directly from sites in the foregut into a host plant during sharpshooter feeding (i.e. probing of the mouthparts, stylets, into the plant). However, despite nearly 70 years of research, no one had associated specific sharpshooter stylet probing behaviors with inoculation until EPG was employed for such research. Development of the third generation (AC-DC) EPG monitor from the first two generations of monitors (AC and DC) helped define the mechanism of *X. fastidiosa* inoculation. EPG and other evidence for the salivation-egestion hypothesis for *X. fastidiosa* inoculation, in which salivation combined with egestion [outward fluid flow] carries bacteria into the xylem, was reviewed. Understanding the inoculation mechanism will aid development of grape varieties resistant to inoculation of *X. fastidiosa* by sharpshooter vectors.

Keywords: Hemiptera, Electrical penetration graph, EPG, feeding, vector, *Xylella fastidiosa*

INTRODUCTION

Studying the feeding, plant damage, and (especially) transmission (i.e. acquisition, retention and inoculation) of plant pathogens by hemipteran insect pests has always been a challenge. This is because their specialized, piercing-sucking mouthparts, the stylets, are probed/penetrated into opaque plant tissues through which the stylets cannot be directly, visually observed during probing. Early studies of hemipteran feeding were forced (by the technical limitations of their time) to study snapshots of feeding, after the fact. For example, one could view deposits of hardened saliva *in planta* left behind after feeding activities had ceased [22], in essence, frozen in time. Or, one could quantify collections of excretory droplets [18] or document transmission of plant pathogens from long-past bouts of feeding [4,29]. However, a researcher could not directly study or quantify hemipteran feeding in real time, as it was occurring, until the invention of are volutionary technology nearly 50 years ago. That technology is electrical penetration graph (EPG) monitoring of insect feeding[3,13,35].

Today, EPG is used in three main ways for development of novel integrated pest management (IPM) tactics for hemipteran pests, especially vectors of plant pathogens. First, in cases where the fundamental mechanisms of feeding damage or transmission of a plant pathogen are unknown, EPG is instrumental in elucidating such information. Second, once such mechanisms are understood, EPG can be used to demonstrate the effects of insecticides, antifeedants, or other chemical compounds on specific feeding behaviors responsible for pathogen transmission. Third, EPG can similarly identify the effects of resistant vs. susceptible varieties of crop plants, including those genetically engineered to express biopesticides. Rapid computerized analysis of EPG data can provide quantitative comparisons of, for example, the responses of vectors to resistant and susceptible plants. A researcher can then predict whether a pathogen will be transmitted from/to a putatively resistant host plant, leading to a novel mechanism for host plant resistance.

The purpose of this paper is to review: 1) the principals and history of EPG, especially development of the new, third-generation AC-DC monitor, and 2), a summary of my own work on the mechanisms of transmission of *Xylella fastidiosa* by sharpshooter leafhoppers. Much of the writing herein is excerpted and/or adapted from Backus [7], to which the reader is referred for more information.

Principles and history of EPG monitoring of insect feeding

Most advances in understanding the role of vector feeding behavior in mechanisms of plant pathogen transmission have been made possible by use of EPG over the last 50 years. As diagrammed in Fig. 1a, the insect is made a part of an electrical circuit by attaching a thin (~10-60 μm) gold wire to its dorsal surface with conductive glue, then connecting the insect to the input of a head stage amplifier attached to a monitor that also electrifies the plant. When the insect's stylets probe the plant tissues, fluids in the stylet food and salivary canals ionically conduct the electrical signal through the insect to the monitor, where it is amplified and outputted to a computerized digital display. Variable biopotentials (i.e., biological voltages) and/or electrical resistances to fluid flow (Fig. 1b) generated by the insect-plant interface instantaneously transform the constant applied signal into a variable-voltage output signal that is graphed as a waveform. The biological meanings of EPG waveforms are defined by correlation with: 1) stylet tip locations in the plant, via histology of salivary sheaths or cut stylets in probed plant tissues, and 2) intricate stylet activities (e.g. stylet movements, salivation, etc.) performed by the insect, via observation in transparent artificial diets and other methods [(35)]. Electrical resistance and biopotentials each produce different parts of the waveforms, depending on the generation of EPG technology used.

First developed in the late 1950's to early 1960's, EPG has advanced over the last 50 years along with the revolution in electronics. The earliest, first-generation EPG monitors, developed by McLean and Kinsey [(23)], used technology typical of the time, i.e., glass-tube amplifiers in the late 1950's, later evolving into early solid-state transistors by the 1960's. They also used AC (alternating current) applied signal, and a low amplifier sensitivity or input impedance (Ri) of 10^6 Ohms[(24)]. Monitors with low Ri outputted signals caused primarily by electrical resistance to/conductance of ionic charges carried in fluids (e.g. saliva, plant fluids) passing through the stylets (modeled as a variable resistor, Ra, in Fig. 1b) [(35)]. Thus, the earliest AC monitors best detected information such as starting and ending of stylet probing, saliva secretion and salivary sheath formation (today termed pathway activities), stylet movements such as extension, retraction, and partial stylet withdrawal, and stylet contact with vascular tissues such as phloem and xylem[(13)].

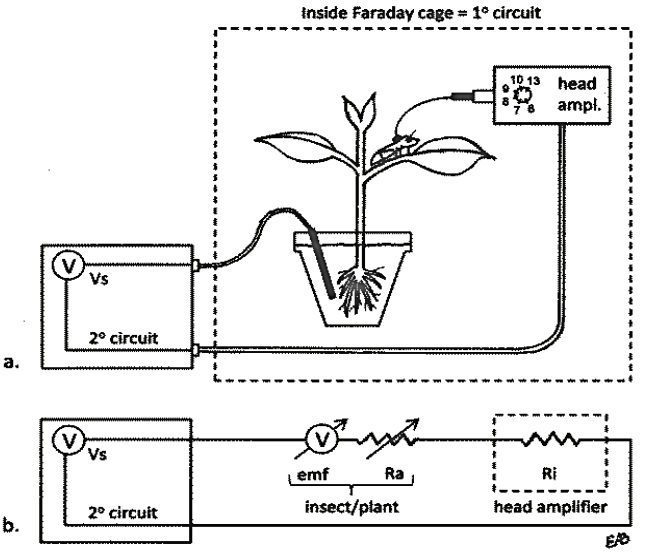


Fig. 1. Diagrammatic representation of the primary (1^o) circuit of an EPG monitor. **a.** Realistic model of the plant and insect. **b.** Electronic block diagram of primary circuit, including variable biopotentials (emf) and variable resistance (Ra). Modified from an original drawing by G. P. Walker [35]. 2^o = secondary circuit (i.e., signal processing circuitry); head ampl. = head stage amplifier; emf = electromotive force (biopotential); Ra = insect (e.g., aphid) resistance; Ri = input impedance of the head amplifier; Vs = source voltage. (Reprinted with permission of the American Phytopathological Society.)

By the late 1970's, electronics had been revolutionized with improved solid-state transistor technology (operational amplifiers, or op amps), so that more sophisticated amplifiers and recording devices were available and affordable. The second-generation (termed DC) monitor, developed by Tjallingii [33], used op amps in simple printed circuits, DC (direct current) applied signal, Faraday cages to control noise, FM tape recorders or rapid-response strip chart recorders as output devices, and (most importantly) higher amplifier sensitivity (Ri of either 10^9 or 10^{13} Ohms) [33; 34]. Tjallingii [33; 34] also established the modern theoretical understanding for EPG science by introducing the concepts of the R (or resistance) component (Ra, described above) and the electromotive force, emf(synonymous with biological voltage orbiopotential) component, blended together in the output signal [35]. The R and emf components are also termed electrical origins of a waveform. There are two known mechanisms underlying the emf component in the plant or plant-insect interface. The first is

disruption of the plant cell membrane's charge separation between external and internal cell environments, by stylet tips breaking living plant cell membranes. Such membrane breakages lead to two electrical effects on waveform output: 1) sudden voltage drops as the stylets puncture a membrane, and 2) positive vs. negative voltage levels that indicate extracellular (apoplastic) vs. intracellular (symplastic) stylet tip positions, respectively. The second biopotential mechanism is streaming potentials, i.e., voltages developed by charge separation that occurs nearly instantaneously in ionic fluids rapidly moving through thin capillary tubes, such as the stylet food and salivary canals [35]. Streaming potentials cause regular-frequency waveforms that result from rhythmic pumping of muscles [18; 35]. Increased amplifier sensitivity in the DC monitor made it possible to detect both R components (previously detected by the AC monitor) and additional emf components in the EPG output waveform. Identifying the electrical origin(s) of a waveform greatly aided in defining its biological meaning.

Another valuable theory of Tjallingii for sharpshooter studies was the sigmoidal R/emf responsiveness curve [33, 34], produced when the proportion (0-100%) of emf in an insect's total EPG output signal is graphed in relation to Ri level (on a scale of 10^6 to 10^{13} Ω) [11]. The lower the Ri level, the smaller the proportion of the total signal that consists of emf (Fig. 2). Because the ratio of R:emf is reciprocal within the total EPG signal (system voltage), the smaller the emf proportion, the larger the R proportion [11,12]. In addition, the position of the R/emf responsiveness curve with respect to Ri can shift with the size of the insect being recorded (Fig. 2; compare small aphids vs. small leafhoppers vs. sharpshooters). Generally, the larger an insect's body (i.e.,with larger-diameter food and salivary canals, therefore greater ionic conductivity), the more the responsiveness curve shifts towards the left on the chart (Fig. 2). Thus, lower Ri levels allow detection of more emf in the signal of large hemipterans like sharpshooter leafhoppers, compared with smaller species like aphids. The maximum number of waveforms, and thus of detectable probing behaviors, will be detected at the 50:50 R:emf balance point. Tjallingii chose an intermediate Ri level of 10^9 Ohms for his (DC monitor) design, to balance R and emf components for small aphids. Thus, the R/emf responsiveness curve explains why an AC monitor (Ri 10^6 Ohms) detects almost no emf component in aphid probing [11], but slightly more in sharpshooter waveforms [13].

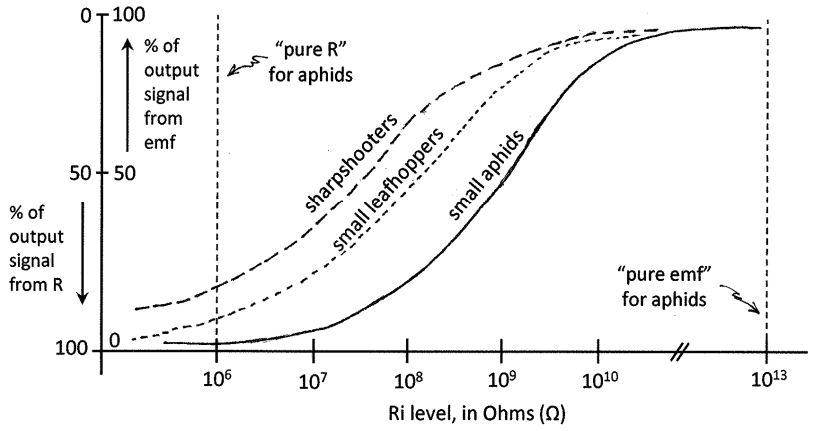


Fig. 2. Theoretical R/emf responsiveness curves for selected example insects. See text for summary, and [11] for more detailed explanation of theory. (Reprinted with permission of the American Phytopathological Society.)

By the mid-1990's, all EPG researchers were using computerized analog-to-digital waveform display, greatly improving waveform fine-structure detail from both AC and DC recordings [31]. Also by that time, EPG had gradually become specialized so that AC monitors were used primarily for medium-to-large insects such as leafhoppers, planthoppers, and other auchenorrhynchans, while DC monitors were used for smaller insects, especially aphids, whiteflies, thrips, psyllids, and other sternorrynchans [5]. In retrospect, the R/emf responsiveness curve explains part of the success of such recordings, because signals from small insects like sternorrhynchans would contain both R and emf at Ri 10^9 Ohms, while recordings of larger insects like auchenorrhynchans would contain both components at lower input impedance (Ri 10^6 Ohms). However, there also may be a difference in tolerance to type of applied signal. Although such differential tolerance is poorly known and an active area of research, anecdotal observations suggest that some leafhoppers (especially sharpshooters) seem to tolerate AC better than DC at different Ri levels, while aphids may tolerate DC better (Backus *unpub. data*). To increase flexibility for all types and sizes of insects, a third-generation EPG monitor (termed AC-DC) was developed by Backus and Bennett [11]. It provides selectable Ri levels of 10^6-10^{10} plus 10^{13} Ohms, choice of AC or DC applied signal, and modernized, up-to-date electronics such as instrumentation-quality op amps on standardized, commercially-manufactured printed circuit boards. This instrument removes artifactual voltages and other problems of older electronics [11, 12]. In addition,

the AC-DC monitor was specifically designed to incorporate all functions of the second-generation DC monitor, while also expanding its capabilities to include greater flexibility of settings. Thus, a researcher can tailor the monitor settings to the specific needs of each insect species recorded. As a result, the AC-DC monitor combines all the advantages of both previous generations of EPG monitor, with none of the disadvantages. Using the AC-DC monitor, it was found that there were no differences in appearance of aphid waveforms based on type of applied signal *per se*, i.e., AC vs. DC. However, large differences occurred based on Ri level, as predicted by the R/emf model [(11)].

