Research Article

Investigating the Impact of Huanglongbing in Citrus in Southern Lao PDR

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Abstract

Citrus has been promoted in the Lao People's Democratic Republic (Lao PDR) as a poverty reduction strategy for at least two decades. However, citrus trees have been in widespread decline for no less than ten years. Since 2010, the authors have observed symptoms on citrus trees consistent with the bacterial disease huanglongbing (HLB). These symptoms included asymmetric leaf mottle, small lopsided fruit, poor fruit production and tree decline. The authors then initiated a long-term study on the occurrence of HLB in southern Lao PDR. Samples of leaf mid-ribs were collected from citrus trees in orchards, nurseries, and backyards across four provinces: Champasak, Sekong, Salavan, and Savannakhet. The presence of '*Candidatus* Liberibacter asiaticus', the putative causal agent of the Asiatic form of HLB, was confirmed in 59 of 109 samples collected in all four provinces. The Asian citrus psyllid, the vector of '*Ca*. L. asiaticus', was also observed on citrus trees and tested positive for the pathogen. The implications of these findings for citrus production in Lao PDR are discussed.

Keywords: graft-transmissible, Asian citrus psyllid, smallholder

Introduction

The Lao People's Democratic Republic (Lao PDR) is a mountainous country in southeast Asia bordered by Việt Nam, Cambodia, Thailand, Myanmar and China (Figure 1). The Mekong River runs through the country from north to south, and the land is prone to damaging storms, floods, landslides, and drought. There are encouraging trends in economic growth and development and the Lao PDR is expected to graduate from Least Developed Country status in 2026 (United Nations 2021). However, poverty, hunger, and malnutrition remain a challenge for the Lao PDR (World Food Programme 2021). Food insecurity is exacerbated by poverty, volatile food prices, land availability, weather extremes and poor productivity. Rain-fed rice is the main staple crop grown on the relatively flat land along the Mekong and in mountainous areas. Horticultural crops have been promoted and have made a significant contribution to poverty alleviation in some areas. However, plant diseases have constrained productivity (Ireland et al. 2014; Ireland et al. 2016; Callaghan et al. 2016; Callaghan et al. 2016).

Citrus has been promoted as a poverty alleviation crop by the government of Lao PDR for at least two decades. Smallholder orchards and backyard citrus are found in the north near Vang Vieng and Luang Prabang, and in the south mainly in the provinces of Champasak, Sekong and Salavan (Figure 1). There is a new citrus area in the Vilabouly District, eastern Savannakhet, and another on the Bolavan Plateau in Paksong District, Champasak Province. Fruit is exported fresh or dried to Việt Nam and Thailand, but there are no accurate records on total production and domestic consumption. There is limited support for the industry, and little is known about citrus disease issues faced by farmers.

The widespread decline of citrus trees in the major growing areas led to a request for assistance to the Australian authors by the Lao PDR Department of Agriculture in 2010. Subsequent investigations found citrus trees with asymmetric leaf mottle that crosses leaf veins, small upright chlorotic leaves with symptoms resembling nutrient deficiencies, raised corky leaf veins, small lopsided fruit, poor fruit production and tree decline. All these symptoms are consistent with the devastating bacterial disease huanglongbing (HLB) (Bové 2006; Gottwald et al. 2007) and were observed in mandarin, lime and pomelo trees affected by severe decline near Vang Vieng in northern Lao PDR in 2010, and in the south in Champasak Province in 2013.

HLB is the most significant threat to global citrus production and is well established throughout southeast Asia (Beattie and Holford 2008). Citrus and other Rutaceae

are affected by HLB, which is associated with endogenous, phloem limited, α-Proteobacteria that include: '*Candidatus* Liberibacter asiaticus' ('*Ca*. L. asiaticus'), '*Ca.* L. americanus' and '*Ca.* L. africanus' (Bové 2006). The Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama: Hemiptera: Sternorrhyncha: Psyllidae), is the vector of '*Ca*. L. asiaticus' (Bové 2006), and both '*Ca*. L. asiaticus' and ACP were previously reported as being present in northern Lao PDR (Garnier and Bové 2000; EPPO 2022a, b).

