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Review

Alleviation of Huanglongbing disease in citrus by foliar application of microelements

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Huanglongbing (HLB), also referred to as citrus greening disease, ranks high among the most destructive diseases in citrus plants worldwide. This disease is caused by the Gram-negative bacterium *Candidatus* Liberibacter species. As a strategy for the appropriate management of this disease has not been established yet, economic cultivation of citrus in the diseased areas has mostly ceased. One of the most conspicuous phenotypic characteristics of this disease is the chlorosis caused by bacterial plaques in the plant phloem systems due to microelement deficiency. Therefore, the effects of the disease may be mitigated with sufficient supply of these nutrients. This may in turn lead to the establishment of a strategy to manage the disease symptoms, even though trees might not completely recover. Such management would at least enhance the longevity of trees and contribute to an increase in their yield. Thus, an approach of reviewing microelement function might provide insights that can be translated into strategies for HLB management.

Key words: Citrus, HLB, nutrient, micronutrient, pathogen.

INTRODUCTION

Citrus huanglongbing (HLB) or citrus greening disease, caused by the Gram-negative, phloem-limited Alphaproteobacteria *Candidatus* Liberibacter species is the most destructive citrus pathosystem across the globe (Jagoueix et al., 1994; Bové, 2006; Duan et al., 2009; Gottwald, 2010; Ghosh et al., 2018). This pathogen comprises three species, that is, '*Candidatus* Liberibacter asiaticus' (CLas), '*Candidatus* Liberibacter africanus', and '*Candidatus* Liberibacter americanus', which are distinguished using 16 rDNA sequencing (Bové, 2006). These bacteria are vectored by hemipteran insects, that is, *Diaphorina citri* Kuwayama (Grafton-Cardwell et al., 2013; Tabatchnick, 2015) or *Trioza erytreae* Del Guercio (Rasowo et al., 2019; Aidoo et al., 2021). HLB caused by these pathogens is distributed in more than 40 countries including major citrus-producing areas, such as China, Brazil, USA, and India (Gottwald, 2010). This disease seems to have potential to expand into unaffected areas

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(Ajene et al., 2020). If curative measures are not developed, the citrus industry could be destroyed globally. In the USA, the disease was first reported in 2005 in Florida, and then spread to Louisiana, South Carolina, Georgia, Texas, California, and Arizona (Gottwald, 2010). It is estimated that the production costs were 40% greater for the management of the vector insect and HLB after the spread than before (Irey et al., 2008). So far, about 80% of citrus trees in Florida were infected with the HLB pathogen, compared to before HLB pandemic, and the average percentage yield loss reaches 41% (Singerman and Useche, 2016). In Asia, HLB was reported in South China in 1943 and in Taiwan in 1951. This destructive citrus disease continued to spread in Southeast Asia and reached Japan in 1988 (Miyakawa and Tsuno, 1989).

Presently, HLB management strategies are limited with respect to both efficiency and efficacy (Bassanezi et al., 2020; Li and Feng, 2020; Zapata et al., 2021). In some countries where HLB is limited to small areas or where HLB invasion is in a relatively early stage, the removal of HLB-infected trees is used as a strategy to eradicate the disease (Bové, 2012; Bassanezi et al., 2013). However, this strategy is laborious and cannot be carried out in large areas where vector invasions are unavoidable or uncontrolled. Another strategy is the use of antibiotics (Zhang et al., 2014; Hu et al., 2018; Chanvatik et al., 2019; McKenna, 2019; Yang et al., 2020), which may reduce the pathogen load in trees, resulting in the disappearance of symptoms. Nonetheless, "re-appearance" or "re-infection" does occur in response to the surviving bacteria or repeated transmission of the pathogen by vectors (Aubert and Quilici, 1984; Zhang et al., 2014; Hu et al., 2018). However, the use of antibiotics is restricted or has been completely eliminated in agriculture. Recently, Huang et al. (2021) revealed that stable antimicrobial peptides from *Microcitrus australasica* killed HLB bacteria and consequently prevented HLB infections (Huang et al., 2021). However, as this technology is still in its initial stages, it cannot be used for HLB management yet. Thus, although some of the measures reported so far may reduce the occurrence of the disease, they are either expensive or labor-intensive. Therefore, practical management measures for citrus growers need to be developed.

