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Contents

1 Acknowledgments

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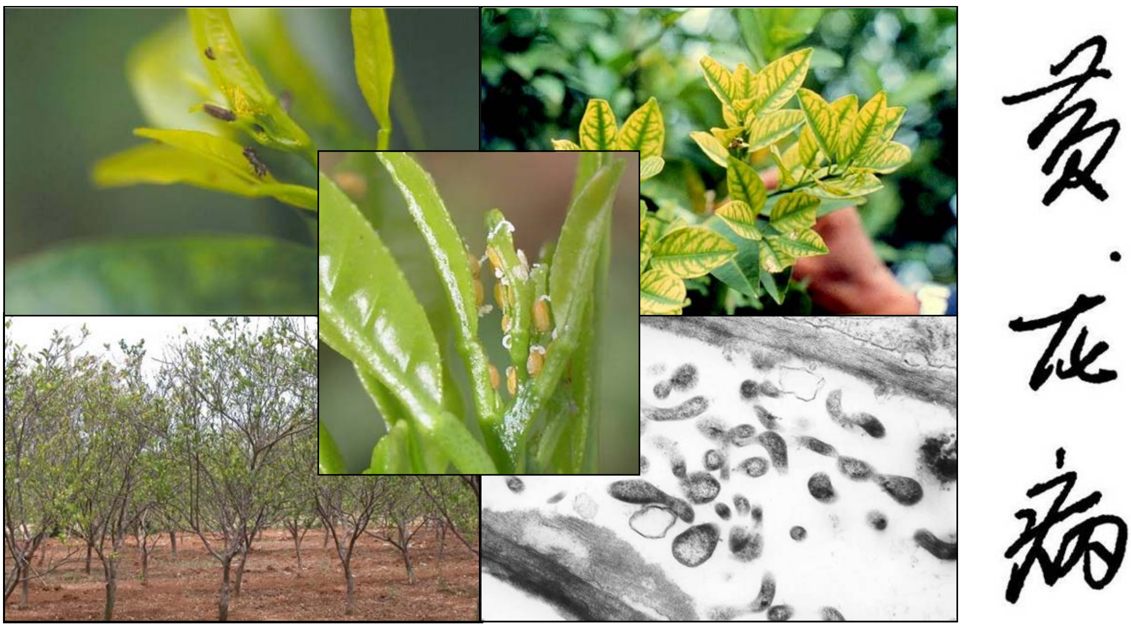

Huanglongbing (calligraphy: Yang Yueping). Photographs clockwise from top right: leaf symptoms (Pat Barkley, China, 1979); bacteria in phloem sieve tubes (Lafleche & Bové 1970), dying 4-5 year-old orchard (GAC Beattie, Indonesia, 2003); adult *Diaphorina citri* and eggs, (GAC Beattie, China, 1979). Centre: *D. citri* nymphs and honeydew (GAC Beattie, Indonesia, 2003).

2 Executive summary

Huanglongbing (HLB) is a disease of citrus caused by phloem-limited, Gram-negative liberibacters (α-Proteobacteria): a heat-tolerant form '*Candidatus* Liberibacter asiaticus', and two heat-sensitive forms '*Ca*. L. africanus' and '*Ca*. L. americanus'. It occurs in: South and Southeast Asia (from the Indian subcontinent to the Philippines, Indonesia, East Timor and Japan) and New Guinea (Papua and Papua New Guinea); the Arabian Peninsula, Mauritius and Réunion where it is caused by both '*Ca*. L. asiaticus' and '*Ca*. L. africanus'; in sub-Saharan Africa where it is caused by '*Ca*. L. africanus'; in the United States of America (USA: Florida, Georgia and Louisiana), Cuba, Belize, Jamaica, the Dominican Republic, Mexico and Honduras where it is caused by '*Ca*. L. asiaticus'; and in Brazil where it is caused by '*Ca*. L. asiaticus' and '*Ca*. L. americanus'. A subspecies, '*Ca*. L. africanus ssp. capensis', occurs in Cape chestnut, an ornamental tree in southern Africa (Beattie & Barkley 2009). The disease was not known to be present in the Americas or New Guinea when the project commenced in 2002. In Asia, New Guinea and the Americas it is transmitted by the Asiatic citrus psyllid, *Diaphorina citri.* In Africa, it is transmitted by the African citrus psyllid, *Trioza erytreae*. In parts of the Arabian Peninsula and in Mauritius and Réunion, it may be transmitted by either or both psyllids. It can destroy orchards within 5 years of planting: 100% infection of initially pathogen-free trees can occur within 2 years of planting. Use of insecticides does not prevent spread of the pathogens by the vectors. It is the most serious impediment to citriculture in Asia and, as a consequence, seriously affects the welfare of farmers.

The disease and its vectors are not present in Australia (Bellis et al. 2005, Beattie & Barkley 2009), but both '*Ca*. L. asiaticus' and *D. citri* now occur in New Guinea (Davis et al. 2005). Inevitable movement of the disease and *D. citri* eastward from Asia, to and through Australasia, will represent a serious threat to the Australian citrus industry, and to the region's biodiversity through the loss of citrus species (50% of true species) and relatives that are endemic to Australasia.

What was a problem restricted largely to Asia when the project commenced is now a world problem that threatens the very viability of commercial citrus production, a major world source of vitamin C. What was a problem for generally poor small-scale farmers in Asia is now a problem for wealthier, larger-scale commercial producers throughout the world.

The over-arching aims of the project were to improve knowledge about the aetiology, transmission and management of HLB in Indonesia and Việt Nam, improve technology transfer and capacity building in both countries, and more widely in Asia, through education and training. Additionally, the project aimed to develop an incursion management plan to protect the Australian citrus industry and native germplasm.

Major outcomes of the project were:

- a comprehensive review of literature pertaining to HLB, its vectors, and hosts of the disease and the vectors;
- increasing biosecurity through resolution of ambiguous host records for both the disease and its known vectors;
- an HLB incursion management plan for Australia: 'Huanglongbing and its Vectors: A Pest-Specific Contingency Plan for the Citrus and Nursery and Garden Industries' (Beattie & Barkley 2009);
- strengthened capacity of Australia to address incursions of the disease and its vectors;

- strengthened capacity of Indonesia and Việt Nam to deal with HLB, particularly through training and co-supervision of seven post-graduate students in Indonesia, and one Vietnamese student in Australia;
- strengthened collaboration with research and extension agencies in Asia and the Americas;
- greater understanding of the impact of weather variables and climate, particularly saturation deficits, on the incidence of *D. citri*, and the disease, and in relation to longitude, latitude, and elevation above sea level;
- confirmation that the fundamental requirements for effective management of HLB are production of pathogen-free trees, planting of these trees in areas free of the disease or with very low incidence of it, regular (four times annually) area-wide monitoring for, and immediate removal of, diseased trees;
- confirmation that pesticides, including systemic insecticides, and natural enemies may slow spread and incidence of the disease and of *D. citri*, but not to the extent of preventing rapid decline and loss of orchards, even when pesticides are used at rates and frequencies that lead to unacceptable residues in fruit, and to detrimental impacts on the environment and on the health of farmers and their families;
- the outcome of a trial comparing the efficacies of mineral oils, and systemic and contact insecticides was affected by a decision not to remove guava trees before planting citrus trees for the experiment: this decision led to results that confirmed observations made in the Mekong Delta by Vietnamese farmers and researchers on the impact of guava interplants on the incidence of huanglongbing and *D. citri* in orchards
- disclosure and open discussion of the impact of guava interplants at an international conference in 2006 led to three patent applications in the United States of America for use of guava volatiles in slow release formulations for suppressing *D. citri* populations;
- results indicated that mineral oils can be as, or more, effective than synthetic insecticides for suppressing populations of *D. citri*, and for simultaneously suppressing of some other citrus pests and of citrus greasy spot (*Mycosphaerella citri*): no oil-induced phytotoxicity was observed;
- studies in Indonesia that showed that *D. citri* adults can detect oil volatiles and are repelled by them: this repellency leads to reduced feeding and oviposition (egg laying) by the psyllid;
- studies in Việt Nam that showed that spray deposits of aqueous mineral oils have significant impacts on survival of psyllid eggs and young nymphs;
- studies in Việt Nam that showed also showed that impact of the systemic insecticide imidacloprid on *D. citri* declined with time after application
- two books on mineral oils, although not formally part of the project, were published in Vietnamese, then Chinese, through support from AusAID and ACIAR;
- molecular and morphological studies in Indonesia presented the first recent evidence to support the view of a minority of botanists and horticulturists that '*Murraya paniculata*', commonly know as orange jasmine (orange jessamine) and the favoured host of *D. citri*, is most probably two species, *M. paniculata* and *M. exotica*, the latter being orange jasmine and the favoured host of *D. citri*;

- in field trials comparing more that 20 *Citrus* and *Citrus* relatives as hosts of *D. citri* and HLB in Indonesia, the favoured hosts from 2006 to 2008 were *M. paniculata* var. *exotica*, then *Swinglea glutinosa*, *M. paniculata* (about one-third of the number recorded on *M. paniculata* var. *exotica*) and *Citrus* × *junos*, and then other varieties and *Citrus* and *Citrus* relatives;
- field and pot trails in Indonesia confirmed that HLB-infected mandarin trees express mineral deficiencies, particularly zinc (Zn) deficiency, and that these symptoms may be ameliorated and tree health improved by application of fertilisers;
- similarities between secondary symptoms of HLB and zinc deficiency, and overlooked publications on mottle yellows caused by zinc deficiency (but attributed erroneously by some authors to HLB) led to studies to determine if catechol, a derivative of salicylic acid and a potentially toxic phenol, occurs at damaging, higher-than-normal, concentrations in HLB infected plants;
- higher levels of catechol did occur on a leaf-weight basis;
- the impact of the higher levels of catechol were not assessed but research led us to propose a sequence of post-infection physiological events leading initially to manifestation of primary, then secondary symptoms, and finally, death of trees as a result of restricted, then no, transport of carbohydrate through dysfunctional phloem, and reduced, then no, uptake of minerals and water as roots die, or fail to form, as a result of carbohydrate deprivation;
- the research on catechol, and observation of seemingly greater tolerance to infection in mature trees than in young trees, and variable reports on the susceptibility of orange jasmine to the disease led us to consider the possibility that mature citrus trees and some citrus relatives may contain compounds that are toxic to liberibacters; and
- recommendations for future research and technology activities were broadly grouped as follow: management; systematics and biogeography; biodiversity and germplasm conservation; resistance to HLB; HLB transmission and host plant interactions; other diseases and disease interactions; regional collaboration, technology transfer and infrastructure; and funding of activities.

3 Background

The project commenced in January 2003. It stemmed from ACIAR projects on integrated control of citrus pests, initially in China (ACIAR CS2/1993/005: Integrated Control of Citrus Pests in China), then in China, Thailand, Việt Nam and Malaysia (CS2/1996/176: Integrated Control of Citrus Pests in China and Southeast Asia), growing concern about the impact of huanglongbing (HLB) in Asia, and the threat that the disease, caused in Asia by '*Ca*. Liberibacter asiaticus' [α-Proteobacteria], and its vectors, particularly the Asiatic citrus psyllid, *Diaphorina citri*, pose to the Australian citrus industry and native species of Citrus in Australasia.

Time required to propagate pathogen-free trees delayed the start of some field experiments by one to almost 3 years. Travel restrictions related to terrorism and natural disasters had an impact in the first 2 years of the project. Increased costs also had an impact on studies on the impacts of latitude and altitude on *D. citri* populations and HLB incidence, and on plant surveys. Work on interactions between scion genotype, rootstock, '*Ca*. L. asiaticus' genotype and *D. citri* in Việt Nam was affected when a prospective student could not pursue a PhD funded by an ACIAR John Allwright Fellowship.

Background information, material and methods, and key results and discussion are summarised under the following headings:

- incursion management plan;
- incidence of *D. citri* and its natural enemies at three altitudes in Central Java;
- hosts of *D. citri* and HLB;
- mineral oil and imidacloprid field and laboratory studies in Indonesia and Việt Nam;
- interactions between plant nutrition and HLB;
- catechol, mottle yellows, starch accumulation and HLB?;
- germplasm tolerance to HLB and *D. citri* in Việt Nam;
- guava interplants in Indonesia;
- observations in Pakistan, Bhutan, and Meghalaya in northeast India; and
- technology transfer and capacity building.

Incursion management plan

Australia required an incursion management plan to address threats posed to its commercial citrus and nursery industries, native vegetation, and home gardens by HLB and its vectors, *D. citri* and *Trioza erytreae*, the African citrus psyllid.

Incidence of *D. citri* and its natural enemies at three altitudes in Central Java

The potential for populations of *D. citri* to exist and flourish is known to decline with increasing altitude and latitude (Bové & Garnier 1984, Aubert et al. 1985, Yang et al. 2006). Aubert et al. (1985) surveyed 29 orchards in Java over 10 days at the end of the dry season in early November 1984. These orchards ranged in age from 1 to 40 years and were located at altitudes ranging from 100 m to 1200 m asl. However, no formal investigation on the effect of altitude was made. Therefore, we assessed populations of the psyllid and its natural enemies at three altitudes in Central Java over 2-3 years.

Major natural enemies of *D. citri* include two primary parasitoids, the ectoparasitoid *Tamarixia radiata* and the endoparasitoid *Diaphorencyrtus aligarhensis*, and predatory ladybirds (coccinellids), lacewings and spiders (Aubert 1987, Sadana 1991, Waterhouse 1998, Halbert & Manjunath 2004, Michaud 2004). On eclosion, *T. radiata* adults leave an exit hole in the thorax of the mummified body of the host, and *D. aligarhensis* adults leave an exit hole in the abdomen (Aubert 1987). Both parasitoids occur in Indonesia (Nurhadi 1987, Istianto et al. 1992) but Aubert et al. (1985) did not observe *T. radiata* in their survey on November 1984. Nurhadi (1988) noted the ability of both parasitoids to suppress considerable numbers of the psyllid at some locations in East Java, but he did not mention levels of parasitism in orchards.

Hosts of *D. citri* and HLB

When the project, commenced there was some doubt about the number of rutaceous genera recorded as hosts of HLB. It was considered likely that the number of genera was higher than the then 3 widely acknowledged commercially important citrus genera, *Citrus*, *Poncirus* and *Fortunella* (all now *Citrus*: Mabberley 1997, 1998, 2001, 2004), and more than 5 citrus relatives, including *Murraya* (see da Graça 1991, Koizumi et al. 1996, Korsten et al. 1996). The number of alternative hosts of the vector was also uncertain, and most probably greater than known. Uncertainties about host susceptibility to *D. citri* and HLB were exacerbated by the doubtful validity of some genera and species of the Aurantioideae, and multiple names for the same plants. We sought to revise and add to records, improve Australia's ability to respond appropriately to incursions of the disease and its vectors in order to protect the Australian citrus industry, and to conserve potentially threatened native germplasm of ecological, agricultural and medical importance.

Distributions of known and potential hosts of both *D. citri* and HLB in Indonesian, Việt Nam, Papua New Guinea and Australia required thorough documentation. *M. paniculata* was considered to be the favoured host, but its systematic status was uncertain, with the possibility that it comprised several forms, including the widely grown ornamental known as orange jasmine, or two species, *M. paniculata* and *M. exotica* with the latter, not the former, being orange jasmine, the favoured host of *D. citri*, and a possible host of '*Ca*. L. asiaticus'. Herein, we refer to *M. exotica* as *M. paniculata* var. *exotica* (*sensu* Huang) (see Huang 1959).

Mineral oil and imidacloprid field and laboratory studies in Indonesia & Việt Nam

The impact of the HLB on citrus production in Asia has led to heavy use of pesticides to reduce populations of *D. citri*, and spread of '*Ca*. L. asiaticus'. Most emphasis has been placed use of on contact and systemic synthetic insecticides to kill psyllid eggs, nymphs or adults, but use of these and other chemicals merely slows the inevitable death of trees from the disease, with loss of orchards comprising initially pathogen-free trees possible within six years of planting. Infected adult psyllids can also transmit the pathogen while acquiring lethal doses of insecticide. Relatively limited emphasis has been placed on strategies such as use of mineral oils (petroleum spray oils) (Rae et al. 1997, Huang et al. 2005) and guava interplants (Hall et al. 2009, Zaka et al. 2009, Cen et al. in press) to slow establishment and spread of the pathogen in orchards by:

- repelling the adult psyllids or by reducing volatiles that attract them to their host plants;
- slowing ingress of psyllids into orchards; and
- reducing feeding and oviposition by adult psyllids.

Evidence of rejection or acceptance by phytophagous insect and mite pests of citrus of host plants treated with mineral oils suggests that impacts of oil deposits on citrus red mite (Cen et al. 2002), two-spotted mite (Liu & Beattie 2002), greenhouse thrips (Liu et al. 2002b), dacine fruit flies (Liu et al. 2002a, Nguyen et al. 2007), citrus leafminer (Liu et al. 2006), and *D. citri* (Rae et al. 1997) may be related to detection by olfactory and/or contact chemoreceptors of repellent oil or plant volatiles, masking of attractant volatiles, or suppression of attractant host plant volatiles.

Evidence that mineral oils, applied at low concentrations (0.3% to 0.5% v/v) at volumes adequate to wet trees to run-off so as to form fine films of oil on sprayed surfaces, can be as effective as synthetic contact and systemic insecticides for management of *D. citri* and

HLB was obtained in Sarawak during ACIAR CS2/1996/176 (Leong et al. 2002) and in subsequent studies (Leong 2006).

In the study reported by Leong et al. (2002), sprays were applied weekly or fortnightly to non-bearing and mature honey tangerine (*Citrus* × *aurantium*) and to mature limau langkat mandarin (*C. reticulata*: syn. *C.* × *suhuiensis*) trees (Siem in Indonesia, Shatangju in China, Sz-wei-kom in the Philippines, and Som-keo-wan in Thailand). For the immature honey tangerine trees, *n*C24 agricultural mineral oil (AMO) sprays were compared with applications of imidacloprid (0.01%) alternating with chlorpyrifos (0.02%) or deltamethrin (0.0025%) in a Department of Agriculture-based program (DOA), and triazophos (0.03%) alternating with a proprietary mix of cypermethrin (0.125%) and chlorpyrifos (0.0125) in a farmer-based program (FP). In the DOA-based program for mature trees, deltamethrin alternated with chlorpyrifos. In FP-based program for mature mandarin trees triazophos alternated with the cypermethrin/chlorpyrifos mix, and in the mature honey tangerine orchard prothiofos (0.1%) alternated with the cypermethrin/chlorpyrifos mix.

In the subsequent study with mature honey tangerine trees (Leong 2006), an unsprayed control was compared with weekly applications of 0.35% (v/v) of the *n*C24 AMO (Ampol D-C-Tron Plus), a spray program based on triazophos (0.03%) alternating with the above mentioned cypermethrin/chlorpyrifos mix, and a spray program based on imidacloprid (0.01%), over 23 months. All sprays were applied thoroughly to runoff every 6-7 days during peak psyllid activity within flush cycles. As in the first study reported by Leong et al. (2002), the AMO treatment was as effective, or more effective, for suppressing *D. citri* populations than the synthetic pesticide treatments. It had significant impacts on reducing oviposition and feeding (Leong 2006). Spread of HLB in this orchard from 1998 to October 2001 was also reported by Leong (2006). Levels of HLB-infection in the initially pathogen-free trees rose quite rapidly over the next 4 years in the unsprayed control and less rapidly in the other treatments. Increases in the AMO and imidacloprid treatments were similar and less rapid than in the triazophos/cypermethrin/chlorpyrifos treatment. Symptoms resembling HLB were present in 80% of control trees in October 2001, and in 40%, 33% and 65% of trees in the AMO, imidacloprid, and triazophos/cypermethrin/chlorpyrifos treatments, respectively. Levels of HLB as determined by PCR were 42%, 11%, 9% and 23%, respectively (Leong 2006).

Gatineau et al. (2006) compared three treatments under high *D. citri* and HLB pressure in three, 0.5 ha, unreplicated king orange (*C.* × *aurantium*) orchards in the Mekong Delta in Việt Nam over 2 consecutive years: untreated, fenobucarb applied fortnightly as a contact insecticide, and imidacloprid applied monthly as a systemic insecticide. All trees were pathogen free when planted. At the end of the experiment, PCR-tests confirmed infection levels of HLB in the initially pathogen trees were approximately 96%, 75% and 24%, respectively. High numbers of adults and nymphs psyllids were observed in the control. Numbers in the two insecticide treatments were more than 90% lower than in the unsprayed control. Very few adults were observed in the imidacloprid treatment and no nymphs reached the final instar in this treatment. The results demonstrated that significant transmission of HLB can occur even when very high levels of suppression of vector populations are achieved. For imidacloprid, it is clear that transmission occurred before lethal quantities of the insecticide were ingested by some adult psyllids that entered the trial site from neighbouring orchards. For fenobucarb, it is clear that fortnightly sprays were less effective than monthly trunk applications of imidacloprid: weekly applications, though impractical, may have given better control, but HLB levels after two years would probably have still been higher than in the imidacloprid treatment. Better results may have been achieved in larger orchards in area-wide management programs, and in regions with lower pest and disease pressure.