Background on *Xylella fastidiosa* and its vectors, sharpshooter leafhoppers

An intimate understanding of the transmission mechanism of *Xylella fastidiosa* (Wells), causative agent of Pierce's disease of grape and numerous other scorch diseases, has eluded scientists for nearly 70 years. The transmission mechanism of *X. fastidiosa* is unique, because the bacterium is the only known arthropod-transmitted plant pathogen that is non-circulative yet also propagative in its vector. Bacteria are acquired and grow as a complex biofilm on the cuticle of the functional anterior foregut in its vectors. Thus, the pathogen is considered foregut-borne [(27)]. The best-studied vectors of *X. fastidiosa* are sharpshooters, comprising two tribes (Cicadellini and Proconiini) in a subfamily (Cicadellinae) of leafhoppers (Cicadellidae) within the suborder Auchenorrhyncha, order Hemiptera.

Bacterial cells are inoculated directly into the plant during sharpshooter stylet probing into xylem vessels. Yet, exactly how such reverse-flow of fluid out of the stylets is possible, and exactly what probing behaviors are responsible, has been an intractable problem until recently. Probing behaviors of salivary sheath-making hemipteran insects like sharpshooters are quite complicated. Therefore, it has been a challenge to answer the "essential question" [(2)]about *X. fastidiosa* inoculation into plants, i.e., *what (specific) probing behaviors are associated with inoculation*? EPG has been vital for the solution.

The anterior foregut in sharpshooters (Fig. 3) consists of two parts; the first is a narrow channel, the precibarium [(15)] that conveys fluid from the food canal in the stylets to the second part, the sucking pump or cibarium. Lining the precibarium are two groups of gustatory (taste) chemosensilla separated by a small, flap-like valve. The precibarial valve occurs in a basin whose structure suggests that fluid is channeled to force the valve

to passively close [15]. Yet, the valve is also powered by a tiny muscle (Fig. 3, vm) whose innervation and location are independent of the cibarial dilator muscles (Fig. 3) that lift the lid of the pump, called thecibarial diaphragm[16]. Fluid is swallowed from the cibarium through the true mouth (Fig. 1, *) into the true foregut, composed of the narrow,short pharynx, then the wider, longer esophagus. The end of the foregut occurs at the non-muscular, passively closing esophageal valve at the entrance to the midgut. The esophageal valve functions in all hemipterans to prevent regurgitation, defined as movement of fluid from the midgut "backwards" into the foregut [19]. Fluid outflow from the stylet tips was thought impossible due to the esophageal valve, until Harris [20] found evidence of (and named) 'egestion' in aphids, and Backus [6] proposed that the egested fluid originated in the precibarium.

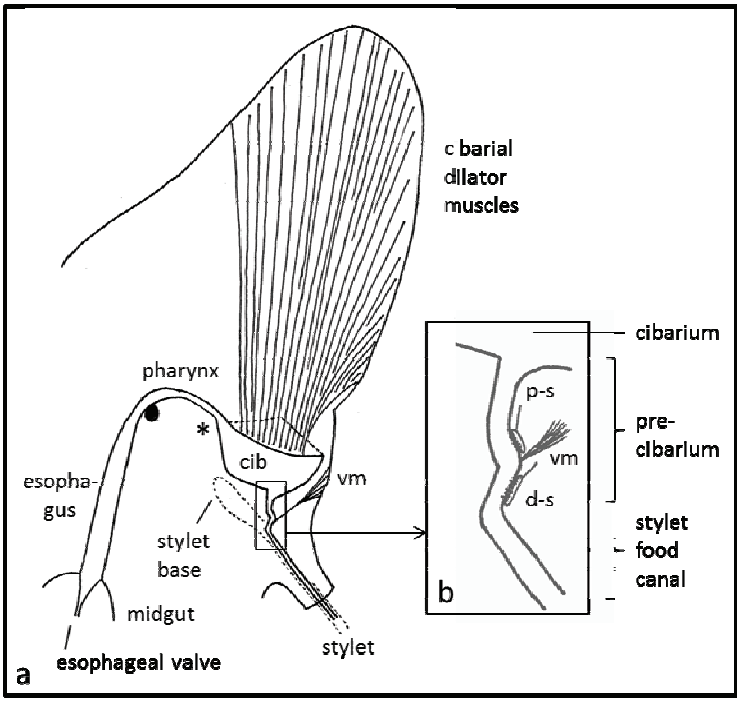


Fig. 3. a. Side view of the head of *H. vitripennis*showing the sizes and positions of structures (described in the text) in the functional foregut of sharpshooters. Small box near the cibarium (cib) is enlarged in inset, part b. *, location of the true mouth; vm, precibarial valve muscle. **b.** Enlargement of the boxed area in part a, showing the fluid-conducting channel formed by the convergence of the stylet food canal, precibarium and cibarium. d-s, neuron cell bodies and nerve from the D (distal)-sensilla; p-s, cell bodies and nerve from P (proximal)-sensilla; black circle, cross section of the tentorium. (Reprinted with permission of the American Phytopathological Society.)

EPG monitors used for studies of sharpshooters

Three of the four EPG-studied sharpshooter species are North American: the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (formerly *H. coagulata*) [32], the smoke tree sharpshooter, *H. literata* Ball, and the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret). The fourth is a tropical Brazilian species, *Bucephalogonia xanthophis* (Berg). The *Homalodisca* spp. are in tribe Proconiini, while *G. atropunctata* and *B. xanthophis* are in tribe Cicadellini.

Seven EPG studies of sharpshooter feeding have been published. *G. atropunctata* was recorded with an electronically updated first-generation (AC) monitor (Ri of 10^6 Ohms) with dynamic noise cancellation [3,10], *B. xanthophis* was recorded with a second-generation (DC) monitor (Ri of 10^9 Ohms) [25], and *Homalodisca* spp. were recorded with a third-generation (AC-DC) monitor (at all Ri levels, but especially 10^6-10^9 Ohms) [7]. AC-DC recordings provided a means of cross-referencing the recordings of the older AC and DC monitors; they revealed waveforms for *Homalodisca* spp. that were nearly identical to those of *G. Atropunctata* and *B. xanthophis* when AC-DCRi levels were set to the same as used for the latter two species, i.e., 10^6 and 10^9 Ohms, respectively. Most importantly, intermediate Ri levels of 10^7 and 10^8 Ohms outputted waveforms intermediate in appearance between older AC and DC monitors. Thus, as predicted, AC-DC recordings bridged the large difference in waveform appearances between the earlier types of recordings of sharpshooters, as also observed for aphid EPG recordings [11].

Mechanism of inoculation of *Xylella fastidiosa* by sharpshooters

EPG evidence reviewed in more detail elsewhere [7] describes experiments that defined the biological meanings of four of the component waveforms of the sharpshooter X wave, i.e., B1w, fB1w, B1s, and C1, showing that they represent the salivation and egestion behaviors of vectors [14]. Based on this EPG evidence, the salivation-egestion hypothesis was proposed. Briefly, it states that inoculation of *X. fastidiosa* occurs when saliva taken up into foregut is egested (via two different mechanisms, rinsing and discharging egestion) into the plant, carrying bacteria.

In more detail, inoculation begins when still-liquid enzymatic gelling saliva[9] is secreted into the plant (represented by B1w), at any point in stylet probing but especially when the stylet tips are in a xylem cell. A portion of that saliva is rapidly brought into the precibarium (via cibarial diaphragm quivering during fB1w; Fig. 4a and inset boxes),

where bacterial cells have already been acquired and colonized. Fluid in the precibarium is then swished back and forth across the distal and proximal precibarial chemosensilla by fluttering of the precibarial valve (during B1s), located between the two sets of sensilla (Fig. 4b). Hypothesized enzymatic degradation by saliva of the matrix cementing *X. fastidiosa*cells to the cuticle [9], combined with mechanical rupture, would cause some cells to be dislodged. Once gustatory sampling is completed, this fluid is not swallowed [14], but instead is swept out through the stylet tipsvia rinsing egestion [17].

Rinsing egestion occurs when the precibarial valve is fully functional, i.e., its rapid movement sweeps material from distal to (below) the valve out of the stylets. Although the precibarium had been proposed as the "staging area" in the foregut from which *X. fastidiosa* bacterial cells are egested [4], the manner and exact precibarial site from which material is egested were not known until a 2011 study [17]. Populations of bacteria in the cibarium remained in equilibrium over six days of acquisition access period (AAP) (Fig 5), when imaged daily[17]. In contrast, populations in the precibarium increased in a progressively more distal (lower) direction from the cibarium, every two to three days. It was striking that virtually no bacterial colonization (only 7 of 50 insects) occurred distal to the valve, and then only when space above (proximal to) the valve was already colonized (e.g., Fig. 5, Day 3). Lack of bacterial biofilm distal to the precibarial valve was interpreted as evidence of fluid turbulence due to valve fluttering, effectively preventing bacterial attachment under normal circumstances. As long as the valve could move, any microbes growing or carried into the area below it would be swept into the food canal and out the stylet tips.

Waveform B1s, and therefore rinsing egestion, is performed in all cell types along the stylet pathway as well as in the xylem [14]. Only a small number of *X. fastidiosa* cells would be rinsed from the area distal to the valve, because the area is small compared with the total length of the precibarium. This explains why small numbers of *X. fastidiosa* have been detected outside of xylem in sheath saliva deposited by *H. vitripennis* on glass [30], in artificial diet [1], and in varying lengths of salivary sheaths in all probed plant tissues (Backus, unpub. data). The presence of bacterial cells in saliva supports the salivation-egestion hypothesis [14]. Symptomatic consequences of inoculation into non-xylem cells are presently unknown, although it is assumed that bacteria cannot survive outside xylem.

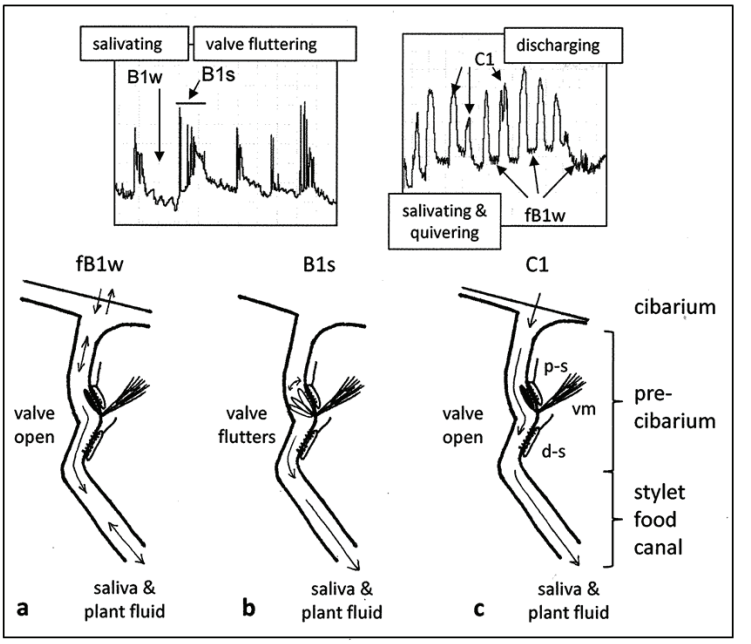


Fig. 4. Illustration of the sequential behavioral steps represented by the parts (inset boxes) of the sharpshooter X wave. **a.** Combined salivation and cibarial quivering during fB1w pulls small amounts of saliva plus plant fluid into and out of the precibarium while the precibarial valve (black oval) is open. **b.** Fluttering of unencumbered precibarial valve (black-lined oval) (and possibly also cibarial quivering, not shown) during B1s causes fluid turbulence and outward push of fluid below (distal to) the valve (rinsing egestion). **c.** Rapid fall of the cibarial diaphragm during C1 causes strong flow of fluid outward (discharging egestion) that may dislodge blockages clogging the valve (black oval) open. d-s = distal (precibarial chemo)sensilla; p-s = proximal sensilla; vm = valve muscle. (Reprinted with permission of the American Phytopathological Society.)

