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Lower Concentrations of Microelements in Leaves of Citrus Infected with '*Candidatus* Liberibacter asiaticus'

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Abstract

Citrus trees affected by greening (huanglongbing, HLB) often develop symptoms that resemble those of Zn or Fe deficiency in their leaves. However, there have been few studies of mineral concentrations in infected leaves. To examine the effects of infection by '*Candidatu*s Liberibacter asiaticus' (the causal organism of the Asiatic form of HLB) on mineral nutrition, *Citrus jambhiri* (Lush.), *C. reticulata* cv. Siem, and *C. depressa* Hayata were patch-grafted with infected bark squares and grown in pots in greenhouses in Japan and Indonesia. In addition, leaves were collected from field-grown *C. tankan* Hayata and *C. unshiu* Marc. in Japan and *C. reticulata* cv. Siem in Indonesia, and their disease status was determined by PCR. Leaf samples were homogenized in 2-(N-morpholino) ethanesulfonic acid buffer and the concentrations of water-soluble Cu, Fe, Mn, and Zn in the macerate were determined using inductively coupled plasma atomic emission spectrometry (Japan) or atomic absorption spectrometry (Indonesia). In general, infected leaf samples had lower Fe and Zn. On average, the concentrations of Fe and Zn in infected plants were approximately half those in healthy plants. Cu was not significantly reduced by infection and Mn was occasionally lower. In *C. unshiu,* the concentrations of Fe and Zn were reduced before chlorosis appeared. These results suggested that the concentrations of particular elements (Fe and Zn) rather than element concentrations in general are reduced by infection by '*Candidatu*s Liberibacter asiaticus'.

Discipline: Plant disease
Additional key words: *Citrus depressa*, Citrus greening, Huanglongbing, Liberibacter

Introduction

Greening (huanglongbing, HLB) is one of the most devastating diseases for citrus in many parts of the world and is caused by non-culturable, phloem-limited bacteria of the *Candidatus* genus Liberibacter. '*Candidatus* Liberibacter africanus' causes the African form of the disease and is mainly transmitted by a species of citrus psyllid, *Trioza erytreae* del Guercio, whilst '*Ca.* Liberibacter asiaticus' (Las) causes the Asian form of the disease and is vectored by another psyllid, *Diaphorina citri* Kuwayama[3,4,7,8]. The African form is found in cooler areas and at higher altitudes, whereas the Asian form is more widely

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spread in warmer lowlands in tropical and subtropical areas[3,4]. Recently, a third species of bacterium causing HLB was found in Brazil and was designated as '*Candidatus* Liberibacter asiaticus' [22]. The vector of '*Ca.* Liberibacter americanus' has recently been shown to be *Diaphorina citri*[24].

In the leaves of infected citrus, the bacteria typically induce chlorosis that resembles the symptoms associated with Fe or Zn deficiency. However, there have been few studies to precisely quantify Fe, Zn, or other microelements in infected plants. Work on the African form of the disease in an unnamed citrus species compared macroelement concentrations in diseased and healthy plants[9]. These studies found that concentrations of potassium were higher in infected plants, whilst calcium and magnesium were lower. A study of infected plants in Réunion[1] found lower concentrations of Ca, Mn, and Zn in citrus trees. However, the species of citrus tested and the number of trees treated were not given, and no statistical inference was made. In addition, on this island, both African and Asian forms of the disease occur[4] and the form infecting the trees was also not reported. The study of Koen and Langenegger[9] and that of Aubert[1] relied on detection using visual symptoms, and the presence of liberibacters was not confirmed by PCR.

The Asian form of the disease is widely distributed throughout Southeast Asia[4,19] and is causing significant losses in citrus production. As the symptoms of HLB closely resemble those of Fe and Zn deficiency, it is important to know whether changes in these elements are commonly associated with infection by HLB. However, since the reports of Koen and Langenegger[9] and Aubert[1], there has been no work on mineral concentrations within HLB-infected trees. Therefore, this study looked at the mineral concentrations in a range of citrus species and cultivars grown in different environments to determine whether infection by '*Ca.* Liberibacter asiaticus' affects plant element composition.