Foliar application of nourishing materials including micronutrients enhances citrus tree vigor against HLB (Wang, 2019; Bassanezi et al., 2011). Recently, two pioneer papers have reported data that can be used to develop practical management strategies for HLB. While one paper reported the curative effects of manganese (Mn) on HLB, particularly the disappearance of the pathogen from the HLB-affected trees (Zambon et al., 2019), the other reported the reduction of both the pathogen population and symptomatic appearances on the plant body (Inoue et al., 2020a). As typical HLB symptoms, such as yellowing of leaves, resemble symptoms that are attributed to micronutrient deficiency (Ohtsu et al., 1998), it may be possible that a sufficient supply of these elements could mitigate the disease symptoms. Although the use of these nutrients to treat the disease is debatable, it is worth reviewing the functions of these elements as potential remedial agents in terms of their interaction with the pathogen and with respect to plant physiology. These elements have a relatively lower cost of use and might be included in strategies used for HLB management. Here, we discuss micronutrients that have been reported in relation to citrus physiology and determine whether they are effective with respect to stemming HLB infection, and their potential for disease management.

HLB TREATMENT BY Mn APPLICATION

In plants, Mn plays an important role in physiological function, that is, acting as an enzyme co-factor or as a metal with catalytic activity in biological processes. As HLB infection lowers the pH in the leaves of satsuma mandarin (*Citrus reticulata*) (Masaoka et al. 2011, Zambon et al. 2019), foliar application of $MnSO_4$ resulted in 45% more yield from HLB-affected trees. This effect of Mn is concentration dependent. A higher concentration, that is, 2-times more, reduced the yield by 25% (Morgan et al., 2016), whereas foliar application of $Mn_3(PO_3)_2$ in a phosphate form resulted in a 25% reduction (Morgan et al., 2016). These results suggest that Mn mitigates the effects of HLB if applied in an appropriate compound form and at an appropriate concentration.

The improvement in physiological conditions in response to Mn application has been reported in other crops. For example, the foliar application of Mn as $MnSO_4$ in cowpea reduced 42.7 and 42.0% of the disease severity caused by *Rhizoctonia solani* and *Rhizoctonia bataticola*, respectively (Kalim et al., 2003). In a sugarcane variety (*Saccharum* species), which is susceptible to orange rust (*Puccinia kuebnii*), a single spray of Mn at a concentration of 0.5 or 1.0% reduced the percentage of diseased leaf area by 2.2 and 0.9%, respectively, which was much lower than the 15% observed in untreated plants (Mesquita et al., 2019). In coffee, the foliar application of $Mn_3(PO_3)_2$ suppressed the coffee rust (*Hemileia vastatrix*)-induced bean damage (Pérez et al., 2020). Chaves et al. (2021) reported that Mn reduced the symptoms of white-mold disease (causative agent, *Sclerotinia sclerotiorum*) in tomato. No physiological and biochemical roles of Mn have been distinguished. The adverse effects of *S. sclerotiorum* infection on photosynthesis have been reported to be mitigated by $MnPO_4$, as evidenced upon the evaluation of the net carbon assimilation rate, stomatal conductance in water vapor, transpiration rate, maximal photosystem II quantum yield values, and concentrations of photosynthetic pigments (Chaves et al., 2021). Excess Mn in plant leaves induces oxidative stress, resulting in toxicity by disruption of photosynthetic electron flow in chloroplasts (Fernando and Lynch, 2015). In addition, *in-vitro* assays showed that $MnPO_4$ inhibited the growth of *S. sclerotiorum* in a dose-dependent manner, indicating that $MnPO_4$ directly affects pathogen growth, in addition to allowing the plant to develop resistance against the disease. TigerSul manganese+, a solution containing 0.16% Mn, has been reported to reduce the pathogenicity/virulence of CLas using quantitative PCR of sweet orange leaves (Zambon et al., 2019); its root application increased the yield by 45% and lowered the HLB-pathogen load to below a qPCR detectable level. However, the therapeutic effects of a mixture of Mn (0.16%) and boron (0.44%) have not been confirmed. No explanations have been provided for the loss of Mn effects when Mn is applied in the form of this mixture. A possible reason is that micronutrients applied in combination including Mn can reduce the acquisition of CLas by *D. citri*, thereby reducing the disease infection (da Silva et al., 2020). We are await further studies on the effects of Mn on citrus HLB.

ALLEVIATION OF HLB SYMPTOMS BY Fe IN BIOAVAILABLE FORMULATIONS

The mechanism of Fe absorption systems in plants are divided into two strategies, strategy I and strategy II (Römheld and Marschner, 1986). Under Fe deficiency, graminaceous plants secrete Fe chelate compounds from their roots, mugineic acids family to uptake Fe(III)-mugineic acids as a complex formulation (Römheld and Marschner, 1986). Non-graminaceous plants secrete reductants or chelate compounds from their roots into the rhizosphere, enhancing proton excretion and increasing their ferric reduction capacity in the root surface and the transport of Fe(II) across the plasma membrane by Fe(II) transporters (strategy I) (Mori, 1999). In contrast, some dicot plants are poorly adapted for Fe limited soil. Citrus plants utilize strategy I, and some plants in the citrus species are susceptible to Fe limited calcareous soil; citrus trees with many commercial rootstocks perform poorly in high-carbonate soils (Castle et al., 2009).