HLB is considered the biggest biosecurity threat to the Australian citrus industry. Knowledge and positive control material gained by Australian scientists through work in Lao PDR forms part of a collaborative effort to prepare Australia for an incursion of HLB. The authors have undertaken regular surveys for HLB in citrus focused on the southern provinces of Lao PDR from 2014 to 2020, and the findings are reported here.

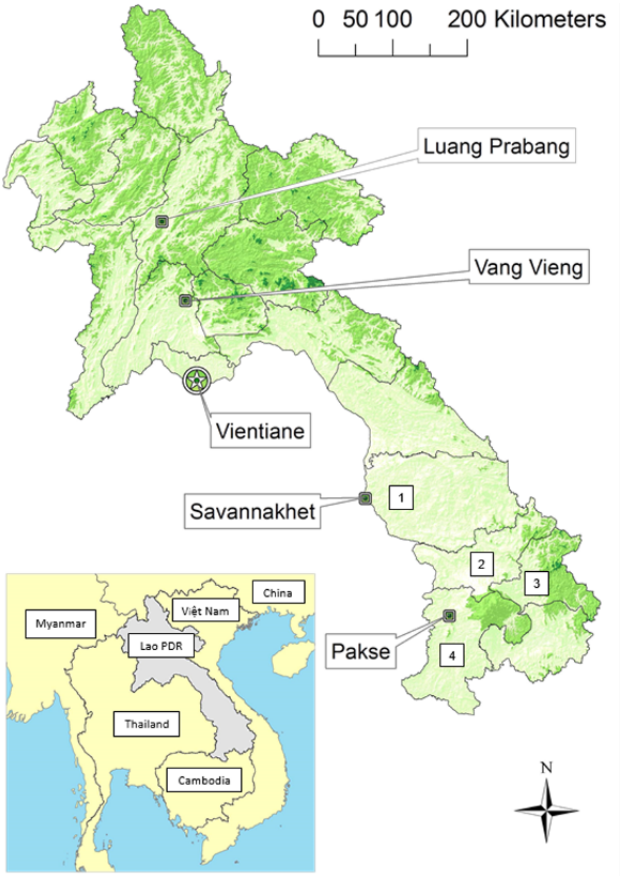

Figure 1. Map of the Lao People's Democratic Republic (Lao PDR) showing the capital Vientiane, key provincial cities, and provincial borders. Numbers indicate the four provinces from where samples were collected: (1) Savannakhet (2) Salavan (3) Sekong and (4) Champasak. The inset map shows the countries bordering Lao PDR. Map modified from original by C. Vote.

Materials and Methods

Citrus trees were inspected in nurseries, orchards, and backyards in the provinces of Champasak, Sekong, Salavan and Savannakhet. The selection of sites was on an *ad hoc* basis given the irregular distribution of plantings or single trees—the objective being to obtain representative samples from across the southern provinces. Samples consisting of five to ten leaves were collected predominantly from symptomatic trees in 2014 (May to August), 2015 (February), 2017 (August, September) and 2018 (February and October), and from both symptomatic and non-symptomatic trees in February 2020. From symptomatic trees, leaves were collected with chlorotic patterns such as asymmetric mottle, or raised, corky leaf veins. Leaves were collected from each quadrant of the canopy of non-symptomatic trees, or from as many access points as possible given the conditions and height of foliage. Leaves from individual trees were pooled as one sample and were kept cool by storing in an insulated container with an ice pack until they were able to be processed. Later, on the day of collection, leaf mid-ribs were excised using a sterile cutting tool and preserved in 70% ethanol. Additionally, adult psyllids were collected, identified morphologically as ACP and preserved in 70% ethanol. Leaf mid ribs and psyllids from the same tree were stored in two separate vials. All samples were transported under permit to Australia for pathogen testing.

Laboratory testing was undertaken at the New South Wales Department of Primary Industries' Elizabeth Macarthur Agricultural Institute in Menangle, Australia. All DNA extracts from the leaf midribs and adult psyllids were tested for '*Ca*. L. asiaticus', the putative causal agent of HLB in Asia. DNA extracts from leaf and psyllid samples collected in 2018 and 2020 were also tested for '*Ca*. L. africanus' and '*Ca*. L. americanus'.