In this project (ACIAR HORT/2000/043), we conducted laboratory studies in Việt Nam and Indonesia to determine the impact of mineral oil deposits on *D. citri* eggs, nymphs and adults, and whether adult psyllids detect mineral oil volatiles. In northern Việt Nam we determined psyllid mortalities for intervals of up to 4 days after adults were released onto sweet orange trees (*C.* × *aurantium*) to which imidacloprid was applied as a soil drench 2-10 weeks earlier. In field experiments in orchards in Central Java (mandarin), and northern (sweet orange) and southern (king orange) Việt Nam, we evaluated efficacy of horticultural mineral oil (HMO) and AMO treatments, use of imidacloprid, and farmer practice treatments for minimising *D. citri* populations and HLB incidence in orchards. The king orange trees in the orchard in southern Việt Nam were planted among young guava (*Psidium guajava*) trees. This followed a request by the farmer who asked if the guava trees should be removed. The decision to leave them was based on income that would be derived from the guava trees over 2-3 years before fruit would be produced by the king orange trees. This decision had a significant impact on the outcome of the experiment in the orchard.

Interactions between plant nutrition and HLB

Little work has been performed of the interactions between plant nutrition and the development of HLB with only two studies being published prior to our research. Work by Koen & Langenegger (1970), using an unnamed citrus species infected with '*Ca*. L. africanus' (the pathogen that causes the African form of the disease) found that concentrations of potassium were higher in infected plants, whilst calcium (Ca) and magnesium (Mg) were lower. Aubert (1979) found that infected plants in Réunion contained lower concentrations of Ca, manganese (Mn) and zinc (Zn). However, the species of citrus tested was not stated and no statistical inference was made. We addressed this deficit in our knowledge of HLB through three sets of studies: a survey of mandarins in Central Java; pot trials and field surveys in Central Java and Japan; and field and screenhouse pot-trial studies at Universitas Gadjah Mada.

Catechol, mottle yellows, starch accumulation and HLB

It is widely, but we believe incorrectly, assumed that HLB originated in China (Bové 2006, Beattie & Barkley 2009). The assumption is partially based on similarities between symptoms of nutrient disorders (particularly 'mottle yellows'), water-logging and HLB. Papers by Reinking (1919), Lee (1921) and Lin (1956) are often cited as evidence for a Chinese origin. However, Reinking (1919) and Lee (1921) did not describe symptoms of HLB (Beattie & Barkley 2009) and studies reported by Reed & Dufrénoy (1935a, 1942) on 'mottle leaf' of sweet orange in California suggest that 'mottle yellows' in the Philippines, as reported by Lee (1921), was caused by Zn deficiency. Moreover, Lee (1921) stated that the type of mottling was entirely the same as that seen in California and attributable to malnutrition. Reinking (1921), in a paper on citrus diseases in Southeast Asia, noted that 'mottled-leaf' in the Philippines was not transmissible from one plant to another, and symptoms in a photograph in his paper of affected leaves resemble Zn deficiency symptoms. Wallace (1978), in reviewing leaf-mottle yellows disease in the Philippines, concluded that 'mottle leaf', as studied by Lee (1921), was caused by Zn deficiency and that the severity of leaf mottle on trees on pomelo was due to the susceptibility of the stock to *Citrus* tristeza virus.

In studies in California, Reed & Dufrénoy (1935a) observed enlarged, hypoplastic palisade cells in the mesophyll of mottled sweet orange leaves, and noted (a) that such abnormalities commenced from an early stage in the buds, which developed slowly with delayed differentiation of meristematic tissue, and (b) that cells in affected buds contained enhanced amounts of phenolic compounds in the vacuolar solution. Severely affected leaves were stunted (Reed & Dufrénoy 1935a). Reed & Parker (1936) reported that orange trees with mottle leaf resumed normal growth when they were sprayed with a $ZnSO_4$-lime mixture. Reed & Dufrénoy (1935b) observed phloem necrosis in mottled orange leaves, even at an early stage of mottling, and Reed & Parker (1936) noted that xylem bands in 14 and 26-month old twigs of Zn-deficient sweet orange plants were significantly thicker than normal, but that phloem bands were not. Reed (1941) observed no necrosis of epidermal or meristematic cells of vegetative buds of Zn-deficient apricot and peach trees.

Reed & Dufrénoy (1942) subsequently reported that the disorder in apricot was associated with abnormally high accumulation of catechol in the vacuoles of post-meristematic cells of root-tips, buds and leaves, and that abnormalities were more evident in perivascular and spongy parenchyma than in palisade cells; cells surrounding xylem contained catechol in several forms. They noted that such accumulations of catechol in citrus and other plants had been linked to nutrient deficiency, 'virus' diseases and attacks by parasitic organisms.

Reed & Dufrénoy (1935a) observed that plastids of palisade cells in green orange leaves showed large starch grains when examined in the morning. Those in mottled leaves contained very thin elongated starch grains that did not seem to fill the cavity of the plastid in which they lay. They postulated that starch in such cells may be translocated faster than it is being formed in the plastids. Iodine was used to detect the starch. Reed & Dufrénoy (1942) observed that plastids in abnormal spongy parenchyma cells in Zn-deficient apricot leaves accumulated starch. Reed (1938) considered it possible that an essential process in carbohydrate metabolism may have been inhibited in plants in which accumulation was obvious. Skoog (1940) observed that abnormally high oxidative capacity of Zn-deficient plants was responsible for rapid inactivation of auxin, and enhanced oxidation of catechol in extracts of Zn-deficient plants.

These studies have been surprisingly overlooked given similarities between symptoms and effects of HLB and those of Zn deficiency as exhibited by species and varieties of citrus. The most obvious similarities are interveinal chlorosis, stunted leaves, hypertrophy of cells, phloem necrosis, and starch accumulation (Matsumoto et al. 1961, Tirtawidjaja et al. 1965, Schneider 1966, 1968): HLB is known as citrus vein-phloem degeneration in Indonesia (Tirtawidjaja et al. 1965, Tirtawidjaja 1981). However, accumulation of catechols, and the significance and consequences of such accumulations, have not been reported for HLB infected plants.

Catechol is a naturally occurring chemical in plants (Reed & Dufrenoy 1942, Andjelković et al. 2006), where it is derived from several sources including salicylic acid, which is a key component of plant responses to infection and of systemic acquired resistance (Shah 2003, Durrant & Dong 2004). An et al. (2001) reported that small quantities of catechol applied exogenously to germinating wheat (*Triticum aestivum*) seeds were highly toxic. It causes production of reactive oxygen species such a hydrogen peroxide and thereby causes DNA damage through breakage of the deoxyribose phosphate backbone and base modification (Schweigert et al. 2001, van Wees & Glazebrook 2003), and oxidised catechols lead to protein inactivation (Schweigert et al. 2001).

In transgenic plants expressing the bacterial gene *Nah*G that encodes salicylate hydroxylase, salicylic acid is converted to catechol (Shah 2003, van Wees & Glazebrook 2003, Alkio et al. 2005). The same enzyme, or a similar one, may convert salicylic acid to catechol naturally in plants subjected to infections that initiate stress responses, and environmental stresses, such as Zn deficiency, that may do likewise. However, there appear to be no reports to explain how Zn deficiency leads to increased levels of catechol in plants as reported by Reed & Dufrénoy (1942). Moreover, catechols and other phenols are chelating agents (Mila et al. 1996, Schweigert et al. 2001, Andjelković et al. 2006), more so for copper (Cu) and iron (Fe) than Zn (Schweigert et al. 2001), and their possible role in sequestering Zn in diseased or environmentally stressed plants is uncertain. Salicylic acid is also a metal chelator in plants (Mila et al. 1996). Mila et al. (1996) hypothesised that plant polyphenols may withhold Fe, Cu and Zn ions essential for the growth of plant pathogens.

Some Florida growers have reported that application of salicylic acid improves productivity and health of HLB-infected citrus trees, and USDA is seeking to ameliorate effects of the disease by manipulating the salicylic acid signalling pathway in citrus (http://www.ars.usda.gov/research/projects/projects.htm?ACCN_NO=416610). However, such strategies in which life of infected trees is prolonged would presumably lead to increased risk of spreading infection spreading within and between orchards, and catastrophic impacts on immature, recently planted, trees adjacent to infected mature trees.

We hypothesised that liberibacters may express a gene that encodes salicylate hydroxylase or an enzyme with similar activity. Alternatively, severe symptoms of HLB in citrus and other symptomatic plants may in part be related to an indirect effect of liberibacters on the normal physiology of the plants: plants may respond to infection, as they may in Zn deficient plants, by over-producing salicylic acid that subsequently leads to abnormal levels of catechols that may become phytotoxic. Evidence to support the second hypothesis comes from published literature cited above and our studies, as summarised below, in Indonesia. Firstly, plants with HLB have lower levels of Zn and Fe. Secondly, plants growing in soil containing higher levels of minerals have greater concentrations of Zn and Fe in their leaves and lower levels of disease. Thirdly, pot trials show interactions between levels of applied Zn and Fe and disease levels.

In this study we sought to determine if catechol accumulates in HLB-infected Siem mandarin leaves and if so, relationships between such accumulations and concentrations of Zn and Fe in the leaves.

Germplasm tolerance to HLB and *D. citri* in Việt Nam

This objective sought to determine distributions citrus germplasm in Việt Nam, the susceptibility of some species/varieties to ‘*Ca*. L. asiaticus’ and *D. citri*, and the impact of some rootstock-scion combinations on susceptibility of trees to ‘*Ca*. L. asiaticus’ and *D. citri*.

Guava interplants in Indonesia

Observations on the impact of guava interplants on *D. citri* populations and the incidence of HLB in citrus orchards in the Mekong Delta led us to evaluate the impact of two varieties of guava, ‘Jakarta’ (seeds with red flesh) and ‘Sukun’ (seedless with white flesh), on *D. citri* populations on Siem mandarin in Central Java. One of these trials is reported here.

Observations in Pakistan, Bhutan and India

During the project, opportunities arose that led to visits to orchards in Pakistan, Bhutan and India. Pakistan is within the region where the Asiatic form of HLB, caused by ‘*Ca*. L. asiaticus’, first occurred and where *D. citri* may have originated in association with a species of *Murraya* or *Bergera* (Beattie & Barkley 2009). Bhutan is a region where *D. communis*, a sister species to *D. citri* originally recorded on curry leaf at Dehra Dun at about 700 m asl in Uttarakhand in northern India and (Mathur 1935, 1975), appears to occur naturally. India is region where another psyllid, *Psylla murrayi* has also been recorded, from curry leaf at Dehra Dun in Uttarakhand (Mathur 1935, 1975) and citrus exclusively at Shillong in Meghalaya, 1625 m asl (Lahiri & Biswas 1980). Comments by Lahiri & Biswas (1980) suggest that it may have been introduced to Meghalaya. They reported that it inflicts great loss to citrus cultivation in Meghalaya: foliage of infested plants became deformed, resulting in a depleted photosynthetic activity over and above the loss caused by intake of plant sap by the growing larvae. Burckhardt (1994) suggested that *P. citricola* and *P. citrisuga*, both reported from citrus in Yunnan, China, may be synonyms of *P. murrayi*.

Technology transfer and capacity building

Australia’s capacity to deal with HLB was limited. The Australian citrus industry required an incursion management plan, and declining expertise within Australia needed to be addressed. Indonesia required training of young scientists and extension workers, and transfer of technology from elsewhere in the region, particularly use of mineral oils for management of citrus pests and diseases. Việt Nam’s requirements were similar to those of Indonesia, but mineral oils were being used in citrus integrated pest management programs before the project commenced due to technology transfer during ACIAR

CS2/1996/176, and an AusAID CARD project. The capacity of botanists, horticulturist, entomologists and plant pathologists in each country to recognise hosts of the disease and *D. citri* also needed to be addressed.

Adult *Diaphorina citri* female ovipositing on *Murraya paniculata* var. *exotica*, South China Agricultural University, Guangzhou, China, 28 June 2007 (GAC Beattie).

4 Objectives

The objectives of the initial three-year phase of the project were to:

- Survey distributions and occurrence of *Diaphorina citri*, related psyllid fauna, and psyllid natural enemies (entomopathogens, predators, and primary and secondary parasitoids) in Indonesia, Việt Nam and Australia;
- Determine distributions of known and potential alternative hosts of *D. citri* and '*Candidatus* Liberibacter asiaticus' in Indonesia, Việt Nam and Australia;
- Develop incursion management plans for the Australian citrus industry;
- Determine the impacts of ambient temperatures at different latitudes, longitudes and altitudes in Indonesia and Việt Nam on the incidence of *D. citri*, and the spread and severity of huanglongbing in different species and cultivars of citrus and alternative hosts;
- Determine impact of feeding behaviour of adult *D. citri*, and host plant phenology on transmission of '*Ca*. L. asiaticus', on sweet orange, mandarin and selected alternative hosts;
- Determine the impact of mineral and plant oil sprays on mortality, and spray deposits on feeding and oviposition behaviour, of *D. citri* and spread of huanglongbing under laboratory and field conditions;
- Explore enhanced use of natural enemies in IPM programs against *D. citri* and '*Ca*. L. asiaticus';
- Determine interactions between scion genotype, rootstock, '*Ca*. L. asiaticus' genotype and vector in Việt Nam; and
- Project workshops/seminars on huanglongbing, citriculture, and the use of horticultural and agricultural mineral oils in citrus integrated crop management programs

The objectives of the 3-year extended phase of the project were to:

- Survey distributions and occurrence of *D. citri*, related psyllid fauna, and psyllid natural enemies (entomopathogens, predators, and primary and secondary parasitoids) in Central Java;
- Determine distributions of known and potential alternative hosts of *D. citri* and '*Ca*. L. asiaticus' in Indonesia, Việt Nam and Australia;
- Determine the impacts of ambient temperatures at different latitudes, longitudes and altitudes in Central Java on the incidence of *D. citri*, and the spread and severity of huanglongbing in different species and cultivars of citrus and alternative hosts;
- Determine impact of feeding behaviour of adult *D. citri*, and host plant phenology, on transmission of '*Ca*. L. asiaticus' on orange, mandarin and selected alternative hosts in Indonesia;
- Determine impact of mineral oils on (a) adult psyllid mortality, feeding & oviposition behaviour, and transmission of huanglongbing under laboratory conditions, (b) psyllid populations & disease transmission under field conditions, and (c) the impact of sprays on foliar diseases in the field, Indonesia and Việt Nam;
- Determine influence of scions and rootstocks on the severity of huanglongbing in Việt Nam;

- Determine the impact of plant nutrition on the development and severity of huanglongbing in Indonesia;
- Impact of guava interplants on the incidence of *D. citri*, and the spread of huanglongbing, in orchards in Việt Nam and Indonesia; and
- Capacity building and technology transfer in Indonesia & Việt Nam.

Within this report, background information, methodologies, and key results and discussion relating to the above objectives are summarised under the following headings:

- incursion management plan;
- incidence of *D. citri* and its natural enemies at three altitudes in Central Java;
- hosts of *D. citri* and HLB;
- mineral oil and imidacloprid field and laboratory studies in Indonesia and Việt Nam;
- interactions between plant nutrition and HLB;
- catechol, mottle yellows, starch accumulation and HLB?;
- germplasm tolerance to HLB and *D. citri* in Việt Nam;
- guava interplants in Indonesia;
- observations in Pakistan, Bhutan, and Meghalaya in northeast India; and
- technology transfer and capacity building.

Diaphorina citri nymphs and honeydew on *Murraya paniculata* var. *exotica* in Kolkata, India, 28 October 2009 (GAC Beattie).

5 Methodology

Incursion management plan

Literature was reviewed and a plan developed (see Beattie & Barkley 2009).

Incidence of *D. citri* and its natural enemies at three altitudes in Central Java

D. citri populations were assessed fortnightly from February 2005 to February 2007 in mandarin orchards established before the project commenced and at Siem mandarin sites established in October 2005. The Siem mandarin rootstock used in this study, and in all other field experiments established during the project, was Japanese citron or Yuzu (*C.* × *junos*). The assessments were made at Purworejo (60 m asl: 7° 42′ S, 109° 56′ E), Grabak (640 m asl: 7° 22′ S, 110° 18′ E) and Ngablak (1300 m asl: 7° 24′ S, 109° 23′).

Monthly incidences of ladybirds and the two primary parasitoids were studied in orchards at Purworejo, Grabak and Ngablak from February 2005 to February 2007. Incidence was based in monthly suction samples from 60 trees (≥ three years old and < 3 m tall) at each location. The role of spiders and lacewings was not assessed.

Monthly levels of parasitism at Purworejo and Grabag were also determined. This was done by identifying parasitoids that emerged from parasitised host mummies collected from host nymph-infested potted mandarin trees placed in the orchards for one week and subsequently held, in the absence of adult parasitoids, in an insect-proof screenhouse.

The five most common ladybirds in the orchards, *Coelophora inaequalis*, *Menochilus* (*Cheilomenes*) *sexmaculatus*, *Harmonia sedecimnotata*, *Orcus janthinus* and *Scymnus* sp., were studied in the laboratory. The most extensive studies were undertaken with *M. sexmaculatus*.

Hosts of *D. citri* and HLB

Host records for *D. citri* and HLB, including a review published by Halbert & Manjunath (2004) after the project commenced, were revised. Information was gathered to prepare distribution maps for Aurantioidae in Australia and Papua New Guinea.

Indonesia. Herbarium and other records of Rutaceae in Indonesia were revised in order to prepare regional distribution maps.

We compared morphological characters of *Murraya paniculata* and *M. paniculata* var. *exotica*, and used RAPD analysis to determine molecular differences. DNA was isolated from plants sourced from living collections located at Bogor (06° 36' S, 106° 48' E), Purwodadi (07° 07' S, 110° 55' E), and Yogyakarta (07° 49' S, 110° 22' E) using CTAB based on Komar (1999). DNA amplification was based on Karsinah et al. (2002). Amplification was conducted in 15 µL of solution consisting of 7.5 µL of PCR master mix, 1.5 µL of primer, 4.5 µL of MilliQ water, and 1.5 µL of template DNA using My Cyclber thermocycler (BioRad). The primers used for amplification were OPU-3, 6, and 7, and OPN-16 and OPW-19. An initial denaturation was carried out at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 30 s, and extending at 72°C for 80 s. A last cycle with extension at 72°C for 10 min was then performed. Electrophoresis was performed at 100 V for 25–30 min in agarose gel to which ethidium bromide had been added. The amplified DNA fragment patterns were visualized using a UV transilluminator and photographs were taken.

PCR, based on OPU primers OPU 3, OPU 6, OPU 7, OPN 16 and OPW 19, was used to determine the susceptibility of *M. paniculata* var. *exotica* to '*Ca*. L. asiaticus'.

Field trials evaluating some 30 (less than the 80 envisaged) species and varieties of *Citrus* and *Citrus* relatives as hosts of *D. citri* and '*Ca*. L. asiaticus' were undertaken at Purworejo (~50 m asl) in Central Java, in a region where the incidence of the disease is high. Seedlings of 20 species or varieties in Trial 1 were planted 2 m apart in each of 16 blocks (replicates). Border rows of Siem mandarin were planted around each block. Twelve species and varieties were planted 1.5 m apart in March 2007 in 5 blocks: plants included an unknown *Citrus* sp., *Citrus × virgata*, *Atalantia buxifolia*, two *M. paniculata* var. *exotica* selections (from California and Yunnan), *Afraegle paniculata*, three species of *Citropsis* (Aurantieae), and *Cl. excavata* (Clauseneae). Inclusion of species and varieties of plants in these trials was affected by restricted access to plants in Australia.

Việt Nam. Literature and herbarium records were reviewed. Surveys were conducted by Plant Protection Research Institute (PPRI) staff in 2007, 2008 and 2009 in the northern mountainous regions of Hoa Binh (January 2009), Cuc Phuong National Park (November 2007 and March 2008), Cat Ba National Park (July 2008), Tam Dao (February 2008), Yen Bai (December 2008 and April 2009) and Dien Bien (December 2007). Where feasible, cuttings were taken and 5-10 plants of each/species variety were propagated at PPRI. On 29 May 2009, a randomly selected branch on one of each of 21 species/varieties of collected plants was selected and caged with fine mesh. Ten adult psyllids from nymphs reared on HLB-infected plants were released into each cage. Numbers of adults resting and or feeding on the plants were recorded daily for 3 days after they were released.

Mineral oil and imidacloprid field and laboratory studies in Indonesia & Việt Nam

Laboratory-based mineral oil studies in Việt Nam. Several experiments evaluating the impact of deposits of 0%, 0.25%, 0.5%, 1% and 2% v/v aqueous mineral oil emulsions on *D. citri* were conducted at the PPRI in Hà Nội.

In no-choice tests, psyllids were caged with jasmine orange shrubs and/or sweet orange trees sprayed separately with aqueous emulsions of an *n*C21 HMO (Sunspray Ultra-Fine) and/or an *n*C24 AMO (SK EnSpray 99). Similar choice tests were also conducted with both host plants. Additional choice tests were also undertaken to determine the impact on female adults landing and laying eggs on buds ≤ 5 mm long (lengths most suitable for oviposition) and > 10 mm long.

