

Does HLB Disease Prefer Citrus Growing in Alkaline Soil?

Haruhiko INOUE^{1*}, Akiko OKADA², Shigeru UENOSONO³,
Motofumi SUZUKI⁴, Tomoya MATSUYAMA⁴ and Yoshikuni MASAOKA^{2*}

¹ Division of Plant and Microbial Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan

² Graduate School of Biosphere Science, University of Hiroshima, Higashi-Hiroshima, Japan

³ Kagoshima Prefectural Osumi Food Technology Development Center, Kanoya, Japan

⁴ Frontier Research and Development Division, Environment and Energy Innovation Department, Aichi Steel Corporation, Tokai, Japan

Abstract

An epidemiological investigation was conducted in the Amami Islands of Kagoshima Prefecture, Japan, to determine the pathogenesis of citrus greening disease, also known as citrus Huanglongbing (HLB) disease, especially in relation to trace metal nutrition. We sampled 11 sites with acidic soil and 46 sites with alkaline soil in private gardens on the islands of Tokunoshima and Amami Oshima. At the acidic sites, no HLB-infected citrus trees were detected using PCR amplification. In the private gardens, 38.6% of the trees tested were infected with HLB disease. By comparing soil pH values in those private gardens, we found that HLB infection was related to soil pH. Among trees growing in alkaline soil, 47.8% were infected, whereas none of the trees growing in acidic soil in the private gardens were infected. When comparing a distribution map of HLB-diseased trees with a soil component map of the five Amami Islands, HLB disease was not detected in acidic soil, but the distribution of HLB-diseased trees was similar to the location of alkaline soil. Microelement analysis of leaves from trees grown in alkaline soil revealed significantly lower levels of manganese (71.9%), copper (70.1%), zinc (52.3%), and iron (40.9%) compared with the leaves from trees grown in acidic soil. These results suggest that HLB disease prefers citrus growing in alkaline soil where the concentrations of Fe and Zn nutrients are reduced.

Discipline: Agricultural Environment

Additional key words: acidic soil, citrus greening disease, iron, micronutrient, PCR

Introduction

Citrus greening disease or Huanglongbing (HLB) disease is among the most serious citrus diseases in Africa, Asia, and North and South America (Grafton-Cardwell, et al. 2013; Ukuda-Hosokawa et al. 2015). The disease is caused by one of three pathogens: *Candidatus Liberibacter asiaticus*, *Ca. L. africanus*, or *Ca. L. americanus* (Texeria et al. 2005). HLB can be transmitted by grafting or by the citrus psyllid *Diaphorina citri* (da Graca 1991). Infected trees exhibit yellowed and fallen leaves, poor fruit color, and finally death (Texeria et al. 2005). Methods of plant protection have yet to be found for this citrus disease.

HLB was first confirmed in Japan on the island of Iriomotejima in 1988 (Miyakawa & Tsuno 1989). From 1988 to 1997, the disease was found throughout Okinawa

Prefecture except on Minami-Daito Island and Kita-Daito Island (Naito et al. 2001). Later in Kagoshima Prefecture, diseased citrus was confirmed for the first time on Yoron Island in 2002 and in the Amami archipelago, except on Amami Oshima (and the smaller islands of Kikajima, Tokunoshima, and Okinoerabujima), beginning in 2003 (Shinohara et al. 2006).

The disease characteristics of HLB on citrus are similar to the physiological deficiencies of microelements. Thus, much effort has been expended to find the relation between microelement deficiency and HLB (Ohtsu et al. 1998). Leaves of citrus infected with HLB disease resemble leaves showing symptoms of zinc (Zn) and iron (Fe) deficiency. Direct evidence has been obtained showing that diseased leaves have lower levels of both microelements (Masaoka et al. 2011). HLB-infected plants supplied with nitrogen, phosphate, potassium, and

*Co-corresponding authors: e-mail haruhiko@affrc.go.jp, yosimasa@hiroshima-u.ac.jp

Received 31 August 2018; accepted 25 June 2019.

Zn showed no recovery from the disease phenotype, suggesting that there was no relation between HLB and nutritional deficiency (Razi et al. 2011). When Zn was supplied to HLB-infected leaves, the leaves proliferated and the internal microbiota changed (Zhang et al. 2016). The transport of Zn in grapefruit has been analyzed in detail by X-ray fluorescence using a synchrotron (Tian et al. 2014).

