THE LETHAL DISEASE OF COCONUT IN GHANA: DEVELOPING MOLECULAR MARKERS AND PATHOGEN QUANTIFICATION TECHNIQUES FOR THE BREEDING OF RESISTANT OR TOLERANT VARIETIES

By

Egya Ndede Yankey

University of Nottingham, School of Biosciences
Plant Sciences Division
Loughborough, Leicestershire
LE12 5RD, United Kingdom

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy
March 2012

TABLE OF CONTENT

ACKNOWLEDGEMENTS ..... **VII**

LIST OF FIGURES ..... **IX**

LIST OF TABLES ..... **XI**

LIST OF PLATES ..... **XIII**

LIST OF APPENDICES ..... **XIV**

ABBREVIATIONS ..... **XV**

ABSTRACT ..... **XVII**

CHAPTER 1: GENERAL INTRODUCTION **1**

1.1 THE COCONUT PALM AND CONSTRAINTS TO ITS CULTIVATION 1

1.2 ECONOMIC IMPORTANCE OF COCONUT 2

1.3 PHYTOPLASMAS 4

1.3.1 History of discovery *4*

1.3.2 Morphology and genomic characteristics of phytoplasmas *5*

1.3.3 Diseases caused by phytoplasmas *7*

1.3.4 Pathogenesis and impact on hosts *10*

1.3.5 Phytoplasma taxonomy *12*

1.3.6 Transmission of phytoplasmas *15*

1.3.7 Phytoplasma diagnostics *18*

1.4 THE LETHAL YELLOWING DISEASES 22

1.4.1 History and spread of LD in Ghana *24*

1.4.2 Symptoms of LD *28*

1.4.3 Research effort at overcoming LD in Ghana *30*

1.5 ALTERNATE HOSTS OF THE LD PHYTOPLASMA 32

1.6 SEED TRANSMISSION OF LD 33

1.7 MARKER ASSISTED SELECTION IN COCONUT BREEDING 34

1.8 AIMS AND OBJECTIVES 38

CHAPTER 2: DEVELOPING MOLECULAR MARKERS FOR VARIETY VERIFICATION....40

2.1 INTRODUCTION 40

2.2 MATERIALS AND METHODS 47

2.2.1 Plant Materials (coconut tissues) *47*

2.2.1.1 Location of palms and sample numbers 47

2.2.1.2 Sampling method and periods 48

2.2.2 Buffers and chemicals used for DNA extraction *49*

2.2.3 DNA extraction protocols *50*

2.2.3.1 CTAB extraction (Daire *et al.* 1997) 50

2.2.3.2 DNA clean-up with polyvinyl polypyrrolidone (PVPP) 51

2.2.4 PCR analysis *52*

2.2.5 Agarose gel electrophoresis *53*

2.2.6 Cloning *53*

2.2.6.1 Ligation reactions 54

2.2.6.2 Transformation of competent cells 54

2.2.6.3 PCR assessment of colonies 55

2.2.7 Purification of PCR products *55*

2.2.8 Melt curve analyses *55*

2.2.9 Real-time LAMP *56*

2.3 RESULTS 58

2.3.1 Investigating an array of SSR markers for possible linkage to tolerance/resistance or susceptibility in coconut varieties *58*

2.3.1.1 Improving resolution of allele size differences using melt curve analyses 60

2.3.2 Developing SNP markers based on genes encoding WRKY transcription factors *67*

2.3.2.1 Development of LAMP assays to detect SNPs in coconut varieties ... 67

2.3.2.2 Real -time LAMP (RT-LAMP) detection of SNPs 71

2.3.2.3 Testing specificity of assays for amplifying WRKY-05 on mixed DNA samples 74

2.3.3 Developing markers based on single nucleotide polymorphisms using the candidate gene approach targeting the NBS-LRR Class R-genes *76*

2.3.3.1 Oligonucleotide primers and PCR approach 76

2.3.3.2 Sequence analyses of amplified fragments 79

2.4 DISCUSSION 83

CHAPTER 3: STUDYING THE GENETIC BASIS OF TOLERANCE/ RESISTANCE OF COCONUT GERMPLASM TO LD AND THE SEASONAL QUANTIFICATION OF PATHOGEN TITRES IN DISEASED PALMS 87

3.1 INTRODUCTION 87

3.1.1 Influence of the distribution of phytoplasmas and environmental factors on the development of LY/LD *87*

3.1.2 Quantification of nucleic acids by real-time PCR *88*

3.1.3 Field based diagnostic methods for LD *91*

3.2 MATERIALS AND METHODS 94

3.2.1 Q-PCR primers and preparation of standards *94*

3.2.2 Q-PCR assay and quantification method *95*

3.2.3 PCR diagnosis of LD-infection *96*

3.2.4 Detection of LAMP products using lateral flow devices (LFD) *97*

3.2.5 Extraction of DNA using LFD *98*

3.3 RESULTS 100

3.3.1 Assessing the efficacy of the DNA extraction protocol using multiplex PCR *100*

3.3.2 Assessing the effectiveness of different PCR assays in detecting the LD phytoplasma *102*

3.3.3 Evaluating a real-time LAMP assay as a diagnostic method for LD *105*

3.3.4 Assessment of LD-infection and disease development characteristics among the selected coconut varieties *112*

3.3.5 Seasonal quantification and distribution of phytoplasma levels in infected palms *117*

3.3.5.1 Performance characteristics of the real-time PCR assay 117

3.3.5.2 Assessing the levels of phytoplasmas in infected palms and their seasonal fluctuations 122

3.4 DISCUSSION 128

CHAPTER 4: SEARCHING FOR ALTERNATE HOSTS OF THE LD PHYTOPLASMA.....133

4.1 INTRODUCTION 133

4.2 MATERIALS AND METHODS 135

4.2.1 Plant materials, locations and sampling method 135

4.2.2 Sample preparation 136

4.2.3 DNA extraction 137

4.3 RESULTS 138

4.3.1 Assessing the diversity of the sampled flora 138

4.3.2 Assessing the effectiveness of the extraction process 143

4.3.3 Assessment of plant samples for LD phytoplasma infection 144

4.4 DISCUSSION 145

CHAPTER 5: INVESTIGATING THE POSSIBILITY OF PHYTOPLASMA TRANSMISSION FROM SEEDS OF LD-INFECTED PALMS TO PROGENY PLANTS 147

5.1 INTRODUCTION 147

5.2 MATERIALS AND METHODS 150

5.2.1 Sampling of coconut seeds 150

5.2.2 Establishment of seed nursery 151

5.2.3 Extraction of solid endosperm cylinders containing the embryos 152

5.2.4 Surface sterilisation of embryo cylinders and excision of embryos used for plumule culturing 155

5.2.5 Preparation of PCA Y3 culture medium 157

5.3 RESULTS 160

5.3.1 Assessment of seedlings derived from diseased palms for the presence of phytoplasma DNA 160

5.3.1.1 Confirmation of LD-infection in sampled palms and assessment of embryos for the presence of phytoplasma DNA ahead of germination tests 160

5.3.1.2 Determining the germination rates of seeds from infected versus healthy palms and assessment of seedlings for phytoplasma DNA 163

5.3.2 Assessment of embryo sections for the presence of phytoplasma DNA ahead of embryo culturing..... 164

5.4 DISCUSSION 167

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS.....170

REFERENCES 183

APPENDICES 207

ACKNOWLEDGEMENTS

I would like to say thank you to Prof Matthew Dickinson, who was my main supervisor, for his patience, advice and encouragement during the period of this research and the writing of this thesis. I am also grateful to Dr Joseph Nipah, my second supervisor for his support and for his guidance on the field work. My heartfelt appreciation to Dr Phil Swarbrick, a postdoctoral researcher who worked along with me on this project and taught me almost all the molecular techniques used in this work. Data was shared between us on the marker development and pathogen quantification work, which was done in collaboration.

I acknowledge the contributions of my colleagues at the Oil Palm Research Institute's (OPRI), Coconut Research Programme in Sekondi, Ghana. Special mention is made of Robert Quaicoe, Sylvestre Obeng-Darko, Richard Affail and Joseph Opoku for their help with the field sampling.

Funding for this study was largely provided by the Biotechnology and Biological Sciences Research Council (BBSRC-UK) and the Department for International Development (DFID-UK). The aspect on the development of field-based diagnostics was funded by the Leverhulme-Royal Society Africa Award.

I appreciate the support of my wife, Ophelia and son, David who had to endure my absence from home during the period of my study – thanks for your understanding and patience.

Finally, I thank God for granting me strength and good health to go through this period.

LIST OF FIGURES

1.1 Host cycle of phytoplasmas 17

1.2 LD distribution in Western, Central and Volta Regions of Ghana 27

2.1 Schematic organisation of LAMP primers 45

2.2 Amplification of SSR DNA from coconut at loci CnCirH4 (A) and CAC65 (B) 59

2.3 Melt analyses of VTTs and WATs at locus CnCirC12 61

2.4 Melt analyses of marker CnCirC12 showing clustering around four melt peaks (alleles): the SGDs and VTT1&2; VTT 6; the MYDs and the WATs 62

2.5 Melt analyses of SSR DNA at locus CAC65 62

2.6 Further analyses of locus CAC65 with more samples of each variety type 64

2.7 Further analyses of locus CnCirC12 with more samples of each variety type 65

2.8 Further analyses of WAT and SGD samples for possible WAT specific allele 66

2.9 RT-LAMP amplification and output for SNP containing sequences using assays based on WRKY-05 72

2.10 Alignment of sequences from tall and dwarf varieties at locus CnWRKY-05 73

2.11 Agarose gel showing PCR amplification of RGAs from WAT sample 78

2.12 Phylogenetic relationships amongst the amplified coconut RGAs and some database RGAs 82

3.1 PCR amplification of coconut and phytoplasma DNA using CSPWDSecAFor2/Rev2 and CnCirF3 F/R 101

3.2 RT-LAMP outputs: (A) A plot of fluorescence against time (isothermal amplification) showing the time (min) at which amplification starts (indicated by arrows) and (B) A melt plot used to validate amplification results by comparing the melting temperature of amplicons to that of a control DNA (B1) 106

3.3 RT-LAMP output of analyses using DNA extracted with LFD 111

3.4 Agarose gel analyses of PCR amplified plant and pathogen standards 118

3.5 Standard curves of plant (A) and (B) pathogen standards 120

3.6 Gel analyses of real-time PCR products 121

3.7 Seasonal variation of phytoplasma titres in infected palms 122

3.8 Distribution of phytoplasmas in the trunk of infected palms 124

3.9 Variation of phytoplasma levels in different tissues of the palm 125

3.10 Pathogen titres in palms at different stages of LD-infection 127

4.1 Genomic DNA extracted by the modified Daire *et al.* (1997) CTAB protocol 143

5.1 P1/P7 PCR amplification of phytoplasma DNA from trunk samples and nested PCR (P1/P7 followed by GH813f/AwkaSR) detection of phytoplasma DNA in coconut embryos (from first collection) 161

