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Final report

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1 Acknowledgments

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2 Executive summary

The project HORT/2012/113 - Integrated disease management strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia had the goal to contribute to economic growth in the Philippines through increased income and improved livelihoods of smallholder farmers, including tropical fruit growers in the southern Philippines. This project delivered this goal by building research capacity, developing skills and undertaking comprehensive studies on the epidemiology of bacterial crown rot (BCR) and phytoplasma diseases to help generate sustainable management approaches.

The specific objectives of this project were:

1. To characterize the *Erwinia* sp. associated with BCR and its survival (Philippines).
2. To develop and evaluate sustainable management practices for BCR (Philippines).
3. To develop and evaluate sustainable management practices for dieback of papaya (Australia and Philippines)
4. To disseminate a package of technology (Integrated Disease Management (IDM) strategies) for papaya (Philippines and Australia).

Bacterial Crown Rot

Molecular techniques were used and standardized to recover bacterial crown rot (Philippines) and papaya phytoplasma from diseased plants, planting material, weeds and insects. These techniques helped provide a better understanding of papaya disease epidemiology, insect vector relationships and effective disease management strategies. *Erwinia mallotivora* has been confirmed as the causal organism of BCR in the Philippines and has shown a 99.2% similarity to the BT - MARDI strain that causes BCR in Malaysia. The results of targeted gene sequencing of 16s rDNA, gyrB, rpoB, and recA have also shown no genetic variation among the many *Erwinia mallotivora* isolates recovered from BCR affected papaya in the Philippines.

Six isolates of *Erwinia mallotivora* have been sequenced at PCARI-SGCL using whole genome sequencing and these will be deposited at the NCBI database. Access to these sequences will allow for rapid and precise identification, which will help determine the origin and spread of the BCR pathogen. BCR tolerant lines tested under glasshouse conditions have shown significant differences in fruit weight, length and width, TSS, flesh colour, peel thickness, titratable acidity, firmness and percent edible portion. The field screening of a few selected tolerant lines showed a high incidence of BCR recently under wet weather conditions and indicated that the F2 population is still segregating and requires further work to stabilise the material. Foliar applications of Phosphro and Bacillus *QST 719* were found to be more effective at managing BCR than copper fungicides. Regular sprays with these products could be integrated into the disease management plan.

Phytoplasma:

Real-time and nested Polymerase chain reaction (PCR) were consistent in the identification of phytoplasma from papaya plants, weeds and insect samples. The phytoplasma groups were identified using 16 sRNA sequencing and phylogenetic analysis. *Ca* .P. *aurantifolia,* which belongs to the 16 Sr II group of phytoplasmas, was identified as the causal organism of a previously unidentified phytoplasma disease in Mindanao, the Philippines.

An extensive survey of papaya growing in commercial plantations using molecular techniques showed that three phytoplasma species were associated with papaya dieback, yellow crinkle disease and mosaic disease in Australia. These were *Candidatus*

Phytoplasma *australiense* (dieback; *PpDB*), *Candiadatus* Phytoplasma *australasia* (yellow crinkle and mosaic disease; *PpYC and PpM) and Candidatus* Phytoplasma *aurantifolia* (Australian Lucerne Yellows, *ALuY*). In Australia '*Ca* P. *aurantifolia*, and '*Ca.* P. *australasia* has been found associated with yellow crinkle and mosaic disease in northern Queensland. Another phytoplasma disease Australian Lucerne Yellows (ALuY), which is closely related to yellow crinkle and mosaic, was recovered from one papaya plant with dieback symptoms. Six weeds/plant species; Praxelis, Sand Spurge, Wire weed, Shrubby Stylo, Sun-hemp and one unidentified weed, which were collected in late summer in Australia, had little leaf and leaf proliferation symptoms and were positive for yellow crinkle and mosaic. Two weeds; Praxelis and Shrubby Stylo were also positive for Australian lucerne yellows (ALuY) phytoplasma. These weeds were found in and around a phytoplasma-infected papaya plantation and were a potential food source for insect vectors and a reservoir of phytoplasma disease.

The leafhopper *Orosius orientalis* (Cicadellidae) and a lace bug (Tingidae) collected from QLD tested positive for the 16 Sr II group of phytoplasma *'Ca. P. aurantifolia* and '*Ca*. P. *australasia*. This is the first report of phytoplasma detection from a lace bug and needs further study to determine if it is a vector of phytoplasma diseases. This finding also indicates the need to explore other groups of phloem feeding insects, not only the known phloem feeding vectors of phytoplasma leafhoppers/plant hoppers and psyllids.

Papaya sticky disease caused by Meleira virus (P MeV) has been recorded for the first time in Australia. Papaya researchers have gained a better understanding of phytoplasma and Meleira virus diagnostic techniques through collaborations with virologists at the Department of Agriculture and Fisheries, Eco sciences Precinct in Brisbane. Attendance at National Biosecurity Training Meetings and a workshop on in field molecular diagnostic techniques (LAMP) improved our technical capacity to detect plant pests and diseases and, in doing so, helped reduce risk and provided security for crop production.

This project improved facilities and equipment in both countries and provided opportunity for three University of the Philippines, Los Banos (UPLB) students to complete their graduation and master's research studies in plant breeding, plant pathology and entomology. The project team members were also given support to attend and present their research at both national and international conferences and symposia. The workshops, training sessions, field visits and grower meetings with researchers, student, project collaborators, Del Monte Philippines Inc. (DMPI) growers and the Australian papaya industry conducted throughout the project also helped strengthen partnerships between researchers and farmers.

Future ACIAR funding for papaya breeding in the Philippines would allow them to develop Papaya ringspot virus (PRSV) tolerant lines with improved flavour by backcrossing to the desired cultivars. They could also be hybridised with the BCR tolerant lines to develop other hybrids with resistance to both PRSV and BCR, then backcrossed to another cultivar to infuse a delayed ripening trait. In addition, there is also the opportunity to explore further the use of the metabolites of endophytic bacteria, which have been shown to enhance resistance to diseases such as BCR and Phytophthora root rot.

3 Background

Papaya is an important and continually expanding crop in the Philippines and is ranked 6th in the world in terms of area planted and 5th in terms of volume produced (157,906 metric tonnes in 2011 (Bureau of Agricultural Statistics, 2011)). Of the volume of fruit produced, 95% is consumed locally as fresh food and about 5% is exported fresh or processed as either fruit salad or dried. Papaya is grown throughout the Philippines in commercial plantations, backyards and occasionally in mixed cropping systems. In Mindanao, papaya is climatically well adapted with yields of up to twice the national average; that is 30% of the national production is produced from just 16% of the total national area under papaya.

However, in recent years, pests and diseases have heavily impacted on production in the Mindanao growing region. The most important of these is the disease bacterial crown rot (BCR) with losses of 50–100% of trees being recorded in many parts of Mindanao following periods of heavy rainfall. Grower surveys of northern Mindanao in November 2009 (ACIAR HORT 2007/067-3) identified BCR as the most important disease in the region. More recently, the disease has been identified in the Davao region where plant losses are on the increase. There were also major concerns that BCR will soon be found in the new papaya growing areas of South Cotabato. Current management practices involve the removal of infected leaf and the use of prophylactic sprays of copper once an outbreak is detected. There is no evidence that these practices are having any effect in reducing the incidence and spread of the disease.

The Australian papaya industry is only very small compared to the Philippines with the mean annual value of production being around $25 million and with a volume of 13,000 t/yr. Most of the production (95%) is located in Queensland with minor production occurring in the Northern Territory, Western Australia and New South Wales. For Australian growers, the disease 'dieback' is of increasing concern. Dieback, a phytoplasma-related disease, is known to cause plant losses of between 10 and 100% in any one season.

In the previous project 'ACIAR HORT 2007/067-3', the focus was to provide Australian and southern Philippine papaya growers with an increased revenue return by introducing an integrated approach to pest and disease management and implementing sustainable nutrient management strategies. The current project; HORT/2012/113 (*Integrated Disease Management (IDM) strategies for the productive, profitable and sustainable production of high quality papaya fruit in the southern Philippines and Australia)* follows-on from pathology-related work conducted in the previous project. Moreover, this was the primary recommendation of ACIAR HORT 2007/067- 3 and the external review of the C3 fruit component.

This project was designed to investigate further potential for integrated disease management strategies to control BCR to overcome a major constraint in the development of the Philippine papaya industry. A slight variation to the project was approved during the mid-term project review in 2015 due to a high incidence of phytoplasma disease in Mindanao and a severe outbreak of yellow crinkle and mosaic disease in Australia. Consequently the phytoplasma study was included in the Philippines research component.

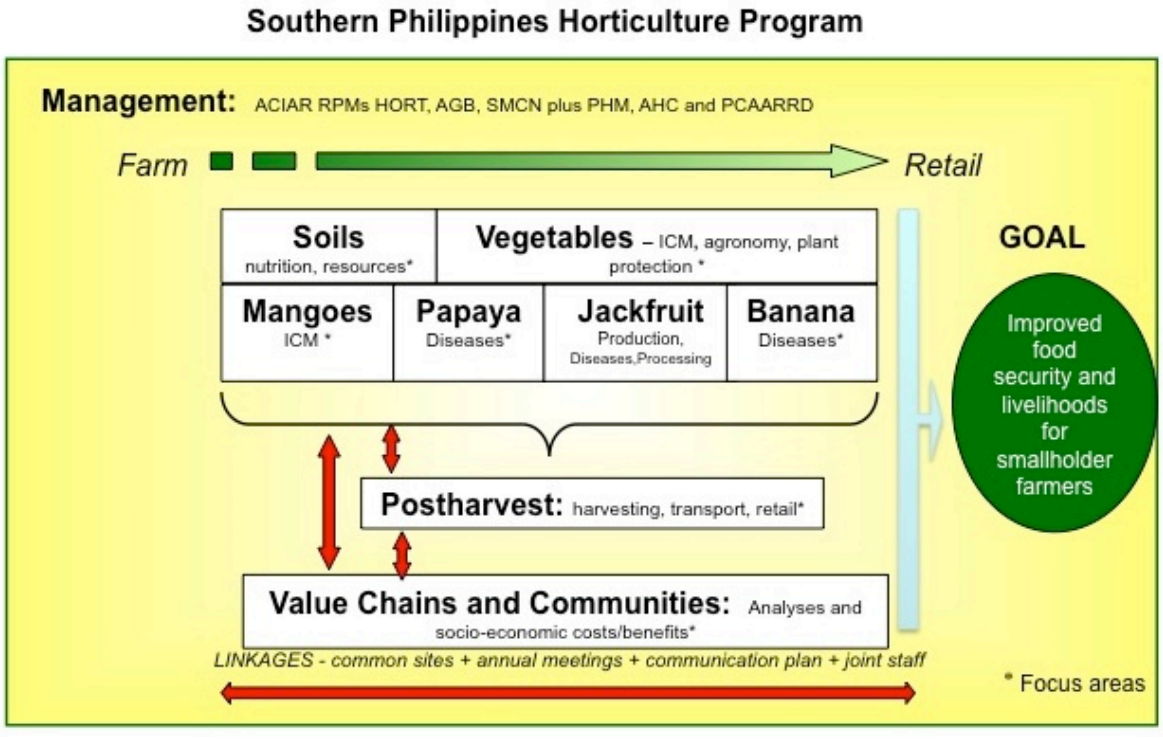
The following questions were included to clarify the research findings and to strengthen the BCR and phytoplasma IDM strategies.

- What pathogenicity differences and genetic diversity exits within the *Erwinia* sp. recovered from BCR affected plants?

- Can the BCR pathogen be transmitted in/on seed or by insects, does it have alternate hosts and does it survive in soil?
- Are seed treatments effective and is there papaya germplasm with resistance/tolerance to the disease?
- Does resistance to BCR in papaya germplasm in the greenhouse still persist in the field and are there morphological markers in papaya associated with resistance or tolerance to BCR.
- What are the phytoplasma groups/sugroups present in the Philippines?
- What are the symptomatic and asymptomatic alternate hosts of the phytoplasma *Candidatus* Phytoplasma *australiense*?
- Can BCR and dieback management options be designed for growers from varying backgrounds and capacity?

The fruit projects of the Philippines Horticulture program aimed to enhance the profitability of the mango, papaya, jackfruit and banana industries by the removal of the key constraints of pests and diseases, supply chain issues and production problems. The chosen fruit commodities and research and development priorities were identified by stakeholders at priority setting meetings with reference to national, regional and local strategies established by government agencies e.g. PCAARRD.

This project and others, involving mango, jackfruit and banana, with elements such as post-harvest constraints and value chains and communities, have the common goal of improving food security and the livelihoods of smallholder farmers in the southern Philippines. Pest and disease management in the fruit program has provided opportunity to integrate and to develop linkages between plant pathologists and plant biotechnologists within the various commodity groups. Collaborative partnerships have also developed between commercial and agri-business partners, such as Del Monte Philippines Inc., Sumifru and the Tupi growers association. These partnerships assisted in the implementation and commercialisation of project outcomes. The alignment of this project with other components of the Philippines Horticulture program is described in the following diagram.



The intent of the ACIAR horticulture program was to contribute to economic growth in the Philippines through increased income and improved livelihoods of smallholder farmers, including tropical fruit growers in the southern Philippines. Consequently, one of the targeted outcomes of the PCAARRD Tropical Fruit Industry Strategic Program Science & Technology Plan from July 2012 was to increase the incomes of smallholder papaya growers by reducing the negative impact of BCR on papaya production by improved IDM technology. Outcomes of this research provided Australian growers with knowledge and information for managing dieback and improving Australia's capacity to prevent the entry and establishment of the exotic disease BCR.

4 Objectives

The aim of this project was to increase the profitable and sustainable production of papaya fruit in the southern Philippines and Australia through the development of integrated disease management (IDM) strategies for the control of bacterial crown rot and dieback. The specific objectives were:

1. To characterize the Erwinia sp. associated with BCR and phytoplasma-like symptoms in papaya and their survival

- *Collect isolates of Erwinia sp. from BCR affected plants and assess their pathogenicity, aggressiveness and morphological and biochemical variations*
- *Determine the genetic diversity and distribution of these isolates using PCR techniques*
- *Develop species specific markers that can be used for disease detection*
- *Identify cause/causal strains of phytoplasma-like symptoms of papaya in the Philippines*

2. To develop and evaluate sustainable disease management strategies for BCR

- *Investigate the epidemiology of BCR of papaya*
- *Develop and perform studies on seed and insect transmission*
- *Develop and perform studies on the importance of alternate hosts and the survival of the BCR pathogen in soil*
- *Investigate possible seed treatments (e.g. heat and chemical)*
- *Screen papaya cultivars for resistance to BCR under greenhouse and field conditions and identify morphological markers for BCR resistance*
- *Develop disease management strategies based on knowledge gained*

3. To develop and evaluate sustainable disease management strategies for dieback

- *Collect and analyse samples of symptomatic and asymptomatic papaya for the dieback phytoplasma*
- *Collect samples of asymptomatic alternate hosts (weeds, native plants, adjacent crops) and use PCR techniques to determine the presence of Candidatus* Phytoplasma *australiense*
- *Develop disease management strategies based on knowledge gained*

4. To devise and disseminate a package of technology (IDM strategies) for papaya

- *Conduct regional collaborator/contractor workshops to help plan and review research activities and adoption processes*
- *Implementation of agreed extension programme*

5 Methodology

Objective 1. To characterize the Erwinia sp. associated with BCR and Phytoplasma like symptoms of papaya in the Philippines

1.1 Collect isolates of Erwinia sp., from BCR affected plants in papaya growing regions
An extensive survey of major papaya growing regions of the Philippines (Mindanao and Luzon) was held and samples were collected from BCR-infected and healthy papaya plants. These samples were analysed at UPLB using different media (KB, YDC, YPGA, NA and CPG) to determine the possible presence of BCR strains from different growing areas.
Samples from plants with Phytoplasma-like symptoms were collected from the Philippines (Laguna, Batangas, Cavite, Negros Occidental, Bukidnon, Davao, South Cotabato) from 2015-2018 and characterized using molecular techniques.

1.2. Assess the morphological and biochemical variations of these isolates
A number of tests were conducted to determine if morphological and biochemical variation existed among isolates and to confirm the identity of the bacterial isolates. These tests included; growing isolates on Kings medium B, Tetrazolium Chloride (TZC) medium and Yeast Extract Dextrose CaCO3 (YDC) medium, Gram stain, Hugh and Leifson test, carbohydrate (melibiose, lactose, l-arabinose, and mannitol) and citrate utilization tests. Acid production from different carbohydrates were tested. Fourteen carbon sources were utilized in this test (i.e. lactose, maltose, D-raffinose, trehalose, glucose, fructose, D-mannose, mellibiose, D-cellobiose, L-arabinose, mannitol, sorbitol, myo-inositol, and citrate). The carbohydrate utilization test (except for citrate) was observed from agar slants (Hayward, 1976) [$NH_4H_2PO_4$, 1.0g; KCl, 0.2g; $MgSO_4.7H_2O$, 0.2g; peptone,1.0g; bromothymol blue, 0.12g; agar, 3.0g; add water, 1L]. The stab inoculated agar slants were examined two days after inoculation or until the agar slants turn from olive green to yellow, which indicates acid production. For the utilization of citrate, Simmon's citrate agar [sodium citrate (anhydrous), 2.0g; $MgSO_4.7H_2O$, 0.2g; $NH_4H_2PO_4$, 1.0g; K_2HPO_4, 1.0g; NaCl, 5.0g; agar, 20.0g; bromothymol blue, 1% (w/v) in 50% ETOH 15.0mL; add water, 1.0L] was used. The streak inoculated plated media were examined two days after inoculation or until a change of color (green to blue) on medium was observed, which indicates the utilization of citrate.

1.3. Determine the pathogenicity and aggressiveness of the BCR isolates
Two month old 'Solo' papaya plants were artificially inoculated using a bacterial suspension 10^{8} cfu/ml obtained from a 24 hr old culture by the leaf clipping method under controlled glasshouse conditions (28-30 °C; RH - 85%). One week after the inoculation, BCR symptoms were observed and the aggressiveness of the isolates was scored using 0-4 disease rating scale. Koch postulates using papaya seedlings cv Solo and the hypersensitive reaction on tobacco plants were used to confirm the isolates were pathogenic (95).

The leaf abrasion inoculation technique was used to assess the BCR tolerance/susceptibility among different papaya varieties/lines under controlled plant-house conditions and in field tests. Twenty-five milligrams of Celite® (SiO2), a mild abrasive, was added to 100 ml of the bacterial suspension. The mixture was gently rubbed on the upper surface of the first and second youngest, fully expanded leaves. The disease was scored using the 0-4 disease rating scale described by Dela Cueva *et al*. (2017).

1.4. Determine the genetic diversity and distribution of the BCR isolates using PCR-based techniques
The DNA of *Erwinia* sp. BCR isolates was extracted and used for downstream applications, such as the detection of the bacterial pathogen, genotyping, and DNA

sequence analysis. The DNA extraction method was based on the manufacturer's instruction using QIAamp DNA mini kit (QIAGEN). The quality of the extracted DNA was observed on 1.2% (w/v) agarose gel, and the quantification of the extracted DNA was done by spectrophotometry.
Another genomic DNA extraction method (De Boer and Ward, 1985) was used for extracting the genomic DNA of *Erwinia* sp. BCR isolates *in planta*, as well as, *in vitro.* The isolates were sequenced for 16s rDNA (40), gyrB gene (35) and rpoB (24) and recA (24) genes.

16s rRNAgene sequence analysis of Erwinia sp. isolates

The following primer sequence was used for the amplification of the 16s rRNA region.

Primer name	**Primer sequence (5' - 3')**
27f	AGA GTT TGA TCM TGG CTC AG
1492r	TAC GGY TAC CTT GTT ACG ACT T

The PCR amplification was made using reagents; 1X PCR Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, .05 µM forward and reverse primer (27f and 1492r), 1 unit of Taq Polymerase. 38 cycles were performed in thermo cycler with the conditions: initial denaturation at 95ºC for 2 min, denaturation at 95ºC for 30 sec, primer annealing at 55 ºC for 30 sec and 72ºC extension for 2 min with a final 72ºC extension for 10 min.
A sequence of the 16S rDNA gene, BOX, REP and ERIC primers was used to determine genetic variation among BCR isolates from different regions.