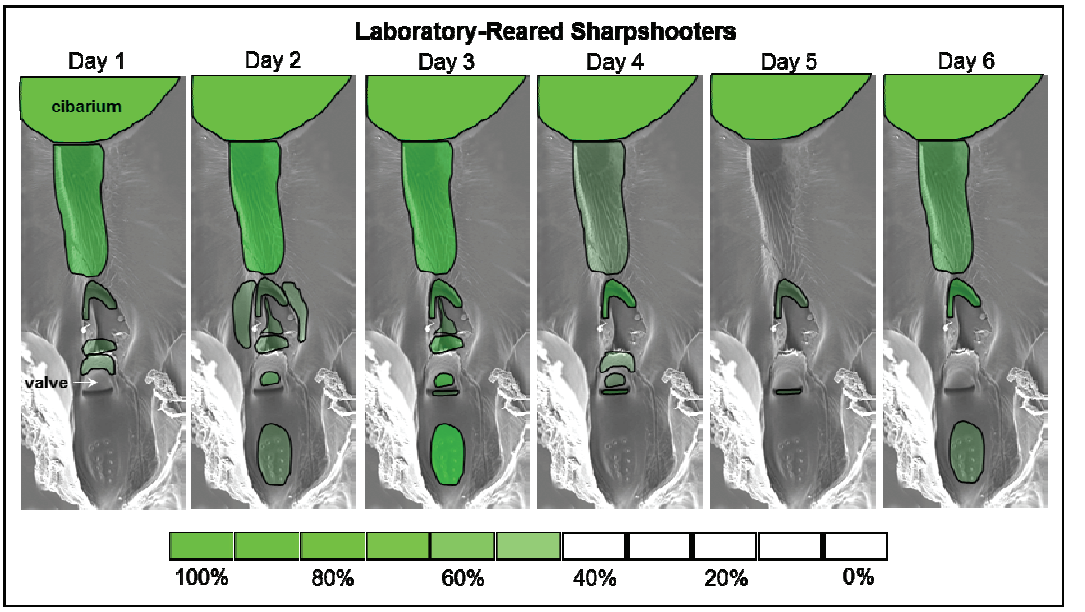


Fig. 5. Pictorial representation of the percentage of 10 laboratory-reared sharpshooters for each of 6 days of AAP that showed any amount of *X. fastidiosa* bacteria in each location in the precibarium or cibarium. Data are visually represented by green shapes superimposed over a scanning electron micrograph of a clean precibarium, as an anatomical reference. Each shape represents one of nine different locations in the precibarium. Degree of transparency of shape denotes percentage, as shown in the key on the bottom. For example, the most opaque area (the cibarium) had a probability of 100% bacterial occupancy (10/10 insects), whereas the most transparent green area (under the precibarial valve, day 4) had a probability of 10% occupancy. Modified from [17]; for names of precibarial locations, see original figure. (Reprinted with permission of the American Phytopathological Society.)

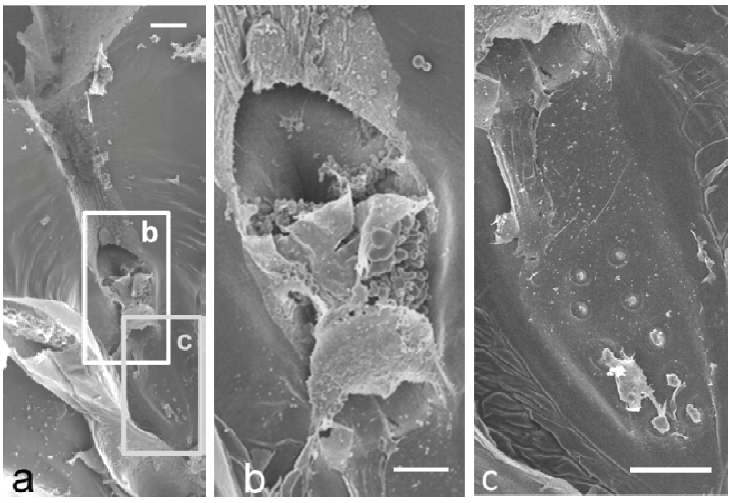


Fig. 6. Scanning electron micrographs of microbes on the floor of the precibarium of field-collected *H. vitripennis*. **A.** Overview, showing microbial biofilm in the cibarium that has grown into the trough of the precibarium. Boxes b and c are enlarged in parts b and c. Scale bar, 25 µm .**b.** Close-up of unknown, rounded microbes in biofilm matrix that overlays the precibarial valve and part of the basin around it; the valve appears to be affixed open by the accumulated biofilm. Scale bar, 10 µm. **c.** Close-up of the D sensilla field distal to the precibarial valve showing biofilm deposits extending from the affixed valve, and encroaching on the D-sensilla. Scale bar same as part b. (Reprinted with permission of the American Phytopathological Society.

Discharging egestion occurs when accumulations of microbes (either *X. fastidiosa* or some other microbial species) cover or clog the precibarial valve, causing it to be affixed open (Fig. 6). Such valve malfunction would likely cause fluid turbulence in the distal end of the channel to be greatly diminished, and allow microbial colonization to occur below the valve. Once the full length of the precibarium becomes heavily colonized by microbes (e.g., Fig. 5, Day 3), the insect must clear such obstructions; otherwise, it will be unable to taste its food or swallow. The only way to mechanically scrub away obstructing material in the precibarium would be sudden, rapid (snap-like) release of the cibarial diaphragm, before the cibarium is completely full. Such rapid cibarial release has been correlated with waveform C1 [(7)]. Rapid drop would forcefully propel fluid into the precibarium and out the stylets (Fig. 7c); this propulsion is termed discharging egestion.The sudden disappearance of all *X. fastidiosa* accumulations along the full length of the precibarium on the fourth to fifth days of AAP (Fig. 5, Day 5) provides evidence of discharging egestion from the precibarium [(17)]. If they were being swallowed, the bacteria would not disappear from below (distal to) the precibarial valve.

Also, the density of bacteria in the cibarium, as well as precibarium, would significantly (and simultaneously) decrease; yet the cibarium remains fully occupied (Fig. 5).

Discharging egestion is probably the most epidemiologically significant inoculation behavior performed by sharpshooter vectors. Based on the above EPG evidence, it is proposed that discharging egestion occurs nearly exclusively in xylem cells [14]. If a vector's precibarium is heavily loaded with *X. fastidiosa* cells, stronger and more frequent events of discharging egestion must be performed within multiple xylem cells, both rejected and accepted cells [14]. Accordingly, the largest number of *X. fastidiosa* cells would be inoculated into the largest number of xylem cells from the most heavily clogged precibaria, probably every two to three days. This may be one reason why AAPs lasting at least two days in source plants have the highest single-probe success of inoculation[21] (Backus, unpub. data). It also supports previous findings that success of acquisition in large part determines success of inoculation [6].

When a sharpshooter secretes and then takes up saliva into its precibarium during the X wave, the bulk of material egested will be saliva, and thus any egested bacteria may be embedded in a bolus of (probably hardened) sheath saliva in the xylem, such as that shown in Fig. 7a. Would such bacteria be permanently entrapped, or can they escape from the saliva to initiate the infection process? To begin to address this question, a small study was performed to examine the interaction between bacteria and saliva in grapevine petioles [8]. Eight *H. vitripennis* were restricted to a 5 cm length of stem for 24 hrs, to produce numerous salivary sheaths in the stem. After removal of insects, green fluorescent protein-expressing *X. fastidiosa* (*Xf* GFP) were mechanically inoculated into the same area; tissues from the insect-probed, inoculated stems were excised 30-60 min later, prepared for immunohistology, and 10 salivary sheaths near needle inoculations of bacteria were examined via confocal microscopy. Four of the ten sheaths had entered xylem vessels that later became filled with *Xf* GFP transported from distant needle punctures. Thus, there was no physical needle-damage to the hardened sheath saliva therein. Most bacteria in the vessels were clearly outside the sheath saliva (Fig. 7a and b). However, a small number were inside the saliva, near the edge of the bolus (Fig. 7b). Several lines of evidence [8] support that the *Xf* GFP were able to penetrate into the portion of hardened saliva left by feeding sharpshooters in the xylem vessel, by some unknown mechanism, perhaps bacterial secretion of enzymes. Such penetration into semi-solid enzymatic gelling saliva suggests that *X. fastidiosa* should be able to move out of soft, newly-secreted saliva before it had fully stiffened, thus providing indirect

evidence for the salivation-egestion hypothesis.

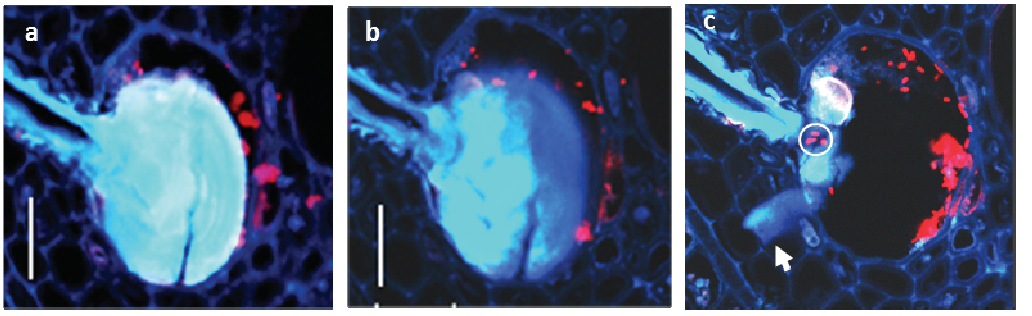


Fig. 7. Confocal microscopy image of a bolus of gelling saliva (pale teal blue) injected into a grapevine stem xylem cell at the end of a probe by *H. vitripennis*, several hours prior to mechanical inoculation of *X. fastidiosa* into the same stem. Inoculated bacteria (red) were translocated upward in xylem into the vicinity of this previously secreted, undamaged salivary sheath. **a.** Full, 10 µm-thick section, showing immunostained bacteria in the xylem cell, but outside the salivary bolus. **b.** An optical section 0.5 µm thick, through the middle of the same salivary bolus, showing red-immuno-stained bacteria inside the outer edge of the bolus (arrows). **c.** An optical section 0.5 µm thick, through the side of the same salivary bolus, showing three red-immunostained bacteria (white circle), possibly wild-type, near the stylet entry point. All three images are the same magnification; both scale bars 25 µm. (Reprinted with permission of the California Department of Food and Agriculture.)

In addition, the immunohistology study of *H. vitripennis*-probed grape stems described above detected three *X. fastidiosa* cells embedded in the salivary bolus near what appears to be the stylet entry point, far from the bacteria that penetrated the exterior of the bolus (Fig. 7c) (8). These cells may have been wild-type bacteria (also immunostained, similar to the mechanically inoculated bacteria) injected into the xylem vessel by the sharpshooter, known to have acquired *X. fastidiosa*. This finding also supports the salivation-egestion hypothesis. However, this putative saliva-inoculation event was not correlated with EPG waveforms.

The salivation-egestion hypothesis is, in some respects, a combination of the past and present hypotheses for vector inoculation of non- and semi-persistent pathogens, i.e., the egestion hypothesis [20] and the salivation hypothesis [28]. The salivation-egestion hypothesis may be applicable to all known or suspected cases of foregut-borne, non-circulative pathogen inoculation[27], such as *Maize chlorotic dwarf virus* by *Dalbulus* spp. leafhoppers [36] or *Cauliflower mosaic virus* by *Brevicoryne brassicae* L.

aphids, when given long AAPs [26]. The general idea that pathogens colonizing the precibarium can be loosened by a combination of mechanical turbulence and saliva in the precibarium, then egested out the stylet tips, seems applicable to multiple vector-pathogen systems. Because no experimental evidence contradicts the salivation-egestion hypothesis, it is likely that EPG was the breakthrough technology that solved the 70-year old mystery of the inoculation mechanism of *X. fastidiosa*.

The above-summarized basic research on the mechanism of *X. fastidiosa* inoculation has direct impact for applied problems in integrated pest management, also thanks to EPG. The sharpshooter X wave is a distinctive, readily recognized waveform that can be easily quantified. Present research in the Backus laboratory uses EPG to record vector behaviors on resistant vs. susceptible plants. Recent preliminary research (Backus, unpub. data) has shown that significantly fewer and shorter X waves are performed by insects on *X. fastidiosa*-resistant grapevines, compared with susceptible. Thus, it may be possible to select for grapevines that are resistant to vector inoculation behaviors, as well as *X. fastidiosa* growth and spread in the plant. Research is underway to eventually use quantitative analysis of X wave performance to develop an "inoculation behavior resistance index" that can be used by grape breeders to develop commercially viable, resistant grape with a diverse collection of *X. fastidiosa*-resistance traits. Thus, basic EPG research seamlessly evolves into applied research to solve real-world problems in management of vector-borne plant diseases.

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Tospoviruses and Thrips-is There an Evolutionary Relationship?

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ABSTRACT

Evidence from evolutionary relationships among Thysanoptera suggest that the associations between a few unrelated species of these insects with the Bunyaviridae. Tospoviruses does not have a long evolutionary history. Since tospoviruses are fully dependent for their existence on the few vector thrips species, the implication is that tospoviruses are a recent evolutionary phenomenon. The suggestion is made that variation in vector ability might be a more useful avenue for research than plant breeding to control these essentially vertebrate viruses.

Keywords: tospoviruses, *Frankliniella*, *Thrips*, vector ability, inheritance

INTRODUCTION

The infection of a plant with a Tospovirus leads to necrosis of the plant tissues, distortion of fruits, serious yield reduction in some crops, and sometimes death of entire plants. The 800 or more species of plants that have been recorded as being infected by tospoviruses include many crops, such as tomatoes, capsicums, watermelons, lettuce, and groundnuts, as well as flower crops such as chrysanthemums, irises, and impatiens. Tosposviruses occur in every major agricultural area around the world, and they rank among the ten most detrimental plant viruses worldwide. That is, for anyone involved in horticultural crop production, tospoviruses are important.