Screenhouse-based mineral oil studies in Indonesia. Four studies were conducted. The first two were undertaken to determine choice and non-choice feeding responses of *D. citri* adults to plants treated with aqueous emulsions of Sunspray Ultra-Fine and SK EnSpray 99. In the third study we determined the response of adult psyllids with oil-contaminated tarsi to Sunspray Ultra-Fine and SK EnSpray 99 deposits on leaves. The oils were applied as 0.5% v/v aqueous emulsions to mandarin leaves, and responses of adults determined after they walked on surfaces saturated with either distilled water or 2% v/v aqueous emulsions of the oils. In the fourth study we used single linear-track olfactometers, with an airflow rate of 140 mL/min, to determine responses of adult psyllids to host-leaf volatiles, mineral oil volatiles emanating from leaves dipped separately in 2% v/v aqueous emulsions of each oil, and similarly treated filter paper paired with water-dipped leaves.

Screenhouse-based imidacloprid studies in Việt Nam. The insecticide was applied as four soil drench treatments to two-year old potted sweet orange trees in a screenhouse at the PPRI in Hà Nội. There were four treatments (50 mL of 0%, 0.2%, 0.4% or 0.6% w/v solutions), each with 6 replicates and with each replicate comprising 5 trees. Adult psyllids (20 per tree) were exposed to treatments at 6 intervals (2, 4, 6, 8 and10 weeks) after application of the insecticide. Adult mortality was determined 1, 2, 4 and 6 hours after their release of adults, then daily for up to 4 days. Mortality in the insecticide treatments was corrected for control (no insecticide) mortality.

Field comparisons of mineral oils, imidacloprid and farmer practices in Indonesia and Việt Nam. Experimental protocols for field studies were prepared in March 2004. In Việt Nam, two sites were established; one at Cai Be (3 m asl: 10° 20′ N, 106° 00′ E) in Tien Giang Province south west of Ho Chi Minh City in the Mekong Delta was established in July 2004, and the other at Cao Phong (280 m asl: 20° 41′ N, 105° 19′ E) in Hoa Binh Province south west of Hà Nội was established in October 2004. One site was established in Indonesia in October 2005 at Purworejo (68 m asl: 07° 42′ S, 109° 56′ E) in the vicinity of Yogyakarta in Central Java. Six treatments were evaluated at each location between 2004 and 2009:

- 0.5% Sunspray Ultra-Fine sprays and conventional fungicides (e.g., sprays for citrus canker, *Xanthomonas citri* subsp. *citri*) applied to initially '*Ca*. L. asiaticus' free trees (pathogen-free trees – PFTs);
- 0.5% SK EnSpray 99 sprays and conventional fungicides applied to initially PFTs;
- farmer's choice of synthetic pesticides (insecticides, acaricides & fungicides) applied to initially PFTs;
- imidacloprid (Confidor: initially applied to ground around the drip-line of each tree and then to each trunk) and conventional fungicides and acaricides (as required) applied to initially PFTs;
- no sprays applied to initially PFTs, other than possible use of conventional fungicides, acaricides, low rates of abamectin and $\leq$ 0.15% SK EnSpray 99 if required; and
- previous treatment with non-PFTs that were carefully selected from local nurseries to ensure minimal levels of '*Ca*. L. asiaticus' infection.

At **Cai Be**, The site of the experiment comprised an area of about 0.65 ha, with 4, 150 m long, 7 m wide raised beds surrounded by 3-4 m wide canals. Trees (400 PFTs and 80 non-PFTs), all king orange on volkameriana rootstocks, were planted in July 2004. Each of the 6 treatments comprised four 7 m × 22.5 m replicates of 20 trees, with one replicate within each of four blocks (= raised beds). Trees in each replicate were planted on an offset 2.5 m grid on a 1:1 ratio between existing < 1 year old guava trees. In 2005, *Melaleuca cajuputi* trees were planted along edges of each raised bed, just above the water level in the surrounding canals. These trees were 2-3 m high in April 2006. They were for shade to reduce sun damage to king orange fruit. They also formed windbreaks. Felled trees were used to support heavy-bearing king orange branches.

The Cai Be site in Tien Giang, Việt Nam, with one year-old guava, shortly before King orange trees were planted. Mr Le Van Bay is standing on the left, back to camera: 4 March 2004 (GAC Beattie).

Confidor 100 SL (imidacloprid) was applied to soil beneath each tree in May (3 mL/tree), August (1.5 mL/tree) and September (1.5 mL/tree) 2004 before treatments were formally applied. Confidor 100 SL was then applied at 2 mL/tree (0.2 mL imidacloprid/tree) to the trunks of trees in treatment 4 on two additional occasions in 2004. Six oil and 6 insecticide (alternating applications of 0.19% fenobucarb; and 0.00625% lambda cyhalothrin) sprays were applied respectively in the oil and farmer practice treatments from October to December 2004. In 2005, oil sprays were applied 15 times in the oil treatments, 0.19% fenobucarb and 0.00625% lambda cyhalothrin were each applied 12 times successively in the farmer practice treatment, Confidor 100 SL was applied on 12 occasions to the trunks of trees at 2 mL/tree in treatment 4, and a Bt (*Bacillus thuringiensis* endotoxin) and abamectin spray was applied to treatments 5 and 6 on 3 occasions. Kasuran (2% kasugamysin and 45% copper oxychloride) was applied on 4 occasions in 2005 for control of citrus canker. Confidor 100 SL was applied to the trunks of trees at 2 mL/tree in treatment 4 on 3 occasions in 2006, in January, February and March. No further applications of Confidor were made in this treatment. Insecticides (either 0.19% fenobucarb or 0.00625% lambda cyhalothrin) were applied in treatment 3 on 5 occasions between early January and late March 2006. No further applications of these insecticides were made in this treatment. A Bt/abamectin spray was applied to treatments 5 and 6 in March 2006. Thereafter, these, and no other biological or synthetic pesticides, were applied to treatments 5 and 6. From April to December 2006 SK EnSpray 99 was applied to treatments to treatment 3, 4, 5 and 6 on 9 occasions, Sunspray Ultra-Fine and SK EnSpray 99 were applied in treatments 1 and 2, respectively, on 12 occasions during the year. In 2007 and 2008, Sunspray Ultra-Fine was applied 12 times annually in treatment 1, and SK Enspray 99 was applied 12 times annually to treatments 3, 4, 5 and 6. From January to June 2009, Sunspray Ultra-Fine was applied to all treatments on 6 occasions.

From May 2004 to December 2005, assessments were based on the four central trees within each treatment replicate. Tree phenology was assessed weekly during flushing cycles, and fortnightly between flushes. These observations determined the number of flushes in each immature flush using the following categories: open buds ≤ 5 mm long; immature flushes 6 mm to 10 mm long; and immature flushes > 10 mm long. Incidence of *D. citri* was assessed during these assessments. Numbers of *D. citri* adults on mature leaves and immature flushes within each of the above categories, numbers of immature flushes in each category with eggs present, and numbers of immature flushes in each category with nymphs present (any stage, live or dead) were assessed. Tree height (up to 5 m or more) made it impractical to undertake these assessments from January 2006 to May 2009, when observations ceased. Incidence of HLB was assessed annually from 2005. Each tree was checked for symptoms of the disease and presence of the pathogen confirmed by PCR. Dead trees were also recorded. Levels of HLB infection in adjacent orchards were also determined in 2006.

At **Cao Phong**, a 2.3 ha site of sweet orange trees on 'chap' (probably Rangpur lime: *C.* × *limon*) rootstocks was established on a gently sloping hill on 29 July 2004. The site was divided into 4 plots, each with one replicate of the six treatments. Each replicate comprised 36 trees planted on a 4 m × 4 m grid. All trees were treated with 3 mL Confidor 100 SL (0.3 mL imidacloprid/tree) on 20 August and 2 December. On 4 November 2004, 0.5% Sunspray Ultra-Fine and SK EnSpray 99 were applied to treatments 1 and 2, respectively, and 0.073% propargite was applied to all other treatments.

In 2005, 0.5% sprays of Sunspray Ultra-Fine and SK EnSpray 99 were applied in treatments 1 and 2, respectively, on 4 occasions, on 1 March, 15 and 22 June, and 8 July. Propargite was applied at 0.1% to treatments 3 and 4 on 1 March. Cypermethrin was applied at 0.0375% to all treatments on 7 May, and methidathion was applied to all treatments at 0.04% on 28 May. Copper hydroxide was applied at 0.077% to all treatments on 15 and 22 June and on 8 July. Profenofos was applied at 0.05% to treatment 3 on 16 and 22 June, and on 8 July, and Sunspray Ultra-Fine and SK EnSpray

99 were applied at 0.15% to treatments 5 and 6 respectively on the same dates. Confidor 100 SL was applied at 3 mL per tree in treatment 4 on 7 April and 8 July.

In 2006, oils, insecticides, miticides and/or fungicides were applied on 7 occasions, on 16 January, 23 May, 14 July, 14 September, 6 October, 15 November and 4 December. Sunspray Ultra-Fine and SK EnSpray 99 were applied at 0.5% respectively in treatments 1 and 2 on 6 occasions, on 16 January, 23 May, 14 July, 14 September, 6 October and 4 December. Propargite was applied at 0.073% in treatments 3, 4, 5 and 6 on 16 January, and in treatments 3 and 4 on 4 December. Matrine, a tetracyclo-quinolizindine alkaloid, was applied at 0. 054% to treatments 3 and 4 on 23 May. Copper hydroxide was applied at 0.077% to all treatments on 14 July, and then at 0.1% to all treatments on 14 September and 6 October. Methidathion was applied at 0.04% to treatment 3 on 14 September and 6 October. SK EnSpray 99 was applied at 0.15% to the control treatments (treatments 5 and 6) on 14 July, 14 September, 6 October and 4 December. Confidor 100 SL was applied at 3 mL per tree in treatment 4 on 14 September and 15 November.

In 2007, treatments were applied on 19 occasions, on 4 and 18 January, 8 February, 3 and 25 March, 7 and 24 April, 10, 16, 23 and 30 May, 7, 15, 22 June, 9 and 28 July, 4 August, 12 October and 10 December. DS 98.8 EC (an nC24 mineral oil) at 0.5%, and 0.001% imidacloprid and 0.00045% abamectin were applied to all treatments on 4 January and 8 February. SK EnSpray 99 at 0.5% was applied with cypermethrin at 0.025% and abamectin at 0.0027% on 7 April, and with copper hydroxide at 0.1% and abamectin at 0.0018% on 23 April. Sunspray Ultra-Fine and SK EnSpray 99 were then applied at 0.5% respectively in treatments 1 and 2 on 12 occasions, on 10, 16, 23, and 30 May, 7, 15 and 22 June, 9 and 28 July, 4 August, 12 October and 10 December: 0.1% copper hydroxide was applied with these sprays on all but the last of these 12 occasions. Propargite at 0.073% and validamycin A at 0.001% were applied to all treatments on 18 January. Fenpiroksimate at 0.005% and beta-cyfluthrin at 0.025% were applied to all treatments on 3 March. Abamectin at 0.0027% and cypermethrin at 0.0375% were applied to all treatments on 25 March. Cypermethrin at 0.0375% was applied with copper hydroxide at 0.1% in treatment 3 on 10 May without copper hydroxide, with 0.1% copper hydroxide on 30 May, 7 and 15 June, 9 and 28 July, 4 August, and 12 October, and without copper hydroxide on 10 December. Methidathion at 0.04% was applied to treatment 3 on 16 May, and to the same treatment with copper hydroxide at 0.1% on 22 June. Cypermethrin at 0.0375% was applied to treatment 4 on 10 May and 16 May without copper hydroxide, and on 22 June with 0.1% copper hydroxide. Confidor 100 SL was applied in this treatment as a soil drench at 3 mL per tree 23 May, 4 August and 10 December. It was applied as a 0.01% imidacloprid spray on 9 and 28 July. Copper hydroxide at 0.1% was also applied to this treatment on 23 and 30 May, 7, 15, 22 June, 9 and 28 July, 4 August, and 12 October. SK EnSpray 99 was applied at 0.15% with copper hydroxide at 0.1% in treatments 5 and 6 on 10, 16 and 30 May, 9 and 28 July, 4 August, and 12 October, and without copper hydroxide on 7 and 15 June, and 10 December.

In 2008, treatments were applied on 8 occasions, on 18 February, 17 March, 16 April, 2 and 25 June, 23 July, 17 August and 18 September. Sunspray Ultra-Fine and SK EnSpray 99 were applied on each occasion at 0.5% respectively in treatments 1 and 2: copper hydroxide at 0.1% was applied with these sprays on 2 June, 23 July and 18 September, and zineb was applied at 0.32% with the oils on 17 August. Abamectin was applied at 0.0027% to treatments 3, 4, 5 and 6 on 18 February. Profenofos and cypermethrin were applied together (as Polytrin P 440 EC) to treatment 3 at 0.04% and 0.004%, respectively, with propargite at 0.0.073% on 17 March and 16 April and with copper hydroxide at 0.1% on 2 June. Abamectin was applied at 0.0027% to treatment 3 on 25 June with 0.32% zineb. These applications were followed by application of 0.05% copper hydroxide and 0.01% phosmet on 23 July, 0.0027% abamectin, 0.02% acetamiprid and 0.32% zineb on 17 August, and 0.5% SK Enspray and 0.1% copper hydroxide on 18 September. Propargite at 0.073% and methidathion at 0.04% were applied to treatment 4 on 17 March and 16 April, and methidathion at 0.04% was applied with 0.1% copper hydroxide in the same treatment on 25 June. Thereafter, applications in this treatment were identical to those in treatment 3. SK Enspray was applied at 0.15% in treatments 5 and 6 on 17 March and 16 April, and at the same rate with 0.1% copper hydroxide on 2 June. Thereafter, applications in these treatments were identical to those in treatments 3 and 4.

In 2009, no mineral oil sprays were applied from January to the end of May. The spray program for all treatments involved identical applications of trichlorfon, cypermethrin, acetamiprid, abamectins and zineb.

All assessments were based on the four central trees within each treatment replicate. Tree phenology was assessed fortnightly. Observations determined the number of flushes in each immature flush using the following categories: open buds ≤ 5 mm long; immature flushes 6 mm to 10 mm long; and immature flushes > 10 mm long. Incidence of *D. citri* was assessed during the phenology assessments. Numbers of *D. citri* adults on mature leaves and immature flushes within each of the above categories, numbers of immature flushes in each category with eggs present, and numbers of immature flushes in each category with nymphs present (any stage, live or dead) were assessed. Incidence of HLB was assessed annually. Each tree was checked for symptoms of the disease and presence of the pathogen confirmed by PCR. Gold-dust weevil (*Hypomeces squamosus*), citrus leafminer (*Phyllocnistis citrella*), citrus red mite (*Panonychus citri*), chilli thrips (*Scirtothrips dorsalis*), brown and/or black citrus aphids (*Toxoptera aurantii* and/or *T. citricida*) armoured scales (e.g., *Aonidiella aurantii*) and whiteflies (*Dialeurodes citri*) infestations, citrus canker, black mildew (*Meliola citricola*), citrus greasy spot (*Mycosphaerella citri*) and powdery mildew infections (*Oidium* sp.), and the presence of foliicolous lichenised fungi were also assessed. Tree heights and canopy circumferences were measured annually. Fruit numbers were assessed in 2008.

The site of the experiment at **Purworejo** was located in an area that was relatively isolated from other orchards. The nearest was about 200 m from the site, and trees within it were severely infected with HLB in 2006. The experiment comprised 256 PFTs and 128 non-PFTs. All trees were Siem mandarin. Each treatment, as at Cai Be and Cao Phong, comprised four replicates of 16 trees, with one replicate within each of four blocks. Trees were planted on a 4 m grid within blocks, and blocks were 8 m apart on a square, slightly terraced, area of land.

Oil sprays (0.5% v/v) were applied 7 day-intervals during flush cycles from bud-burst, when buds were < 5 mm long, until buds were about 40 mm long. The farmer's practice entailed monthly applications of 0.025% lambda cyhalothrin and 0.05% profenofos. Applications of imidacloprid, as Confidor 200 SL, commenced two weeks after planting. In the first year, 50 mL of 0.6% solution of the product was applied every 3 months to soil around the drip-line under each tree. It was then applied monthly and undiluted to the trunks of each tree at the rate of 1 mL per tree (0.2 mL imidacloprid/tree). No oil or insecticide sprays were applied to the control PFT and non-PFT treatments. Propineb at 0.7% and mancozeb at 0.8% were applied monthly to all plants. All sprays were applied evenly to foliage to the point of initial run-off. The upper and lower side of leaves, twigs and branches in both the inner and outer parts of each canopy were sprayed thoroughly.

All assessments were based on the four central trees within each treatment replicate. Tree phenology was assessed weekly during flushing cycles, and fortnightly between flushes. Observations determined the number of flushes in each immature flush using the following categories: open buds ≤ 5 mm long; immature flushes 6 mm to 10 mm long; and immature flushes > 10 mm long. Incidence of *D. citri* was assessed during the phenology assessments. Numbers of *D. citri* adults on mature leaves and immature flushes within each of the above categories, numbers of immature flushes in each category with eggs present, and numbers of immature flushes in each category with nymphs present (any stage, live or dead) were assessed. Incidence of HLB was assessed annually. Each tree was checked for symptoms of the disease and presence of the pathogen confirmed by PCR.

Interactions between plant nutrition and HLB

Mandarin survey in Central Java. Three orchards were surveyed: a coastal site (Site 1), 5 m asl near Purwodadi (7° 49′ S, 109° 59′ E), with a sandy soil and in which manure and NPK fertiliser was applied twice annually; a site (Site 2), 50 m asl near Purworejo, with a clay-loam soil and in which manure and NPK fertilisers were applied twice annually, and foliar fertiliser once annually; and another site (Site 3), 50 m asl near Purworejo, with a clay-loam soil and in which manure and NPK fertilisers were applied twice annually. Leaves and soil were collected for mineral analysis from beneath the canopies of nine infected (PCR positive) and nine PCR negative trees at each site. The symptom severity of PCR-negative trees at all three sites was low and reflected nutrient deficiencies rather than disease. In contrast, the average disease severity of infected trees varied from 36-65%. There was a good agreement between the visual assessment of symptoms and identification of the pathogen status of the trees by PCR.

Pot trials and field surveys in Central Java and Japan. To examine the effects on mineral nutrition on '*Ca*. L. asiaticus' infected citrus, rough lemon (*C*. × *taitensis*: syn. *C*. × *jambhiri*), Siem mandarin and flat lemon (*C. depressa* Hayata, a form of *C. reticulata*) plants were patch-grafted with '*Ca*. L. asiaticus'-infected bark squares and grown in pots in greenhouses in Japan and Indonesia. In addition, leaves were collected from field-grown tankan (*C*. × *aurantium*, also called *C. tankan*) and Satsuma mandarin (*C. reticulata* but also called *C. unshiu*) trees in Japan, and from Siem mandarin trees in Indonesia. For infected plants, leaves showing symptoms (chlorosis) were sampled separately from symptomless ones. Infection status of the plants was determined by PCR and aqueous extracts of leaf tissue were assayed for concentrations of Cu, Fe, Mn and Zn.

Field and screenhouse pot-trial studies at Universitas Gadjah Mada. These studies were undertaken to determine physiological and biochemical alterations in HLB-infected citrus trees related to nutrient treatment. Several experiments were undertaken in a field trial and in a screenhouse trial. The field experiment consisted of HLB-infected trees with typical symptoms and trees that were PCR-negative. Equal numbers (5 trees) of these trees were then subjected to the following treatments: $FeSO_4$, $ZnSO_4$, $Mg(NO_3)_2$ applied separately as sprays, and a treatment in which a general foliar fertiliser sprayed onto the leaf and triple superphosphate broadcasted onto the soil.

In the pot trial, healthy and infected Siem mandarin trees were grown in Hoagland's solution deficient in Zn and Fe and plants were then supplied with these elements via their roots or leaves (Trial 1); in Hoagland's solution containing zero Fe, half-strength Fe, full-strength Fe (16.11 µM) or ten fold Fe (Trial 2); or in Hoagland's solution containing zero P, half-strength P, full-strength P (6.0 mM) or ten fold P. Material from these trials were subjected to the following analyses or assessments: the presence of '*Ca*. L. asiaticus' in trees was assayed by PCR; leaf samples were analysed for Fe, Zn, Mg, P and chlorophyll concentrations and peroxidise activity; the number of branches, flushes, leaves, canopy width were counted and the trunk diameter and height of the plants were made at monthly intervals; and the ratio of root dry weight to shoot dry weight and extent of phloem disruption determined at the end of experiment.

Catechol, mottle yellows, starch accumulation and HLB

Mature leaves were obtained from healthy plants, the PCR-positive HLB-infected plants, PCR-negative plants with HLB symptoms. Catechol concentrations were determined by high performance liquid chromatography. Zn and Fe concentrations were determined by atomic adsorption spectrophotometry. Light microscopy was used to examine transverse sections of leaf midribs for the presence of catechol following staining with neutral red. The research was undertaken at Universitas Gadjah Mada.