Marker name	**Marker sequence (5'-3')**
REP2-1	ICG ICT TAT CIG GCC TAC
REP1R-1	III ICG ICG ICA TCI GGC
ERIC2	AAG TAA GTG ACT GGG GTG AGC G
ERIC1R	ATG TAA GCT CCT GGG GAT TCA C
BOXA1R	CTA CGG CAA GGC GAC GCT GAC G

The PCR reaction contained 1X PCR Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 1uM Forward primer (REP2-1, ERIC2), 1uM Reverse primer (REP1R-1, ERIC1R, BOXA1R), 1.25 units of Taq Polymerase. 38 cycles were performed in thermo cycler with the conditions: initial denaturation at 95ºC for 2 min, denaturation at 94ºC for 30 sec, primer annealing at 44ºC (REP) and 52ºC (BOX and ERIC) for 30 sec, extension at 65ºC for 1 minute with a final 65ºC extension for 10 minutes.
A neighbour joining tree and cluster analysis was performed by using UPGMA (Unweighted Pair-Group Method of Arithmetic average).

1.5. Develop species specific markers that can be used for disease detection
The marker genes 16s rRNA, gyrB, recA, rpoB and Whole Genome Sequencing (WGS) were used to differentiate/identify of BCR isolates. The sensitivity of the primer sets was tested on purified genomic DNA of BCR isolates and the specificity and detection limits of the primer sets were tested against *Erwinia* species, from artificially and naturally infected papaya plants.
Primer sequences derived from the 16s rDNA sequence of the Erwinia mallotivora.

Primer name	**Primer sequence (5' - 3')**
Forward primer	CCT GGA CAA AGA CTG ACG CI
Reverse primer	CGC TTC TCT TTG TAT GCG GC
Esp-for	CGA TCC CTA GCT GGT CTG AG
Esp-rev	GTT AGC CGG TGC TTC TTC TG
Emal-for	GTG GTG AGG TTA ATA ACC TT
Emal-rev	TTC CCG GCC GAA CCG ATG G

Primers derived from the gyrB, recA, and rpoB DNA sequence of the Erwinia mallotivora

Primer name	Primer sequence (5' - 3')
RPOB1F	CCT GAC GTT GCA TGT TTG CA
RPOB2F	GCC AAT CTC TGC TGC GGT GA
RPOB1R	GAA CAA CCC RCT GTC WGA GA
RPOB2R	GTA ACC AGG TCA TCG ACA AAG TG
RECA1F	GTG AAG ACC GCA CTA TGG AT
RECA1R	GAC TTC GTC ACC TTC TTT TA
GYRB1F	ACT AAG CGT GAC TGG CGA AA
GYRB1R	TAT TCG CCC AGC AAT TCA ATT

1.6. Characterize Phytoplasma like symptoms associated with papaya
The molecular based techniques 16srRNA gene sequencing and nested PCR were used to confirm the presence of phytoplasma in an outbreak of disease in papaya.

Objective 2. To develop and evaluate sustainable disease management strategies for BCR of papaya

Activities

2.1. Investigate the epidemiology of BCR of papaya
The controlled greenhouse and field experiments studied the influence of climate, cultivar, host, soil type, pathogen strain, vector and cropping practices on BCR incidence and severity. In glasshouse experiments, papaya seedlings (cv Sunrise Solo), seeds, soil and weeds were artificially inoculated and disease incidence/severity was monitored at weekly intervals. These tests were conducted to confirm the identity of the BCR pathogen. Disease monitoring surveys for BCR were conducted throughout the project period (2014-2018) to define the spread and progression of the disease in the Philippines commercial papaya growing areas. Multiple trial-sites (Laguna, Davao, Cagayan De Oro) were artificially inoculated to reduce the risk of failure due to a lack of disease. The monitoring of one field trial in Mindanao for BCR was linked to weather recordings in in the area.

2.2 Develop and perform studies on seed transmission
Most bacterial diseases are spread by contaminated seeds and planting material. The seeds from BCR infected plant were collected to determine if seed is the primary source of inoculum. The different parts of the infected seeds; sarcotesta (mucilage), seed coat and embryo were plated on KB media for the recovery of BCR pathogen.
Naturally infected and artificially inoculated seed were potted-up in the glasshouse under overhead misting, which is considered favourable for disease development.
Naturally infected fruit and artificially inoculated seed (12 hour soaking in a bacterial suspension) were used in these tests. The seedlings were observed daily for symptom development.

2.3 Determine the importance of alternate hosts to the survival of the BCR pathogen
Samples from symptomatic and asymptomatic weeds and other hosts growing in close association with BCR infected papaya plantations were analysed at UPLB for the BCR pathogen using semi-selective culture media. Species of monocots and dicots (tomato, guava, pineapple, *Synedrella* sp., *Portulaca* sp., *Commelina* sp., *Vernonia* sp., *Amaranthus* sp,) were assayed under glasshouse conditions for their ability to support epiphytic populations of the BCR pathogen by spray inoculation. Isolations were made in an attempt to recover the pathogen over time.

2.4 Determine if the BCR pathogen can survive in soil
Soil samples were taken at the base of BCR infected plants and analysed for the presence and populations of BCR pathogen. A pot experiment was set up in the

glasshouse under controlled conditions. Sterilized and non-sterilized soil was artificially inoculated with the BCR pathogen and periodic isolations (onto KB media) in an attempt to recover the pathogen from soil.

2.5 Determine the role of insects in disease transmission
The role of 'visiting' insects in initiating BCR symptoms was investigated to determine their part in BCR survival and transmission. Bacteria are known to overwinter in the gut of insect vectors. Therefore this study was conducted to determine if the BCR pathogen can survive within insects and be transmitted via feeding wounds, infected mouthparts or frass. Mites and mealy bugs were fed on artificially infested (BCR) papaya plants for three (3) weeks and then transferred to the healthy papaya plants. These plants were monitored daily for symptom expression and KB media was used for the recovery of the BCR pathogen and from insects.

2.6 Investigate possible seed treatments (e.g. heat and chemical)
The artificially inoculated seeds were treated with hot water at 50 and 52ºC for 10 and 15 minutes respectively and evaluated to determine their efficacy in the eradication of the BCR pathogen from papaya seed (being externally and/or internally seed-borne). 'Solo' papaya fruits bought from supermarkets were used for artificial seed inoculation. Seeds from the fruit were harvested and soaked in a bacterial suspension of *E. mallotivora*. After inoculation, the seeds were directly sown into sterile potting mix and allowed to germinate. Two weeks after sowing, the germination rate of uninoculated seeds was compared with the germination rate of inoculated seeds. Isolations from seed and newly germinated seedlings were made onto KB media. PCR tests were also conducted on this material.

2.7 Screen papaya germplasm for resistance to BCR under greenhouse and field conditions and identify morphological markers for BCR resistance
Experiments under field and screen house conditions were conducted to evaluate a wide range of papaya-inbred lines, segregating breeding populations and wild species of papaya for susceptibility to BCR. Resistance or tolerance to BCR was determined using a 0-5 disease rating scale (Dela Cueva *et al*. 2017). Individually selected plants/lines were hybridized to combine and increase the plant resistance to BCR.

Regrowth plants (plants which continued to grow following BCR infection) were subjected to scanning electron microscopy to assess the presence or absence of bacterial cells in or on the plant. Three different treatments were examined. These plants used were a one-month old regrowth plant, a BCR-infected plant and a healthy control. Thin cross sections of the stem of each treatment were prepared by cutting it with a sharp sterile scalpel blade. Two replicates for each sample were prepared.

The stem sections, about 5 mm thick, were collected from the three treatments mentioned above and subjected to primary fixation overnight using 2.5% glutaraldehyde, followed by buffer washing (5X) using 0.1M phosphate buffer, pH 7.2. Post-fixation using 1% osmium teroxide for 2 hours was done followed by buffer washing (5X) at 15-30 minute intervals. To gradually remove the water in the tissues, the sample was subjected to dehydration at 4ºC in ethanol series *i.e.*, 50%, 70%, 80%, 90% and 100%. After the second immersion in 100% ethanol under room temperature, the dehydrated samples were immersed in hexamethyldisilazane (HMDS) for about 10 minutes, and this was done twice. After which, the HMDS was decanted from the sample vial, and the sample was left inside the dessicator to air-dry overnight at room temperature. The dried samples were then mounted onto a scanning electron microscope sample stub with a double sided carbon tape. The samples were then sputtered with gold-palladium using an ion sputter (JEOL, JFC 1100). Scanning electron microscope (SEM) imaging was done using SEM-Phenom XL atr 10-15 kV accelerating voltage (Phenom-World B.V., Dillenburgstra at 9E, 5652 AM Eindhoven, The Netherlands) in Katrin Field Inc., Biñan, Laguna, Philippines.

2.8 *In vitro* propagation of BCR tolerant lines for in field assessment in major papaya growing areas
Plants of BCR tolerant lines (Solo regrowth '552' and '601') to be evaluated under greenhouse and in field conditions in Mindanao were multiplied up by tissue culture and virus indexed at UPLB in Luzon to satisfy the legal requirement of moving planting material from Luzon to Mindanao, which is a PRSV free area. In a field trial at BPI, Davao the breeding lines were assessed for yield and BCR tolerance. 'Solo' papaya (PPY 14) was included in the trial as the susceptible control plant.

2.9 Develop disease management strategies based on knowledge gained
The knowledge gained from objectives 1 and 2 were used to formulate an integrated disease management strategy for bacterial crown rot disease. Investigations were conducted into the epidemiology of the BCR pathogen, the agronomic practices which influenced disease development, the identification of disease tolerant/resistant material, the efficacy of different chemical and biocontrol agents and the development of commercially acceptable cultivars. The outcomes of this research were used to formulate an integrated disease management strategy. The development of this strategy involved all stakeholders and was evaluated in trials conducted at UPLB, BPI and commercial papaya plantations.
Recommendations developed in these trials were disseminated at grower field days and workshops in collaboration with the commercial grower organisations. PowerPoint presentations and brochures outlining the management options were distributed at field days and workshops.

Objective 3. To develop and evaluate sustainable disease management strategies for dieback of papaya

3.1. Collect and analyse samples of symptomatic and asymptomatic papaya
Eleven (11) commercial papaya plantations on the wet tropical coast and the Atherton Tableland-growing region of far north Queensland were assayed between June 2014 and November 2018 for *Ca.* P. *australiense* and *Ca*. P. *australasia* following the outbreak of disease (Appendix 5 Table 10). The papaya samples received from central Queensland and Western Australia were also tested for the phytoplasma diseases. Crop loss assessments was made to determine the level of infestation. A total of 60-80 mg of midrib or leaf sample was used for each genomic DNA extraction. Samples were screened for the presence of phytoplasma using real time and nested PCR procedures. The procedures for sample collection, preparation, DNA extraction and PCR protocol and PCR purification are described in the Papaya phytoplasma manual (Appendix 5).

3.2 Collect samples of alternate hosts (weeds, native pants and adjacent crops)
Between June 2014 and November 2018, 183 samples of weeds, native plants, and adjacent crops, representing at least 76 different species, growing adjacent to symptomatic and asymptomatic, commercially grown, papaya plants were collected from the field (Appendix 5 Table 11). Identification of alternate papaya phytoplasma hosts aims to provide a better understanding of papaya disease epidemiology to develop effective disease management strategies.

Samples were botanically identified and the midrib dissected out for genomic DNA extraction where possible. Whole leaves were used on samples with no midrib or where leaves were too small to dissect out the midrib. The procedures for sample collection, preparation, DNA extraction and PCR protocol, and PCR purification are described in the Papaya phytoplasma manual (Appendix 5).

3.3 Collect *and screen insect vectors from papaya plantations and surrounding vegetation*

Yellow sticky traps and a commercial, petrol-driven Stihl vacuum-blower (with a fine nylon mesh collection bag fitted to the intake tube) were used to collect putative insect vectors from the field. Phloem-feeding insects were targeted for phytoplasma detection (Appendix 1 Table 12). Insect samples were stored in 100% ethanol at room temperature prior to DNA extraction and voucher specimens of leafhoppers have been deposited at the Orange Agricultural Institute. The procedures for sample collection, preparation, DNA extraction and PCR protocol, PCR purification and insect barcoding are described in the Papaya phytoplasma manual (Appendix 5).

3.4 Develop disease management strategies based on knowledge gained
The knowledge gained from activities 3.1, 3.2 and 3. 3 were used to formulate an integrated management strategy for dieback, yellow crinkle and mosaic disease of papaya. Plant mortality counts and yield assessments were made to highlight the benefits of the disease management strategy.

Objective 4. To devise and disseminate a package of technology (IDM strategies) for papaya

Activities
4.1 Conduct stakeholder planning, review and adoption meetings
Meetings with regional collaborators, commercial growers and contractors were organised with the aim of reviewing the current knowledge of BCR, possible BCR management strategies and production technology in papaya. This involved discussions with different stakeholder groups operating in Davao, collaborators from Del Monte, Sumifru, UPLB and farmers. Researchers and interested farmers participated in disease identification and management workshops and co-operated with researchers in the development of BCR management practices. It is expected that these farmers will mentor other growers in their communities and facilitate the dissemination of best practices for the management of BCR of papaya. For smallholder farmers, improvements in simple farming practices were considered the most appropriate way of managing plant disease in the short term. The establishment of trials on farms with collaboration from farmers, researchers and extension officers enabled the growers to understand the biology and epidemiology of the control measures and observed changes in the incidence and severity of the disease. The on-farm trials improved the communication between farmers and extension staff and have strengthened relationships for future collaborations.

4.2 Implementation of agreed extension program
Activities included regional disease detection and management training for technicians, growers, contractors and consultants. A training manual and posters were prepared and information was delivered to growers through highlighting the research results, demonstration (best bet) of management practices, training and mentoring. Commercial partners Del Monte and Sumifru actively participated and contributed improvements in the management of BCR and phytoplasma diseases. The research outcomes were discussed in national workshops, conferences and paper(s) published in reputed scientific research journals.

6 Achievements against activities and outputs/milestones

Objective 1: To characterize the Erwinia sp.associated with BCR of papaya in the Philippines

No.	Activity	Outputs/ milestones	Due date of output/ milestone	Comments
1.1	Collect isolates of *Erwinia* sp. from papaya growing regions (PC)	24 papaya growing areas were surveyed and 95 *Erwinia* isolates were recovered	January 2018	Major papaya growing regions of the Philippines; Mindanao and Luzon surveyed for Bacterial Crown Rot disease (BCR). **Mindanao:** Bukidnon, Misamis Oriental, Davao del Sur and South Cotabato. **Luzon:** Cagayan, Isabela, Nueva Vizcaya, Quirino, Batangas, Cavite, Laguna, and Quezon. 95 isolates collected from different BCR-infected plants and soils d from different provinces identified as *Erwinia* sp. BCR incidence observed in Batangas, Cavite, Laguna, Quezon, Bukidnon, Misamis Oriental, Davao del Sur, and South Cotabato.
1.2	Assess the morphological and biochemical variations of isolates (PC)	63 out of 92 isolates tested to determine variation among isolates	January 2018	No morphological and biochemical variation observed among 63 BCR isolates. White, smooth, round colonies produced on KB medium. Isolates tested KOH positive and showed ability to utilize glucose, melibiose, l-arabinose, mannitol, citrate but not lactose. No blue pigment produced on KB medium (an indicator of *Erwinia papaya*)*;* but occasional blue pigmentation observed in refrigerated stored cultures. The BCR pathogen can grow at 27 –36 ^{0} C. Biochemical tests identified the Philippines BCR pathogen as being closely related to the Malaysian isolate (MARDI strain) *Erwinia mallotivora.*
1.3	Determine the pathogenicity and aggressiveness of isolates (PC)	Tested pathogenicity of 95 isolates under controlled conditions	January 2018	Sixty-four isolates identified as *E. mallotivora* found pathogenic on papaya. Inoculated susceptible variety; 'Solo' showed symptoms 2-4 days after inoculation. Isolates showed variation in aggressiveness but no correlation was found in relation to their source or geographic location. No soft rotting observed on carrot and potato slices. None of the other tested crops, tomato, corn, banana, melon, cassava, and guava grown near papaya were found to be alternate hosts of the BCR pathogen.
1.4	Determine the genetic diversity and distribution of isolates (PC)	16 s rDNA sequencing of 4 BCR isolates confirmed an identity of BCR disease (PHP) BOX, REP and ERIC primers optimized for	January 2018	4 isolates from BCR affected plants from Laguna, Batangas and South Cotabato were sequenced and showed 99% similarity to the Malaysian strain (*E. mallotivora*) of BCR**.** Little variation was observed among the tested isolates (EP25, EP27, EP59 and EP61) using BOX, REP and ERIC primers. In addition, gene coding for 16s rRNA, gyrB, recA and rpoB were amplified using primers developed from sequences obtained from

		genetic variation studies		NCBI. No nucleotide variation was observed among *E. mallotivora isolates* The 16s rRNA gene of *Erwinia* isolates were amplified using 27f/1492r primers by conventional PCR. The amplified products were sequenced at AITBIOTECH facility. BLAST analysis of 16s rRNA showed 99% similarity to the Malaysian *E. mallotivora* BT-MARDI strain of papaya BCR.
1.5	Develop species specific markers to use in BCR detection (PC)	DNA from 60 BCR isolates have been extracted and 2 primers are synthesised and being validated.	January 2018	DNA from 60 BCR isolates was extracted and 2 primers were synthesised and evaluated for accurate BCR diagnostics. Primers developed from sequences of gyr B proved a promising marker for the specific detection of *E. mallotivora*.
1.6	Characterize Phytoplasma like symptoms (PC)	Nested / qPCR performed	November 2018	Incidence of Phytoplasma disease in Mindanao was confirmed using nested PCR. 16S rRNA gene results and blast analysis showed the association of 16Sr II group; "*Candidatus* .P. *aurantifolia*, with a known Philippine strain of Phytoplasma causing papaya crown yellows. This is a reoccurring disease in the area due to drier weather conditions and an increase in vector activity. Phytoplasma dieback disease was not recorded.

PC = partner country, A = Australia

Objective 2: To develop and evaluate sustainable disease management strategies for BCR of papaya

No.	Activity	Outputs/ Milestones	Completion date	Comments
2.1	Investigate the epidemiology of BCR of papaya (PC)	Information and data recovered assisted in the development of BCR management strategies	October, 2018	Wet summer season i.e. rain at night providing prolonged leaf wetness and warm temperatures are critical for BCR development. The average monsoon rainfall dropped three fold in the major papaya growing area (June - Sept 2014 -1145 mm to 403 mm in 2018) and resulted in a low or no BCR; this negatively affected in-field experiments. https://www.worldweatheronline.com/cagayan-de-oro-weather-history/cagayan-de-oro/ph.aspx
2.2	Develop and perform studies on seed transmission (PC)	The information and data recovered assisted in the development of BCR management strategies	January, 2018	Seed transmission studies conducted at UPLB were unable to confirm that BCR can be transmitted in or on papaya seed. Numerous studies including extraction of seed from naturally infected and artificially inoculated fruit and the culturing of different seed parts on culture media were unable to recover the pathogen. Seedlings grown from seed from infected fruit and artificially inoculated seed did not express BCR symptoms and the pathogen could not be detected using molecular tests.

2.3	Determine the importance of alternate hosts to the survival of the BCR pathogen (PC)	The information and data recovered assisted in the development of BCR management strategies	January, 2018	The BCR pathogen was not recovered from weeds in and around papaya plantations. No BCR symptoms were expressed on artificially inoculated test weeds (8) and other crops (7 two weeks after inoculation. However the BCR pathogen was recovered from *Amaranthus spinosus, Amaranthus viridius, Syndrella nodiflora,* and *Achelpha indica.and Commelina benghalensis* 4 days post inoculation. However, the recovery of BCR colonies was reduced by half in *Commelina benghalensis*, which suggested that the BCR pathogen could survive on weeds but the survival rate may vary with different hosts.
2.4	Determine if the BCR pathogen can survive in soil (PC)	The information and data recovered assisted in the development of BCR management strategies	October, 2018	None of the plant; with and without root injury expressed BCR symptoms when transplanted to pots containing soil mixed with chopped BCR-infected leaves and stem. However, more colonies of the BCR pathogen were recovered from soil containing chopped BCR plant material indicating the BCR pathogen can survive longer in plant tissues compared to bare soil.
2.5	Determine the role of insects in disease transmission (PC)	The information and data recovered assisted in the development of BCR management strategies	October,2018	Healthy papaya seedlings developed BCR symptoms after exposure to red spider mites and mealy bugs which were reared on BCR infected plants.
2.6	Investigate possible seed treatments (PC)	An effective seed treatment method identified	October, 2018	Hot water treatments had no adverse effect on the germination of inoculated and non-inoculated seed. Hot water seed treatment (52 ºC for 10 minutes) is recommended to eliminate the possibility of the BCR pathogen being transmitted in seed.