Little is known about how Fe is involved in HLB disease, however. In its reduced state as in paddy field soils, Fe is in contact with plant roots in a soluble ferrous ion form, but in an upland soil environment where citrus grows, Fe is in the oxidized form, existing as ferric ion. Plants cannot use the ferric form as FeOH_3 because this form precipitates in soil, especially in calcareous alkaline soils due to the high pH (Guerinot & Yi 1994). Therefore, plants must have an Fe acquisition strategy, the so-called strategies I and II (Marschner et al. 1986). In strategy II, some plants release a chelator to solubilize Fe and absorb it as an Fe complex (Kobayashi & Nishizawa 2012). In contrast, citrus plants use strategy I to acquire Fe (Wulandari et al. 2014). The model plant *Arabidopsis*, also a strategy I plant, upregulates the expression of the *FRO2* gene in roots, thereby increasing the reductase activity on the root surface (Robinson et al. 1999). Fe is changed into the highly soluble ferrous ion and becomes absorbed by the divalent iron transporter IRT1 (Eide et al. 1996; Vert et al. 2002). The reductase activity in roots is a strong indicator of the tolerance to Fe deficiency in strategy I plants (Castle & Nunnallee 2009). In addition, plants resistant to HLB (e.g., *Murraya exotica*) have higher root reductase activity for Fe than susceptible plants (e.g., *Poncirus trifoliata*) (Wulandari et al. 2014, Ramadugu et al. 2016). Therefore, the low Fe content in leaves of HLB-infected trees (Masaoka et al. 2011) strongly suggests a relation between Fe-deficiency tolerance and HLB resistance.

Japan has primarily acidic soils, although some soils in the southwestern islands are alkaline. On the island of Tokunoshima in Kagoshima Prefecture, the soil taxonomy varies from acidic to alkaline, reflecting the different soil types in the northern and southern parts of the island. In this study, we conducted an epidemiological investigation of the incidence of HLB disease on citrus in acidic and alkaline soils on two Kagoshima islands, and examined the relation between micronutrition and HLB disease.

Materials and methods

1. Field investigations and samplings of plants and soils

Citrus fields were investigated on Tokunoshima in December 2006 and on Amami Oshima in February 2007; we sampled soil and plants from a total of 71 locations, 57 private gardens, and 14 commercial sites. On Tokunoshima, we collected infected trees and adjacent uninfected trees in the southern part of the island where infected trees had been previously identified. In the northern area, HLB infection had not been previously identified; therefore, we chose random sampling sites to avoid bias. On Amami Oshima, HLB infection had not been reported; therefore, samples were collected from the entire island. Many of the trees looked healthy, but in areas where the soil pH was high, some trees had yellow leaves. Nearly identical numbers of samples were collected from trees with yellow leaves, which we suspected were infected, and from trees with healthy green leaves.

For soil sampling, we removed foreign substances other than plants and soil on the surface 15 cm away from the citrus tree trunk. Soil samples were collected at depths of 0-10 cm (on Tokunoshima), 10-20 cm, and 20-30 cm from the ground surface, and were well mixed with all depths (on Amami Oshima). Samples were air-dried and used for microelement analysis as described below. Average values were obtained for each soil depth and used as the component value for the sampling location. To sample the plants, we collected fully expanded leaves of native citrus plants grown in private gardens or Tankan mandarin (*C. tankan* Hayata) grown at commercial sites on Tokunoshima. Plant samples were collected from trees with heights of 2 to 3 meters. About 20 to 30 leaves were randomly sampled from each branch at a height of about 1 to 2 m above the ground for genomic DNA extraction and microelement analyses. For genomic DNA extraction, we sampled about 0.3 g of the middle ribs of five leaves. For microelement analysis, we sampled five to six whole leaves.

2. DNA isolation

Genomic DNA isolation followed a previously reported protocol (Dellaporta et al. 1983) with slight modifications. Fresh leaf samples (0.1 g) were crushed into small pieces in 1 ml of buffer 1 (0.2 M Tris-HCl (pH 8.0), 1.0 M NaCl, 2% (w/v) 2-mercaptoethanol). Then 1 ml of buffer 2 (0.1M EDTA (pH 8.0), 2.5% (w/v) SDS, 6.6% (w/v) polyvinylpyrrolidone) was added to the sample. After incubation at 65°C for 10 min., 0.5 ml of 5M potassium acetate was added and the tube was

vigorously vortexed. The sample was then incubated on ice for 30 min. followed by centrifugation at 9,100 x g for 10 min. The supernatant liquid (1.25 ml) was transferred to a new Eppendorf tube and mixed with 0.75 ml isopropyl alcohol. Then the sample was gently mixed and incubated on ice for 30 min. After centrifugation for 10 min. at 4°C, the pellet was dissolved in 0.4 ml of distilled water. The dissolved sample was added to 1 ml 99.5% ethanol and 40 µl 3M sodium acetate (pH 5.2), and then stored at -80°C for 30 min. Finally, the sample was centrifuged at 9,100 x g at 4°C for 10 min., and the pellet was dissolved in 200 µl of distilled water.