Germplasm tolerance to HLB and *D.citri* in Việt Nam

Natural severity of HLB symptoms in citrus species, varieties and relatives in the Mekong Delta. Surveys were undertaken from 2006 to 2008 to rank the severity of HLB symptoms (- no symptoms, negative PCR; + low, ++ average, +++ heavy, ++++ severe, positive

PCR) manifested in citrus species, varieties and relatives growing in orchards in the Mekong Delta.

Evaluation of *Limonia acidissima* and *Feroniella lucida* as rootstocks. *L. acidissima* was evaluated as a rootstock to improve tolerance of pomelo, sweet orange and King orange scions to HLB. Seven Namroi pomelo, 2 Daxanh pomelo (*C. maxima*), 2 King orange and 1 sweet orange scions were evaluated by exposing the plants to natural infection in the field in Vinh Long and Tien Giang provinces in the Mekong Delta. PCR was used to confirm the presence or absence of '*Ca*. L. asiaticus' in the plants after 12 months. In another trail, *L. acidissima*, *F. lucida* and volkameriana were evaluated as rootstocks for king orange in Tien Giang. The trees were planted in 2006.

Psyllid transmission of HLB to sweet orange and 4 citrus relatives. Psyllid adults reared on HLB-infected sweet orange plants were used to determine whether the adults could transmit HLB to sweet orange, orange jasmine (*M. paniculata* var. *exotica*), *Triphasia trifolia*, *L. acidissima* and *F. lucida*. Three plants of each type were caged separately with 20 psyllids for 3 days, after adults had fed on infected plants for 24 hours. PCR was used to confirm the presence or absence of '*Ca*. L. asiaticus' in the plants 60 and 75 days after the psyllids were released into the cages.

HLB tolerance of scions on volkameriana rootsocks following graft transmission of HLB. Pathogen-free scions of sweet, Tieu and Sanh mandarins, Tuong lemon, sweet orange, Namroi pomelo, calamondin, trifoliate orange (flying dragon), *L. acidissima* and *F. lucida* were grafted onto volkameriana rootstocks (3 replications, with 3 scions per replicate). After 30 days, HLB-infected sweet orange buds were grafted onto each scion. The development of HLB symptoms on the plants was monitored and PCR was used to confirm the presence or absence of '*Ca*. L. asiaticus' in leaves of each scion 80, 102, 180 and 240 days after grafting.

Guava interplants in Indonesia

A 0.12 ha site was established on flat land at Purworejo, Central Java, in November 2006. Three treatments were compared: Siem mandarin alone; Siem mandarin interplanted a red seeded variety of guava ('Jakarta'); and Siem mandarin interplanted with a white seedless variety of guava 'Sukun'. There were 6 replicates of each treatment. Each replicate comprised a 6 m × 4 m plot in which 5 mandarin trees were planted 1.5 m apart within each of two rows planted 3 m apart. In each of the two guava treatments, the rows of mandarin trees were planted between three rows of guava trees planted 3 m apart, with each row of guava trees comprising 5 trees planted 1.5 m apart. The distance between plots was 2 m. The guava trees were planted in November 2006 and the mandarin trees in March 2007. *D. citri*, citrus leafminer and brown citrus aphid populations were assessed monthly from April 2007 to May 2009.

Observations in Pakistan, Bhutan and India

Orchards were visited in the Punjab and North West Frontier Province of Pakistan in July 2006, the Puna Tsang river valley in Bhutan from just north of Punakha southwards to Damphu in Tsirang in May 2009, and in the Garo Hills of Meghalaya in northeast India in October 2009.

Technology transfer and capacity building

Activities focused on: joint supervision of postgraduate students Indonesia; journal publications; participation in conferences, workshops and field days; publication of texts on mineral oils; assisting the marketing of mineral oils.

6 Achievements against activities and outputs/milestones

Objective 1: To survey distributions and occurrence of D. citri*, related psyllid fauna, and psyllid natural enemies (entomopathogens, predators, and primary and secondary parasitoids) in Central Java*

No.	Activity	Outputs/ milestones	Completion date	Comments
1.1	Survey, collect and identify natural enemies of *Diaphorina citri* in Central Java	Survey and collection records tabulated and interpreted and summarised in biannual reports to project leaders.	biannually	Surveys completed and results incorporated into draft thesis and a paper
1.2	Compare seasonal occurrence of natural enemies at three altitudes (50 m, 650 m and 1300 m asl)	Seasonal abundance of natural enemies at the 3 altitudes summarised and interpreted within biannual reports to project leaders.	biannually	Surveys completed and results incorporated into draft thesis and paper
1.3	Biology of coccinellid predators of the psyllid	Data on the biology of the coccinellids analysed, interpreted and summarised within biannual report to project leaders.	biannually	Studies completed and results incorporated into draft thesis.

PC = partner country, A = Australia

Objective 2: To determine distributions of known and potential alternative hosts of D. citri *and 'Ca. L. asiaticus' in Indonesia, Việt Nam and Australia*

No.	Activity	Outputs/ milestones	Completion date	Comments
2.1	Continue surveys and validation of herbarium records in Việt Nam and Indonesia	Details of plants collected and identified summarised for biannual reports	biannually	Extensive revision of host records of huanglongbing and its vectors completed in April 2008: work on identities of host and non-host flora continuing
2.2	Morphological, propagation and molecular studies to resolve uncertainties about the status species of *Clausena* and *Murraya* (Indonesia)	Outcomes of progressive studies summarised for biannual reports.	biannually	Initial studies in Indonesia indicated that 'orange jasmine' is not a single species, *Murraya paniculata*, but two species, *M. paniculata* and *M. exotica*. This work became part of PhD studies at the University of Western Sydney by a John Allwright Fellowship (JAF) PhD student, Nguyen Huy Chung, from Việt Nam.

2.3	Variation in phenotypes, phenologies and growth rates of specific species within these genera (Indonesia)	Data for studies on phenotype, phenological, and growth rate variations analysed results summarised for biannual report.	biannually	See above: research being undertaken by a UWS PhD student
2.4	Comparison of the phenotypes and leaf and fruit morphologies of *Murraya paniculata* and *Murraya exotica* (Indonesia)	Differences summarise in phenotypes and leaf and fruit morphology summarised within a biannual report.	biannually	See above: results suggest that '*Murraya exotica*', the widely grown ornamental and favoured host of *D. citri*, is not *M. paniculata*. The studies also indicate that what was considered a single species by many botanists may be 3 species.
2.5	Molecular studies to support identification and relationships between species of *Citrus* other Aurantioideae in Việt Nam (will depend on John Allwright Fellowship).	Progress with molecular studies, and tentative conclusions, reported biannually (if JAF funded)	biannually	See above: results suggest that '*Murraya exotica'* is not *Murraya paniculata.* DNA was collected from *Feroniella lucida* and *Limnocitrus littoralis* in southern Việt Nam and arrangements are being made for the identities of 4 Citrus species/hybrids and 16 Citrus relatives from Cuc Phoung National Park near Ha Noi to be confirmed/determined by Profs Mabberley (UK) and Bayer (USA*).*

PC = partner country, A = Australia

Objective 3: To determine the impacts of ambient temperatures at different latitudes, longitudes and altitudes in Central Java on the incidence of **D. citri***, and the spread and severity of huanglongbing in different species and cultivars of citrus and alternative hosts (Indonesia)*

No.	Activity	Outputs/ milestones	Completion date	Comments
3.1	Monitor growth of plants and seasonal incidence of *D. citri* on its hosts at three altitudes	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	The psyllid was detected at the low (60 m asl) and medium altitude (600 m asl) sites but not at 1300 m asl.
3.2	Monitor the incidence and spread of huanglongbing at these altitudes	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	Huanglongbing was detected at the low (60 m asl) and medium altitude (600 m asl) sites but not at 1300 m asl. Powdery mildew was a severe impediment to citrus production at 1300 m asl.
3.3	Determine rates of huanglongbing symptom expression and development on orange and mandarin seedlings under at three temperature ranges under controlled conditions	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	This work was not been undertaken.

3.4	Model temporal and spatial patterns of climatic variables in Java	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	Progress was reported in January 2008 but the thesis was not been completed due to the role of the student in Universitas Gadjah Mada administration and business activities
3.5	Model impact of weather variables on the incidence of the disease and its vector	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	Progress was reported in January 2008 but the thesis was not been completed due to the role of the student in Universitas Gadjah Mada administration and business activities
3.6	Predict rates disease development at locations throughout Java, and possibly more broadly	Conduct studies as required to resolve these issues; report details to project leaders biannually	biannually	Progress was reported in January 2008 but the thesis was not been completed due to the role of the student in Universitas Gadjah Mada administration and business activities

PC = Partner Country, A = Australia

Objective 4: To determine impact of feeding behaviour of adult **D. citri***, and host plant phenology on transmission of* **'Ca.** ***L. asiaticus*'***, on orange, mandarin and selected alternative hosts (Indonesia)*

No.	Activity	Outputs/ milestones	Completion date	Comments
4.1	Determine the impact of the feeding behaviour of adult psyllids on transmission of huanglongbing to orange, mandarin and alternative hosts in laboratory and controlled environment choice and no-choice studies.	Conduct studies as required to resolve these issues; report details to project leaders biannually.	biannually	Activities were based on field studies involving more than 20 plant species/varieties, including *Citrus* and *Citrus* relatives: results indicated marked differences in host suitability for the psyllid; presence of the disease became apparent in some hosts in 2008. A poster summarising results to late 2008 was presented at an international conference in Florida in December 2008. The site is being maintained and monitored until 2011.

PC = Partner Country, A = Australia

Objective 5: To determine the impact of mineral oil sprays on (a) adult psyllid mortality, feeding and oviposition behaviour, and transmission of huanglongbing under laboratory conditions, (b) psyllid populations and disease transmission under field conditions, and (c) the impact of sprays on foliar diseases in the field (Indonesia and Việt Nam)

No.	Activity	Outputs/ milestones	Completion date	Comments
5.1	Impact of spray deposits on feeding, oviposition and transmission in laboratory and screenhouse experiments	Impacts of sprays determined and interpreted. Results reported to project leaders biannually.	biannually	Studies were completed and results incorporated into a draft thesis and a paper

5.2	Impact of sprays of psyllid populations and transmission of huanglongbing in field experiments.	Impacts of sprays determined and interpreted. Results reported to project leaders biannually.	biannually	Results were incorporated into a draft thesis and a paper: assessments will continue until 2011.
5.3	Impact of sprays on the incidence on foliar algal and fungal pathogens in the field.	Impacts of sprays determined and interpreted. Results reported to project leaders biannually.	biannually	Comparisons were made: assessments will continue until 2011.

PC = Partner Country, A = Australia

Objective 6: To determine influence of scions and rootstocks on the severity of huanglongbing (Việt Nam)

No.	Activity	Outputs/ milestones	Completion date	Comments
6.1	Liaisons with Southern Horticulture Research Institute (SHRI) staff.	Knowledge of scion/rootstock combinations on disease severity. Results reported to project leaders biannually.	biannually	Work on this objective was affected by loss of a key staff member at SHRI. The most notable outcome was that heavily huanglongbing infected and stunted king orange scions on *Limonia acidissima* rootstocks in January 2008 were growing well in June 2009, with less evident symptoms of the disease. In the same field experiment, scions on *Feroniella lucida* that were also heavily symptomatic in January 2008 had died by June 2009, but the rootstock were alive and symptomless. Observations elsewhere by Vietnamese colleagues suggest that *F. lucida* may confer some tolerance to the disease in orange scions.

PC = Partner Country, A = Australia

Objective 7: To determine the impact of plant nutrition on the development and severity of huanglongbing (Indonesia)

No.	Activity	Outputs/ milestones	Completion date	Comments
7.1	Screenhouse trials with potted plants to determine the impact of different levels of nutrition using different rates of complete fertilisers on the development of disease symptoms in potted mandarin seedlings.	Impacts determined and interpreted. Results reported to project leaders biannually.	biannually	Results were incorporated into draft thesis and draft and published papers

7.2	Impact of selected micronutrients on disease development in screenhouse and field experiments involving application of fertilisers to young (one-year-old) and mature (five-year-old) mandarin trees.	Impacts determined and interpreted. Results reported to project leaders biannually.	biannually	Results were incorporated in draft thesis and draft and published papers
7.3	Trials involving N or P on seedlings planted at different altitudes.	Impacts determined and interpreted. Results reported to project leaders biannually.	biannually	Data from pot trials were inconclusive
	Observations on the proliferation of the disease above and below inoculation site.	Impacts determined and interpreted. Results reported to project leaders biannually.	biannually	Not achieved: replaced in 2007/2008 by new activity determining relationships between Zn deficiency, huanglongbing severity and catechol accumulation in leaves. The results suggested that catechol levels are abnormally high in huanglongbing infected leaves but the more important outcome was a better understanding of how Citrus is affected by the bacteria that cause HLB.
	Observations on relationships between proliferation of the bacterium, anatomical changes to phloem (vein-degeneration caused by proliferation of phloem and then death of these cells), starch accumulation, metabolite, proline and nutrient concentrations.	Impacts determined and interpreted. Results reported to project leaders biannually.	biannually	Observations were reported.

PC = Partner Country, A = Australia

Objective 8: To determine the impact of guava interplants on the incidence of **D. citri***, and the spread of huanglongbing, in orchards (Việt Nam and Indonesia)*

No.	Activity	Outputs/ milestones	Completion date	Comments

8.1	Conduct two experiments in Indonesia to determine the impact of guava interplants on populations of *D. citri* and the incidence of huanglongbing (Indonesia RICSH & UGM).	Experiments established from mid 2006, citrus trees planted in early 2007 and incidence of *D. citri* and huanglongbing determined on citrus thereafter	biannually	Results indicated that two varieties of guava can reduce psyllid populations in interplanted orchards and that one of the varieties evaluated was more effective than the other.
8.2	Survey orchards in Việt Nam to determine the impact of guava interplants on the incidence of *D. citri* and huanglongbing (Việt Nam SHRI)	Psyllid populations and sampled in carefully selected groves with citrus monocultures and likewise, groves with guava interplants, 4 times annually in 2007 and 2008.	biannually	Surveys during visits to farms in Tien Giang and nearby provinces in the Mekong Delta continued to indicate low populations of *D. citri* in the presence of guava and high populations of the psyllid in the absence of guava

PC = Partner Country, A = Australia

Objective 9: Capacity building and technology transfer (Indonesia & Việt Nam)

No.	Activity	Outputs/ milestones	Completion date	Comments
9.1	Annual workshops at Universitas Gadjah Mada.	Project activities and findings reported to leading farmers, Indonesian research and extension personnel, and international participants.	Dec 07	Workshop held in January 2008.
9.2	Biannual distribution of fact sheets to local Indonesian farmers, and more widely to Indonesian research and extension personnel	Project activities and findings reported in fact sheets twice annually from January 2007.	biannually	An extension document was prepared in 2007. Other publications have not been published as differences between treatments in field experiments are still not clear due to slow establishment of psyllid populations and ingress of the disease. Clear differences should be apparent by 2011. Mineral oils have not been registered in Indonesia.
9.3	Distribution of project fact sheets to extension agencies and personnel in Việt Nam at completion of the project	Project activities and findings reported in fact sheet in December 2008.	May 09	Use of guava interplants and mineral oils to suppress psyllid populations and ingress of huanglongbing into orchards in southern Viet Nam is now quite widespread. New extension facilities have been constructed at SHRI and the Japan International Cooperation Agency (JICA) is reported to be investing US $ 3.8 million on promoting pomelo production and guava interplanting in the Mekong Delta.

9.4	Project field days and demonstration of disease-free tree propagation, pest and disease monitoring procedures, and spray application procedures to farmers in Indonesia.	Demonstrations held during annual reviews and where feasible visits to farming communities	Dec 07	Farmers participated in field trials and extension activities at Purworejo and Purwokerto in Central Java in July 2008.
9.5	Modification and translation of AusAID/ACIAR 'Vietnamese/Chinese' citrus IPM and mineral oil books for publication in Bahasa Indonesia.	Two books translated to Bahasa Indonesia (at no cost to the project)	Dec 07	Modification of existing text was delayed pending (a) clear differences between treatments in field experiments in which establishment of psyllid populations has been slow and (b) registration of products (see above).

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7 Key results and discussion

Incursion management plan

The plan 'Huanglongbing and its vectors: A pest-specific contingency plan for the citrus and nursery and garden industries' was submitted to Horticulture Australia Limited and Citrus Australia Limited in November 2008. Version 2 (276 pages), with minor errors corrected and additional text was, prepared in February 2009 (Beattie & Barkley 2009). Version 4 of the plan will be made accessible on the internet in mid 2010.

The plan includes comprehensive information about: HLB and its vectors, including hosts, biology, epidemiology; transmission, symptoms, detection and survey methodologies; origins of the disease, the vectors and the genus *Citrus*; potential impacts on commercial citrus production, nurseries, home gardens and native ecosystems; pre- and post-incursion quarantine; and control of vectors and the disease.

Incidence of *D. citri* and its natural enemies at three altitudes in Central Java

Ingress of *D. citri* in to the Siem mandarin sites established in October 2005 at Purworejo (60 m asl), Grabag (640 m asl) and Ngablak (1300 m asl) differed. Adults were first observed at Purworejo in December 2005, again in February 2006 but not regularly until September 2006. They were first recorded at Grabag in 21 March 2007, but not regularly until October 2007. No psyllids were recorded at Ngablak and observations in nearby orchards suggest that infestations will not occur. HLB was recorded at Purworejo in January 2008. It was not recorded at Grabag or Ngablak. It was not present in any of the surveyed orchards at Ngablak. Trees at Purworejo flushed more prolifically and, axiomatically, grew faster than at the other locations. The interval over which observations were possible during the project was not long enough to draw accurate conclusions about the impact of temperature, relative humidity and rainfall on psyllid populations. However, heavy rainfall appeared, as in the literature (e.g., Aubert et al. 1985), to reduce populations by dislodging eggs and nymphs from immature flush growth, but on occasions populations increased rapidly towards the end of the dry season and declined before the wet season commenced.

Aubert et al. (1985) attributed low or absent populations of *D. citri* at high altitudes in Java during surveys in November 1985 to the direct impact of rainfall and to high relative humidities that favour entomopathogens. Our observations in Pakistan (at ambient temperatures ≥ 45°C at 160 m to > 600 m asl), Bhutan (36°C at 1300 m to 1500 m asl), Indonesia, Malaysia, Thailand, Việt Nam and China, and those of Aubert et al. (1985) in Indonesia and Bové & Garnier (1984) in Saudi Arabia and Yemen, suggest that high saturation deficits favour the development of D. citri populations and under such conditions it can flourish at altitudes above 1500 m and ambient temperatures above 40°C. In contrast, low saturation deficits (high relative humidities) favour populations the African citrus psyllid (Catling 1969, Green & Catling 1971). Contrary to laboratory results reported by Liu & Tsai (2000) and Tsai & Liu (2000), the psyllid can thrive at ambient temperatures above 33°C—providing relative humidity is not 'high' and host plant leaves are turgid. Survival of the psyllid under these conditions may be related to evaporative cooling of leaves and psyllid nymphs and adults (Beattie & Barkley 2009). Rapid rises in leaf temperatures, possibly above 45°C, in full sunlight immediately after showers during tropical rainy (monsoon) seasons may lead to high mortality of *D. citri* eggs and nymphs, and to seasonal titres of liberibacters in leaves. Overhead irrigation, including misting of water onto leaves, could have similar effects, and heavy overhead irrigation would most probably dislodge eggs and nymphs.

At Grabag and Ngablak the major impediment to tree growth and bearing was powdery mildew, in this instance *Oidium citri*, not *O. tingitaninum*. Infections at Ngablak were very serious. Production of citrus at altitudes above 600 m in Java could be greatly improved through use of wettable sulphur and/or mineral oils, neither of which is available in

Indonesia. This should be addressed. Citrus leafminer was also serious above 600 m, and would be easily controlled with mineral oils if they were available, and if used at high altitude did not cause phytotoxicity. Application of mineral oils for control of powdery mildew during the project caused leaf drop but it was not clear if this was due to application of sprays at 10× the concentration intended. The trial is being repeated and at this point no phytotoxicity has been observed.

Both parasitoids were recorded at Purworejo and Grabag, where populations increased following increases in populations of their host at the onset of the rainy season (October-January). They were more abundant at Purworejo than at Grabag. Neither species occurred at Ngablak, where their host was not recorded. In general, *D. aligarhensis* was more abundant than *T. radiata*.

The highest levels of parasitism of fourth instar host nymphs from which the parasitoids emerged were 8% for *D. aligarhensis* and 4.7% for *T. radiata*. The parasitoids were most abundant during the rainy seasons of 2005-2006 (October to January) and 2006-2007 (November to February). The lowest levels of parasitism (0.8%) occurred during the dry season in August and September 2007.