2.7	Screen papaya germplasm for resistance to BCR (PC)	Papaya cultivars/breeding lines showed considerable tolerance to BCR under glasshouse and field conditions	2017	Ten regrowth and seven non-regrowth BCR tolerant breeding lines were selected and these were self and sib-pollinated in the screen house before being grown in the field.
		Scanning electron microscopy used to differentiate between tolerant and susceptible genotypes	2017	Scanning electron microscopy was used to check for the presence of the BCR pathogen in regrowth tolerant selections and susceptible and healthy controls. Vascular tissues of regrowth lines were found to be bacteria free; however, the vascular tissue of susceptible/infected plants was clogged with bacterial cells.
		Hybridization of BCR tolerant lines and PRSV tolerant lines	2018	S1 lines were evaluated, selected and self- or sib- pollinated. S2 lines were produced and these were planted in the field. S1 Solo lines were embryo cultured virus indexed and sent for inclusion in the IDM trial to BPI, Davao.
		Evaluation of the genotypes under field conditions	2018	Five F1 hybrids, (BCR x PRSV), two three-way crosses and two BC1F1 papaya genotypes were evaluated and advanced to a field trial. Five F2 lines were evaluated for horticultural traits and BCR tolerance. The lines were found moderately tolerant to PRSV but showed a differential reaction to BCR. The disease incidence ranged between 60-100% during the rainy season in 2018.This suggested that lines were segregating and require further selection and stabilisation. Many infected plants showed regrowth at flowering (November 2018) and at the writing of this report are being self-pollinated to advance and stabilise the selected F2 lines.

2.8	Develop disease management strategies based on knowledge gained (PC)	BCR tolerant/ Solo (PPY14) and solo regrowth tested for BCR disease at BPI, Davao ID Field trial IDM Del Monte Manolo Fortich, Bukidnon research station Planting material raised through tissue culture and virus indexed for Field trial (Solo regrowth 552 and 601) at BPI, Davao	2017 2017 2018	Four field trials on BCR management were conducted between 2016-2018 at Del Monte Camp Philips and the BPI research facility in Davao. All trials included a farmer's practice which was compared with treatments designed by DAF,UPLB,Del Monte,and BPI. No BCR infection was observed in the 2016-17 field trial due to prolonged dry weather in Mindanao. Plants in the guard rows of the trial were artificially inoculated one week before planting the datum rows in the 2017-18 field trial to ensure BCR infection. As a result of this work, foliar sprays of *Bacillus subtillus* QST 713 and phosphorous acid were recommended as prophylactic sprays to control BCR at field days, growers meetings and the ACIAR-PACCRRD annual meetings and workshops.

PC = partner country, A = Australia

Objective 3: To develop and evaluate sustainable disease management strategies for dieback of papaya

No.	Activity	Outputs/ Milestones	Completion date	Comments
3.1	Identify weeds, native plants and crops growing close to papaya (A)	Plant hosts identified	October 2018	Between June 2014 and October 2018, 118 phytoplasma positive samples, from eleven locations, were selected and sequenced (16S rRNA gene sequence) for phytoplasma identification. Three phytoplasma-causing organisms belonging to two 16 Sr phytoplasma groups; 16 Sr II and 16 Sr XII were identified from symptomatic papaya. • *16 Sr XII* *Ca.* P. *autraliense (PDB)* • *16 Sr II* *Ca.* P. *australasia (PpYC , PpM and ALuY)* *Ca.* P. *aurantifolia (Chick pea little leaf)*

3.2	Screen plants for 'dieback' (PDB) Phytoplasma (A)	Alternate host/s of the 'dieback'/yellow crinkle and mosaic phytoplasma identified	October 2018	Between June 2014 and October 2018, 183 samples of weeds, native plants, and alternate crop plants, representing at least 76 different species, growing adjacent to symptomatic and asymptomatic, commercially grown, papaya plants, were collected from the field. Samples were assessed for the presence of phytoplasma using real-time PCR and confirmed through nested PCR and sequencing. Fifteen symptomatic weeds tested positive for phytoplasmas associated with papaya diseases. These weeds have been identified as potential alternate hosts for papaya phytoplasma diseases. ***PDB:*** *Portulaca pilosa and Solanum nigrum/americanum* ***PpYC and PpM:*** *Peripleura diffusa, Cajanus cajan, Crotalaria juncea*, *Phyllanthus fuernrohrii*, *Praxelis clematidea*, one weed from the Lamiaceae family and one from the Fabaceae family ***ALuY*****:** *Cajanus cajan, Praxelis clematidea, Stylosanthes scabra* and one sample from the Lamiaceae family. One weed, A weed sample of *Stylosanthes scabra* was positive for an unidentified 16 S group of phytoplasma causing 'Stylosanthes little leaf disease' (StLL).
3.3	Screen possible vectors for dieback disease (A)	Vector of the 'dieback'/yellow crinkle and mosaic identified	October 2018	Three insect samples, from 17 sampling times, detected positive for phytoplasma using molecular techniques. ***PDB*****:** Leafhopper *Orosius sp found identical to PDB* ***PpYC and PpM:*** Leafhoppers and plant hoppers Cicadellids: *Cicadulina bimaculata and Baclutha incisa* (composite sample using real time PCR). Delphacids: *Falcotoya aurinia* Fennah *Orosius orientalis* and Lace Bug (*Tingidae).* ***AluY*****:** *Orosius orientalis* *Orosius* sp are confirmed vectors, while others insect vectors detected positive for the first time for papaya phytoplasma diseases. Insect vectors require transmission studies to confirm if they are vectors/carriers of PpYC and PpM.

3.4	Develop disease management strategies based on knowledge gained	Advice on disease control given to growers	October 2018	**BCR IDM strategies:** The research findings suggested that the BCR pathogen has a limited host range and poor survival in soil and on plant surfaces. The BCR pathogen survives in infected plant parts and requires continuous wet weather and high humidity for disease development and dissemination. Bacteria enter through natural openings and wounds and are spread by wind-blown rain and insect-pests. The optimum temperature for disease development ranges between 18-34 ºC. Infection does not occur above 35 ºC. IDM strategies for BCR include; the use of clean/tolerant planting material, avoiding the use of stem cuttings from infected field plants, the use of a hot water seed treatment (52 ºC for 10 minutes) by seed suppliers), the rouging of infected plants and removal of plant debris and the management of insect pests and weeds. The use of prophylactic copper sprays, crop hygiene and sanitation foliar sprays of *Bacillus subtillis* QST 713 strain and phosphorous acid, and timely r applications of fertiliser is recommended. **Phytoplasma IDM strategies:** A Phytoplasma disease survey and the use of molecular techniques confirmed the presence of dieback, yellow crinkle, mosaic and Australian lucerne yellows phytoplasma with papaya symptomatic plants. The association of Australian lucerne yellows with papaya is probably a new disease record in north Queensland Australia. The positive detection of papaya phytoplasma causing organisms from weeds and other crops and insect vectors showed the potential for a disease reservoir within papaya plantations and in papaya growing areas. The IDM strategies for phytoplasma includes; use of disease free planting material, rogueing of infected plants, weed management, regular monitoring for crop and insect vector populations in the area, and the timely application of insecticide sprays. Other management options discussed with growers include the use of border trap crops, crop netting, physical barriers to control insect movement, barrier sprays to prevent the vector feeding and reflective mulches to deter the insect vector.

PC = partner country, A = Australia

Objective 4: To disseminate a package of technology (IDM strategies) for papaya

No.	Activity	Outputs/ Milestones	Completion date	Comments
4.1	Conduct annual stakeholder planning meetings (PC & A)	Meeting with project collaborators and stakeholders were conducted twice a year. Research outcome discussed and research activities planned.	October 2018	**Philippines:** The training of technicians and students was conducted to assist in the evaluation of germplasm, phytoplasma diagnostics and bioinformatics. At meetings conducted twice a year, research findings were discussed with the Tupi papaya growers, students and industry partners East-West Seeds, Del Monte and Sumifru. IDM trials were conducted at the Del Monte and BPI research facilities at Camp Philips and Davao respectively. **Australia:** Field visits, field days, seminars and workshops informed papaya growers and researchers of methods for the early field diagnosis of phytoplasma related diseases, accurate detection methods and knowledge of disease management options targeting insect vectors and alternate hosts. Mentoring, training, phytoplasma diagnostics services provided through-out the project.
4.2	Implementation of agreed extension program (PC & A)	Formed a framework agreed by stakeholders. Activities planned/ developed revised feedback gathered. Research activities clarified and agreed to Two seminar workshops were conducted at UPLB and at Cagayan de Oro for research update and symptom identification	November 2018	Two workshops were conducted at UPLB and at Cagayan de Oro for a research update and advice on disease identification. Workshop manuals, posters, PowerPoint presentations and grower information reports were produced. Mentoring, field visits and the training of junior staff was conducted throughout the project. Field days, seminars and a workshop were organised for papaya growers and researchers to aid in the early field identification of phytoplasma related diseases, accurate detection methods and knowledge of disease management options targeting insect vectors and alternate hosts. 40 meetings (monthly) with growers, and Papaya Australia, Biosecurity Queensland and HIA were conducted at Mareeba, Cairns, Brisbane and South Johnstone Research Station. Updated research findings and increased grower's knowledge in relation to symptoms, epidemiology, planting time, monitoring of alternate hosts, insect vector populations and the timing disease management options targeting insect vectors and alternate hosts.

4.3	Coordination of final report (PC & A)	Reports completed on time	Jan, 2019	Compiled project reports and made presentations on time for project reviews.

PC = partner country, A = Australia

7 Key results and discussion

Bacterial Crown Rot (BCR) disease survey and sample collection

The survey of major papaya growing areas in the Philippines showed the incidence of BCR in Luzon (Batangas, Cavite, Laguna, Quezon) and in the Mindanao region (Bukidnon, Misamis Oriental, Davao del Sur, and South Cotabato).

Initial symptoms of BCR were dark-green water-soaked lesions on almost all parts of the papaya plant including the stem, fruit, petiole and leaves (Figure 1). These early symptoms later developed into the typical rot and decapitation of the crown, commonly referred to as the 'pencil-like symptom' or 'toppling down' of the upper portion of the papaya plant (Figure 2).

In some cases, yellowing of the leaves was observed on BCR-infected plants. It was also noticed that some papaya plants recovered from the disease and produced side shoots (Figure 3). The BCR symptoms in the Philippines were observed as being similar to the papaya bacterial diseases dieback, decline and canker, which are reported in other papaya producing countries and caused by an *Erwinia spp.*

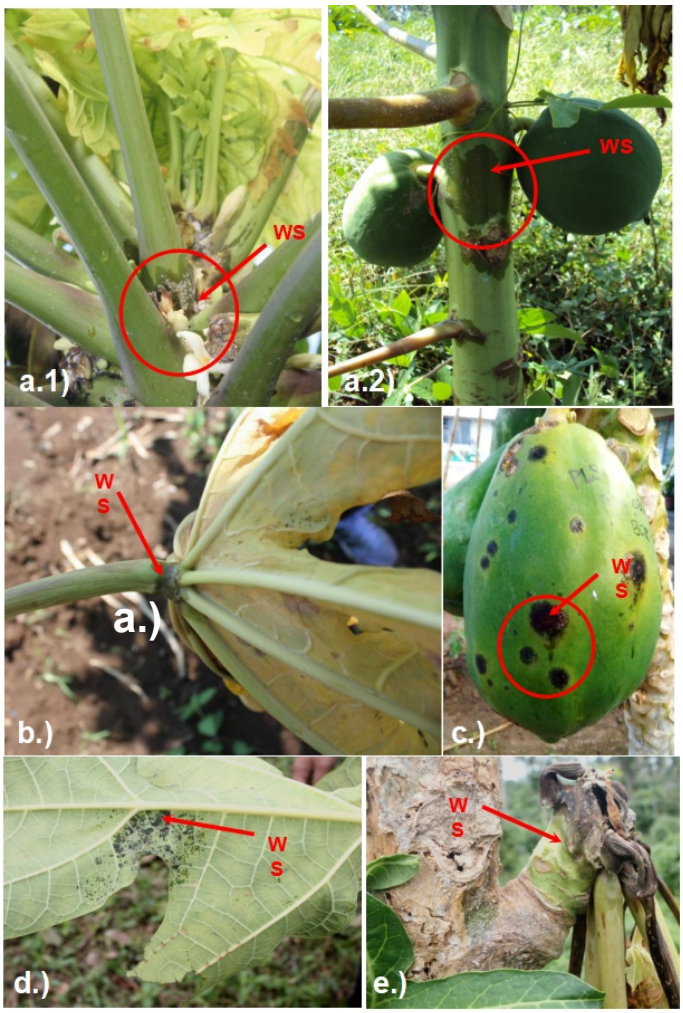


Figure 1 Dark green, water-soaked (WS) lesions on BCR infected papaya plant.1) stem (between leaf axil and stem, a.2) internode, b) petiole, c) fruit, d) leaves, and in some cases, e) side shoots

Figure 2 Crown rot of BCR infected papaya plant a) toppling down of the stem b) forms 'pencil-like' appearance

Figure 3 BCR infected papaya plant showing decapitation and side shoot growth

Characterization of the BCR isolates

Ninety five (95) bacterial isolates were subjected to genus identification tests, which included; growth on Yeast extract-Dextrose-$CaCO_3$ (YDC) medium, Gram staining, Hugh and Leifson test, and (Schaad et. al; 2001) hypersensitive reaction response (tobacco plant), potato soft rot test followed by Koch's postulate test on papaya (cv. Solo). Out of 95 BCR isolates, only 64 isolates were confirmed as *Erwinia sp.* These isolates produced white colonies with an entire margin on YDC medium, were Gram negative and showed a fermentative (Hugh and Leifson test) reaction (Figure 4).

Papaya seedlings inoculated with a suspension of the *Erwinia* sp. isolates showed water-soaked lesions around the point of inoculation two days after inoculation. Some leaves of infected plants were observed to turn yellow and fell off. Other leaves were found to turn brown when the green, water soaked lesions coalesced. The premature abscission of leaves and cessation of apical growth caused "pencil-point symptom" to occur. The same bacterium was re-isolated from inoculated papaya seedlings and the infected papaya stem. This confers with the results of other authors (Gardan *et al.,* 2004; Olabiyi, 2010), which showed pathogenicity of their test isolates and confirmed Koch's postulate.

The remaining 31 isolates were not able to infect papaya seedlings. Only 64 *Erwinia* isolates elicited a hypersensitive reaction on tobacco leaves. Potato slices inoculated with the bacterium failed to develop soft rot after 3 days indicating that the BCR pathogen belongs to non-soft rot group of *Erwinia* species. Only a light discoloration (browning) around the inoculation site was observed on papaya fruit slices (Figure 5).

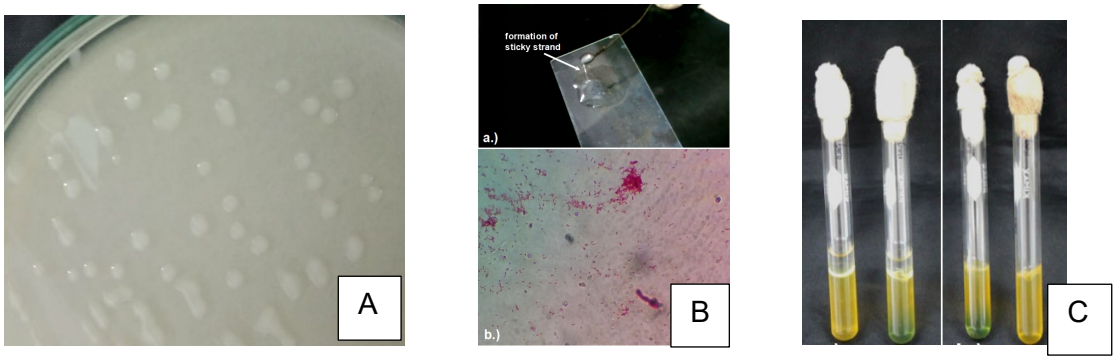


Figure 4 BCR isolate characterization A. white colonies on YDC medium B Gram negative Gram negative reaction a) formation of sticky strand from KOH test and b) a reddish pink-stained bacterial cells C. Glucose utilization of *Erwinia* sp. isolates in both a) anaerobic (with mineral oil) and b) aerobic (without mineral oil) conditions on Hugh and Leifson (O/F) test.

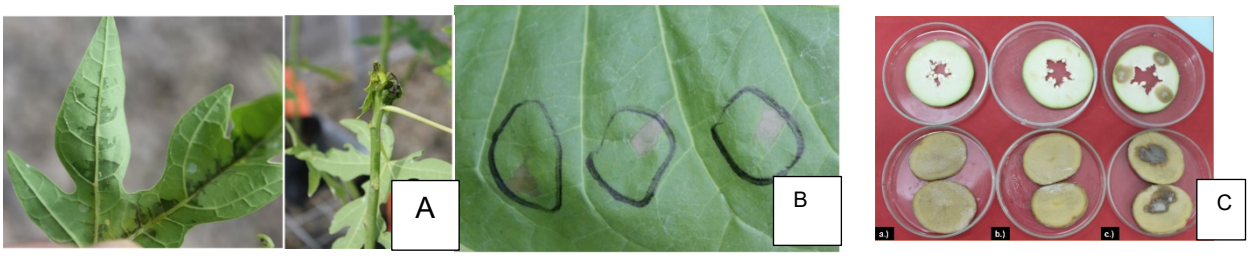


Figure 5 Pathogenicity test; Dark green water-soaked lesions on inoculated seedling (Cv. Solo); A. leaves and stem B. Necrotic lesions elicited by the *Erwinia* sp. isolates on tobacco leaves showing hypersensitive reaction C. soft rot test on sliced papaya and potato a) water (negative check); b) inoculated with *Erwinia* sp.;and c) Inoculated with *E. carotovorum*.

Assess the morphological and biochemical variations among BCR isolates

The colony morphology of 64 BCR isolates of *Erwinia sp.* was assessed using different bacteriological media e.g. King's medium (KB), yeast-peptone-glucose-agar (YPGA), yeast-dextrose-$CaCO_3$ (YDC) medium, nutrient agar (NA) and casein-peptone-glucose (CPG) medium.

KB medium was used to confirm if the *Erwinia* sp. isolated from BCR-infected papaya in the Philippines f was *Erwinia papayae,* a novel species of bacterial canker of papaya, which produces a non-diffusible blue pigment on KB medium. No variation in colony morphology was observed among the BCR isolates *Erwinia* sp. (Figure 6). The colony characteristics of the BCR isolates on bacteriological media were as follows:

> KB – the colonies were opaque, white, glistening, convex, round, and had an entire margin. None of the BCR isolates were found to produce non-diffusible blue pigment on KB media.
>
> YDC medium –colonies were fluidal and white in color, sometimes with brownish tinge, with an entire margin.

YPGA medium - colonies were white in color, convex, with an entire margin. Some colonies were round to irregular in shape.

NA medium - colonies were large, white in color, somewhat resemble an egg and had irregular shape.

CPG medium - colonies were small, white in colour, convex, round, with an entire margin.

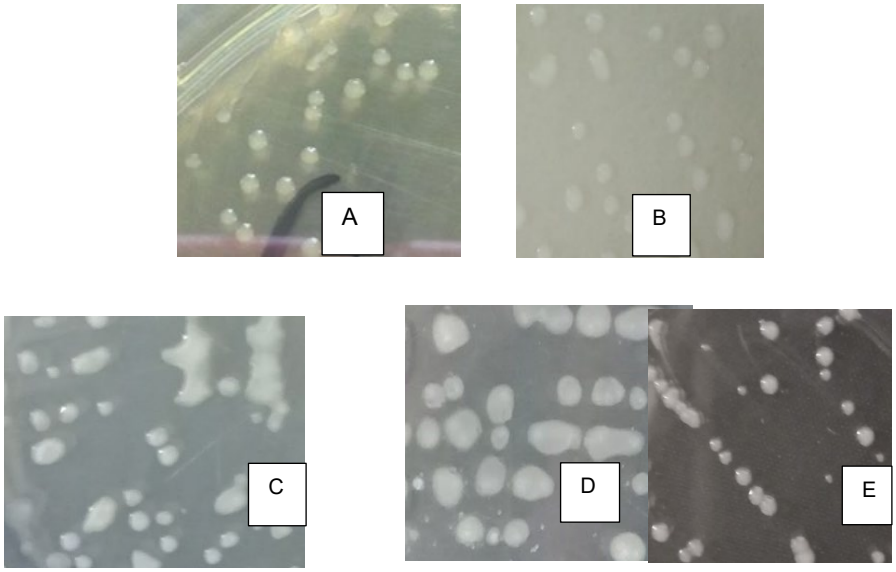


Figure 6 Three day-old colonies of a representative *Erwinia* sp.on different media; **A.** KB **B.**YDC **C.**YPGA **D.**NA **E.**CPG

Assess biochemical variations among the Erwinia sp. isolates

The results of carbohydrate utilization and citrate utilization tests revealed no biochemical variation among the BCR isolates. All isolates utilized mannitol, myo-inositol, L-arabinose, D-mannose, glucose, mellibiose, and trehalose (Figure 7 A and Table 1) after 2 days; however delayed acid production was observed from myo-inositol after 4 or 5 days. All BCR isolates showed the ability to utilize citrate as a carbon source, which was indicated by a change in colour of the media from green to blue (Figure 7 B).