Materials and methods

1. Plant taxonomy

A number of systems for classifying species of citrus exist, and the taxonomy of the genus is under review. The citrus species used in this study were: the rough lemon (*Citrus taitensis* Risso., syn. *C. jambhiri* Lush), the flat lemon (*C. reticulata* Blanco cv. Depressa, syn. *C. depressa* Hayata), the Siem mandarin (*C. reticulata* cv. Siem), the Tankan mandarin (*C. reticulata* cv. Tankan, syn. *C. tankan* Hayata), and the Satsuma mandarin (*C. reticulata* cv. Satsuma, syn. *C. unshiu* Marc). As most readers are likely to be more familiar with the classifications of Swingle and Reece[18] and Tanaka[21], the names used by these authors will be employed throughout the remainder of this article.

2. Pot trials

For tests of plants grown in the greenhouse, seedlings of rough lemon (*Citrus jambhiri* Lush.) and seedlings of flat lemon (*C. depressa* Hayata [a variety of mandarin]) were grown at the National Agricultural Research Center for Kyushu and Okinawa Region, Koshi, Japan, in pots filled with one liter of a commercial mix of soil and peat moss (Kenbyo, Yae Company, Nagasaki, Japan). In Indonesia, Siem mandarin (*C. reticulata* cv. Siem) plants grafted on rough lemon seedlings were grown at the University of Gadjah Mada (UGM), Yogyakarta, in pots filled with 5 kg of a 1:1:1 mixture of quartz sand, cocopeat, and composted lamb/goat dung. For each species, four to five plants were inoculated with '*Ca.* L. asiaticus' (Las) and a further five plants acted as healthy controls. All the plants were about 20 months old when they were graft-inoculated. In Japan, plants were watered with an equal volume of water, typically 100 ml per day, and were fertilized equally every month with 0.5 g of a commercial chemical fertilizer (N:P:K 6.5:6:19). In Indonesia, a slow release fertilizer NPK 15:15:15 was used at 5 g per pot. In both countries, the temperature of the greenhouses was 26–35°C during the day and 25–30°C at night.

For infection in Japan, a rough lemon plant harboring Las strain OC94–31 served as the source of inoculum. Budsticks were taken from the source plant and side-grafted to the stem of recipient plants about 50 mm above the ground. In Indonesia, budsticks were obtained from three field-grown, infected trees of Siem mandarin. The bark was removed from the budsticks to give patches of approximately 5 × 10 mm, and grafted onto the receptor Siem mandarin trees on rough lemon rootstocks. Each Siem mandarin tree was grafted with three bark patches that were separately taken from each infected plant. About 10 leaves were taken from each potted citrus plant for analyzing microelements.

3. Field investigations

Survey trips to citrus fields in Okinawa, Japan, were made in June 2005. Fully expanded leaves of Tankan mandarin (*C. tankan* Hayata) and Satsuma mandarin (*C. unshiu* Marc) were collected. For Tankan mandarin, three to four shoots at shoulder height were evenly chosen from each plant, and 15 to 20 leaves were sampled at random from middle leaf position of each shoot. For Satsuma mandarin, leaves from healthy (PCR-negative) plants were sampled in the same way as for Tankan. However, on infected (PCR-positive) plants, leaves showing symptoms (chlorosis) were sampled separately from those without symptoms. Since spring shoots appear in March in Okinawa, the age of the leaves was estimated to be about three months. About 10 leaves that were collected from a shoot of each branch were used for extracting microelements. In Indonesia, leaves were collected from Siem mandarin trees growing in a farmer's field in the Kulonprogo District, Yogjakarta Province, Java, in October 2006, following the sampling method established for Tankan and Satsuma mandarin in Japan. Before analyzing microelements from these leaves, all plants in both countries were tested for the presence of Las by PCR using other leaves of the same trees. Four randomly chosen leaves were tested for PCR detection, and a tree was determined to be positive for Las when at least one leaf tested positive by PCR.