Coccinellids found in surveys included *Chilocorus politus*, *Chilocorus nigrita*, *C. inaequalis*, *Cryptogonus orbiculus*, *Curinus coeruleus*, *Harmonia sedecimnotata*, *Heteroneda billardieri*, *Illeis* sp., *Menochilus sexmaculatus*, *Micraspis frenata*, *O. janthinus* and *Scymnus* sp. The five most common were *C. inaequalis*, *M. sexmaculatus*, *H. sedecimnotata*, *O. janthinus* and *Scymnus* sp., and all five species fed on *D. citri* in laboratory studies. *M. sexmaculatus*, *C. inaequalis* and *O. janthinus* were common at Purworejo, *H. sedecimnotata* was common at Grabak, and *C. inaequalis, O. janthinus and H. sedecimnotata* were common at Ngablak. *Scymnus* was common at each location. Species of *Harmonia* are primarily aphidiphagous but they also feed on scales, psyllids and whiteflies (Semyanov & Vaghina 2001, Hodek & Honěk 2009). In Indonesia *O. janthinus* has been recorded feeding on soft scales (Leefmans 1929). *C. inaequalis*, *M. sexmaculatus* and *Scymnus* spp. are polyphagous predators, whose prey includes aphids and psyllids. In laboratory studies, *M. sexmaculatus* was the most voracious of the five most common predators of *D. citri*. However, at 27°C, the dry-weight of *M. sexmaculatus* adults bred from larvae fed on *D. citri* nymphs was about half that of adults that emerge from larvae reared on cowpea aphid (*Aphis craccivora*), and successful completion of the life cycle was > 10 less than on the aphid.

Hosts of D. citri and HLB

Host records for *D. citri*, *T. erytreae* and the HLB pathogens were revised (Beattie & Barkley 2009). Distribution maps for Australian Aurantioideae were prepared but superseded by official maps and records.

Indonesia. These studies focused on a review of records of *Citrus* and *Citrus* relatives in Indonesia, preparation of distribution maps for Rutaceae in Indonesia, molecular and morphological studies to determine the systematic status of *M. paniculata* and *M. paniculata* var. *exotica*, and susceptibility of the latter to '*Ca*. L. asiaticus', and two short-term field trials that evaluated the *Citrus* and *Citrus* relatives as hosts of *D. citri* and '*Ca*. L. asiaticus',

Herbarium and other records indicated that 18 genera and 44 species/varieties of Aurantioideae have been recorded in Indonesia: within the Aurantieae, *Aegle marmellos*, *Atalantia buxifolia*, *At. ceylanica*, *Citrus amblycarpa* (nasnaran mandarin), *C.* × *aurantium* (sweet and sour oranges), *C. hystrix* (kaffir lime), *C. japonica* (kumquats: as *Fortunella* spp.), *C.* × *junos*, *Citrus* × *microcarpa* (calamondin), *C.* × *limon* (lemons), *C.* × *macrophylla* (alemow), *C. reticulata* (mandarins, several varieties), *C. maxima* (pomelo: as *C. maxima* and *C. grandis*), *C. medica* (citron as *C. papaya*), *Citrus* 'spp.' ('*C. limettioides*', '*C. volkameriana*'), *Feroniella lucida*, *Naringi* (syn. *Hesperethusa*) *crenulata*, *Limonia* (syn. *Feronia*) *acidissima*, *Limnocitrus littoralis* (as *Pleiospermium littorale*), *Luvunga eleutherandra* (record requires validation), *L. sarmentosa*, *Merope angulata*, *Merrillia* *caloxylon*, *M. paniculata*, *M. paniculata* var. *exotica* (*sensu* Huang), *Pleiospermium dubium*, *Swinglea glutinosa* and *Triphasia trifolia*; within the Clauseneae, *Bergera crenulata*, *B. koenigii* (curry leaf), *Clausena anisata*, *Cl. anisum-olens*, *Cl. brevistyla*, *Cl. engleri*, *Cl. excavata*, *Cl. harmandiana*, *Cl. indica*, *Cl. lansium* (wampee or huangpi), *Glycosmis citrifolia*, *G. cochinchinesis*, *G. elongata*, *G. pentaphylla*, *Micromelum minutum* and *Mi. pubescens*. Maps reflecting geographical distributions of the plants were prepared.

Some species of *Clausena* e.g., *Cl. excavata* and *Cl. lansium*, are apparently widely distributed in Indonesia, as they occur in Java, Sumatra, Kalimantan, Sulawesi, Bali, and Nusa Tenggara (the Lesser Sunda Islands to the east of Bali). The records, and other sources of information, suggest that four species are endemic to Indonesia: *Cl. engleri* (confined to North Sumatra), *Cl. excavata* (regional with Southeast Asia), *Cl. harmandiana* (distributed in the East Java, the Kangean Islands, Bali and Lombok) and *Cl. anisum-olens* (Kalimantan).

The records and other sources also suggest that *Cl. anisum-olens* was introduced to Java from Kalimantan and the Philippines, and that *Cl. anisata*, *Cl. indica*, and *Cl. lansium* are introduced species. A record of '*Cl. pinnata*' in the Purwodadi Botanical Gardens in East Java is erroneous: the plant listed is apparently *Bergera crenulata*. The identities of all species, particularly *Cl. anisata* and *Cl. lansium*, need to be confirmed and one species, *Cl. brevistyla*, which is endemic to the eastern islands of the archipelago and Papua is not listed in botanic garden records. Also missing from records is *C. halimii* from Sumatera, where it may no longer occur.

PCR based on OPU primers OPU 3, OPU 6, OPU 7, OPN 16 and OPW 19 showed that *M. paniculata* var. *exotica* can be infected with '*Ca*. L. asiaticus'.

In the taxonomic studies on *M. paniculata* and *M. paniculata* var. *exotica*, RAPD analysis with OPM-16 and OPW-19 primers produced DNA banding patterns that supported classification of the two forms as distinct species, *M. paniculata* and *M. exotica*. The shapes of fruit and seeds, and the nature of the inflorescences also varied. However, as the origins of the material in Indonesia are unknown, the possibility exists that it may have come from one original introduction. Therefore, further RAPD analysis was also conducted using primers OPU-3, OPU-6 and OPU-7. Amplification with these primers gave variable results and there was no consistency in the banding patterns produced from DNA extracted from different accessions of the two species. Phytochemical differences between the two forms in China were reported by Li et al. (1988), and they considered the two forms to be distinct species. Mou (2009) recently treated the two forms as separate species. However, Ranade et al. (2006) concluded from molecular studies based on ITS, RAPD and DAMB analysis that *M. paniculata* var. *exotica* is a synonym of *M. paniculata*—but they presented no data to support this conclusion. Our efforts to resolve this issue are being continued by Nguyen Huy Chung, an ACIAR John Allwright Fellow and PhD student at the University of Western Sydney, who is determining morphological and molecular differences between plants in Asia, Australasia and the Americas (California, Florida and Brazil).

Differences between the two forms continue to have important implications for management of HLB. It is clear that *M. paniculata* var. *exotica*, cited by authors (e.g., Li & Ke 2002, Lopes et al. 2006, Silvio Lopes, Fundecitrus, pers. comm., December 2006, Deng Xiaoling, South China Agricultural University, pers. comm., July 2007) as M. paniculata, appears to be transient asymptomatic and symptomatic host of liberibacters, whilst the status of *M. paniculata* (*sensu* Huang: Huang 1959, 1997) as a symptomatic or asymptomatic, either temporarily or permanently, host is not known.

Host records for *D. citri* on 'orange jasmine' also require clarification. Most, perhaps all, orange jasmine plants observed by us and our colleagues in Guangzhou in China resemble *M. paniculata* var. *exotica*, and plants cultivated from seeds from the Xishuangbanna Tropical Botanic Gardens and the University of California, Riverside, for our field studies, although labelled *M. paniculata*, were, according to their morphology and molecular biology, *M. paniculata* var. *exotica* (Inggit Puji Astuti, Bogor Botanic Gardens, pers. comm., 2005: Nguyen Huy Chung, University of Western Sydney, pers. comm., 2010). We suspect that many host records for D. citri on *M. paniculata* in China (see Yang et al. 2006) and elsewhere (see Beattie & Barkley 2009) pertain to *M. paniculata* var. *exotica*.

In the first (Trial 1) of two field trials at Purworejo in Central Java in which Citrus and Citrus relatives were exposed naturally to *D. citri* and HLB from 2006 to 2008, the incidence of *D. citri* adults was highest on *M. paniculata* var. *exotica*, then *S. glutinosa*, *M. paniculata* (about one-third of those recorded on *M. paniculata* var. *exotica*) and *Citrus* × *junos*, and then, in order of declining incidence, and more than 90% lower than on *M. paniculata* var. *exotica*, *B. koenigii*, *C*. × *aurantium* 'Natsudaidai' (neck orange), *C. reticulata* 'Grabak', *C. reticulata* 'Leter', *F. lucida*, *T. trifolia*, *C. reticulata* 'Siem', *L. acidissima*, *C. maxima*, *C*. × *aurantium* (sour orange), and *Aegle marmelos* (bael). No adults were observed on *C. hystrix*, *Cl. lansium*, *Cl. harmandiana* and *Glycosmis pentaphylla*. The incidence of nymphs was highest on *M. paniculata* var. *exotica*, then *S. glutinosa*, *C*. × *junos*, *C. hystrix*, *M. paniculata* (almost 90% fewer than on *M. paniculata* var. *exotica*), *B. koenigii* (90% lower than on *M. paniculata* var. *exotica*), *C*. × *aurantium* 'Natsudaidai' (93% lower), and *T. trifolia* (97% lower), then, in order of declining incidence, and more than 99% lower than on *M. paniculata* var. *exotica*, *C. reticulata* 'Leter', *F. lucida*, *C. reticulata* 'Grabak', *L. acidissima*, and *A. marmelos*. No nymphs were recorded on *C*. × *aurantium* (sour orange), *C. maxima*, *C. reticulata* Siem, *F. lucida*, *Cl. lansium*, *Cl. harmandiana* and *G. pentaphylla*. The incidence of eggs was highest on *M. paniculata* var. exotica, then, *C*. × *junos* (but only marginally lower), *S. glutinosa* (50% lower), *T. trifolia* and *B. koenigii* (both > 98% lower). No eggs were observed on the other species and varieties. PCR confirmed the presence of '*Ca*. L. asiaticus' in Natsudaidai in early 2007, and in Siem mandarin in late 2007. In late 2008, chlorosis was visible on the foliage of 55 plants, but HLB-positive PCR results were only obtained for Natsudaidai, pomelo (*C. maxima*) and *C. reticulata* 'Grabag'.

In the second (Trial 2) field trial at Purworejo in which additional species and varieties to those in Trial 1 were evaluated from early 2007, no *D. citri* adults, nymphs and eggs were observed in 2007. Adults and nymphs were recorded in 2008, the vast majority on *M. paniculata* var. *exotica* selections from the University of California, Riverside, United States of America, and Xishuangbanna Tropical Botanic Gardens, Yunnan, China. Adults were also recorded on *Afraegle paniculata* (ex Riverside), *Cl. excavata*, and a species of *Clausena* from West Sumatera. No HLB symptoms were observed in the trial in either 2007 or 2008.

In these choice studies, *M. paniculata* var. *exotica* was the most favoured host of *D. citri*. It grew and flowered more prolifically than *M. paniculata*. *C*. × *junos* (a possible hybrid of *C. cavaleriei* and *C. reticulata*) was the preferred *Citrus* host, but as for *Murraya*, differences were, aside from phytochemistry, influenced by growth rates and frequency and extent of flush cycles. The psyllid has not been previously been observed on *F. lucida*. Subandiyah et al. (2008) reported this record but erroneously mentioned *Cl. harmandiana* and *Gl. pentaphylla* as hosts. *Cl. lansium* did not grow well at the trial site and was probably the reason why the psyllid was not recorded on it, as it is known host of both *D. citri* and '*Ca*. L. asiaticus' (Beattie & Barkley 2009). *Af. paniculata* and *Cl. excavata* have also been recorded as hosts of *D. citri* (Beattie & Barkley 2009). Observations will continue until December 2010.

Việt Nam. The reviews indicated that there are 169 species of Rutaceae within 32 genera listed as being present in Việt Nam. However, these records largely predate recent major revisions: 37 species/varieties of Citrus were noted, most synonyms. Of the 169 species, 59 occur in the northern mountainous region followed by 55, 19, 19, 15, 12 and 5 in the southeast, northern coastal region, southern coastal region, the Red River Delta, the central highlands and the Mekong River Delta, respectively.

Trips to Hoa Binh, Cuc Phuong National Park, Cat Ba National Park, Tam Dao, Yen Bai and Dien Bien resulted in collection of 33 species/varieties within 11 genera. Most species/varieties were collected from Cuc Phuong National Park (21 of 30 listed). It was not possible to link distributions to altitude, topography, soil types and climate with any degree of certainty. Plant densities were low and generally reflected the impact of human activities. No *D. citri* eggs, nymphs or adults were observed on any of these plants and symptoms of HLB were not evident. The number of each of these plants found was low and their distributions were scattered.

The following 21 species/varieties were propagated at PPRI: *Atalantia guillauminii*, *At. roxburghiana*, pomelo (*C. maxima* ×2), mandarin (*C. reticulata* var. *depressa*), lemon (*C.* × *limon*), orange jasmine (*M. paniculata*, not *M. paniculata* var. *exotica*) [Aurantioideae: Aurantieae], *Cl. excavata*, *Cl. lansium* (×2), *Cl. indica*, *Clausena* sp., *Glycosmis lucida* (syn. *G. cymosa*), *G. parviflora* (syn. *G. citrifolia*), *G. pentaphylla*, *G. petelotii*, *Micromelum hirsutum* [Aurantioideae: Clauseneae], *Melicope pteleifolia*, *Zanthoxylum*. *cucullatipetalum*, *Z. cuspidatum* and two forms of *Z. nitidum* [Rutoideae].

Adult psyllids were observed resting and feeding on 9 of the 21 species/varieties on which 10 adults from HLB-infected plants were released into mesh cages attached to one plant of each species/variety on 29 May 2009, and observed for 3 days. Numbers of psyllids observed respectively on the first, second and third days were: 2, 7 and 7 on *At. guillauminii*; 1, 2 and 4 on *At. roxburghiana*; 1, 1 and 3 on *C.* × *limon*; 2, 3 and 4 on *C. maxima* (first of 2 plants); 0, 1 and 2 on *C. maxima* (1 of 2 plants); 2, 4 and 5 on *C. reticulata* var. *depressa*; 0, 0 and 1 on *Cl. lansium* (1 of 2 plants); 0, 0 and 1 on *Z. cucullatipetalum*; and 0, 0 and 1 on *Z. nutidum*. These are first records for records of *D. citri* adults resting or feeding on *At. roxburghiana*, *Z. cucullatipetalum* and *Z. nutidum*. No adults were observed on 12 species: *Cl. excavata*, *Cl. indica*, *Cl. lansium* (second of 2 plants), *Clausena* sp., *Gl. lucida*, *G. parviflora*, *G. pentaphylla*, *G. petelotii*, *Me. pteleifolia*, *Mi. hirsutum*, *M. paniculata* and *Z. avicennae*. Initial PCR tests for '*Ca*. L. asiaticus' on 8 June 2009 were negative. Observations are continuing.

Plants collected but not exposed to psyllids included a lemon type, king orange, an orange type, *Atalantia buxifolia* (syn. *At. bilocularis* and *Severinia buxifolia*: Chinese box orange), *Murraya alata* [Aurantioideae: Aurantieae], *Cl. dunniana* (syn. *Cl. dentata*, syn. *Cl. anisata*?), *Cl. heptaphylla*, *Micromelum falcatum* [Aurantioideae: Clauseneae], *Acronychia* sp. (possibly *Ac. pedunculata*), *Tetradium fraxinifolium*, *Toddalia asiaticus* and *Z. myriacanthum* [Rutoideae]. Further work is required to confirm the observations.

Plants that were not collected included: sweet and sour oranges (*C.* × *aurantium*), lime (*C.* × *aurantiifolia*), kaffir lime (*C. hystrix*), kumquat (*C. japonica*), citron (*C. medica*), calamondin (*C.* × *microcarpa*), *L. littoralis*, *F. lucida*, bael (*A. marmelos*), *At. monophylla*, *L. acidissima* and *T. trifolia* [Aurantioideae: Aurantieae].

Mineral oil and imidacloprid field and laboratory studies in Indonesia & Việt Nam

Laboratory-based mineral oil studies in Việt Nam. In the no-choice tests at PPRI in which orange jasmine shrubs were sprayed with aqueous SK EnSpray 99 emulsions, mortality of the psyllids in the 2% treatment was significantly higher than in the 0.25% to 1% treatments. After 7 days, mortality in all oil treatments was significantly higher than in the control. In similar tests with sweet orange trees, significant mortality occurred in the oil treatments within 12 hours of the psyllids being released into cages. After 3 days, mortality exceeded 90% in the 0.5%-2% treatments. Similar trends were evident when plants were treated with Sunspray Ultra-Fine. In these experiments, adults tended to settle on petioles rather than on leaves, especially in the higher oil-concentration treatments.

In choice tests at PPRI, numbers of adults landing on trees sprayed with SK EnSpray 99 emulsions only differed significantly from the control in the 2% oil treatment over the first 5 of 13 days over which the experiment was undertaken. Results for Sunspray Ultra-Fine were similar over the first 7 days. As above for the no-choice experiment, adults tended to settle on petioles rather than on leaves, especially in the higher oil-concentration treatments.

In the PPRI choice studies on the impact of oil deposits (of both Sunspray Ultra-Fine and SK EnSpray 99) on landings by adult psyllids on orange trees with new ≤ 5 mm long buds, numbers of landings tended to be lower for 5 days post-treatment in the ≥ 0.5% oil treatments than in the control and 0.25% treatment. Numbers of eggs laid on the buds were significantly lower in all oil treatments than in the controls, with an average reduction of 75% for the ≥ 0.5% Sunspray Ultra-Fine treatments and 83% for the ≥ 0.5% SK EnSpray 99 treatments.

In similar choice studies at PPRI with > 10 mm long expanded buds, numbers of psyllids landing on the buds sprayed with the oils (both Sunspray Ultra-Fine and SK EnSpray 99) were lower 2-6 hours after of sprays being applied than in the control. For oil sprayed plants, the landings were scattered on petioles, leaves and trunks. Numbers landing on these > 10 mm long buds, each with clearly visible loosely packed immature leaves, were lower than on the shorter ≤ 5 mm long compact buds. Landings over 5 days in the SK EnSpray 99 treatments were significantly lower for all oil concentrations than in the control. These trends were not evident in a similar experiment with Sunspray Ultra-Fine. Numbers of eggs laid on the buds were significantly lower in all oil treatments than in the controls, with an average reduction of 77% for the ≥ 0.5% Sunspray Ultra-Fine treatments and 94% for the ≥ 0.5% SK EnSpray 99 treatments.

The impacts of treatments on egg hatch were variable, but combined mortality for eggs and nymphs within 24 hours of hatching averaged 56.3% and 57% for deposits of ≥ 0.5% emulsions of Sunspray Ultra-Fine and SK EnSpray 99, respectively.

Laboratory-based mineral oil studies in Indonesia. In the first study undertaken to determine feeding responses of *D. citri* adults to plants treated with aqueous emulsions in choice tests, the proportion of *D. citri* adults landing on the plants treated with 0%, 0.25%, 0.5%, 1% and 2% oil emulsions was 49%, 18%, 17%, 9.4% and 6.4%, respectively. Proportions of *D. citri* landing on control and treated plants were significantly different. A second application of oil after 7 days increased the number of adults avoiding treated plants. Avoidance was more noticeable in the second study based on no-choice tests.

Mortality of adults 1 day after they were caged in no-choice tests with plants sprayed with 0.25%, 0.5%, 1% and 2% oil emulsions was 22.5%, 37.5%, 40%, and 65% respectively: all adults died within 2 days of being caged with plants treated with 2% oil. Mortality appeared to be related to reluctance of the psyllids to feed on plants sprayed with oil.

In the third study in which oils were applied as 0.5% v/v aqueous emulsions to mandarin leaves, and in which responses of adults determined after they walked on surfaces saturated with either distilled water or 2% v/v aqueous emulsions of the oils, deposits of both oils significantly reduced the proportion of adults attracted to citrus leaves. Both oils (Sunspray Ultra-Fine and SK EnSpray 99) had similar effects, and prior exposure to oil deposits did not appear to influence responses exhibited by adults. This outcome suggested that the response of adults was related to detection of oil volatiles by antennal olfactory receptors.

In the fourth study, in which responses of adult psyllids to host-leaf volatiles, mineral oil volatiles emanating from leaves dipped in 2% aqueous emulsions of each oil, and similarly treated filter paper paired with water-dipped leaves were determined in single linear-tube olfactometers.