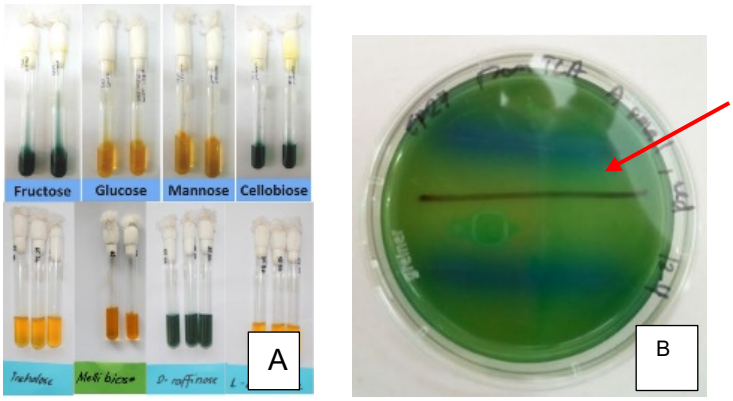


Figure 7 A. Carbohydrate utilization **B.** Citrate utilization test of *Erwinia* sp.

Table 1 Carbohydrate utilization by *Erwinia* sp. isolates

Carbohydrate	Reaction	Carbohydrate	Reaction
mannitol	+	fructose	-
myo-inositol	+	mellibiose	+
sorbitol	-	glucose	+
L-arabinose	+	D-raffinose	-
lactose	-	trehalose	+
maltose	-	cellobiose	-
D-mannose	+	citrate	+

The morphological and biochemical tests revealed that *Erwinia* sp. isolated from BCR-infected papaya plants in the Philippines does not produce non-diffusible blue pigment on KB medium similar to *Erwinia mallotivora* and *Erwinia caricae*, a novel species coined by Obrero (1980). There were some noticeable differences between the *Erwinia* sp. (Philippine isolate) and *E. caricae* and and *E. mallotivora* in the utilization of certain carbohydrates (Table 2).

Table 2 Comparison of *Erwinia* isolates subjected to a range of biochemical tests (Amin et al 2011).

Characteristics	*Erwinia* sp.	*Erwinia mallotivora*	*Erwinia papayae*	*Erwinia caricae*	*Erwinia psidii*
	(Philippine isolate)	(D-BiTE)	(Gardan et al., 2004)	(Obrero, 1980)	(D-BiTE)
Blue pigment on KB medium	-	nd	+	nd	nd
Carbohydrate utilization					
Fructose	-	nd	+	nd	nd
Glucose	+	+	nd	+	+
myo-Inositol	+	-	nd	+	+
L-arabinose	+	-	+	+	nd
Lactose	-	-	nd	-	-
Maltose	-	-	-	+	nd
Mannitol	+	+	-	+	+
Sorbitol	-	-	-	-	+
Trehalose	+	+	+	+	-
D-Raffinose	-	-	-	-	-
Mellibiose	+	-	-	+	nd
Cellobiose	-	-	nd	+	-
D-Mannose	+	+	+	+	+
Carbon source					
Citrate	+	+a	-	+	nd

Evaluation of inoculation techniques for the screening of papaya accessions/germplasm for BCR tolerance/susceptibility

Different inoculation techniques; pricking, leaf abrasion, stem injection and drenching were evaluated on papaya seedling (cv. Solo). All inoculation techniques reproduced the BCR symptoms after two days of inoculation. However, the leaf abrasion technique reproduced uniform and similar symptoms to BCR-infected papaya plants in the field and was used in germplasm screening for BCR tolerance or susceptibility. Inoculated plants showed both small pin-prick sized and angular, dark green, water soaked lesions on the surface of the leaves (site of inoculation) two days after inoculation (Figure 8). At 4-9 days, the dark green, water soaked lesions enlarged and the disease progressed from the leaves to the petiole,

and eventually to the stem (Figure 9, Table 3). The crown of the plant toppled down and complete rotting was observed from 10-11 days after inoculation.

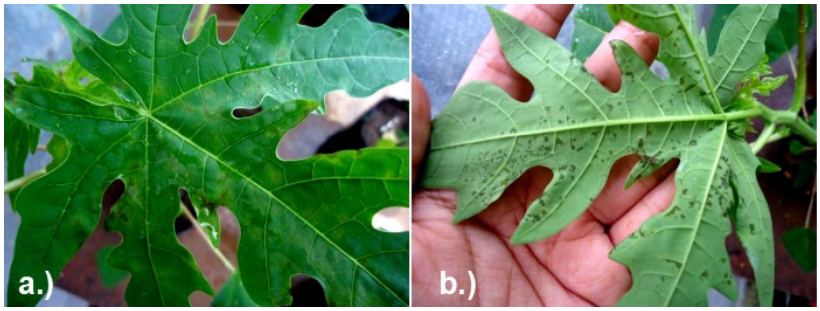


Figure 8 Inoculated leaves of papaya by leaf abrasion showing dark green, water soaked lesions on the a) adaxial (top) and b) abaxial (bottom) surface at 4 days after inoculation.

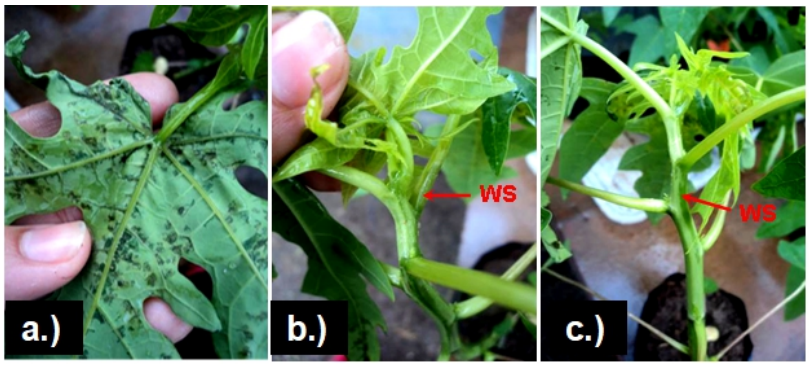


Figure 9 Disease progress observed on the a) leaves, b) petiole, and c) stem at 7 days after inoculation.

Table 3 Evaluation of different inoculation techniques used in the screening of papaya germplasm for bacterial crown rot tolerance/susceptibility

Method	Initial symptom after inoculation	Complete rotting after inoculation
Injection	2 DAI	9 DAI
Pricking	2 DAI	9 DAI
Leaf Clipping	2 DAI	9 DAI
Drenching	9 DAI	14 DAI
Leaf abrasion	2 DAI	11 DAI

Determine the genetic diversity among BCR Erwinia sp. isolates

The genotypic diversity of the *Erwinia* sp. BCR isolates was determined through rep-PCR using REP, ERIC, and BOX markers. The repetitive element sequence-based PCR (rep-PCR) markers (e.g. BOX, REP, and ERIC) used to amplify repetitive elements that occur in multiple copies throughout the genome. The rep-PCR was proven a discriminatory tool for the delineation or genotyping of bacterial species.

The REP, BOX, and ERIC fingerprints of representative isolates did not show much fingerprint variation among the *Erwinia* sp. BCR isolates (Figure 10).

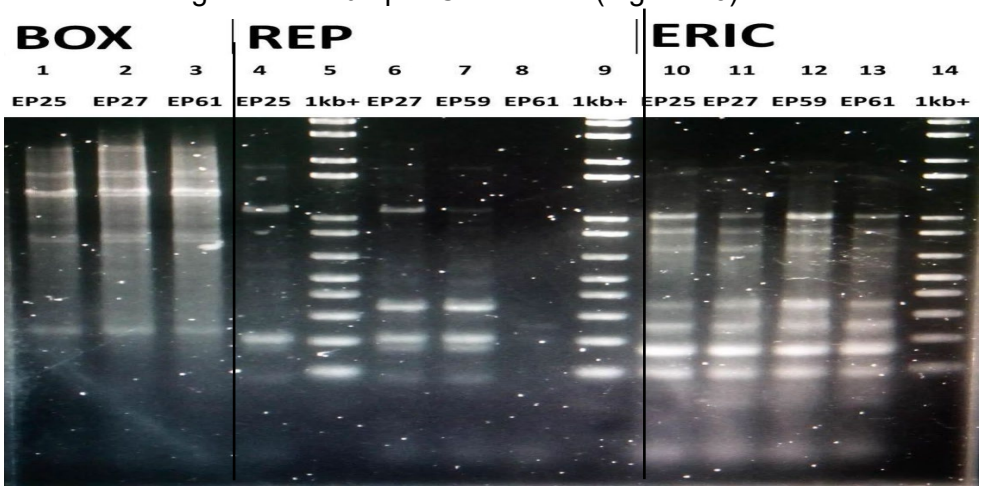


Figure 10 BOX, REP, and ERIC fingerprints of representative *Erwinia* sp. isolates (EP25, EP27, EP59, and EP61)

Amplification and sequence analysis of 16s rRNA on *Erwinia* sp. isolates
The 16s rRNA region of the *Erwinia* sp. BCR isolates using 27f/1492r (universal primers) was successfully amplified and showed expected near full-length band size of **~1,500bp (Figure** 12). The 16S rRNA gene is used in reconstructing phylogenies of bacteria, due to the slow rates of evolution of this region. The attachment site of 27f/1492r primers is shown in Figure 11.

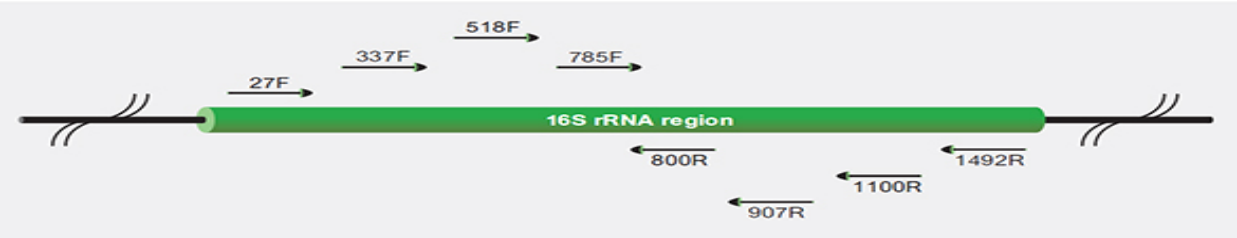
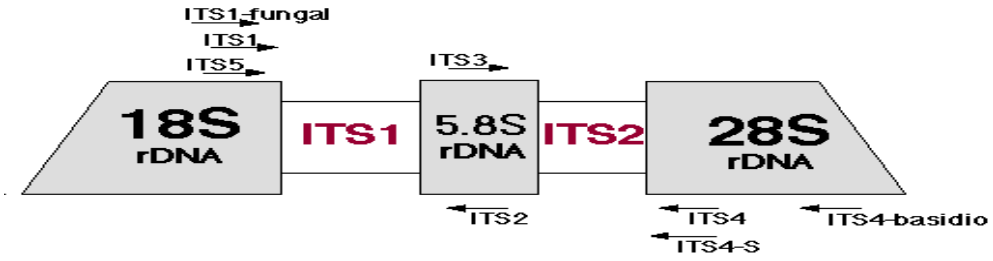


Figure 11 Attachment site on the 16s rRNA region* of different universal primers for the amplification of the 16s rRNA region

*Image taken from:
http://foreign.macrogen.co.kr/eng/images/business/img/bacteria_img.jpg



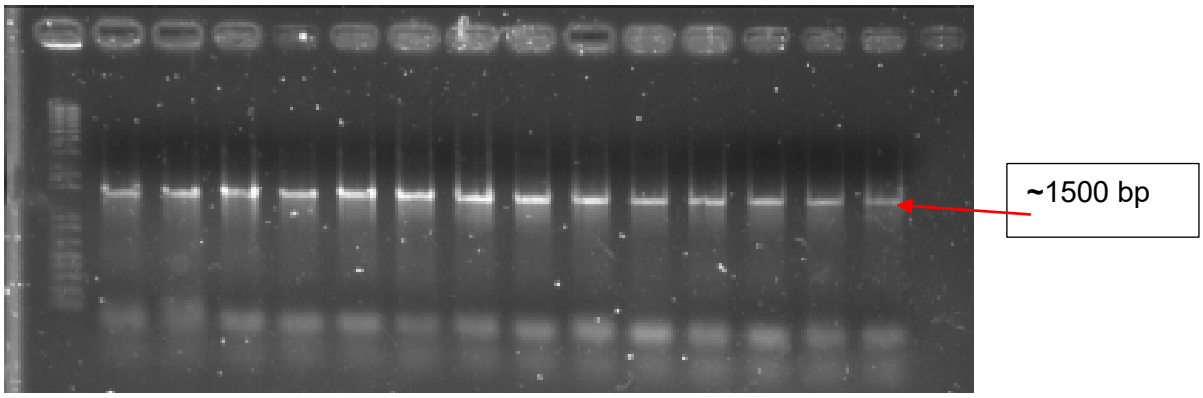


Figure 12 PCR amplification of the 16s rRNA region using 27f/1492r primers

The PCR product was sent to AITBiotech for capillary sequencing and sequence analysis The raw sequences were analysed using Geneious R9 (software). *De novo* assembly was done on all forward and reverse sequences to create a consensus sequence, and the ends of the consensus sequence were trimmed. The resulting consensus sequences of the 16s rRNA region ranges from 1,100-1,300 bp. BLAST sequence analysis of the 16s rRNA region of the *Erwinia* sp. isolates revealed that the causal pathogen of the BCR disease of papaya in the Philippines is, indeed, belonging to the genus *Erwinia*. BLAST results (Appendix 1 Table 2) revealed that the pathogen is similar to *Erwinia mallotivora* (99.9%), *Erwinia papayae* (99.8%), and *Erwinia psidii* (99%). Initial characterization of the pathogen through biochemical characteristics supports the identity of the pathogen as *Erwinia mallotivora*. However, low nucleotide diversity was observed on the 16s rRNA region of the *Erwinia mallotivora* (Philippine) isolates (Table 4).

Table 4 Similarity co-efficient of 16s rRNA gene, gyrB, recA and rpoB sequences of the Philippines BCR *Erwinia* sp to the BLAST results.

Amplified Gene	Base Pair Size	Closest Blast Hit	Accession Number	% Pairwise Identity
16s rRNA	1,192 bp	Erwinia mallotivora BT-MARDI	HQ456230	99.0 - 99.2
gyrB	591 bp	Erwinia mallotivora LMG 2708	HQ393601	98.8
recA	544 bp	Erwinia mallotivora	AY217064	97.2
rpoB	446 bp	Erwinia papayae	HQ393624	99.6

Development of a species-specific primer for the detection of Erwinia mallotivora, Philippine BCR pathogen

Three primer pairs were derived from the aligned 16s rDNA sequences of the *Erwinia* sp. BCR isolates and a small segment of the target 16s rDNA (Figure13) was amplified. However, when tested on the DNA of *Dickeya chrysanthemi* (former *Erwinia chrysanthemi*) and *Ralstonia solanacearum*, a PCR product was also generated using the primer set.

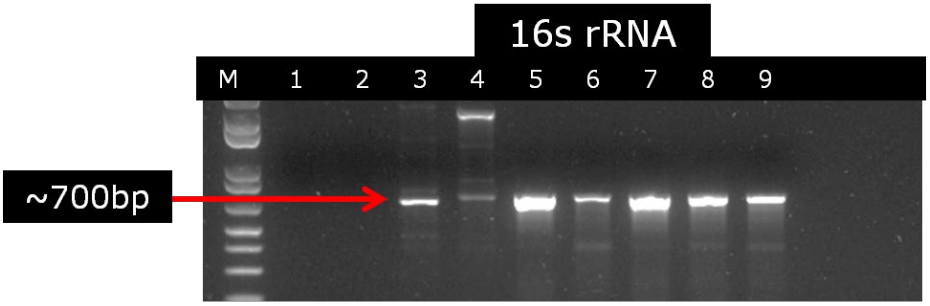


Figure 13 Gel electrophoresis of product from 16s rRNA amplification. (M=1kb+ ladder, 1=Blank, 2=DEPC water, 3=*Ralstonia solanacearum*, 4=*Erwinia chrysanthemi*, 5-9=*Erwinia mallotivora*)

B. Species-specific primers derived from the gyrB, recA, and rpoB DNA sequence of the Erwinia mallotivora

Markers derived from gyrB, rpoB, and recA gen were used for PCR detection. Results for each of the markers were reproducible. Using the designed rpoB and recA markers, nonspecific band, however faint, was amplified from the DNA of *Ralstonia solanacearum* and *Erwinia chrysanthemi* (Figure14). For the results of gyrB, no band was observed from *Ralstonia solanacearum* and *Erwinia chrysanthemi* (Figure14). Results showed that gyrB was the most promising marker for the detection of *Erwinia mallotivora*. However, it should be tested against other closely related species of *Erwinia*.

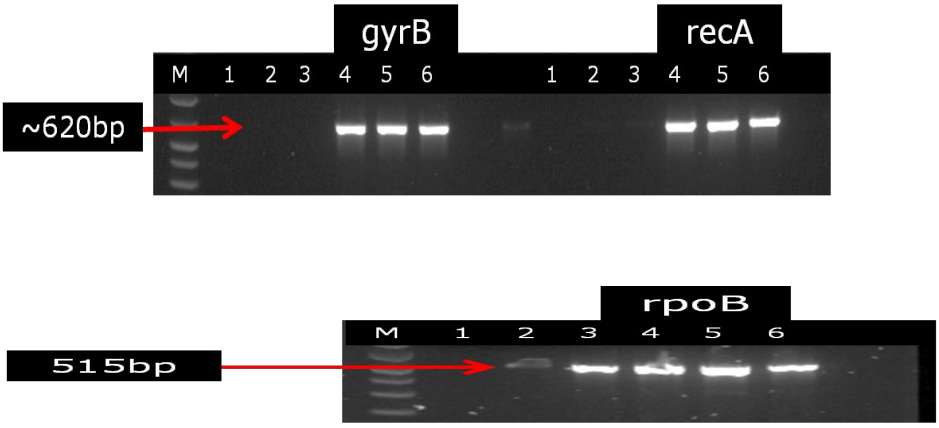


Figure 14 Gel electrophoresis of product from three markers: gyrB, recA and rpoB. (M=1kb+ ladder, 1=DEPC water, 2=*Ralstonia solanacearum*, 3=*Erwinia chrysanthemi*, 4-6=*Erwinia mallotivora*)

Primer sensitivity of gyrB marker

The starting DNA concentration of 50 ng/µL and a further serial dilution was made to test the sensitivity of primer pair gyrB 1F/1R. Results showed that the primer pair gyrB 1F/1R is highly sensitive and can detect DNA at lower concentration of 1:1,000, which is equivalent to 5 pictogram. However, faint bands were generated from the DNA concentrations at 1:10,000 and 1:100,000 (Fig. 15A). The result was reproducible (Fig.15B).

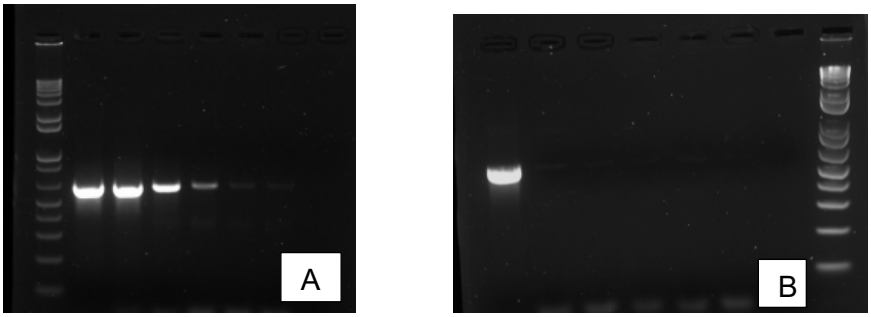


Figure 15 Gel electrophoresis of product from amplification of *E. mallotivora* gyrB DNA using different concentrations of DNA. A) Lane 1 - 1kb+ marker, Lane 2 - 50ng, Lane 3 - 1:10, Lane 4 - 1:100, Lane 5 - 1:1,000, Lane 6 - 1:10,000, Lane 7 - 1:100,000, Lane 8 - DEPC. B) Lane 1 - 50ng, Lane 2 - 1:100,000, Lane 3 - 1:1M, Lane 4 - 1:10M, Lane 5 - 1:100M, Lane 6 - 1:1T, Lane 7 - DEPC, Lane 8 - 1kb+ ladder.