4. Measurements of microelements in leaves

A batch of about 10 leaves was used for analyzing microelements in the leaves of each shoot. The microelement concentration of one main upright shoot was considered as the concentration of each potted plant, whereas the average of three to four shoots was used to estimate the microelement concentration of each plant in the field. Leaves were washed several times with tap water, three times with distilled water, and then moisture was gently removed by clean paper towels. The leaves were then bisected along the main vein. One half of each leaf was dried in an oven at 70°C for 48 h to determine leaf dry weight. The other half of each leaf was weighed and then homogenized in 20 ml of 2-(N-morpholino) ethanesulfonic acid (MES) buffer (1 mM, pH 6.0). The macerate was centrifuged at 10,000 rpm for 15 min, and then the concentrations of Cu, Fe, Mn and Zn in the supernatant were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES, Japan) or atomic absorption spectrometry (AAS, Indonesia). The concentrations of the microelements were then expressed on a dry weight basis for each tree.

5. DNA extraction and detection by PCR

Total DNA was extracted from about 0.2 g of leaf midribs using cetyltrimethylammonium bromide[12] and suspended in 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). PCR was performed to amplify a portion of the nusG-rplKAJL gene cluster[15] using primers MHO353 (5'-GTGTCTCTGATGGTCCGTTTGCT-TCTTTTA-3') and MHO354 (5'-GAACCTTCCACCAT-ACGCATAGCCCCTTCA-3') reported by Hoy *et al*[6]. In Japan, the PCR reaction mixture (20 µl) consisted of 200 µM of each dNTP, 0.25 µM each of each primer, 0.1 unit of *ExTaq* (Takara, Bio Inc., Shiga, Japan), 1 × Ex Taq buffer supplied by the manufacturer and an arbitrary amount of DNA template (approximately 100 ng). The amplifications were performed in a DNA Thermal Cycler 9700 (Applied Biosystems, Foster City, CA) for 40 cycles under the following conditions: 30 s at 94°C, 30 s at 56°C and 60 s at 72°C with an initial denaturation step of 2 min at 94°C. In Indonesia, the PCR reaction mixture (15 µl) consisted of 200 µM of each dNTP, 0.22 µM each of each primer (MHO353 and MHO354), 0.1 unit of *Taq* polymerase with anti-Taq monoclonal antibodies (Microzone Ltd, Haywards Heath, West Sussex, UK), 1 × manufacturer's buffer and 1 µl of DNA extract. The amplifications were performed in a DNA Thermal Cycler (MyCycler, BioRad) for 35 cycles under the following conditions: one cycle; 10 s at 94°C, 30 s at 65°C and 60 s at 68°C with an initial denaturation step of 5 min at 94°C.

6. Statistics

Analyses of variance were performed using STATISTICA for Windows, Version 6 (StatSoft, Inc., Tulsa, OK).

Results

1. Measurement of microelements in potted plants

The yellowing and mottling of leaves appeared on shoots of the rough lemon, the flat lemon, and Siem mandarin plants eight to twelve months after graft-inoculation. All graft-inoculated trees tested positive by PCR for Las: all uninoculated trees were PCR-negative (data not shown). In the trial using rough lemon, both the average concentrations of Fe and Zn were reduced in infected plants by approximately a half compared to levels in healthy plants (Fig. 1, Table 1). The average concentrations of Cu and Mn were not significantly affected by infection (Table 1). In the trial using flat lemon, all tested microelements except for Cu were significantly reduced by infection (Fig. 1, Table 1). For Siem mandarins, only the concentration of Fe was affected by infection by Las, with levels in infected plants again being half those of the healthy controls (Fig. 1, Table 1).

