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Management of Huanglongbing (HLB) by an Intensive Vector and Disease Control in the Surroundings of the Orchard, in Addition to Planting HLB-free Trees in Okinawa, Japan

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Abstract

Citrus greening disease, huanglongbing (HLB), is one of the most destructive citrus diseases worldwide. The causal agent, *Candidatus* Liberibacter asiaticus (Las), is transmitted by the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae). In Okinawa, in the beginning of 2000, HLB was widespread. Local control and planting of HLB-free trees were considered necessary measures to manage HLB. In 2006, 25 HLB-free trees were planted at an experimental field in Okinawa. Then, insecticides were sprayed in the experimental field according to the spray calendar (during the experimental period: imidacloprid 20%, thiamethoxam 10%, dinotefuran 20%, and clothianidin 16%; each application rate was 0.1 L/m^2). In addition, "local control," that is, insecticide spraying [fenitrothion 50% twice a year from 2006 to 2008, mineral oil 97% once, and methidathion 40% (June-July) once after 2008; each application rate was 0.1 L/m^2] and removal of HLB-positive trees in the residential area, were continued. Until 2010, psyllids were very few, and none of the 25 planted trees were infected with HLB in the experimental field. Even in 2011, only 3 of the 25 trees were infected, indicating that the HLB infection rate was low when greening-free trees were initially planted and local control (pest control and removal of infected trees in the residential area) was effective at an early stage of cultivation. This result suggests that even in Japan, local control and planting HLB-free trees are effective in managing HLB.

Discipline: Plant protection
Additional key words: Asian citrus psyllid, *Citrus depressa*, *Diaphorina citri*, citrus greening disease, *Candidatus* Liberibacter asiaticus (Las)

Introduction

Citrus greening disease, huanglongbing (HLB), is one of the most destructive citrus diseases worldwide (Gottwald 2010). The causal agent is *Candidatus* Liberibacter asiaticus (Las) in Asian countries, including Japan (Iwanami et al. 2009, Bové 2006), and related species, *Ca*. L. africanus and *Ca*. L. americanus, are the causal agents in Africa and the Americas, respectively (Bové 2006). In the USA, HLB was first found in Florida in 2005 and in California in 2012 (CDFA 2012). In Japan, diseased trees were found for the first time in 1989 on Iriomote Island (Miyakawa & Tsuno 1989), and HLB quickly spread to Okinawa, Tokunoshima, and

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Kikai Islands (Kawano et al. 1997, Toguchi & Kawano 1997, Ooishi et al. 2006, Horie & Totokawa 2014, Naito et al. 2001, Shinohara et al. 2006). However, HLB was eradicated from Kikai Island, and the northern limit of HLB in Japan is Tokunoshima Island (Ministry of Agriculture, Forestry and Fisheries 2012).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a vector of the pathogens that cause HLB (Hall et al. 2013). *D. citri* is indigenous to tropical and subtropical Asian countries and has spread worldwide (e.g., CABI 2011, Halbert & Manjunath 2004, Hall et al. 2013). In Japan, *D. citri* is distributed to the south of Amami Island (Azuma et al. 2002).

Because the infected trees can be an inoculum, removal of diseased trees and reducing the vectors are the main practical methods to control HLB. The incidence of HLB infections within surrounding regions greatly affects the probability and efficacy of slowing the epidemic (Gottwald 2010). HLB is responsible for the economic losses of the entire citrus orchards within five or six years following the initial spread of epidemics (Aubert 1988). Therefore, regional control and planting of HLB-free trees have been recommended (Gottwald 2010). In Japan, the importance of Gottwald's proposal is empirically recognized, but the citrus cultivation system or scale in Japan, especially in the Nansei Islands, is quite different from that in many other citrus growing areas, such as in North and South America. In the Nansei Islands, citrus orchards are small (hundreds of square meters) and located in hilly and mountainous areas. Therefore, the proposal should be verified in orchards in Japan.