Phylogenetic analysis

The phylogeny of *Erwinia spp.* was determined using the three markers (gyrB, recA, and rpoB). Available sequences from the GenBank were used to generate the phylogenetic tree. All markers used produced different results.

For the results of the rpoB markers, it was interesting to note that the soft rot *Erwinia spp.* were separated from the non-soft rot *Erwinia spp.* (Figure16). The soft rot *Erwinia spp.group* included *Dickeya chrysanthemi*, *Brenneria* species, and *Pectobacterium carotovorum* which were previously known as *Erwinia spp.* The non-soft rot *Erwinias* included *Erwinia papayae* and *Erwinia mallotivora*. Both pathogens formed a distinct cluster from the other non-soft rot *Erwinia spp* (Figure17).

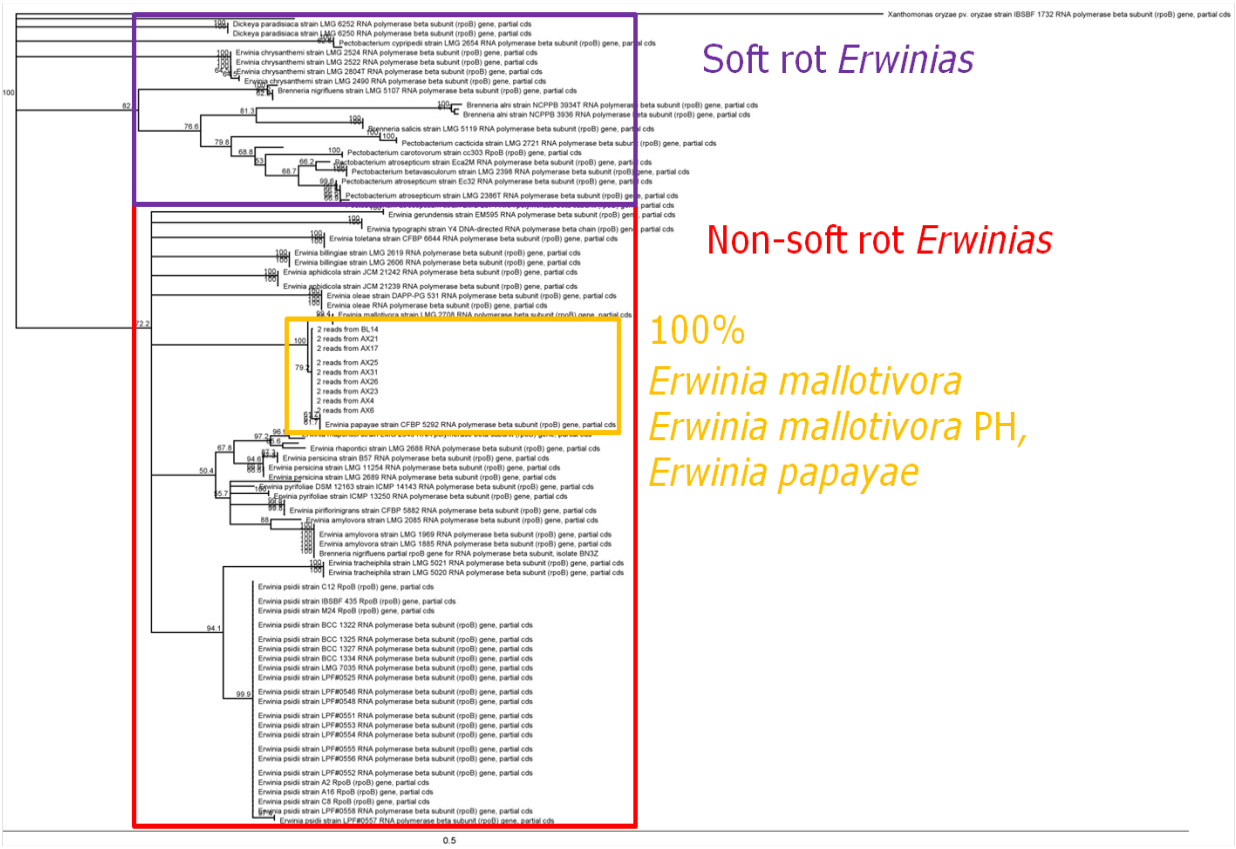


Figure 16 Phylogenetic tree generated from rpoB markers. Purple = soft rot *Erwinias*; Red = non-soft rot *Erwinias*; Yellow = BCR pathogens (*Erwinia mallotivora* and *Erwinia*

papayae).For the results of the recA markers, the soft rot *Erwinias* were not distinctly separated from the non-soft rot *Erwinias* .The BCR pathogen, *Erwinia mallotivora*, formed a distinct cluster (Fig. 16).

Figure 17 Phylogenetic tree generated from recA markers. Purple = *Erwinia mallotivora* isolates.

For the results of the gyrB markers, the soft rot *Erwinias* were not distinctly separated from the non-soft rot *Erwinias* (Figure.17). However, it was observed that *Erwinia psidii* and *tracheiphila* formed a cluster with the BCR pathogens (*Erwinia mallotivora* and *Erwinia papayae*). The four *Erwinia* species were reported to be closely related species. In addition, it was interesting that *Erwinia mallotivora* ATCC 29573 which was the type strain of *E. mallotivora* (isolated from Mallotus japonicus in Japan) was separated to the BCR isolates from the Philippines (Figure18). It was not clear if the separation is geographical or by host.

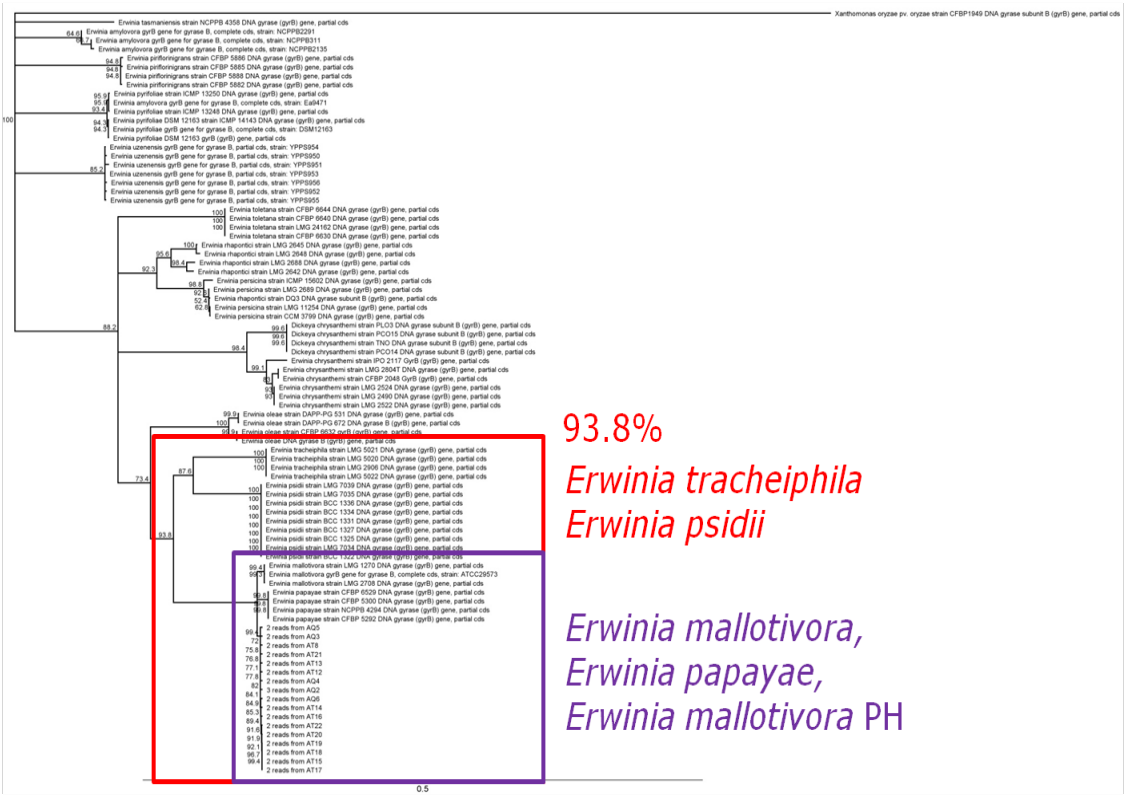


Figure 18 Phylogenetic tree generated from gyrB markers. Red = *E. tracheiphila* and *E. psidii*; Purple = BCR pathogens (*Erwinia mallotivora* and *Erwinia papayae*).

Whole genome sequencing

Six isolates of *Erwinia mallotivora* were sent to PCARI-SGCL for whole genome sequencing. The service includes sample preparation (DNA extraction, 16s rDNA identification and sequencing), library preparation (Nextera DNA Flex library preparation), sequencing which will use MiSeq Flow Cell, and bioinformatics services (annotation).

Screening of papaya cultivars for resistance to BCR under greenhouse and field conditions

Field evaluation of different papaya genotypes

78 papaya genotypes were evaluated for BCR tolerance / susceptibility in the field. Of these, 52 inbreds and 20 genotypes did not express even a mild symptom of bacterial infection. Three genotypes (acc 415, 344-B and 5893-A) and two lines (097 - S1 – 1 and 4174 - S1 – 1) were rated resistant with infection ranging from 4 to 10%. Twenty-five genotypes expressed an intermediate reaction, 15 were moderately resistant and 10 were moderately susceptible. Two genotypes (385 - S1 – 1 and 5649 - S3 – 1) were susceptible. Typical symptoms of bacterial infection viz., water soaked lesions on leaves and stem, premature defoliation and pencil-like structure were observed on susceptible genotypes. Less severe infection was noted from papaya hybrid genotypes. Percent infection ranged from 0 to 40%, hence, no hybrid was rated susceptible or highly susceptible (Table 5).

Table 5 In-field evaluation of Papaya genotypes

Inbred

Disease Reaction	% Disease Severity	Genotypes (Number)	Genotypes
Highly Resistant	0%	20	374, 382, 413, 414, 421, 098-A, 100-A, 234-A, 336-A, 336 S1-1, 345-A, 349 S1-1, 380 S1-1, 382 S1-1. 387 S1-1, 4172-A, 4173-A, 4174-A, 5650-B, 5650 S3-1
Resistant	4-10%	5	415, 097 S1-1, 344-B, 4174 S1-1, 5893-A
Moderately Resistant	11.1-25%	15	5647 S1-1, 5648-A, 417, 097-A, 097-B, 234-B, 234 S1-1, 292 S4-1, 336-B, 384 S1-1, 4172-B, 4173 S3-1, 5893-B, 5893 S14-1, 62-A
Moderately Susceptible	32.2-50%	10	344-A, 344 S1-1, 349-A, 383 S1-1, 4172 S14, 4175 S1-1, 5648-B, 5649-A, 62 S1-1, Sinta
Susceptible	55.6-62.5%	2	385 S1-1, 5649 S3-1

Hybrids

Disease Reaction	% Disease Severity	Genotypes Number	Genotypes
Highly Resistant	0	14	100x5648, 234X4172, 234X5650, 336X5648,345X5648, 4173X5648,4174X5648, 5648X097, 5648X336, 5648X5650, 5893X234, 5893X336, 5893X344, 5893X5648
Moderately Resistant	11.1 - 25	9	62X5648, 097X4172, 136X5648, 234X5648, 349X5893, 4172X5650, 5648X344, 5649X4172, 5893X097
Moderately Susceptible	33.3 - 40	3	344X5648, 349X548, 4172-B (5649X) OH

Screening of promising papaya genotypes under glasshouse conditions

Differences in disease onset and symptom expression were observed among fifty-nine artificially inoculated (leaf abrasion) genotypes. Initial symptoms that were recorded three to five days after inoculation included pinhead size water-soaked lesions that eventually turned into necrotic lesions, which also started to appear on stems and petioles. In the advanced stage of the disease development, stems showed a pencil-like appearance (Figure 19).

Figure 19 BCR screening of promising papaya genotypes under greenhouse conditions (left); Pencil-like symptoms on susceptible papaya genotypes (right)

Of the fifty-nine genotypes tested, three accessions, one inbred and one papaya hybrid were rated resistant to bacterial crown rot with a disease rating of 4-10% (Table 6). Moderate resistance to infection was expressed by six (6) accessions, five (5) hybrids and four (4) inbred lines.

Table 6 Evaluation of papaya germplasm under glasshouse condition

Disease Reaction	Disease Severity (%)	No. of genotypes	Genotypes
Resistant	7-10%	5	5893 (B), 4174 (A), 336 (B), 4174X5648, 234 S1-1
Moderately Resistant	11-24%	15	416, 5650 (B), 414, 413, 100-A, 344 (A), 5893X5648, 4173X5648, 345X5648, 5648X5650, 234X5648, 5647 S1-1, 5650 S3-1, 382 S1-1, 4175 S1-1
Moderately Susceptible	30-51%	15	234 (A)X5650(B), 234X4172, 383 S1-1, 344 (B), 4173 (A), 336 (A), 380 S1-1, 349X5648, 5648X344, 4174 S1-1, Solo, 5893X336, 5893X344, 345 (A), 5648X097
Susceptible	52-75%	18	4172 (A), 415, 234 (A), 234 (B), 4172 (B), 382, 097X4172, 5648X336, 097 S1-1, 344 S1-1, 5648 (B), 5648 (A), 336X5648, 234X5650, 4172X5650, 100X5648, 384 S1-1, 5649X4172
Highly Susceptible	77-88%	6	62 (A), 4172 S1-1, 374, 097 (A), 5893X234, 421

Comparative disease reaction of selected papaya genotypes (in-field screening vs. Greenhouse screening)

The varieties that expressed a high level of disease resistance during field evaluation succumbed to infection when exposed to high disease pressure through artificial inoculation under planthouse conditions. It was interesting to note, that genotypes with intermediate (MR-MS) reaction to the disease in the field showed promising resistance (R to MR) under glasshouse conditions (Annexure, 1 Table 4). All plants that survived under high disease pressure in the planthouse were further evaluated under field conditions for horticultural traits e.g. fruit quality (Annexure Table). Greenhouse screening incited symptoms similar to infection of papaya in the field. The inoculation method that was developed to screen breeding lines proved effective in ascertaining the reaction of different breeding lines.

Figure 20 Replicated field trial of regrowth selections and hybrids between BCR-and PRSV-tolerant lines and row planting of backcrosses in Tranca, Bay, Laguna, Philippines (14°09'13.5" N, latitude; 121°15'42.52" E, longitude) August 2017.Note the semi-dwarf nature and the prolific fruiting habit of the trees

The eight BCR tolerant regrowth selections planted in large plastic bags were successfully grown to maturity until fruiting and harvesting stages in the screen-house (Figure 21). F_1 hybrids 5893x234 and 5648x336 were prolific bearers of medium-sized fruits weighing 0.6-1.0 kg. Selections 345 and 5648x336 had the biggest fruit weighing 1,150.0 g and 1,032.5 g, respectively while selection 382-S_1 and 234x5648 had the smallest fruit with 340.0 g and 225.0 g, respectively (Table 7). Selections 5648x336 and 4174x5648 had the thickest flesh of 3.65 and 2.9 cm, respectively while 234x5648 had the thinnest flesh of 1.8 cm. Selections 382-S_1 and 345 had the sweetest flesh with TSS of 13-14◦Brix while 336 had the lowest TSS of 7◦Brix. Flesh color of the different selections ranged from yellow to red (Figure 21). In addition, selections 5893x234 and 336 have the highest edible portion of 89.84 and 88.26%, respectively while 345 had the lowest edible portion of 69.91%. Overall, selection 5648x336 was the best genotype among the tolerant lines for having big fruit (1,032.5 g), thick flesh (3.65 cm), sweet flesh (12◦Brix), and a high edible portion (85.26%).

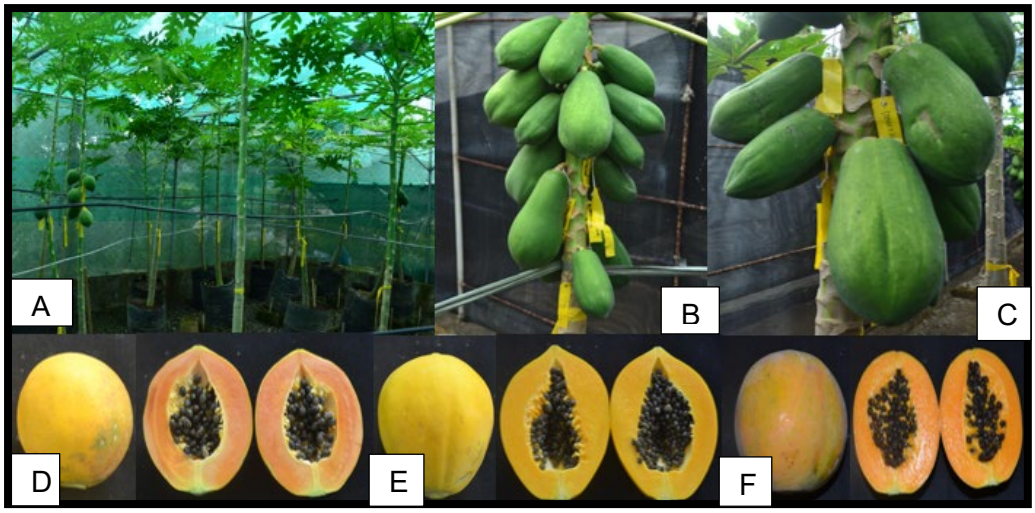


Figure 21 BCR-tolerant regrowth selections growing in screenhouse (A),prolific fruiting habit of F_1 hybrids 5893x234 (B) 5648x336 (C) the ripe fruits of 234x5648 (D), 4174x5648 (E) and 382-S_1 (F)

Table 7 Mean values of fruit quality parameters of the selected screenhouse-grown regrowth papaya lines that were previously inoculated with the BCR pathogen in the screenhouse

Regrowth lines	Fruit weight (g)	Fruit length (cm)	Fruit width (cm)	Flesh thickness (cm)	TSS (◦Brix)	Edible Portion (%)
5648x097	560.0±0.5	13.80±0.9	10.50±0.6	2.80±0.5	12.0±1.2	83.75±2.5
5648x336	1,032.5±0.4	22.20±0.6	10.60±0.7	3.65±0.5	12.0±1.8	85.26±3.2
5893x234	647.0±0.5	18.90±0.6	8.58±0.5	2.32±0.6	12.6±1.4	89.84±2.2
234x5648	225.0±0.8	9.30±0.4	7.55±0.7	1.80±0.5	11.0±1.9	78.54±1.9
4174x5648	619.0±0.6	13.30±0.2	10.62±0.5	2.90±0.4	11.0±2.1	82.77±2.1
382-S_1	340.0±0.3	10.53±0.5	8.50±0.4	2.10±0.9	14.0±0.9	75.53±2.5
336	605.0±0.2	23.69±0.4	7.40±0.4	2.10±0.8	7.0±0.8	88.26±2.7
345	1,150.0±0.7	23.50±0.3	14.50±0.2	2.80±0.6	13.0±1.2	69.91±2.3

Note: These individual selections stood, despite BCR infection through regrowth, so there was no replication done. Instead, the standard deviation of the samples were indicated.

Screening of promising papaya genotype

The Philippine papaya germplasm collection was screened under greenhouse conditions for BCR *(E. mallotivora)* resistance. Forty-one breeding lines were screened in August 2015 and 56 lines from January 2016 to November 2016.

In 2015, four lines; 546, 549, 550 Yellow Davao and 560 were rated as resistant and 27 lines were rated as moderately resistant to BCR. From January 2016 to November 2016; line 492X was evaluated as a resistant line that had the least disease severity of 6.8%, and 8 lines 415, 474, 465, 481, 476, 475, 473, 493 were rated as moderately resistant to BCR disease. Disease severity in these lines ranged from 14 - 22% (Annexure 1 Table 5-9).

The surviving plants from the screening were maintained in the greenhouse. Isolations were done on different parts of the surviving plants to assess if there was bacteria present. Sections from the basal stem, middle stem (trunk, hollow) and crown were dissected and used in the isolation. *Erwinia mallotivora* was not recovered from the surviving plants from the first screening. In addition to this, yellow and white bacterial colonies were recovered from the papaya genotypes that showed non-regrowth.