However, tospoviruses cannot exist in nature on their own. They are neither seed transmitted nor root transmitted, and thus they cannot move from plant to plant, whether across a single field, or within a field from one season to the next. Under field conditions tospoviruses are transmitted from one plant to another only by particular species of thrips. Moreover, this association is highly specific, in that for an adult to be able to transmit a tospovirus, it must have obtained that virus during its development at the second instar larval stage [(4)]. Adult thrips cannot acquire and then transmit a

tospovirus. As a result of this association, Tospoviruses do not have an independent existence-they are fully dependent on just a few particular species of thrips for their continued existence in nature. In the laboratory, plant pathologists transmit tospoviruses by rubbing sap from an infected leaf into an uninfected leaf, but this does not happen in nature. The other essential fact to be considered is that Tospoviruses are members of the Bunyaviridae-a family of viruses that are associated with animals and are not plant viruses.

Thus we have a remarkable situation. How did one or more virus in an animal come to be associated with, and dependent on, a few species of insects that feed on plants, and how have these animal viruses come to cause serious damage to plant cells. Accepting that nothing makes sense in biology except in the light of evolution, the essential question thus becomes-what was the origin of the associations between thrips and tospoviruses? Did one plant-feeding thrips acquire from some unknown animal a particular bunyavirid species, and was this then modified within the thrips and inserted into a plant? Such a route seems unlikely, but the alternative is even less likely. This would involve an animal infected with a bunyavirid browsing on and infecting a plant with that virus. The bunyavirid would then need to have been modified within the plant, and subsequently ingested by a thrips that then transmitted the disease to another plant. Considering the second of these two scenarios, there is a good probability that many animals infested with bunyavirids will browse on plants, but the rest of this transformation route seems too tortuous for serious consideration. In contrast, some of the thrips species that are tospovirus vectors are predatory on other arthropods, and adults of several species of thrips probe the skin of vertebrates, including man. Moreover, the typical thigmotactic behaviour of adult thrips commonly results in these insects being found in dark places, including the nests of birds and mammals. A potential pathway thus exists for a bunyavirid from a vertebrate directly into a thrips-but this is still a bunyavirid, not a tospovirus.

Leaving such conjectures concerning the origin of tospoviruses for consideration by future molecular biologists, the next important question concerning the thrips association with tospoviruses becomes – did the association evolve once only, or did it evolve several times? That is, did the genus *Tospovirus* evolve from Bunyaviridae just once and subsequently diversify into a series of species, progressively acquiring the diverse thrips vectors that are now recognised? Or did the association between thrips and these viruses arise several times, possibly even from different Bunyaviridae, and in

association with different thrips species? This second possibility involves the genus *Tospovirus* being polyphyletic, with several virus species having evolved independently in association with different thrips species, possibly even on different continents.

Tospovirus specialists will consider the molecular evidence relevant to such questions, but the present discussion is concerned with the evidence from thrips. First, only a few thrips species are tospovirus vectors, and these species are not all closely related. Thus only five of the 160 species of *Frankliniella*, and only 3 of the 280 species of *Thrips* genus, are known to be tospovirus vectors (Table 1). These two large genera are not sister genera, and are not considered to be particularly closely related within the subfamily Thripinae of the Thripidae [(1)]. The genus *Frankllíniella* is essentially New World, whereas *Thrips* is primarily Old World with some species in North America but no species native to South America. The two genera thus possibly evolved around the time that the Atlantic Ocean was developing between these two land masses. Was the association between thrips and tospoviruses in existence at that time, such that the ancestors of *Frankliniella* and *Thrips* were already vectors? If so, then either there must be many species in these two genera that are unrecognised vectors of unrecognised tospoviruses, or else most species in these genera have lost the ability to be vectors. There is no evidence to support either of these scenarios, and neither of them seems likely. Moreover, if this discussion is extended to consider the three unrelated vector thrips in the genera *Ceratothripoides*, *Dictyothrips*, and *Scirtothrips*, the situation becomes even more complex. This would then involve an assumption that the ancestor of the family Thripidae was a tospovirus vector, and that more than one thousand species in this family have lost the vector ability.

Table 1. Generic relationships of tospovirus vector species

Genera of Thripinae	Species described in genus	Species recorded as vectors
Ceratothripoides	5	1
Dictyothrips	1	1
Frankliniella	230	5
Scirtothrips	100	1
Thrips	280	3

Inoue and Sakurai [(2)] examined the phylogenetic relationships between a series of tospovirus species and a series of thrips species. They concluded that there was

considerable congruence between the phylogeny of thrips and that of tospoviruses, and they were able to recognise three different clades among tospoviruses (Fig. 1). One of these was associated with species of genus *Thrips* as vectors, and these viruses are found primarily in plants of the Cucurbitaceae and Liliaceae. A second clade of tospoviruses was associated with species of *Frankliniella* as vectors, and these viruses are found mainly in Solanaceae and Asteraceae. A third clade was associated with *Scirtothrips* as vector, and these viruses are found on Fabaceae. This study suggests the possibility that the associations between groups of tospoviruses and different genera of thrips vectors have originated independently. Unfortunately, this type of investigation is complicated by the fact that several pest thrips, and many crop plants and their viruses, have been disseminated widely around the world by human trade. *Thrips tabaci* probably evolved in the eastern Mediterranean in association with the genus *Allium*. But the Romans took onions and garlic across Europe to Britain, and Alexander's army presumably did the same across Asia to India. Later, the Spanish took onions and their thrips to South America, the Portuguese to India, the Dutch to Indonesia, and the British to Australia. The areas of origin of the tospoviruses are thus likely to be more difficult to predict than the areas of origin of the known vector species. This translocation of vectors and of viruses between continents has presumably facilitated the ability of a widespread species such as *Thrips tabaci* to vector relatively unrelated tospovirus species.

Table 2. Geographical areas of origin of tospovirus vector species

Vector species	American continent	Asia	Eurasia
Frankliniella bispinosa	+		
Frankliniella fusca	+		
Frankliniella occidentalis	+		
Frankliniella schultzei	+		
Frankliniella zucchini	+		
Ceratothripoides claratris		+	
Scirtothrips dorsalis		+	
Thrips palmi		+	
Thrips setosus		+	
Dictyothrips betae			+
Frankliniella intonsa			+
Thrips tabaci			+

Five of the vector species originated unequivocally on the American continent, whereas four of the vectors are equally clearly Asian in origin, and three vector species seem to be of European or Eurasian origin (Table 2). However, these three areas of origin of the vector species need not reflect the areas where a particular thrips species became associated with one or more tospoviruses. Moreover, since some vector species transmit more than one tospovirus, it is possible that for such species the ability to transmit one virus might have facilitated the acquisition, and ability to transmit, other tospoviruses. Two parts of the world are noticeably absent from this discussion, Australia and tropical Africa. Studies on the insects and viruses of Africa are not extensive, but the thrips fauna of Australia has been intensively studied in recent years, and there is no evidence that any Australian native thrips species is a tospovirus vector. Moreover, despite many plant species that have been introduced to Australia suffering from tospovirus infections, where the viruses are vectored only by non-native thrips species, there is no evidence that any native Australian plant is infected by a tospovirus when growing in its natural habitat.

Because tospoviruses affect plants, much of the research on these organisms is carried out by plant pathologists and plant breeders. As a result the major research target is on producing tospovirus resistance in plants. In contrast, much of the success in controlling human diseases has been, not through breeding disease resistant humans, but though reducing the rates of disease transmission. In this way, the population of a pathogen is reduced below a critical level for continued transmission. Crop entomologists emulate this strategy by insecticide spraying, with the hope of reducing thrips populations and hence virus transmission. However, a more effective long-term solution might be to exploit the known variation in vector ability among populations of thrips species [(5)]. Such variation investigated by Jacobson & Kennedy [(3)] suggests that thrips might carry genes that are involved in limiting the efficiency with which individuals of some populations act as vectors. Breeding from thrips individuals with reduced vector capacity, and releasing such populations under controlled conditions, might well drive down the rate of transmission and thus hold the possibility of eliminating locally a targeted tospovirus. However, biological approaches such as this, involving critical studies on the behaviour and genetics of the insect vectors, are at present less likely to attract research funding than molecular studies on viruses.

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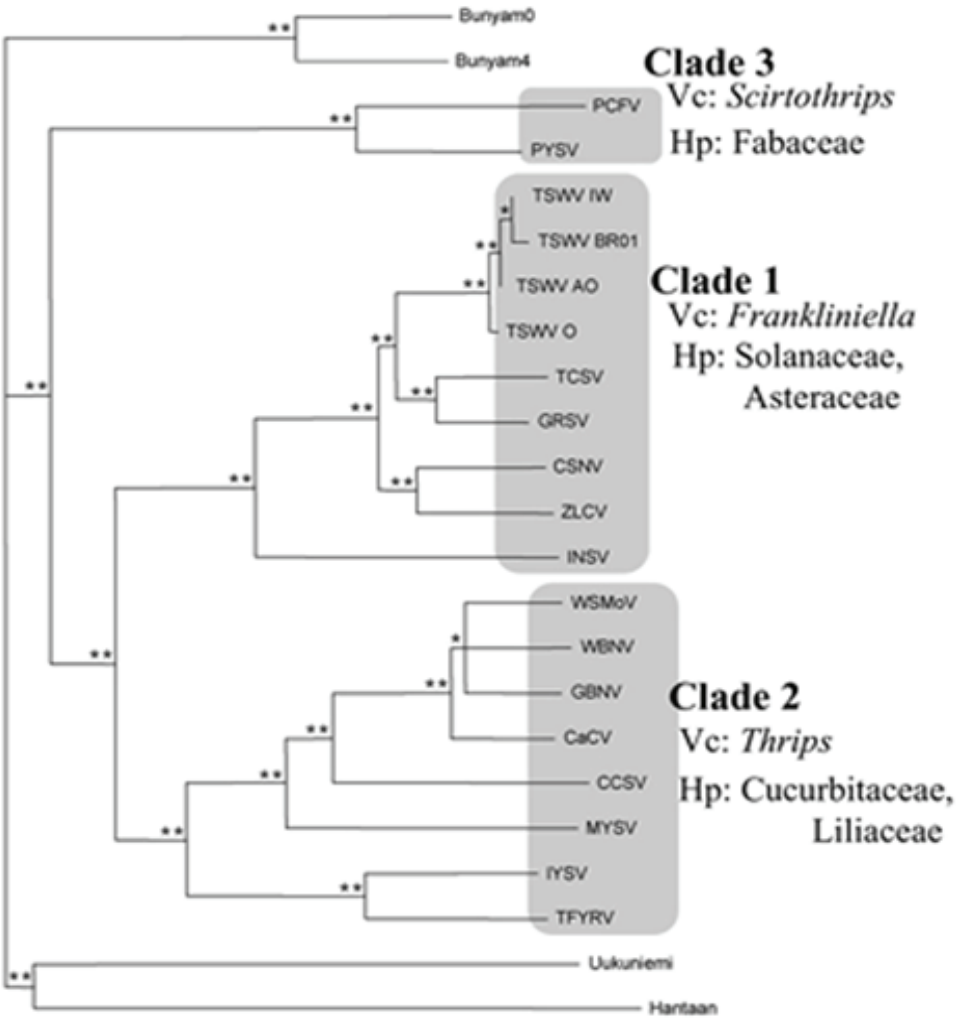


Fig. 1. Phylogenetic relationships between tospoviruses and thrips - after Inoue and Sakurai [(2)].

Tomato Leaf Curl Disease in Taiwan and Breeding for Resistance Against it

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ABSTRACT

In the past few decades, whiteflies have become more important as agricultural pests in many areas. The whitefly-transmitted geminiviruses (begomoviruses) have also become major constraints to tomato production worldwide. A tomato-infecting begomovirus, *Tomato leaf curl Taiwan virus* (ToLCTWV), was first detected in Taiwan in 1981, and was endemic throughout the island by the early 1990's. Since then, three other tomato-infecting begomoviruses have been detected in Taiwan; *Ageratum yellow vein Hualien virus* (AYVHuV) and *Tomato leaf curl Hsinchu virus* (ToLCHsV) have been detected only occasionally from tomato, whereas *Tomato yellow leaf curl Thailand virus* (TYLCTHV), which was probably imported to Taiwan in about 2005, is now widespread throughout Taiwan. TYLCTHV is a bipartite and mechanically transmissible begomovirus, whereas ToLCTWV is monopartite and not mechanically transmissible. Through monitoring the begomoviruses in the tomato production areas over time, it is apparent that the introduced TYLCTHV is now displacing ToLCTWV in most areas. The tomato leaf curl resistance gene *Ty-2* was identified in Taiwan using ToLCTWV, and was incorporated into tomato cultivars to help manage the leaf curl disease. However, tomato cultivars carrying the *Ty-2* gene are not resistant to TYLCTHV which causes severe disease symptoms on them. The combining (pyramiding) of different *Ty* resistance genes in new tomato cultivars shows potential for resistance to leaf curl disease in Taiwan. The combined use of resistant cultivars and cultural methods to exclude vector whiteflies and prevent virus infection is the most effective and sustainable strategy to manage the tomato begomovirus complex in Taiwan and elsewhere.