Total DNA was extracted from the leaf mid ribs using a RedExtract-N-Amp kit (Sigma-Aldrich, Australia) or an ISOLATE II Plant DNA Kit (Meridian Biosciences, Ohio, United States). Sections up to 10 mm in length were excised from up to 5 mid ribs per sample to produce a weight of 50 mg of plant material for extraction. DNA was extracted from individual psyllids using a DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). Quantitative polymerase chain reactions (qPCR) were performed on DNA extracts from leaf and psyllid samples as per Li et al. (2006) using primers and probes specific to '*Ca*. L. asiaticus' (HLBas/HLBp/HLBr), '*Ca*. L. africanus' (HLBaf/HLBp/HLBr) and '*Ca*. L. americanus' (HLBam/HLBp/HLBr), with COX internal controls (COXf/COXp/COXr) for leaf samples and, as per Manjunath et al. (2008) targeting the *wingless* gene (*wg*) of *D. citri* (DCf/DCp/DCr) for psyllid samples. All reactions were performed in duplicate, and the data was analysed using Rotor-Gene® Q software (QIAGEN). Conventional PCR was performed as per Boykin et al. (2012) to confirm the psyllid identity as *D. citri* using the primer pair DCITRI COI-L/DCITRI COI-R.

DNA extracts from representative *D. citri* and leaf samples were tested for '*Ca*. L. asiaticus' as per Morrow et al. (2020) using the generic liberibacter primer pair 484F/1124R. The purified, DNA amplification products

were bidirectionally sequenced at the Australian Genome Research Facility, Sydney Australia.

Results

Most citrus trees were propagated from seed or marcottage (air-layering). Grafted trees were only observed in significant commercial plantings. Symptoms consistent with HLB were observed on trees of West Indian lime (*Citrus* × *aurantiifolia* var. *aurantiifolia*), Tahiti lime (*C.* × *latifolia* var. *latifolia*), leech lime (*C. hystrix* DC), pomelo (*C. maxima* (Burm.) Merr), sweet orange (*C.* × *aurantium* var. *sinensis* L.) and mandarin (*C. reticulata* Blanco). Symptoms included asymmetric blotchy mottle on leaves (Figure 2a), small upright chlorotic leaves with patterns resembling nutrient deficiencies, raised or corky leaf veins, lopsided fruit shape (Figure 2b) and tree decline (Figure 3a). The symptoms of raised corky leaf veins, lopsided fruit and tree decline could also be induced by some variants of citrus tristeza virus (CTV).

Adult psyllids, identified morphologically as ACP and confirmed by PCR, were collected from West Indian lime trees in Khong and Paksong Districts, and a sweet orange tree in Khong District. Also, psyllids were collected from an ornamental garden plant of *Murraya paniculata* (L.) Jack. in Pakse town. Psyllid adults and nymphs with waxy tubules were observed on young citrus flush in Sansomboun District (Figure 4).

Figure 2. (a) Symptoms of huanglongbing (HLB) expressed as asymmetric blotchy mottle on West Indian lime leaves in Khong District, Champasak Province; (b) asymmetrical pomelo fruit from a HLB positive tree, Phatoumphone District, Champasak Province

Figure 3. Citrus trees in Khong District, Champasak Province: (a) symptomatic West Indian lime tree, '*Candidatus* Liberibacter asiaticus' ('*Ca*. L. asiaticus') positive; (b) healthy pomelo tree in a mixed garden, '*Ca*. L. asiaticus' not detected

Figure 4. Asian citrus psyllid (ACP) adults and late-instar nymphs feeding on West Indian lime, Sanasomboun District, Champasak Province

'*Ca*. L. asiaticus' was detected using qPCR in 59 citrus leaf samples collected from 109 trees from 2014 to 2020 (Table 1). Additionally, '*Ca*. L. asiaticus' was detected in six of seven psyllids collected from one symptomatic West Indian lime tree on Don Khong island; the leaf sample from that symptomatic tree also tested positive. Furthermore, adult psyllids from a lime tree in Paksong District tested positive for '*Ca*. L. asiaticus'; no corresponding leaf sample was collected. The sequences from *D. citri* and leaf samples collected from a symptomatic West Indian lime tree in Khong District, were found to be 100% identical to published sequences for '*Ca*. L. asiaticus'. The sequences from both leaf and psyllid samples were lodged with GenBank (accession numbers MW386759 and MW386760 respectively). '*Ca*. L. africanus' and '*Ca*. L. americanus' were not detected in any samples using qPCR.