2. Measurement of microelements in citrus in the field

Detection of Las by PCR showed that there were four infected and three healthy trees of Satsuma mandarin and three infected and twelve healthy trees of Tankan mandarin in the orchards in Okinawa, Japan, whereas there were three infected and three healthy trees of Siem mandarin in Java, Indonesia. For the Tankan and Siem mandarins, concentrations of Zn were lower in diseased than in healthy plants with concentrations being a third to

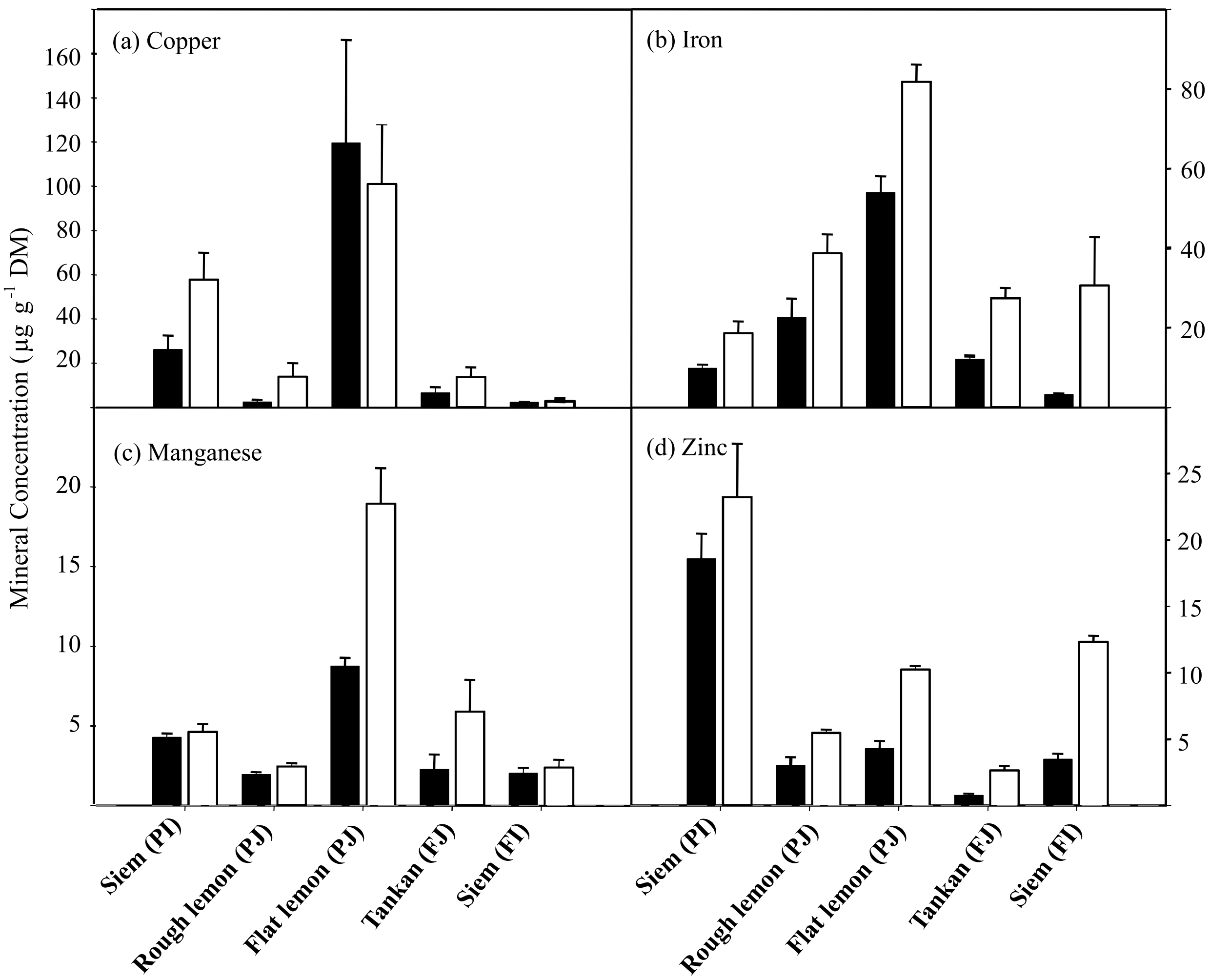

Fig. 1. Mean levels of microelements in dry matter (DM) found in healthy and HLB-infected citrus. The plants were either growing in the field (F) or were from pot trials (P)

Bars represent standard error. Abbreviations: PI, pot trial in Indonesia; PJ, pot trial in Japan; FJ, field trial in Japan; FI, field trial in Indonesia.