Keywords: Begomovirus, Ty resistance, resistance breeding, disease management

INTRODUCTION

Over the last 30 years, the whiteflies (*Bemisia tabaci*) have emerged as major pests in agricultural areas worldwide, and as a consequence, plant diseases caused by whitefly-transmitted viruses such as the begomoviruses have also been increased [18]. In tropical and subtropical tomato production areas, begomoviruses often caused severe disease epidemics [25, 30, 37, 38, 40]. The disease are commonly known as tomato leaf curl or tomato yellow leaf curl disease as the major symptoms are leaf curling, interveinal and marginal yellowing, interveinal crinkling and internode shorting (Fig. 1) [40]. The disease can cause significant yield loss; up to 100% loss is frequent [29, 30, 37]. The tomato-infecting begomoviruses are genetically diverse, and so far more than 100 species have been delineated based on the International Committee on Taxonomy of Viruses (ICTV) species demarcation criteria [38, 39, 41]. This diversity compounds the difficulty in managing the diseases, especially where several different species are present at the same time. More than 12 tomato-infecting begomovirus species have been reported in China, five in Indonesia, four each in India, the Philippines and Taiwan, and at least two each in Bangladesh, Iran, Japan, Laos, Thailand and Vietnam [37, 38, 39, 41]. While some tomato-infecting begomovirus species are only present in small areas, others are more widely distributed across several countries. For example, *Tomato leaf curl Taiwan virus* (ToLCTWV) has been detected in China and Taiwan [26, 39], while *Tomato yellow leaf curl Thailand virus* (TYLCTHV) has been detected in China, Myanmar, Taiwan and Thailand [12, 13, 31, 33, 38]. The widely distributed *Tomato yellow leaf virus* (TYLCV) has spread from its likely origin in the middle East (Israel) to become the dominant tomato-infecting begomovirus in many countries including China, Dominican Republic, Israel, Italy, Japan, South Korea, Spain, USA and islands of the Indian Ocean [5, 6, 9, 30, 32, 41].

Tomato-infecting begomoviruses are monopartite with a single circular single-stranded DNA (ssDNA, DNA-A like) genome, or bipartite having a genome of two circular ssDNAs, DNA-A and DNA-B [21] (Fig. 2). The DNA-A or DNA-A like component is essentially for virus reproduction. They contain two open reading frames (ORFs; AV1 and AV2) in viral sense and four (AC1, AC2, AC3 and AC4) in complementary sense. Viral DNA-B component contains an ORF each in viral sense (BV1) and complementary sense (BC1). The AV1 encodes the coat protein (CP) and AV2 is associated with virus movement. The AC1 is the viral DNA replication associated protein. The AC2 protein is a transcriptional activator. AC3 protein enhances

viral DNA replication. The AC4 can determine symptom development. The BV1 and BC1 are the nuclear shuttle protein and the movement protein, respectively. AC2 and AC4 can also act as suppressors of gene silencing [4, 44].

The deployment of natural resistance is considered the most sustainable strategy for controlling tomato leaf curl disease. No effective resistance was discovered in the cultivated tomato (*Solanum lycopersicum*), so the tomato wild relatives such as *S. chilense*, *S. habrochaites* f. *glabratum*, and *S. peruvianum* have been screened for resistance [10]. Five resistance genes (designated *Ty-1*, *2*, *3*, *4* and *5*) have been identified and deployed to greater or lesser extents [1, 14, 16, 17, 45]. The first to be identified, *Ty-1*, originated from *S. chilense* (LA1969) and was mapped to chromosome 6 [45]. Next, *Ty-2* was derived from *S. habrochaites* (B6013) and mapped to chromosome 11 [14]. The alleles *Ty-3* and *Ty-3a* originated from *S. chilense* LA2779 and LA1932, respectively, and map to chromosome 6 [16]. *Ty-1* and *Ty-3* have recently been shown to be allelic [42]. Also from *S. chilense* LA1932, *Ty-4* has been mapped on chromosome 3, but is less effective than the other genes [17]. Recently, *Ty-5* was identified from breeding line TY172 (originating from *S. peruvianum*) and mapped to chromosome 4 [1]. In addition, resistances to the vector whiteflies have been identified in *S. pennellii* and *S. habrochaites* [2, 3, 27].

In this review, the tomato-infecting begomoviruses of Taiwan, as well as the possible effect the resistance breeding efforts have had on the population dynamics will be described.

The tomato-infecting begomovirus in Taiwan

In Taiwan, the tomato leaf curl disease was first observed in 1981 in Tainan County. Later on, the causal agent was confirmed as a begomovirus based on the observation of geminate particles with an electronmicroscope, and a positive reaction with an antibody against tobacco leaf curl virus, a begomovirus form Japan [11]. By the 1990's, the disease had spread throughout the island of Taiwan and was causing significant yield losses of up to 84% [34, 36]. In 1997, the causal virus was identified by viral genomic sequence as *Tomato leaf curl Taiwan virus* (ToLCTWV) based on the species delimitation criteria of the ICTV [21]. The host range of ToLCTWV was limited to tomato (*Solanum sp.*), *Datura stramonium*, *Lonicera japonica*, *Nicotiana benthamiana*, *Petunia hybrida*, *Physalis floridana* and *S. melongena* [11]. Later on, ToLCTWV was detected in Southeast China [26]. The ToLCTWV Taiwan isolates were confirmed as a

monopartite begomovirus since no DNA-B was detected in samples that tested positive for ToLCTWV alone [38]. Agroinoculation of tomato plants with an infectious clone of a ToLCTWV DNA-A like component alone also generated similar symptoms as a field isolate. The ToLCTWV Taiwan isolates were classified into 3 strains [38]. The strain A is commonly found throughout the country, whereas the strains B and C were restricted to East and West Taiwan, respectively.

In 2000, a second tomato-infecting begomovirus, *Tomato leaf curl Hsinchu virus* (ToLCHsV) was detected in Hsinchu area of Taiwan [38]. However, the distribution of ToLCHsV was limited to the Hsinchu area with low detection frequency; only four of 14 begomovirus-positive samples collected in 2000 and two of 39 samples in 2001. ToLCHsV was not detected after that in Taiwan. However, after ToLCHsV was detected in Taiwan, a high genetic identity virus was detected in plants of Ramie (*Boehmeria nivea* L.) in China, and hence the name Ramie mosaic virus (RamMV) was given. The ToLCHsV/RamMV in China was found to infect tobacco also, but has not been detected in tomato in the field in China. Since RamMV was found to infect ramie in many provinces of China, it may be that ramie represents the original host of ToLCHsV [23].

The third tomato-infecting begomovirus detected in Taiwan was *Ageratum yellow vein Hualien virus* (AYVHuV) **[38]**. The virus was only detected in two samples collected in Hsinchu area in 2003, and these isolates were identified as likely recombinants between ToLCTWV and AYVHuV. The virus background was AYVHuV ageratum isolate and the recombination region (607 nucleotides in length) including the complete AC4 ORF, the 5' half of AC1 (Rep) ORF and left part of the IR was most similar (92.8%) to the corresponding region of the ToLCTWV strain B tomato isolate.

The forth tomato-infecting begomovirus detected in Taiwan was *Tomato yellow leaf curl Thailand virus* (TYLCTHV). TYLCTHV had previously been detected in south China, Myanmar and Thailand **[12, 24, 33]**, but its first detection in Taiwan was in 2005 in Central West Taiwan suggesting it was only a recent introduction to Taiwan **[38]**. After 2005, TYLCTHV spread rapidly in West Taiwan and then in East Taiwan (Table 1). TYLCTHV Taiwan isolates was found to infect not only tomato but also pepper plants in the field **[35]**. This indicated pepper was a natural host for TYLCTHV, but not for ToLCTWV. TYLCTHV has been considered as a bipartite begomovirus since it is always detected as a combination of DNA-A and DNA-B, and it can be mechanical transmitted **[38]**. Although agroinoculation of an infectious clone of the DNA-A alone

results in development of symptoms; symptoms are mild and the development is much delayed in comparison to when both components are present, and infection with DNA-A alone is not mechanically transmissible.

Mixed infection of tomato plants with two begomoviruses was commonly detected in Taiwan, including with the less common species, AYVHuV or ToLCHsV. Because of the relatively high genetic diversity of ToLCTWV and its predominance before the emergence of TYLCTHV, it was considered to be the endemic tomato-infecting begomovirus in Taiwan [(38)]. However, since it was first detected in Western Taiwan in 2005, TYLCTHV rapidly spread to become prevalent across all tomato-growing regions of Taiwan. Virus survey results show that TYLCTHV is likely to displace ToLCTWV in many parts of Taiwan (Table 1). Whether this dynamic change of tomato-infecting begomovirus species affects the tomato production and breeding effort in Taiwan or vice-versa will be discussed below.

Resistance breeding against tomato-infecting begomoviruses in Taiwan

Since the emergence of tomato leaf curl disease caused by begomovirus in the early 1980s, the disease has become prominent and a major limiting factor for tomato production in Taiwan by the early 1990s. The reduction in tomato productivity in Taiwan from 35-52 tonnes/ha (1983-1992) to 24-26 tonnes/ha (after 2005) is attributed in part to the increased prevalence of leaf curl disease (Table 2). Since deployment of tomato cultivars carrying resistance is considered the most effective and sustainable method for managing tomato (yellow) leaf curl disease, especially for resource-poor growers in developing countries. AVRDC- the World Vegetable Center started to try to identify sources of resistance and include them in the tomato breeding program soon after leaf curl disease became a problem in Taiwan. Resistance to ToLCTWV was identified in accessions of *S. chilense*, *S. habrochaites* f. *glabratum*, *S. lycopersicum* and *S. peruvianum* [(10)]. Based on screening with ToLCTWV, leaf curl disease resistance identified from the tomato line H24, derived from *S. habrochaites* B6013, was included in the breeding program in 2000, and commercial varieties carrying this resistance were released in Taiwan in 2003. The gene responsible for resistance was subsequently mapped and named *Ty-2* [(14)].

When TYLCTHV was first detected in Taiwan in 2005, it caused severe disease symptoms in tomato lines carrying the *Ty-2* gene [(15)]. This indicates the *Ty-2* gene is not effective against TYLCTHV. All other resistance sources identified by screening with

ToLCTWV did not provide effective resistance to TYLCTHV. Recently, the tomato line FLA456 was identified as being able to provide a good level of tolerance to TYLCTHV, and two major and two minor QTLs were identified as involved in the resistance [20]. Pyramiding of several resistance genes in a variety can provide broader and more durable disease resistance in crops [19]. Combining two resistances from wild species provided increased resistance to TYLCV [43]. The pyramiding of resistance genes has been also tested for controlling tomato-infecting begomovirus disease in Taiwan. Tomato lines combining *Ty-2* and *Ty-1*/*Ty-3* were generated and identified as being able to provide the greatest resistance to leaf curl disease in the field in Taiwan where both ToLCTWV and TYLCTHV may present [28]. However, combining resistances to virus and its vector whitefly into tomato cultivars have the potential to generate more substantial resistance to tomato-infecting begomovirus disease in Taiwan. As well as resistance to the begomoviruses, resistances against the vector whitefly (*B. tabaci*) have been identified in accessions of *S. pennellii* and *S. habrochaites* [2, 3, 27]. Combining resistances to the virus and its vector in tomato cultivars has the potential to provide better leaf curl disease control and improve the durability of the resistance against the virus. Thus, AVRDC- the World Vegetable Center is also in the process of pyramiding begomovirus resistance with whitefly resistance into improved tomato breeding lines.

Management of tomato-infecting begomoviruses

The management of tomato leaf curl disease caused by begomoviruses is difficult and methods used are often unsuccessful. Various cultural practices have been used to control tomato leaf curl disease [27]. These include methods for reducing the sources of virus inoculum and reducing vector whitefly population. Virus inoculum can be reduced by good field sanitation including roguing diseased plants and cleaning the field of crop debris after final harvest. The whitefly population can be controlled by application of appropriate pesticide, deployment of sticky yellow traps, intercropping with non-host plant species, and using biological and physical barriers. Controlling the weeds, especially alternative hosts of the virus and/or whitefly, can also contribute to leaf curl disease management. Whiteflies transmit begomoviruses in the persistent manner; they remain viruliferous for life after virus acquisition. Since young tomato plants are a preferred host for whiteflies, protecting tomato seedlings, for example within a whitefly-proof net cage, will prevent them from becoming infected during this very vulnerable stage. However, it is difficult to convince growers to strictly keep the net

cages closed and thus keep the whiteflies out since this interferes with their routine cultural practices, and because it takes weeks for disease symptoms to develop after inoculation, so the link between whiteflies and leaf curl disease is not obvious. Tomato plants infected at the seedling stage will often not develop pronounced leaf curl symptoms until several weeks after transplanting, by which time the virus has been transmitted to neighbouring plants and it is no longer possible to control the disease.

Although the deployment of resistant or tolerant tomato cultivars is considered likely to be the most effective and sustainable means of controlling leaf curl disease, the strategy should be implemented with care to ensure that it really is effective and sustainable. Not all identified resistance is effective against all tomato-infecting begomovirus species **(10)**. Pyramiding many different resistance genes into a single tomato cultivar should provide protection against a wide range of begomovirus species, but runs the risk of selecting for virus, perhaps via recombination, that can overcome all the resistances included. A more sustainable approach might be to only deploy one or two resistances that are effective against the local begomoviruses, while retaining other resistances in reserve in case the deployed resistances are overcome. Use of good cultural management practices to prevent or at least delay infection with begomovirus will help increase the durability of deployed resistances. A field trial with seedling protection at AVRDC- the World vegetable Center in 2004 showed that 89 days after transplanting, there was less than 2.5% of a resistant/tolerance cultivar became infected under the net protection (60 mesh), whereas 47.5% of the same cultivar were infected in the open nursery (Fig. 3). In the same experiment, 77.1% of a susceptible variety raised under the net protection were infected 89 days after transplanting, but all of the similar plants raised in the open nursery were infected 42 days after transplanting. Combining the use of leaf curl disease resistant tomato cultivars with good plant protection such as the net protection from the seedlings stage is effective in controlling leaf curl disease in Taiwan and is likely to be effective elsewhere.