3. PCR detection of HLB

To determine whether a plant was infected with HLB, we used PCR detection using 2 µl of template DNA. Primers for the PCR amplification (Jagoueix et al. 1994) were as follows: OI2C 5'-GCC TCG CGG CTT CGC AAC CCA T-3' and OI1 5'-GCg CGT ATG CAA TAC GAG CGG CA-3'. PCR was conducted by following the manufacturer's protocol for TaKaRa rTaq (Takara Bio, Shiga, Japan). The PCR cycle was 94°C for 5 min., followed by 40 cycles of 94°C for 30 s and 68°C for 2 min. After PCR amplification, a 1,160 bp band was detected by 1.0% (w/v) agarose gel electrophoresis and ethidium bromide staining.

4. Microelement analysis

Five leaves from each tree were sampled and washed with distilled water. The leaves were blotted dry with paper towels and cut into smaller pieces with scissors. Then 20 ml of 1 mM MES buffer solution (pH 6.0) was added to 5 to 6 g of fresh leaves and homogenized into a slurry at 8,000 to 10,000 rpm with a Polytron CH-6010 homogenizer (Switzerland). The samples were centrifuged at 4,550 x g at 5°C for 15 min. to remove cellular debris, and the supernatant liquid was adjusted to a constant volume with 1 mM MES buffer (pH 6.0). Each sample was evaluated with an ICAP-575II high-frequency plasma emission spectrometer (Nippon Jarrell-Ash, Kyoto, Japan).

5. Statistics

Analyses of variance were conducted using js-STAR, version 9.0.4j (<http://www.kisnet.or.jp/nappa/software/star/info/new.htm>).

Results

Figure 1 b and c show the analyzed soil pH values from the soil samples collected on Amami Oshima and Tokunoshima, respectively. Soil pH at the commercial

sites (open circles inside the squares) on both islands was almost acidic, and no infected trees were detected on either island (Fig. 1). More than 90% of the plants at the commercial sites were growing in soil below pH 7, and more than 60% were growing in soil below pH 5 (Fig. 1). On Amami Oshima, the pH was slightly higher in the northern area, but low throughout the rest of the island. PCR detection of HLB from plant samples obtained on Amami Oshima showed 0/2 (PCR positive/PCR negative) at Wano, 0/1 at Akaogi, 0/1 at Ogachi, 0/1 at Kawachi, 0/1 at Gusuku, 0/1 at Miyamae, 0/1 at Seisui, 0/1 at Nesebe, and 0/1 at Asajin. Thus, no HLB disease was detected from trees growing in private gardens and at the commercial sites on Amami Oshima. Although our study was conducted in February 2007, our findings are consistent with the recent report by Horie & Totokawa (2014). As of 2018, HLB disease has not been detected on Amami Oshima.

Figure 1 c shows the analyzed soil pH values from the Tokunoshima samples. Overall, the soil pH was low in the northern and eastern parts of the island, and consistently increased from the western to southern locations. PCR tests to detect HLB from plant samples obtained on Tokunoshima found no evidence of the disease in the northern part: 0/5 at Tete, 0/5 at Okamae, and 0/5 at Asahigaoka. And in the southern part, the incidence of PCR-positive to negative samples was 7/11 at Kinen, 5/5 at Higashi-Metegu, 0/1 at Higashi-Omonawa, 5/5 at Saben, 1/1 at Nishi-Metegu, and 0/5 at Inutabu. Although our investigation was conducted in December 2006, the HLB disease incidence survey was consistent with our results until 2012 (Horie & Totokawa 2014), indicating that prevalence of the disease had not changed for at least six years in the private gardens.

Based on the relation between soil pH and the incidence of HLB from plants on the islands of Amami Oshima and Tokunoshima, HLB was positively detected by PCR in citrus trees growing in the private gardens where the soil pH values were more than 7, but not in soil with pH values lower than 7 (Fig. 2, Table 1). However, in soil where no HLB was detected, more than 30% of the plants in private gardens were growing in soil below pH 7. Taken together, these data indicate that 38.6% of all tested samples collected from the private gardens had detectable HLB. Thus, plants growing in acidic soil showed no evidence of HLB, and 100% of the HLB-positive plants were growing in alkaline soil.

Table 2 shows the relation between the occurrence of HLB and soil component analysis on the islands of Tokunoshima and Amami Oshima. The PCR results were negative for all samples from the commercial sites. In contrast, negative and positive PCR results were obtained