5.2 Nested PCR (P1/P7 followed by GH813f/AwkaSR) amplification of phytoplasma DNA from trunk and embryo DNA samples (second collection) 162

LIST OF TABLES

1.1 Edible and non-edible uses of various parts of the coconut palm 3

2.1 WRKY SNP containing sequences identified in coconut 68

2.2 LAMP primers used in assays developed for amplifying WRKY sequences 70

2.3 RT-LAMP output of assays for amplifying CnWRKY-05 using mixed and unmixed DNA samples 75

2.4 Oligonucleotide combinations used to amplify RGAs from coconut DNA in second round PCR 77

2.5 BLAST searches for closest matches of amplified coconut RGAs 80

3.1 Comparative performance of different PCR assays at detecting the LD phytoplasma 103

3.2 Comparison of real-time LAMP, LAMP detection by LFDs and PCR assays for LD diagnosis 108

3.3 Further comparison of the RT-LAMP assay with conventional PCR assays (including both single round and nested PCR assays) 109

3.4 Summary of disease incidence among the selected palms 114

3.5 Survival rate of infected WAT palms 116

4.1 List of plant species, their families and number of samples analysed 139

4.2 Plant families known to host phytoplasmas and whose members were sampled 141

5.1 Seeds harvested for planting and for assessing phytoplasma DNA presence in coconut embryos 152

5.2 Composition of PCA Y3 medium 158

5.3 Germination percentages of seeds of healthy and infected palms 164

LIST OF PLATES

1.1	Electron micrographs of cross sections of sieve tubes showing phytoplasmas with varying shapes and sizes	6
1.2	Some symptoms of phytoplasma mediated-diseases	9
1.3	Sequence of symptom development of LD	29
3.1	Extraction of coconut DNA using LFD	110
4.1	Plants showing symptoms of phytoplasma infection and 'tell tale' symptoms of disease	142
5.1	Extraction of endosperm cylinders from coconut seeds	154
5.2	Excision of embryos from endosperms for plumule cultures	156
5.3	Plumule cultures showing differentiating leaves	166

LIST OF APPENDICES

1. Primers used to amplify coconut microsatellite DNA 207

2. Oligonucleotide primers used to amplify RGAs from coconut DNA 209

3. LAMP Primers for LD assay 210

4. Photographic documentation of plant species sampled for alternate hosts studies 211

ABBREVIATIONS

AFD	Agence Francaise de Development
BIP	Backward inner primer
BL	Backward loop primer
BNARI	Biotechnology and Nuclear Research Institute
bp	Base pairs
COGENT	Coconut Genetic Resources Network
CSDP	Coconut Sector Development Programme
CSIR	Council for Scientific and Industrial Research
CSPWD	Cape St. Paul wilt disease
CT	Cycle threshold
FIP	Forward inner primer
FL	Forward loop primer
g	Relative centrifugal force
HRM	High resolution melt
ICSB	International Committee on Systematic Bacteriology
IRPCM	International Research Program for Comparative Mycoplasmology
kb	Kilobases
LAMP	Loop–mediated isothermal amplification
LD	Lethal disease
LFD	Lateral flow device
LY	Lethal yellowing
LYD	Lethal yellowing-like disease
MLO	Mycoplasma-like organisms
MYD	Malayan Yellow Dwarf
PCR	Polymerase chain reaction

PNT	Panama Tall
Q-PCR	Real-time quantitative PCR
RGA	Resistance gene analogues
RPM	Revolutions per minute
RT-LAMP	Real-time LAMP
SGD	Sri-Lankan Green Dwarf
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism
ssDNA	Single-stranded DNA
SSR	Simple sequence repeats
T_m	Melting temperature
VTT	Vanuatu Tall
WAT	West African Tall
WRKY	WRKY transcription factors
w/v	Weight to volume (expressed as a percentage)

ABSTRACT

Lethal diseases (LD) of coconut caused by phytoplasmas have destroyed millions of palms globally and pose a serious threat to the coconut industry in Ghana. This study investigated the genetic basis of resistance/tolerance of coconut varieties and hybrids to LD. The study was also aimed at developing molecular markers for cultivar verification to be used for the sustainable breeding of high value varieties and hybrids. Using PCR diagnosis and monitoring of symptoms of LD over a three year period, the study determined that escapee palms of the West African Tall ecotype (WAT) found in LD-devastated fields die once infected and do not represent resistant/tolerant sub-populations. Six monthly observations of symptoms and PCR diagnosis showed that LD-infection and symptom development occurred all year round. Quantification of phytoplasma amounts using novel quantitative real-time PCR methods did not reveal a pattern in pathogen disease titres between the rainy and dry seasons or between plant parts. Due to a lack of LD-infection in the sampled SGDxVTT and MYDxVTT palms, the basis of their resistance/tolerance could not be determined. Out of 44 microsatellites markers assessed for their usefulness in differentiating between the Ghanaian breeding materials, only two of the markers, CnCirC12 and CAC65 initially appeared to be associated with alleles specific for the susceptible 'West Africa Tall' variety but screening with further samples showed these two markers also to not be specific . The study found that, palms of each variety did not show consistent genotypes for variety-specific SSR markers to be identified. Diagnostic assays based on the LAMP technique (DNA amplification at a single temperature using *Bst*

polymerase) were assessed for their potential for in-field use. The simplicity of the technique and the rapidity with which results are obtained (<30 min) demonstrated that this non-PCR technique could be a future method of choice for field diagnostics in Ghana and in Africa at large. Seeds from LD-infected palms were assessed for their ability to transmit the LD phytoplasma to progeny plants. Out of 105 coconut seedlings derived from LD-infected palms, none was found to be infected or developed LD after six monthly PCR diagnosis for 18 months. This study concludes that LD is unlikely to be seed transmitted and that the DNA fragments detected in coconut embryos may not represent a viable organism.

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE COCONUT PALM AND CONSTRAINTS TO ITS CULTIVATION

Coconut (*Cocos nucifera* L.) belongs to the *Arecaceae* family (formerly *Palmaceae*) and the *Coicoidea* sub family. The palm has no close relatives and is the only species of the genus *Cocos*. Coconut is a diploid and has 32 chromosomes (Teulat *et al.*, 2000; Foale, 2005; Perera *et al.*, 2008). Coconut is a crop of the humid tropics and grows in over 86 countries in four continents (Perera *et al.*, 2000). It is believed to have originated in the Indo-Malayan Region (Indonesia, Malaysia and the Philippines) from where it spread driven by ocean currents to tropical coasts where it became established (Rajesh *et al.*, 2008). It is thought to have been introduced to Africa by European navigators (Lebrun *et al.*, 1998).

The palm has a woody trunk which grows erect if not caused to bend leeward by strong winds. The trunk terminates in a long and broad crown of green pinnate leaves arranged at different angles all around the trunk, with the lowest leaves on the whorl being the oldest. The extensive adventitious roots of the palm hold it firmly in the ground (Ohler, 1984; Bourdeix *et al.*, 2005). The palm is usually divided into two distinct groups, the 'talls' and the 'dwarfs'. The talls are usually outcrossing and the dwarfs are autogamous, however the two groups can successfully cross to form hybrids (Foale, 2005).

Coconut is a small holder crop and usually grown by resource poor farmers mostly in developing countries of the tropics. Global production of coconut has been on the decline in recent years and in the last decade (1998 – 2007); global production of coconut did not see any significant growth in terms of the area under cultivation, which is estimated at 12 million hectares (Legoupil and Courbet, 2008). The decline in farm productivity can be attributed to a number of factors including competition against coconut oil (the main commercial product of the palm) from other oil seed plants such as sunflower, soya bean and rapeseed which are short term crops (Batugal *et al.*, 2005; Legoupil and Courbet, 2008). Coconut is a perennial crop and therefore difficult to programme production to respond to market demand. Other factors include the incidence of pests and diseases, unavailability of affordable high yielding and adapted varieties, the lack of resources to invest in innovations to improve yields and incomes and the lack of maintenance of coconut farms (Batugal *et al.*, 2005).

1.2 ECONOMIC IMPORTANCE OF COCONUT

As a result of the myriad of benefits man has derived from coconut, man has showered the palm with several praises. These include; "the tree of life, a tree of a hundred uses, tree of paradise and the milk bottle on the doorstep of mankind" (Ohler, 1984; Bourdeix *et al.*, 2005). The palm is of special importance to rural communities since all parts of the palm are used for food and non-food purposes (Table 1.1).

Table 1.1 Edible and non-edible uses of various parts of the coconut palm

Edible uses	
Sap obtained by tapping inflorescence	• Toddy (unfermented) • Sugar (by boiling) • Alcohol (by fermentation and distillation) • Vinegar (by fermentation)
Immature husk and shell (endocarp)	• Chewed like sugar cane
Water from immature fruit	• Sweet, refreshing, uncontaminated drink; some with aromatic flavour
Mature endosperm	• Coconut flour • Milk & cream • Edible oil (solid at low temperature or hydrogenated to ghee and margarine
Copra cake	• Animal feed
Industrial uses	
Coconut oil	• Lubricant (and anti-rust) • Illuminant (stearine candles) • Fuel (direct substitute for diesel with or without esterification) • Ingredient for soap, shampoo, cosmetics
Shell	• Charcoal (excellent for activation in gold recovery & gas production) • Directly burnt as fuel • Half shell as a container (collecting latex when rubber tapping etc.)
Husk	• Cocopeat for horticultural soil mixtures • Fibre (coir) for ropes, mats & geotextiles
Stem	• Timber (lumber, flooring, furniture etc)
Medicinal uses	
Water from immature fruit	• Naturally sterile, isotonic substitute for blood plasma in emergency surgery • diluents in artificial insemination • athletic rehydration drink

Source: Adapted from Harries *et al.* (2004).

In spite of these tremendous advantages derived from the cultivation of the crop, several factors impact negatively on its production; prominent among them are diseases, particularly a phytoplasma-mediated disease referred to as lethal yellowing (LY) in the Americas and the Caribbean region and lethal disease (LD) or lethal yellowing-like disease (LYD) in Africa (Eden-Green, 1997; Ofori and Nkansah-Poku, 1997).

1.3 PHYTOPLASMAS

1.3.1 History of discovery

Phytoplasmas lack a cell wall and are a group of prokaryotic plant pathogens that are generally found in the phloem of plants and the salivary glands of their insect vectors (Lee *et al.*, 1998; Christensen *et al.*, 2005). They were discovered by Doi and co-workers in 1967 who consistently observed these pleiomorphic bodies in leaves and shoot preparations of the mulberry tree and other plants infected with yellows disease. Before the discovery of phytoplasmas, the etiological agents of plant yellows diseases were thought to be viruses because the diseases were infectious, produced similar symptoms to viral diseases and were transmitted by insects (Bertaccini and Duduk, 2009; Kunkel, 1926, see Gasparich, 2010).