Table 1. Number of '*Candidatus* Liberibacter asiaticus' ('*Ca*. L. asiaticus') detections by quantitative real-time polymerase chain reaction (qPCR) in citrus leaf samples collected in the southern provinces of the Lao People's Democratic Republic (the first number is the number of positive samples and the second number is the number of samples tested)

Sample location	Number of '*Ca*. L. asiaticus' detections / Number of samples tested				
	lime[1]	leech lime	pomelo	orange	mandarin
Savannakhet Province					
Savannakhet District	3/4				
Vilabouly					2/3
Sepon	1/1			0/1	
Salavan Province					
Lakhonepheng	1/1		1/1	0/1	
Sekong province					
Sekong				1/1	
Thateng	0/2		0/2		
Champasak Province					
Khong	4/5	1/1	4/6	0/7	
Phatoumphone	2/7	3/4	2/2		0/1
Pakse		1/1	2/2	1/1	
Bachieng	1/1	3/4			
Paksong	4/19	1/1	9/11	0/3	2/5
Sanasomboun	9/9				
Champasak		1/2			

[1] All samples were identified as West Indian lime, except for 1 sample from Savannakhet District identified as Tahiti lime

Discussion

HLB was found in all four provinces, and ACP was recorded in three districts of Champasak Province up to an altitude of approximately 900 m above sea level (ASL). The only positive detections of '*Ca*. L. asiaticus' on the Bolavan plateau (above 900 m ASL) were in trees imported to the plateau from neighboring countries, and ACP is not known to occur on the plateau. '*Ca*. L. africanus' and '*Ca*. L. americanus' were not detected, consistent with reports on the distribution of these pathogens (EPPO 2022 c, d). Other pathogens were detected in a limited number of leaf samples, such as viroids (Donovan et al. 2020) and CTV (Donovan et al. 2021). HLB was most common and is likely to be the major contributor to the decline of citrus trees in southern Lao PDR.

Most citrus trees in Lao PDR are seedlings or propagated by marcottage (air-layering). Seeds in fruit from HLB affected trees are typically aborted, or small and dark. There have been conflicting reports on seed transmission of '*Ca*. L. asiaticus'. Reports range from infection confirmed by PCR in seedlings grown from infected seed, with or without symptom expression, and typically transient and in a low percentage of seedlings (Graham et al. 2008; Shatters 2008; Benyon et al. 2009; Bagio et al. 2020), to no symptoms and no PCR detections (Hartung et al. 2010). However, the findings of Bagio et al. (2020) do not support transmission of viable '*Ca*. L. asiaticus' cells from seeds to seedlings. All graft-transmissible citrus pathogens can be spread by marcottage, and '*Ca*. L. asiaticus' can be introduced to citrus plants via psyllid vectors, such as ACP.

The citrus trees in Vang Vieng Province in northern Lao PDR were in severe decline in 2010 and, by the 2014 survey, many trees had already died. In one village, Ban Somsuvad, no HLB symptoms were observed on young, replacement West Indian lime trees, and samples did not test positive (data not shown). This could be because they were seedling trees, or were produced via marcottage from healthy trees, or the trees were infected at titers below detectable levels. Farmers commented that citrus plants were not shared between villages because of the mountainous terrain and that few farmers owned motorbikes, serving as unintentional yet effective local quarantine.

In the south, repeat visits enabled the authors to track the decline in some orchards, although individual trees were not monitored. A smallholder in Sanasomboun District planted West Indian lime trees in individual concrete pipes (diameter 800 mm) around 2015. Symptoms consistent with HLB of asymmetric leaf mottle, nutrient deficiency and tree dieback were observed on trees in February 2018, but samples did not test PCR positive. The orchard was revisited in September 2018 and February 2020; symptoms had progressed, and all trees sampled tested positive for '*Ca*. L. asiaticus' by qPCR. In addition, adults and nymphs of ACP were observed in the orchard in 2018, 2019 and 2020. In contrast, in the Phatoumphone District, although one West Indian lime orchard was devastated by HLB with declining trees testing positive for '*Ca*. L. asiaticus', nearby plantings of West Indian lime did