When given a choice to move towards oil or towards water dipped leaves, some 60% of adults (1:1 sex ratio) did not move in the oil treatments compared to 22% in the controls. When given a choice to move towards leaves dipped in either Sunspray Ultra-Fine or SK EnSpray 99, some 42% of adults did not move in either treatment. When given a choice between oil treated leaves and a treatment comprising an untreated leaf and a piece of oil treated filter paper, more than 52% of adults did not move towards the treated leaves compared to less than 30% in the other treatment. When presented with a choice of no stimulus (no leaf, filter paper or oil) and an untreated leaf, > 65% of adults did not move towards the former compared to about 20% in the latter. Lastly, when treatments comprising water-dipped leaves plus filter papers dipped in emulsions of either of the two oils were compared with water dipped leaves, some 30% of adults did not move in the oil treatments compared to about 15% in the other treatment with just water dipped leaves. These results suggest that adult *D. citri* can detect oil volatiles (possibly more readily from deposits on leaf surfaces than on paper) and that this leads them to avoid oil treated surfaces for feeding and oviposition. Alternatively, application of oils to leaves may lead to (a) suppression of plant volatiles that attract *D. citri* adults or (b) to the release of plant volatiles that they avoid. Detection of volatiles appears to be through antennal olfactory receptors.

Screenhouse-based imidacloprid studies in Việt Nam. Two weeks after treatment, dead psyllids were found within 2 hours of release in the 0.4% and 0.6% imidacloprid treatments, and 4 hours after release in 0.2% treatment. Mortality after 4 hours was 6%, 11.2% and 12.3% in the 0.2%, 0.4% and 0.6% treatments, respectively. All adults died within 2 days of release in the 0.4% and 0.6% treatments, and within 3 days in the 0.2% treatment. However, 24 hours after their release, 19.6%, 12.2% and 8.5% of adults were still alive in the 0.2%, 0.4% and 0.6% treatments, respectively.

Four weeks after treatment, dead psyllids were found within 2 hours of release in 0.4% and 0.6% imidacloprid treatments, and 4 hours after release in 0.2% treatment. Mortality at 4 hours, 6 hours, 1 day and 2 days after release was less than at same intervals 2 weeks after application of the insecticide. All adults died within 4 days of release in the 0.4% and 0.6% treatments. At this point, mortality in the 0.2% treatment was 96.9%. Rain during the assessments may have reduced psyllid mortality.

Six weeks after treatment, dead psyllids were found within 1 hour of release in 0.4% and 0.6% imidacloprid treatments, and 2 hours after release in 0.2% treatment. Mortality in all imidacloprid treatments 2 hours, 4 hours, 6 hours, 1 day and 2 days after release was greater than at the same intervals in the assessments 4 weeks after application of the pesticide. Complete mortality was observed within 3 days in all imidacloprid treatments but, 24 hours after their release, 50.2%, 37% and 26.4% of adults were still alive in the 0.2%, 0.4% and 0.6% treatments, respectively.

Eight weeks after treatment, mortality of adult psyllids within 1 hour of their release in the all imidacloprid treatments was < 2%. Other results were similar to those recorded 6 weeks after treatments were applied. Complete mortality was observed within 3 days in the 0.4% and 0.6% imidacloprid treatments. At this point, mortality in the 0.2% treatment was 99.6%. However, 24 hours after their release, 47%, 31.2% and 27.8% of adults were still alive in the 0.2%, 0.4% and 0.6% treatments, respectively.

Ten weeks after treatment, dead psyllids were found within 1 hour of release in 0.4% and 0.6% imidacloprid treatments, and 2 hours after release in 0.2% treatment. The mortality of D. citri at 6 hours, 1 day and 2 days after release on 10-week treated plants were similar to rates recorded for assessments 8 weeks after treatment. Complete death of psyllids was observed within 3 days in all imidacloprid treatments but, 24 hours after their release, 41.7%, 28.7% and 16.2% of adults were still alive in the 0.2%, 0.4% and 0.6% treatments, respectively.

These results were published by Quach et al. (2008).

Field comparisons of mineral oils, imidacloprid and farmer practices in Indonesia and Việt Nam. At Cai Be, with the exception of two adult psyllids observed in June 2004 in treatment 5, no psyllids were observed during fortnightly assessments in any treatment from when trees were planted in June 2004 until 31 December 2005. Flushing was almost continuous with 3-4 major cycles per year. The trees grew rapidly under the tropical conditions and by early 2007 they were 4-5 m high. From early 2006 it became impractical to adequately assess psyllid populations, and from March 2006 to the end of December

2008 Sunspray Ultra-Fine was applied to the *n*C21 HMO treatment about 12 times per year (≈ 3 sprays per flush cycle) and *n*C24 SK Enspray 99 was applied likewise to all other treatments: no phytotoxicity was observed. No synthetic insecticides were applied from March 2006. In 2007, the guava trees were topped in mid-2007 to reduce competition with the citrus trees, but in retrospect this was not necessary. No psyllids were observed in the orchard during 2-3 visits annually by UWS staff from October 2004 to April 2009. The low incidence of the psyllid and our inability to determine differences in psyllid populations between treatments was attributed to presence of the guava interplants. Psyllids were present in surrounding groves in the absence of guava interplants, and in 2006 levels of HLB infection in orchards to the north, east, south west of the site were 37.5%, 72.7%, 34.4% and 57.1%, respectively.

In early 2007, some 21% of trees were infected (PCR-positive) with HLB, but levels of infection would have been higher, perhaps double (see Irey et al. 2006a, b), as only symptomatic trees were sampled for PCR. The percentage of infected trees varied among treatments: 11%, 52%, 9%, 20%, 26% and 15% in treatments 1 (Sunspray Ultra-Fine), 2 (SK Enspray 99), 3 (farmer practice), 4 (imidacloprid), 5 (fungicide only PFTs) and 6 (fungicide only with non-PFTs), respectively. However, treatment replicates on the eastern and southern sides of the site had higher levels of infection elsewhere in the block and the level of infection in the SK Enspray 99 treatment was related to this bias and the principal direction of ingress of *D. citri* adults into the orchard.

In 2008, 5.2% of trees were dead and 23.3% of live trees were PCR-positive. In 2009, 20.6% of trees were dead and 28.6% of live trees were infected: 34.3% of the dead trees were PCR-positive for HLB in 2008. Most deaths were attributed to phytophthora root and collar rots caused by *Phytophthora* sp., gummosis caused by *Diplodia natalensis*, and / or pink disease caused by *Corticium salmonicolor*. Observations indicated that very few deaths, perhaps none, were caused by HLB alone. Clearly, research and technology transfer programs are required to address the impact of these diseases on citriculture in the Mekong Delta, and in similar regions in Southeast Asia. However, although the impact of diseases was serious, more than 25 t of fruit were harvested from the orchard in 2009 and sold for about AUD $2/kg.

In contrast to the incidence of HLB observed in the orchard, Gatineau et al. (2006), as noted above, recorded approximately 96%, 75% and 24% infection respectively of 2 year-old king orange trees in 0.5 ha plots that were untreated, sprayed fortnightly with fenobucarb, or treated monthly with imidacloprid applied to the soil or trunks of trees. High numbers of adults and nymphs psyllids were observed in the control. Numbers in the two insecticide treatments were more than 90% lower than in the unsprayed control. Very few adults were observed in the imidacloprid treatment and no nymphs reached the final instar in this treatment. Although both insecticide treatments may have been more effective if applied to larger areas, these results demonstrate, as is widely evident in the literature and in commercial orchards in Asia, that very effective suppression of psyllid populations (e.g., > 95%) does not prevent rapid spread of HLB. The manner in which synthetic pesticides are used for 'control' of *D. citri* in Asia would not be feasible or acceptable in Australia due to withholding periods, maximum residue levels, environmental and health impacts, and risk of resistance. For example, use of imidacloprid for control of citrus pests in Australia is limited to one application per year with a 20 week withholding period for trees up to 4 m high. Furthermore, direct and indirect costs (e.g., disruption of existing, highly effective, integrated pest management programs) would be prohibitive.

Observations during several surveys in the Mekong Delta indicated that psyllid populations were noticeably higher in absence of guava interplants than in interplanted orchards: easily found compared to difficult. Such differences have been verified in field studies by staff at the Southern Horticultural Research Institute (SHRI). Related laboratory studies in China showed that guava volatiles repel adult *D. citri* and that citrus grown in the presence of guava may be less attractive to the psyllid (Zaka et al. 2009, Syed Muhammad Zaka, South China Agricultural University, pers. comm., 2009).

Visits to some orchards in the Mekong Delta suggested that the most significant impact of interplants may be in the first few years after establishment of orchards. In one such orchard that was 17 years old in 2008, guava interplants were removed 3-4 years after the orchard was established. Management thereafter was based on 8-12 applications of mineral oils (initially *n*C24 Caltex D-C-Tron Plus, then SK Enspray 99) per year, mainly during major flush cycles, and no phytotoxicity was observed. Imidacloprid and abamectin were also used occasionally, and the grower said that he removed branches or trees with symptoms of HLB. Survival of these trees in the absence of guava, 3-4 years after planting, may have been related to several factors. Firstly, citrus trees in the region grow quickly and the trees are densely planted. *D. citri* adults, if present, would therefore be more common in upper canopies. Secondly, flushes become less abundant in relation to tree size as trees grow. Larger numbers of psyllids in proportion to canopy volumes would be attracted to younger trees, with more prolific flush growth, than to older trees flushing less prolifically. Thirdly, older trees are less prone to infection than younger trees (Lin 1963) and rates of spread of the pathogens in mature citrus trees are reported to be slower than in young trees (Lin 1956, Fraser & Singh 1969, Moll 1977, Bassanezi & Bassanezi 2008). It seems that movement of the pathogen in a tree may be related to the rate it can multiply and move within phloem before the sieve elements it resides in cease to function as extant phloem ages naturally before being incorporated into the dead outer bark. Thus, infection may be required on multiple occasions, and at several locations, before a tall tree succumbs to the disease. Lateral movement within phloem may be rare. Alternatively, as trees age changes in phytochemistry may influence liberibacter survival, Also, if movement of liberibacters in mature citrus trees is relatively slower, and if production of new phloem continued, supply of carbohydrate to roots and supply of nutrient and water to leaves would continue to sustain tree growth (production of new growth and vascular tissue) until the infection, presumably originating at several points, spread widely within the canopy and to the roots (see further discussion below for 'Catechol, mottle yellows and HLB'). (Progress of the disease in this manner could give the impression that plants are less susceptible than others to the disease and give false hope for selection of tolerant germplasm.)

News about the impact of guava interplants at Cai Be led to considerable interest in the possible use of similar strategies within Việt Nam, and elsewhere including, China, Indonesia and Florida where potential use is being assessed. It is likely that outcomes will depend on achieving and maintaining effective concentrations of guava volatiles within orchards. This will be more easily achieved in warm, humid climates, where orchards are planted with windbreaks on flat land, than in hilly and windy regions. Alternatives to guava should be assessed and use of guava varieties as interplants or cultivated and mowed within interrow spaces needs to be assessed. Vietnamese farmers were the first to observe the impacts of guava interplants on *D. citri* and HLB, and any improvements and patents stemming from these observations should acknowledge their intellectual property.

At Cao Phong, *D. citri* populations between planting in October 2004 and 2009 were low and possibly related to low incidence of the psyllid in neighbouring orchards where the thoroughness of mineral oil and pesticide applications increased after ACIAR CS2/1996/176. Only 3 adults were recorded in 2005 and no psyllids were recorded from then until June 2009. The sweet orange trees grew relatively slowly. In 2008, trunk circumferences and tree heights ranged from 26 to 32 cm and 284 to 336 cm, respectively. Average numbers of fruit per tree varied from 21.5 to 41.4. Trees in treatment 4 (imidacloprid) grew faster and bore more fruit in 2008 than in the other treatments. Slowest growth and lowest numbers of fruit were recorded in treatment 6 (non-pathogen free control trees) but differences between treatments were not significant. Major flushes occurred in February/March and May/June followed by less extensive flushing in September. Heavy use of pesticides did not prevent ingress of HLB into the orchard. In June 2009, 3.7% of trees in the orchard exhibited symptoms of the disease, and 2.4% of trees (66% of those with symptoms) were PCR-positive; 2.1%, 1.4%, 2.8%, 2.8%, 2.1% and 3.5% of trees in treatments 1-6, respectively. The distribution of infected trees did not appear to be related to their proximity to neighbouring orchards or to trees within the replicates of the non-PFT treatment. The level of infection in relation to very low psyllid populations suggests that the percentage of PCR-positive trees could rise to more than 20% by mid 2011.

With the exception of gold dust weevil, the mineral oils were as or more effective than the synthetic pesticides against citrus leafminer, citrus red mite (induced by the use insecticides, particularly pyrethroids and organphosphates), citrus whitefly and black and/or brown citrus aphids.

At Purworejo, the Siem mandarin trees grew well but the ingress of *D. citri* into the orchard was slow and eggs, nymphs or adults were not observed until July 2007, 20 months after the trees were planted. Some 20 sprays were applied in each oil treatment between late 2004 and April 2009. No oil-induced phytotoxicity was observed but some minor, not readily noticeable, transient and inconsequential oil soaking was observed. Trees in the imidacloprid treatment did not appear to be any better than trees in other treatments. Average numbers of adult psyllids observed per tree from July 2007 to April 2009 were in the Sunspray Ultra-Fine, SK Enspray 99, farmer practice, imidacloprid, control PFT, and control non-PDF treatments were 0.9, 1, 3, 1, 11.6 and 4.8, respectively. Symptoms of HLB were evident in 6.3%, 6.3%, 0%, 12.5%, 43.8% and 25% of trees, respectively, in April 2009. The percentage of PCR-positive trees in each of the treatments was 6.3%, 6.3%, 0%, 12.5%, 25% and 6.3%, respectively. Citrus greasy spot was less common on oil sprayed trees than on trees in the other treatments. The experiment will continue at this site until late 2010.

Interactions between plant nutrition and HLB

Mandarin survey in Central Java. On average, the foliar concentrations of N, Mg and Fe were 12, 21 and 42% lower in infected trees, respectively, than in PCR-negative trees. However, no differences were found in concentrations of K, P and Zn. Soil analysis showed no difference between samples taken from under the tree canopies of infected or PCR-negative trees. Therefore, the differences in foliar mineral concentrations between infected and PCR-negative trees were not due to differences in the soil. Symptom severity was affected by fertiliser treatment. At Site 2, where the plants were given foliar application of fertilisers, infected plants had a severity score of 36%: this was ~40% less than the trees given soil-applied fertilisers only. No difference was seen in symptom severity at the other two sites despite the large difference in the composition of the soils. There was little difference between disease severity exhibited by trees grown on the sandy and clay loam soils, despite the large difference in the fertility of the two soil types.

Pot trials and field surveys in Central Java and Japan. This study determined the impact of element concentrations in citrus plants infected with '*Ca*. L. asiaticus'. Except for one marginal case in the field samples of Siem mandarin in Indonesia, infected leaves had lower Fe concentrations. Likewise, with the exception of one pot trial of Siem mandarin in Indonesia, infected leaves also contained significantly lower concentrations of Zn. On average, concentrations of Fe and Zn in infected plants were approximately half those in healthy plants, irrespective of the absolute concentrations of these elements in the plants. Cu was not significantly decreased in any of the trials, and Mn was lower occasionally 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 infected with '*Ca*. L. 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 with these pathogens. It was 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. However, caution must be taken when considering mineral levels in HLB-infected trees. In infected trees, non-structured carbohydrate accumulation may have an apparent dilution effect on nutrient concentrations.

Field and screenhouse pot-trial studies at Universitas Gadjah Mada. Preliminary analysis of data indicated interactions between mineral nutrition and the effect of '*Ca*. L. asiaticus' on growth. Healthy plants grown under nutrient deficient conditions had expected reductions in growth; however, infected plants often had severe symptoms including the death of leaves and branches. There appeared to be little difference in plant growth or symptomatology between plants given foliar or root applied minerals as was suggested by field surveys of mandarins in Central Java.

The results of the first of these 3 trials suggested to us that roots of the HLB-infected plants were affected by the disease and not able to transport minerals efficiently (Pustika et al. 2008). The results of the second trial indicated that, in addition to causing phloem degeneration, the '*Ca*. L. asiaticus' may also affect re-translocation of element through production of a toxin or siderophore that chelates Fe and Zn, but following our subsequent work on catechol and mottle yellows we consider other reasons more likely (see below).

The results of the first and second both indicated that applications of foliar fertilisers would significantly reduce disease severity and increase tree life. Such impacts have recently been reported (http://www.theledger.com/article/20090819/news/908195031?Title=Rebel-With-A-Cause-May-Yield-Greening-Breakthrough) but the results of the third trial (see above) suggested limited scope for such an approach. Moreover, prolonging the life of infected trees would increase the probability of transmission of the pathogen by *D. citri* and might only be advisable for small farms in Asia in the absence of effective area-wide management strategies for the disease.

Catechol, mottle yellows, starch accumulation and HLB

Fe and Zn concentrations were reduced and concentrations of catechol were higher in asymptomatic infected and symptomatic infected plants, compared to the healthy ones. Catechol accumulation was also evident in sections of infected petioles stained with neutral red. Thus, catechol accumulated in Zn deficient and HLB infected plants. We did not determine if the levels we observed induced cell abnormalities or if its accumulation was directly related to levels of salicylic acid. Instead, as the study progressed, we considered more broadly impacts of Zn and other mineral deficiencies on citrus and, given that there is no evidence to suggest that plants within the genus *Citrus* are original hosts of liberibacters that cause HLB (Beattie et al. 2008, Beattie & Barkley 2009), the relationships among the bacteria, the disease they cause, and *Citrus* and *Citrus* relatives. The original hosts must have been, or are, asymptomatic and not killed by the bacteria, and the pathogens were possibly transmitted from them to *Citrus* and other plants by insect vectors or parasitic plants in the recent past (< 1000 years) (Beattie et al. 2008, Beattie & Barkley 2009), presumably on at least three occasions. These circumstances led us to consider the possibility that HLB could be an over-reaction to liberibacters by unnatural hosts, particularly species and varieties of *Citrus* and some *Citrus* relatives, rather than a direct impact of toxins that may be produced by the bacteria. In essence, HLB may be the result of severe mineral deficiencies stemming from over-reaction to an infection that deprives roots of carbohydrate in the absence of an effective physiological response that limits spread of the disease with plant s. Genes involved in metal transport are known to be up-regulated in plants suffering from mineral nutrition (David Ellsworth, University of Western Sydney, pers. comm., 12 February 2010): the effect of root starvation is discussed below.

Impacts on plant physiology and ultrastructure support this possibility.

- Lin (1956) and Lin & Lin (1990: an English translation of Lin 1956) noted that initial yellow shoot symptoms of HLB and decay of feeding roots were followed by a premature off-season blooming during the second year and then Zn, Mn and boron (B) deficiency symptoms combined with twig dieback.
- Matsumoto et al. (1961) recorded starch accumulation in HLB-infected leaves.

- Salibe & Cortez (1966) reported that diseased trees had poorly developed root systems. They also observed starch accumulation in the rootstock portions (we assume above ground level) of the diseased trees and in mottled leaves, and no degeneration of rootstock tissues (we assume above ground level) below bud unions.
- Tirtawidjaja et al. (1965) described vein phloem collapse (hyperplasia and necrosis) and noted that it was associated with accumulation of starch in leaves;
- Schneider (1966, 1968) observed parenchyma cells loaded with starch granules in some HLB-affected leaves. These leaves had a leathery appearance and retained moisture when removed from the tree.
- Schneider (1968) observed localised pockets of necrotic phloem scattered through the leaf vascular system of orange infected with '*Ca*. L. africanus'. The necrosis embraced the entire cross-section of the phloem of the leaf veinlets, but usually only a portion of the phloem in the main lateral veins, the midvein, the petiole, the leaf's base in the node, and occasionally in stems. The necrotic tissue was eventually compressed by enlargement (hypertrophy) of parenchyma cells. There was massive accumulation of starch in the plastids, aberrations in cambial activity and in the subsequent differentiation of cambial derivatives, thickening of cytoplasm and increased staining of cell walls indicating a build up of hemicellulose and excessive phloem formation (hyperplasia) (Schneider 1968)
- Schneider (1968) suggested that grana and chlorophyll of the chloroplasts may be destroyed by being stretched due to enlargement of the starch granule within, and by being crowded against enlarging granules in other chloroplasts, giving rise to the yellow blotching symptom. Pockets of phloem necrosis did not appear in chlorotic shoots until leaves approached maturity. He stated that HLB (then called citrus greening) kills mature phloem.

Wu & Zhang (1985) observed that the major cytopathic effect in the leaf vein of citrus infected with '*Ca*. L. asiaticus' was the presence of many cytoplasmic vesicles formed by enlarged invaginations of plasma membranes that occurred in all cell types and varied significantly in appearance. They also observed aberrations of the thylakoid membrane systems of chloroplasts (grana lamellae were not well developed) and collapse of mitochondria.

Aubert (1988) observed that '*Ca*. L. asiaticus' caused large reductions in the weight of new leaves, young fruits and green twigs and similar reductions in the size of feeder roots. He noted that, as opposed to strict 'physiological mineral deficiencies' that show up within entire canopies, HLB-driven Zn and Mn deficiencies in infected trees always occurred in sectors: in individual leaves as non-symmetrical blotchy mottling in the lamina, in canopies as non-symmetrical yellow shoots, and finally as patchy dieback in the branches.