Phytoplasmas were initially referred to as mycoplasma-like organisms (MLOs) because of their morphological and ultrastructural similarities to mycoplasmas, which cause diseases in animals and humans and like mycoplasmas, they were sensitive to treatment with the antibiotic

tetracycline (Doi *et al.*, 1967). The name MLOs was used until 1994 when the sub-committee on the taxonomy of mollicutes of the International Committee on Systematic Bacteriology (ICSB) applied the trivial name 'Phytoplasmas' to reflect their evolutionary distance from other bacteria of the class mollicute. They indicated that by their phytopathogenic properties and habitat in phloem sieve tubes, these organisms were neither mycoplasmas nor MLOs (ICSB, 1993, 1997).

1.3.2 Morphology and genomic characteristics of phytoplasmas

When observed under an electron microscope, phytoplasmas appear as rounded to filamentous pleiomorphic bodies and have varying sizes ranging from 200 to 800 nm (Plate 1.1) (Lee *et al.*, 2000; Bertaccini, 2007). The filamentous forms may be branched. It has been suggested that the diverse sizes of phytoplasmas observed in their plant hosts may represent their developmental stages. They have a single unit cell membrane and lack a rigid cell wall (Haggis and Sinha, 1978; Lee *et al.*, 2000). Phytoplasmas have varying genome sizes ranging from 530 kb (which is the bacterium with smallest genome determined) to 1350 kb; the Bermuda grass white leaf phytoplasma and a tomato strain of the stolbur phytoplasma represent the smallest and largest genomes known, respectively (Marcone *et al.*, 1999; Lee *et al.*, 2000; Firrao *et al.*, 2007). Variability in genome size also exists among closely related phytoplasmas in a sub-group: the Rape virescence phytoplasma and the Hydragea phyllody (Belgium strain) of the aster yellows subgroup have genome sizes of 1130 kb and 660 kb respectively (Marcone *et al.*, 1999).

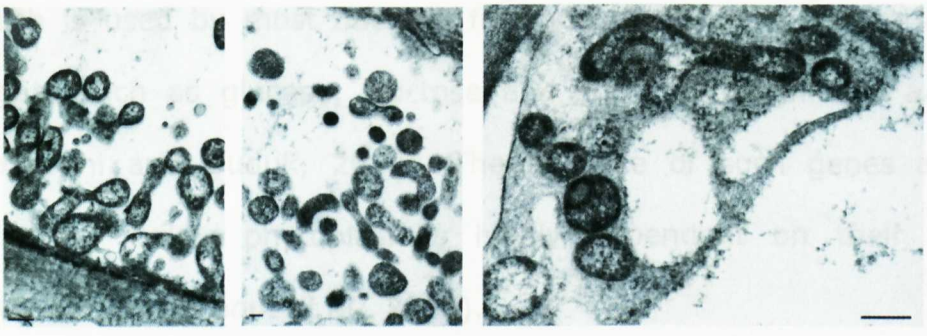


Plate 1.1 Electron micrographs of cross sections of sieve tubes showing phytoplasmas with varying shapes and sizes. Source: Adapted from Bertaccini and Duduk (2009). Bar = 200 nm in each figure.

This heterogeneity of genome size has been attributed to the presence of clusters of repeated gene sequences called potential mobile units (PMUs) (Bai *et al.*, 2006) or sequence variable mosaics (Jomantiene and Davies, 2006) and these are thought to enable phytoplasmas to adapt to the diverse plant and insect hosts in which they live (Razin, 2007). Similar to other mollicutes, phytoplasmas have a guanine plus cytosine (G+C) content as low as 23.0–29.5 mol% (Razin *et al.*, 1998, Bertaccini and Duduk, 2009).

Phytoplasmas have been a poorly studied group of plant pathogens despite the numerous diseases they cause because of their non-amenability to *in vitro* culturing. This phenomenon is thought to be due to the lack of essential genes and their functions (Razin, 2007). Phytoplasmas, like other mollicutes, lack the genes for the biosynthesis of amino acids, fatty acids and nucleotides (Oshima *et al.*, 2004;

Christensen *et al.*, 2005). However, phytoplasmas lack other metabolic pathway genes including, the absence of a phosphotransferase system, which is used by most bacteria for the import and phosphorylation of sugars such as glucose, fructose and sucrose (Oshima *et al.*, 2004; Bertaccini and Duduk, 2009). The absence of such genes and their functions makes phytoplasmas highly dependent on their host for nutrients (Hogenhout *et al.*, 2008).

1.3.3 Diseases caused by phytoplasmas

Phytoplasmas cause diseases globally in hundreds of plants including grasses, vegetables, ornamental plants, orchards and fibre plants (Lee *et al.*, 2000; Bertaccini and Duduk, 2009; Gasparich, 2010). In Brazil for example, phytoplasmas are reported to infect plants belonging to 28 botanical families (Montano *et al.*, 2007). The aster yellows phytoplasmas alone are reported to cause diseases in about 85 plant species in all continents (Lee *et al.*, 2000). Heavy losses of phytoplasma infected plants have been variously reported for crops such as lime in Oman and Iran; peach and cherry in the United States; rice in several regions of south-eastern Asia, potato and corn in Central and South America; grapes in Europe and Australia; legumes in Asia; elms in Europe and North America and coconut in Africa and the Carribean region (Eden-Green, 1997; Bertaccini and Duduk, 2009). However, in some Poinsettia (*Euphorbia pulcherrima*) cultivars, a phytoplasma is reported to be the agent responsible for free branching, a desirable characteristic, because

of the plant's floral uses (Lee *et al.*, 1997; Nicolaisen and Horvath, 2008) (Plate 1.2 E).

Phytoplasma infected plants exhibit various symptoms depending on the stage of disease development and the type of phytoplasma involved. Some plants are, however, able to harbour the phytoplasma without showing any symptoms. Symptoms induced by phytoplasmas include: stunting (Plate 1.2A & B), yellowing of leaves (Plate 1.2C), virescence (the development of green flowers and the loss of normal flower pigments) (e.g. Plate 1.2D), phyllody (development of floral parts into leafy structures), sterility of flowers, abnormal elongation of internodes resulting in slender shoots, proliferation of auxiliary or axillary shoots resulting in 'witches' broom' appearance, leaf curl, bunchy appearance of growth at the ends of stems and generalized decline (Lee *et al.*, 2000; Choi *et al.*, 2004; Hogenhout *et al.*, 2008; Bertaccini and Duduk, 2009).

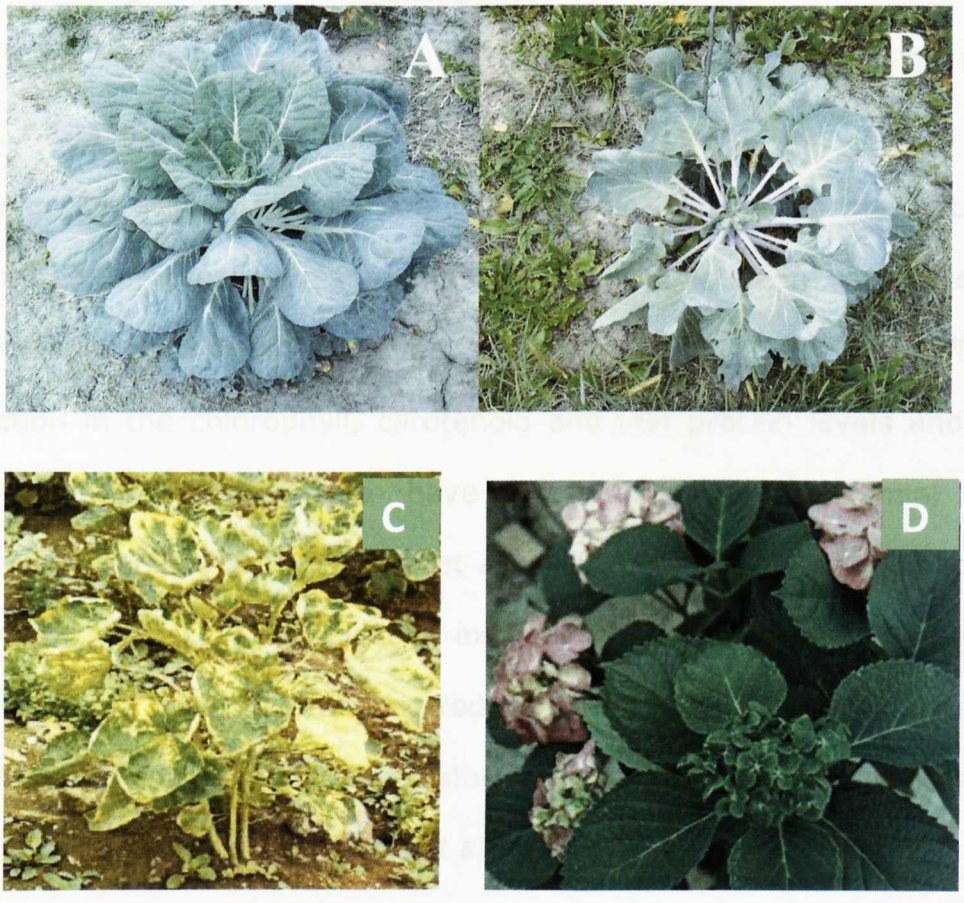


Plate 1.2 Some symptoms of phytoplasma-mediated diseases. Healthy kale (A) and infected plant exhibiting stunted grwoth (B) (Trkulja *et al.*, 2011); leaf yellowing in okra (C) (Gungoosingh-Bunwaree *et al.*, 2011); infected hydrangea showing a virescent flower (D) (Bertaccini, 2007); Healthy *E. pulcherrima* (Ea) with long internodes and infected plants showing branched internodes (Eb) (Nicolaisen and Horvath, 2008).

1.3.4 Pathogenesis and impact on hosts

The exact mechanism by which phytoplasmas induce disease in their plant hosts has not been elucidated. The symptoms they induce can, however, be associated with some physiological processes that become disordered in diseased plants. Photosynthesis has been shown to be impaired in phytoplasma infected grapevines and apples; a significant reduction in the chlorophyll, carotenoid and leaf protein levels and the overall photosynthesis rate have been observed (Bertamini and Nedunchezhian, 2001; Bertamini *et al.*, 2003). Stomatal conductance, a parameter necessary for gaseous exchange and for water and nutrient transport has been shown to reduce progressively as the disease developed to complete closure at stage 3 (a late stage of the disease) in LY-affected coconut palms (Leon *et al.*, 1996).

Inhibition of phloem transport leading to the accumulation of higher levels of carbohydrates in mature leaves and a marked reduction in sink tissues such as young leaves and roots has been reported in phytoplasma infected tobacco and *C. roseus* (Lepka *et al.*, 1999). In coconut, Maust *et al.* (2003) proposed a model for the development of symptoms in LY-infected palms which stated partly that the reduced carbohydrate in the sink organs leads to reduced respiration in the roots, which, affects ion uptake and the synthesis of some plant growth regulators causing physiological changes in the canopy and premature senescence and abscission of fruit and leaves.