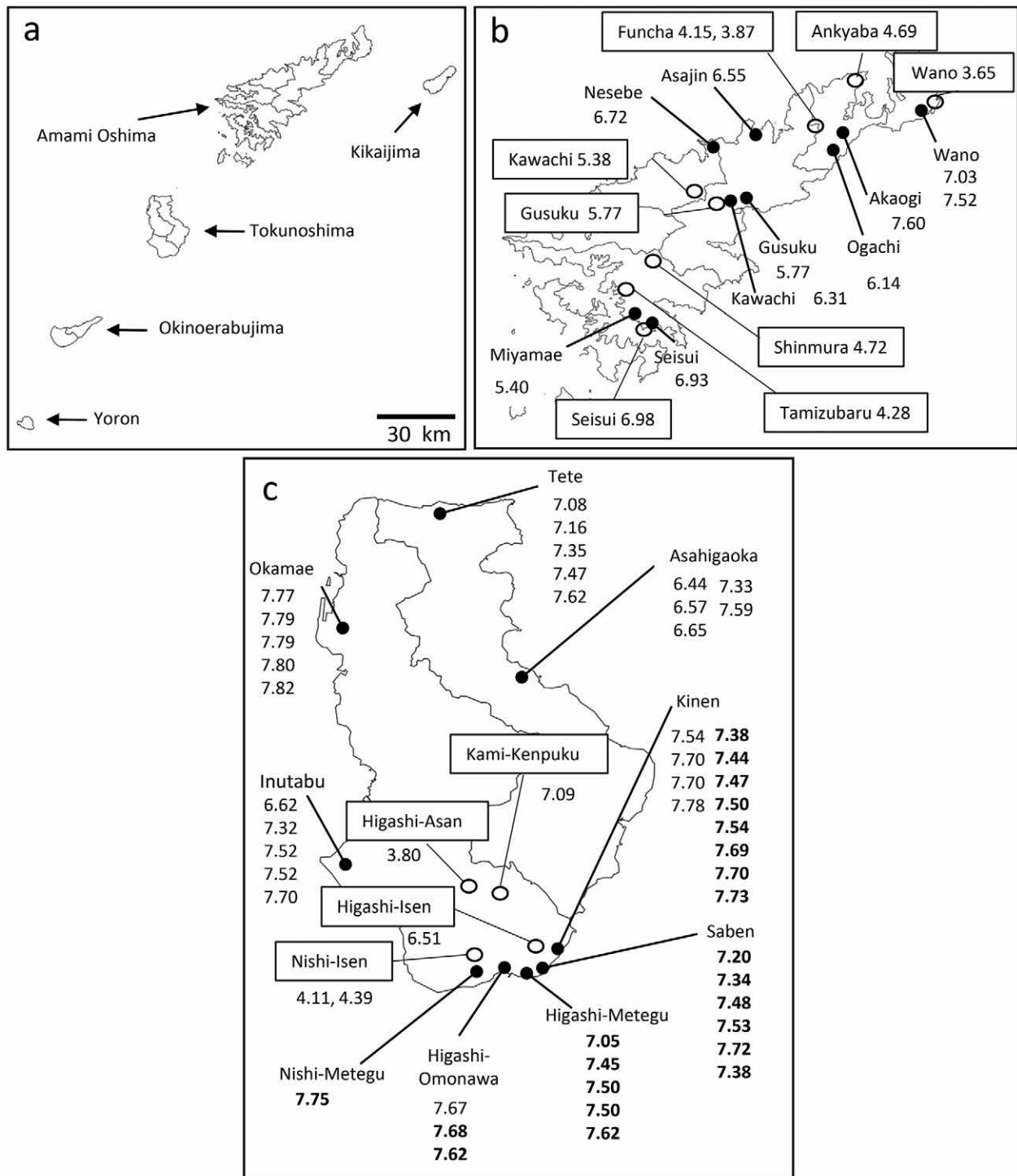


Fig. 1. Location maps

a: Overview of the Amami Islands, which are part of Kagoshima Prefecture in southern Japan. The scale bar is equal to 30 kilometers.

b: Enlarged view of Amami Oshima

c: Enlarged view of Tokunoshima

Sampling locations are indicated with measured pH values. Numbers in bold font indicate the pH values of HLB-positive trees as determined by PCR detection. Open circles inside the squares indicate commercial sites. Filled circles indicate private gardens.

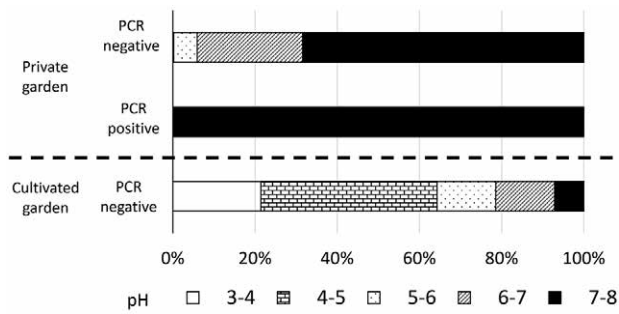


Fig. 2. Distribution of diseased citrus trees and healthy trees by soil pH values on Amami Oshima and Tokunoshima

PCR negative: no HLB disease was PCR-amplified from the samples grown in private gardens (n=35). PCR positive: HLB was detected in the samples (n=22). Commercial sites: samples were collected from commercial sites (n=14). Each bar indicates the percentage of samples having soil pH values from pH 3 to pH 8.