Su & Huang (1990) observed starch accumulation in the chloroplasts of HLB-infected leaves, and progressive accumulation of starch grains in xylem and phloem parenchyma cells.

Achor et al. (2008) found that starch accumulation occurred after phloem plugging and cell collapse and, therefore, localised phloem carbohydrate deficiency may have been a factor. Starch packing of chloroplasts did not rupture the outer membranes, but the inner granal structure was disrupted thus leading to chlorosis, but only in parts of leaves where phloem plugging occurred. Sieve elements were obstructed by both amorphous and filamentous materials and both occurred in significant amounts, but bacteria were insufficient to cause plugging directly. The amorphous material was positively identified as callose by immunoassay with gold labelling. The filamentous material was presumed to be a phloem protein.

Albrecht & Bowman (2008) detected transcripts for a phloem-specific lectin PP2-like protein in infected symptomatic sweet orange leaves 13-17 weeks after graft inoculation of seedlings with '*Ca*. L. asiaticus' but not 5–9 weeks after graft inoculation or in infected by symptomless plants. They concluded that expression of the protein appears to be directly linked to symptom development and a possible attempt by the host plant to seal the sieve tubes as a barrier against increasing populations of bacteria. They noted that transcript levels for a second phloem-specific lectin PP2-like protein increased more than four-fold during this time.

Etxeberria et al. (2009) observed abnormally high levels of starch in leaves, petioles, stems and bark (30 cm above the scion-rootstock union) in HLB-infected, 10-year-old Valencia orange (*C*. × *aurantium*: syn. *C. sinensis*) in contrast to almost no starch in the roots. Lipid inclusions in HLB-affected leaf cells were more numerous and visibly larger than in healthy leaves. They (Etxeberria et al. 2009) hypothesised that substantial changes in carbohydrate partitioning observed in the trees was in itself a cause of rapid decline and death of infected trees as a result of root starvation. Only under Zn deficiency and girdled branches did they observe high accumulation in leaves in non-HLB infected trees. However, in neither case were starch levels comparable to those observed in HLB-infected trees (Etxeberria et al. 2009).

Kim et al. (2009) attributed starch accumulation in symptomatic sweet orange leaves infected with '*Ca*. L. asiaticus' to a combination of the restriction of photosynthate movement by phloem plugging and the up-regulation of the starch biosynthesis genes in response to infection. Vahling et al. (2010) considered it possible that a perturbation of ATP levels within a cell resulting from the import of ATP via NttA may play a role in increased starch production in infected plants, either by directly affecting the regulation of starch biosynthesis genes or by indirectly altering the processes that govern glucose production.

- Based on the above, the sequence of events after infection, some possibly concurrent, as the bacteria multiply and spread from points of infection, mostly in immature growth, to other aerial parts of the host and eventually to the roots appears to be:
- P-protein (slime bodies), lectin and callose (1-3, β-glucopyranose, an insoluble glucan) are overproduced within sieve elements of initially infected tissues, then subsequently in adjacent leaves, petioles, stems and new growth, as the bacteria multiply and spread;
- callose deposition in the sieve elements blocks sieve plates and plasmodesmata of affected phloem;
- glucans synthesised by phloem form hemicelluose that thickens cell walls of sieve elements;
- phloem transport in the sieve elements of affected tissues is blocked;
- salicylic acid is produced as a response to infection;
- proliferation (hyperplasia) and enlargement (hypertrophy) of phloem parenchyma cells leads to crushing of phloem sieve elements;
- **primary symptoms** (Schneider 1968, McClean & Schwarz 1970), a blotchy-mottling, appear in infected mature leaves as starch accumulates in chloroplasts and plastids, and as chlorophyll content falls and / or fewer chloroplasts are produced;
- starch accumulation occurs in chloroplasts and plastids of all infected shoot tissues (mesophyll, epidermis and vascular parenchyma of leaves, epidermis and parenchyma of stems and bark);
- the presence of starch in the xylem parenchyma of stems indicates that these cells obtain carbohydrate prior to phloem collapse, as infection spreads towards the roots;

- extensive phloem collapse in stems as a result of hyperplasia and hypertrophy of phloem parenchyma severely impedes or prevents transport of carbohydrate to roots;
- feeder roots produced in the previous season of growth consume available carbohydrate and starch ceases to accumulate in roots;
- production of new feeder roots ceases in the absence of carbohydrate;
- uptake of minerals declines to negligible levels;
- enzyme activity in newly-forming leaves, and elsewhere, is severely disrupted and normal metabolic processes and growth fail rapidly;
- **secondary symptoms** (Schneider 1968, McClean & Schwarz 1970) develop in new leaves as they are produced;
- the leaves fail to develop normally—they become small, leathery, upright and exhibit severe mineral deficiency symptoms (particularly Zn);
- carbohydrate transport is further retarded;
- lipid accumulation occurs in plastids of mesophyll;
- this indicates excessive storage of carbohydrate and possibly catechol;
- thickening of phloem walls with hemicellulose continues;
- abnormal cell development occurs in fruit;
- branches die;
- the tree dies.

Siem mandarin (*C. reticulata*: syn. *C.* × *suhuiensis*) leaves with **primary symptoms** of HLB: Purworejo, Central Java, Indonesia, 18 February 2010 (Siti Subandiyah & Achmed Himawan)

Steps in this proposed sequence of events need to be confirmed and revised as additional information becomes available. For example, in microarray analyses reported by Kim et al. (2009) salicylic acid and jasmonates that play critical roles in plant defence responses were not significantly regulated by HLB infection. This led the authors (Kim et al. 2009) to suggest that salicylic acid and jasmonates may not have a prominent role in response of citrus to HLB infections.

Gradual death of roots stemming from reduced supply of carbohydrate, then ultimately none, leads to reduced, then finally no, supply of minerals and water required for normal metabolic processes. This results in accumulation of abnormal levels of starch, lipids, catechol and other substance in leaves and other aerial tissues.

Infected marcotted citrus trees decline rapidly after planting, because infection is likely to be wide spread within phloem, possibly even in the roots, at planting. Young, initially pathogen-free, citrus trees decline rapidly if they become infected soon after planting, particularly if infections occur at multiple points and spread quickly to the roots over relatively short distances before new vascular tissue can be produced to translocate carbohydrate and minerals to and from roots. Observations reported by van Vuuren (1993) suggest that liberibacters in such plants move at least 50 cm downwards towards the roots within one year of infection.

Rates of spread of the disease in mature citrus trees are reported to be slower than in young trees (Lin 1956, Fraser & Singh 1969, Moll 1977, Bassanezi & Bassanezi 2008). This, in part, may be due to translocation of carbohydrates and minerals in pathogen-free vascular tissue produced after infection, as the bacteria move downwards over relatively long distances from points of infection to the roots in phloem formed before infection. This possibility is supported by recent DIISR-funded research by Zhang Pei at South China Agricultural University. By increasing leaf temperatures she was able to significantly reduce titres of ‘*Ca*. L. asiaticus’ in small, severely infected, potted mandarin trees (in this instance *C. reticulata* ‘Sunki’: syn. *C. sunki* Hort ex. Tanaka) to a point where the bacteria in leaves expressing severe symptoms of HLB could no longer be detected by PCR; the trees started to grow, relatively rapidly, again, presumably after production of new vascular tissue and/or after reductions in deposits of callose and P-protein. Moreover, as trees mature their physiological responses to infection may be become more effective and slow bacterial spread, thus limiting the severity of infection and effects of the bacterium ‘diluted’ by the greater mass of tissue. These possibilities, generation of new vascular tissue and changes in physiology, need to be considered in surveys for tolerant germplasm.

Siem mandarin (*C. reticulata*: syn. *C.* × *suhuiensis*) leaves with severe Zn deficiency-like **secondary symptoms** of HLB: Purworejo, Central Java, Indonesia, 1 October 2003 (GAC Beattie)

Transient infections of *M. paniculata* var. *exotica*, as observed in China (Deng Xiaoling, South China Agricultural University, pers. comm., 2007), in which only primary-like symptoms were expressed, indicate that some citrus relatives can kill HLB pathogens, and then resume normal growth. If so, forms of *Murraya* may be a source of genes that, as suggested by Guo & Deng (1998), may be possible to incorporate into *Citrus*. However, Guo & Deng (1998) attempted, but failed, to transfer an assumed HLB resistance trait from *M. paniculata* to Page tangelo by somatic hybridisation. They chose somatic hybridisation because Swingle & Reece (1967) had reported that efforts to create viable sexual progeny and grafted plants between *M. paniculata* and *Citrus* had failed due to sexual incompatibility and limited graft compatibility.

The status of *Murraya* as a host of HLB has been controversial for more than 20 years, with some reports claiming it is not a host and others that it is a symptomatic host. However:

- there are no reports of infected trees dying;
- Miyakawa (1980) observed no visible signs of infection following tissue inoculation;
- Tirtawidjaja (1981) reported stunting and suspicious external and internal symptoms after transmission by *D. citri*;
- Aubert (1988) reported that it can harbour disease; Garnier & Bové (1993) reported that there was no evidence for the presence of the pathogen in plants;
- Koizumi et al. (1996) observed no symptoms, but mistakenly said that Miyakawa (1980) could not confirm the presence of the pathogen in inoculated *M. paniculata* with electron microscopy;
- Guo & Deng (1998, 2001) said that Chen & Liao (1982) said *M. paniculata* was immune to HLB but Chen & Liao did not mention this;
- Toorawa (1998) considered it as a non-host;
- Li & Ke (2002) reported detection of the pathogen in *M. paniculata* plants in city streets and near orchards, and also in psyllids fed on infected *M. paniculata*;
- Li & Ke (2002) referred to it as a 'cryptic symptom host'; Siti Subandiyah & Achmed Himawan (UGM, pers. comm., 2002) detected the pathogen in symptomatic plants;
- Dai et al. (2005) could not graft transmit '*Ca*. L. asiaticus' it from infected citrus;
- (Deng et al. 2007) reported that symptomatic leaves collected from trees near an infected mandarin orchard and within Guangzhou city were PCR positive but asymptomatic leaves from the same plants were not;
- Deng et al. (2007) concluded that *M. paniculata* is a host of '*Ca*. L. asiaticus', but bacterial titres may be low;
- Zhou et al. (2007) transmitted the disease from *M. paniculata* to citrus via dodder;
- Manjunath et al. (2008) recorded PCR-positive nymphs;
- Lopes (2006) and Lopes et al. (2005) reported symptomatic PCR-positive trees in Brazil.

To the best of our knowledge, based on research at UWS by Nguyen Huy Chung (an ACIAR John Allwright Fellow), all forms of *M. paniculata* studied or mentioned by the cited authors were M. paniculata var. exotica.

The presence of antibacterial alkaloids and proteins in species of *Clausena* (Chakraborty et al. 1995ab, Begum et al. 2006) and Bergera (Ramsewak et al. 1999, Rahman & Gray 2005, Shivkanya et al. 2009, Ningappa 2010) within the Clauseneae, and *Murraya* (El-Sakhawy et al. 1998) within the Aurantieae suggests the possibility that these, and other, Citrus relatives may contain phloem translocated substances that may be toxic to liberibacters. The phytochemistry of species *of Clausena*, *Bergera* and *Murraya,* and the seemingly transient nature of liberibacter infections in *Murraya*, suggests that these plants may have developed chemical defence responses to counter bacterial infections, including phloem-limited bacteria. If so, such responses would influence our understanding of possible co-evolution of liberibacters, their psyllid vectors and host plants. Beattie & Barkley (2009) speculated that co-evolution of liberibacters may have occurred in Africa with species of *Vepris* [Rutoideae] and *T. erytreae* but at this point there is no evidence, other than recent detection at the University of Western Sydney of '*Ca*. L. asiaticus' in *M. paniculata* that may be related to contamination of *Murraya* DNA samples in laboratories, that these bacteria have co-evolved with species of *Diaphorina* and Aurantioideae in Asia.

Guava interplants in Indonesia

Populations of *D. citri*, citrus leafminer and brown citrus aphid differed in the three treatments: Siem mandarin alone; interplanted with seeded red-fleshed guava ('Jakarta'); and interplanted with seedless white-fleshed guava ('Sukun'). No psyllids were observed from April to October 2007, but infestations rose rapidly in the control in December 2007. At this point, *D. citri* populations were about 60% and 90% lower in the 'Jakarta' and 'Sukun' treatments, respectively, than in the control, and lowest in the 'Sukun' treatment. Populations from January to April 2008 were markedly lower in the 'Sukun' treatment than in the other two treatments. Thereafter, until May 2009, populations in all treatments were lower than from November 2007 to April 2008. Even so, populations in the guava treatments were still markedly lower than in the control in January and February 2009. From April 2007 to May 2009, infestations in the 'Sukun' and 'Jakarta' treatments were on average 81% and 46% lower, respectively, than in the control.

Citrus leafminer was present from March 2007 and more common at the end of the year: average infestations in the guava treatments in 2007 were > 50% lower than in the control, and lowest in the 'Jakarta' treatment. However, from January 2008 to May 2009, infestations were some 40% lower in the control than in the two guava treatments. Aphid populations rose slowly until August 2007, then relatively rapidly: average infestations in the guava treatments from August to December 2007 were > 50% lower than in the control, and lowest in the 'Sukun' treatment.

Symptoms of HLB appeared in mid 2008, and the incidence of PCR+ trees rose slowly in the guava treatments to reach 3.3% and 8.3% of trees in the 'Sukun' and 'Jakarta' treatments, respectively, in February 2009. Infections rose more rapidly in the control, reaching 26.6% in February 2009. By May 2009, the 10%, 15% and 43.3% of trees were PCR+ in the 'Sukun', 'Jakarta' and control treatments, respectively.

Despite the small size of the replicates, these results clearly support reports from Việt Nam about the impact of guava interplants on ingress of *D. citri* and HLB into orchards (see above). Impacts may have been greater in larger-scale experiments.

The results also suggest that the volatile profiles and/or concentrations of different guava cultivars vary, and that some varieties will have greater impact on the behaviour of *D. citri* than others. Identification of volatiles that have both direct (repellent) and indirect (reduced attractiveness of citrus) effects on *D. citri* should be a key focus of future research, and, as noted above, any commercialisation of products or strategies arising from this research should acknowledge the initial role of Vietnamese farmers.

Germplasm tolerance to HLB and *D. citri* in Việt Nam

Natural severity of HLB symptoms in citrus species, varieties and relatives in the Mekong Delta. In surveys undertaken from 2006 to 2008, rankings of citrus species, varieties and relatives for their susceptibility to '*Ca*. L. asiaticus' were ++++ for sweet mandarin, Tieu mandarin and King orange, +++ for sweet and Soan oranges, ++ for lime (*C.* × *aurantiifolia*), Eureka lemon and lemon, +/++ for Namroi, Daxanth, Long and Banhue pomeloes, and Sanh mandarin, and + for calamondin. Data were not obtained for Sen orange, Con lemon, Tuong lemon and sour orange. Observations are continuing.

Evaluation of *Limonia acidissima* and *Feroniella lucida* as rootstocks. PCR tests did not detect '*Ca*. L. asiaticus' in any of the pomelo and orange scions grafted onto *L. acidissima* 12 months after the plants were exposed to natural *D. citri*-transmitted infection in either

Vinh Long or Tien Jiang. In January 2008, clear differences were evident in the Tien Giang trial comparing between *L. acidissima*, *F. lucida* and volkameriana as rootstocks for King orange. The 2year-old trees on volkameriana were > 2 m tall, thinly conical (even though widely spaced) and HLB symptoms were not readily noticeable during a brief visit. Scions on *L. acidissima* were relatively small, about 1 m tall, with severe mottling. Those on *F. lucida* were stunted, < 500 mm tall, with severe mottling and vein corking, and with the symptomless-rootstock growing. These observations suggested that severity of the disease was related to the rootstock-scion combinations, and to rate of tree growth and rate of spread of the pathogen.

Psyllid transmission of HLB to sweet orange and 4 citrus relatives. The pathogen was only transmitted by *D. citri* from sweet orange to sweet orange. These plants were PCR-positive 60 days and 75 days after infected psyllids were caged with them. Infected adults did not transmit '*Ca*. L. asiaticus' from sweet orange to *T. trifolia*, *L. acidissima* and *F. lucida* during the experiment.

HLB tolerance of scions on volkameriana rootsocks following graft transmission of HLB. '*Ca*. L. asiaticus' was detected in scions of 8 of the 11 citrus species, varieties or relatives 80, 120, 180 and 240 days after HLB-infected sweet orange buds were grafted onto the scions: in sweet, Tieu and Sanh mandarins, Tuong lemon, sweet orange, Namroi pomelo, and calamondin. The pathogen was not detected in *L. acidissima* or *F. lucida* scions 80, 120, 180 and 240 days afterwards, or in trifoliate orange scions after 80 and 120 days (samples were not taken on days 180 and 240 because leaves were absent).

Observations in Pakistan, Bhutan and India

In Pakistan in July (mid summer) 2006, *D. citri* adults and eggs were abundant on lemon trees (*C.* × *limon*) citrus trees observed in the Malakand Valley (circa 34° 34′ N, 71° 56′ E, 600 m asl). The ambient temperature was 47°C (50°C the day before) and such temperatures are common in this region for 3 to 4 months each year. The infestations of ACP were the highest observed by the project leader during extensive travel in Southeast Asia since 1979. The abundance of the psyllid under such conditions led to revision of current models that indicated relatively poor survival in the hottest citrus growing regions of Australia. The observations suggested that survival of the psyllid under such conditions with high saturation deficits is related to evaporative cooling of leaves and of psyllid nymphs and adults, providing trees have access to sufficient soil moisture. The observations led to research funded by the Department of Innovation, Industry, Science and Research (DIISR) in China to investigate the impact of monsoon rains and leaf temperatures on seasonal titres of '*Ca*. L. asiaticus' in leaves and adult psyllids, and mortality of psyllid eggs and nymphs.

Citrus seedlings in an open-air nursery near Sargodha (32° 04′ N, 72° 40′ E, 185 m asl) in the Punjab were covered by a species of dodder (*Cuscuta* sp.), species of which have been used to transmit '*Ca*. L. asiaticus' in laboratory studies. Budwood was being sourced from HLB-infected mother trees in an adjacent mature grove. These observations suggested that dodder transmission of plant pathogens may occur naturally.

In Bhutan in May 2009, *D. citri* and HLB was observed at higher altitudes (up to 1400 m asl) than in Java during the project. This was attributed to higher saturation deficits at high altitudes (800 to 1500 m) in Bhutan than in Java. Ambient temperatures at the altitudes visited were up to 36°C and humidity was low. During the visit another psyllid, *D. communis*, was observed feeding on HLB-infested citrus at Baychu (27° 18′ N, 89° 58′ E, 763 m asl), Kamichu (27° 16′ N, 90° 02′ E, 637 m asl) and at Lower Suntaley (27° 02′ N, 90° 06′ E, 950 m asl) in Tsirang, generally on trees in close proximity to curry leaf, B. koenigii. It was more common on curry leaf than on citrus. Adults were collected to determine if they harboured '*Ca*. L. asiaticus'.

In the Garo Hills of Meghalaya in northeast India in October 2009, psyllid nymphs and adults (4 females) that were initially assumed to be *P. murrayi* were collected from a pomelo tree at Dura Sakalgre (25° 30′ N, 90° 17′ E, 1190 m asl). The adults were subsequently identified as possibly *Cacopsylla* (syn. *Psylla*) *citrisuga* (Yang & Li), a species known from citrus in Yunnan (Yang & Li 1984), by Dr Daniel Burckhardt (Naturhistorisches Museum, Basel, Switzerland). This record, and a comments made by Lahiri & Biswas (1980), suggest that the psyllid studied by Lahiri & Biswas (1980) may have been *Ca*. *citrisuga*, not *P. murrayi*. The climate of Dura Sakalgre and similar localities in Meghalaya is humid. This suggests that the psyllid is adapted to low saturation deficits. *D. citri*, which is adapted to high saturation deficits, was not observed above 400 m, and although the visit was short, the environment suggested that it would not occur at altitudes higher than 500 m asl. HLB was not obvious at the higher altitudes but thorough surveys should be undertaken. Powdery mildew appeared to be the main disease limiting production above 500 m. Seedlings of the rare *Citrus indica* were being propagated under open conditions at Dura Selbalgri (25° 35′ N, 90° 17′ E, 485 m asl) for planting at higher altitudes. Such propagation could lead to infection by D. citri-transmitted '*Ca*. L. asiaticus' and subsequent spread by *Ca*. *citrisuga* but it is not known if *Ca*. *citrisuga* can transmit the pathogen.

Diaphorina communis on mandarin, though more common on curry leaf, Kamichu, Bhutan, 24 May 2009 (GAC Beattie)

8 Impacts

8.1 Scientific impacts – now and in 5 years

The incursion management plan is widely regarded a valuable source of information about HLB and its vectors, and strategies to deal with incursions. It will remain so if it is revised annually.