Some typical phytoplasma-mediated disease symptoms such as nut drop (fruit abscission) in coconut and other general symptoms such as out of season flowering, phyllody, premature bud opening and other growth disorders are suggestive of hormonal imbalance in the plants (Kuske and Kirkpatrick, 1992; Leon *et al.*, 1996; Chang, 1998). In coconut, nut drop was induced in symptomless palms by the application of ethephon, the ethylene releasing agent and a temporal increase in EFE, the ethylene forming enzyme has been demonstrated suggesting a possible involvement of ethylene in fruit abscission (Leon *et al.*, 1996). Cytokinin levels were observed to increase markedly in virescent flowers of infected *C. roseus* as compared to uninfected plants (Davey and Staden, 1981). In the internodes of infected *Euphorbia pulcherrima* plants, out of four genes that were differentially expressed, three were known to be associated with phytohormone action (Nicolaisen and Horvath (2008).

Phytoplasma concentration in infected plants is also considered an important pathogenicity factor (Berges *et al.*, 2000). Results from the quantification of phytoplasmas in infected *E. pulcherrima* by Christensen *et al.* (2004) using real-time PCR (Q-PCR) assays seem to suggest that phytoplasma concentration is directly proportional to the severity of symptoms. Kuske and Kirkpatrick (1992) using DNA hybridization assays also demonstrated that phytoplasma concentration and distribution is directly correlated with severity of symptoms.

Inter- and intra-plant fluctuations in phytoplasma concentration and distribution in infected plants have been reported (Siddique *et al.*, 1998; Christensen et *al.*, 2004; Wei *et al.*, 2004; Bertaccini and Duduk, 2009) and such knowledge may be useful in the choice of plant tissue to sample for phytoplasma purification. Seasonal variations in phytoplasma levels are also known. In some deciduous woody hosts such as *Malus* spp. and *Pyrus* spp. phytoplasmas are reported to overwinter in the roots and emerge during spring to re-colonise the aerial parts (Schaper and Seemuller, 1982).

1.3.5 Phytoplasma taxonomy

Phytoplasmas belong to the Superkingdom Prokaryota; Kingdom Monera; Domain Bacteria, Phylum Firmicutes; Class Mollicutes; *Candidatus* (*Ca.*) genus Phytoplasma (Hogenhout *et al.*, 2008). Mollicutes are derived from Gram positive bacteria and are considered to be closely related to bacteria such as *Bacillus*, *Clostridium* and *Streptococcus* (Razin, 1985; Razin *et al.*, 1998; Hodgetts *et al.*, 2008). Other mollicutes include the Mycoplasmas, Acholeplasmas and Spiroplasmas. Phytoplasmas comprise a monophyletic clade that arose from the *Acholeplasma* branch and share similar properties such as codon usage, metabolic requirements and the absence of a functional phosphotransferase transport system (PTS) (Lee *et al.*, 2000; Oshima *et al.*, 2004; Bai *et al.*, 2006).

Phytoplasma classification has mainly been based on analysis of genes encoding the highly conserved 16S rRNA. RFLP analysis of PCR amplified 16S rDNA products has been used to classify and assign newly identified phytoplasmas to groups and subgroups in a scheme referred to as the 16Sr groups. The method involves the use of a suite of restriction enzymes (15-18) to generate RFLP profiles of representative phytoplasma strains associated with several diseases. The resultant patterns are compared and used to assign the phytoplasmas to groups and subgroups (Lee *et al.*, 1993, 1998, 2000). Lee *et al.* (1998, 2000) constructed a 16S rRNA classification scheme comprising 14 groups and about 40 subgroups; the list continues to be updated and presently there are 19 16Sr groups and about 50 sub-groups (Lee *et al.*, 2010). The 16Sr classification system has been shown to be consistent with phylogenetic groupings based on analysis of the 16S rRNA gene sequences (Bertaccini and Duduk, 2009; Lee *et al.*, 2010).

As a result of difficulties with maintaining representative strains for RFLP analysis, a computer simulated RFLP analysis of 16S rDNA sequences was developed (Wei *et al.*, 2008; Zhao *et al.*, 2009). The virtual RFLP scheme has been used to classify phytoplasmas, including previously unclassified ones into 30 groups and more than 100 sub-groups (Zhao *et al.*, 2010). This classification system, however, relies on the availability of sequence information and therefore the lack of equipment and facilities can hinder the classification of new phytoplasmas in some labs (Hodgetts *et al.*, 2010). In such instances, actual RFLP can be used as a preliminary

classification method to characterise unknown phytoplasma strains (Lee *et al.*, 2010).

Due to its highly conserved nature, the 16S rRNA gene is limited in its use for finer differentiation of closely related but distinct strains of phytoplasmas in a 16Sr subgroup and other less conserved genes such as the ribosomal protein (rp), 23S rRNA, 16-23S rRNA intergenic spacer region (ISR), *tuf* gene, *secY* and *secA* genes have been used as diagnostics (Lee *et al.*, 1998; Garcia-Martinez *et al.*, 1999; Lee *et al.*, 2000; Hodgetts *et al.*, 2007; Hodgetts *et al.*, 2009). These additional genes have provided finer resolution in some 16Sr groups and subgroups. The phytoplasma associated with the LY/LYD, for example belongs to group 16Sr IV which has two subgroups, 16Sr IV-A and 16Sr IV-B (Lee *et al.*, 1998, 2000). It has, however, been shown that strain differences exist among members of 16Sr IV-B which are associated with coconut in East and West Africa and was therefore further subdivided into 16Sr IV-B and 16Sr IV-C respectively (Mpunami *et al.*, 1999; Hodgetts and Dickinson, 2010). This finer resolution is supported by the *in silico* classification by Wei *et al.* (2007) who placed the Nigerian strain of the phytoplasma (which has a similar 16S rRNA sequence identity to the LD causing phytoplasma in Ghana) into a distinct group, 16S rRNA XXII. The sequence analysis of the *secA* gene of the coconut associated phytoplasma by Hodgetts *et al.* (2008) also supports the distinction between the phytoplasmas in the three subgroups.

Parallel to the 16Sr groups is another classification system proposed by the International Research Program for Comparative Mycoplasmology (IRPCM) in 2004, in which phytoplasmas were assigned to a provisional genus referred to as *Candidatus* (*Ca*) Phytoplasma. In this system, each species or group is represented by the 16S rRNA gene sequence of a reference strain (> 1200 bp) and strains within a species share at least 97.5% sequence identity with the reference strain. A strain is considered as a new *Candidatus* species if its 16S rRNA gene sequence is less than 97.5% similar to the sequence of any previously described species. It was, however, recognised that it is possible for two or more phytoplasmas to share >97.5% sequence similarity and be ecologically distinct from one another. In such instances other factor such as host and vectoring status are used to separate the species. Using the *Candidatus* (*Ca*) system, 28 'species' have been identified (Zhao *et al.*, 2010).

1.3.6 Transmission of phytoplasmas

In nature, phytoplasmas are transmitted mainly by insect vectors belonging to the families Cicadelloidea (leafhoppers) and Fulgroidea (planthoppers) (Lee *et al.*, 1998). Transmission can also occur through vegetative propagation through cuttings, storage tubers, rhizomes or bulbs but they cannot be introduced into hosts by artificial inoculation (Kirkpatrick, 1992; Lee *et al.*, 2000). For an insect to plant transmission, the insect acquires the pathogen through the stylet during its feeding activities. The phytoplasma then attaches itself to the mid gut of the insect and invades the mid gut. The phytoplasma traverses the gut to

multiply in other tissues of the insect being carried by the haemolymph, after which it crosses the salivary gland cells into the saliva of the insect for onward introduction into a host plant during feeding. The period of colonization of the insect vector by the phytoplasma to an infection level is known as the latency period and may take up to three weeks (Christensen *et al.*, 2005; Hogenhout *et al.*, 2008). In plants, phytoplasmas are found mainly in the phloem elements including sieve tubes with or without nuclei, with the highest concentrations found in the mature ones (Christensen *et al.*, 2004). Phytoplasmas use the extensive sieve elements to spread systemically in their plant hosts; however, the exact mechanism of this translocation has not been elucidated (Christensen *et al.*, 2005). The host cycle of phytoplasmas is illustrated in Figure 1.1

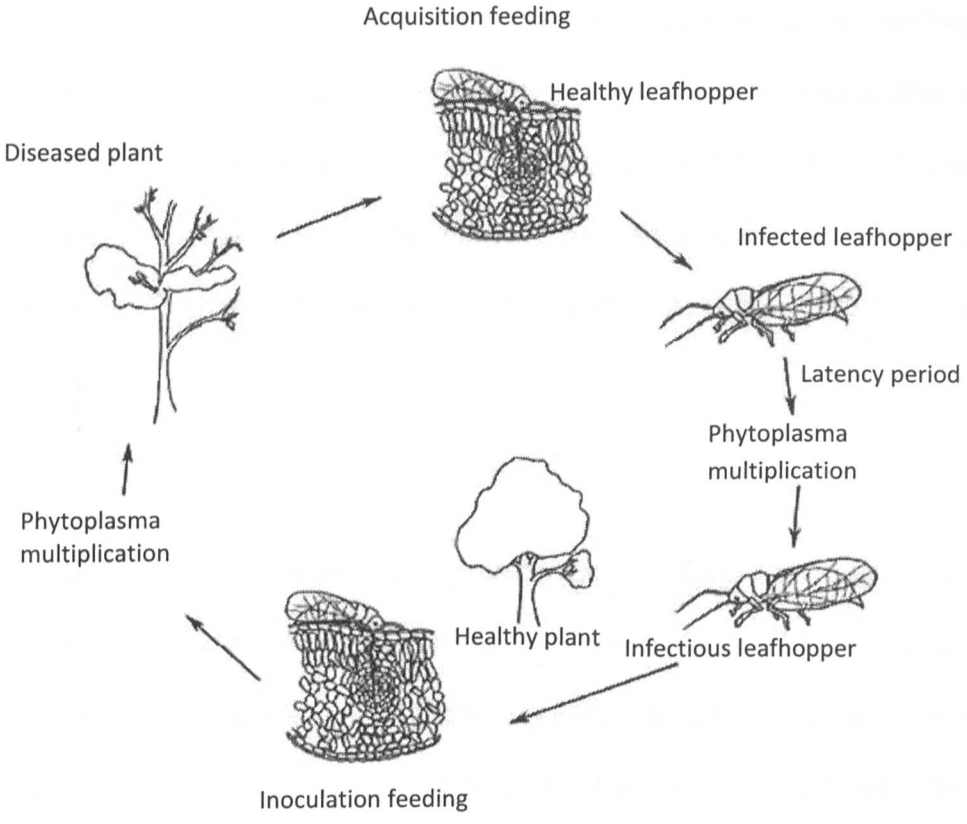


Figure 1.1 Host cycle of phytoplasmas. A healthy leafhopper feeds on a phytoplasma-infected plant (acquisition feeding). A latency period, during which the phytoplasmas multiply within the insect, is necessary before the insect can transmit phytoplasmas to a healthy plant (inoculation feeding). Multiplication and spread of phytoplasmas in the host plant is often accompanied by the appearance of disease symptoms. (Adapted from Christensen *et al.*, 2005).