Materials and methods

1. Planting HLB-free trees in the experimental field

The village of Ogimi is located in the northern part of Okinawa Island and is the main citrus production area in the Okinawa Prefecture. The native citrus fruit, *Citrus depressa* Hayata (local name: Shiikuwasha) (Rutaceae), is widely grown. The occurrence of HLB in this area was first detected in 2000 (Takaesu 2001). In 2006, an experimental field (hereafter called field A) was established at Taminato, Ogimi village, Okinawa Prefecture (26°40′07.1″ N, 128°07′17.5″ E, alt. approx. 5 m). Residential area in Taminato faces to Shioya Bay in the south, and the east, north, and west sides are surrounded by forests and are separated by neighboring residential areas (Fig. 1). The size of field A was approximately 500 m^2, and the east and south sides of the field were bordered by hedges of hibiscus, *Hibiscus rosa-sinensis* L. (Malvaceae). The west side of the field bordered on a private house, and the north side continued to a slope with *C. depressa* trees. These *C. depressa* trees were once planted by local people and were left untreated afterward. Twenty-five *C. depressa* trees were planted in field A. These trees were confirmed as HLB-free by multiplex PCR (Urasaki et al. 2007). Rootstocks were produced in a screened nursery house at Ogimi and were grafted at the Okinawa Prefectural Agricultural Research Center. At the time of planting, these trees were 3 or 4 years old, and the tree height and crown diameter of these trees were 50-90 and 30-80 cm, respectively. After the establishment of field A, HLB-infected trees were surveyed in residential areas and the neighboring orchards (Fig. 1). As a result, 17 of 1,100 citrus trees were confirmed to be infected with HLB.

There was another citrus field, 50 m north-west of the experimental field (hereafter called field B; Fig. 1). Twenty-two trees were planted by an owner, with no confirmation whether these trees are HLB-free. The size of this field was similar to the experimental field, approximately 500 m^2. This field was observed in 2011 for comparison with field A.

2. Pesticide application and investigation of HLB-infected trees and *D. citri*

After planting the HLB-free trees, intensive local management, that is, pesticide application and

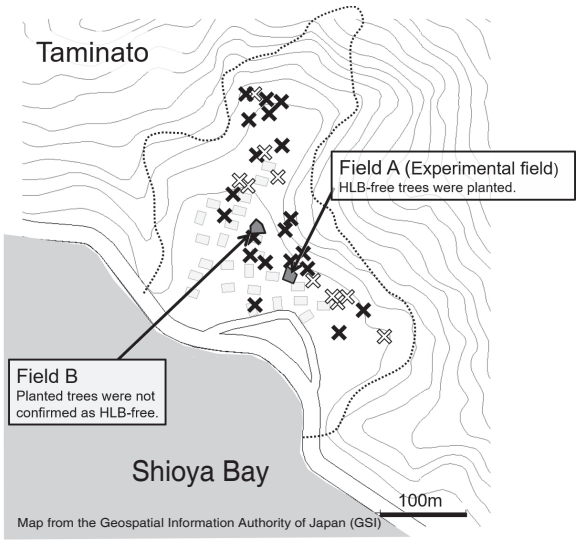

Fig. 1. Location of the experimental field in Taminato, Ogimi, Okinawa Prefecture
Black crosses indicate infected trees discovered until 2008, two years after planting the HLB-free trees. White crosses indicate diseased trees discovered in 2010 by another survey (Okinawa Prefecture, unpublished data), four years after planting the HLB-free trees. All of the diseased trees died or were cut down and removed until 2011. Dotted line indicates the extent of the survey area, including a residential area and citrus orchards.

investigation of HLB-infected trees and *D. citri* were practiced until 2008. Pesticides were periodically sprayed in field A following a spray calendar (conventional control) of Okinawa Prefecture at that time (imidacloprid 20% in late January, thiamethoxam 10%, dinotefuran 20%, and clothianidin 16%; each application rate was 0.1 L/m^2; Table 1). In addition, at the residential area, fenitrothion 50% emulsion (MEP emulsion) was applied twice a year in 2006, 2007, and 2008 as part of local management. Each application rate was 0.7 L/m^2. In parallel, the occurrence of HLB-infected trees and *D. citri* adults in the experimental field, residential area, and citrus orchards was monitored (Fig. 1) in April and May in 2006, 2007, and 2008. These months are good for detecting HLB because the density of the pathogen in plants and in insects becomes highest in a year, and detecting infection by symptoms and PCR assay is easy (Iwanami et al. 2009, Ukuda-Hosokawa et al. 2015). The surveyed area was approximately 6000 m^2. In 2007, all of the trees in this area (1300) were investigated. Many trees were approximately 10 years old or older. In 2008, small nursery trees were excluded from the investigation because of two reasons: the nursery trees were not infested by *D. citri* and not infected with HLB either (if small trees were infected, symptoms appeared within several months and were easy to find), and some nursery trees were transferred from the nursery field to other areas for planting. Therefore, 1,000 trees were investigated in 2008. If trees were thought to be infected with HLB (i.e., based on observations of HLB symptoms), we collected several leaves from those trees for DNA extraction and analysis. Leaves were sampled from every five branches, with more leaves sampled from larger trees (with more branches) and fewer from smaller trees (with fewer branches). From the collected leaves, total DNA was extracted using the CTAB method, and the extracted DNA was analyzed by PCR methods as described in Naito et al. (2001). The primers and conditions for PCR were described by Jagoueix et al. (1997). These infected trees were cut down and removed within several years.

