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project

Bogia coconut syndrome and related phytoplasma syndromes in Papua New Guinea: developing biological knowledge and a risk management strategy

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Contents

Acronyms

16Sr	16S ribosomal (a component of the prokaryotic ribosome that binds with the Shine-Dalgarno sequence and that is used to define phytoplasma groups)
ACIAR	Australian Centre for International Agricultural Research
BCS	Bogia Coconut Syndrome
BWAP	Banana Wilt Associated Phytoplasma
CCI	Coconut and Cocoa Institute (later KIK)
CLY	Coconut Lethal Yellowing
CRB	Coconut Rhinoceros Beetle
CSPWD	Cape Saint Paul Wilt Disease
CSU	Charles Sturt University
DNA	Deoxyribonucleic acid
FAFU	Fujian Agriculture and Forestry University
IPDM	Integrated Pest and Disease Management
KIK	Kokonas Indastri Koporesen (formally CCI)
LAMP	Loop-mediated isothermal amplification
LYD	Lethal Yellowing Disease
NAQIA	National Agriculture Quarantine and Inspection Authority
NARI	National Agricultural Research Institute
NBPOL	New Britain Palm Oil Limited
PCR	Polymerase chain reaction
PNG	Papua New Guinea
PNG OPRA	Papua New Guinea Oil Palm Research Association
UQ	University of Queensland
USQ	University of Southern Queensland

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1 Executive summary

Lethal yellowing diseases are a global threat to coconut production and the communities that rely on them. Most knowledge about lethal yellowing diseases, such as Bogia Coconut Syndrome (BCS) (and the very closely related Banana Wilt Associated Phytoplasma (BWAP)) has only recently become known as the technology to detect the pathogens has advanced. Phytoplasmas are hosted by both plants and insects, and use each host to multiply and spread. Managing phytoplasma diseases therefore requires knowledge of the host plant range and insect species that can vector the pathogen.

This project has detected the DNA of BCS/BWAP in multiple plant and insect species with cage tests proving a phytoplasma etiology. Several thousand insect and plant samples were tested for phytoplasma with a combination of nested PCR and LAMP and about 170 sequences have been obtained showing high sequence similarity to BCS/BWAP.

The LAMP assays developed during the project were unable to discriminate between BCS and BWAP phytoplasma DNA because these are genetically so similar. Accordingly, two LAMP primers were used; one specifically for BCS/BWAP and a second for phytoplasmas in general. Integrating the results from both tests for a given sample indicates whether it is positive for BCS/BWAP, positive for other phytoplasmas, positive for both, or negative for both. The present results are reported as positives or negatives for detection of BCS/BWAP DNA. Nested PCR followed by sequencing were performed to confirm LAMP results. During surveys, detections of DNA of BCS/BWAP were recorded from coconut, banana, betel nut, taro and small numbers of individuals of other plant species.

Insects sampled from BCS-affected areas were dissected and DNA extracted from the heads was subjected to LAMP assays to test for the presence of phytoplasma DNA. Whilst a range of taxa were consistently negative, members of several other taxa commonly gave positives: all were members of the families Ricaniidae, Flatidae, Derbidae or Lophopidae. More detailed studies complemented assays of the heads of insects. These used assays of saliva collected from individual live insects. The results from this component of the study led to a prioritised list of species that were considered most likely to be vectors: *Lophops saccharicida* and *Zophiuma pupillata* (Lophopidae), an unidentified Ricaniidae species, *Taparella amata* and *Colgar* sp. (Flatidae), an unidentified Zoraidini species (Derbidae). These were selected for further study based on the incidence of positive BCS/BWAP detections in their heads and saliva combined with their incidence on BCS/BWAP affected sites. Each putative vector was used as a treatment in a randomized, replicated cage trial for which hundreds of field-collected individuals of each taxon were introduced into the assigned cages over a prolonged period. During this time, oil palm, coconut, betel nut, Cavendish banana and Kalapua cooking banana were growing as single plants within each cage, these plant species constituted sub-plot treatments within each main plot insect species treatment. Plants were subsequently sampled for LAMP assays. Results indicate that two species of Hemiptera are competent vectors: *L. saccharicida* and *Z. pupillata.* BCS/BWAP DNA was detected in coconut, banana (Cavendish and Kalapua), betel nut and oil palm but inferring that all of these crop species are

hosts of BCS/BWAP under field conditions on the basis of cage trial results alone is not reliable.

Work on the spread of BCS focused on testing the possibility that the pathogen was seed borne. Studies of dissected coconut drupes (nuts) led to the detection of phytoplasma in various tissues including the embryo. A further study was conducted in which 50 coconut and 60 betel nut drupes were collected from BCS-symptomatic trees. These propagules were placed into insect-proof cages and treated repeatedly with insecticide and fungicide until the plants germinated. Leaf samples were then taken from the germinating seedlings and subjected to molecular assays. Nested PCR, LAMP and sequencing results showed evidence of possible vertical (seed) transmission in both coconut and betel nut. Because the fundamental and applied implications of this finding are major, confirmatory studies must be a priority.

The present body of results represents a significant advance in our understanding of key aspects of the biology of BCS and related phytoplasmas. One paper on vectors has already been published in an open access journal and others are in preparation. This new information has been integrated with a comprehensive synthesis of the literature which itself was published in an open access journal. All this work was presented at a stakeholder workshop in early 2020 and the recommendations from that workshop were integrated with the project's findings to generate nine recommendations for management and containment measures. Key features of the recommendations are: (i) the re-establishment of a check point on the highway to prevent spread of BCS out of Madang Province in the short-term; (ii) well-coordinated surveillance that will provide information on disease spread and allow prompt removal and destruction of symptomatic plants in BCS-affected areas in the medium-term; and (iii) further research investment that will build on new knowledge established by this project and facilitate a wider range of control options (e.g. resistant varieties) in the longer-term.

2 Background

Bogia Coconut Syndrome (BCS) is a plant disease that has caused severe losses to coconut palms in Bogia, Sumkar and Madang districts of Madang Province of Papua New Guinea (PNG). Following reports of dying palms that date back to the 1990s, molecular testing in 2008 of palm samples from the Bogia region established the presence of a phytoplasma closely related to a group of phytoplasmas (16SrIV) that is associated with Coconut Lethal Yellowing (CLY) (Kelly et al., 2011). Coconut growing areas around the world have been repeatedly devastated by CLY diseases since the early 1900s (Kelly et al., 2011;Gurr et al., 2016). This was the first time a CLY disease has been found in the Oceania region and concerns were raised for the PNG coconut industry. Previous outbreaks of this disease in other parts of the world caused widespread death of millions of coconut and other closely related palm trees (Eziashi and Omamor, 2010).

Concurrent to the early reports of BCS in the Madang region, surveys by plant pathologists from PNG and Australia revealed yellowing symptoms in bananas. Initial testing was for fungal pathogens but in 2008, a sample tested positive for the presence of a phytoplasma. In response, PNG's National Agriculture Quarantine and Inspection Authority (NAQIA) and Australia's Department of Agriculture, Fisheries and Forestry (DAFF) carried out a banana survey across the Madang, Morobe, Milne Bay, East Sepik, Western and North Solomons Provinces in 2008 and 2009. Samples from yellowing and dying banana plants showed a phytoplasma, which was named Banana Wilt Associated Phytoplasma (BWAP), but was found to be genetically similar to the BCS-associated phytoplasma (Davis et al., 2012).

Symptoms of BCS start with premature nut fall, followed by leaf yellowing and collapse of the crown leading to the death of the plant. The disease spreads rapidly to neighbouring palms. Kelly et al. (2011) cited farmer reports of over 5,000 palms being lost in the Bogia district. By 2020, drone surveillance of palm plantations in Madang Province revealed that BCS had spread with a preliminary estimate of 190,000 palms killed. Although bananas with BWAP infection have been found in Madang and other PNG provinces, BCS has only been detected in Madang Province leading to the conclusion that, although they are closely related, they are separate pathogens, and speculation that BCS is a mutation of BWAP.

Coconut provides a staple food and serves as a cash crop in many developing countries; copra (coconut "meat") being one of the few sources of cash income for many households (Bourke and Harwood, 2009). Coconut is not only a major source of food but coconut vegetation is also used in building houses (Figure 1) and the coconut shells are used for common household use such as bowls and fuel (Ovasuru, 1994). Coconut is a very important crop for the mainland coastal and island regions of PNG with exports of copra and coconut oil bringing in US$27 million and US$43 million per year. In many developing countries, such as PNG, the ability to produce a cash crop for domestic and overseas export markets is closely linked with relieving rural poverty and aiding development (Bourke and Harwood, 2009). In the Madang Province, most of the population (290,000) rely on coconut as a source of food and income. Many people from the highlands region drive to Madang to buy coconuts from local village growers. A shortage of coconut

in this area has meant that the highlanders have to drive further and pay more to source this staple food.

Figure 1. Coconut is used for building material as well as being an important food commodity. Dead trees such as in this image are a common sight around Bogia and Madang.

Early responses to the discovery of BCS included the establishment of a highway check point, managed by the NAQIA, preventing the movement of coconuts that had not been de-hulled as well as other vegetative material such as banana seedlings from the Madang Province. An extensive awareness program was also initiated in the affected areas.

Internationally, there are many phytoplasmas associated with multiple of diseases affecting wild and cultivated plants (Musetti and Pagliari, 2019). Phytoplasmas can be transmitted by insects but it is well known that the conclusive identification of phytoplasma vector species is a difficult undertaking. An ACIAR-funded pilot study (PC/2011/056) surveyed various plant and insect species and detected phytoplasma in some tissue samples but did not provide evidence of vector capacity, confirm causality of a phytoplasma, or define the range of host plants affected.

To fill remaining knowledge gaps, ACIAR commissioned the present project with a multi-national team to undertake biological studies to develop a clearer understanding of BCS and related phytoplasmas. The results of these studies, including BCS host range and vector identification, would provide the scientific basis for recommendations on risk management.

3 Objectives

The specific objectives of the work were to:

1) **Develop an understanding of the biology of BCS**
   i) Establishing the range of crop and non-crop plant species affected by BCS.
   ii) Testing whether the phytoplasma associated with BCS is the causal agent.
   iii) Determining which insect species are capable of vectoring BCS.
   iv) Assessing the types of plant material in which BCS can be spread.
   v) Undertake a whole genome analysis of BCS and BWAP (this objective was added late in the project).

2) **Develop a management strategy to address the BCS problem in PNG**. Integrating the biological knowledge with information from the wider literature and from workers in the field to generate a best practice strategy for BCS.

4 Methodology

Where was the work done?

Plant and insect sampling were carried out in Madang Province in areas that had been affected by BCS including Siar, Mobdu and Bogia from 2016 to 2018 (Figure 2). Transmission experiments were conducted at Siar and Mobdu close to Madang from 2018 to 2019. Seed transmission experiments were conducted in 2017-2018 at Stewart Research Station close to Madang. In addition to plant and insects sampled from the Madang Province, later in the project (2019) additional material (banana and insects) was obtained from the Markham Valley for whole genome sequencing work.

All plant DNA was extracted at the Dr Ghodake Biotechnology Centre at NARI in Bubia (Morobe Province) and insect DNA extracted at PNG OPRA Plant Pathology Laboratory at the Dami Oil Palm Research Station (West New Britain Province) before being sent for molecular analysis in Australia (USQ) or China (FAFU). DNA was extracted from plant samples and insect heads using the DNeasy Mini Plant kit (Qiagen, Australia). Whole genome sequencing preparatory work was performed in Australia at USQ.

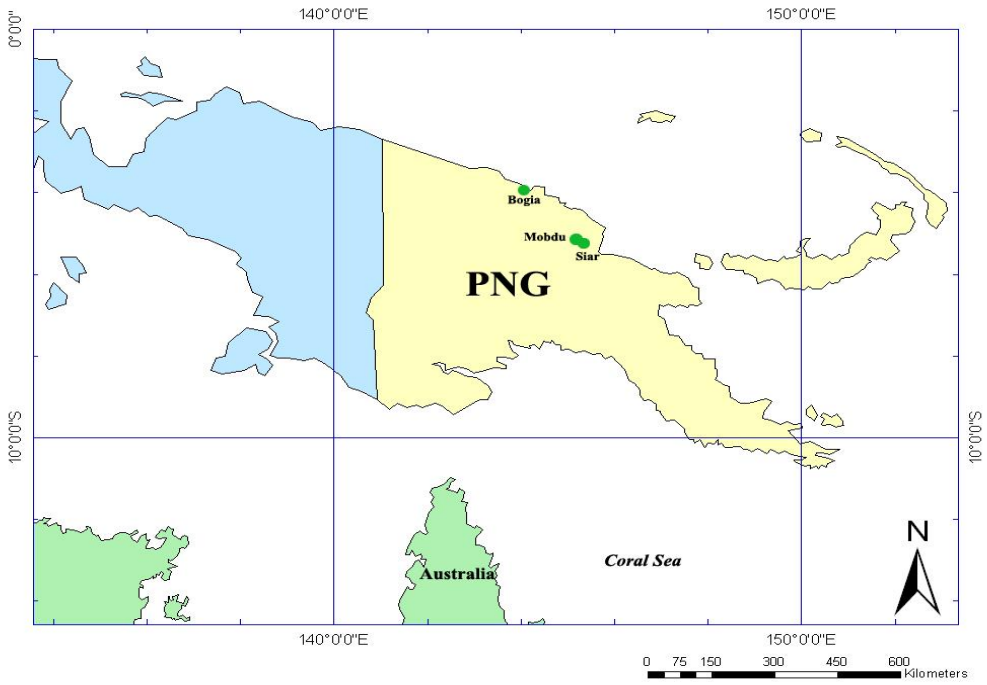

Figure 2. Bogia, Mobdu and Siar in the Madang region of PNG.

Detecting and identifying the BCS phytoplasma

Two Loop Mediated Isothermal Amplification (LAMP) assays were designed, one to detect phytoplasmas in general (Phyto) and a second more specific primer set (OptiBCS) that could detect DNA of BCS/BWAP, aimed at excluding other phytoplasmas. Accordingly, the present results are reported as positives or negatives for detection of BCS/BWAP.

Primers for the LAMP assay were designed based on the partial sequence of the 16S ribosomal RNA gene region of coconut (*Cocos nucifera*) tissue testing positive for BCS (GenBank sequence KP053907.1, 1808 bp). DNA extracted phytoplasma samples were obtained from Richard Davis at NAQS, Cairns, Australia. These served as both positive (BCS) and negative controls (for example DNA from affected plants showing witches broom, lethal yellowing, little leaf) for the PCR and LAMP assays. The positive control was DNA originated from diseased coconut palm tissue, which was collected in Dugumar Village, Bogia District, Papua New Guinea in June 2008.