1.3.7 Phytoplasma diagnostics

As in humans and animals, the accurate and timely diagnosis of diseases in plants is important for the effective control or management of the disease. Accurate diagnosis allows for the identification of the etiological agents involved, their classification, disease management and quarantine (Dickinson, 2003). This is even more necessary for plant pathogens like phytoplasmas which cannot be cultured outside their hosts and therefore cannot be inoculated into their hosts artificially. Uncertainties about incubation periods in some plant hosts and about the insect vectors involved further distinguish phytoplasma diseases from other plant diseases.

Following their discovery, phytoplasma diagnosis was based on symptomatology and confirmation of diagnosis was obtained by the differential response of infected plants to penicillin and tetracycline treatment and electron microscopy (Kirkpatrick *et al.*, 1987; Harrison *et al.*, 1992; Oropeza *et al.*, 2005). Symptomatology, though an inexpensive method, is not reliable since some phytoplasma infected plants do not develop symptoms and some symptoms begin to appear in later stages of infection. Stage 1 of LD-infection, for instance, can easily be missed since frond yellowing, the main symptom of the disease may start at stage 2 (Section 1.4.2). Electron microscopy also often produced false negative results as a result of the low concentrations and uneven distribution of phytoplasmas especially in woody hosts (Thomas, 1979). The use of the DNA staining dye 4'6-diamidino-2-phenylindole (DAPI) and

electron microscopy was, however, used with success to detect phytoplasma in woody host plants; nevertheless microscopy cannot differentiate between phytoplasmas from different groups (Harrison *et al.*, 1999; Oropeza *et al.*, 2005).

Serological techniques such as enzyme linked immunosorbent assay (ELISA) were also developed for detecting phytoplasmas. Although serological techniques are more rapid and specific than microscopy, there are often problems with cross reactivity of polyclonal antibodies with host plant antigens. Monoclonal antibodies, which are more specific, largely overcame the problem of non-specific reactivity with contaminating host plant antigens, but because they are designed against a single phytoplasma epitope, they are limited in their use for classifying phytoplasmas at the species level (Kirkpatrick *et al.*, 1987; Firrao *et al.*, 2007).

Currently the polymerase chain reaction (PCR) including RFLP, discussed above (Section 1.3.5) is routinely used to detect and identify phytoplasmas in their plant and insect hosts and various PCR-based assays have been developed for this purpose (Heinrich *et al.*, 2001; Firrao *et al.*, 2007). The PCR technique involves the repeated cycling of three main steps to make many copies of a specific DNA fragment from a minute quantity of template DNA which can then be visualized and identified by gel electrophoresis. The first step, denaturation, involves the thermal dissociation of a double stranded DNA into single strands at

temperatures greater than 90°C. A pair of synthetic oligonucleotide primers designed to have complementary regions on the target DNA hybridize to their complementary strands in the annealing step, which usually occurs at temperatures between 50-75°C. The hybridised primers act as templates for the enzyme *Taq* polymerase to build a new strand by the sequential addition of deoxynucleotides and this step is referred to as the extension step. The extension step takes place optimally between 72-78°C. Theoretically the number of copies of the target DNA is doubled at the end of each cycle and so there is an exponential increase in the amount of the template at the end of each cycle; in practice, however, this is dependent on the efficiency of each cycle (Guatelli *et al.*, 1989; Mackay *et al.*, 2002).

Sometimes to increase the sensitivity of phytoplasma PCR diagnostic assays because of their unusually low concentrations particularly in their plant hosts, a nested PCR is performed (Lee *et al.*, 1994; Dickinson, 2003; Nicolaisen and Bertaccini, 2007). It involves performing two rounds of PCR in which the product of the first round PCR is used as the template in the second PCR. Often a universal primer pair (amplifies sequences from a broad range of phytoplasmas) is used in the first round and then a group specific primer pair (designed to amplify sequences of members of a specific group of phytoplasmas) is employed in the second round. Nested PCR, however, can be fraught with cross–over contaminations because of the numerous handling steps involved and it is time consuming.

These constraints are largely eliminated in real-time PCR (Q-PCR) assays which do not require post amplification detection and identification of amplicons. The closed system in which Q-PCR is performed minimises the potential for carry-over contamination and increases the speed of the diagnosis by using sensitive detection systems while eliminating post amplification procedures such as electrophoresis. In Q-PCR, a fluorophore is included in the reaction and the accumulation of amplicons is detected as a fluorescence signal. The ability to monitor the complete amplification process gives the Q-PCR technique the advantage of being a quantitative tool and not just a qualitative one as conventional PCR (Ginzinger, 2002; Mackay *et al.*, 2002). Various Q-PCR assays have been designed for detecting and quantifying phytoplasmas in infected plants (Christensen *et al.*, 2004; Angelini *et al.*, 2007; Hren *et al.*, 2007; Hodgetts *et al.*, 2009). The theory behind Q-PCR and nucleic acid quantification is discussed in Section 3.1.2.

Molecular techniques have been found to be faster, more reliable and specific compared to the other methods for diagnosis of phytoplasmas. On the other hand, PCR-based molecular techniques are expensive and often require a lot of technical competence to analyse and interpret complex results. Molecular diagnostics are difficult to use on a sustainable basis in most labs in Africa including Ghana, because of the lack of equipment and resources to buy the necessary consumables. Non-PCR-based methods that circumvent the need for expensive laboratory facilities would greatly enhance the work of African scientists who depend

largely on foreign sponsored projects for the supply of consumables. One such method developed is the 'Loop-Mediated Isothermal Amplification' (LAMP) assay developed by Notomi *et al.* (2000). The technique uses *Bst* polymerase which has a strand displacement activity in conjunction with 4-6 specially designed primers that recognise 6-8 regions of the target DNA and is run under isothermal conditions (60-65 °C) (LAMP primers are discussed in Section 2.1). The ability to achieve amplification at a single temperature, the rapidity with which results are obtained and the several amplicon detection methods used makes LAMP suitable for in-field diagnosis of plant diseases. LAMP assays have been developed for detecting several plant pathogens (Liu *et al.*, 2010; Tomlinson *et al.*, 2007) and together with emerging rapid DNA extraction techniques, a new and exciting page in the area of field-based diagnostics seems to be opening. Such methods, however, would need to be trialled and fine tuned to make them adaptable for their use in the field in remote parts of Ghana and Africa in general.

1.4 THE LETHAL YELLOWING DISEASES

Lethal yellowing (LY) is a highly destructive disease of coconut and about 30 other palms such as *Veitchia merrilli*, *Pritchardia thurstonii* and *Phoenix dactylifera* (Harrison *et al.*, 1999). LY is caused by phytoplasmas and transmitted by insect vectors. The disease is more associated with coconut because its effect on the palm has been more prominent than in the other palms. Lethal yellowing is the name given to the disease in the Americas and the Caribbean and when diseases showing similar

symptoms as LY were observed in Africa, they were called lethal diseases or lethal yellowing-like disease (LD/LYD). The first scientific report of the disease was from Jamaica in the Caribbean Region in 1891 (Fawcett, 1891, see Eden-Green, 1997 p. 9). The disease subsequently spread to other areas in the Caribbean such as Cuba, Hispaniola, Haiti, the Dominican Republic and parts of the Bahamas Islands. In Africa, LYD can be found in Ghana (Cape Saint Paul wilt disease), Nigeria (Awka wilt), Togo (Kaincope), Cameroon (Kribi disease), Tanzania (Lethal disease) and in Kenya and Mozambique (Eden-Green, 1997). In the Americas, it occurs in Mexico, Honduras, Belize and Florida (USA) (Eden-Green, 1997; Legoupil and Courbet, 2008). Natuna and Kalimantan wilts are lethal diseases of coconut in Indonesia, however, the phytoplasmas involved do not belong to the same 16S rDNA group as the phytoplasmas involved in the African and Caribbean disease (Dollet *et al.*, 2009).

LD/LY is considered as the single most important threat to the coconut industry because it spreads so fast and kills susceptible palms within a short time (Harries, 1978). The destructive nature of the disease is well documented: in Jamaica, about five million palms of the 'Jamaica Tall' variety died from the disease in the main coconut growing areas over a 20 year period (Lebrun *et al.*, 2008). In mainland Tanzania, the disease destroyed about 38% of the total palm population over a 30 year period (Schuiling *et al.*, 1992; Mpunami *et al.*, 1999). In Ghana, the disease assumed epidemic levels and virtually destroyed all the palms in the Volta Region; coconut growing areas in the Western and Central Regions

were also badly affected and by 1995 about 5000 ha of palms had been destroyed (Ofori and Nkansah-Poku, 1997). The economic impact of the disease has been devastating for the mostly rural communities dependent on the palm for their sustenance. In most of these areas, the lands are poor and therefore difficult for farmers to shift to the cultivation of annual crops which requires considerable amounts of investment in fertilizers and labour.

1.4.1 History and spread of LD in Ghana

LD is locally referred to as Cape Saint Paul wilt (CSPWD) disease in Ghana and it is so called because it was first observed in Woe, near Cape Saint Paul in the Volta Region. It is the bane of the coconut industry in Ghana and is the major factor responsible for the decline of the industry for well over 70 years (Dery *et al.*, 1997; Ofori and Nkansah-Poku, 1997). The destructive nature of the disease became apparent soon after its detection in the country in 1932. Within the first nine years, it had destroyed most of the palms in the Cape Saint Paul area. The disease spread relentlessly and by 1970, palms in areas such as Dzelukope, Keta, Denu, Aflao, Agbosome, Tegbi, Adina and Aflao had been affected. Copra production in the region began to plummet and in 1977, a vegetable oil mill in the Region had to be closed down due to inadequate supply of copra (Ofori and Nkansah-Poku, 1997). The disease became dormant after this period until 1993 when it re-emerged, threatening the replanted palms. LD virtually caused the collapse of the once vibrant coconut industry in this Region.

While the disease wreaked havoc in the South-Eastern part of the country, in 1964, it was detected at Cape Three Points in the South-Western part of the country, which is about 450 km from Cape Saint Paul. The pattern of rapid decimation of palms was repeated in the Western Region and in just four years (by 1968) about a hectare of palms had been killed. In 1976, the disease jumped to Princess town, a distance of 10 km from Cape Three Points. From this time the disease spread to affect areas such as Adjoa, Funko, Apowa, Amanfulkuma, Ajembra, Awuku near Axim, Banso, Beahu and Tumentu. In 1995, a major disease outbreak was observed at Ampain, 14 km west of River Ankobra in the direction of Cote d'Ivoire (Ofori and Nkansah-Poku, 1997).