from samples collected from the private gardens. The soil pH was significantly different between locations where HLB occurred and locations where HLB was absent (Table 2). In addition, when comparing the analyzed components with the presence/absence of HLB, a great difference in the effectiveness of microelements was observed as compared to macroelements such as potassium. Among the parameters, the concentration of Fe in the soil was significantly lower at locations where HLB-diseased trees were found; differences in the Fe content reduced the ratio of PCR positive to PCR negative plants to 0.56 (Table 2). Next, we examined the influence of HLB infection and soil pH on leaf elemental composition (Table 3). As some of these trace elements become insoluble and nonfunctional in plants, we extracted leaf tissues to obtain a water-soluble functional fraction (MES-extracted fraction) (Cakmak & Marschner 1987). The element content of healthy leaves of plants

Table 1. PCR detection of HLB from citrus growing in acid or alkaline soil in private gardens on two islands

Sampling locations	Amami Oshima		Tokunoshima		Percent PCR-positive	
	Number of Samples	PCR -positive	Number of Samples	PCR -positive	Number of Samples	Ratio (%)
Acid	7	0	4	0	11	0
Alkaline	3	0	43	22	46	47.8
Total	10	0	47	22	57	38.6

Note: Alkaline soil means samples with pH values greater than pH 7; acid soil means samples with pH values less than pH 7.

Table 2. Soil component analysis from the sampling locations on two islands

PCR detection at different sampling locations	pH (H ₂ O)	Exchangeable cations (cmol/kg)			DTPA-extractable (mg/kg)				
		K	Ca	Mg	Fe	Mn	Cu	Zn	
Private garden PCR negative (n=35)	AV	7.17a	0.91a	29.92a	3.58a	1.92a	4.29a	0.79a	10.99a
	SE	0.11	0.23	2.02	0.31	0.37	1.16	0.14	2.91
Private garden PCR positive (n=22)	AV	7.51b	0.79a	30.36a	4.15a	1.07b	2.38a	0.72a	13.21a
	SE	0.04	0.16	1.73	0.23	0.12	0.39	0.3	2.52
Commercial site (n=14)	AV	4.96c	1.1a	8.91b	2.98a	38.91c	10.88a	2.13b	1.27b
	SE	0.31	0.26	2.33	0.87	8.95	3.25	0.59	0.3
Ratio of PCR positive/negative in private gardens	1.05	0.86	1.01	1.16	0.56	0.56	0.9	1.2	
	**	NS	NS	NS	*	NS	NS	NS	

Notes: The ratio of PCR indicates the percentage (%) of the average pH value, exchangeable cations, and DTPA-extractable elements in PCR positive/PCR negative samples collected from private garden sites.

Values with the same letter and same treatments were not significantly different ($P < 0.05$). * and ** denote significant differences ($P < 0.05$ and $P < 0.01$) between two treatments, and NS denotes not significant at $P < 0.05$.

Table 3. Leaf component analysis at private garden sites from the sampling locations on two islands

		Number of samples	Soil pH (H ₂ O)	MES soluble microelements (mg/g dry weight) in the leaves				
				Fe	Mn	Cu	Zn	
(1)	PCR negative	11	AV	6.37a	34.09a	8.3a	8.97a	8.46a
	Acidic soil		SE	0.14	6.17	1.54	1.46	1.52
(2)	PCR negative	24	AV	7.55b	18.52b	7.65a	9.79a	5.59b
	Alkaline soil		SE	0.05	1.94	0.89	1.42	0.44
(3)	PCR positive	22	AV	7.53b	13.95c	5.97b	6.29b	4.43c
	Alkaline soil		SE	0.04	1.82	0.84	0.96	0.29
Percentage of (2) / (1) (%)					54.3	92.1	109.1	66.1
Percentage of (3) / (1) (%)					40.9	71.9	70.1	52.3

Note: Values with the same letter and same treatment were not significantly different ($P < 0.05$).

grown in alkaline soil was lower than that of leaves collected from trees growing in acidic soil: Mn (92.1%), Cu (109.1%), Zn (66.1%), (Fe 54.3%) (Table 3). Moreover, the micronutrient content of diseased leaves was lower than that of healthy leaves from trees grown in acidic soil: Mn (71.9%), Cu (70.1%), (Zn 52.3%). Most notably, there was much less Fe (40.9%); the decrease in Fe concentration to one-half or less (Table 3) was similar to that reported previously (Masaoka et al. 2011).

Discussion

HLB is a serious citrus disease in Africa, Asia, and North and South America. At nearly the same time, this disease has also invaded the Japanese islands south of Kyushu and poses a very serious problem that needs prompt resolution. HLB was initially detected in Japan in Okinawa Prefecture in 1988, but since that time, HLB was found on the southwest islands during our investigation in 2006 and continues to be present.