LAMP Designer 1.13 (Premier Biosoft International) was used to design the 6 primers consisting of the forward outer (F3), backward outer (B3), forward inner (FIP), backward inner (BIP) and the forward and reverse loop (Loop F and Loop B, respectively). Primers were synthesized by GeneWorks Pty Ltd, Australia or Macrogen, Korea and are detailed in Table 1.

The LAMP assays were continuously optimised (manipulating volumes of DNA and primers) throughout the project and were performed in duplicate or triplicate where necessary.

Table 1. Primers used for the detection of phytoplasma DNA by LAMP.

Primer	Sequence (5'-3')
Phyto^ F3	CGCCACATTAGTTAGTTGGTA
Phyto B3	TTCATCGAATAGCGTCAAGG
Phyto FIP	GTTTGGGCCGTGTCTCAGTGCCTACCAAGACGATGATG
Phyto BIP	TACGGGAGGCAGCAGTAGGAGTACTTCATCGTTCACGC
Phyto LoopF	GTGGCTGTTCAACCTCTCA
Phyto LoopB	AACTCTGACCGAGCAACG
OptiBCS* F3	GTAGCCTAACTACGCAAGTAG
OptiBCS B3	TCCTTCATCGGCTCTTAGT
OptiBCS FIP	CTTAGAAAGGAGGTGATCCATCCCGATCCGTCTAAGGTAGGGT
OptiBCS BIP	TTAGAGCACACGCCTGATAAGCCGAGTACCTTATGCTGGTG
OptiBCS LoopF	GGATACCTTGTTACGACTTAACC
OptiBCS LoopB	GTCGGTGGTTCAAGTCCAT

^'Phyto' refers to the generic LAMP primer set designed to amplify many phytoplasma groups

*'OptiBCS' refers to the optimised LAMP primer set designed amplify only BCS/BWAP

A nested PCR protocol was used on samples to verify LAMP results using the

generic phytoplasma primers below. Nested PCR was performed in duplicate or triplicate. PCR (1st round) used primers R16mF2 CATGCAAGTCGAACGGA / R16mR1 CTTAACCCCAATCATCGAC and (2nd round) used the primers R16F2n GAAACGACTGCTAAGACTGG / R16R2TGACGGGCGGTGTGTACAAACCCCG as described in Lu et al. (2016).

Good laboratory practice was used for the extraction of DNA from plant and insect tissue. All equipment (spatulas, mortar and pestles) were cleaned thoroughly by scrubbing them in water and with 70% ethanol and paper towel. Likewise for the nested PCR and LAMP, all samples were tested using good laboratory practice in a sterile laminar flow cabinet. Nested PCR positive products were sent externally for sequencing.

Identifying the host plant range for the BCS phytoplasma

Sampling in 2015 focused on crop plants with typical yellowing symptoms but also included non-symptomatic plants growing close to symptomatic plants in order to detect plant species that may be potential 'silent hosts', which could also serve as controls for the molecular tests (Table 2). Fifty samples were collected in 2015 from Mobdu, Siar, Kelebabal, Baroidig, Stewart Research Station (Murunas), Malala Secondary, and the coconut Genebank. Samples from coconut were taken by drilling into the trunk (Figure 3) and by taking a tissue sample from the meristem. Other plants were sampled by taking part of or whole plants. A larger round of sampling was conducted in 2017-2018 (ca. 200 samples) from Bogia, Mobdu and Siar and in 2019, another 100 samples were collected from Mobdu, Kananam and the Stewart Research Station (Table 2). The tools used to sample plant tissue were cleaned thoroughly with 70% ethanol and paper towel between samples to avoid cross contamination.

Figure 3. Palm sampling using an electric drill to collect 'wood dust'.

Table 2. Plants collected for the host range study.

Common name	Species name	No. samples by year				Total plants sampled
		2015	2017	2018	2019	
African tulip	*Spathodea campanulata*		1	1	2	4
Aibika	*Abelmoschus manihot*			2	2	4
Asthma weed	*Euphorbia hirta*				1	1
Banana Cavendish	*Musa* sp.	8	3	2	18	31
Banana Daru	*Musa* sp.		4	5		9
Banana Kalapua/cooking	*Musa* sp.		4	2		6
Banana Yawa	*Musa* sp.		4	3		7
Banana Wild	*Musa* sp.		1	1		2
Bean	Unknown			1		1
Betel nut	*Areca catechu*	5	2	5	4	16
Cassava	*Manihot esculenta*		4	4	4	12
Cherry	Unknown				1	1
Cocoa	*Theobroma cacao*		1	4	4	9
Coconut	*Cocos nucifera*	28	2	4	13	47
Creeper vine	Unknown		1			1
Croton	*Croton* sp.		2	2		4
Daka	*Piper betel*		2	2	2	6
Fern	Unknown		2	1		3
Ginger	*Zingiber* sp.		2	3		5
Goats weed	*Ageratum conyzoides*		1		2	3
Hibiscus	*Hibiscus* sp.		1			1
Kunai grass	*Imperata kunai*		1	2	2	5
Limbum palm	Unknown		2	2	2	6
Mami (lesser yam)	*Dioscorea esculenta*		4			4
Marita pandanus	*Pandanus conoideus*	1				1
Milk weed	*Euphorbia heterophylla*		1	1	2	4
Mimosa	*Mimosa pigra*+		2	2	2	6
Oil palm	*Elaeis guineensis*		1	1	1	3
Pandanus palm	*Pandanus* sp.		2		2	4
Passionfruit wild	*Passiflora* sp.		2	1	1	4
Pawpaw	*Carica papaya*		4	2	1	7
Peperomia	*Peperomia* sp.				1	1
Phyllan tree	*Phyllocladus* sp.+				1	1
Pitpit	*Saccharum edulae*		4	3	2	9

Common name	Species name	No. samples by year				Total plants sampled
		2015	2017	2018	2019	
Rat's tail grass	*Sporobolus* sp.+			1		1
Sago	*Metroxylon sagu*		2	2	2	6
Snake weed	Unknown				1	1
Sneeze weed	*Helenium autumnale*				1	1
Sugarcane	*Saccharum officinarum*		2	3	4	9
Sweetpotato	*Ipomoea batatas*		5	10	4	19
Arrowleaf elephant ear	*Xanthosoma sagittifolium*	2	2			4
Taro	*Colocasia esculenta*	9	4	10	6	29
Thurston grass	*Urochloa* sp.		2			2
Yam	*Dioscorea alata*			3	8	11
Yam African	*Dioscorea* sp.			1		1
White head weed	Unknown				1	1

+ Denotes tentative identification.

Identifying the vector species for BCS phytoplasma

Vector transmission is the most important route for phytoplasma dispersion (Rashidi et al., 2014). Despite a great deal of work, the vectors of many phytoplasmas have not been identified (Weintraub and Beanland, 2006). Transmission tests of LYD vectors are especially difficult, involving caging mature palms during the incubation period and capturing thousands of insects that have been previously exposed to phytoplasma infected palms (Howard et al., 1983). Though conventional vector transmission trials for LYD in palms have been carried out since the 1960s, very few have been successful (Tsai, 1980; Eden-Green, 1995; Bila et al., 2019), so knowledge of vectors is incomplete and tentative for the vast majority of phytoplasma pathosystems. Reflecting the logistical challenges involved in transmission tests, another form of vector testing was adopted that involves using a sucrose-based feeding medium. The vector is held in a small vessel and can access the feeding medium by piercing a Parafilm® barrier to access the medium held in a second compartment (Figure 4). Later, the medium can be tested for phytoplasma DNA. This shows that the insect is producing the phytoplasma in saliva and able to transmit it when feeding so is strongly suggestive of a capacity to transmit the pathogen when feeding on a plant host (Tanne et al., 2001).

A range of methods was used to sample insects that included sweep netting, aerial traps suspended from palm trees, and digging into soil and leaf litter. Insects were collected from a wide range of plants in BCS-affected areas: mile a minute vine (*Mikania micrantha*), *Imperata* spp., coconut, banana, betel nut, a local dicot shrub snake diwai, valangur (a local cultivated herb), African tulip, and cocoa.

The insect heads were subject to LAMP assays (to test for phytoplasma in the

salivary glands and exclude the possibility of false positives arising from phytoplasma DNA in the gut from feeding on infected tissue). A Fujian Agriculture and Forestry University-based master student (Hengyu Lu) spent one month in Madang in early 2015 and again in 2016. He worked on field collections and experiments in which suspected vector insects fed on a sucrose solution inside the cap of a 1.5 ml tube, contained by thinly stretched Parafilm® through which the hemipteran could insert its stylet. These solutions were subsequently assayed for the presence of phytoplasma DNA using LAMP at USQ in Australia and nested PCR at FAFU in China to determine which hemipteran species injected phytoplasma during feeding. The full version of the methodology is described in Lu et al. (2016).

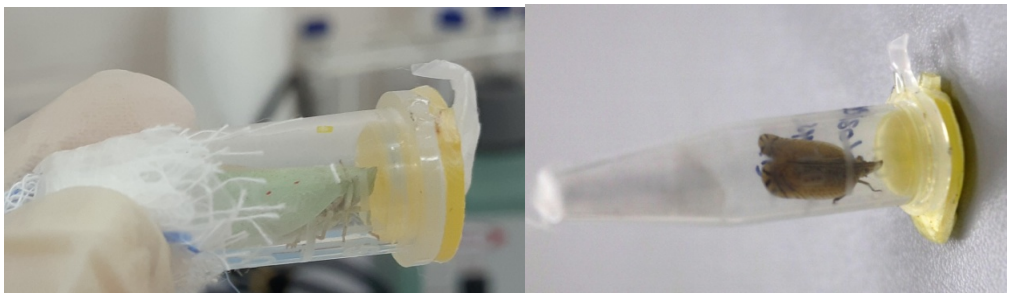

Figure 4. Live Hemiptera adults in apparatus designed to collect saliva from insects for molecular testing to assess potential vector competency.

Confirm the causality of BCS and assess the efficacy of vector species

Large (2m^3) cages for transmission experiments were constructed at Siar (10 cages) and Mobdu (15 cages) villages close to Madang (Figure 5). The cages were constructed using a fine, insect proof mesh to contain suspected vector insects on test plants in single species treatments (Table 3). The land around the cages was cleared of vegetation and treated with insecticides.

Figure 5. Cages used for testing competency of putative vector species of BCS in

PNG.

Table 3. Treatments for cage trials to determine vectors of BCS.

Treatment	Treatment details	No. of replicates	Notes
T1	Control	2	No insects. Karate® insecticide
T2	*Lophops saccharicida*	3	-
T3	*Zophiuma pupillata*	3	-
T4	*Zoraidini* sp.	3	-
T5	*Taparella amata*	3	-
T6	*Colgar* sp.	3	-
T7	Ricaniidae	3	-
Total cages		20	

The prioritised list of insect species was selected based on the incidence of positive BCS detections in their heads and saliva samples, combined with their incidence on BCS affected sites. These were: *Lophops saccharicida* and *Zophiuma pupillata* (Lophopidae), an unidentified Ricaniidae species, *Taparella amata* and an unidentified *Colgar* species (Flatidae), and an unidentified *Zoraidini* species (Derbidae) (Figure 6).

Lophops saccharicida

Zophiuma pupillata

Zoraidini sp.

Taparella amata

Colgar sp.

Unidentified Ricaniidae species

Figure 6. Putative vectors of Bogia Coconut Syndrome.

Field-collected candidate vectors were introduced into the treatment cages over a six-month-long period during which oil palm, coconut, betel nut, Cavendish banana and cooking (Kalapua) banana were grown as single plants within each cage

(Figure 5). The test plant seedlings were raised in a shade house at the Stewart Research Station (an area thought to be free of BCS). Plants in cages were sampled for BCS after being placed in the cages prior to the introduction of vectors, to exclude the possibility of existing phytoplasma infection. Plants were left to acclimatize for a period of two weeks before insect releases commenced. Individual insects were collected from symptomatic and non-symptomatic plant species in the area immediately around the cage site at Mobdu where BCS affected coconut and BWAP affected bananas were common. Initial attempts were made to rear insects in a large breeding cage and subsequently inoculating them with the BCS phytoplasma by allowing them to feed on infected planting material before release into the treatment cages. This method, however, proved too stressful for the insects resulting in more deaths than releases. Accordingly, field-collected insects were directly released into the cages. This was done with the knowledge that rates of phytoplasma detection within the population of all these species were relatively high, exceeding 10% for all taxa. Over the six-month period, at least 542 adults were released into each treatment with a total of 6,407 insects released (Table 4).

Table 4. Numbers of insects released into cage test treatments.

Cage No.	Insect Species	Apr-17	May-17	Jun-17	Jul-17	Aug-17	Sep-17	Oct-17	Totals	Species total
1	Control	0	0	0	0	0	0	0	0	
13	Control	0	0	0	0	0	0	0	0	**0**
5	*Zoraidini* sp.	9	23	74	55	97	112	76	437	
14	*Zoraidini* sp.	9	23	94	55	97	108	88	465	**1364**
18	*Zoraidini* sp.	6	20	94	55	97	108	88	462	
8	*T. amata*	2	7	68	43	79	89	69	355	
12	*T. amata*	2	7	48	39	79	89	69	331	**1007**
17	*T. amata*	2	6	40	38	79	89	69	321	
3	*Colgar* sp.	18	14	58	41	71	93	68	345	
10	*Colgar* sp.	9	14	35	38	71	99	68	325	**994**
16	*Colgar* sp.	10	12	35	38	71	99	69	324	
6	*L. saccharicida*	8	22	66	56	75	108	89	416	
9	*L. saccharicida*	8	22	52	54	75	108	88	399	**1221**
11	*L. saccharicida*	8	22	63	54	75	104	88	406	
2	Ricaniid	8	22	112	59	69	108	72	442	
4	Ricaniid	8	22	113	57	69	108	72	441	**1279**
20	Ricaniid	8	22	73	54	67	108	72	396	
7	*Z. pupillata*	8	22	80	29	32	21	13	197	
15	*Z. pupillata*	8	24	59	22	31	21	13	170	**542**

Cage No.	Insect Species	Apr-17	May-17	Jun-17	Jul-17	Aug-17	Sep-17	Oct-17	Totals	Species total
19	*Z. pupillata*	8	22	58	29	32	21	13	175	

Control cages received no insects and were treated with lambda-cyhalothrin (Karate®) insecticide to kill any insects that may have been accidently introduced to the cage as sampling was undertaken. Visual assessment of BCS symptoms commenced shortly after the first batches insects were released in June of 2018. Visual symptoms were first observed a few months from the first insect release in coconuts and cooking bananas (in two of the *Zophiuma pupillata* cages, despite this treatment receiving the lowest numbers of insects). Plant tissues were sampled on two occasions, one in the first months of BCS symptom observations and another at the end of the year.