■ : Diseased, □ : Healthy.

Table 1. Statistical significance of the difference between the microelement concentrations found in healthy and HLB-infected citrus

Citrus	Copper		Iron		Manganese		Zinc	
	F[a]	*p*[b]	*F*	*p*	*F*	*p*	*F*	*p*
Siem (pot trial in Indonesia)	4.495	0.071	**7.95**	**0.022**	0.363	0.564	1.084	0.328
Rough lemon (pot trial in Japan)	3.451	0.100	**5.832**	**0.042**	3.700	0.090	**12.687**	**0.007**
Flat lemon (pot trial in Japan)	0.243	0.640	**18.706**	**0.005**	**11.43**	**0.014**	**110.317**	**<0.0001**
Tankan (field material in Japan)	0.568	0.460	**8.123**	**0.014**	0.752	0.401	**5.995**	**0.0293**
Siem (field material in Indonesia)	0.406	0.558	5.114	0.087	0.349	0.586	**211.567**	**0.0001**

Significant differences are in bold.
The plants were either growing in the field or were from pot trials. The mean concentration of each element is shown in Fig. 1.
a) *F* value, b) Significance level.

a half those in healthy material. Fe was also significantly lower in Tankan mandarin (Fig. 1, Table 1). The average concentrations of Fe in Siem mandarin were 3.25 and 30.65 μg g^{-1} in dry matter (DM) in the symptomatic and healthy plants, respectively (Fig. 1). However, despite a roughly 10-fold difference in concentration due to the large variance of Fe concentrations in the symptomless material, this difference fails to be statistically significant (Table 1). No statistically significant differences were found in Cu and Mn concentrations in these two varieties (Table 1).

In Satsuma mandarin, concentrations of Fe, Mn, and Zn were significantly higher in leaves from healthy plants than from diseased ones (Fig. 2, Table 2). However, there was little difference in concentrations of these elements between symptomatic and asymptomatic leaves from the same infected trees (Fig. 2). No significant difference was found in Cu concentrations of the leaves from healthy trees and those from infected trees (Table 2).

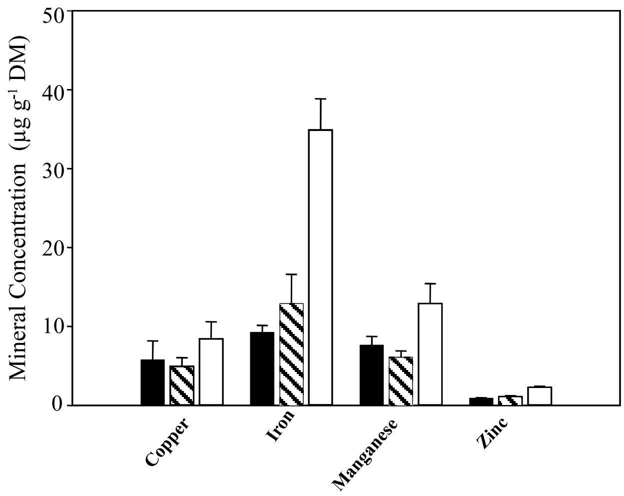

Fig. 2. Mean levels of microelements in dry matter (DM) found in leaves of healthy plants of Satsuma mandarin growing in the field in Japan and infected plants that were either showing symptoms or were symptomless

Bars represent standard error.
■ : Infected, symptomatic,
▨ : Infected, non-symptomatic,
□ : Healthy.

Table 2. Statistical significance of the difference between the microelement concentrations found in the leaves of healthy plants of Satsuma mandarin growing in the field in Japan and those of infected plants that were either showing symptoms or were symptomless

	F^{a}	p^{b}
Copper	0.766	0.496
Iron	**18.846**	**0.0009**
Manganese	**5.565**	**0.031**
Zinc	**36.471**	**0.0001**

Significant differences are in bold. The mean concentrations for each element are shown in Fig. 2.
a) *F* value, b) Significance level.