This epidemiological study of HLB showed that trees in the Amami Islands are infected with HLB; however, the disease was more common in citrus grown in alkaline soil. PCR positive plants were only found in private gardens. We focused on these garden sites to elucidate the most significant factors for promoting HLB infection. In alkaline soil, the degree of disease development was 47.8%, whereas no disease was detected in acidic soil (Table 1). These findings are consistent with reports on the distribution of diseased sites found from 2002 to 2012 (Horie & Totokawa 2014), which reported the incidence of HLB disease found to date in comparison with the Japanese soil inventories of Amami Oshima,

Tokunoshima, and Okinoerabujima (<https://soil-inventory.dc.affrc.go.jp/figure.html>) (Fig. 3).

The left side of Figure 3 shows the sampling locations where PCR surveys were conducted to detect HLB disease. HLB was not detected in Amami Oshima (data not shown), but was detected in the western and southern parts of Tokunoshima (Fig. 3 a) and the northern and western parts of Okinoerabujima (Fig. 3 c). As shown in the soil component map, the entire island of Amami Oshima has Haplic argic red-yellow soil, an almost acidic soil. The custom of spreading sea sand in gardens is a common practice on these islands that probably affects soil pH. On Tokunoshima (Fig. 3 e), acidic soil is a mixture of Haplic argic red-yellow soil and Paralithic terrestrial regosols, and Haplic Cambic red-yellow soil is present in the southern area, but the alkaline soil found there is composed of Argic calcaric dark red soil (Fig. 3 e).

The areas of HLB disease incidence and locations of alkaline soil overlapped significantly (Fig. 3 a and e). In addition, Okinoerabujima also contained Argic calcaric dark red soil, and the degree of HLB disease was similar to that of Tokunoshima (Fig. 3 c and g). Yoron Island, where HLB was detected throughout the island, also had an alkaline soil (Fig. 3 d and h). Interestingly, on Kikaijima where HLB disease was eradicated in 2012, the location where HLB disease occurred also had alkaline soil (Fig. 3 b and f). Although there is a correlation between HLB detection and alkaline soil through this epidemiological study, a more extensive future investigation of other locations where HLB is detected and experimental proof in the laboratory are warranted.