Vertical (seed) transmission of BCS

In India, root wilt disease in coconut, a 16SrXI group phytoplasma, has been found in coconut embryos (Manimekalai et al., 2014) and positive detection of 16SrIV lethal yellowing phytoplasma was reported in plantlets through *in vitro* germination of zygotic embryos from the drupes of infected coconut palms in Mexico (Oropeza et al., 2017).

Figure 7: Initial dissection of coconut drupes prior to collection of tissue samples.

To determine if BCS was potentially spread by seed, 30 coconut and 30 betel nut drupes were collected from the infected coconut palms and infected betel nut palms growing in three BCS-affected sites at Mobdu, Siar and Bogia (10 drupes per site) in Madang Province in PNG. The coconut drupe was dissected into five parts: husk, shell, flesh, embryo and juice and the betel nut drupe into two parts: husk and flesh (Figure 7). The tools used to dissect and sub-sample each plant part were cleaned thoroughly with 70% ethanol and paper towel within and between samples to avoid cross contamination. The plant samples from each drupe were placed individually into 10 ml labelled vials containing 100% ethanol

and transported on ice to the laboratory. Genomic DNA was extracted from the plant tissue and samples were tested for the presence of phytoplasma by nested PCR. Good laboratory practice was adhered to for the extraction of DNA from plant tissue. All equipment (spatulas, mortar and pestles) were cleaned thoroughly by scrubbing them in water, followed by wiping with 70% ethanol using paper towel. Likewise for the nested PCR and LAMP, all samples were tested using good laboratory practice in a sterile laminar flow cabinet using calibrated pipettes and filter tips. Nested PCR positive products were sent externally for sequencing.

Further collections of 39 coconut and 60 betel nut drupes were made from the field. Coconuts were from BCS-symptomatic trees in five BCS hotspots with an additional group collected from non-symptomatic palms (cv. Karkar Tall) located at the coconut Genebank, which was believed to be free of BCS at the time. Betel nut drupes were collected from two symptomatic palms at the Mobdu cage trial site where BCS symptoms were common in coconut. Drupes were placed in insect proof cages (Figure 8) and treated with fungicide and insecticide. Leaf and petiole samples were taken after six months, the DNA extracted and sent for nested PCR, LAMP testing and sequencing.

Figure 8. Preparation of coconut drupes for placement in cages to test for seed transmission.

Undertake a whole genome analysis of BCS and BWAP

DNA was extracted from symptomatic banana and coconut plants using the DNeasy mini and maxi plant kits (Qiagen, Australia). DNA was extracted from insect-fed sucrose solutions and insect heads, after insects had fed on banana pseudostem or coconut meristem tissue. Banana tissue was specifically sampled from the Markham Valley area in the Morobe Province, away from coconuts, to avoid the potential of cross contamination between BWAP in banana and BCS in coconut. Approximately 80 samples were sent to Australia to test for BCS/BWAP, as well testing the quality and quantity of DNA. Many samples from insect feeding (*Z. pupillata*) were pooled before further analysis because the volume and

concentration of DNA was so low. Samples for genome work were tested for BCS/BWAP using LAMP, nested PCR and the presence of the phytoplasma was confirmed with sequencing of the amplified gene region.

DNA from banana, coconut and betel nut was concentrated and purified and sent for whole genome sequencing in collaboration with the University of Queensland (UQ). Long-read sequencing using Oxford Nanopore Technologies' PromethION technology will be used to acquire data for genome assembly.

Who was involved in the work?

The project involved personnel representing PNG Oil Palm Research Organisation (PNGOPRA), Cocoa and Coconut Institute (CCI) (later to become Kokonas Indastri Koporesen (KIK)), National Agricultural Research Institute (NARI), National Agriculture and Quarantine Inspection Authority (NAQIA), and New Britain Palm Oil Limited (NBPOL). With the exception of Sharon Woruba of CCI who was funded by ACIAR to work full time on the project for its duration and Hengyu Lu who was funded by Fujian Agriculture and Forestry University to conduct a two-year master's project, all personnel contributed fractions of their time (<20%) to project activities. Molecular diagnostics was conducted by Dr Bree Wilson at USQ supervised by Prof Gavin Ash. Project administration and research assistance from Mrs Anne Johnson, CSU. The project was led by Professor Geoff Gurr through the Graham Centre of Agricultural Innovation at CSU. Hengyu Lu was supervised by Profs Geoff Gurr and Guang Yang (FAFU) with support from Prof Gavin Ash and Bree Wilson.

5 Achievements against activities and outputs/milestones

Objective 1: Develop an understanding of the biology of BCS so that key, practical issues are adequately understood.

no.	activity	outputs/ milestones	completion date	comments
1.1	Identify the range of plant species affected by BCS.	Output: Technical report on results from molecular assays (A). Milestone: Field sampling completed (PC).	30 Nov 2019	Two LAMP assays were developed: one to detect phytoplasmas in general and a second more specific assay to detect BCS/BWAP. Plants positive for BCS/BWAP were coconut, betel nut, banana, taro and very small numbers of other plants including sweetpotato, cocoa, milk weed and *Imperata* sp. (NB see also section 1.3). Technical report provided as Appendix 1 (but see main text for updated results).
1.2	Identify the insect species capable of vectoring BCS phytoplasma.	Output: Technical report on results from molecular assays (A). Milestone: Field sampling completed (PC).	30 Nov 2019	Multiple Hemiptera species sampled from BCS affected areas had detectable BCS/BWAP DNA in their head tissues. Six species were shortlisted as likely vectors (because phytoplasma DNA was detectable in their saliva) and used in further tests (see section 1.3). Technical report provided as Appendix 1 (but see main text for updated results).
1.3	Confirm the causality (rather than current 'association') of the coconut lethal yellowing phytoplasma in BCS and to definitively assess the efficacy of each putative vector species.	Output: Technical report on results from molecular assays (A). Milestone: Field experiment completed (PC – based at CCI).	30 Nov 2019	A cage test showed that *Zophiuma pupillata* and *Lophops saccharicida* were competent vectors; leading to phytoplasma DNA detection in plant tissue samples from palms exposed to these Hemiptera. Technical report provided as Appendix 1 (but see main text for updated results).
1.4	Determine mode/s of spread for BCS.	Output: Technical report on results from vector dispersal and plant material experiments (A). Milestone: Field studies completed (PC – based at CCI).	30 Nov 2019	This work provided evidence that BCS/BWAP can be vertically transmitted (seed borne) in coconut and betel nut. Technical report provided as Appendix 1 (but see main text for updated results).

no.	activity	outputs/ milestones	completion date	comments
1.5	Undertake a whole genome analysis of BCS and BWAP.	Understanding of the evolutionary and taxonomic relationship between BCS and BWAP and does this account for the ore limited geographical extent of BCS compared with BWAP? (A)	30 Nov 2019	This work commenced but technical and logistical challenges prevented it being completed. It is being pursued by USQ in collaboration with UQ and results will be published.

PC = partner country, A = Australia

Objective 2: Develop a management strategy to address the BCS problem in PNG.

no.	Activity	outputs/ milestones	completion date	comments
2.1	Conduct a review of the available scientific and applied literature on the control of similar plant diseases worldwide.	Output: Published review article integrating and synthesising information from all project activities (A).	1 March 2016	Published in an open access journal: Gurr GM, Johnson AC, Ash GJ, Wilson BAL, Ero MM, Pilotti CA, Dewhurst CF, You MS (2016) Coconut Lethal Yellowing Diseases: A Phytoplasma Threat to Palms of Global Economic and Social Significance. *Front Plant Sci* 7 (1521). doi:10.3389/fpls.2016.01521
2.2	Synthesise the results of the project with information from the literature review and prepare a detailed technical report with recommendations for BCS and BWAP.	Milestone: Technical report available (PC).	28 February 2020	A technical committee meeting at Madang in 2020 brought together a range of stakeholders with the project team to discuss results and implications. The present report incorporates the views of that group along with the advice from ACIAR-appointed project reviewers. Workshop report provided as Appendix 2.

PC = partner country, A = Australia

6 Key results and discussion

6.1 Identify the range of plant species affected by BCS.

In 2015, detections of DNA of BCS/BWAP were recorded from coconut tissues (wood dust, meristem, flower and inflorescence sheath) as well as in samples of banana, taro and betel nut (Figure 9). Testing used nested PCR (Figure 10), LAMP (Figure 11) and sequencing. The LAMP assay is semi-quantitative and in the example in Figure 11, sample 371 (lighter blue) has less phytoplasma than the other samples and the control (red). For banana, seven of eight samples were positive for BCS/BWAP for Mobdu and Siar, as were seven of the nine taro samples (Figure 12). One sample of betel nut meristem (from Mobdu) and one sample of betel nut wood dust (from Kelebabal) was positive for BCS/BWAP.

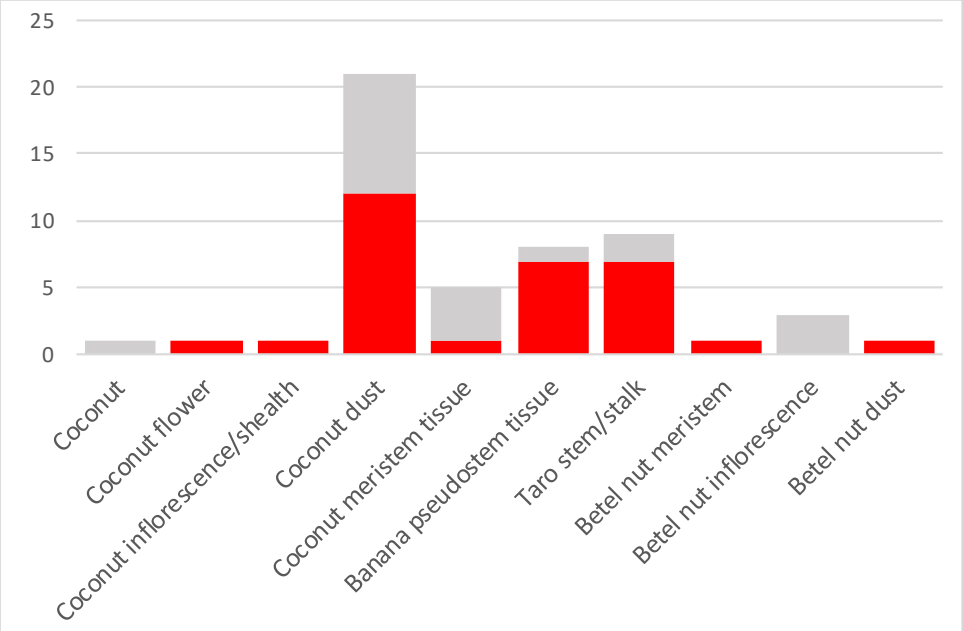

Figure 9. Numbers of coconut, banana, taro and betel nut plant samples infected with BCS/BWAP (in red) or phytoplasma free (grey).

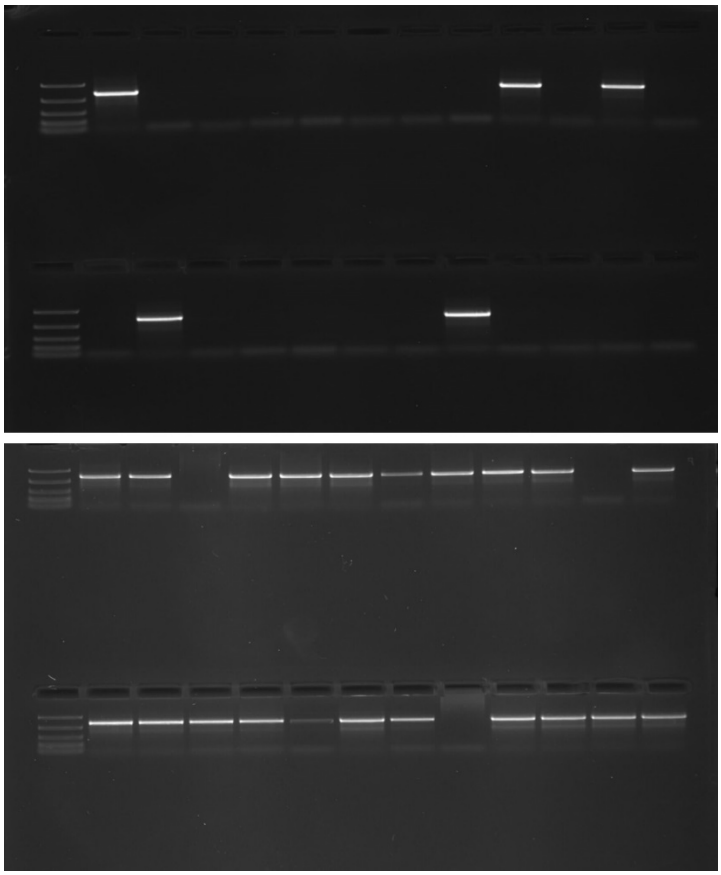

Figure 10. Example of nested PCR with primers R16F2n/R2 for the detection of phytoplasma (expected 1200 bp product) in mixed plant samples. The first column on each gel is the molecular marker.