To find *D. citri* adults, trees were beaten by a wooden stick from four directions, namely, east, west, north, and south. Under the trees, a white tray (27 cm × 37 cm) was set to catch adult *D. citri* in the trees. The collected adults were tested for HLB using the PCR assay following Naito et al. (2001).

In 2009, intensive local management was stopped because the research project (18040, see Acknowledgments) was over. After that, insecticide spraying (mineral oil 97% once and methidathion 40% in June or July; each application rate was 0.7 L/m^2) in the residential area of Taminato was continued. Also, in fields A and B, chemical control was continued by owners. The detailed history of the chemical control performed by owners is unknown, but it is presumed that insecticides in the control calendar were used when many *D. citri* were sighted.

3. Follow-up survey in 2011

In 2011, the occurrence of HLB-infected trees and *D. citri* adults in field A was surveyed. Two of the 25 trees originally planted in the experimental field were excluded from detection because they died owing to infestation with white-spotted longicorn beetle (probably *Anoplophora macularia*). Several leaves (the number of collected leaves depends on the size) were collected randomly from every five branches of the 23 remaining trees (branches were selected randomly). From the collected leaves, total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The samples were analyzed by PCR using a primer set MHO353/354, following the methods described by Hoy et al. (2001). The *D. citri* adults collected were tested for HLB using PCR methods, following Naito et al. (2001). (Note that, until 2009, during the research project (18040), PCR analysis was performed at the Okinawa Prefectural Experimental Station. In 2011, PCR analysis was performed at the Institute of Fruit Tree and Tea Science in Tsukuba, Ibaraki. Both protocols are suitable for the detection of HLB; see Hoy et al. (2001) and Naito et al. (2001)).

Results and discussion

At field A, in 2007, no trees were infected with HLB, and only three *D. citri* adults were observed (Table 2). In the residential area, 16 out of 1,300 trees were found to have symptoms of HLB (Fig. 1). These trees were confirmed to be HLB-positive by PCR assay. They apparently had been infected for at least several years because they had been adjacent to infected trees in the

Table 1. Insecticides and application methods used in the experimental field from 2006 to 2008

	Late January	Early March	Early May	Early June
Insecticide	Imidacloprid 20%	Thiamethoxam 10%	Dinotefuran 20%	Clothianidin 16%
Application rate	0.1 L/m^2	0.1 L/m^2	0.1 L/m^2	0.1 L/m^2
Application method	spraying	spraying	spraying	spraying

past. Since the flight ability of *D. citri* is poor (Arakawa & Miyamoto 2007) and its dispersal distance is several meters (Kobori et al. 2009), trees adjacent to infected ones are easily infected. In 2008, HLB-infected trees and *D. citri* adults were not observed in the experimental field (Table 2). In the residential area and orchards, 4 out of approximately 1,000 trees in the village were found to be infected. These infected trees were estimated to be almost 50 or 60 years old. Therefore, it was considered that these were not newly infected trees, but that they had been overlooked in the survey before 2008. It is because HLB has a latency period of three months to multiple years before symptoms are evident (ITP-USDA, 2010). In 2011, Las was detected from three trees in field A (Tables 2 and 3). However, the pathogen was not always detected in all of the collected five branches (Table 3). In addition, some fluorescent bands in electrophoresis were very faint ("false-positive" in Table 3). These results indicate that Las was maldistributed in the tree body, and the density of the pathogen was very low, that is, at the early stage of infection. Vector insects were found in 2011, but Las-positive insects were not found (Table 2).

In field B, 18 trees survived (Table 4) in 2011, and nine trees were smaller than before. It is supposed that some trees had weakened or died by HLB and were removed, and new trees were planted instead by the owner. Among the 18 trees that survived, six were HLB-infected (Table 4). PCR assay showed very dense bands in electrophoresis ("positive" in Table 4), indicating that a certain period of time had passed after infection. Comparing the number of negative trees at fields A and B, it is suggested that using HLB-free trees would delay the onset of the infestation.