The project helped to resolve inaccuracies in host records for HLB, *D. citri* and *T. erytreae*. The assumed origins of *Citrus* (India and Southeast Asia) and HLB (China) were questioned. Australasia was proposed as the region where *Citrus* originated, and the Indian subcontinent as the possible origin of the Asiatic form of HLB. These assumptions will almost certainly be tested over the next 5 years.

Strategies based on sole use of insecticides for control of HLB and *D. citri* were shown to be ineffective: advances in insecticide chemistry will not improve these strategies.

Field studies in the Mekong Delta and in Indonesia provided supporting evidence for the impact of guava interplants on rates of ingress of HLB and *D. citri* into orchards. Participation in conferences led to world-wide interest in the possible impact of guava volatiles on *D. citri*. Work in China showed that guava volatiles repel the psyllid and three patents on use of slow-release formulations of volatiles are pending in the United Sates of America. Guava interplanting is being promoted in the Mekong Delta and should be promoted in Indonesia and elsewhere in Southeast Asia, where feasible.

The potential for applications of fertilisers to prolong life of HLB-infected trees was demonstrated In Indonesia. It may be useful strategy to increase yields in situations where area-wide control involving removal of infected trees cannot be achieved.

The project verified that the incidence of HLB and *D. citri* declines with increasing altitude. This suggests that impacts of HLB on citriculture in some regions of Southeast Asia would be reduced by moving production to higher altitudes.

Conclusions related to the impact of HLB on *Citrus* and possible 'resistance' of some *Citrus* relatives to HLB led us to consider the possibility of integrating traits from these relatives into *Citrus* in order to minimise multiplication of liberibacters in *Citrus*. The existence of the traits needs to be confirmed and the feasibility of using them determined.

8.2 Capacity impacts – now and in 5 years

The incursion management plan has greatly enhanced Australia's capacity to deal with HLB and its vectors. It is being is used overseas as a reference document by research and extension personnel. This use will increase when it is placed on the world-wide-web in mid 2010, and if revised annually, it will continue to be a valuable reference document for several years. Although Australia does not have HLB or its vectors, it is acknowledged as a world-leader in expertise related to the disease and the insects. Maintenance of this expertise will require support from governments and relevant institutions.

Seven scientists were trained at Universitas Gadjah Mada in Indonesia, and one Vietnamese scientist is being trained in Australia. In addition to HLB, capacity was also increased in entomology, plant pathology, botany, and plant nutrition. It is hope these young scientists, will contribute to on-going capacity building in Indonesia through universities and other institutions.

Research collaboration with Indonesia, Việt Nam, Pakistan, Malaysia, China, Japan, the United States of America and Brazil was strengthened, and should continue.

Capacity of scientists to recognise *Citrus* and *Citrus* relatives Indonesia and Việt Nam was enhanced. The project contributed to efforts to revise names of Rutaceae in Việt Nam and Indonesia and to reduce ambiguity and inaccuracies stemming from use of invalid names.

Capacity to increase citrus yields in Việt Nam was enhanced through publicity about the use of guava interplants to slow ingress of HLB into orchards. The project site at Cai Be was used for some of this publicity, but the credit for initial observations and reports belongs to Vietnamese farmers and researchers. Use of guava interplants should spread more widely in the next 5 years through funding (US $3.6 million) by the Japan International Cooperation Agency (JICA) to enhance pomelo production and use of guava interplants in southern Việt Nam. Given promising results in Indonesia towards the end of the project, it may be possible with additional data and support for technology transfer, to promote the technology for adoption by Indonesia farmers. Trials on the use of guava interplants commenced in China in 2007 and work has been conducted in China on the impact of guava volatiles. In the United States three, patents are pending on the use of guava volatiles in slow release formulations.

Use of mineral oils in Việt Nam was enhanced during the project through publication of two books and links with oil companies. This outcome was not achieved in Indonesia because mineral oils are (surprisingly) not registered there. Implementation of biorational management practices is encumbered by over-reliance and over-emphasis on the use of synthetic pesticides.

Capacity building for farmers during the project, and during preceding projects, has not been effectively improved management of HLB (though reasonable and increasing success has been achieved for general integrated control of citrus pests in China and Việt Nam). Top down approaches through seminars and field days have limited scope for training large numbers of farmers. Farmer field schools tend to be more effective, but they are over-rated and their impacts are still vastly inadequate. In terms of training, the model recently established at the Southern Horticultural Research Institute (SHRI) in southern Việt Nam may prove to be more effective. SHRI has established facilities, including a plant clinic, where researchers, extension personnel but particularly key ('certified') farmers with local knowledge and experience all play a role in training. The facility, with short-term accommodation for 50 farmers, has the capacity to train 100s of farmers annually.

8.3 Community impacts – now and in 5 years

8.3.1 Economic impacts

Economic impacts were not measured during the project. No cure was found for the disease. No cure has been found elsewhere. Excessive and costly use of pesticides does not prevent rapid spread of the disease. Most orchards in Indonesia and in Việt Nam, particularly in the south, succumb to the disease within a few years.

The economic well-being of farmers in the Mekong Delta may be improved over the next five years if guava interplanting with citrus can be successfully promoted by JICA.

This possibility is partially supported by data on yields and income in 2009 from the project's 0.5 ha field site at Cai Be in the Mekong Delta where king orange trees were planted between guava trees in 2004. During a visit to the site in June 2009, the owner, Mr Le Van Bay, said he had recently harvested 25 t of fruit from the block. He sold the fruit for VND 24,000/kg (US $1.40/kg: in 2008 prices were lower: VND 14,000 to VND 19,000). All fruit had not been picked. Income was also derived from the sale of guava fruit and guava marcotts from 2004-2008. Comparative data were not obtained from other farms but the incidence of HLB in the orchard was less than 30% in 2009 compared to 100% in nearby farms with no guava interplants.

Given initial results in Central Java, intercropping may benefit farmers in Indonesia by reducing the impact of HLB and providing additional income from the sale of guava. This will require effective technology transfer programs.

For Australia, economic benefits of the project should stem from improved capacity to deals with incursions of the disease and its vectors.

8.3.2 Social impacts

Social impacts were not measured during the project. However, comments by farmers using guava interplants, mineral oils and reduced levels of synthetic pesticides in the Mekong Delta suggested that their exposure to synthetic pesticides had declined markedly and that their health had improved. Such impacts should be more widespread if use of guava interplants is successfully promoted by JICA.

8.3.3 Environmental impacts

The project demonstrated that it is possible to use mineral oils and guava interplants as alternatives to broad-spectrum synthetic pesticides. Use of the latter should, therefore, decline and lead to lower levels of pollution of soil and waterways and lower impacts on biodiversity and human health.

The project raised awareness about the potential impact of HLB on loss of valuable germplasm, and biodiversity some of which is rare and already endangered.

8.4 Communication and dissemination activities

Technology transfer and capacity building

Postgraduate training. Seven project personnel in Indonesia undertook or are completing postgraduate studies at Universitas Gadjah Mada:

- Mr Mofit Eko Poerwanto, PhD, The impact of horticultural and agricultural mineral oils to the feeding and oviposition behaviour of *Diaphorina citri* Kuwayama
- Mr Rachmad Gunadi, PhD, The potential influence of tropical climate in Java on citrus vein phloem degeneration (CVPD) disease and its vector
- Mr Tris Haris Ramadhan, PhD, The distribution of *Diaphorina citri* and its insect natural enemies in Central Java and the potency of coccinellids as biological control agents
- Ms Arlyna Budi Pustika, PhD, Development of huanglongbing disease in relation to plant nutrition
- Ms Inggit Puji Astuti, MSc, The study on taxonomy of *Murraya paniculata* in Java based on morphological and molecular characteristics
- Mr Achmad Himawan, PhD, Penularan '*Candidatus* Liberibacter asiaticus' penyebab CVPD melalui vektor *Diaphorina citri*
- Ms Sri Widinugraheni, MSc, Interactions between catechol, mineral nutrition and huanglongbing in citrus

International events prevented an Australian student being placed at Universitas Gadjah Mada to undertake a PhD on HLB and *D. citri*.

Mr Nguyen Huy Chung, an ACIAR John Allwright Fellow from PPRI, Ha Noi, is undertaking his PhD 'Circumscription of *Murraya* and *Merrillia* (Sapindales: Rutaceae: Aurantioideae) and susceptibility of species and forms to huanglongbing' at the University of Western Sydney.

Meeting, field days and related activities. In 2002, a project planning meeting was held in February in Ha Noi; Dr Beattie also attended a Food & Fertilizer Technology Center workshop on huanglongbing in Vinh in Việt Nam.

In 2003, aspects of the project were incorporated into a UWS AusAID CARD project 'Extension of Citrus IPM in Việt Nam'. Drs Beattie and Holford attended the Sixth International Conference on Plant Protection in the Tropics in Kuala Lumpur, Malaysia, and participated in an Agriculture Forestry Fisheries Australia (AFFA) sponsored international workshop on diagnostic standards for huanglongbing in Kuala Lumpur; and Dr Beattie contributed to an international training course on 'Production of Diseased Free Citrus Nursery Trees in Humid Tropics' at Prince of Songkhla University in Thailand from 4-5 March 2003.

In 2004, a HLB workshop was held as scheduled in Yogyakarta in November; it was attended by project colleagues from Việt Nam, China and Japan.

In 2005, Dr Beattie attended the Fifth Việt Nam National Conference on Entomology in Ha Noi, Việt Nam, Mr Himawan (UGM) attended the 'Second Asian Conference on Plant Pathology', National University of Singapore, several members of the project team attended the 'First International Conference of Crop Security', Brawijaya University, Malang, Indonesia. Drs Paul Holford and Zamir Hossain were plenary speakers. Drs Beattie and Holford contributed to a paper presented at the 'Second International Citrus Canker and Huanglongbing Research Workshop' in Orlando, Florida.

In 2006, Dr Beattie gave seminars at Universitas Gadjah Mada, South China Agricultural University, and at the Southern Fruit Research Institute (now Southern Horticulture Research Institute). He also presented a paper at the International Workshop for Prevention of Citrus Greening Disease in Severely Infested Areas, in Ishigaki, Japan.

In 2007, Dr Beattie was invited speak at the annual University of Florida Citrus Expo in Fort Meyers, Florida, in August. He gave seminars at the University of Florida, Lake Alfred, and at United States Department of Agriculture, Fort Pierce, Florida, in August; to students and staff at the Northwest Agriculture & Forestry University, Yangling, Shanxi, China on 7 November 2007; to postgraduate students at South China Agricultural University. Dr Holford attended a field day on HLB and citriculture with UGM staff and students at Wonotawang in Central Java on 7 July 2007.

Dr Siti Subandiyah addressing villagers at Wonotawang, Central Java, on 7 July 2007 (P Holford)

In 2008, a project review and extension meeting was held at Universitas Gadjah Mada in January. The meeting was attended by UGM staff and students (total about 10), Dr Lily Eng from the Sarawak Department of Agriculture (SDOA), and 39 extension personnel, most from Java, but also from Kalimantan, Sulawesi and Bali. Farmers were also present. A field day was held at Porwokerto in Java in July. In other activities, Dr Beattie was invited by the National Research Council (NRC) of the US National Academy of Sciences to participate in a meeting in April to determine US priorities for research on HLB; he contributed to a paper presented at an 'International Workshop on Citrus Huanglongbing ('*Candidatus* Liberibacter asiaticus') and the Asian Citrus Psyllid (*Diaphorina citri*)' in Mexico in May; He gave a seminar to postgraduate students at South China Agricultural University, attended the Sixth Vietnam Conference on Entomology, 9-10 May 2008, Ha Noi, Việt Nam as an invited speaker; presented a seminar on HLB at ACIAR headquarters in Canberra on 17 December 2008; and he was an invited keynote speaker at an 'International Research Conference on Huanglongbing' in Orlando, Florida, in December. Dr Subandiyah also attended this meeting and presented a poster on project research comparing hosts of *D. citri* and HLB.

Universitas Gadjah Mada students, Ms Arlyna Budi Pustika (PhD) and Ms Sri Widinugraheni (MSc), demonstrating the iodine-starch test for HLB to farmers and extension personnel at Porwokerto, Central Java, on 16 July 2008 (GAC Beattie).

Use of mineral oils. During ACIAR CS2/1993/005 ('Integrated Control of Citrus Pests in China') and CS2/1996/176 ('Integrated Control of Citrus Pests in China and Southeast Asia') mineral oils manufactured by Ampol Limited, Caltex (Australia) Pty Ltd and the SK Corporation were registered in China, Thailand and Việt Nam use in citrus integrated pest management programs. Links with the companies, particularly the SK Corporation, led to increased use of the products in these countries, particularly in China and Việt Nam. Books were published in Chinese and Vietnamese to aid adoption of the technology:

- Rae DJ, Beattie GAC, Nguyen VT, Nguyen VC, Pham VL, Broadbent P. 2003. A Guide for Using Mineral Oils in Vietnamese Citrus IPDM. Ha Noi Agricultural Press, Ha Noi, Việt Nam. 136 pp.
- Rae DJ, Beattie GAC, Nguyen VT, Nguyen VC, Pham VL, Duong AT. 2003. Use of Horticultural and Agricultural Mineral Oils in Citrus IPDM. Ha Noi Agricultural Press, Ha Noi, Việt Nam. 136 pp.

- Huang MD, Rae DJ, Beattie GAC, Ouyang GC, Yang YP, Broadbent P. 2005. Green Technology for Citrus Pest and Disease Control. Guangdong Science & Technology Press: Guangzhou, China.
- Rae DJ, Beattie GAC, Huang MD, Yang YP, Ouyang GC. 2006. Mineral Oils and Their Use: Sustainable Pest Management and Green Agriculture. Guangdong Science & Technology Press, Guangzhou, Guangdong, People's Republic of China. 106 pp.

This success was not achieved in Indonesia where, despite interest stemming from project activities, products have not been registered for use on any crops.

Guava interplants and volatiles. Presentation in Ishigaki, Japan, in 2006, and subsequently in Florida in 2007, about the impact of guava interplants on *D. citri* population and incidence of HLB in orchards Tien Giang in Viet Nam led to widespread interest, and some scepticism, in Asia and the Americas about the impacts. Interplant trials were subsequently established in Indonesia (see this report), China (South China Agricultural University) and Florida (United States Department of Agriculture). Laboratory studies in China have shown the impact of the interplants on the psyllids is partly due to repellent volatiles produced by guava leaves. Studies in Florida led to three patent applications (including the University of Florida and Tropicana Fruit Products) for slow release formulations of guava volatiles, without due collaboration with partners in this project, or acknowledgement of the intellectual property of Vietnamese farmers and researchers.

9 Conclusions and recommendations

9.1 Conclusions

Australia's ability to deal with HLB and its vectors was greatly enhanced through development, with co-funding from Horticulture Australia Limited, of an incursion management plan that included: (a) a thorough review of the literature for use by researchers, extension, biosecurity and quarantine personnel; and (b) review and augmentation of host records for the disease and its vectors. However, if the disease and *D. citri* become established in Australia, the long-term future of the Australian citrus industry will be bleak unless an effective area-wide control can be found that will prevent the disease, or limit its impact to minor status.

The situation in Asia where HLB occurs remains dire. Despite research undertaken as part of the project, and research elsewhere, the major constraint to improving economic returns for citrus growers in Asia is the absence of effective area-wide management programs that we believe should be implemented and enforced by national, provincial and local governments. Unless this is done, the economic well-being of citrus growers in HLB-affected regions in Asia may never improve. Two less acceptable but partially viable alternatives are: (a) rotation cropping for 7-8 years (with harvests over 4-5 years) with minimal inputs, perhaps applications of mineral oils during major flushes and systemic insecticides between major flushes (the latter within withholding periods); or, (b) if feasible, rotation cropping over 12-18 years based on interplanting with guava in the first 3-4 years and use of mineral oils during major flushes and systemic insecticides between them, and within withholding periods. These options must be based on the use of pathogen-free trees produced in insect-proof screenhouses. Production and sale of marcotts and trees produced in open nurseries must be banned.

Urgent action must be taken to prevent loss of wild populations of true species of *Citrus* in Asia and Australasia.

9.2 Recommendations

Management

- For effective management of HLB, governments (national, state/provincial and county) in Asia need to impose mandatory, uniform, area-wide management practices for citrus growers to follow.
- Area-wide management should be based on initial removal of HLB infected plants (*Citrus* and *Citrus* relatives) within the area, strict quarantine, subsequent planting of pathogen-free trees, use of common practices to minimise populations of *D. citr*i, surveying 4 times annually for diseased trees, and immediate removal of diseased trees detected in or between surveys.
- Plants other than guava should be screened for substances that may repel *D. citri*, or reduce the attractiveness of citrus foliage to *D. citri*.
- Repellent volatiles should be identified and evaluated for incorporation in slow-release formulations for distribution in citrus orchards or direct application to citrus trees.
- Optimum planting densities for guava interplants in citrus orchards, and impacts of climate and topography, should be determined in South and Southeast Asia.

- Studies should be undertaken to the impact of saturation deficits and leaf temperatures on seasonal survival of *D. citri* and '*Ca*. L. asiaticus', and include evaluation of strategies to artificially increase leaf temperatures in summer.
- Management programs should be developed in Asia for control of powdery mildew and greasy spot in relation to elevations above sea level where these diseases are severe.
- Mineral oils and sulphur should be registered in Indonesia for control of pests and diseases of citrus and other crops.
- Collaboration with oil companies is required to assist marketing of mineral oils in the region.

Systematics and biogeography

- The systematics and biogeography of species of *Clausena*, *Bergera*, *Murraya* (including *Merrillia*) and *Citrus* and other Malesian, Australian and Pacific Aurantioideae should be resolved.
- The plethora of names (about 1000) for true species of *Citrus* (about 25) and hybrid forms must be correctly typified, synonymised and be reduced to produce a workable system of recognisable taxa with validly published names e.g., all mandarins should be cited as *C. reticulata* with cultivars such as Satsuma mandarin (*C. unshiu* (Mack.) Marc) cited as *C. reticulata* Blanco 'Satsuma', and *C. suhuiensis* Hort. ex Tanaka (Canton mandarin, Shatangju, Sz-wei-kom, Som-keo-wan, Siem, Xiem, Limau langkat) as *C. reticulata* Blanco 'Common Name' or *C*. × *suhuiensis* Hort. ex Tanaka.

Biodiversity and germplasm conservation

- Strategies must be developed to protect Australasian (Australia, New Guinea and New Caledonia) species of *Citrus*, some rare and endangered from potential loss.

Resistance to HLB

- Surveys should be undertaken to determine if liberibacters occur naturally in species of *Clausena*, *Bergera* and *Murraya*.
- The status of species of *Vepris* in Africa as hosts of liberibacters and *T. erytreae* should be determined.
- Species of *Clausena*, *Bergera* and *Murraya* in Asia and Australasia, and *Vepris* in Africa should be screened for antibacterial substances that may be active against liberibacters in phloem.
- Cape chestnut (*Calodendrum capense*) should be screened for antibacterial substances.
- *Citrus* species and cultivars should be screened for the screened for antibacterial substances in young and mature trees.
- Successful production of hybrids between *Citrus* and *Citrus* relatives for the intergression of resistance to HLB.

HLB transmission and host plant interactions

- Studies should be undertaken to determine liberibacters can be transmitted by *Diaphorina communis*, *Psylla murrayi*, *Cacopsylla citrisuga* and *Ca. citricola*: principal and alternative hosts of these species and their distributions should be determined.
- The vascular anatomy of citrus stems and branches should be studied in relation to movement of liberibacters within phloem of young and mature trees.

- Rates of movement of liberibacters, in relation to confirmed presence by PCR, symptom development and impact, in young and mature trees following single and multiple liberibacter infections should be determined.

Other diseases and disease interactions

- The impact of phytophthora root and collar rots caused, diplodia gummosis, and/or pink disease in humid low-lying high-water table orchards in Asia should be determined, compared to the impact of HLB, and effective management programs determined.
- Research is required on the prevalence of citrus viruses, viroids and phytoplasmas, their symptoms, and their interactions with HLB.

Regional collaboration, technology transfer and infrastructure

- Funds should be allocated for annual, regional (Asia and Australasia) meetings to discuss research outcomes and directions, and to foster technology transfer.
- National HLB research, technology transfer and development of quarantine protocols and strategies should be coordinated and funds allocated to permit this to occur.
- Funds should be allocated for annual revision of the Australian incursion management plan for HLB and its vectors.
- Australian governments and agencies should collaborate to ensure that expertise is maintained within Australia to address the threat posed by HLB.
- Regional facilities for mineral analyses should be improved.

Funding of activities

- It is recommended that links be formed between ACIAR and other agencies, institutions and foundations, nationally and internationally, to fund the activities summarised above.

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10.2 List of publications produced by project

Journal publications

Hall DG, Gottwald TR, Nguyen MC, Ichinose K, Le QD, Beattie GAC, Stover E. 2009. Greenhouse investigations on the effect of guava on infestations of Asian citrus psyllid in grapefruit. Proceedings of the Florida State Horticultural Society 121: 104–109.

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11 Appendixes

11.1 Appendix 1:

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