Characteristics of selected BCR-tolerant regrowth plants using scanning electron microscopy

Scanning electron microscopy (SEM) of the BCR regrowth tolerant selection, the susceptible and healthy controls were conducted to check the presence of the BCR pathogen in the host tissues (Figure 22). The results showed that the epidermis and vascular tissues of the BCR tolerant regrowth were free from bacteria. This result suggested that there are no bacterial cells in the vascular bundles of the BCR tolerant regrowth plant that shows a hypersensitive reaction, wherein the point of infection had healed from infection. This further suggests that the bacterial cells are no longer present in the tissues of the BCR tolerant regrowth selection. It has been pointed out that both virulent and avirulent strains of a bacterial pathogen could multiply exponentially inside the host plant until necrosis *i.e.,* hypersensitive response reaction occurs on the host and subsequently the bacterial population decline. The decline in the population could be attributed to the accumulation of antimicrobial compounds, and in the case of papaya, latex is the suspected reservoir of various antimicrobial compounds that causes death of BCR bacterial cells.

On the other hand, the BCR susceptible test plant had plenty of bacterial cells on the surface of the stem (Figure 22 B.1) and a close-up of this shows that these cells are going to the natural wounds on the stem, like the cracks that are evident on the surface of the stem. Eventually, these bacterial cells grew and entered into wounds and cracks on the stem going into the plant system. In addition, the bacteria (Figure 22 B.2) invaded the phloem and xylem vessels of the susceptible test plant.

In contrast, the vascular conducting tissues of the BCR-free healthy control is free of bacteria (Figure 22 C). A close-up of this shows that the phloem and the xylem are indeed devoid of bacterial cells. This situation is the same with the BCR-tolerant regrowth plant sample.

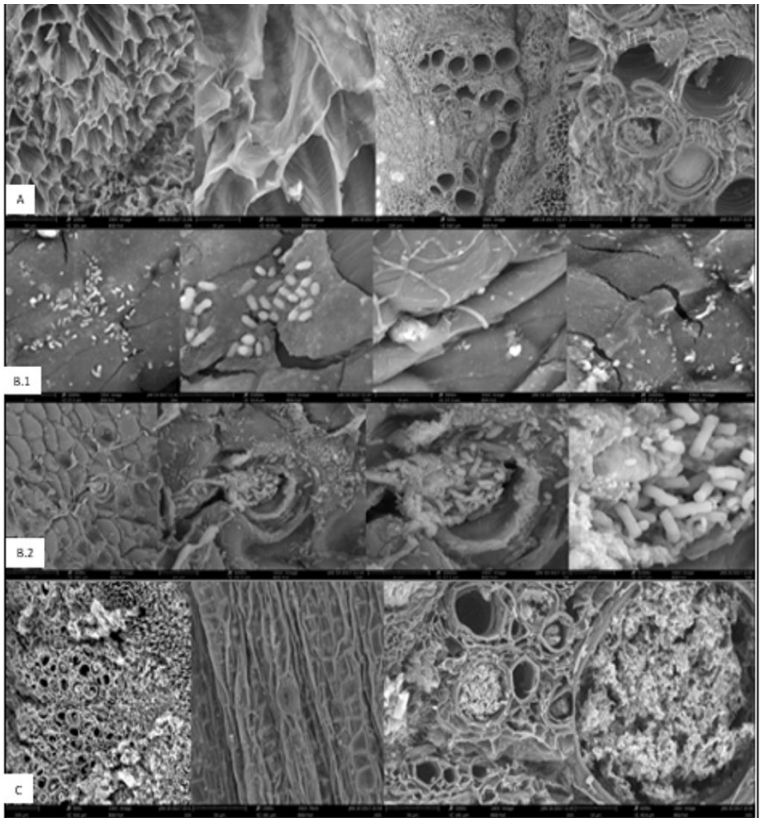


Figure 22 Scanning electron micrograph used for the evaluation of BCR pathogen on stem surface and vascular tissues of regrowth. **A** cross section of regrowth showing absence of bacterial cells in vascular system **B1** regrowth from susceptible or BCR-infected sample showing the presence of bacterial cells on stem surface and B.2**,** in vascular tissues **C** negative control; showing absence of bacterial cells in vascular system close up of longitudinal cross-section of phloem and xylem vessels.

Inhibition Assay on the Growth of *Erwinia mallotivora* Using Papaya Latex Water-Soluble Fraction

Papaya latex extract, in which papain is the main component proteinase enzyme, was tested in many studies for possible antagonistic properties against some bacterial and fungal pathogens of both plants and humans. Extracts from blended unripe papaya were shown to have bacteriostatic and antioxidative properties against enteropathogens, such as *Bacillus subtilis*, *Enterobacter* and *Escherichia coli*. No study has been conducted yet that tested the antimicrobial effect of the latex extract on the growth of *E. mallotivora* in *in vitro* conditions. The assay used in this study was successful in qualitatively showing *E. mallotivora* inhibition by exposure to latex water-soluble crude enzyme extracts. This assay is first of its kind and originally developed in this project. The assay conducted in this project, using the filter-sterilized water-soluble fraction of the papaya latex, demonstrated the probable role of the latex in eliminating *E. mallotivora* infection leading to disease recovery.

The effect of papaya latex at different concentrations and exposure time in the multiplication of *E. mallotivora* cells was investigated. No inhibition of bacterial growth was observed at 18 h in any latex concentration, except for 100% latex concentration, wherein there is only slight inhibition. Partial inhibition was observed starting 30 h of exposure, while complete inhibition was observed at 48 to 66 h with 50% water-soluble latex concentration. These results indicated the probable role of the papaya latex in bacterial growth inhibition during the early stage of BCR infection. Proteases are the major component of papaya latex and previous studies have shown the antibacterial and antifungal properties of the papaya latex and its

component proteases and proved its antimicrobial properties against common plant pathogens (Shindo and van der Hoorn 2008).

Develop and evaluate sustainable disease management strategies for BCR

Disease progression and Seed transmission studies

Fruit on a healthy papaya plant was artificially inoculated to determine if BCR is a seed-borne disease. The artificial inoculation made during the dry season showed dark green, water-soaked lesions on the fruit surface 14 days after inoculation (Figure 23).

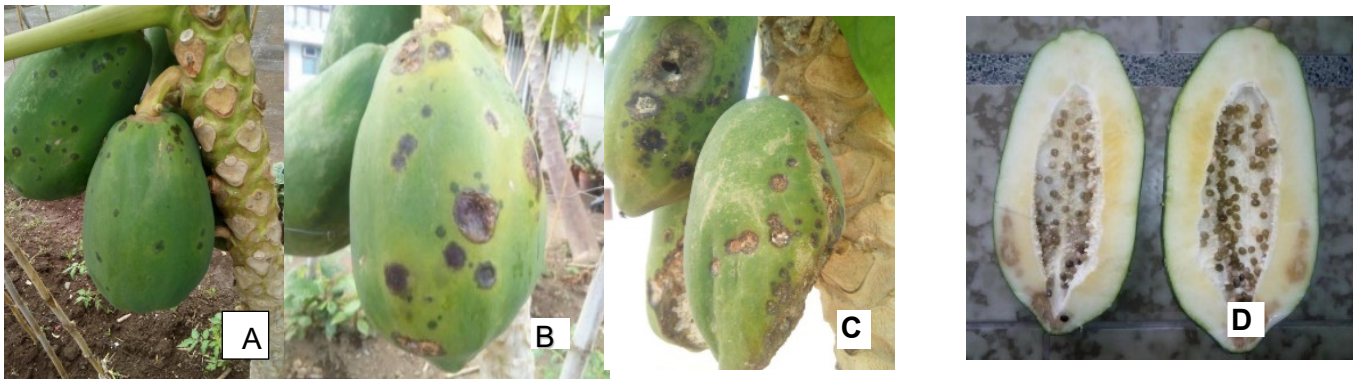


Figure 23 BCR symptoms on artificially inoculated papaya fruit **A**. at 14 days **B.** at 28 days, **C.** at 40 days and **D** at 140 days after inoculation.

The water-soaked lesions continued to enlarge and formed a depressed lesion 28 days after inoculation. At 140 days after inoculation, the lesions had coalesced and were colonized fungal mycelium. The disease did not progress to the peduncle of the fruit or to the trunk of the papaya tree. However, other uninfected fruit in close contact with the infected fruit soon developed similar symptoms. Fruit dissection 140 days after inoculation showed a brown discoloration of fleshy tissues. The seeds were harvested and sown on soil to allow germination. BCR symptoms were not observed in the germinated seedlings six months after germination.

Artificial seed inoculation

'Solo' papaya fruits bought from supermarkets were used in the artificial inoculation of seed. Seeds were harvested from fruit and soaked in a bacterial suspension of *Erwinia mallotivora* for 24 hours. The inoculated and uninoculated seeds were sown in sterile soil and observed for seed germination and BCR symptoms. Two weeks after sowing, inoculated seeds (94%) showed a higher seed germination compared to uninoculated seeds (92%). No BCR symptoms were observed from the germinating seedlings from the inoculated seeds.

Similar results were obtained when soaking hours in bacterial suspension were extended to 48 hours to ensure that the BCR pathogen penetrated into the sarcotesta and the seed coat of the papaya seed. However, average germination rate from all tests of artificially inoculated seed was 68% and healthy or uninoculated seeds was 72%. Destructive sampling of the artificially inoculated seeds did not recover bacterial colonies of *Erwinia mallotivora* on KB medium.

Destructive sampling of seedlings from artificially inoculated seeds

Three-month old papaya seedlings were subjected to destructive sampling to determine the presence of Erwinia mallotivora on seedlings germinated from artificially inoculated seeds. The samples were divided into three parts: top (crown part), middle, bottom (near roots). The

results from this test did not show any presence of the BCR pathogen from the sampled parts of the seedlings. However, yellow coloured colonies were recovered from top portion and light brown and white fluidal colonies were recovered from the bottom part of the seedlings.

Evaluation of seed transmission from naturally infected BCR fruits from known BCR infested fields in Mindanao

Five hundred and thirty-eight seeds were collected from BCR-infected Red Lady fruit (432 mature; and 106 immature seed) at Lunokan, ManoloFortich and Bukidnon. One hundred and thirty seeds were evaluated for BCR seed transmission. The observations were made on a regular basis and none of the seedlings expressed BCR symptoms after six months.

In another study, 50 seeds from BCR-infected papaya fruit collected from two locations in Bukidnon were tested to determine if BCR was seed borne. The seeds were separated into three parts: sarcotesta, seed coat, and embryo and each part was plated on KB medium and MS medium. All plated parts showed bacterial and fungal growth on both the media but no *Erwinia mallotivora* was isolated from the seed parts.

Five bacterial species were consistently isolated from the seed parts and these were identified using 16S rDNA gene sequence analysis as Microbacterium sp, *Brevundimonas* sp. and *Chryseobacteriu*m sp. These bacteria were consistently isolated from the sarcotesta (sac/mucilage) and esclerostesta (seed coat/black cover). *Microbacterium sp.* and *Methylobacterium* sp. were consistently isolated from the embryo.

Two hundred remnant seeds per fruit were planted in the greenhouse. The plants were observed for BCR symptom expression under greenhouse conditions. As of this writing, the plants are in the reproductive stage and not even a single tree exhibited BCR symptoms.

Detection of BCR pathogen by PCR from naturally infected seeds

Different parts of seeds extracted from naturally infected BCR fruit viz. sarcotesta (sac/mucilage) and esclerotesta (seed coat/black cover) were tested using PCR. Results revealed that all parts of seed was free of the BCR pathogen, This area of research requires further study to determine if BCR is, in fact, seed-borne.

2.3 Determine the importance of alternate hosts to the survival of the BCR pathogen

Isolations were made from water-soaked lesions on papaya, Amaranthus and pineapple, and from soil underneath symptomatic papaya plants (Red Lady). Samples were collected from DMPI and Bukidhnon. The isolations from water soaked lesions and serial dilution from soil samples were made using KB media. *Erwinia mallotivora* was not recovered from any tested plants and soil samples.

Isolation of BCR pathogen from artificially inoculated weeds

Different weed species were collected from the field and brought to the Plant Pathology Laboratory, Institute of Plant Breeding, UPLB. The weeds species included *Amaranthus spinosus*, *Amaranthus viridis*, *Commelina benghalensis*, *Euphorbia heterophylla*, *Euphorbia hirta*, *Synedrella nodiflora*, *Portulaca oleracea* and *Vernonia cinerea*. Other crops included tomato (*Solanum lycopersicum*), melon (*Cucumis melo*), guava (*Psidium guajava*), cowpea (*Vigna unguiculata*), banana (*Musa* sp.) and corn (*Zea mays*). Guava (*Psidium guajava*) was included in the inoculation to determine if it is an alternate host for *E. mallotivora*. *E. mallotivora* and *E.psidii*, the causal organism of bacterial blight of guava, are two closely related species. Papaya (*Carica papaya*) cv. Solo was used as a positive control. The

control papaya plants expressed BCR symptoms after two days however, none of the other inoculated plants expressed BCR symptoms.

Isolation of BCR pathogen from artificially inoculated Amaranthus viridis

The inoculated *Amaranthus* did not show BCR-like symptoms, but vein clearing, leaf spot, and water-soaked lesions were noted one month after inoculation. Isolations were made from symptomatic parts and bacterial colonies other than *E mallotivora* were recovered. A mixture of yellow and pink colonies were isolated from *Amaranthus viridis* with vein clearing symptom whereas flat, dry, white bacterial colonies were isolated from water-soaked lesions on the *Amaranthus viridis* plant.

Epiphytic populations from selected weed species

Results revealed that *Erwinia mallotivora* can survive on weeds for some 4 days after inoculation (Table 8). It was observed that *E. mallotivora* produced visible symptoms on *Carica papaya* 3 days after inoculation. At 4 days, *Carica papaya* also had the greatest number of *E. mallotivora* colonies counted on KB medium followed by *Acalypha indica,*The number of bacterial colonies increased in *Amaranthus viridis*, *Amaranthus spinosus*, and *Synedrella nodiflora*. *Amaranthus spinosus* had the least count of bacterial colonies.

Table 8. Epiphytic population from selected weed species

WEED SPECIES	Bacterial colonies	
	2 DOI	4 DOI
Amaranthus spinosus (AS)	14	28
Amaranthus viridis (AV)	77	121
Synedrella nodiflora (SN)	89	181
Commelina benghalensis (CB)	92	42
Acalypha indica	>300	>300
Carica papaya (CP)	>300	>300

Determine if the BCR pathogen can survive in soil

Incorporation of BCR-infected papaya in soil and the recovery of E. mallotivora

An isolate E.mallotivora was used to inoculate papaya plants. After fourteen days, plants with advanced disease symptoms were chopped into five different parts: roots, stem near roots, middle stem, leaves and crown. The different parts were then placed in a sterilized flask containing 100g of sterile damp soil and incubated for 15 days. Isolations was made to determine the survival of *Erwinia mallotivora* in the soil (Table 9).

Erwinia-like colonies were isolated, purified and re-inoculated to papaya plants. 'Solo' papaya plants inoculated with Erwinia-like colonies did not develop symptoms associated with bacterial crown rot caused by *E. mallotivora.*

Different methods employed in determining the survival of BCR pathogen in soil

1. Survival of Erwinia mallotivora isolates in soil

Table 9. Colony counts of *E. mallotivora* at 1, 3, and 5 DAI

Treatment	Colony Count 1 DAI	Colony Count 3 DAI	Colony Count 5 DAI
Control	0 c	0 c	0 b
EP25 (Mindanao)	5.6 b	0.27 b	1.40 a
EP26 (Mindanao)	10.40 a	0.27 b	1.40 a
EP60 (Luzon)	11.73 a	0 c	0.93 ab
9EP64 (Luzon)	4.13 b	0.87 a	0.80 ab

The study revealed the significant differences in pathogen population among the isolates but showed no difference between geographical origins one day after inoculation. After three and five days, the Mindanao isolates showed a higher pathogen population compared to the Luzon isolates. The pathogen population decreased significantly after three days, At 5 DAI, a number of *Erwinia* like colonies were isolated and pathogenicity tested on Solo papaya. Isolated colonies did not infect the Solo papaya. This indicated that *E. mallotivora* populations decrease with time and could not survive long in soil.

2. Survival of Erwinia mallotivora in free soil and amended soil

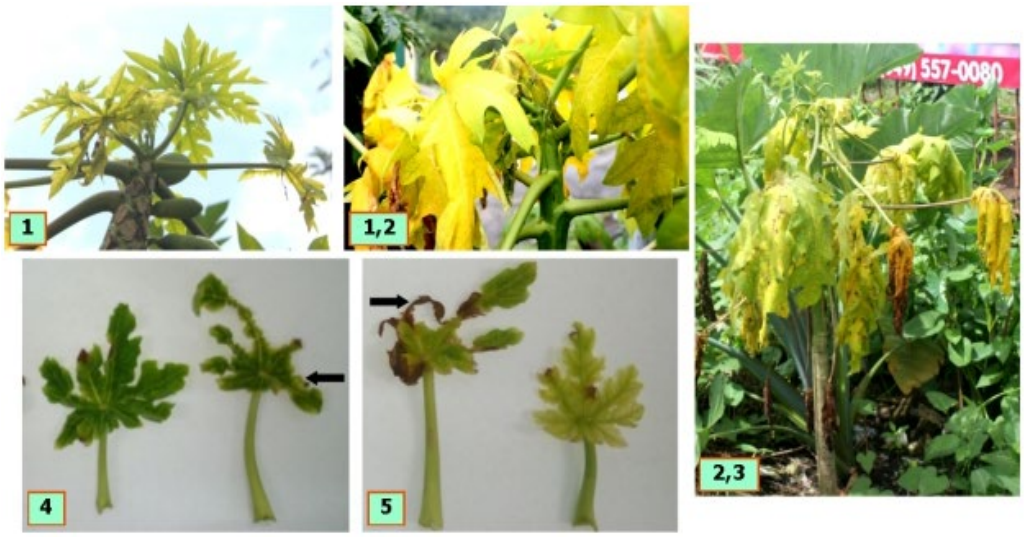
Ten one-month old 'Solo' papaya seedlings were inoculated with the BCR pathogen. One week after inoculation infected seedlings were chopped into the various plant parts, infected leaves, stem, uninfected stem and roots. These plant parts were then incorporated with sterile soil into six pots and mixed. The pots were then watered until water capacity was attained. Three pots were then planted with one-month old papaya seedlings with wounded roots and another three pots were planted to one-month old papaya seedlings without wounded roots. Results showed both the unwounded and the wounded roots did not show any BCR symptoms.

Table 10 Possible colony count of *E. mallotivora* at 1, 3, and 5 DAI

Treatment	Colony count 1 DAI	Colony count 3 DAI
Drenched and amended with BCR-infected papaya (T1)	4.47 a	3.27 a
Drenched alone (T2)	4.13 a	0.87 b

Characterization of Phytoplasma disease in the Philippines:

Out of eighty-nine symptomatic samples, eighteen were d found to positive for phytoplasma using Nested PCR .The samples that tested positive for phytoplasma infection were collected from Mindanao (Table 11; Figure 24).



Six

Figure 24 Symptoms associated with papaya phytoplasma disease in Mindanao the Philippines; 1. yellowing of young leaves, 2. bending of the stem tip 3. shrivelling of leaves 4. translucent leaf margins 5.browning/necrosis of leaf margins

Table 11 Summary of infected papaya leaf sample collection used in the study

Place	Year /No. of sample collection	Positive Phytoplasma Sample
Laguna	2015 (6), 2016 (5), 2017 (12), 2018 (3)	0/26
Batangas	2015 (2), 2016 (4), 2018 (3)	0/9
Cavite	2016 (3), 2018 (3)	0/6
Negros Occidental	2017 (2)	0/2
Bukidnon	2016 (22), 2017 (5)	9/27
Davao	2016 (2), 2017 (6)	2/8
South Cotabato	2015 (4), 2016 (7)	7/11

Phylogenetic analysis of phytoplasma DNA sequences

Based on NCBI BLAST analysis, the phytoplasma causing the unknown papaya disease in the Philippines belongs to the phytoplasma 16srII group. Notable closest BLAST hit from the

16srII group for the phytoplasma samples used in this study was '*Ca*. Phytoplasma *aurantifolia*'. Billones *et al*. (2005) was the first to characterize the Philippine papaya phytoplasma as '*Ca*. Phytoplasma *aurantifolia*' SPLL-V4 strain by restriction fragment profile. The '*Ca*. Phytoplasma *aurantifolia* SPLL (sweet potato little leaf)' is a representative strain of the 16srII group, subgroup D (2). This is a strain of '*Ca*. Phytoplasma *aurantifolia*' known to cause papaya yellow crinkle and papaya mosaic disease.