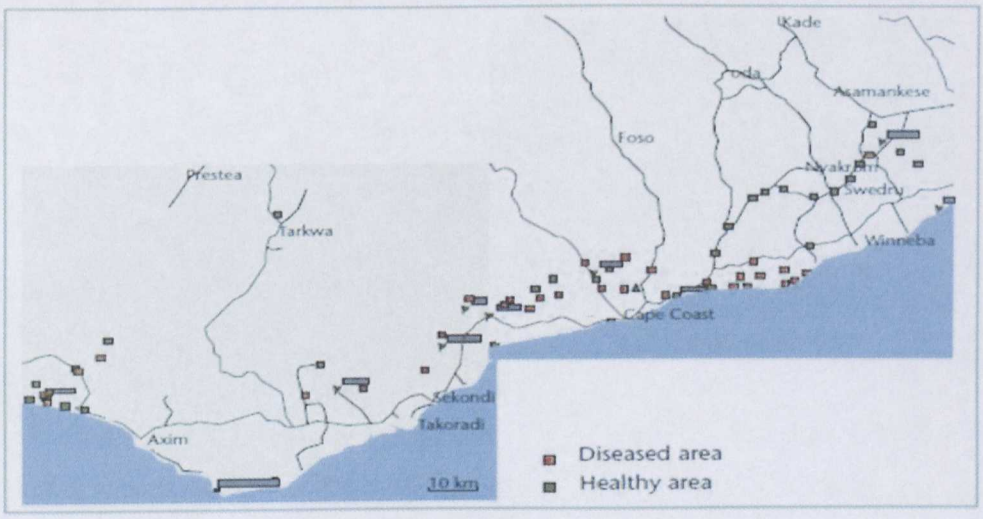
The disease was spotted in the Central Region, the third coconut producing Region in 1983 at Ayensudo in the Komenda Edena Eguafo (KEEA) District and in about five years, 500 palms had been destroyed. In 1989, the disease jumped to Eduma, a distance of about 35 km east of Ayensudo. By 1990, several foci had been established in the KEEA district and in 1995, the disease jumped to Apam, a distance of about 80 km from Ayensudo (Ofori and Nkansah-Poku, 1997).

In a recent survey by Nkansah-Poku *et al.* (2009), it was observed that in the Volta Region, the disease had been less aggressive and of the estimated 1300 ha of palms, less than 1% had been lost since the resurgence of the disease in 1995. In the Western Region, the spread of the disease after the last survey in 1995 had been by expansion of

existing foci rather than by 'jump spread' to new areas. It was revealed that the worst affected areas were in the Ahanta West District which had lost about 70% of its palm population; however, the rate of spread of the disease was observed to have slowed down in the area. The most active foci were found in the Nzema East District, with the Asanta focus which appeared in 1992, expanding to destroy about 90% of the palms in the area by 2006. The focus had spread northwest to Nyamebekyere by 2006, crossing the Ankobra River to merge with the Awuku focus established in 1990, which had spread westwards to Axim. The Ampain focus, detected in 1995, and is of strategic importance because of its closeness to the Ivory Coast was observed to have expanded the least despite the loss of about 70% of its original 25 ha planting. All together the district had lost between 20-30% of it 1200 ha palms. Other active disease foci were found in Botogyina and Daboase in the Shama Ahanta East and Mpohor Wassa East districts respectively.

In the Central Region, the recent survey showed that one of the extensive coconut groves in the Agona District, previously disease-free, was under threat. The area is a major supplier of fresh nuts to Accra, the capital city of Ghana. In the KEEA district, an area that has lost a lot of palms to the disease, infected fields with varied damage levels were observed. The distribution of the disease as of 2008 in the Central, Western and Volta Regions are shown in Figure 1.2.

A



B

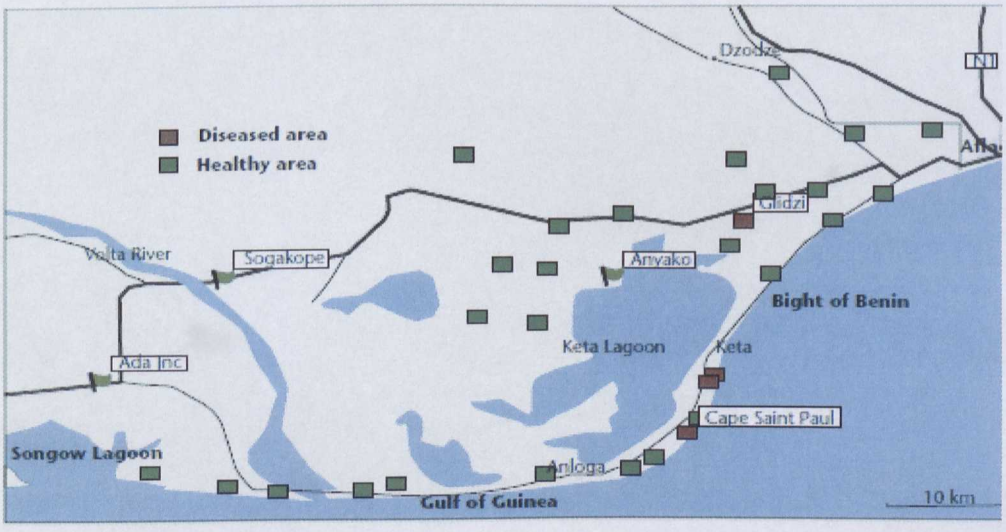


Figure 1.2 LD distribution in (A) Western and Central Regions and (B) Volta Region of Ghana (Source: Nkansah-Poku *et al.*, 2009).

1.4.2 Symptoms of LD

The first visible sign of the onset of LD is the dropping of both ripe and unripe nuts with or without yellowing of the leaves. Nut drop is followed by blackening of immature inflorescences and yellowing of the leaves, starting from the oldest and progressively to the youngest leaves. In some instances the leaves take a brown colour rather than the usual yellowing. Yellowing of palms, however, does not always connote LD as other factors can induce yellowing. Yellowing can be a consequence of ageing in the oldest fronds, which may eventually fall off. Yellowing can also be seen in palms growing in swampy areas, on limestone soil and palms suffering from drought. The spear leaf and the immediate younger leaves begin to rot rapidly before the yellowing reaches the youngest leaves. The yellow leaves turn brown and eventually fall off. In the final stages of the disease, only a few of the yellow leaves, looking smaller than normal remain but eventually fall off due to progressive rotting or being blown off by the wind, leaving a crownless palm standing (telegraph pole condition) (Eden-Green, 1997; Dery *et al.*, 2008; Dollet *et al.*, 2009). Dery and Philippe (1997) suggested four stages of LD-infection as follows:

Stage 1: Premature nut drop, blackening of the inflorescence with or without one or two leaves yellow.

Stage 2: More than two leaves, but less than half the canopy, yellow.

Stage 3: More than half the canopy yellow but with some green leaves still present.

Stage 4: All leaves in canopy yellow.

The sequence of symptom development of LD is shown in Plate 1.3 below.

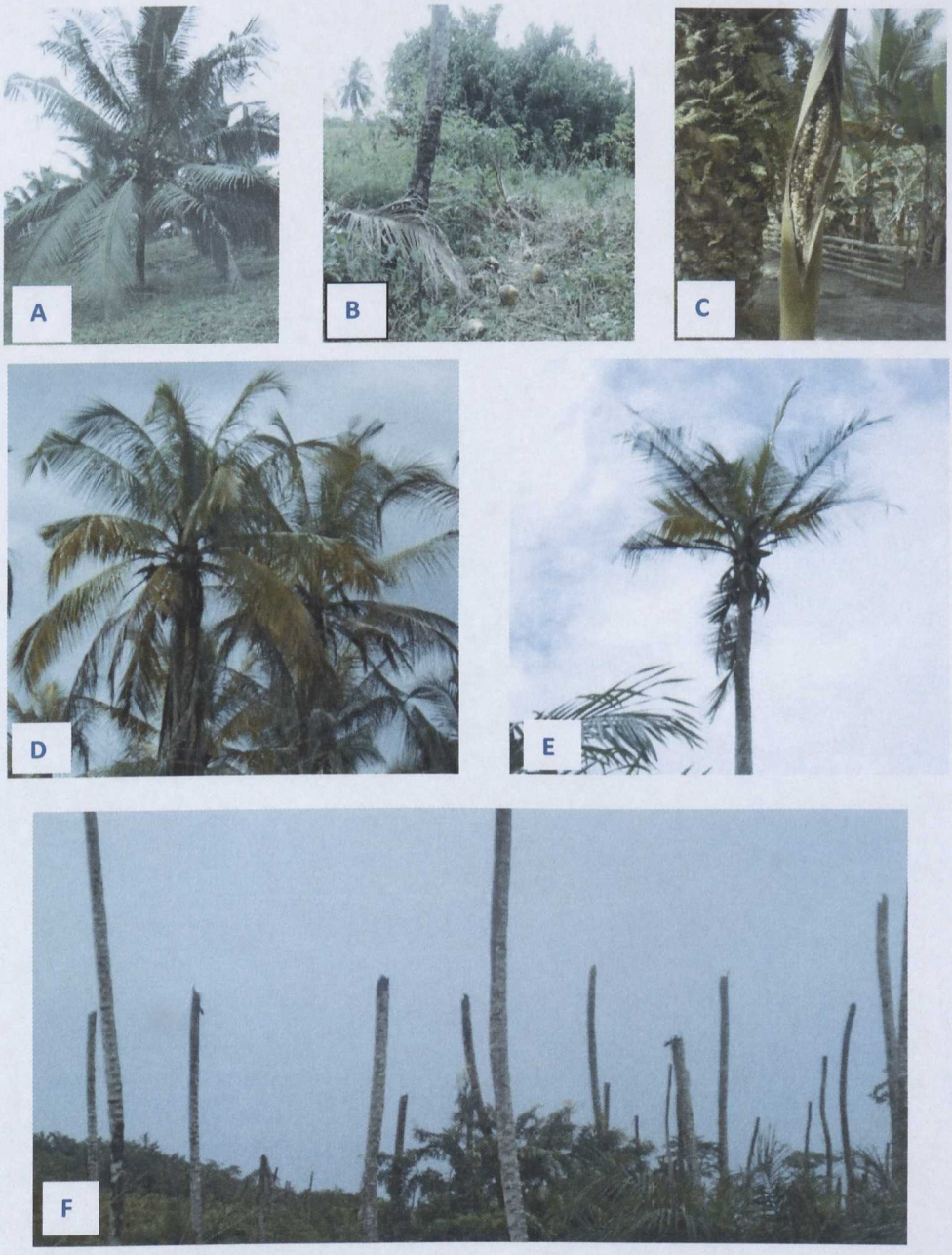


Plate 1.3 Sequence of symptom development of LD. Healthy palm (A); Premature dropping of nuts (B); Blackening of inflorescence (C); Yellowing of lower (D), and upper fronds (E) and bare trunks or telephone poles (F).