In the southern part of Tokunoshima where the soil

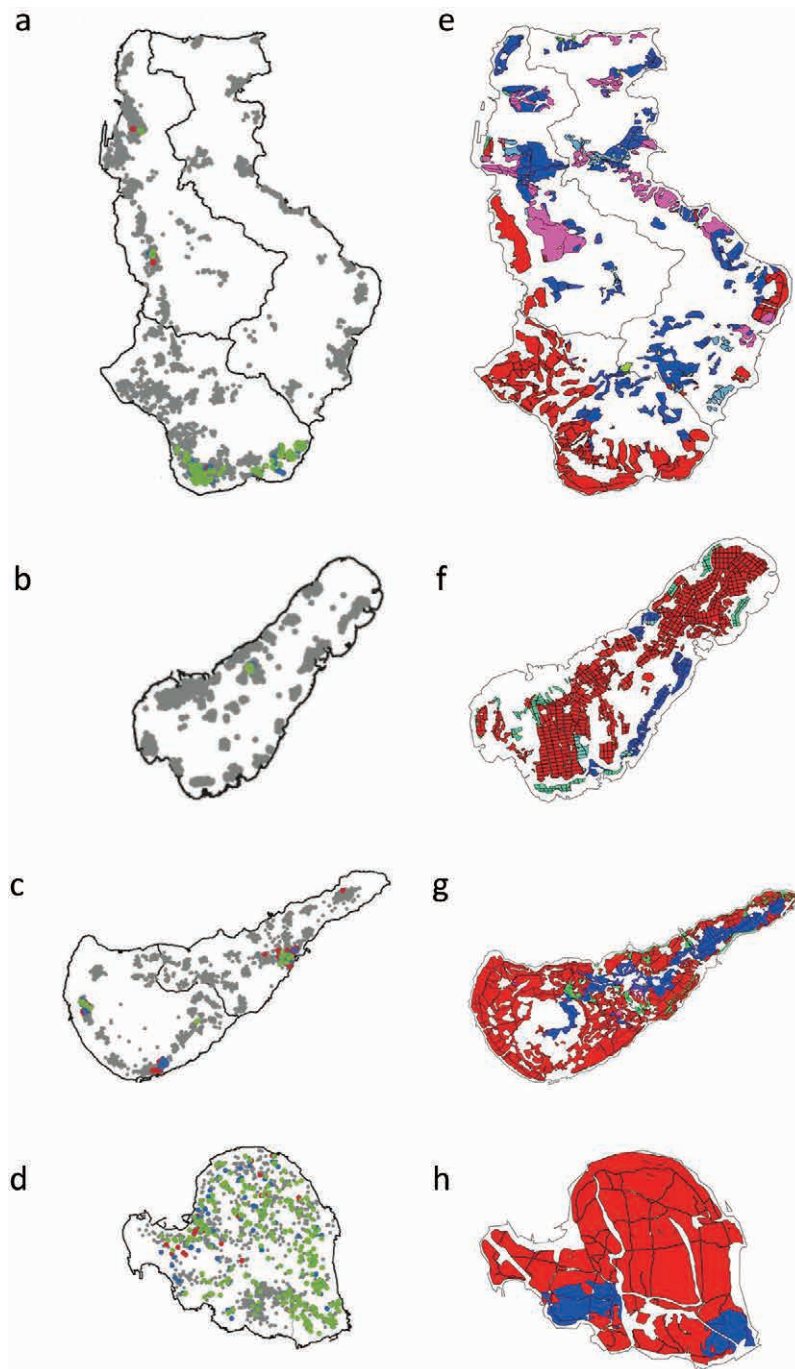


Fig. 3. A comparison between the soil distribution map and locations of HLB-diseased trees on the Amami Islands

Tokunoshima (a, e), Kikaijima (b, f), Okinoerabujima (c, g), and Yoron island (d, h). Fig. 3 a-d: modified maps from the previously published maps reported in Horie and Totokawa (2014). Gray indicates the locations of planted citrus trees. Colored portions indicate the locations of confirmed HLB-infected citrus trees found in the 1st survey (2002-2006: light green), 2nd survey (2006-2009: blue) and 3rd survey (2009-2012: red). Fig. 3 e-h show the distribution of soil types in a map published by the National Agriculture and Food Research Organization, Japan (<https://soil-inventory.dc.affrc.go.jp/>). The map shows the distribution of each soil type (blue: Haplic Argic red-yellow soil; pink: Haplic Cambic red-yellow soil; light green: Haplic Volcangenous Regosols; light blue: Haplic brown lowland soil; and red: Argic Calcaric dark red soil).

is alkaline, no HLB was detected at the commercial sites (Fig. 1 c). At those commercial sites, the soil is more fertile compared to that in private gardens, a feature that also correlates with reduced HLB infection. These observations suggest that it is not only important to protect trees against infected citrus psyllids using disinfection, but it may also be necessary to acidify the soil when growing citrus. HLB was possibly found on Kikajima because the citrus psyllids did not fly from Tokunoshima, but flew directly from Amami Oshima. Residents of Amami Oshima acidified the soil where citrus trees were growing, thereby allowing inorganic nutrients to be solubilized in the soil environment. To keep the bacterial density relatively low (i.e., not detectable by PCR), planting citrus such as the HLB-resistant citrus tree *Murraya exotica* could be recommended in case of infection. In the southern part of Yoron Island, disease spots of HLB were also found in trees growing in acidic soil (Fig. 3 h). This finding may result from significant increases in the number of trees growing in alkaline soil being infected, thereby causing the HLB density to increase and resulting in the infection of trees growing in acidic soil.

In Japan, tree felling is undertaken as soon as HLB-diseased citrus trees are found by the Plant Protection Office of Japan's Ministry of Agriculture, Forestry and Fisheries. This eradication project will continue mainly in the Amami Islands of Kagoshima Prefecture. The method requires PCR detection of HLB by randomly sampling citrus, a costly, time-consuming, and laborious procedure. Therefore, we propose that analyzing soil components could be an effective indicator of potential sites for HLB infection. Soil testing should make it possible to identify places where the potential onset of HLB disease is high, thereby reducing the number of citrus plants to be sampled. Eradication could consequently be achieved earlier. Graham et al. (2017) reported citrus growing in a high soil pH environment in Florida, USA, where the HLB disease progressed rapidly, leading to remarkable levels of tree weakness. These investigators hypothesized that the citrus root system developed poorly in high pH soil, leading to decreases in micronutrient absorption. This report also confirms that as soil pH increases, the risk of HLB infection of citrus also increases. Moreover, alkaline soil lowered the effective concentrations of microelements, resulting in particularly significant Fe immobilization. This investigation also proposes that HLB disease is closely related to reductions in citrus Fe content.

Acknowledgement

We wish to thank Akiko Hamashima of the Kagoshima Prefectural Institute for Agricultural Development, Fruit Tree Division, for the PCR detection of HLB.