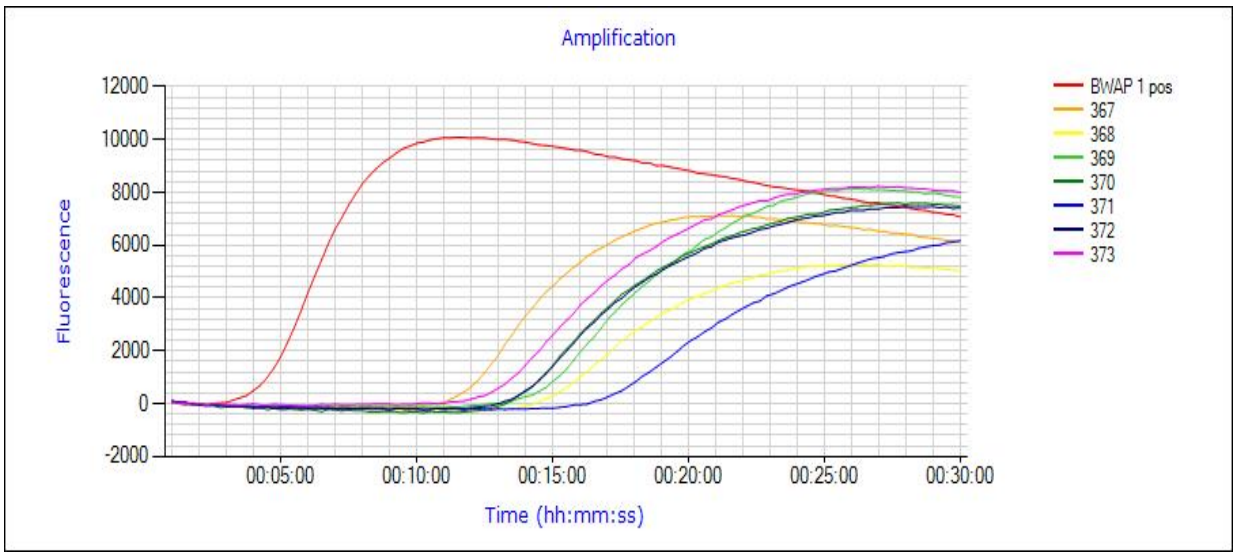

Figure 11. Example of LAMP assay for the detection of phytoplasma in banana samples.

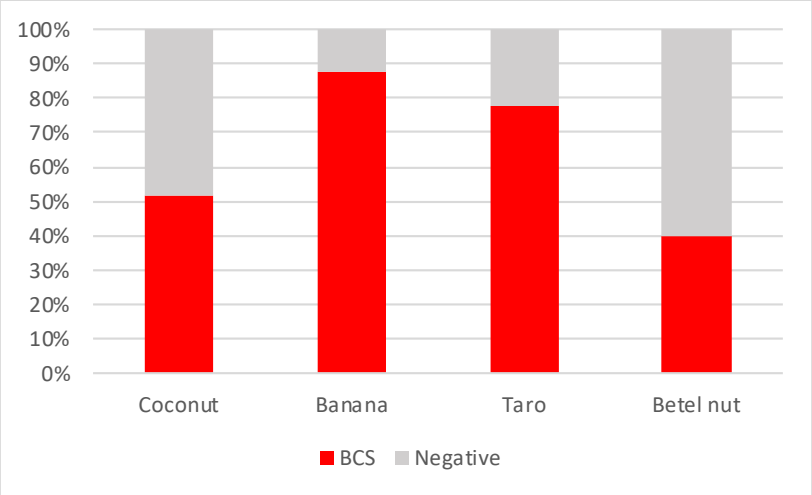

Figure 12. Percentage detection of BCS/BWAP (in red) or phytoplasma free (grey) in crop plants in initial survey in 2015 (total = 50 plants).

Additional plant surveying was performed during 2017-2018 in Bogia (Bom Kalawa), Mobdu and Siar. Samples collected in Siar in 2017 yielded only 3 positives for BCS/BWAP: petioles from two cooking bananas and one Daru banana. In Mobdu (2017), positive BCS/BWAP detections were from a wider range of plant species (Table 5). In 2018, two plants sampled from Bom Kalawa tested positive for BCS/BWAP: a petiole sample from symptomatic native, wild banana and a sample from the dust of a symptomatic coconut palm

Table 5. Mobdu collected plants and their tissue types that yielded positive results for BCS/BWAP.

Plant	No. positive/total plants sampled for that species	Tissue type
Cooking banana	2/3	Petiole
Banana Daru	1/2	Petiole
Banana Yawa	1/3	Petiole
Banana wild	1/1	Petiole
Coconut dust	1/1	Dust (stage 4 palm)
Imperata kunai	1/1	Young leaf
Sweetpotato	1/3	Shoot
Taro	1/2	Petiole

In the final plant survey, another 100 samples were collected. Of these, 35 tested positive for BCS/BWAP with nested PCR. However, LAMP and nested PCR results were not always in consensus for this set of samples (Table 6). It should be noted that the concentration and quality of DNA in these samples was lower and poorer than in previous samples obtained during the project, potentially caused by a delay during transit. Towards the end of the project, efforts concentrated on

avoiding potential false negatives. Accordingly, many rounds of PCR were completed with varying concentrations of DNA. For many of these samples an equivalent depth of optimisation was not completed for LAMP and further optimisation may reveal a consensus between all results with nested PCR and LAMP.

Table 6. Plant detections of BCS/BWAP using nested PCR and LAMP in 2019 plant samples

Common name	Nested PCR	LAMP (BCS or 'Other'*)
Sweetpotato	+	-
Betel nut	+	+
Cocoa	+	other
African tulip	+	-
Milk weed	+	-
Pandanus	+	other
Yam	+	+
Cherry	+	-
Sneeze weed	+	-
White head weed	+	-
Sago	-	+ and other
Aibika	-	+

*In these samples, a different melt curve was observed in the LAMP assay

Positive results for nested PCR confirmed the presence of BCS/BWAP in several plant hosts. At the time of the final report, sequencing results were available for all samples except for aibika, cherry, milk weed, sago, sneeze weed and yam. New samples of these plants were requested from PNG as the existing tubes were exhausted from earlier LAMP testing (which revealed detection of BCS/BWAP). However, this DNA was only received into Australia on the 26/5/2020 due to the COVID-19 pandemic. Nested PCR is being repeated for these samples and sequencing data will be reported in a later publication.

Available sequencing results all showed very high similarity (>99.8%) to BCS/BWAP accessions (for example GenBank accession LC228757.1, BCS isolated from coconut in Siar or GenBank accession KP053901 BWAP isolated from banana in Madang). The sample sizes of some of these plant species was small so it is not clear how common phytoplasma infection is in plants such as sweetpotato, yam, cocoa, and African tulip. Additional sampling and molecular analysis is required to better understand the full host range of BCS/BWAP. It is unlikely that the BCS/BWAP positives in these plants were due to contamination during plant sampling: strict hygiene measures were adhered to in all processing steps. If there was contamination amongst samples, either in the field or subsequently in the laboratory, much higher numbers of positives would be expected with patterns of positives more random across species and sites. Plants that consistently return negative results for BCS/BWAP may play a role in BCS

management by serving as non-hosts to grow around areas of susceptible plants, or for replanting efforts in areas where coconuts have been killed by BCS.

6.2 Identify the insect species capable of vectoring BCS phytoplasma.

In 2015, approximately 175 insects (Flatidae, Derbidae, Lophopidae and other unidentified insects) were collected in BCS-affected areas and were used to test the first set of LAMP generic phytoplasma primers designed. Of these insects, approximately 78% tested positive for BCS/BWAP with LAMP and nested PCR and showed high sequence similarity to BCS/BWAP accessions isolated from coconut in Madang (GenBank accession KP053907).

After the initial work above, also in 2015, more detailed studies were conducted with assays of saliva collected from individual live insects and this led to a prioritised list of species that were considered most likely to be vectors (Table 7). These were: *Lophops saccharicida* and *Zophiuma pupillata* (Lophopidae), an unidentified Ricaniidae species, *Taparella amata* and *Colgar* sp. (Flatidae), an unidentified *Zoraidini* species (Derbidae). Nested PCR, LAMP and sequencing was used to test the samples and the insects were ranked as BCS vectors based on proportional rate of phytoplasma DNA in feeding media and insect head tissues (Table 8).

Table 7. Numbers of Hemiptera taxa collected from BCS-affected sites in Madang Province in PNG. Published in Lu et al. (2016)

Taxon	Site			Total
	Mobdu	Siar	Bogia	
Lophopidae				
Zophiuma pupillata	17	5	36	58
Lophops saccharicida (adult)	38	2	0	40
Lophops saccharicida (nymph)	18	5	2	25
Derbidae				
Unidentified Zoraidini species	70	24	40	134
Unidentified Derbidae: Derbinae: Cenchreini	20	0	0	0
Ricaniidae				
Unidentified Ricaniidae species 1	46	5	4	55
Unidentified Ricaniidae species 2	1	2	0	3
Flatidae				
Taparella amata (Walker)	21	86	0	107
Colgar sp.	73	46	11	130
Unidentified Flatidae species 1	0	0	50	50
Cicadellidae				
Unidentified Cicadellidae species 1	4	0	0	4
Paramesodes sp.	0	0	1	1

In 2016, a further 1,600 insects were sampled from BCS-affected areas (Figures

13 and 14) and the heads were subject to LAMP assays to test for the presence of phytoplasma DNA. Two primers were used: a generic set for screening (to be sure that no phytoplasmas were missed) and the specific set designed to exclude phytoplasmas other than BCS/BWAP. Whilst a range of taxa were consistently negative, members of several hemipteran families commonly gave positives including Ricaniidae, Flatidae, Derbidae and the Lophopidae species *Zophiuma pupillata* and *Lophops saccharicida*.

Table 8. Insect taxa ranked in descending order for likelihood as BCS vectors based on proportional rate of phytoplasma DNA in feeding media and insect head tissues. Published in Lu et al. (2016)

Insect taxon	Number tested	Proportion positive insects (no. of positive in heads)	Proportion positive in the feeding medium of positive insects (n)
L. saccharicida (adult+ nymph total)	65	0.231 (15)	0.333 (5)
L. saccharicida (nymph)	25	0.200 (5)	0.600 (3)
L. saccharicida (adult)	40	0.250 (10)	0.200 (2)
Z. pupillata	58	0.534 (31)	0.290 (9)
Unidentified Ricaniidae	55	0.145 (8)	0.250 (2)
Taparella amata	107	0.187 (20)	0.200 (4)
Zoraidini sp.	134	0.224 (30)	0.133 (4)
Colgar sp.	130	0.077 (10)	0.100 (1)
Unidentified Flatidae sp. 1	50	0.420 (21)	0 (0)
Unidentified Ricaniidae sp. 2	3	0.667 (2)	0 (0)
Unidentified Derbinae: Cenchreini	20	0 (0)	—
Unidentified Cicadellidae species 1	4	0 (0)	—
Paramesodes sp.	1	0 (0)	—
Chi square (for n, excluding taxa with zero positives)		64.890	4.266
P (for n, excluding taxa with zero positives)		<0.001	0.512

The presence of BCS/BWAP was confirmed with nested PCR, and insect head samples showed >98-99.8% similarity to BCS/BWAP accessions (for example GenBank accession LC228757.1, BCS isolated from coconut in Siar or GenBank accession KP053901 BWAP isolated from banana in Madang).

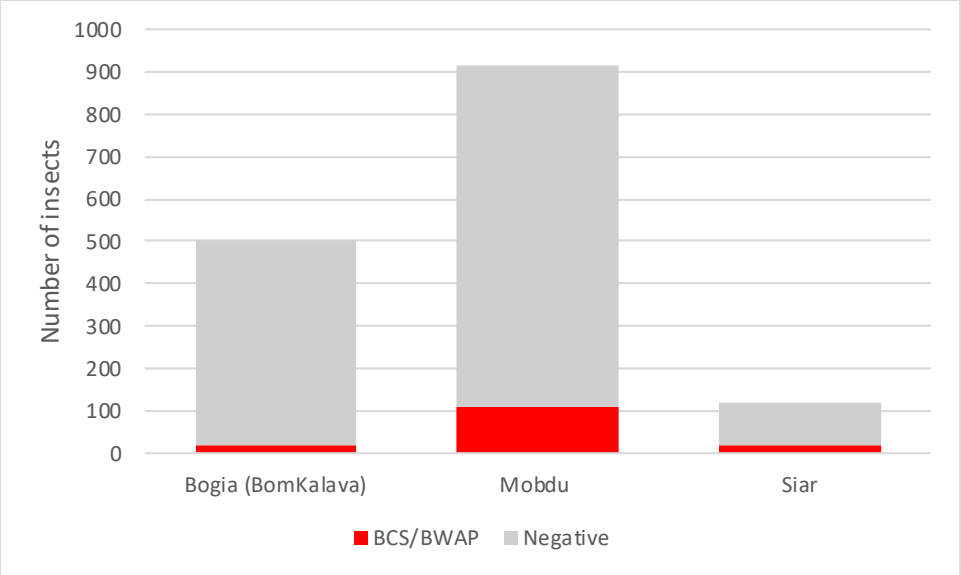

Figure 13. Initial study of DNA in the excised heads of insect samples collected from field sites in PNG. Incidence of LAMP assay detections of BCS/BWAP (red) and phytoplasma negative samples (grey)

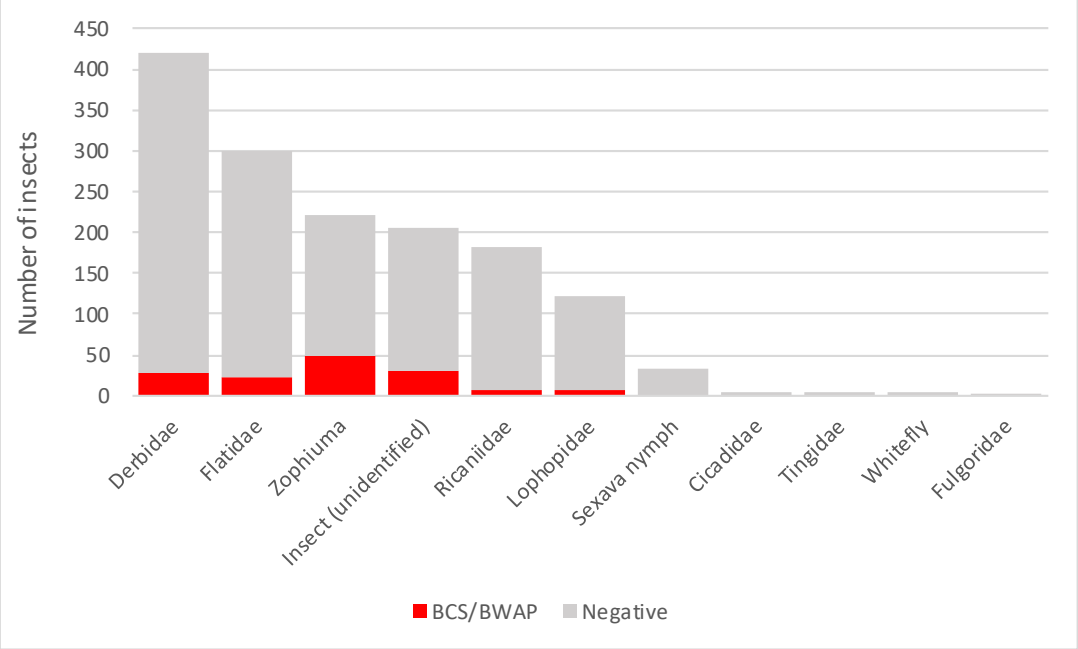

Figure 14. Initial study of incidence of LAMP assay detections of BCS/BWAP (red) DNA in the excised heads of insect samples collected from field sites in PNG broken down by insect taxa across all sites. (The 'insect' category contains unidentified taxa).