1.4.3 Research effort at overcoming LD in Ghana

Research effort at overcoming LD has been on-going since 1942, when the Department of Agriculture of the Gold Coast began investigations on the causative agent and mode of spread of the disease (Chona and Adansi, 1970; Dery *et al.*, 2008). Failing to achieve these research objectives, further research concentrated on the identification of resistant germplasm and this has been ongoing with support from various funding agencies and by various local and foreign collaborating scientists to the present time. From 1956 to 1969, three Malayan Dwarf varieties (red, yellow and green), because of their resistance to the disease in Jamaica were introduced into the country from Jamaica, Malay (now Malaysia) and the Ivory Coast for screening but the disease killed these palms (Chona and Adansi, 1970; Addison, 1980). Another trial with the above three varieties in addition to Veitchia, Phoenix, some wild oil palm undergrowths and using the local West African Tall (WAT) ecotype as controls were planted at Cape Three Points in 1977. Again, the disease wiped out the coconut palms but the other palm species were unaffected (Addison, 1980). Between 1981 and 1983 the Ministry of Food and Agriculture, under the France-Ghana-Cote d'Ivoire Coconut Project also set up seven variety trials with 27 varieties in the Western Region including Cape Three Points. The results of these trials revealed one variety, the Sri Lanka Green Dwarf (SGD) as very promising with regards to its resistance to LD. Eleven more varieties were imported and planted at two sites in the Western Region of Ghana in 1995 (Cape Three Points and Tumentu). Based on the performance of the palms as of 1995, two hybrids, the Malayan Yellow Dwarf (MYD) and the Vanuatu Tall (VTT)

varieties MYD x VTT and the Sri-Lanka Green Dwarf and VTT (SGD x VTT) were recommended as suitable materials for rehabilitating the coconut industry in Ghana. As a result of its availability, the MYD x VTT was chosen although its resistance was not the best. This led to the start of an Agence Francaise de Development (AFD) sponsored rehabilitation programme, the 'Coconut Sector Development Programme' (CSDP) covering 1,300 ha (Dery *et al.*, 2008; Quaicoe *et al.*, 2009). Although this hybrid has suffered some losses to LD, the severity is very low compared with the response of the local susceptible WAT ecotype. A number of recommendations have been made by Dery *et al.* (2008) on the choice of hybrid to use for replanting and were stated as thus:

1. In the immediate vicinity of an active CSPWD focus, it is preferable to avoid planting coconut, since the risk of transmission of the disease is high, even for partially resistant cultivars.

2. At some distance from these foci, the risk remains appreciable and the SGD x VTT hybrid is recommended.

3. Wherever the risk of contamination is low, the preference is given to the MYD x VTT, due to its favourable agronomic performances and to the fact that supplies of the other hybrid are limited (Dery *et al.*, 2008).

A lot of research efforts have also been made at identifying the vector of LD in Ghana but these have proved unsuccessful (Philippe *et al.*, 2007;

Philippe *et al.*, 2009). So far it is only in Florida that the Cixiidae, *Myndus crudus* has been identified as the vector of LY (Howard *et al.*, 1983).

1.5 ALTERNATE HOSTS OF THE LD PHYTOPLASMA

Many palm species as well as several grass species have been identified as alternate hosts of the American LY phytoplasma (Harrison *et al.*, 1999; Brown *et al.*, 2008). The implication is that disease transmission in a coconut plantation may occur not just from one coconut to another but from an alternate host which can serve as a reservoir of the phytoplasmas. In Ghana, no alternate hosts are known, however, the history of the disease at Cape Three Points in the Western Region where successive plantings of coconut in a disease screening plot have been killed by the disease gives a strong indication of the possibility of alternate hosts participating in the spread of LD (Section 1.4.3). A recent study by Yankey *et al.* (2009), failed to identify any such plant from a collection of 57 plant species belonging to 27 botanical families. This study was the first major attempt in Ghana to identify alternate hosts but was limited in scope as it considered only two locations and dealt with sample sizes that were not large enough because of time constraints. It is therefore still worthwhile to consider including other areas of LD-infection, screening new plant species and even screening some of the plants that were assessed in the above mentioned work to increase the sample sizes of the collections.

1.6 SEED TRANSMISSION OF LD

Seed transmission of phytoplasma diseases has been considered unlikely, because in plants phytoplasmas are found in the phloem sieve elements which lack any connection with the embryo. However, past and recent research results have demonstrated the presence of phytoplasma DNA in plant embryos. Botti and Bertaccini (2006) suggested seed transmission of phytoplasmas in seedlings of tomato, winter oil seeds and lime. Cordova *et al.* (2003) found phytoplasma DNA in the embryos of coconut with both universal phytoplasma primers as well as in nested PCR with LY specific primers. These results were further confirmed by the detection of phytoplasma fragments by *in situ* PCR using oligonuleotide primers for two genes. Nipah *et al.* (2007) also detected phytoplasma DNA in floral parts of coconut as well as in the embryo.

All put together, it is becoming evident that phytoplasmas may reside in other parts of the plant other than in the phloem sieve elements. The conventional PCR and *in situ* assays used in the above experiments involving coconut embryos did not distinguish between a viable and non-viable phytoplasma fragment and therefore made it inconclusive as to whether the phytoplasma containing embryos would have grown into diseased seedlings. In both experiments, the embryos had to be crushed and DNA extracted for the subsequent PCR, and therefore it was not possible to grow the embryos using tissue culture techniques to determine if they would have grown into diseased plants. Seed transmission of LY/LD, if demonstrated, would open a new page in our

knowledge of the epidemiology of the disease. It would also have huge implications for the movement of seed within and between countries. In Ghana for example, all the varietal resistance screening experiments are carried out in areas with intense disease pressure and therefore it may not be possible to move seeds from these areas to disease free zones. It may not also be possible to sell or even exchange seeds with institutes in other countries (Nipah *et al.*, 2007).

1.7 MARKER ASSISTED SELECTION IN COCONUT BREEDING

The reproductive biology of coconut makes the application of conventional breeding approaches a slow and labour intensive process that also requires large portions of land. The palm may take up to seven years to come into bearing, has a low multiplication rate and clonal propagation has had limited success (Chan *et al.*, 1998). It is estimated that a minimum of 60 years is needed for the development of homozygous inbred lines for hybrid production (Perera *et al.*, 2008). These challenges with conventional breeding are further exacerbated by the threat of LY/LD in some countries.

The use of marker assisted selection has long been recognised as a means of assisting in and accelerating coconut breeding (Lebrun *et al.*, 2001; Cardena *et al.*, 2003; Perera *et al.*, 2003).

There are three types of markers that are used to assist in selection by breeders: morphological, biochemical and molecular markers (Collard *et al.*, 2005; Semagn *et al.*, 2006; Modini *et al.*, 2009). Morphological markers are visually characterised phenotypic traits such as flower colour, seed shape, pigmentation and growth habits. Morphological markers have the advantage of being directly linked to agronomically important traits and the characterisation of these traits does not require expensive equipment. However, they have disadvantages of being limited in number and being influenced by the environment. Large tracts of land and long periods of characterisation are needed and can therefore in the long term make this type of marker expensive (Rao *et al.*, 2005; Rajesh *et al.*, 2008; Modini *et al.*, 2009). The general classification of coconut into either tall or dwarf types is based on morphology. Coconut fruit characteristics have been employed in diversity studies to reveal differences between cultivars (Asburner *et al.*, 1997; Zizumbo-Villareal and Pinero, 1998).

Biochemical or protein markers involving the use of isozymes and polyphenols have been used to describe diversity in coconut. The major drawback of protein markers, however, is that there are a limited number of enzymes which usually have low polymorphisms in coconut and therefore reduces their capacity to resolve diversity (Cardena *et al.*, 1998; Lebrun *et al.*, 2005).

Molecular markers are designed to reflect differences between individuals or populations at the DNA level and therefore represent the most accurate way of distinguishing between or certifying the identity of an individual (Karp *et al.*, 1997; Agarwal *et al.*, 2008). Molecular markers are usually selectively neutral in the sense that they do not impact on the phenotype of a plant and can rather be considered as constant landmarks in the genome (Jones *et al.*, 1997; Collard *et al.*, 2005). Unlike morphological and biochemical markers, molecular markers are abundant and are not affected by the environment or the developmental stage of the plant (Jones *et al.*, 1997; Lebrun *et al.*, 2005).

In plant genetic studies, molecular markers are used in the construction of linkage maps which are used to identify chromosomal locations containing genes or quantitative trait loci (QTLs) that control particular traits, for identification of individuals and for genetic diversity studies (Semagn *et al.*, 2006; Lammerts van Bueren *et al.*, 2010). Many molecular markers have been developed and many more that combine various aspects of existing ones continue to appear in the literature. Generally they are categorized based on the marker detection technique used; hence they may be described as PCR or non-PCR-based or sometimes as Restriction-Hybridisation or PCR / DNA sequence based markers. They may also be described as dominant or co-dominant markers, with the difference being that the latter can distinguish between a homozygous and a heterozygous genotype (Karp *et al.*, 1997; Agarwal *et al.*, 2008). The choice of a marker system to use should, however, be

based on the question being addressed and the available resources (Karp *et al.*, 1996).The most established markers include Random Amplified Polymorphic DNA (RAPD); Simple Sequence Repeats (SSR); Amplified Fragment Length Polymorphism (AFLP); Restriction Fragment Length Polymorphism (RFLP) and Single Nucleotide Polymorphism (SNP) and these have been used in coconut breeding programmes and diversity studies (Lebrun *et al.*, 1998; Lebrun *et al.*, 2001; Cardena *et al.*, 2003; Perera *et al.*, 2003; Lebrun *et al.*, 2005; Mauro-Herrera *et al.*, 2006; Rajesh *et al.*, 2008).

In Jamaica, the heavy losses suffered by the MayPan hybrid which was planted extensively as a control of LY has been partly explained by the genetic contamination of the Panama Tall (PNT), the pollen parent, with pollen from the susceptible Jamaican Tall ecotype and a large percentage of off-types observed in the MYD mother palms (Broschat *et al.*, 2002; Baudouin *et al.*, 2008; Lebrun *et al.*, 2008). The need to ensure genetic purity in breeding programmes is therefore important and this requires having the appropriate tools such as molecular markers for cultivar verification.

The use of molecular markers on promising ecotypes and hybrids identified in the Ghanaian breeding programme will help to determine how heterogenous the parental materials are and which genotypes should be used for future breeding programmes. The identification of markers linked to resistance and tolerance in the promising ecotypes and WAT

escapees will also provide valuable information for future mapping work and increase our understanding of the inheritance of resistance in coconut.

1.8 AIMS AND OBJECTIVES

The overall aims of the study were therefore:

1 To confirm the distinction between resistance and tolerance to LD.

2 To establish strategies and markers for the sustainable breeding of resistant/tolerant varieties.

3 To provide conclusive scientific evidence about the risks of phytoplasma transmission through movement of coconut germplasm.