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Table 1. The percentage of ToLCTWV and TYLCTHV detected in begomovirus-positive tomato samples collected in Taiwan

Year	Virus detection[a]					
	Western Taiwan			Eastern Taiwan		
	TH	TW	TH+TW	TH	TW	TH+TW
2005	1%	99%	0%	0%	100%	0%
2007	35%	16%	49%	2%	84%	14%
2008-2010	50%	2%	48%	74%	15%	11%
2012-2013	54%	10%	33%	80%	14%	6%

[a]TW indicates percentage of samples only positive for ToLCTWV by PCR using specific primer pair TW1978/PAR1c715H. TH indicates percentage of samples only positive for TYLCTHV using specific primer pair TH1978/PAR1c715H. TW + TH indicate percentage of samples positive for both of ToLCTWV and TYLCTHV. Percentages indicate the proportions of viruses detected in the begomovirus-positive samples. Detailed information of primers was described previously (Tsai *et al*., 2011).

Table 2. The tomato production in Taiwan since 1980

Year	Planted area, ha	Yield, kg/ha	Production, m.t.	Year	Planted area, ha	Yield, kg/ha	Production, m.t.
1980	10,947	30,075	329,232	1997	4,090	29,157	118,818
1981	8,561	23,734	203,188	1998	3,798	25,690	96,875
1982	10,450	31,685	331,106	1999	3,831	28,398	108,554
1983	12,248	39,389	480,945	2000	4,392	28,692	124,727
1984	12,394	51,741	641,127	2001	4,459	26,197	116,171
1985	11,212	43,181	483,756	2002	5,200	29,461	153,081
1986	9,682	42,591	396,201	2003	5,128	27,858	142,703
1987	9,047	40,415	365,436	2004	5,043	28,532	143,889
1988	6,821	36,516	249,029	2005	4,762	24,906	118,422
1989	7,122	42,596	298,997	2006	4,597	25,975	119,275
1990	6,369	41,111	257,920	2007	3,936	24,627	96,841
1991	5,434	39,726	215,728	2008	4,535	24,428	110,662
1992	4,375	35,186	151,062	2009	4,104	24,264	99,491
1993	4,525	31,822	143,962	2010	4,734	24,520	116,034
1994	4,094	31,361	127,960	2011	4,817	25,524	122,870
1995	4,367	30,358	132,444	2012	4,501	24,751	111,361
1996	4,385	31,387	137,394				

Source: Agriculture and Food Agency, Council of Agriculture, Executive Yuan, Republic of China

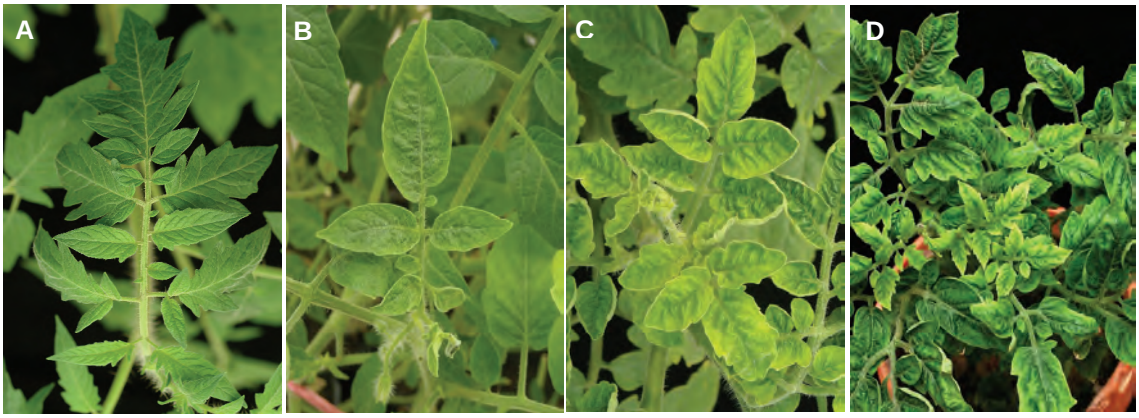


Fig. 1. Symptoms of tomato infected with begomoviruses on *Solanum lycopersicum*. A: symptomless healthy plant leaf; B: mild yellowing and leaf curling symptoms, C: yellowing and leaf curling symptoms, and D: severe yellowing, leaf curling and stunting symptoms.

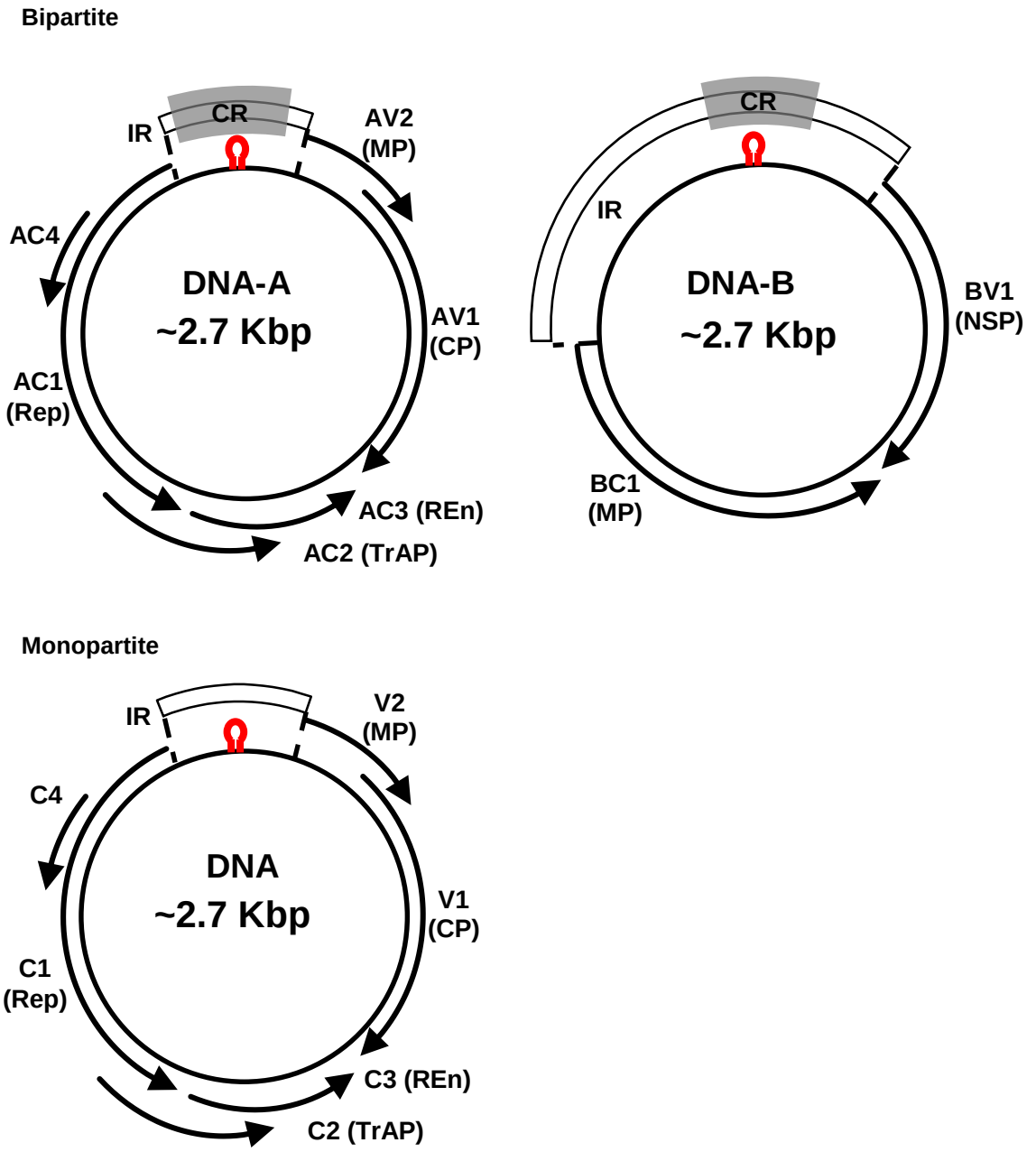


Fig. 2. Genome organization of tomato-infecting begomoviruses. The gray box indicated the common region (CR). The geminivirus conserved stem-loop structure has represented in red color. Arrows represent open reading frames of virus and complementary sense. IR: intergenic region; CP: capsid protein; Rep: replication-associated protein; TrAP: transcriptional activator protein; REn: replication enhancer; MP: movement protein; NSP: nuclear shuttle protein.

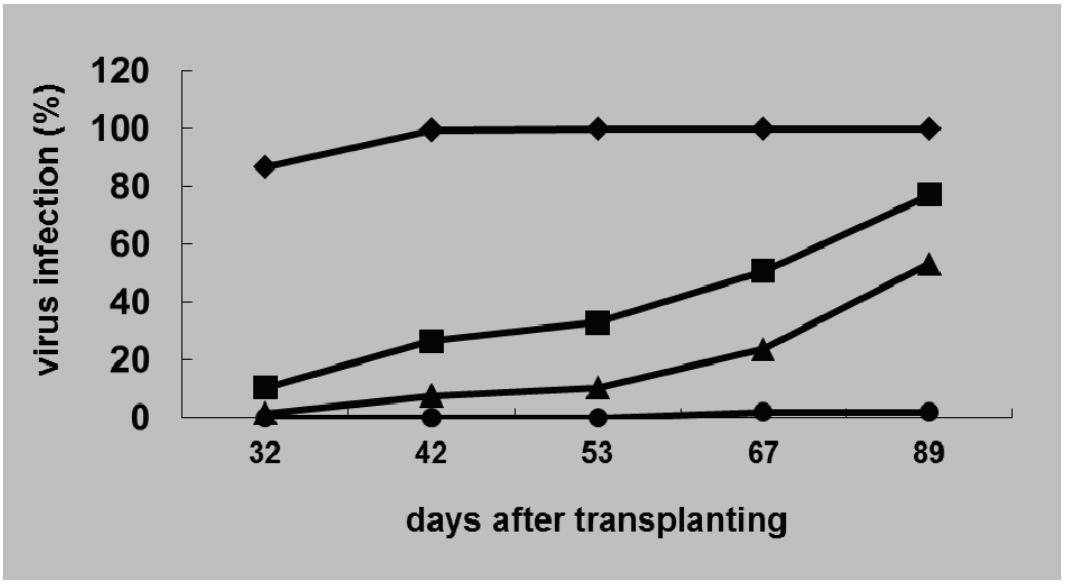


Fig. 3. Time course of begomovirus infection in resistant and susceptible tomato lines grown under the net (60 mesh) protection and in the open field prior to transplanting to the open field. ◆: susceptible tomato without net protection; ■: susceptible tomato with net protection; ▲: resistant tomato without net protection; ●: resistant tomato with net protection.

Insect Transmission of Tomato Yellow Leaf Curl Viruses

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ABSTRACT

Whitefly-transmitted tomato yellow leaf curl disease (TYLCD) is associated with many phylogenetically related viruses named tomato yellow leaf curl viruses that belong to the genus *Begomovirus* of the family *Geminiviridae*. Begomoviruses are mainly transmitted by the sweet potato whitefly, *Bemisia tabaci* (family Aleyrodidae, order Hemiptera), which is also a species complex with many biotypes. TYLCD is one of the most devastating viral diseases affecting tomato production in tropical and temperate areas worldwide, and the invasion of begomoviruses into a new region always accompanies the expansion of *B. tabaci* population. It is always thought that *B. tabaci* transmits begomoviruses in a persistent-circulative transmission mode; however, some studies showed that begomoviruses not only circulate in the hemolymph of *B. tabaci* but also replicate in it. In addition, *Tomato yellow leaf curl virus* Israel isolate (TYLCV-Is) was reported to be transovarially transmitted from virus-infected females to their progeny. TYLCV-Is is a well-studied begomovirus, and its transmission by *B. tabaci* has been examined in details. The acquisition access period and inoculation access period of TYLCV-Is transmission seems to be much shorter than those characteristics of typical persistent-circulative transmission mode. The vector transmission of TYLCV-Is requires a short latent period, and the virus persists and replicates in its insect vector. After reviewing related studies, we propose that few begomoviruses (at lease TYLCV-Is) are transmitted by *B. tabaci* in a special type of persistent-propagative transmission mode. More studies on the vector transmission of other begomoviruses are needed.

Keywords: *Begomovirus*, *Bemisia tabaci*, transmission mode, transovarial transmission, insect vector

INTRODUCTION

The feeding of sap-sucking insects always accompanies the transmission of plant viruses, and the feeding damage and viral disease cause great economic loss [40]. Many viral diseases of tomato are transmitted by the sweet potato whitefly, *Bemisia tabaci*, and the diseases often cause yield loss of tomato. *Bemisia tabaci*, an insect vector, transmits more than 111 virus species that induce disease symptoms, e.g. mosaic, yellowing, leaf curling, crinkling and even stunting on plants (Fig. 1) [45].