6.3 Confirm the causality of BCS and to definitively assess the efficacy of each putative vector species.

The putative vector species prioritised as described above were used as treatments in a randomized, replicated cage trial. In this work, multiple, field-collected individuals of each taxon were introduced into the appropriate cages over a prolonged period during which oil palm, coconut, betel nut, Cavendish banana and a cooking banana were grown as single plants within each cage. These plant species constituted sub-plot treatments within each main plot insect species. Plants were subsequently sampled for molecular analyses.

LAMP, nested PCR and sequencing was used to examine 231 samples taken from the young palms in mid-2018 for evidence of phytoplasma. Plants in only 4 cages tested positive for BCS/BWAP: two cages treated with *Lophops saccharicida* and two cages treated with *Zophiuma pupillata*. No other phytoplasmas (or bacteria) were detected in sequence analyses of the various samples including those from the control cages. In all four cages detailed below (2 x *L. saccharicida* and 2 x *Z. pupillata*) and two other cages (1 x *Colgar* sp. and 1x Ricaniidae species) the coconut plants had previously died and were therefore not sampled.

***Box 1: Lophops saccharicida* treatment**

In one cage, one Cavendish banana leaf sample showed very high sequence similarity (99.72%) to BWAP accession KP642659 isolated from banana in Debepari PNG. In the same cage, one coconut sample (unopened leaf) showed high similarity 98.96% to BCS/BWAP: for example, LC228757, BCS isolated from coconut in Siar and GenBank accession KP053901 BWAP isolated from banana in Madang. Again in the same cage, three betel nut and two oil palm samples were positive for LAMP and nested PCR. Although sequencing results are pending, it is likely that these samples are also BCS/BWAP. In another *L. saccharicida* treatment cage, three oil palm samples (unopened leaf and petioles) showed 99.81% similarity to BCS/BWAP: for example, LC228757 and KP053901.

***Box 2: Zophiuma pupillata* treatment**

In one of these cages, three Cavendish banana samples (petiole, leaf and unopened leaf) showed high sequence similarity (99.71%) to accessions LC228757 and KP053901. In the other cage, two samples of Cavendish banana and one Kalapua banana showed high 99.69% similarity to BCS/BWAP: for example, LC228757 and KP053901.

The foregoing results (Box 1, Box 2) show that among the insect species selected as the most likely vectors of BCS, *Z. pupillata* and *L. saccharicida* are competent vectors.

Cage samples were collected on a second occasion in late 2018 and these also underwent LAMP, nested PCR and sequencing. Compared to the first sampling, there was a greater number of positive samples across cages and treatments (Table 9). There was a consensus with LAMP and nested PCR in the majority of cases. Whilst these results are useful for indicating which plant species became positive for phytoplasmas, the fact that positives were recorded from the control cages (11 out of a total 25 samples) indicates that this round of samples cannot be used to infer vector status of the insect species used as the experimental treatments. Rather, it is likely that infections resulted from 'wild' insects feeding from the *outside* of the cages on expanded leaves/fronds that had grown to the extent that they were in contact with the mesh. This would explain the positives from the control treatment but this effect could also have applied to the other treatments in which insects were placed inside cages. The cage study took place in an area known to be affected by BCS and many plants in the vicinity were expressing phytoplasma infection symptoms. In contrast to this problem with the second (later) round of sampling, the absence of positives in control cages in the first round of sampling, when plants were younger, and the leaves/fronds smaller, indicates that those earlier results on vector status are valid.

The detection of BCS/BWAP DNA in oil palm needs to be interpreted with a caveat before this can be considered a host plant. Cage test conditions inevitably led to unnatural conditions these may have affected vector behaviour and plant susceptibility. It is important to note that oil palm samples were not positive in the field survey of BCS-affected areas. Furthermore, no symptoms of BCS were evident among oil palm plants.

Table 9. Summary of BCS/BWAP detected across cages for the second sampling date, from which potential host range of phytoplasma but not vector competency can be inferred (see text for detail).

Treatment	Plant samples with BCS/BWAP	% positive within treatment	Total number of samples analysed	Nearest GenBank accession and % similarity
Flatid *Colgar* sp.	All	56	36	>99% similarity to BCS LC22857
Flatid *Taparella amata*	All except oil palm	45	33	As above*
Derbid	All	45	40	As above
Zophiuma	Cavendish banana, betel nut, oil palm	39	23	As above^
Lophops	Oil palm, both bananas, betel nut	38	29	As above
Ricannids	All except Kalapua banana	34	35	As above
Control	All	*44*	25	>99% similarity to BCS LC22857 and BWAP KP642659

*Two samples in cage 8 (*Taparella amata*): one coconut and one Kalapua banana showed sequence similarity to an uncultured bacterium.
^one coconut sample in cage 15 (*Zophiuma*) showed sequence similarity to an uncultured bacterium.

6.4 Determine mode of spread for BCS.

Initial studies of dissected coconut drupes (nuts) led to the detection of phytoplasma in various component tissues including the embryo (Table 10). No phytoplasma was detected in 30 samples of betel nut but assays detected phytoplasma DNA in all the coconut plant parts other than the juice.

Table 10. Results Trial 1: Detection of BCS phytoplasma in different tissues of infected seeds collected from PNG using nested PCR.

Tissue	Incidence of BCS/BWAP DNA detection (Proportion in brackets)			
	Mobdu	**Siar**	**Bogia**	**Total**
Coconut husk	4/10	3/10	3/10	10/30 (0.333)
Coconut shell	1/10	3/10	1/10	5/30 (0.167)
Coconut flesh	4/10	1/10	0/10	5/30 (0.167)
Coconut embryo	1/10	2/10	1/10	4/30 (0.113)
Coconut juice	0/10	0/10	0/10	0/30
Betel nut husk	0/10	0/10	0/10	0/30
Betel nut flesh	0/10	0/10	0/10	0/30
Chi-Square tests (for df =3, N=120, excluding group with zero positives)				**4.583**
P (for df =3, N= 120, excluding group with zero positives)				**0.205**

Sequencing of phytoplasmas recovered from coconut tissues at three gene regions showed a single phytoplasma type across samples and showed high sequence similarity (>99%) with *'Cocos nucifera'* BCS and BWAP sequences from GenBank and with the phytoplasma detected in insect samples (Figure 15). If this presence of phytoplasma DNA is due to the presence of live phytoplasma cells that lead to infection of the seedling growing from phytoplasma-positive nuts, it would suggest that the earlier biosecurity measure of de-husking coconuts for transport from Madang may not be sufficiently stringent. Accordingly, a follow-up study was carried out to test the possibility of vertical transmission from parent to offspring.

Nested PCR and LAMP analysis of the tissue samples taken from young palms that germinated from drupes held in insect-proof cages revealed evidence of possible seed transmission in coconut and betel nut. Overall, 12 of 60 betel nut seedling samples were positive for phytoplasma. Of the 10 samples successfully sequenced, 9 samples showed high sequence similarity (>98%) to BWAP accession KP642659 isolated from banana in Debepari PNG and the other sample showed very high sequence similarity (>99%) to BCS/BWAP: for example LC228757, BCS isolated from coconut in Siar and GenBank accession KP053901 BWAP isolated from banana in Madang.

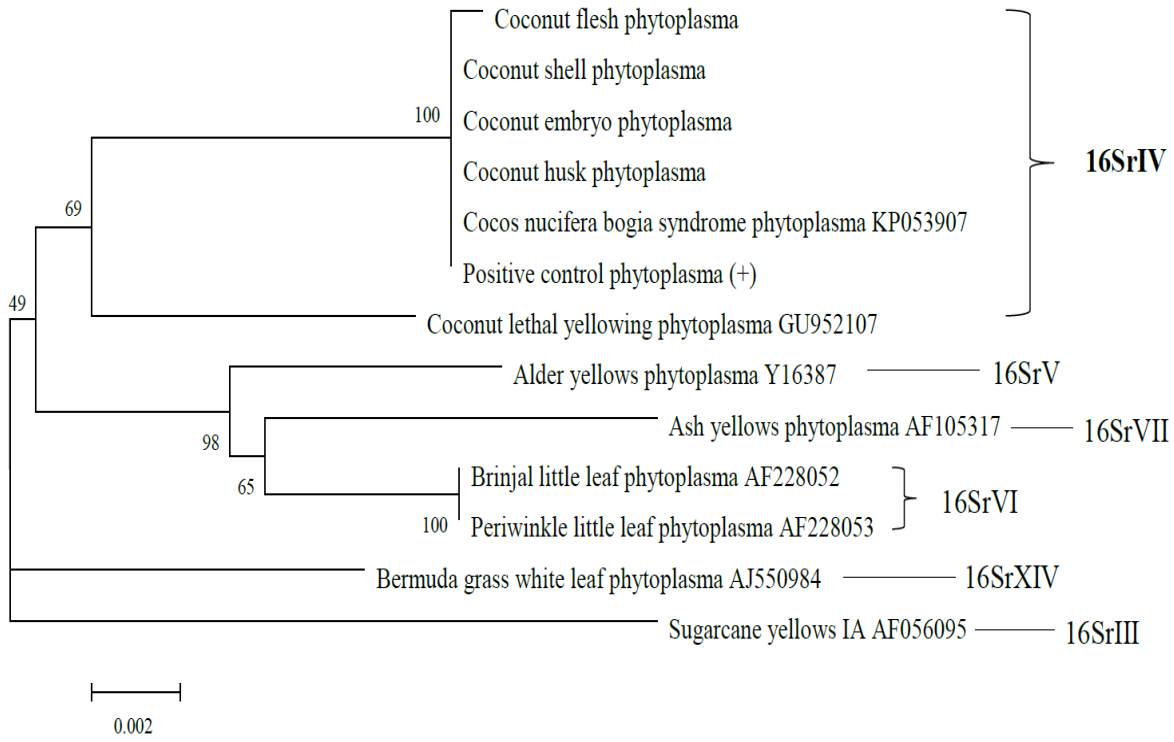

Figure 15. Phylogenetic tree based on the sequences of the husk, shell, flesh, embryo tissue phytoplasma and selected 16Sr phytoplasma groups using the neighbor-joining method. Numbers above the branches are confidence values obtained from 1000 bootstrap replicates. Sampled tissues phytoplasma in PNG is shown as a member of 16SrIV group enclosing the *'Cocos nucifera'* Bogia syndrome

For coconut, 10 of 39 seedling samples tested positive. All of these samples showed high sequence similarity (>99%) to BWAP accession KP642659. The fact that the coconut germplasm samples originated from differing sites allows the rate of positive samples to be partitioned. Positives were evident among samples from Muad and Sugar but not for samples from Rivo Road or OAD (Table 11). Notably, however, four of the 12 samples from asymptomatic trees from the coconut Genebank (thought to be outside the BCS-affected area) were positive. Accordingly, caution is required in the handling of germplasm collected from this location and used in any curation for future germplasm repositories.

Table 11. Breakdown of positive and negative PCR results for tissue samples of seedlings germinated from coconuts collected from differing sites.

	Genebank	Muad	OAD	Rivo Road	Sugar	Total
Total	18	9	5	2	5	39
Positive	4	3	0	0	3	10

Overall, these results provide evidence for vertical (seed) transmission that is strong enough to merit: (i) that this mode of spread needs to be considered possible when formulating containment and management plans for BCS, and (ii)

that there is a need for more detailed studies, especially to determine whether this is possible in other crop species that host BCS/BWAP. Whilst vertical transmission of *other* phytoplasmas has recently been reported in a range of crop species by other teams, this remains contentious in the discipline.

6.5 Undertake a whole genome analysis of BCS and BWAP.

Technical challenges prevented this work being fully completed. There was a considerable delay in Australia receiving plant and insect DNA samples from PNG and an unforeseen delay being granted a new permit to import samples. The samples required to initiate the sequencing were received by Australia on 24 December 2019.

To avoid having plant DNA 'contaminating' the phytoplasma samples and complicating downstream analysis of whole genome sequencing, PNG partners isolated DNA from the sucrose-feeding solution method tested in this project. However, despite active feeding by insects on infected tissues (banana and coconut), the majority of the samples received were negative for phytoplasma (nested-PCR tested in Australia).

To provide additional material, DNA was extracted at the same time from plants symptomatic for BWAP on banana in the Markham Valley and BCS on coconut in Madang. Markham Valley banana tissue was selected for DNA extractions because it is possible that that BWAP/BCS in these bananas is different to the BCS/BWAP seen in the Madang region, thus a good comparison for whole genome sequence and downstream analysis and use of that sequence information for LAMP/q-PCR. All plant samples prepared for whole genome sequencing showed high sequence similarity to BCS or BWAP.

Protocols were implemented to enrich the phytoplasma DNA using known, yet complicated methods. Staff changes at USQ meant that the personnel contracted to conduct the whole genome sequencing left employment. Thereafter, liaison with the UQ group working on BWAP in banana revealed a method approach that avoided the need for the complicated enrichment step.

USQ is now collaborating with UQ to complete the whole genome sequencing of BCS and BWAP. Approximately 30 additional DNA extractions of banana and coconut tissue were tested for the presence of phytoplasma using LAMP, nested PCR and sequencing. A selection of these samples, along with several betel nut samples have been concentrated and purified for long-read sequencing. Accordingly, the originally planned studies will continue despite the project ending. If differences are detected between the phytoplasmas, new LAMP primers can be designed to allow for a rapid laboratory diagnostic in PNG, and this information will be published after the current DNA collection is screened.

6.6 Conduct a review of the available scientific and applied literature on the control of similar plant diseases worldwide.

As the first step of the project, the team carried out an extensive review of the

literature on lethal yellowing-like diseases, their classification, detection and identification, vectors and spread as well as best practice management in other parts of the world (Gurr et al., 2016).