The phylogenetic analysis (Figure 25) showed the relationship between the Philippine papaya phytoplasma from 16sr XII group represented by papaya dieback (Y10095.1), and from 16srII group represented by papaya yellow crinkle (Y10097.1) and papaya mosaic (Y10096.1). The high value obtained from the UPGMA indicated that the Philippine papaya phytoplasma in this study belongs to 16srII group. The reoccurrence of the disease in the Mindanao region is believed to be due to drier weather conditions and an increase in vector population.

Figure 25 Phylogenetic tree shows the relationship of the Philippine papaya phytoplasma from 16sr XII group represented by papaya dieback (Y10095.1), and from 16srII group represented by papaya yellow crinkle (Y10097.1) and papaya mosaic (Y10096.1).

Development of integrated Disease Management strategies (IDM)

In commercial plantations control measures in the papaya operation have focused on the rogueing and burning of infected plants, and the use of prophylactic sprays of copper fungicide. Unfortunately, little success has been achieved in suppressing phytoplasma disease in papaya. Bacillus and phosphorous acid have been shown to give a level of disease control. *Bacillus,* aside from competition on colonization sites, are known to produce antimicrobial metabolites like antibiotics that can antagonise bacterial and fungal pathogens. Phosphorus acid, on the other hand, activates plant resistance once absorbed by the plants. Compounds such as these were tested at the Del Monte research station, Cagayan De Oro and BPI-DNCRDPSC research station Davao.

Efficacy of Bacillus spp., phosphorous acid, and copper fungicide on bacterial crown rot of Papaya

Table 12. Disease severity index of papaya plants in Research Cpd

Treatment2/	**Disease Severity Index, %1/**
	Week after treatment

		1	2	3	4	5	6	7	8
A.	Bacillus subtilis QST 713, 100mL/900mL water	33.3	32.5	22.5	4.2	15.8	31.7	55a	68.3 ab
B.	Bacillus amyloliquefaciens strain D747, 0.027 g/L	33.3	33.3	27.5	6.7	17.5	39.2	67.5a	86.7 a
C.	Bacillus subtilis, 5x1010cfu/g), 0.0249 g/L	32.5	32.5	30	5.8	15	32.5	60a	81.7 a
D.	Phosphorous Acid, 1.6g/L	33.3	32.5	17.5	12.5	18.3	32.5	62.5a	73.3 a
E.	Champion 77WP (Copper hydroxide), 3.7 g/L	32.5	33.3	24.2	7.5	20	27.5	55.8a	79.2 a
X1.	Inoculated Untreated Control	35.8	33.3	29.2	7.5	11.7	29.2	63.3a	83.3 a
X2.	Uninoculated Untreated Control	35	34.2	30	3.3	1.7	5.8	16.7 b	32.5b
F-value, α=0.05		ns	ns	ns	ns	ns	ns	**	**

[1/]Disease severity index = ∑nRi x 100/(10)(5); where n = number of plants with Ri score; i = 0 to 3; 10 = number of plants rated; 3 = highest score in rating scheme. Numbers are means of four replicates. . In a column, means with the same letters are not significantly different at 5% level HSD.
Date planted: November 11, 2016
Date of 1st treatment application: 2/1/17; succeeding treatments done weekly
Date of inoculation: 2/4/17
Date of 1st reading: 2/8/17

Table 13 Mortality count of papaya plants in Research Cpd

Treatment2/		**% Mortality (n=10)1/**							
		Week after treatment							
		1	2	3	4	5	6	7	8
A.	Bacillus subtilis QST 713, 100mL/900mL water	0	0	0	0	0	0	22.5	60 ab
B.	Bacillus amyloliquefaciens strain D747, 0.027 g/L	0	0	0	0	0	7.5	32.5	82.5a
C.	Bacillus subtilis, 5x1010cfu/g), 0.0249 g/L	0	0	0	0	0	0	27.5	72.5a
D.	Phosphorous Acid, 1.6g/L	0	0	0	0	0	2.5	40	65ab
E.	Champion 77WP (Copper hydroxide), 3.7 g/L	0	0	0	0	0	2.5	27.5	72.5a
X1.	Inoculated Untreated Control	0	0	0	0	0	0	25	72.5a
X2.	Uninoculated Untreated Control	0	0	0	0	0	2.5	5	17.5b
F-value, α=0.05		ns	ns	ns	ns	ns	ns	ns	**

[1/]Each, number is an average of 4 replicates. In a column, means with the same letters are not significantly different at 5% level HSD.

[1/]Each number is an average of 4 replicates. In a column, means with the same letters are not significantly different at 5% level HSD.
Date planted: November 11, 2016
Date of 1st treatment application: 2/1/17; succeeding treatments done weekly
Date of inoculation: 2/4/17
Date of 1st reading: 2/8/17

The studies conducted at Del Monte research station showed that a foliar spray of *Bacillus* spp., phosphorous acid and copper did not reduce the severity of the BCR disease compared to the untreated treatment. Disease infection on leaves was observed one (1) week after inoculation and continued to develop throughout the 8-week observation period (Table 12 &13).

Bacillus subtilis QST 713 and phosphorous acid significantly lowered plant mortality (60%) compared with the untreated control 8 weeks after the treatment applications commenced

(Table 13). The uninoculated control had only 17.5% mortality at week 8. This result implies that weekly foliar spray *Bacillus subtilis* QST 713 and phosphorous acid may provide a level of control of BCR. None of the chemical treatments was significantly different to the inoculated untreated control.

In another trial conducted at BPI research station, Davao, seven treatments were evaluated for the control of BCR. The percent plant survival was found significantly higher in plants treated with Phospro (85.5%) and the Biological control (serenade 80.9%) followed by foliar fertilizer, Copper fungicide, and Epsom salt (76.2%, 71.4%, 71.4% and 66.6%) respectively. The lowest plant survival was observed in untreated plots (46.6%).

Table: 14. Effect of different treatments on BCR disease management

Treatment	Percent Plant survival
T1 - Phospro (2.2L/ha)	85.5 a
T2 – Copper fungicide (375g ai/ha)	71.4 ab
T3 – Biological control agent (Serenade 2L/ha)	80.9 a
T4 – Epsom salt (4tbsp/16L of water)	66.6 ab
T5 – Foliar fertilizer (16ml/16 L of water)	76.2 ab
T6 – Grower's practice (Copper fungicide)	71.4 ab
T7 – Untreated	46.6 b

Phytoplasma detection in papaya:

Surveys

A phytoplasma survey in papaya plantation (Far North Queensland) was conducted between June 2014 and November 2018. Disease assessment of the papaya trees was based on visual symptoms. The phytoplasma-like symptoms found present included leaf yellowing, mottling, water soaked lesion on stem, lack of sap, bunchy top, phyllody and dieback papaya plants (Annexure 5 Figure 1). The phytoplasma disease incidence of 15-80% was observed in the wet tropical coast (Cardwell and Mossman) and 5-12% in Mareeba and Dimbulah growing areas. However, maximum incidence was observed in younger plantings. The incidence of lepidopterous fruit and stem borer was also observed on phytoplasma-affected plants.

A total of 103 of 536 samples were confirmed positive for phytoplasma by PCR from papaya and other plant species and insects (Appendix 1, Table 10 -12). Phytoplasma was only detected from symptomatic plants. One hundred samples were further sequenced and identified to species level using known groups of phytoplasma standards. The incidence of dieback disease was observed before September 2015 and found less, as compared to yellow crinkle and mosaic in subsequent years.

Phylogenetic analysis revealed the association of two groups of phytoplasmas with north Queensland isolates i.e.16Sr XII; *Candidatus* P. *australiense* causing papaya dieback, and 16Sr II; *Candidatus* P. *australasia* causing yellow crinkle and mosaic and *Ca* P. *aurantifolia;* producing phytoplasma die back-like symptoms and which, was only recovered from Mareeba isolates (Figure 26). One unidentified 16S group of phytoplasma, Stylosanthes little leaf (StLL), was recovered from a stylosanthes sample in north Queensland but no StLL phytoplasma was recovered from papaya samples (Figure 26). The die back and yellow crinkle was found to be prevalent in all plantations, however *Ca* P. *aurantifolia* was only recovered from Mareeba with die back-like symptoms. The majority of symptomatic papaya, weed and insect samples collected during June 2016 and onwards shared a 100% identity

with the Australian strain of papaya yellow crinkle and mosaic (16Sr II-D Y10097). This confirmed that the phytoplasma disease outbreak in Australia was caused by historical PpYC and PpM; sweet potato little leaf strain (Figure 27). However, few symptomatic papaya samples were found to be 100% identical to peanut witches'-broom (PnWB16 Sr II- A) phytoplasma (pigeon pea witches broom, *Celosia argentea*, *Corchorous aestuans* sweet potato little leaf and *Vigna unguiculata* phytoplasma). This indicated that greater genetic diversity exists among yellow crinkle and mosaic strains than previously thought and further validity is required to determine pathogenic variations within groups/subgroups based on geography.

An attempt was made for a finer differentiation of phytoplasmas using additional genetic markers; sec Y gene to characterize phytoplasma isolates. It was interesting to note that species *Ca.* P. *australasia,* which showed 98.9% similarity of their 16s rRNA gene, is separated into two species, *Ca.* P. *australasia* and *Ca.* P. *aurantifolia* with 87.5% sequence similarity. This showed that the papaya isolates used in this study could be further characterised and separated into the different species/subgroups that may represent ecologically separated populations and could be described as a separate species (Appendix 1 Figure 1-4).

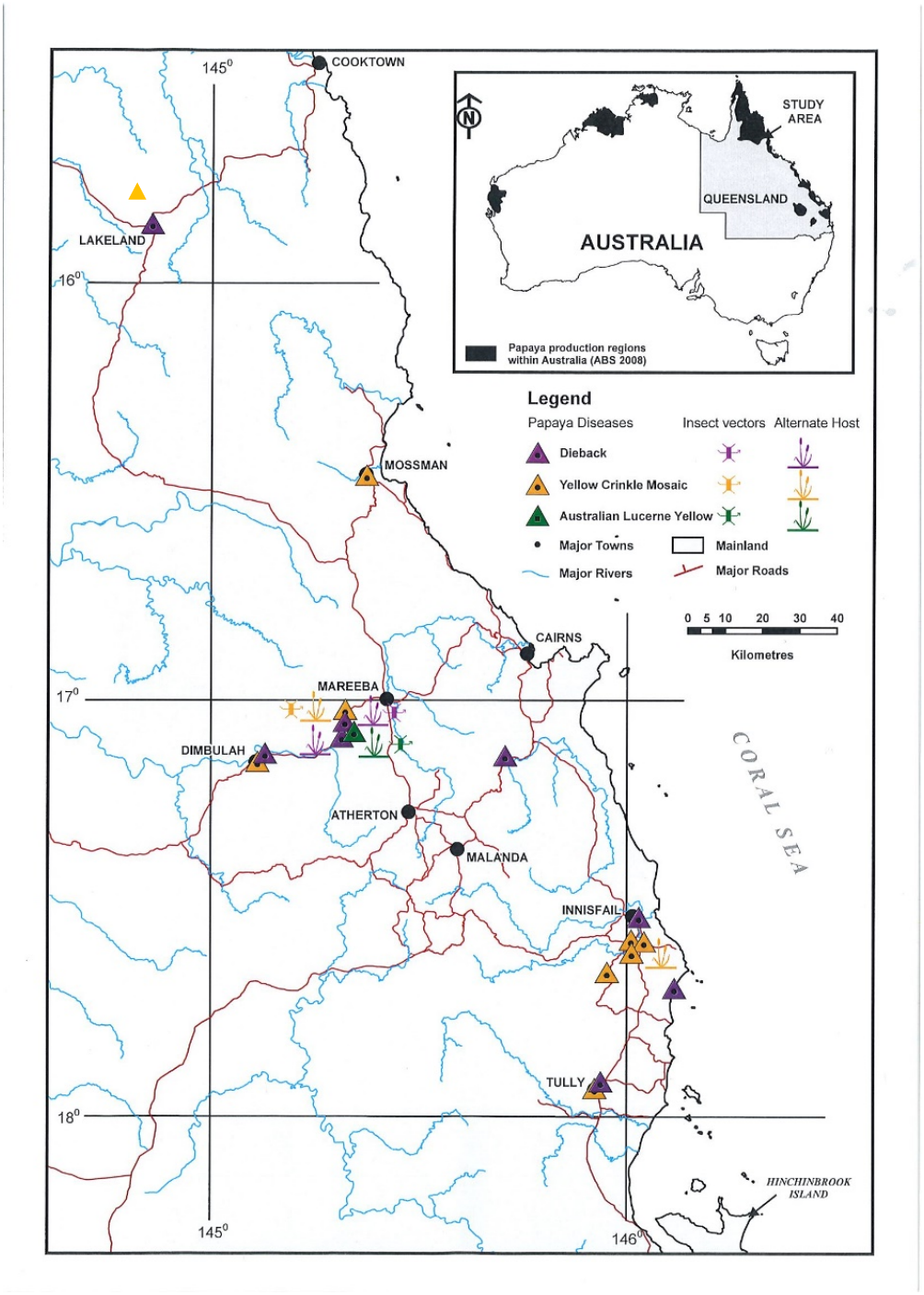


Fig 26 Map showing papaya sampling sites and distribution of Dieback and Yellow crinkle and mosaic diseases in commercial papaya plantations in far north Queensland

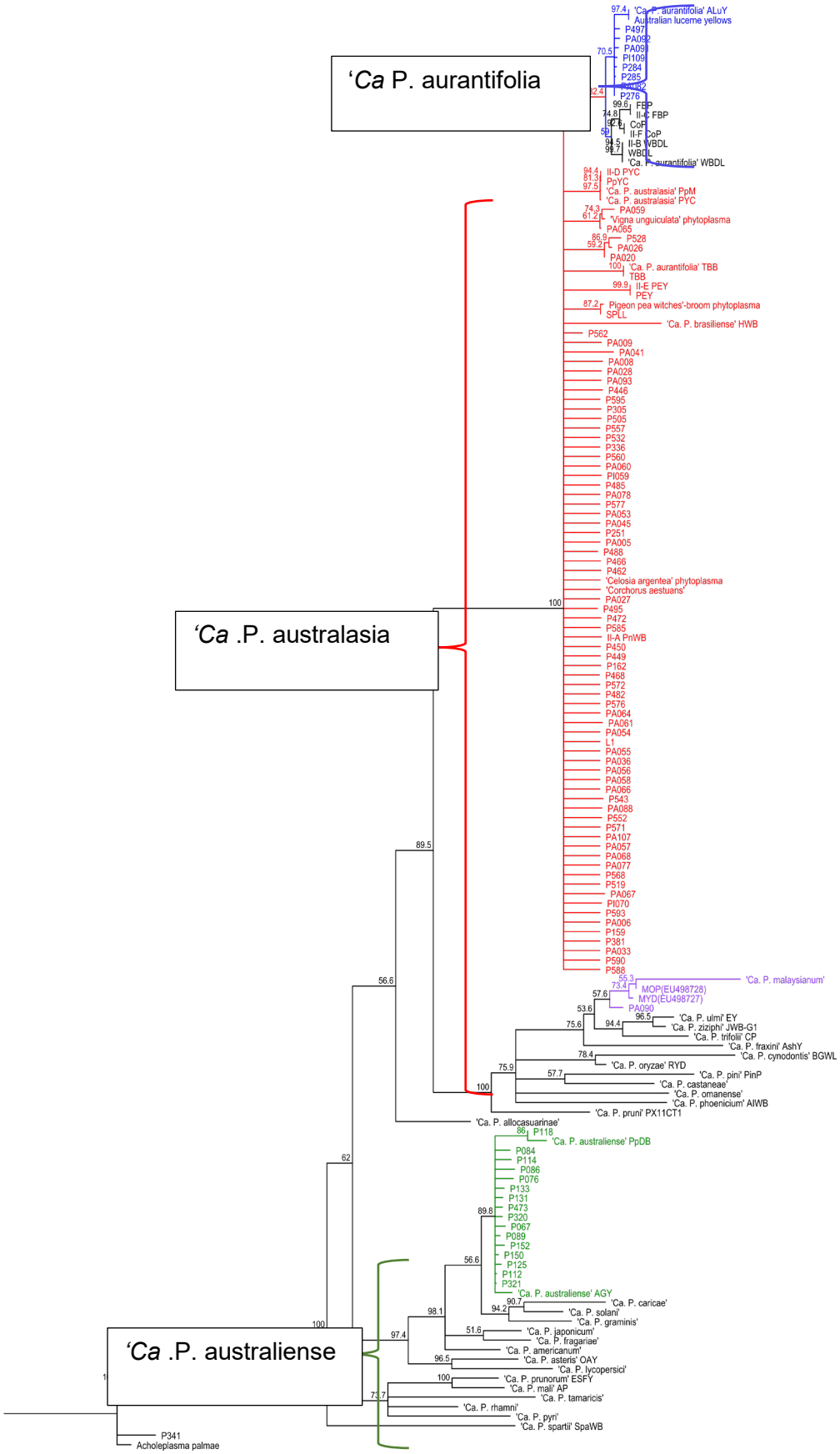

'Ca. P. aurantifolia'
'Ca. P. australasia'
'Ca. P. australiense'
'Ca. P. aurantifolia' ALuY
Australian lucerne yellows
P497
PA092
PA091
PI109
P284
P285
P276
FBP
II-C FBP
CoF
II-F CoP
II-B WBDL
WBDL
'Ca. P. aurantifolia' WBDL
II-D PYC
PpYC
'Ca. P. australasia' PpM
'Ca. P. australasia' PYC
PA059
'Vigna unguiculata' phytoplasma
PA065
P528
PA026
PA020
'Ca. P. aurantifolia' TBB
TBB
II-E PEY
PEY
Pigeon pea witches'-broom phytoplasma
SPLL
'Ca. P. brasiliense' HWB
P562
PA009
PA041
PA008
PA028
PA093
P446
P595
P305
P505
P557
P532
P336
P560
PA060
PI059
P485
PA078
P577
PA053
PA045
P251
PA005
P488
P466
P462
'Celosia argentea' phytoplasma
'Corchorus aestuans'
PA027
P495
P472
P585
II-A PnWB
P450
P449
P162
P468
P572
P482
P576
PA064
PA061
PA054
L1
PA055
PA036
PA056
PA058
PA066
P543
PA088
P552
P571
PA107
PA057
PA068
PA077
P568
P519
PA067
PI070
P593
PA006
P159
P381
PA033
P590
P588
'Ca. P. malaysianum'
MOP(EU498728)
MYD(EU498727)
PA090
'Ca. P. ulmi' EY
'Ca. P. ziziphi' JWB-G1
'Ca. P. trifolii' CP
'Ca. P. fraxini' AshY
'Ca. P. cynodontis' BGWL
'Ca. P. oryzae' RYD
'Ca. P. pini' PinP
'Ca. P. castaneae'
'Ca. P. omanense'
'Ca. P. phoenicium' AlWB
'Ca. P. pruni' PX11CT1
'Ca. P. allocasuarinae'
P118
'Ca. P. australiense' PpDB
P084
P114
P086
P076
P133
P131
P473
P320
P067
P089
P152
P150
P125
P112
P321
'Ca. P. australiense' AGY
'Ca. P. caricae'
'Ca. P. solani'
'Ca. P. graminis'
'Ca. P. japonicum'
'Ca. P. fragariae'
'Ca. P. americanum'
'Ca. P. asteris' OAY
'Ca. P. lycopersici'
'Ca. P. prunorum' ESFY
'Ca. P. mali' AP
'Ca. P. tamaricis'
'Ca. P. rhamni'
'Ca. P. pyri'
'Ca. P. spartii' SpaWB
P341
Acholeplasma palmae

Figure 27 Phylogram of 16-23 rDNA (spacer region sequences) of 24 representative phytoplasma species and *Acholeplasma palmae* (NR_029152) as the out-group compared to north Queensland papaya phytoplasma isolates. The positions of papaya phytoplasma isolates are colour coded; red papaya yellow crinkle and mosaic – *Ca* .P. *australasia* blue- *Ca*. P. *aurantifolia* and green papaya dieback *Ca.* P*. australiense*. Phytoplasma isolates showed closed similarity to accession number of 16 Sr group II (Y 10096; Y10097 and Y08173) and 16 Sr group XII. (Y10095 and L76865).The clade assignment is accordance to Firrao *et.al* (2005) and White *et.al* (1998).