References

- Cakmak, I. & Marschner, H. (1987) Mechanism of phosphorus-induced zinc deficiency in cotton. III. Changes in physiological availability of zinc in plants. *Physiologia Plantarum*, **70**, 13-20.
- Castle, W. S. & Nunnallee, J. (2009) Screening Citrus Rootstocks and Related Selections in Soil and Solution Culture for Tolerance to Low-iron Stress. *Hort. Science*, **44**, 638-645.
- da Grace, J. V. (1991) Citrus greening disease. *Annu. Rev. Phytopathol.*, **29**, 109-136.
- Dellaporta et al. (1983) A plant DNA miniprep: Version II. *Plant Molecular Biology Reporter*, **1**, 19-21.
- Eide, D. et al. (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences*, **93**, 5624-5628.
- Grafton-Cardwell, E. E. et al. (2013) "Biology and management of Asian citrus psyllid, vector of the Huanglongbing pathogens." *Annual Review of Entomology*, **58**, 413-432.
- Graham, J. et al. (2017) Soil and water acidification sustain root density of Huanglongbing-infected trees in Florida. Paper presented at the 5th International Research Conference on Huanglongbing, March 14-17.
- Guerinot, M. L. & Yi, Y. (1994) Iron: nutritious, noxious, and not readily available. *Plant Physiology*, **104**, 815.
- Horie, H. & Totokawa, N. (2014) Survey of Citrus Huanglongbing (greening disease) on the Amami Islands. 3. Transition of diseased tree distribution after the second survey. *Kyushu Pl. Prot. Res.*, **60**, 37-42 [In Japanese with English summary].
- Jagueix, S., J. M. et al. (1994) The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the *Proteobacteria*. *Int. J. Syst. Bacteriol.*, **44**, 379-386.
- Kobayashi, T. & Nishizawa, N. K. (2012) Iron uptake, translocation, and regulation in higher plants. *Annual Review of Plant Biology*, **63**, 131-152.
- Masaoka, Y. et al. (2011) Lower Concentrations of Microelements in Leaves of Citrus Infected with 'Candidatus Liberibacter asiaticus.' *JARQ*, **45**, 269-275.
- Marschner, H. et al. (1986) Different strategies in higher plants in mobilization and uptake of iron. *Journal of Plant Nutrition*, **9**, 695-713.
- Miyakawa, T. & Tsuno, K. (1989) Occurrence of citrus greening disease in the southern islands of Japan. *Japanese Journal of Phytopathology*, **55**, 667-670.
- Naito, T. et al. (2001) Detection of the citrus Huanglongbing (greening disease) by polymerase chain reaction (PCR) assays and distribution in Okinawa, Japan. *Bulletin of the Okinawa Agricultural Experiment Station (Japan)*.

- Ohtsu, Y. et al. (1998) Typical symptoms of citrus greening of mandarin trees in Nepal, supported by detection and characterization of ribosomal DNA of the causal organisms. *Ann. Phytopath. Soc. Japan*, **64**, 539-545.
- Ramadugu, C. et al. (2016) Long-term field evaluation reveals Huanglongbing resistance in Citrus relatives. *Plant Disease*, **100**, 1858-1869.
- Razi, M. et al. (2011) Citrus plant nutritional profile in relation to Huanglongbing prevalence in Pakistan. *Pakistan Journal of Agricultural Sciences*, **48**, 299-304.
- Robinson, N. J. et al. (1999) A ferric-chelate reductase for iron uptake from soils. *Nature*, **397**, 694.
- Shinohara, K. et al. (2006) Survey of citrus Huanglongbing (greening disease) on the Amami Islands, 1: Characteristics of distribution in the Amami Islands [Japan]. *Kyushu Plant Protection Research*, **52**, 6-10 [In Japanese with English summary]
- Teixeira, D. D. C. et al. (2005) First Report of a Huanglongbing-Like Disease of Citrus in Sao Paulo State, Brazil and Association of a New Liberibacter Species, "*Candidatus Liberibacter americanus*," with the Disease. *Plant Disease*, **89**, 107-107.
- Tian, S. et al. (2014) Spatial imaging of Zn and other elements in Huanglongbing-affected grapefruit by synchrotron-based micro X-ray fluorescence investigation. *Journal of Experimental Botany*, **65**, 953-964.
- Ukuda-Hosokawa, R. et al. (2015) Infection density dynamics of the citrus greening bacterium "*Candidatus Liberibacter asiaticus*" in field populations of the psyllid *Diaphorina citri* and its relevance to the efficiency of pathogen transmission to citrus plants. *Applied and Environmental Microbiology*, **81**, 3728-3736.
- Vert, G. et al. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell*, **14**, 1223-1233.
- Wulandari, C. et al. (2014) Effect of iron deficiency on root ferric chelate reductase, proton extrusion, biomass production and mineral absorption of citrus root stock orange jasmine (*Murraya exotica* L.). *Journal of Plant Nutrition*, **37**, 50-64.
- Zhang, M. Q. et al. (2016) Zinc treatment increases the titre of '*Candidatus Liberibacter asiaticus*' in Huanglongbing-affected citrus plants while affecting the bacterial microbiomes. *Journal of Applied Microbiology*, **120**, 1616-1628.