The HLB infection rates (the number of infected trees/planted trees) at field A were zero (0/25) until 2010 (four years after planting) and 12% (3/25) in 2011 (five years after planting). The ratio was lower than that in other reports. In Vietnam, in the experiment with imidacloprid, the levels of HLB infection reached 74% within two years and 96% in the untreated control (Gatineau et al. 2006). Also, in Malaysia, the effect of three chemicals (imidacloprid, mineral oil, and a conventional synthetic insecticide) on HLB infection was investigated (Leong et al. 2012). The levels of HLB infection were 9.4%, 11.4%, and 22.7%, respectively, which were lower than that of 42.2% in untreated (control) trees. Actually, the spray intervals in the experiment were more frequent (mineral oils were sprayed every six to seven days, particularly during the flushing periods; synthetic pesticides were

Table 3. The number of infected/not infected twigs and collected psyllids in 2011 at the experimental field (field A)

Tree No.	Number of twigs			Number of psyllids on the tree**
	positive	false-positive*	negative	
1	0	0	5	0
2	0	0	5	0
3	0	0	5	1
4	0	0	5	0
5	0	0	5	0
6	0	0	5	0
7	0	2	3	0
8	NA	NA	NA	NA
9	1	4	0	1
10	1	0	4	0
11	0	0	5	0
12	0	0	5	0
13	0	0	5	0
14	0	0	5	0
15	0	0	5	3
16	0	0	5	0
17	0	0	5	0
18	0	0	5	0
19	0	0	5	1
20	0	0	5	3
21	0	0	5	0
22	NA	NA	NA	NA
23	0	0	5	0
24	0	0	5	0
25	0	0	5	1

* The fluorescent bands in electrophoresis were very faint.
** All psyllids were negative.
NA: Not available

Table 2. Number of HLB-infected trees, *Diaphorina citri* adults, and the proportion of Las-positive insects from 2006 to 2011 in the experimental field

	2006 HLB-free trees planted	2007 1 year after planting	2008 2 years after planting	2009 3 years after planting	2010 4 years after planting	2011 5 years after planting
No. of diseased trees out of 23	0	0	0	no data	0	3
No. of collected *D. citri* adults	0	3	0	0	0	10
Proportion of Las-positive insects	-	0	-	-	-	0

sprayed biweekly) than those in our research. As a cause of the differences in HLB infection level between these two reports and our results, the presence or absence of a control in the surrounding environment is suggested. In Gottwald (2010), the incidence of HLB infection within the surrounding region greatly affects the probability and efficacy of slowing the epidemic. In our case, we planted controls at the residential area and citrus orchards and removed infected trees, in addition to planting HLB-free trees and controls at field A.

These results indicate that both transplanting HLB-free trees and control of HLB and vectors around the residential area (that is, local management) contributed to reducing infection and delaying the onset of HLB disease. The orchards in the Nansei Islands are small and scattered in residential areas. In residential areas, hedges of orange jasmine (*Murraya paniculata* (L.) Jack) are distributed around houses and orchards, and trees of *C. depressa* are planted in private yards as memorial trees or the places where gods dwell. In addition, native citrus and orange jasmine grow in the surrounding woody areas, where the Asian citrus psyllid dwells. These conditions are different from the vast and uniform citrus orchards in other countries, such as USA and Brazil, that were mentioned in Gottwald (2010). Even under such small and diverse environments, local management and planting HLB-free trees, as proposed by Gottwald (2010), could be effective. Fortunately, the flight ability of *D. citri* is poor (Arakawa & Miyamoto 2007), therefore, ridges and forests between residential areas might be geographical barriers. We would like to conclude that the results of this study can be a successful example of local management in Japan, where HLB occurs.

In addition, we consider that long-term surveys for at least several years (that is, checking planted trees regularly if they are still HLB-free by PCR) are necessary to follow up the effectiveness of the management.

As a secondary effect of performing a local management program, the residents could understand the administration's efforts. Also, residential people gradually realized the importance of HLB control, and came to consent to use HLB-free trees, frequent insecticide spraying and cut down the infected trees.

Currently, control projects are being implemented and continued by municipalities in the Nansei Islands. In the near future, more labor-saving, effective, and sustainable measures are required to sustainably control HLB. Evaluation from a management-like viewpoint, e.g., calculating costs for spraying insecticides and searching infected trees in Belasque et al. (2010), is also necessary.

Table 4. The number of infected/not infected twigs and collected psyllids in 2011 at field B, where the HLB-free trees were not planted

Tree No.	Number of twigs			Number of psyllids on the tree**
	positive	false-positive*	negative	
21	0	0	5	0
22	0	0	5	0
23	0	0	5	0
24	0	0	5	0
25	0	0	5	1
26	0	0	5	0
27	NA	NA	NA	NA
28	0	0	5	0
29	NA	NA	NA	NA
30	1	0	4	0
31	0	1	4	0
101	0	0	5	0
102	0	0	5	0
103	0	0	5	0
104	1	0	4	1
105	2	2	1	0
106	0	2	3	0
107	0	0	5	0
108	4	0	1	0
109	0	0	5	0
110	NA	NA	NA	NA
111	NA	NA	NA	NA

* The fluorescent bands in electrophoresis were very faint.
** All psyllids were negative.
NA: Not available

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