Lethal yellowing diseases (LYD) continue to be serious in the Caribbean and Central America (EPPO/CABI, 1997; Myrie et al., 2006; Myrie et al., 2012; CARDI, 2013; Ntushelo et al., 2013b). Lethal yellowing like diseases are known to be present in Benin, Cameroon, Ghana, Kenya, Mozambique, Nigeria, Tanzania, and Togo (EPPO/CABI, 1997; Eziashi and Omamor, 2010) and recently PNG (Kelly et al., 2011) (Figure 16). The impact of LYD is particularly felt in communities of smallholder and subsistence farmers that rely nutritionally and economically on coconuts (Myrie et al., 2011). Compounding the impact of this group of diseases, the coconut industry is often poorly resourced for carrying out phytoplasma research. In many parts of the world where LYDs occur, research is piecemeal usually due to the intermittent funding inputs through foreign aid (Baudouin et al., 2009; Philippe et al., 2009).

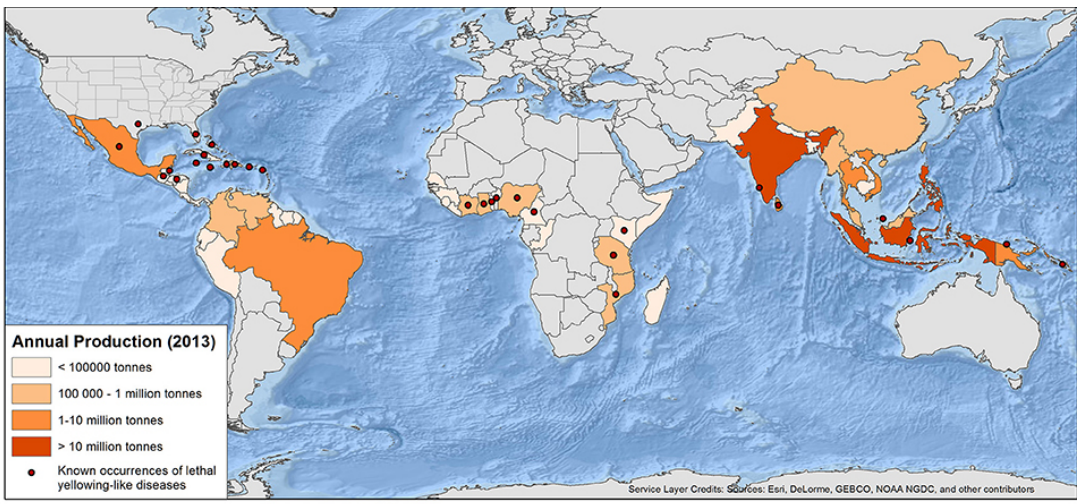

Figure 16. World map of coconut production and reports of lethal yellowing-type diseases of palms. From Gurr et al. 2016, see that open access article for full detail and caveats. (Solomon Islands record is for banana but included because of close genetic similarity to BCS/BWAP (Davis, R. I., Henderson, J., Jones, L. M., McTaggart, A. R., O'Dwyer, C., Tsatsia, F., et al. (2015). First record of a wilt disease of banana plants associated with phytoplasmas in Solomon Islands. Australas. Plant Dis. Notes 10:14. doi: 10.1007/s13314-015-0163-4)).

Vector transmission is considered the main form of local spread of LYD. Phytoplasmas are also known to be spread through the movement of plant materials and when human-aided this can result in rapid, long range movement. Human activity has been an important cause of the spread of LYD, especially because many phytoplasmas have multiple plant host species, some of which are non-symptomatic so transported plant materials can appear healthy (Marcone, 2014). Many different palm species have been identified as hosts of LYD. Few non-palm related species have been positively identified as alternate hosts to LYD associated phytoplasmas.

Most vectors of phytoplasmas are from the Order Hemiptera, sub-order Auchenorrhyncha, except some species of the Family Psyllidae, which are in the

sub-order Sternorrhyncha (Howard, 2001).

The literature review concluded that there were no current cost-effective, curative treatments available for any LYD, meaning that containment is key to limiting impact. An integrated pest and disease management (IPDM) strategy could encompass tools such as surveillance and sanitation, quarantine, management of alternate hosts and replanting with tolerant varieties. There is a range of other theoretical management approaches but this is reliant on the future development of technology and discovery of yet unknown biological and ecological information about plant-disease-vector interactions (Figure 17). The most effective IPDM approach recorded in the literature involves a weekly surveillance of palm plantations with the immediate removal and destruction of symptomatic palms and replacement with an alternate high yielding variety. No variety in the world is yet to be recorded as being fully resistant to LYD but some slow the spread of the disease and allow growers to have up to 10 years of high yields. This approach did not prevent palm death but rapidly slowed the spread and impact of LYD (Myrie et al., 2011; Serju, 2012). No insecticide approaches have been successful in the literature especially compared to destruction of symptomatic palms (Nkansah et al., 2005).

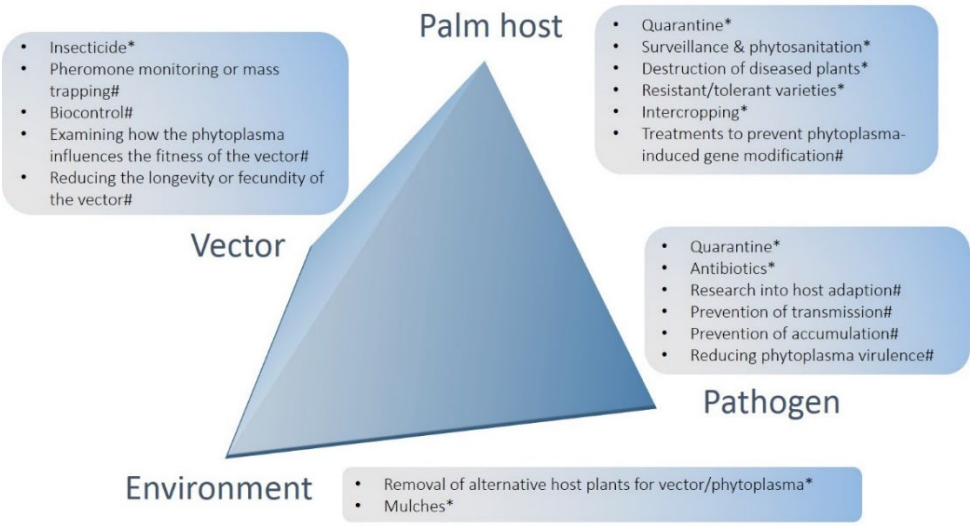

Figure 17. Summary of options for the management of lethal yellowing diseases of palms arranged in relation to crop, pathogen, environment and vector components of the pathosystem. *Denotes options that have had impact in at least some field settings, including methods that have scope for further development and use; #denotes potential future options.

Phytoplasma “resistance” has been described as the absence of symptoms associated with a low pathogen titer in the infected plants whilst phytoplasma “tolerance” is mild symptoms under a high pathogen titer (Jarausch et al., 2013). No genotype of coconut has yet been found to be totally “resistant” to LYD (Baudouin et al., 2009), though Harries (2002) describes “field resistance” as “the ability to plant…and survive long enough to repay the loan, cover costs and allow some profit.” A more formal description is the portion of a population surviving after field exposure to the disease (Been, 1995). In some situations where resistance screening has taken place in coconut, it has been speculated that genetics were

not necessarily the cause of the resistance as populations had succumbed to LYD when grown in different locations. Although it is known that environmental conditions will affect the level of resistance (Baudouin et al., 2009), there is also the possibility that a resistant strain of coconut will encounter a different sub-group of phytoplasmas (or different vector species) when grown in a new location. The most commonly recommended strategy for use is planting a range of resistant varieties in any growing area to reduce the risk of widespread plantation death should plant resistance be eroded by adaptation in the pathogen or vector population. Problems have occurred where varieties that have previously been reported as resistant have shown symptoms indicating a resistance breakdown (Broschat et al., 2002; Been and Myrie, 2005; Quaicoe et al., 2009).

A more general challenge associated with the use of host plant resistance to manage LYDs is that varieties with resistance to the phytoplasma may lack resistance to other serious biotic threats and this might geographically constrain their value. For example, two exotic ecotypes of coconut failed to survive in PNG due to attack by the coconut rhinoceros beetles, *Oryctes rhinoceros* (Linnaeus) (CRB), *Scapanes australis* (Boisduval) (Coleoptera: Scarabaeidae) and the black palm weevil *Rhyncophorus bilineatus* (Montr.) (Coleoptera: Curculionidae) (Ovasuru, 1994).

Notwithstanding the challenges, history does point to the potential of host plant resistance to contribute to LYD management. Cape Saint Paul Wilt Disease (CSPWD) in Ghana has been addressed by resistance screening work using a hybrid of Sri Lanka Green Dwarf (SGD) and Vanuatu Tall (VTT), which is being used for replanting following the disease epidemic (Quaicoe et al., 2009; Bila et al., 2019). Similarly, in Jamaica, the local variety was widely replaced with the Malayan dwarf varieties and MayPan, which led to a recovery of the coconut industry (Been, 1995). However, a later outbreak killed up to two thirds of the new varieties, which triggered research exploring the possibility of the occurrence of a new phytoplasma, a new vector or a change in the virulence of the current phytoplasma. Broschat et al. (2002) cast doubt on the results of the original resistance trials, and showed new evidence that the MayPan and Malayan varieties were not resistant as previously claimed. It is now known that there are several subgroups of phytoplasma present in Jamaica (Ntushelo et al., 2013a).

Climate change is considered to have a significant impact on the spread and establishment of vectors and phytoplasmas associated diseases into areas with a previously unfavourable climate (Foissac and Wilson, 2010; Krishnareddy, 2013). Thuiller (2007) stated that a 1 °C increase in temperature could shift ecological zones by up to 160 km. Increased temperatures have already been shown to result in insect species moving into new areas (Walther et al., 2002; Parmesan and Yohe, 2003) and even new countries. It is also known that elevated temperatures increase the rate of spread of some phytoplasmas either through faster multiplication in the host or higher feeding frequency of the insect vectors resulting in increased transmission opportunities (Maggi et al., 2014).

These cases illustrate the principle that biological information is key to effective management.

6.7 Synthesise the results of the project with information from the literature review and prepare a detailed technical report with recommendations for BCS and BWAP.

This section provides a brief commentary on the nexus between the available information generated by the project's activities (empirical studies and literature review and synthesis) and how these relate to recommendations for BCS/BWAP containment and management. Fuller detail is provided in the Recommendations section (below).

Prevention

Prevention is the best practice because, once plants are infected, BCS is usually lethal. The Tapo check point, previously established to prevent the movement of BCS out of Madang Province, has been dismantled to make way for a proposed new bridge. Dr Richard Davis confirmed that the coconut samples from Brahmann High School, Usino-Bundi District (Madang Province) and Hayfield at Maprik (East Sepik Province) were false positives. Site visits to these two locations by Dr Lastus Kuniata revealed no BCS symptoms in the coconuts there. Accordingly, it was concluded that BCS is still confined to Madang, Sumkar and Bogia districts of Madang Province.

It was agreed that a new check point should be established within Madang along the Madang/Lae/Highlands highway as soon as possible to prevent any spread of BCS from the province. A new proposal has been developed by NAQIA/KIK with a proposed framework for cost sharing by government and private industry.

At the time of finalising the present report (July 2020) plans had been drawn up by KIK to establish a police check-point at Naru, approximately 50km south of Madang on the Ramu Highway (that connects Madang Province with Morobe Province to the east) and well before the intersection with the Highlands Highway that connects coastal areas with highlands provinces.

The February 2020 stakeholder workshop visit to the International Coconut Genebank highlighted the potential risk of losing the genetic material if BCS spread onto the site. BCS has been reported within a few kilometres of the site and, notably, four of the 12 drupes collected from asymptomatic Genebank palms grew into seedlings that were positive in molecular tests for BCS/BWAP DNA suggesting that the disease has reached the Genebank. This is despite the fact that a buffer of cleared land around the site had been established in an attempt to slow the invasion of BCS. Work is currently underway to establish a new Genebank site offshore on an island. This will use newly collected material that will be held at an interim location for several months and tested for BCS before moving to the final Genebank location.

Sanitation

Worldwide, best practice for the management of coconut lethal yellowing

phytoplasma associated diseases is the immediate removal and destruction of infected palms (Gurr et al., 2016; Yankey et al., 2018; Bila et al., 2019). Identification of symptomatic trees can be facilitated by drones and nearby non-symptomatic palms can be tested using LAMP. As noted at the 2020 stakeholder workshop some education of farmers may be needed so that understanding of why non-symptomatic (but infected) palms may need to be destroyed. Various compensation schemes were also suggested such as a 'two-for-one' hybrid seedling replacement.

Sanitation not only slows the spread by vector insects but prevents coconut rhinoceros beetle (CRB), *Oryctes rhinoceros*, from breeding in the dead trunks that can lead to elevated local pest densities and consequent economic impact (Olivier et al., 2019). It was reported at the 2020 stakeholder workshop that CRB is already breeding inside rotting coconut logs and standing poles killed by BCS. In Mozambique, a congeneric of CRB, *Oryctes monoceros* (Oliver) is present. There, the plant-insect-phytoplasma-beetle relationship is a recognised problem and destruction of the felled palms by chopping and burning is recommended (Bila et al., 2019). It was further reported that the identity of the CRB present in Madang Province needs to be determined in case it is the especially damaging Guam biotype (CRB-G). This biotype has high levels of resistance to the biological control agent, *Oryctes rhinoceros* nudivirus (OrNV), which gives high levels of control for other biotypes of this pest.

Alternative crops

In relation to management, several crops appeared to be free of BCS/BWAP and other phytoplasmas including cassava, and sugarcane. These may play a role in BCS management by serving as non-host plants to grow around areas of susceptible plants, or for replanting efforts in areas where coconuts have been killed by BCS. Whilst Kra et al. (2017b) reported that areas that had been replanted with cassava following the devastating impact from the Côte d'Ivoire lethal yellowing phytoplasma from the 16SrXXII-B subgroup were experiencing symptoms in cassava caused by that LYD phytoplasma, this is a different group to the 16SrIV group responsible for BCS.

In areas where coconuts are yet to be affected by BCS, promotion of intercropping with non-host crops can reduce economic losses if BCS does spread.

Resistant varieties

It should be a priority to determine if any high yielding varieties or hybrids are tolerant of BCS. No variety in the world is yet to be recorded as being fully resistant to BCS but some slow the spread of the disease and allow growers to have up to 10 years of high yields. Bila et al. (2019) lists several African coconut varieties that have been used to save coconut industries and the details of several field trials that are still underway.

Banana varieties should also be assessed to determine if any resistance for BWAP exists in the current banana genetics.

Chemical control

Generally, the literature does not suggest pesticide use is economically effective

either as trunk injection to protect individual palms or more broadly for vector population control.