Among several plant diseases transmitted by *B. tabaci*, tomato yellow leaf curl disease is one of the most devastating viral diseases affecting tomato production in tropical and temperate areas worldwide [39, 57]. Tomato yellow leaf curl disease is associated with many phylogenetically related viruses named tomato yellow leaf curl viruses that belong to the genus *Begomovirus* of the family *Geminiviridae* [47]. Evidence shows that the invasion of begomoviruses into a new region always accompanies the expansion of *B. tabaci* population [6, 15, 24]. In Spain, the displacement between two species of tomato yellow leaf curl viruses has been reported [79], so as two tomato begomoviruses in Taiwan [85]. The displacement of tomato begomoviruses in the fields may be due to the invasion of new virus/vector and the different transmission efficiency of these viruses by their insect vectors [79, 85].

It is always thought that *B. tabaci* transmits begomoviruses in a persistent-circulative transmission mode [26, 40]. It means that begomoviruses infect *B. tabaci* and disseminate from alimentary gut to salivary glands without virus replication. However, some studies showed that begomoviruses not only circulate in the hemolymph of *B. tabaci* but also replicate in it [25, 33, 54]. The relationships between the virus, host plant, and whitefly vector are intricate [38, 66]. Whether the persistent transmission of begomoviruses by whiteflies is propagative or circulative is still controversial. We reviewed studies related to the transmission of various begomoviruses by *B. tabaci* and discussed the transmission mode of these begomoviruses.

Bemisia tabaci

Bemisia tabaci belongs to the family Aleyrodidae of the order Hemiptera. Among more than 1,550 species in the family Aleyrodidae, *B. tabaci* is considered to be the most important agricultural pest [13, 27]. *Bemisia tabaci* has high genetic variation within species with a number of recognized biotypes. Over the past decades, the application of molecular markers has distinguished more than 20 biotypes of *B. tabaci* [48, 70].

These biotypes differ in biological characteristics such as host range, virus transmission ability, insecticide resistance, and endosymbionts [3, 13, 41, 80, 90]. Some taxonomists even define *B. tabaci* as a species complex containing at least 28 morphologically indistinguishable species [28, 51].

Within this whitefly complex, B biotype and Q biotype have risen to international prominence [13, 28]. Recently, the spread of *B. tabaci* in greenhouses in temperate areas has been reported [41, 60, 88]. Most of these invasive whiteflies were identified as B biotype of *B. tabaci* that has spread to the areas in America, Africa, Asia, and Australia [9, 28]. Since 1980s, invasive B biotype has risen in status to one of the most damaging pests of crops worldwide [13, 14, 75], and it is currently listed as one of the top 100 invasive species worldwide [13]. Liu et al. [50] further proposed that the asymmetric mating interactions between closely related groups of *B. tabaci* is a driving force contributing to the invasion and displacement of invasive B biotype. In addition, Q biotype of *B. tabaci* has recently become a new invader via the same pathway as B biotype [27, 43].

In recent years, Q biotype of *B. tabaci* has invaded to many countries in Asia, Australia, and America, and caused economic loses of many cash crops [22, 53, 92]. There are four biotypes of *B. tabaci* reported in Taiwan, and invasive B biotype has out-competed endemic Nauru and An biotypes [44]. Q biotype of *B. tabaci* was also found in poinsettia greenhouses, but no field population was found in Taiwan [43].

Bemisia tabaci is a tiny insect (mostly 0.8 mm in length) with high reproductive rate and many generations in a year. The life history of *B. tabaci* is divided into 3 stages: egg, nymph, and adult (Fig. 2). The eggs are pyriform and laid in groups. First instar nymphs, commonly called crawlers, are flat in body shape. The mobile crawlers walk to find a suitable area on the leaf and stay at the same feeding site through whole nymphal stage. Second and third instar nymphs become stationary without any legs. Fourth instar nymphs, also referred to as red-eyes nymphs, turn yellowish in body color and thickening of body shape. Late in fourth instar stage, they stop feeding and subsequently molt to adult. The body of the adult is yellowish and has four membranous wings. The fore legs and hind legs of the adult distribute wax powder over the wings and the rest of body. In general, it takes 20-30 days to develop from eggs to adults in the warm climate and 30-45 days in the cool climate [17]. The lifespan of the adult is 25 to 30 days. There are 8-12 generations of *B. tabaci* in a year, and an adult female can laid up to 300 eggs in her lifespan.

Bemisia tabaci is a phloem-feeding insect and mostly feeds on herbaceous species,

which can result in greater than 50% yield reduction through sap sucking [17]. Plant host range of *B. tabaci* includes *Brassica* spp., tomatoes, eggplants, squash, cucumber, beans, cotton, and poinsettia. *Bemisia tabaci* affects crops with its feeding in three ways: 1) removing plant sap and subsequently reducing the plant vigor and yields, 2) excreting honeydew that fosters sooty mold fungi, and 3) transmitting plant viruses. *Bemisia tabaci* is known to transmit 111 plant virus species including many species of begomoviruses [45].

Begomovirus

Four genera in the family *Geminiviridae* have been established: *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus* [47]. *Begomovirus* is mainly transmitted by *B. tabaci* [15]. Whereas *Mastrevirus* and *Curtovirus* are transmitted by leafhoppers, *Topocuvirus* is transmitted by a treehopper *Micrutalis malleifera* [5, 10, 76]. The genus *Begomovirus* currently includes about 200 formally accepted virus species, and it is the largest genus in the family *Geminiviridae* [64]. Begomoviruses have a very wide host range but are limited to the dicotyledonous plants. The viruses cause a lot of economic damages to important crops such as tomatoes, beans, squash, cassava, and cotton in the world [12, 24, 84]. Begomoviruses are mostly restricted to the phloem of plants and induce disease symptoms, e.g. mosaic, yellowing, leaf curling, crinkling, and even stunting on plants [45]. Tomato yellow leaf curl disease is the most devastating disease caused by begomoviruses that affects tomato crops in tropical and temperate areas worldwide [29, 49, 57].

Members in the family *Geminiviridae* have circular single-strand DNA (ssDNA) genomes, and their virus particles consist of characteristic two incomplete icosahedra joined together [47]. The genome of geminivirus contains six open reading frames (ORFs). According to genome organization, begomoviruses are divided into two groups, monopartite viruses and bipartite viruses. Monopartite begomoviruses contain only one DNA segment (referred to as DNA-A) with genome size at 2.5-2.7 kb; bipartite begomoviruses contain two DNA segments (referred to as DNA-A and DNA-B) with similar genome size (2.5-2.7 kb) [64]. Some species of *Begomovirus* are associated with DNA satellites [65].

Among six ORFs of monopartite begomoviruses, the viral sense V1 gene encodes coat protein (CP), which is the only structural protein of begomovirus particles and is related to virus movement and vector transmission [83]; V2 gene encodes movement

protein; the complementary sense C1, C2, C3, and C4 genes encode replication associated protein, transcriptional activator protein, replication enhancer protein, and RNA silencing suppressor, respectively [31]. In bipartite begomoviruses, there are six ORFs in DNA-A: the viral sense AV1 and AV2 genes encode the CP and silencing suppressor, respectively [31, 37, 59]; four ORFs in the complementary sense, AC1, AC2, AC3, and AC4 genes, encode the same functional proteins as C1, C2, C3, and C4 genes of monopartite begomoviruses [31]. DNA-B contains two ORFs; the viral sense BV1 gene and the complementary sense BC1 gene encode nuclear shuttle protein and movement protein, respectively [31].

Over the past decades, diseases caused by begomoviruses have emerged as serious diseases to the cultivation of a variety of vegetable crops [16]. The host plants of begomoviruses range from more than 10 families of crops to weeds. Weeds serve as reservoirs of begomoviruses, so they play an important role in the emergence of viral diseases of crops [7, 11, 32, 46]. In addition, anthropogenic activity is a key factor of long-distance dispersal of plant viruses. A well-documented case showed that *Tomato yellow leaf curl virus* (TYLCV), a begomovirus, introduced into the Dominican Republic from Israel in 1992 [71, 73].

In Taiwan, there are at least six begomovirus species occurring in the fields including *Ageratum yellow vein Taiwan virus*, *Poinsettia leaf curl virus*, *Sweet potato leaf curl virus*, *Tomato leaf curl Taiwan virus* (ToLCTWV), *Tomato yellow leaf curl Thailand virus* (TYLCTHV), and *Tomato leaf curl Hsinchu virus* [45, 85], and the viruses are transmitted mainly by *B. tabaci*. In the fields, crops are usually infected with more than one species of begomoviruses, and the predominant species belong to tomato yellow leaf curl virus complex [64, 85].

Transmission biology of tomato yellow leaf curl viruses

Insects transmit the majority of described plant viruses. There are more than 1,000 plant viruses proved to be transmitted by insect vectors [40]. Hemipteran insects transmit most insect-borne plant viruses via piercing-sucking mouthparts. The food canal of hemipteran insects is formed by the opposed maxillae held together by a system of grooves, and the maxillae also contain the salivary canal [21]. Plant-feeding hemipteran insects are specialized as phloem, xylem, or mesophyll feeders [21]. Most plant viruses would infect phloem tissues so phloem-feeding hemipteran insects are the main vectors of these plant viruses. Insects transmit plant viruses in three transmission

modes: 1) nonpersistent, 2) semipersistent, and 3) persistent transmission [63]. Depending on the multiplication of virus in its insect vector, persistent transmission is further divided into two types: persistent-circulative and persistent-propagative [63](Table 1).

Bemisia tabaci is a principal insect vector of begomoviruses. *Bemisia tabaci* acquires begomovirus when it feeds the phloem sap and transmits the virus when it feeds on a new susceptible host, thus vectors are often the species that colonize the host plant. *Tomato yellow leaf curl virus* Israel isolate (subsequently referred to as 'TYLCV-Is') is a well-studied begomovirus, and its transmission by *B. tabaci* has been examined in details [33, 34, 78]. It is always thought that *B. tabaci* transmits begomoviruses in a persistent-circulative transmission mode [40], but it still remains a controversial issue. Interestingly, TYLCV-Is was documented to be transmitted from mother to offspring, and the offspring remain infective (i.e. transovarial transmission) [33], and some studies suggested that the virus could replicate in its insect vector [25, 54]. It is necessary to verify the transmission mode of begomoviruses by *B. tabaci*.

Vector transmission of geminiviruses is in a persistent-circulative mode [40]. *Cicadulina mbila*, a leafhopper, transmitted *Maize streak virus* (genus *Mastrevirus*) with an acquisition access period (AAP) of 3 h, and the virus was retained in its insect vector for 35 days [2, 40]. *Circulifer tenellus*, a leafhopper, transmitted *Beet curly top virus* (genus *Curtovirus*) with an AAP of 1 h, but the inoculation access period (IAP) was as long as 5 days for successful transmission of the virus, and the retention time of the virus was 30 days [82]. *Tomato pseudo-curly top virus* was transmitted by *Micrutalis malleifera*, a treehopper, with an AAP of 24 h, and the latent period of the virus transmission was as long as a week [81].

Most persistent-circulatively transmitted viruses require a period of hours to days for successfully acquiring and inoculating viruses. Interestingly, *B. tabaci* transmits begomoviruses with shorter AAP and IAP than the viruses belonging to other genera of *Geminiviridae* (Table 1). TYLCV-Is was detected in 15% of *B. tabaci* after they were fed on virus-infected plants for 30 min, and the frequency of the virus detection increased with the duration of the AAP [91]. The minimum IAP of TYLCV-Is was 15-30 min, and viral DNA was only detected in 10% of test plants [52]. Atzmon et al. [4] also reported that TYLCV-Is was detected in all test plants with an IAP of 30 min after an AAP of 24 h. The similar value of a minimum AAP of 1 h was reported for another begomovirus *Tomato yellow leaf curl Sardinia virus* (TYLCSV)[18]. The same experiment

also conducted with *Squash leaf curl virus* (SLCV), a begomoviruses, and the minimum AAP and IAP were 12 h and 24 h, respectively [72]. According to our unpublished results, *B. tabaci* successfully acquired and inoculated TYLCTHV and ToLCTWV within a couple of hours. In summary, the AAP and IAP of vector transmission of most begomoviruses by *B. tabaci* seem to be much shorter than those characteristics of typical persistent-circulative transmission mode (Table 1).

The other two transmission characteristics, i.e. latent period and retention time, are employed to distinguish persistent transmission mode and non-persistent transmission mode. A latent period is required when virus particles circulate or multiply in insect vector's body until they are ready to be inoculated to a new susceptible host. Most studies proved that TYLCV-Is lurks in *B. tabaci* with a short latent period. Once *B. tabaci* acquired TYLCV-Is, it kept the virus and remained infective through its entire life (~30 days) [25]. The latent period of TYLCV-Is was reported to be 20-24 h [23] or 8 h [35]. The latent period of SLCV and *Tomato leaf curl virus* (ToLCV), two begomoviruses, were 8 h and 6 h, respectively, which were similar with that of TYLCV-Is. [25, 58, 77]. Rubinstein and Czosnek [78] verified the ability of viruliferous *B. tabaci* to transmit TYLCV-Is to tomato plants steadily decreased with age but did not disappear completely until they died. Another study showed that TYLCSV DNA was remained in *B. tabaci* up to 22 days after the end of AAP, but the infectivity of the whiteflies only persisted till 18 days [19]. We also found that TYLCTHV and ToLCTWV needed a short latent period to be transmitted and viruliferous *B. tabaci* kept infective through adulthood (Weng and Tsai, unpublished results). In summary, the transmission of most begomoviruses by *B. tabaci* requires a short latent period, and the viruses persist in their insect vector lifelong. The latent period and retention time of begomoviruses fit the transmission characteristics of persistent-circulative and persistent-propagative transmission modes, respectively (Table 1).