Pytoplasma detection in weeds/other crops:

Ten (10) out of one hundred and seventy (170) symptomatic/asymptomatic weed/other crop host species collected within and around papaya growing areas detected positive for 16Sr II and 16 SrXII group of phytoplasma. The symptomatic weed *Praxelis clematidea* (praxelis), *Crotalaria juncea* (sunn hemp), *Phyllanthus sp* (phyllanthus),*Cajanus cajan (*pigeon pea),*Stylosanthes scabra* (stylo),*Sida acuta* (wire weed) were tested positive to yellow crinkle and mosaic (*Candidatus* Phytoplasma australasia) and shared 100% similarity to papaya yellow mosaic. Two symptomatic weeds *Stylosanthes scabra* (stylo) and *Praxelis clematidea* collected within and around the papaya plantation from Atherton Tablelands were detected positive for Australian Lucerne yellows phytoplasmas. It can be concluded that the weed hosts around and within papaya plantations harbors 16SrII group phytoplasma strains associated with papaya yellow crinkle and mosaic phytoplasma, and are a potential reservoir of the disease. However, only two weeds; *Portulaca pilosa* (pigweed) and Solanum nigrum/americanum were detected positive for papaya dieback disease; *Candidatus* Phytoplasma *australiense.*

Figure 28 Putative weed host for papaya yellow crinkle and mosaic (*PpYC*, *PpM* and Australian Lucerne yellows (*ALuY)* phytoplasma **A** Sun hemp **B** Sand Spurge **C** Wire weed**, D** Shrubby stylo **E** Praxelis **F** Pigeon pea

Phytoplasma detection in insect vectors

Four **(**4) insect samples out of 17 sampling lines detected positive for phytoplasma using molecular techniques. The mixed sample of leafhoppers and plant hoppers Cicadellids: *Cicadulina bimaculata* and *Baclutha incisa* and Delphacids: *Falcotoya aurinia* Fennah collected from PpYC and PpM affected papaya plantation detected positive for phytoplasma using Real time PCR.

Leafhopper*; Orosius sp* was found identical to PDB phytoplasma. However, yellow crinkle and mosaic phytoplasma strain was detected from *Orosius orientalis* and Lace Bug (*Tingidae)* and Australian Lucerne yellows from (ALuY**)** *Orosius orientalis. Orosius* spp are confirmed vectors for papaya phytoplasma diseases and were sampled from the Mareeba blocks in September, October, November, February and April (Appendix 4,Table 13) while others detected positive for the first time for papaya phytoplasma diseases and require further ecological and transmission studies to confirm if they are vector/carriers for PpYC and PpM.

Figure 29 Phytoplasma positive insects Yellow crinkle and mosaic and Australian Lucerne yellows; *Orosius orientalis* (Left) https://en.wikipedia.org/wiki/Orosius_(leafhopper); Yellow crinkle and mosaic; Lace bug (Tingidae) positive (Right) http://www.ozanimals.com/Insect/Sycamore-Lace-Bug/Corythucha/ciliata.html.

8 Impacts

8.1 Scientific impacts – now and in 5 years

The project team has significantly improved the diagnostic techniques for the accurate detection of BCR and papaya phytoplasma diseases. More specifically we have increased the knowledge of papaya pest and diseases for the Philippines and Australia. The identification of the BCR pathogen (*Erwinia mallotivora*) in the Philippines has provided a better understanding of disease epidemiology and the development of disease management strategies to avoid devastating losses from BCR. In addition to this, the identification of the BCR specific marker gene gyrB has allowed for accurate, rapid and early disease diagnoses. Its use has proven highly effective in the screening of alternate hosts, insect vectors, seed and planting material. The submission of the whole genome sequence of the Philippines BCR pathogen and 16 S rRNA sequences of papaya phytoplasma to the NCBI database will provide researchers with a reference for the rapid and precise identification, origin and real time tracking and spread of the disease.

An anticipated benefit of this study is the identification of BCR resistant/ tolerant lines of papaya and the development of a variety or line with resistance to both BCR and PRSV. The availability of these varieties and knowledge of parental lines will benefit the papaya industry at large, especially in countries where other diseases cause extensive plant and yield losses. In addition to this, the identification of regrowth as a mechanism of BCR tolerance and the use of SEM to show an absence of bacterial cells in the vascular tissues of tolerant regrowth plants are important tools in the screening of papaya germplasm for disease tolerance.

The knowledge gained in early BCR symptom recognition, accurate detection techniques and epidemiology of an exotic BCR disease has been shared between researchers, students and growers. In addition, this knowledge has improved the awareness and preparedness of the Australian papaya industry in the event of such a disease incursion in Queensland. A newly emerging technology, such as small-unmanned aerial vehicles (UAVs), was used for the first time for phytoplasma surveillance in Australia and proved an excellent aerial tool for disease estimation and its spatial distribution. The prospects for this technology was shared among researchers and growers (65), and may provide them with the early identification of stress related diseases and disorders to make precise decisions on better crop management.

The information of scientifically detected new insect vectors and alternate weed hosts of dieback and yellow crinkle and mosaic disease in Australia was presented at relevant seminars and workshops and was discussed with the Philippine project collaborators. Additionally, a new disease, papaya Meleira virus, was detected and reported in Australia and discussed with Philippine project collaborators. Two students, Mr. Mark Paul Rivarez and Ms. May Balbin completed their theses on the mechanism of resistance to BCR in regrowth selected lines and on the potential vectors of BCR respectively. Six papers and three posters were presented in national/international conferences and a manual on papaya phytoplasma diagnostic was compiled.

8.2 Capacity impacts – now and in 5 years

This project has enhanced the knowledge and skills of 285 collaborating scientists, technicians, extension workers, students and growers in the field of BCR and phytoplasma symptomatology, disease detection techniques and epidemiology. The project has reduced the impact of these diseases through the intervention of accurate and early disease diagnosis and the implementation of integrated disease management strategies. The project

funds have allowed improvements to research facilities in the Philippines and Australia to be made (laboratory consumables, screen houses, glasshouse facilities (irrigation systems)); supplied technical resources (trainings, information resources, laptops, GPS, PCR machines) and provided job opportunities to students, research assistants and casual workers. Research funds have assisted project staff (15) to participate in a number of annual ACIAR-PCAARRD meetings, two (2) national and international conferences, workshops and two (2) training opportunities. In addition, opportunities for researchers and technical staff to present and publish research papers and articles in scientific journals has been made available. For example, one Filipino researcher visited Australia to attend an international conference at Cairns and one technician attended two weeks training on bacterial disease diagnostic techniques and bioinformatics at DAF Mareeba and James Cook University (JCU), Cairns.

Australian biosecurity is better informed through the accurate diagnosis of BCR causal organisms and knowledge of specific markers for BCR detection that will enable better decision making tools to prevent the entry and establishment of this exotic disease. The participation in national training has increased the knowledge of DAF researchers (2) in a new field molecular diagnostic technique (LAMP). Two researchers have also increased their skills for the diagnostics of newly found papaya Meleira virus disease in Australia.

The phytoplasma workshop conducted at DAF, Mareeba, Queensland has established more linkages between researchers (10) and has strengthened and improved capacity in early and accurate diagnosis of phytoplasma diseases. The discussion held also provided the opportunity to assess various management strategies in relation to insect vectors and alternate hosts. Two Philippine students were able to complete their graduation in the field of plant pathology and plant breeding. Their thesis research was part of this project. The capacity impacts have been substantial to the project collaborators in transferring knowledge to the growers, consultants, technicians, extension workers and students in the area of BCR and phytoplasma symptomatology, disease detection techniques and epidemiology. The increased capacity of collaborating institutes to conduct their own research and teaching will allow better understanding of plant and patho-systems and their knowledge to growers and consultants for better disease management and decision making under changing local and global environmental conditions.

8.3 Community impacts – now and in 5 years

A potential community benefit from strengthening research on papaya productivity was the improvement of communication and trust amongst all stakeholders attributing to greater responsiveness in maintaining quality, delivering on time and adequately sharing resulting profits. The growers were keen to learn more on disease symptoms, mode of disease spread and management strategies. The researchers submitted project proposal to continue research on disease resistant breeding to strengthen the management strategies for new and reoccurring papaya diseases. The Philippines Department of Agriculture has included a project proposal on management of papaya diseases in their 2016-2022 Research, Development and Extension Agenda.

8.3.1 Economic impacts

Papaya is grown throughout the Philippines in commercial plantations, backyards and occasionally in mixed cropping systems. It has been estimated that in seasons favourable for BCR development, lost production could be as high as $US10 million per year (20% of Philippine papaya production). The research outcomes of this project have revealed that the BCR disease requires continuous periods of intense and prolonged wet weather (generally

associated with typhoon activity) to cause extensive plant damage. The infected plant residue and infected plants (regrowth) are the primary source of pathogen survival and windblown rain and insects play an important role in secondary spread of the pathogen. The knowledge gained through this project has been used to formulate the disease management strategies, such as hot water seed treatment, use of clean planting material, removal and disposal of infected plant and planting material, timely use of prophylactic sprays for diseases and insect - pests management. The evaluation of BCR tolerant varieties are underway and will be delivered to smallholder farmers. The integration of these strategies into practice will reduce disease impact and increase yield by 20%, reduce costs, provide environmentally sustainability and thus reduce the impact of the disease by at least 40%.

Bacterial crown rot is an exotic disease in Australia and that has potential to threaten the $30 million fast growing Queensland papaya industry. Knowledge obtained from this research is of significant importance to Australian biosecurity, allowing earlier detection of the bacterial crown rot disease; subsequently, reducing the risk of outbreak and damage to regional production and trade. The continued research on the development of BCR and PRSV tolerant varieties in the Philippines could benefit the Queensland industry by providing better disease tolerance and yield.

The identification of a new papaya disease (Australia), papaya Meleria virus (PMeV), during the project has allowed the industry to develop clean seed and planting material protocols. The economic benefits will arise from an enhanced marketable yield, fruit quality, increased export opportunity and market share, due to improved reliability of production. All sectors of the papaya supply chain will benefit from this research and we believe that reducing disease will have a significant impact on grower incomes. Therefore, the project will deliver improved food security and livelihoods for smallholder farmers.

8.3.2 Social impacts

Smallholder farmers and the commercial organisations envisage a significant social impact through change in attitude. These stakeholders were informed on how to increase productivity and improve their livelihoods through the IDM practices. Periodic meetings and updating of research outcomes to farmers and private commercial organisations, Del Monte, Dole, Sumifru and Tupi papaya growers of South Cotabato, have helped to connect with project activities, learn more about papaya diseases and management strategies. Del Monte papaya actively contributed to field trials (BCR) and the development of management strategies.

In Australia, fortnightly field visits were made and a monthly papaya grower meeting was attended in North Queensland. This direct interaction with growers have improved grower knowledge and a change in their farming practices. Growers have already begun to consider the importance of clean seeds, removal of infected plants, disease monitoring and the practice of sanitation and clean cultivation. The growers have developed in-field key indicators to distinguish phytoplasma-infected plants, thereby rouging of infected plants to reduce the source of inoculum eg, a lack of sap and peach moth damage to fruits and plants. Growers have already begun to consider the importance of insect-vector management, especially during the dry periods of the year when surrounding vegetation is dry and leafhopper shifts to the weeds in papaya plantation. A more strategic application of insect vector during the dry and early spring seasons will allow a reduction in the vector population and thereby disease incidence.

The routine field surveys of papaya plantations also identified some of the new physiological and pathological issues (papaya Meleria Virus). The industry shared their concern about the

availability of quality seed/planting material, new/tolerant varieties and the need to explore new export markets for profitability and strengthening of the papaya industry.

An overall increase in papaya production will lead to improved livelihoods of smallholders through increased revenue flow, while increased productivity by larger landholders is likely to increase employment opportunities for landless rural labourers. Papaya production is widely dispersed, grown mostly by back-yard scale growers. Some 80% of all growers in the Philippines have less than 3 hectares, meaning gains from this project in coming years will benefit many smallholder farmers.

8.3.3 Environmental impacts

In the Philippines, prophylactic sprays with copper-based fungicides, such as copper hydroxide and copper oxychloride, commence when BCR is detected. A recent trial conducted at Del Monte research station proved that these chemical sprays provide little benefit in controlling BCR as compared to other options like the use of phospro (phosphorous acid) and Bacillus formulation. The recommendation of using a hot water seed treatment will allow eradication of the pathogen and will ensure the seed is free from the BCR pathogen. In Australia, management of alternate hosts; *Praxalis clematidae, Sida acuta, Stylosanthes scabra, Crotolaria spp, Cajanaus cajan, Portulaca pillosa, Solanum nigrum and Phyllanthus spp.* in and around papaya plantations could have a positive environmental impact, through reducing the focus on insecticide reliance to control the insect vector of phytoplasma diseases.

A reduction in overuse of these chemical inputs will benefit the consumer by reducing residues in fruit, and the farmer by reducing health and safety issues. Reductions in the use of chemicals will also alleviate pollution of soil, water and aerial environments. Use of tolerant /moderately tolerant varieties to BCR and PRSV in the Philippines and information of tolerant parental lines will advance the Queensland industry by providing better disease tolerance and yield. Such improvements increase grower productivity, profitability, whilst reducing environmental, health and safety impacts.

8.4 Communication and dissemination activities

The meetings and workshops were conducted with all project members, commercial grower organisations and a growers group to review the project progress, update research outcomes and to plan for project activity implementation. The project work involved close collaboration with Del Monte, Sumifru and the Tupi Papaya Growers Association of South Cotabato, which utilised their existing extension and dissemination networks. The main strategies used for communication and dissemination were grower participatory trials, demonstrations, field days and the development of papaya disease information resources. The regular field visits and field days were conducted to address and disseminate the project research outcomes with small and commercial papaya growers, collaborating researchers, students and the papaya industry. For example, the BCR and PRSV resistant breeding trial was used as a learning site for UPLB Plant Breeding and Plant Pathology students, project collaborators, researchers and industry partners East-West Seed Company at Tranca, Bay, Laguna. The IDM research trials at IBP, Davao and Camp Philips, Bukidnon were used to demonstrate the effectiveness of foliar application of antimicrobial agents and resistance inducing chemicals in BCR disease management. Similar activities have been conducted in Australia to deliver phytoplasma management strategies. The information gained from this project was used to design and implement BCR and phytoplasma management research options.

The Philippines project collaborators, Dr Fe Dela Cueva, Dr Pablito Magdalita, Ms Valeriana Justo and Australian collaborators Mr. Lynton Vawdrey, Dr Nandita Pathania, Dr Natalie

Dillion and Ms Donna Chambers delivered project outcomes during field days, growers meetings, ACIAR - PCAARRD annual meetings and in national and international conferences. In Australia, at the time of the severe outbreak of yellow crinkle and mosaic disease in far North Queensland, Mr Lynton Vawdrey addressed papaya growers and papaya industry officials regarding phytoplasma epidemiology and their management options. The information was published in the local newspaper “Port Douglas Gazette” entitled, “Paw paw crop peril”, and “Leafhopper insect wreak havoc on Qld papaya industry”, February 2017. The project outcomes will continue to disseminate and communicate with growers through collaborative organisation websites of UPLB, DAF, PCAARRD, ACIAR and the Australian industry magazine Papaya Press.

A summary of the project communication activities are as follows:

- 22 students, technician, collaborative researchers Del Monte, EWS and Sumifru attended a BCR diagnostic workshop in the Philippines.
- 483 farmers attended workshop and field days in the Philippines and Australia
- 20 PowerPoint presentations
- 3 technicians trained in phytoplasma diagnostics at UPLB and QDAF.
- 14 researchers (Australian and Filipinos) gained knowledge in phytoplasma diagnostic techniques
- 4 oral paper, 4 poster presentation and 1 research paper published in national/international journals conferences
- 1 BSc thesis and 1 MSc thesis
- 1 Papaya Phytoplasma diagnostic manual

9 Conclusions and recommendations

9.1 Conclusions

Bacterial crown rot

This project has successfully identified the causal organism and the marker gene for the early and accurate diagnosis of bacterial crown rot. It has also delivered a better understanding of the epidemiology of the disease, enabling the development of improved disease management strategies.

Research results showed that high rainfall and warm weather conditions favour a high incidence and severity of the disease. Tests also showed that the pathogen does not survive well in soil. The host range was confined to papaya, although pathogens were known to survive on the leaves of *Acalypha indica, Amaranthus viridis*, *Amaranthus spinosus*, and *Synedrella nodiflora* after 4 days of inoculation. Research showed that eight (8) common weed species and six (6) cultivated crops (tomato, melon, guava, cowpea, banana and corn) grown near papaya fields in the Philippines that were inoculated with *E. mallotivora* were not susceptible to the BCR pathogen. The scanning electron microscopy studies showed seasonal survival of the pathogen on infected plant surfaces and in vascular tissues of infected plants. Seed transmission studies were unable to prove that BCR is seed borne. However, Obrero (1980) was able to isolate papaya bacterial crown bacterium from the seed of infected papaya fruit. Furthermore, pathogens were still present in seed after the extraction and air drying process and storage at 10ºC for 30 days. Therefore, as a precaution, it is recommended that growers use a hot water seed treatment to ensure that seed is free from the pathogen. The insects of red spider mites, mealy bugs and scavengers were shown to cause the secondary spread of disease. Foliar applications of Phospro® and *Bacillus* QST 719 strain proved effective in reducing the severity of BCR compared to copper fungicides.

The parent lines 5893 and 5648 were identified as being tolerant to BCR in artificial inoculation tests conducted in the plant-house. However, in recent field evaluation tests, a high level of disease has been observed in crosses with these parent lines indicating that further purification of the F2 lines is required. The research also showed that papaya has mechanisms, which can defend against the BCR infection. These include innate properties such as tissue regeneration and pathogen disinfection by plant latex. Acquired resistance by virtue of the metabolites of beneficial endophytic bacteria and plant associated *Bacillus* species may also enhance disease resistance.

Based on research outcomes, the following best-bet IDM strategy was developed for controlling BCR. It includes the use of clean planting material, the rogueing of infected plants, the ploughing-in of plant debris, insect pest and weed management, the use of Phospro®, *Bacillus subtillis* QST 713 strain and copper sprays, crop hygiene and sanitation. The timely and judicious use of nutrients/fertilizer is also recommended.

Phytoplasma:

A survey of papaya for phytoplasma diseases using molecular techniques revealed that there are three species of phytoplasma causing diseases in north Queensland; i.e. Candidatus P. australiense causing papaya dieback, and Candidatus P. australasia and Ca P. aurantifolia causing yellow crinkle and mosaic disease.

An increased incidence of papaya yellow crinkle and mosaic disease was observed in newly established papaya plantations near native vegetation. It is known that the insect vectors are attracted to papaya to feed when the surrounding vegetation dies during the dry spring/summer period. Therefore, by avoiding the establishment of new plantings during the drier time of the year, papaya plants may escape a high disease incidence.

The recovery of dieback, yellow crinkle and mosaic phytoplasma from a number of symptomatic weeds and other crops revealed that they have a wide host range and are a potential reservoir for these diseases. Therefore, area wide monitoring for phytoplasma diseases, weed control and insect vector management using timely insecticide applications will help reduce the risk of phytoplasma epidemics. The recovery of yellow crinkle and mosaic phytoplasma from all parts of the affected plant, including regrowth following ratooning, indicates that the total removal of the infected plant is an important management tool to help reduce inoculum and the further spread of the disease. Consequently, the use of high-density plant populations has been shown to be effective in maintaining production in the event of a phytoplasma disease outbreak.

Because of this project, papaya growers in Australia and in the Philippines have increased their capacity to identify these diseases and increased their knowledge of insect-pest epidemiology and management strategies of papaya diseases. The outcome of implementing a best-bet strategy will mean increased returns to the grower as the costs associated with lost production due to these diseases will be significantly reduced. This project will therefore deliver improved fruit quality, security and livelihoods for smallholder farmers.

9.2 Recommendations

- The hot water treatment (50°C for 20 minutes) is a standard recommendation (AQIS, 2010) for decontamination of seed for the BCR infested countries and is being followed in Malaysia. The application of hot water treatment in the Philippines will allow for the eradication of the pathogen and ensuring disease free status of seed.

- The development of resistant/tolerant varieties is the most economical, sustainable and environmentally friendly measure for the control of diseases. Therefore, future ACIAR funding would allow for the continuation of the papaya disease resistance breeding work in the Philippines and Australia, with the aim to stabilise and improve the performance of BCR and PRSV tolerant lines. In addition to this, new varieties with desired fruit quality traits could also be developed.

- The use of in-field, molecular diagnostic tools is recommended as this would increase our capacity to quickly identify plant disease in its early stage of development, thus preventing major disease outbreaks.

- The weed and vector controls are the only management strategies for the phytoplasma diseases that are being applied so far. The role of entophytic bacteria and their metabolites should be explored and developed to improve disease management practices.

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