Discussion

This study has looked at mineral concentrations in citrus plants infected with Las. Infected leaves had lower Fe concentrations except for one marginal case in the field samples of Siem mandarin in Indonesia. Infected leaves also contained a significantly lower concentration of Zn except for one pot trial of Siem mandarin in Indonesia. On average, concentrations of Fe and Zn in infected plants were approximately half those in healthy plants. Cu was not significantly reduced in any of the trials, and Mn was occasionally lower in infected leaves. These results suggested that the concentrations of particular elements (i.e., Fe and Zn) rather than element concentrations in general are affected by infection by '*Ca.* Liberibacter asiaticus'. Given the similarity of symptoms caused by '*Ca.* L. africanus' and '*Ca.* L. americanus', it is likely that similar disturbances in elements occur during disease development after infection by these pathogens.

The material used in this study comes from four cultivars of mandarin (Tankan, Satsuma, flat lemon, and Siem) and an assumed hybrid (rough lemon) between *C. reticulata* and either *C. medica* L. (the citron) or *C. limon* (L.) Osbeck (the lemon)[11,13]. In Réunion, Aubert[1] also found lower concentrations of Zn, together with reductions in Ca and Mn in citrus trees. The plants used in our study and in that of Aubert's[1] were grown in a range of different conditions and included field- and greenhouse-grown plants. In addition, different isolates of Las in Japan and Indonesia were used and would presumably be different from the strain(s) present in Réunion. Thus, reductions in Fe and Zn appear to be a common feature of infection by Las. The citrus cultivars chosen for our study are widely grown in Japan and other Asian countries. Therefore, these insights into the pattern of mineral concentrations should be broadly applicable to commercial citrus in Asia.

The symptoms associated with Fe and Zn deficiencies in citrus include interveinal chlorosis, and the symptoms induced by HLB infection can be mistaken for these deficiencies. Fe is a cofactor for at least 139 enzymes that catalyze a wide range of biochemical reactions including catalase, peroxidase, and lipoxigenase, chlorophyll metabolism, respiration and insufficient Fe supply reduces protein synthesis, particularly membrane proteins[14]. Zn is also involved in many biochemical reactions including sugar, amino acid, and IAA metabolism[2,5,17]. Thus, our results provide an explanation for the similarity of the symptoms of HLB with those caused by Fe and Zn deficiencies.

It is surprising that only Fe and Zn concentrations in infected plants were affected. Any general dysfunction of the vasculature resulting from infection would be likely to affect all elements. It is known that infection by Las disrupts the phloem[23]; therefore, infection has the possibility of affecting element retranslocation within the plant. However, there is no feature of the chemistry of Fe and Zn that sets these two elements apart from Cu and Mn, and all of these elements are relatively immobile within the plant[16]. It has been proposed that Las produces a toxin, as cell-free extracts from infected plants cause chlorosis when injected into healthy citrus or tobacco[20]. In addition, several plant pathogenic bacteria produce metal chelating siderophores[10]. Therefore, it is possible that the toxin or siderophores interact with Fe and Zn so reducing their availability to the plant.

The results of this study have a number of practical implications. Currently used molecular assays that amplify fragments of the genomes of the organisms that cause HLB suffer from problems caused by the uneven and sporadic distribution of the pathogens within infected trees. This leads to problems with false negative results. Analyses of mineral concentrations may be used to supplement these methods of detection. The reduced levels of Fe and Zn in non-symptomatic leaves of the Satsuma mandarin suggest that reductions in mineral concentrations may precede the development of chlorosis. It may be possible that the lower Fe and or Zn concentrations in non-symptomatic shoots can be used to make a diagnosis of greening. However, this diagnosis method requires knowledge of the normal element status of plants and might not be applicable when most of the citrus trees in the field have low Fe concentrations due to lime-induced Fe deficiency. Furthermore, as Fe and Zn deficiencies appear early in the development of the disease, applications of these elements may reduce symptom expression and increase tree life.

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