Cultural or biological control

Improved knowledge of the biology and ecology of the vectors would assist with the development of cultural and biological control tools. Cultural practices could, for example, be aimed at breeding sites of the vectors. Management of phytoplasma diseases via vector control is not generally effective but advances in knowledge and the development of new technologies may change this (Gurr et al., 2016; Bila et al., 2019).

7 Impacts

7.1 Scientific impacts – now and in 5 years

The project has had immediate scientific impact in terms of both impact within the scientific community and the science of the project impacting BCS management. At the time of finalising the present report (July 2020), action has already been taken in response to project findings and the associated recommendation from the stakeholder workshop (held in February 2020) to re-establish a biosecurity check point on the highway from Madang. Plans have been drawn-up by KIK to establish a police-controlled check-point at Naru. This is located approximately 50km south of Madang on the Ramu Highway (that connects Madang Province with Morobe Province to the east) and well before the intersection with the Highlands Highway that connects coastal areas with highlands provinces. The check point will prohibit the passage of planting material. De-husked coconuts and unripe betel nuts will, reportedly, be allowed to pass – reflecting the important commerce associated with these commodities. The check point will be in place indefinitely, reflecting the recent declaration of Bogia, Sumkar and Madang districts as diseased areas from which plant material movement is restricted. KIK is currently developing the budget to cover costs of building the boom gate, house with power and water supply, and area for (wood-fired) incineration of confiscated plant material, as well as travel allowances for attending personnel but this measure is considered locally as a relatively low cost strategy in relation to the likely benefit of reducing the risk of BCS spreading.

A second impact of this project is that a buffer area of cleared land was established around the site of the International Coconut Genebank at the Stewart Research Station outside Madang. This was intended to slow the invasion of BCS into the Genebank. Despite this measure, molecular assays for BCS/BWAP DNA suggest that infection may have already occurred, though palms are asymptomatic. The February 2020 workshop visit to the Genebank highlighted the risk of losing the genetic material if BCS spread onto the site though this event would represent an opportunity relevant to pre-breeding for disease resistance (see below).

Project outputs that underpin the impacts are listed below.

Gurr GM, Johnson AC, Ash GJ, Wilson BAL, Ero MM, Pilotti CA, Dewhurst CF, You MS (2016) Coconut Lethal Yellowing Diseases: A Phytoplasma Threat to Palms of Global Economic and Social Significance. Front Plant Sci 7 (1521). doi:10.3389/fpls.2016.01521

Reflecting its scientific impact, the Gurr et al. (2016) review conducted by the project team has had over 20,000 views. Further, its Altmetric score of 39, puts it in the top 5% of all research outputs scored by Altmetric (https://frontiers.altmetric.com/details/13053907#score). Altmetric counts tweets, social media coverage and other impact aside from traditional citations. This article has had more views than 97% of all Frontiers articles, irrespective of their publication date, so is having significant impact. On Research Gate it has been read 1,633 times and the project has 37 followers. It has been cited 32 times.

Lu H, Wilson BA, Ash GJ, Woruba SB, Fletcher MJ, You M, Yang G, Gurr GM (2016) Determining putative vectors of the Bogia Coconut Syndrome phytoplasma using loop-mediated isothermal amplification of single-insect feeding media. Sci Rep 6:35801. doi:10.1038/srep35801

This method of using a sucrose solution to detect phytoplasmas in insect saliva is a relatively new method of screening phytoplasma vectors. This successful work has advanced our knowledge detecting phytoplasma vectors and contributed significantly to the selection of candidates for the cage trial experiments. This rapid detection method will significantly aid future work in detecting phytoplasma vectors.

The cage trials are a significant scientific advancement in our knowledge of BCS. This is the first time since the 1980s that caged palm disease vector trials have been successfully carried out for a coconut lethal yellowing disease.

Members of the project team also co-authored a broader opinion piece in *Frontiers in Plant Science* that draws attention to the threat that phytoplasmas pose for Australian agriculture. This paper is also in the top 40% for views of papers published by *Frontiers*.

Liu, J., Gopurenko, D., Fletcher, M., Johnson, A., & Gurr, G. M. (2017). Phytoplasmas – the 'crouching tiger' threat of Australian plant pathology. Front Plant Sci, 8(599). doi:10.3389/fpls.2017.00599

Three additional papers are in preparation and will be submitted for publication to appropriate journals later in 2020. One of these reports detection of phytoplasma DNA in coconut germplasm tissues. It will be supplemented by the newly acquired results on phytoplasma detection in seedlings grown from coconut and betel nut collected from symptomatic palms. Two further manuscripts will report on the cage test, that confirms a phytoplasma etiology and vector competence; and plant host range.

These outputs provide a longer legacy of impacts form the project. Specific advances in knowledge about this pathosystem that have arisen from the research for development projects are as follows.

1. The cage transmission test provided strong evidence of a phytoplasma etiology for BCS and BWAP.

2. Molecular assays identified six putative vectors that were proven to expel BCS/BWAP DNA when feeding. *Lophops saccharicida* and *Zophiuma pupillata* (Lophopidae) were demonstrated to be competent vectors. This is noteworthy because the literature review confirmed that very few phytoplasma pathosystem studies had been able to establish experimentally the vector involved and demonstrate its competency.

3. Field surveys found BCS/BWAP phytoplasma DNA in large numbers of coconut and banana samples. Positives were also detected in betel nut and taro as well as in small numbers of individual samples from sweetpotato

and *Imperata* sp. Several crop species were consistently negative in phytoplasma assays and these might be used in replanting programs.

4. Studies of young plants grown from the drupes of BCS-symptomatic coconut and betel nut trees and germinated in insect-proof cages led to the detection of BCS/BWAP DNA, consistent with vertical transmission of phytoplasma. This phenomenon is contentious within the field of phytoplasmology though it has been reported in some annual crops and phytoplasma DNA has previously been reported to be detectable in coconut drupes. Our finding that it is detectable in the young plants grown from propagules held inside insect proof cages is a major advance in what is likely to be an eventual overturning of the old orthodoxy that phytoplasmas cannot be seed-borne.

5. A review of the available scientific and applied literature on the control of similar plant diseases worldwide was completed early in the project. Notably, two book chapters by other authors published since the publication of the team's Frontiers paper have come to the same general conclusions in terms of best practice management of these phytoplasma diseases (Yankey et al., 2018; Bila et al., 2019).

Further detail on these bullet points is in provided in the Conclusions section (below).

7.2 Capacity impacts – now and in 5 years

Partner country capacity has been enhanced in this project by the development of a general LAMP assay to detect the presence of any phytoplasma, as well as a more specific assay that targets BCS.

Partner country capacity has been enhanced by (i) providing equipment and training for molecular assays to the National Agricultural Research Institute and (ii) by involving staff from multiple agencies in joint publication of results in scientific journals. Prior to this project, there was no human or physical capacity within PNG for LAMP work. The Genie II apparatus for running LAMPs was delivered by Prof Gurr (CSU) to Dr Sar (NARI) in Lae early in the project. Considerable hands on experience has been gained in field sampling and DNA extraction from insect and plants and the use of LAMP technology. These skills will be put to further use in planned studies of the biology and ecology of BCS/BWAP. Ms Gou Rauka from NARI is using the LAMP diagnostic for an independently funded mini-project on BWAP and the use of LAMP in PNG will also be expanded. A particular example is that Ms Rauka of NARI is involved with training for pest and disease LAMP assays associated with ACIAR's program of research on sweetpotato (Figure 18).

Figure 18. Left and Centre: Technical staff were trained in sampling techniques and DNA extraction. Right: Gou Rauka (left) from NARI received training and mentoring from Dr Bree Wilson (right) USQ to develop expertise in molecular plant pathology.

Mr Lu Hengyu – funded entirely by project partner Fujian Agriculture and Forestry University (FAFU) – who successfully completed his master's degree with high marks in 2016 continued his association with the project. Reflecting his strong performance and technical skills, he was employed at FAFU to provide technical support to the project including a repeat visit to Madang March-April 2017 during which he played a leading role in setting up the seed transmission and vector screening studies. He has since entered employment at BGI Group (formerly Beijing Genomics Institute) in China.

Capacity in Australia has been enhanced by Dr Wilson (USQ) developing progressively more advanced skills both at the bench and in providing mentoring to developing country colleagues. Prof Gurr developed greater capacity in project management, dealing with a series of major challenges in the present project including the sudden, tragic death of the partner country lead investigator early in the project (Dr Kakul) followed by disrupted staffing arrangements; a protracted restructure of the partner country lead organisation (CCI/KIK); and slower than expected refinement of molecular diagnostics.

7.3 Community impacts – now and in 5 years

The aim of this project was to develop new biological information that would inform recommendations for containment and management of BCS and related phytoplasmas. It was not planned to develop, test or roll-out any IPDM measures since these were to be informed by an end-of-project stakeholder workshop (Appendix 2). Accordingly, the extent of community impacts at this time is limited.

7.3.1 Economic impacts

Since the stakeholder workshop in February 2020, selected project staff attended a meeting at KIK in Madang. Following one of the main recommendations of the earlier stakeholder workshop, KIK made a submission to the Government of PNG for funding to establish and maintain a check point at Naru further inland than the earlier check point at Tapo, about 50 km from Madang (further details in section 7.1, above). Treasury officers attended the meeting to assess the project. This is considered in PNG to be a *relatively* simple and inexpensive phytosanitary

measure that would provide enduring economic and societal benefits by minimising the possibility of spread in planting material of BCS and related phytoplasmas more widely in PNG. Whilst this measure will have little or no effect on the movement of vector insects, it being concerned with plant material, there is good reason to suspect that spread by planting material is important in longer range 'jumps' in the distribution of BCS. Additional detail is provided in the Recommendations section below and the appended report of the stakeholder workshop.

Longer term impacts, over the next five years and beyond will require further investment (see Recommendations) in order to fully convert the project's recommendations into future impacts for the community. The need for this is great because, according to discussion at the stakeholder workshop, drone surveillance of palm plantations in the Madang Province conducted in 2020 (separate to the present project) revealed that BCS had spread. It was estimated that 190,000 palms were killed. It is unknown how many banana plants have been found with BWAP in Madang but during the recent visit at the time of the stakeholder workshop, yellowing symptoms were widespread in banana plants from the air and the ground.

It was not within the scope of this project to collect data to allow an estimate of the economic impact to date of BCS, nor to forecast the potential economic losses of not confining its distribution. Very clearly, however, BCS has continued to increase its extent and resultant impact of smallholder livelihoods. At the stakeholder workshop, Dr Kuniata reported that before BCS, a household owning 3ha with 450 coconuts could typically produce 4.2 tonnes of copra per year, earning about Kina 4,620. After BCS affected an area, annual production and income reportedly reduced by 54% to 1.9 tonnes and Kina 2,090. In some cases, 100% loss of production and income occurred. He also reported a survey in the Furan area showing that numbers of dead coconut palms increased over a five-year period from just over 20% in July 2011 to approximately 75% in March 2015.

It is imperative that containment measures (see Recommendations section, below) are implemented as soon as possible and further recommendations are followed to reduce losses in areas already affected as well as to fill remaining knowledge gaps. Paramount among these is to better understand the relationship between BCS and BWAP, these being caused by phytoplasmas that are extremely closely related. This will allow the development of a molecular diagnostic able to discriminate between the two phytoplasmas in plant and insect tissue. Such a tool may have immediate economic (commercial) value. Moreover, this understanding will shed light on the paradox that BCS and BWAP are not only caused by similar phytoplasmas and both prevalent in Madang Province (in coconut and banana, respectively), yet elsewhere only BWAP is known to occur. There is clear economic importance associated with resolving this. The collaboration between USQ personnel and UQ that started as a result of this project is likely to resolve the molecular part of this conundrum whilst further field surveys planned by NAQIA representatives in the project will extend to the Highlands and Kar Kar Island to develop a more complete understanding of the extent of co-occurrence of these diseases. Together, these two activities arising from the present project will determine whether BCS arose in Madang Province as a mutation of the BWAP phytoplasma, allowing a host shift from banana to coconut. If this hypothesis were to be supported, it would point to a wider risk of such shifts by these or other

phytoplasmas that would necessitate surveillance in order to provide early detection to minimise economic losses.

7.3.2 Social impacts

In line with the opening explanation in section 7.3, the extent of social impacts as a direct consequence of the project is minimal at this time. Rather the project results will inform and direct future interventions. Notwithstanding this, the social impacts of BCS (as opposed to the BCS project itself) to date are significant (see above) and will only increase. Coconut provides a staple food and serves as a cash crop being one of the few sources of cash income for many households (Bourke and Harwood, 2009). Coconut is not only a major source of food but coconut vegetation is used in building houses and the coconut shells are used for common household use such as bowls and fuel (Ovasuru, 1994). Coconut is a very important crop for mainland coastal and island regions of PNG with exports of copra and coconut oil valued at US$27 million and US$43 million per year.

The recent publication of results from Africa showing that cassava may be a potential alternative host for the Côte d'Ivoire cassava phytoplasma (16SrXXII-B cluster) (Kra et al., 2017a) are also instructive. This reinforces the previously known general phenomenon that a given phytoplasma can affect host plants from widely different plant families (Liu et al., 2017), and illustrates this to be the case for lethal yellowing phytoplasmas with potentially dramatic impacts for smallholder farmers in locations such as PNG. It is, however, worth repeating the caveat from Section 6.7 that BCS phytoplasma is assigned to 16SrIV group rather than the 16SrXXII-B subgroup reported from Côte d'Ivoire.

In many developing countries, such as PNG, the ability to produce a cash crop for domestic and overseas export markets is closely linked with relieving rural poverty and aiding development (Bourke and Harwood, 2009). In the Madang region, the majority of the population of approximately 290,000 rely on coconut as a source of food and income. Many traders from the highlands areas also drive to Madang to buy coconuts from Madang growers to re-sell. A shortage of coconut in this area has meant that the highlanders have to drive further to secure sufficient supply and pay higher prices. Whilst these statements of impact relate to BCS and related phytoplasmas rather than specific impacts of the project, it is clear that there is great potential for the findings and recommendations from the project to have impact in the next five years. In particular, the possibility of seed transmission could have great economic significance especially on the movement of germplasm (e.g., coconut and betel nut). Certainly, the movement of planting materials needs to be more tightly controlled (by the check point in the first instance) despite sharing of crop propagules being a common cultural practice.