The HLB pathogen causes interveinal chlorosis in leaves, which reduces the activity of basic chemical reactions in the photosynthesis of plants. Masaoka et al. (2011) compared the composition of metal elements in leaves between healthy trees and HLB-infected ones in two mandarin plants: satsuma mandarin in Japan (*Citrus unshiu* Marc.) and Siem in Indonesia (*C. reticulata*). They revealed similar deficiency of Fe, Zn, and Mn in HLB-infected trees, especially Fe (Nwugo et al., 2013; Manzanilla-Ramírez et al., 2019; Zambon et al., 2019). These studies suggested that HLB led these three elements to be reduced in plant leaves. Therefore, supplying these citrus bioavailable microelements may help overcome HLB disease symptoms.

Fe deficiency of citrus may have traits similar to those of HLB disease resistance. As Fe in an insoluble form (Fe_2O_3) cannot be used directly by plants grown on neutral to alkaline soils, these plants suffer from Fe deficiency and experience disorders in essential physiological reactions in their body such as photosynthesis, respiration, oxygen transport, and gene regulation (Marschner, 2011). Therefore, plants have evolved Fe acquisition strategies, such as strategy I and II (Marschner 2011), of which citrus plants use strategy I (Wulandari et al., 2014). Graham et al. (2017) reported that symptoms of HLB developed rapidly in citrus grown on high-pH soils in Florida, where Fe precipitated easily into the soil. Similarly, HLB-infected trees are not found in low-pH soils and are common in high-pH soils in Tokunoshima, Kagoshima Prefecture, Japan (Inoue et al., 2020b), suggesting that citrus trees in alkaline soil are more vulnerable to HLB pathogenicity. In addition, cultivars resistant to HLB, such as *Murraya exotica* (Ramadugu et al., 2016), have higher root Fe reductase activity than susceptible cultivars, such as *Poncirus trifoliata* (Wulandari et al., 2014). In summary, citrus plants that can make efficient use of Fe are resistant to HLB disease.

Physiological functions of Fe in the plant body have been reported for their antagonistic effects on plant diseases. Foliar spray of Fe reduces the pathogenicity of the disease, resulting in the disappearance or paling of symptoms (Aznar et al., 2015; Peris-Peris et al., 2017; Nobori et al., 2018). The expression of Fe reductase oxidase genes (*FROs*), which turn ferric ion into highly active ferrous ion that is involved in Fe acquisition, were partially suppressed in HLB-affected citrus (Zhong et al., 2015). In other words, if FROs are activated by supplying bioavailable Fe in the plant body, then the plant may recover from HLB or the effects of the disease. Among these Fe chelate solutions, the most effective for cure HLB-affected tree can sustain the divalent Fe state via X-ray absorption fine structure analysis (Inoue et al., 2020a). The possible Fe impact on pathogen survival is supported by experiments on the model plant *Arabidopsis thaliana* in which a wide variety of siderophores secreted by the pathogenic *Pseudomonas syringe* pv. tomato DC3000 could be controlled by divalent Fe (Nobori et al., 2018). The authors suggest a competitive function of Fe with the microorganisms in the plant, assuming that microorganisms may be able to use Fe in their own biological processes, in turn raising the competition for Fe uptake between plants and pathogens.

Adverse effects of extra-applied Cu due to ionization tendency over the other metals

Copper (Cu) is an essential element in plants used as a growth stimulant. Camp and Fudge (1939) first revealed the nutritional role of Cu. However, Cu overdose has been recognized as being toxic by citrus growers. Nevertheless, Cu has been used as a nutritional element or a fungicide over the past 80 years in Florida (Driscoll, 2004). Extra Cu applied on the plant provides no hints on the aerial parts of the plant body but causes serious damage in the subterranean systems, especially on the fine root growth (Adrees et al., 2015). The expression of overused Cu might be due to the competitive behavior of this metal with others in the soil or by prevention of physiological functions of other elements in the leaf.