not have HLB symptoms, have not tested positive for '*Ca*. L. asiaticus' and were producing significant quantities of fruit despite minimal management. Further south in the Khong District, not all trees with HLB-like symptoms tested positive for '*Ca*. L. asiaticus' in August 2014. By September 2018, most trees exhibited symptoms of the disease and tested positive for the pathogen. During the February 2020 survey, it was challenging to find live citrus trees. Trees in larger citrus plantings, surveyed in previous years, had died. Most remaining trees were unthrifty and tested positive for '*Ca*. L. asiaticus', aside from an isolated, large pomelo tree (Figure 3b). There are conflicting reports about the susceptibility of citrus varieties to HLB, potentially due to differences in host or liberibacter genetics, the environment and management practices (Ramadagu et al. 2016). This contradiction has been highlighted for pomelos in Asia, and this is not the first time that the authors have observed healthy pomelo trees in areas where other citrus trees have succumbed to HLB.

Note that the latency of '*Ca*. L. asiaticus' in citrus plants (time from inoculation to when the infected plant is a source of inoculum) can be highly variable, but it is reported to be shorter than the incubation period (Canale et al. 2020). The incubation period (time from infection to symptom expression) for '*Ca*. L. asiaticus' in citrus can range from a few months to several years, and is shorter in young, vigorous trees. Furthermore, there is a delay from infection to successful PCR detection of '*Ca*. L. asiaticus', although typically '*Ca*. L. asiaticus' can be detected using PCR prior to symptoms appearing (Gottwald 2010; Canale et al. 2020).

In the early survey years in southern Lao PDR, the proportion of trees with symptoms resembling HLB did not correlate well with detection of '*Ca*. L. asiaticus' by qPCR; half of the trees with symptoms tested '*Ca*. L. asiaticus' positive and the other half did not. By early 2018, this had increased to two thirds of symptomatic trees testing positive to '*Ca*. L. asiaticus'. In 2020, all trees with HLB symptoms tested positive for '*Ca*. L. asiaticus', and the pathogen was not detected in trees without symptoms. Whilst HLB is a systemic infection, the '*Ca*. L. asiaticus' bacterium is irregularly distributed through the vascular system of infected trees, and titer is dependent on the season and temperature. '*Ca*. L. asiaticus' titers are reduced under higher temperatures (Gottwald et al. 2007; Lopes et al. 2013), with samples collected during late summer-autumn-early winter most likely to contain the highest bacterial loads and test '*Ca*. L. asiaticus' positive by PCR (Canale et al. 2020). This means that not all samples from infected trees will contain '*Ca*. L. asiaticus' at detectable levels. Also, the HLB-like symptoms observed on the trees may have been due to other causes. For example, lopsided fruit, chlorotic leaves, raised and corky leaf mid-veins and tree decline can also be associated with CTV (Moreno et al. 2008), which has been detected in Lao PDR (Donovan et al. 2021). Mixed infections of '*Ca*. L. asiaticus' and CTV were only detection in three of 109 trees: two declining lime trees in Sanasomboun with asymmetric leaf mottle symptoms indicative of HLB and one asymptomatic mandarin tree in Paksong District. Tree decline in Lao PDR could also be caused or exacerbated by other management challenges including the limited access to resources faced by many smallholders.

There is no cure for HLB, and management options are limited. One option for industry survival in Lao PDR is to establish new citrus orchards at a higher elevation above the normal range for ACP. Om (2017) and Devi et al. (2020) reported that the incidence of '*Ca*. L. asiaticus' declined at higher elevations, and Om (2017) found a concomitant reduction in populations of ACP. However, this proposed strategy requires further surveys to determine the elevations at which ACP is found, and the presence of other psyllids, such as *Cacopsylla citrisuga* Yang & Li, which has been reported to acquire and inoculate '*Ca*. L. asiaticus' (Cen et al. 2012a, b). Also, the success of this strategy relies on planting new orchards with pathogen-free trees, either seedlings of scion varieties or grafted trees propagated from a health-tested source. Nursery plants need to be grown at high elevation in the absence of psyllid vectors of '*Ca*. L. asiaticus' or in bio-secure facilities to avoid HLB infection via transmission by vectors. High health status propagation material and grafted nursery trees are not readily available to smallholders in Lao PDR and would need to be imported.

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