7.3.3 Environmental impacts

An immediate concern with the gradual and ongoing spread of BCS infections is that the regional Genebank collection of coconuts at Stewart Research Station is now threatened. Symptomatic coconut and banana plants are apparent within a few kilometres of the site and recent results suggest that asymptomatic palms in the Genebank may already be infected by BCS. This escalating threat has prompted action by relevant Government agencies and partners to establish a new

Genebank collection on a more remote site using newly-collected germplasm.

More generally in relation to environmental impacts, it is hoped that the biological knowledge generated in this project will help avoid future widespread use of broad-spectrum insecticides and help develop sustainable disease and vector management options.

7.4 Communication and dissemination activities

Results have been communicated freely within the partner organisations involved in the project, allowing findings to influence their respective policy, technical and outreach efforts.

The publication activity listed above has disseminated project findings widely beyond the partner organisations and especially to the scientific audiences. The citation and Altmetric statistics are evidence of the impact. To quote from Altmetric.com: “Altmetrics are metrics and qualitative data that are complementary to traditional, citation-based metrics. They can include (but are not limited to) peer reviews on Faculty of 1000, citations on Wikipedia and in public policy documents, discussions on research blogs, mainstream media coverage, bookmarks on reference managers like Mendeley, and mentions on social networks such as Twitter.”. The Gurr et al. paper has an Altmetric score of 39, putting it in the top 5% of all research outputs.

Dissemination specifically to partner country actors took place at the stakeholder workshop held in February 2020. The influence of stakeholder views on the project’s recommendations are explained in Section 8.2. A report on the workshop is provided in Appendix 2.

8 Conclusions and recommendations

8.1 Conclusions

This project has, in line with its first overarching aim, developed a greater understanding of BCS biology as well as the closely related BWAP-associated phytoplasma.

1. CAUSALITY. The cage transmission test provided strong evidence of a phytoplasma etiology for BCS and BWAP. This builds on previous knowledge of an *association* between phytoplasmas and these plant diseases by demonstrating *causality*.

2. VECTORS. Molecular assays of the heads of field collected insects advanced earlier understanding of the possible vectors of BCS/BWAP by testing only the heads of insects rather than the whole body (that would contain stomach contents, potentially giving false positives). This was complemented by a second method involving testing for phytoplasma DNA in sucrose solution in which the insect has inserted its stylet and fed. That work identified six putative vectors that were proven to expel BCS/BWAP DNA when feeding. These Hemiptera were used in the cage transmission test as experimental treatments. *Lophops saccharicida* and *Zophiuma pupillata* (Lophopidae) were demonstrated to be competent vectors under cage conditions. The other species cannot be discounted as potential additional vectors in nature.

3. HOST RANGE. The headline conclusion regarding host range is that BCS/BWAP is not confined to coconut and banana. Field surveys in Madang Province of crop and non-crop plants found BCS/BWAP phytoplasma DNA in large numbers of coconut and banana samples but also in multiple samples of betel nut (a commonly cultivated, traded and transported commodity), taro (a significant food crop), as well as in small numbers of individual samples from other species including sweetpotato and kunai grass. Whilst oil palm tested positive for BCS/BWAP DNA in the unnatural conditions of the cage transmission test it was not found to be positive in the field survey. Several crop species were consistently negative in phytoplasma assays. The role of non-host plants in phytoplasma management is that they can be used as alternative crops in replanting efforts after diseased plants have been removed. It is, however, of generic importance that such plants are truly non-hosts of the phytoplasma rather than being asymptomatic ‘silent’ hosts that could act as a reservoir of the pathogen.

4. SPREAD. BCS/BWAP DNA was detected in various tissues of coconut drupes (“nuts”), but not in those of betel nut, suggesting the

possibility of vertical ("seed borne") transmission of BCS. Studies of young coconut and betel nut, germinated in insect-proof cages, led to the detection of BCS/BWAP DNA in both plant species, consistent with vertical transmission of phytoplasma. During the course of the project, the area of extent of BCS-symptomatic coconuts expanded markedly to the extent that the Genebank collection at Stewart Research Station is under threat and may already have infected, asymptomatic coconut palms. Efforts have been initiated to abandon the site and recollect to establish a new Genebank in another location.

The second overarching aim of the project was to develop a management strategy to address the BCS problem in PNG. This had two components.

1. A review of the available scientific and applied literature on the control of similar plant diseases worldwide was completed early in the project. This was published in an open access journal to allow unrestricted access to readers including those in developing countries. This established that containment and quarantine efforts are key to minimising disease impact. Within affected areas, effective surveillance to rapidly detect symptoms is important to minimise the risk of infection spread by vectors. Symptomatic plants need to be replaced, potentially with other, non-host plant species or (when available) less susceptible varieties of the focal crop. Efforts to control vectors, with insecticide sprays or trunk inactions for example, had not proven practicable except for particular high value trees in setting such as resort hotels. Whilst host plant resistance to phytoplasma diseases is the ultimate solution, this is a long term undertaking and even pre-breeding was beyond the scope of the present project. Notably, two book chapters published after the team's Frontiers paper have come to the same general conclusions in terms of best practice management of these phytoplasma diseases (Yankey et al., 2018; Bila et al., 2019).

2. Ultimately, the results of the project were to be synthesised with information from the literature review, leading to a detailed technical report with recommendations for BCS and BWAP. This involved a stakeholder workshop in Madang in February 2020 and this underpinned the development of the present document in which recommendations are made (below).

8.2 Recommendations

i) A **check point** restricting movement of planting material was recommended by the stakeholder workshop where it was reported that the previous Tapo check point had been dismantled to make way for a proposed new bridge. The workshop concluded that BCS is still confined in Madang, Sumkar and Bogia districts of Madang Province. Dr Richard Davis confirmed that the coconut samples from Brahmann High School, Usino-Bundi District (Madang Province) and Hayfield at Maprik (East Sepik Province) were false BCS positives. Additionally, site visits to these two locations by Dr Lastus

Kuniata revealed no BCS symptoms in the coconuts there. Whilst this information could be taken to suggest that a check point is not necessary (because dismantling the earlier one has not led to a marked, noticeable spread in BCS), the intensity of surveillance has been modest and the development of BCS symptoms by palms in an affected area is not immediate. For example, Dr Kuniata presented results at the stakeholder workshop showing that numbers of dead coconut palms at Furan increased slowly over a five-year monitoring period from just over 20% in July 2011 to approximately 75% in March 2015. Accordingly, it was agreed at the stakeholder workshop that a new check point should be established within Madang Province along the Madang/Lae/Highlands highway as soon as possible to prevent the spread of BCS to other provinces. Whilst BWAP is present outside Madang Province and genetically very similar to BCS, there are significant differences in the biology of BWAP (it apparently being confined to banana and not affecting coconut outside of Madang Province). A check point is a relatively inexpensive, immediate measure compared with either (i) allowing BCS to spread further, or (ii) other management and containment efforts (see below) that, whilst also important and meritorious, require a still greater investment and, in some cases (e.g. breeding for resistance) will take longer to bring impact. Whilst a check point will have little or no effect on the movement of vector insects, it being concerned with plant material, there is good reason to suspect that spread by planting material is important in longer range 'jumps' in the distribution of BCS. The stakeholder workshop heard a recount of the anecdote that the initial jump of BCS from Bogia district to Sumkar and Madang districts was associated with a marital merger of families that led to planting material (most likely of banana) being brought south from Bogia to the new home of the couple. Careful consideration needs to be given to the types of plant materials that the check point should prohibit. Originally, the focus was on un-shelled 'green' coconuts but the results of this project reinforce the need to control movement of other materials, especially banana 'suckers'. Since the stakeholder workshop, a proposal has been developed and submitted by NAQIA/KIK. Details are provided in Section 7.3.1. This step is considered to be tractable, immediate and relatively low cost compared with other measures (see below) that will provide benefit in the longer-term and will require greater investment and ongoing costs. A check point is key to containment, a principle that is consistent with the project's review of international literature on effective management of phytoplasma diseases (Gurr et al. 2016) and summarised in Sections 6.6 & 6.7. At the time of finalising the present report (July 2020) plans had been drawn up by KIK to establish a police check-point at Naru, approximately 50km south of Madang on the Ramu Highway (that connects Madang Province with Morobe Province to the east) and well before the intersection with the Highlands Highway that connects coastal areas with highlands provinces. The check point will prohibit the passage of all planting material. De-husked coconuts and unripe betel nuts will, reportedly, be allowed to pass – reflecting the important commerce associated with these commodities. It is recommended that the range of plant materials allowed passage at the check point be kept under review to respond to new future evidence on the host range of BCS/BWAP. This will ensure an appropriate balance between the demands of biosecurity whilst minimising disruption to commerce, noting that coconut and betel nut being

transported to the Highlands are overwhelmingly for consumption rather than propagation.

ii) Knock-on effects for **coconut rhinoceros beetle** were discussed at the stakeholder workshop. It was reported that CRB is breeding inside rotting coconut logs and standing poles left after a palm is killed by BCS. This may lead to increased local population density and exacerbated damage to live/surviving palms. At the time of the workshop a need was stressed to precise identity of the CRB in Madang Province to determine whether it is not the damaging Guam biotype (CRB-G). By the time of finalising this report (July 2020), NAQIA confirmed the presence of CRG-G in Madang and was preparing documentation for the Minister of Agriculture to declare the National Capital District, the Central, Gulf and Madang provinces as CRB-G affected. This discovery heightens the need for prompt destruction of palms that are felled as part of a future management effort (see below) as well as the palms that are killed by BCS. It is recommended that protocols are developed to destroy these dead palms to prevent them serving a breeding sites for CRB, including CRB-G,

iii) The visit by stakeholder workshop participants to the **International Coconut Genebank** highlighted the potential risk of losing the material when BCS arrives and results obtained late in the project suggest that some coconut palms in the Genebank may already be infected though they appear asymptomatic. Clearing of a buffer area around the sites had been undertaken in an attempt to slow the invasion of BCS. This needs to be well maintained, especially to remove plant species that are potential hosts of BCS/BWAP in order to minimise spread of BCS into and within the Genebank. The area may also need to be quarantined to restrict public access in case of movement of infected planting materials. There may be a need to use insecticides to suppress vector populations in this zone and project findings show that any such efforts should particularly target *Lophops saccharicida* and *Zophiuma pupillata*. Fortunately, both of these species are relatively large and easily recognisable. However, the additional putative vectors used in the cage tests of this project cannot be entirely discounted as additional vectors. Further work on this is necessary but logistically demanding, even if the cages constructed by the present project are employed. The great height of many of the coconut palms in the Genebank collection precludes effective use of insecticide sprays and may demand the use of trunk-injected systemic insecticides. Protocols for this need to be developed based on the literature. Further molecular testing of samples from the Genebank will determine the extent of infection among these palms but any removal of germplasm from the Genebank needs to take a precautionary stance and assume that BCS is present in the material. Work on these aspects might be undertaken by a sub-set of the partners involved in the present project.

iv) Prompt **removal of symptomatic plants** in BCS-affected areas is important in reducing infection of nearby plants and is consistent with the project's review of international literature on effective management of phytoplasma diseases (Gurr et al. 2016) and summarised in Sections 6.6 & 6.7. It is, however, expensive because of the large area of extent and likely need to compensate growers. The recent advent of inexpensive unmanned aerial vehicles ("drones") carrying video cameras opens the valuable possibility of readily surveying areas on a regular (e.g. monthly) basis to detect early symptoms in plants. Government

regulations controlling the use of drones need to be consulted and, in conjunction with the relevant literature, a protocol for this be developed and considered by NAQIA for implementation as soon as possible. This might be undertaken by a sub-set of the partners involved in the present project.

v) **Pre-breeding** needs to be initiated to identify tolerant, less susceptible, and (ideally) resistant varieties of coconut. This recommendation is strongly supported by the project's review of international literature on effective management of phytoplasma diseases (Gurr et al. 2016) and summarised in Sections 6.6 & 6.7. This is a complex, medium- to long-term, and expensive undertaking but needs to be initiated as soon as possible. One early step is screening the varieties in the Genebank. The current Genebank collection can be monitored as BCS spreads as a natural experiment in which useful traits might become apparent and breeding lines identified. This might be undertaken by a sub-set of the partners involved in the present project. Ultimately, plant breeding may play a wider role in the management of BCS/BWAP by extending to crop species other than coconut.

vi) As less susceptible or resistant lines of coconuts become available, these need to be provided to landholders to replace removed, symptomatic trees. Until this time, **replanting efforts** should be focused on the use of crop species that the present work has found to be unaffected by BCS/BWAP. Accordingly, banana and coconut removed for BCS management could be planted with crops such as cassava and sugarcane. A protocol for this work needs to be developed, led by NARI and potentially supported by sub-set of the partners involved in the present project.

vii) An ongoing **surveillance** effort should be established to asses more fully the extent of the BCS affected area in Madang Province and, in other provinces, to establish the extent of BWAP and monitor for potential transfer of symptoms to coconut and possibly other plant species. Because only small numbers of samples were tested of some plant species in the present study, future surveillance should extend over multiple crop and non-crop species and provide larger total sample size. This will establish with more certainty the full host plant range of BCS/BWAP. Determining whether oil palm is a host is a related priority. Results will be useful in informing the plant materials to be prohibited from passing any new check point and which plant species to be covered by any future program of symptom surveillance and removal-replanting efforts.

viii) In association with a new surveillance program, genomic studies should be continued for isolated phytoplasmas, not limited to BWAP and BCS. This will allow the development of more precise molecular diagnostics that can discriminate BWAP from BCS and help understand the development of the relatively recent BCS problem. For example, whether the BCS phytoplasma is a mutated strain of the BWAP phytoplasma was discussed at the stakeholder workshop. Progress was made during the project in USQ-led studies of this aspect, but it did not lead to conclusive results. A collaboration established with UQ will pursue this important unknown. More widely, future surveillance using improved diagnostic tools will prepare PNG for future phytoplasma problems.

ix) Evidence for the presence of phytoplasma DNA in seedlings grown from the drupes of symptomatic (and, in some cases, asymptomatic) coconut and betel nut in insect-proof cages constitutes evidence for **vertical transmission**. The management and economic implications of this are large so an urgent priority is to assess the reproducibility of this finding by a further round of studies. This might be undertaken by a sub-set of the partners involved in the present project. The results from that work will inform – among other things – the types of planting material that can pass through the check point (see 1, above). It is important also to determine whether vertical transmission is possible for other host plants of BCS/BWAP.

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9.1 List of publications produced by project

Note that these articles are published in open access journals, so both are readily accessed free of charge.

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10 Appendices

10.1 Technical report to ACIAR (Nov 2019).

10.2 Report on Madang Stakeholder Workshop (Feb 2020).