Owing to its lower ionization tendency, excess Cu is precipitated out of soil in the form of a cation and leaches out of the soil, while other metals remain ionized in the soil. Thus, the chemical interaction of Cu results in the deficiency of the elements in the plant (Marschner, 2011; Kopittke and Menzies, 2006). Similar interactions of Cu may occur in leaves, and the overuse of Cu results in high concentrations of Cu reducing yields of citrus production (Bakshi et al., 2013; Behlau et al., 2010; Fan et al., 2011). The overuse of Cu leads to the reduction of microelements in HLB-affected trees of *Citrus sinensis*, although not statistically significant (Ebel et al., 2019). These effects of Cu may be seen in Florida, where the land suffered from severe deficiency in micronutrients due to excessive Cu application (Driscoll, 2004). This is partly explained by the following reasons: Cu has a lower ionization tendency than other heavy metals, which promotes ionization of other metals. Therefore, it is considered that a deficiency of metals other than Cu is caused by yield reduction of citrus fruits. Gottwald et al. (2012) succeeded in removing the Cu effects on micronutrient deficiency and increased citrus fruit yield by supplying the deficient elements through foliar applications or soil drench. Therefore, excessive supply of Cu may have adverse effects on citrus plants.

ZINC TRANSPORT SYSTEM OF HLB-AFFECTED CITRUS MAY BE HIJACKED BY HLB-BACTERIA FOR ITS PATHOGENICITY

Zinc (Zn) that is trivially absorbed through root is indispensable in plants. According to comprehensive reviews on the nature and biochemistry of elements by Broadley et al. (2007), Haydon and Cobbett (2007), and Marschner (2011), HLB-affected citrus trees appear to have much higher Zn requirements than healthy trees. After one year, HLB-affected citrus showed typical HLB symptoms and significantly reduced Zn concentrations in leaves. Micro-XRF imaging of Zn and other nutrients showed that preferential localization of Zn is observed in the stems and leaves collected from healthy grapefruit plants, but lower signal is from HLB-affected samples. Zn concentration in the phloem of veins in healthy leaves is more than 10 times higher than that in HLB-affected leaves (Tian et al., 2014). Albrecht and Bowman (2008) revealed differential expression of the Zn transporter Z1P1 (AT3G12750 in the AGI number system) in the microarray of healthy or HLB-infected *C. sinensis* trees. The *ZIP1* gene was up-regulated by 13.2-fold in HLB-affected trees compared to that in healthy trees. Aritua et al. (2013) reported that the Zn transporter ZIP1 and putative Zn transporter genes were upregulated by 3.76- and 1.48-fold, respectively (in the value of a digit Log2), in HLB-infected trees. Shahzad et al. (2020) performed RNA-seq analyses and suggested that the expression of Zn transporter genes in sweet orange was homologous to the genes. The authors confirmed the expression of Zn transporter10 (ZIP10 orange 1.1g018585) by real-time PCR. Their results suggested that HLB-affected citrus trees had an increased requirement for Zn according to the gene expression level. Treatment of HLB-infected trees with Zn thus augments the pathogenicity of the bacteria in the trees (Zhang et al., 2016). This indicates that the pathogen is hardly controlled by the application of Zn.

Zn is an essential micronutrient for bacteria (McDevitt et al., 2011) and modifies the function of about 100 different proteins including enzymes (Ma et al., 2009). The genome sequence analyses of HLB-infected trees revealed a high-affinity Zn in the uptake system (Duan et al., 2009; Vahling-Armstrong et al., 2012). Molecular studies showed that the Zn cascade encoded by znuABC in plant cells regulated Zn metabolism by importing the element in insufficient amounts due to HLB infection (Vahling-Armstrong et al., 2012). In HLB-infected trees, higher levels of Zn are observed, which may be due to plant~~s~~ physiological changes or Zn related gene expression above (Razi et al., 2011; Zhang et al., 2021). The shortage of Zn may be caused by the Zn uptake by HLB bacteria that overrules a number of plant functions for their survival in the plant (Zhang et al., 2015; Shi et al., 2016). Therefore, the bacteria are compared to a hijacker in the metal transport system of the plant, which consequently develops virulence to the host.

CONCLUSION

Plants treated with Mn are protected from severe attacks by pathogens with recessing HLB disease symptoms (Zambon et al., 2019; Kwakye et al., 2022). This report does not refer to the changes in Fe dynamics in plants by Mn application. The mutual or antagonistic relationships between Mn and Fe, particularly their synergic functions in disease therapeutics, need further study. Other elements may be involved in the interaction of the two microelements. An application of zinc sulphate in combination with manganese sulphate can enhance the vigor and quality of citrus fruits against citrus greening disease (Hussain et al., 2022). The mechanism of element usage can contribute to the development of HLB control. Although the costs associated with the use of these agents must be taken into account for the establishment of HLB management, the application of micro nutritional elements has not been studied so far. Therefore, this review could facilitate future research to address these issues.

CONFLICT OF INTERESTS

The authors have not declared conflict of interests.

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