The replication of begomoviruses in *B. taabci* has been examined by several research groups. Czosnek et al. [25] reported that TYLCV-Is DNA was detected by Southern blot hybridization in viruliferous *B. tabaci* after a 1 h-AAP, and the amount of viral DNA steadily increased after a lag period of 8 h and reached maximum level 16 h after and decreased thereafter. In another study, TYLCV Egypt isolate multiplied in *B. tabaci* starting from the end of 12 h-AAP to 108 h, and the virus titer stayed in a stable level from 108 to 180 h [54]. Following the acquisition of TYLCSV,

accumulation of viral DNA was not observed after an AAP of 12 h [19]. Therefore, the propagation of begomoviruses in their insect vector remains a controversial issue.

Many studies have been focused on the expression of viral genes in *B. tabaci* after feeding on begomovirus-infected plants [1, 36, 56, 68, 80] Vial gene expression indirectly implies the replication of begomoviruses in their insect vector. Real-time RT-PCR assay proved that the transcripts of V1, V2, and C3 genes of TYLCV-Is increased after feeding on virus-infected plants and then transferred to nonhost plants, but the transcripts of AV1, BC1, and BV1 genes of *Tomato mottle virus* (ToMoV) rapidly became undetectable [80]. The transcripts of TYLCV-Is C2 gene in the B and Q biotypes of *B. tabaci* increased along with the feeding periods of 6-72 h on virus-infected tomato plants [68]. The transcripts of TYLCV-Is V1 gene were localized in filter chamber and descending midgut of *B. tabaci* [36]. In summary, virus replication or viral gene transcription are proved in some begomoviruses (e.g. TYLCV-Is), but other begomoviruses do not replicate in their insect vector (e.g. ToMoV). The replication of TYLCV-Is in *B. tabaci* fits the transmission characteristics of persistent-propagative transmission mode (Table 1).

Infection and dissemination of begomoviruses in *Bemisia tabaci*

The infection and dissemination of begomoviruses in *B. tabaci* were mostly studied with TYLCV-Is and TYLCSV. The infection of tissues and organs of viruliferous *B. tabaci* was examined by immunohistological methods using antibodies raised against the CP of begomoviruses. TYLCV-Is was immunolocalized to descending midgut, filter chamber, and primary salivary glands [15, 26]. TYLCV Kisozaki isolate [87], which is closely related to TYLCV-Is, also infected the caecum of midgut, filter chamber, and ascending/descending midgut of *B. tabaci* [67]. Uchibori et al. [86] also verified TYLCV-Is overcame with the barriers of midgut and salivary gland by the way of interacting with vesicle-like structures and accumulating in the epithelial cells of descending and ascending midgut of *B. tabaci*. TYLCSV also showed the similar infection status as TYLCV-Is; the virus was detected in midgut microvilli and primary salivary glands [20, 55]. Although viral DNA fragments have been amplified from ovary tissue of *B. tabaci* that acquired TYLCV-Is via feeding on virus-infected plants [33], no specific labeling of the CP by immunohistology in ovaries was observed [20]. SLCV was reported to infect a number of organs and tissues of *B. tabaci* including midgut and salivary glands [69]. ToMoV and *Cabbage leaf curl virus*, two

begomoviruses, were also detected in salivary glands of viruliferous *B. tabaci* [55].

The dissemination of begomoviruses in viruliferous *B. tabaci* has been studied by PCR assay for TYLCV-Is and SLCV. TYLCV-Is DNA was firstly detected by PCR in the head of *B. tabaci* 10 min after the end of AAP, in the midgut 40 min after, in the hemolymph 90 min after, and in the salivary glands 5.5 hours after [35]. In another study, SLCV was firstly detected in the hemolymph of *B. tabaci* 2 h after the end of AAP, and in the saliva and honeydew 8 h after [77]. More studies focusing on the dissemination of begomoviruses in viruliferous *B. tabaci* are expected.

Transovarial transmission

Transovarial transmission is important in plant disease epidemiology. When viruliferous insect vectors pass virus from mother to their progeny, the progeny are ready to transmit the virus without virus acquisition and latent period. Transovarial transmission has been reported in many insect borne plant viruses [30, 42, 61, 62]. It is important to clarify whether transovarial transmission occurs in tomato yellow leaf curl viruses because it would affect the prediction of disease epidemiology.

Most studies showed that tomato yellow leaf curl viruses could be vertically passed from mother to their offspring (i.e. transovarial passage) (Table 2), but only Ghanim et al. [33] documented that TYLCV-Is not only transovarially passed to the offspring of viruliferous *B. tabaci* but also was ready to be transmitted to new susceptible plants by the whitefly offspring. However, TYLCSV was only passed to the offspring of viruliferous *B. tabaci*, but the offspring did not retain the infectivity [8]. The transovarial transmission of two TYLCV isolates, which are closely related to TYLCV-Is was also examined; the whitefly offsprings inherited the virus from their mothers, but the offsprings did not retain the ability to transmit the virus [8, 68]. The transovarial transmission of TYLCV-Is and *Tomato yellow leaf curl China virus* (TYLCCNV) by the B and Q biotypes of viruliferous *B. tabaci* has also been examined. The results showed that small proportion of the offspring inherited the viruses, but none of them kept the virus until adulthood except that 3% of Q biotype adult retained TYLCV-Is [89]. Apparently, the transovarial transmission was only observed in TYLCV-Is but not in other species of begomoviruses [8, 67, 74]. We also examined the transovarial transmission of TYLCTHV and ToLCTWV that are predominant begomoviruses in Taiwan. TYLCTHV could be transovarially passed to a small proportion of progeny of viruliferous *B. tabaci*, and the progeny were still infective

(Wang and Tsai, unpublished results). However, ToLCTWV could not be passed from the mother to the progeny, and the transovarial transmission did not occur for the virus. In summary, transovarial transmission only occurs in some begomoviruses (e.g. TYLCV-Is and TYLCTHV), and most tomato yellow leaf curl viruses examined failed to be transmitted through transovarial transmission.

CONCLUSION

Begomoviruses cause a lot of economic damages to important vegetable crops such as tomatoes and squash in the world [12, 24, 84], and they are mainly transmitted by *B. tabaci* [15]. The invasion of begomoviruses into a new region always accompanies the expansion of *B. tabaci* population [6, 15, 24]. *Bemisia tabaci* is a tiny insect with high reproductive rate and many generations in a year. Both begomoviruses and *B. tabaci* have very wide host ranges. The alliance of begomoviruses and *B. tabaci* poses a threat to the production of important vegetable crops worldwide. The disease induced by tomato yellow leaf curl viruses is the most devastating disease associated with begomoviruses that affects tomato crops in tropical and temperate areas [29, 49, 57].

It is always thought that *B. tabaci* transmits begomoviruses in a persistent-circulative transmission mode [40], but it still remains a controversial issue. TYLCV-Is is a well-studied begomovirus, and its transmission by *B. tabaci* has been examined in details [33, 34, 78]. The AAP and IAP of vector transmission of most begomoviruses (including TYLCV-Is) by *B. tabaci* seem to be much shorter than those characteristics of typical persistent-circulative transmission mode. The transmission of most begomoviruses (including TYLCV-Is) by *B. tabaci* requires a short latent period, and the viruses persist in their insect vector lifelong. The latent period and retention time of begomoviruses fit the transmission characteristics of persistent-circulative and persistent-propagative transmission modes, respectively.

Virus replication or viral gene transcription in insect vector are demonstrated in some begomoviruses (e.g. TYLCV-Is), but other begomoviruses (e.g. ToMoV) do not replicate in their insect vector. The replication of TYLCV-Is in *B. tabaci* fits the transmission characteristics of persistent-propagative transmission mode. Interestingly, the transovarial transmission was only observed in TYLCV-Is and TYLCTHV but not in other species of begomoviruses [8, 67, 74]. According to the abovementioned studies, it seems that few begomoviruses (e.g. TYLCV-Is) are transmitted by *B. tabaci* in a special type of persistent-propagative transmission mode. More studies on the vector

transmission of other begomoviruses are needed.

The interactions between whitefly and begomovirus are intricate. Despite the economic importance of tomato yellow leaf curl disease, little information for the begomovirus-whitefly interaction is known. Many questions remain to be answered such as the replication and transcription of begomoviruses in *B. tabaci*, the transovarial transmission of begomoviruses, and the deleterious effects of begomovirus infection in its insect vector. Lacking information about virus-vector interactions impairs the efficiency of insect-borne disease control because insect vector is the best target for controlling the diseases. Detailed knowledge of the interrelationship between whiteflies and begomoviruses may be able to help us develop novel strategies to protect crops from begomovirus-incited diseases.

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Fig. 1. Symptoms on tomato (*Lycopersicon esculentum*) caused by tomato yellow leaf curl disease. Leaves of the plant infected with *Tomato yellow leaf cur Thailand virus* exhibited symptoms of mosaic, yellowing, leaf curling, crinkling, and stunting. This photograph was taken in a field in Changhua, Taiwan, in 2010 (Photo by C. W. Tsai, National Taiwan University).

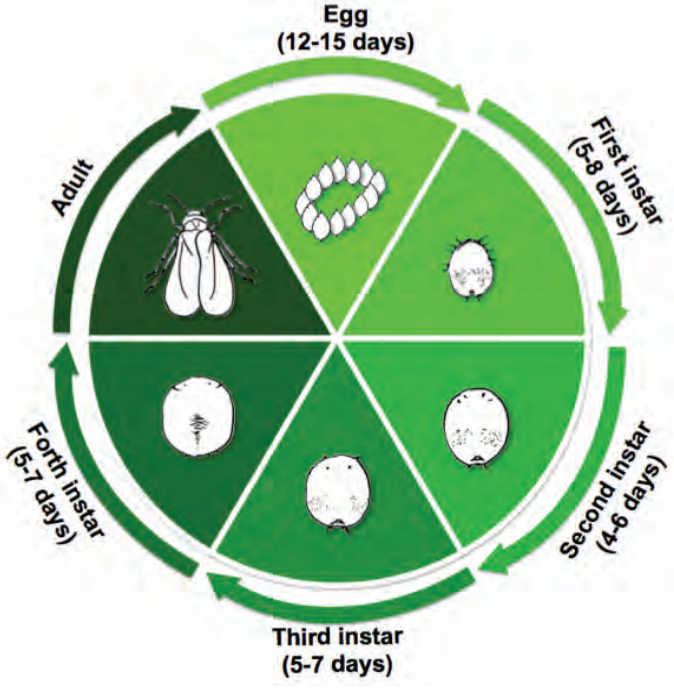


Fig. 2. Graphic representation of the life cycle of *Bemisia tabaci*.

Table 1. Transmission characteristics of *Tomato yellow leaf curl virus* Israel isolate

Transmission characteristics[a]	**Persistent-circulative**[b]	**Persistent-propagative**[b]	**TYLCV-Is**	**References**
Acquisition access period (AAP)	Hours-days	Hours-days	30 min	Zeidan and Czosnek, (1991)
Inoculation access period (IAP)	Hours-days	Hours-days	15-30 min	Mansour and Al-Musa, (1992)
Latent period	Hours-days	Days-weeks	8 h	Gahnim et al. (2001)
Retention time	Days-weeks	Lifelong	Lifelong	Rubinstern and Czosnek, (1997)
Virus multiplication in hemolymph	No	Yes	Yes	Czosnek et al. (2001)
Transovarial transmission	No	Often	Yes	Gahnim et al. (1998)

[a] The definitions of these terms can be found in Nault (1997).
[b] The data of transmission characteristics are derived from Hogenhout et al. (2008).

Table 2. Transovarial passage of tomato yellow leaf curl viruses

Virus	**Biotypes of *Bemisia tabaci***	**Life stage**			**References**
		Egg	**Nymph**	**Adult**	
TYLCV-Is	B	81%	37%	57%	Ghanim et al. (1998)
TYLCV-Is	B	0%	0%	0%	Polston et al. (2001)
TYLCV-Is	B	0%	0%	0%	Bosco et al. (2004)
TYLCSV	B	9%	29%	2%	Bosco et al. (2004)
TYLCV-Is	B	28%	8%	0%	Wang et al. (2010)
TYLCV-Is	Q	17%	15%	3%	Wang et al. (2010)
TYLCCNV	B	19%	11%	0%	Wang et al. (2010)
TYLCCNV	Q	1%	1%	0%	Wang et al. (2010)
TYLCV-Is	B	30%	11%	0%	Pan et al. (2012)
TYLCV-Is	Q	50%	17%	0%	Pan et al. (2012)

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