4 To develop faster, simpler, specific molecular methods for improved detection of phytoplasmas for use in the field.

5 To identify the presence of alternate hosts of the LD phytoplasma.

The specific objectives were:

1 To quantify the levels of phytoplasmas in individual palms of putative tolerant and resistant varieties over three years to track infection and determine whether the environment (wet and dry seasons) affect these levels.

2 To identify markers that can be used to track tolerance/resistance traits in coconut breeding programmes using existing markers and providing new markers based on resistance gene analogues.

3 To determine whether phytoplasma diseases are transmitted through coconut seed.

4 To test, modify and optimise non-PCR-based molecular diagnostic methods in Ghanaian coconut plantations alongside conventional techniques.

5 To use PCR to determine the ability of some sampled flora growing in and around LD-affected fields to host the LD phytoplasma.

6 To provide improved decision support on varieties for distribution to coconut communities in Africa.

CHAPTER 2: DEVELOPING MOLECULAR MARKERS FOR VARIETY VERIFICATION

2.1 INTRODUCTION

Molecular/DNA markers reveal sites of variation in the DNA of species. They arise from different classes of mutations in the genome such as rearrangements (insertions or deletions), substitutions or errors in replicating tandemly repeated DNA (Schlotterer and Tautz, 1992; Modini *et al.*, 2009). DNA markers are abundant and can be used to distinguish many more phenotypes than morphological and biochemical markers can. Molecular markers generally follow Mendelian inheritance patterns although segregation distortions are known; they can therefore be used to predict inheritance patterns and subsequently help to accelerate selection times in breeding programmes (Xu *et al.*, 1997). It must, however, be mentioned that molecular markers do not replace morphological characterisation, but rather complement results of phenotypic characterisation (Karp *et al.*, 1997). Many molecular markers have been developed for plant genetic studies and an ideal molecular marker is expected to have among others the following features: be polymorphic and evenly distributed throughout the genome; provide adequate resolution of genetic differences; be simple, quick and inexpensive; need small amounts of tissues and DNA samples; have linkage to distinct phenotypes and require no prior information about the genome of an organisms (Agarwal *et al.*, 2008; Modini *et al.*, 2009). The choice of a molecular marker system to use depends on the questions being addressed and the resources available.

Simple Sequence Repeats (SSR) also referred to as microsatellites are short tandemly repeated nucleotide motifs (1-6 base pairs (bp)) found in the genome of all prokaryotes and eukaryotes (Zane *et al.*, 2002). Dinucleotide repeats, however, account for majority of SSRs in many species (Li *et al.*, 2002). Microsatellites are highly polymorphic and the polymorphisms are believed to arise from slippage events during DNA replication resulting in the gain or loss of one or more repeat units (Semagn *et al.*, 2006; Agarwal *et al.*, 2008). While the rates of mutation within microsatellite regions are high, the flanking regions are conserved across individuals of the same species and sometimes of different species thereby providing a means of amplifying microsatellite DNA using PCR with primers based on these regions (Selkoe and Toonen, 2006). Microsatellite markers have been used extensively for various genetic studies in coconut (Teulat *et al.*, 2000; Lebrun *et al.*, 2001; Meerow *et al.*, 2003; Konan *et al.*, 2007). They were first isolated in coconut by Rivera *et al.* (1999) who developed 38 informative SSR markers and evaluated their ability to detect genetic diversity among coconut germplasm. Perera *et al.* (2000; 2003) developed and used SSR markers to study genetic diversity among 130 individuals comprising both 'tall' and 'dwarf' varieties collected from around the world. The Coconut Genetic Resources Network (COGENT) has also developed a microsatellite kit comprising 14 markers and standardized methods for diversity studies and cultivar identification (Lebrun *et al.*, 2005).

Single nucleotide polymorphic (SNP) markers have alleles that are characterised by a single nucleotide change in a DNA sequence. SNPs are the most abundant markers and can be found in both coding and non-coding regions of the genome (Agarwal *et al.*, 2008). In coconut, SNP markers have been developed based on WRKY (transcription factor) gene sequences (Mauro-Herrera *et al.*, 2006).

Synteny between closely related species and conservation of the genetic structure of genes which perform the same function even in unrelated species has facilitated the cloning of many genes including resistance genes (Kanazin *et al.*, 1996; Baudouin *et al.*, 2005). The conservation of amino acid sequences of genes involved in plant defence to pathogens has enabled the isolation of gene analogues from different species with degenerate primers (Collins *et al.*, 1998; Shen *et al.*, 1998). Using such approaches, SNPs based on WRKY gene sequences have been developed in cocoa and coconut (Borrone *et al.*, 2004; Mauro-Hererra *et al.*, 2006). Resistance genes that have been cloned have been grouped into about nine different classes based on their predicted protein structure. The largest class contain genes that encode a putative nucleotide-binding site and a stretch of leucine-rich repeats (NBS-LRR) (Dickinson, 2003). Such genes have been isolated from plants such as pepper, maize and lettuce using PCR (Collins *et al.*, 1998; Shen *et al.*, 1998; Egea-Gilbert *et al.*, 2003) and the availability of such primers presents the opportunity to develop potentially functional markers based on RGAs in coconut.

Several methods are available for detecting polymorphisms at a marker locus. They include agarose gel electrophoresis using ethidium bromide, polyacrylamide gel electrophoresis and autoradiography. While ethidium bromide is a cheap method, its sensitivity is low. The use of radioactive labels in autoradiography requires special facilities, and there are concerns with disposal when silver staining is used with polyacrylamide gels (Christensen *et al.*, 1999; Creste *et al.*, 2001). Single-strand conformation polymorphism (SSCP) is used to detect mutations in PCR amplified fragments based on the differential migratory patterns of single-stranded DNA (ssDNA) on a gel arising from conformational differences in polymorphic fragments (Orita *et al.*, 1989). High resolution melt (HRM) analyses involve the use of the melting/dissociation profiles of double- stranded DNA fragments to characterise amplicons (Corinne and Hachler, 2001). The melting temperature of a PCR amplicon is influenced by its sequence composition and length and therefore sequences differing in size and even SNPs can be differentiated using HRM (Ishikawa *et al.*, 2010). HRM analyses are often carried out on high resolution melting instruments such as a LightScanner machine which uses specialised softwares to normalise data and to magnify differences in the shapes of the melting plots (Ishikawa *et al.*, 2010). In the absence of such software, in this work, polymorphisms at an SSR locus were determined by performing a melt analyses on a Q-PCR machine with the aim of finding large differences in the melting profiles between alleles.

To ensure that the technologies developed in this study are suited to the facilities available to the coconut research programme in Ghana, assays based on the Loop-mediated isothermal amplification (LAMP) technique were investigated for their ability to detect allele differences resulting from single nucleotide differences. LAMP uses a set of 4-6 specially designed primers designated as outer primers (F3 and B3); inner primers (forward inner primer (FIP) and backward inner primers (BIP)) and the optional loop primers (forward loop (FL) and backward loop (BL) primers) (Figure 2.1). LAMP uses *Bst* polymerase which has a strand displacement activity to achieve amplification of DNA at a single temperature. A series of sequential strand synthesis and displacement by the inner and outer primers, respectively, yields a large amount of DNA at the end of the reaction (Notomi *et al.*, 2000; Njiru *et al.*, 2008). The loop primers are added to accelerate the reaction, as they bind to DNA loops of the incorrect orientation (Nagamine *et al.*, 2002).

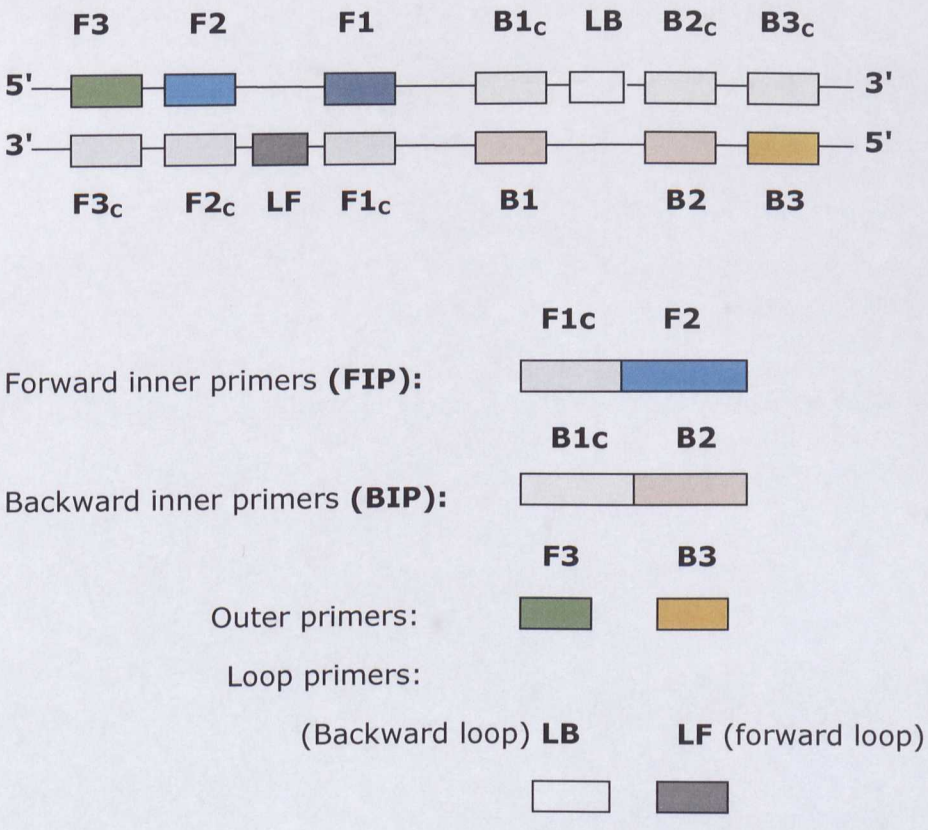


Figure 2.1 Schematic organisation of LAMP primers. Primers F3, B3, FIP, BIP and the optional loop primers (LB & LF) are used in the LAMP assays. $F3_C$, $F2_C$, $F1_C$, $B1_C$, $B2_C$ and $B3_C$ represent the complementary sequences of the DNA template.

The objectives of this section of the study were therefore:

1. To screen an array of SSR markers in an attempt to identify SSRs that have unique alleles associated with tolerance/resistance or susceptibility in the varieties used in the Ghanaian coconut breeding programme; specifically markers unique to resistant/tolerant varieties.
2. To develop methods based on the LAMP technique that can be used to detect SNPs in coconut using WRKY gene sequences as a model.
3. To isolate resistance gene analogues in coconut and attempt to develop useful markers based on SNPs.

2.2 MATERIALS AND METHODS

2.2.1 Plant Materials (coconut tissues)

2.2.1.1 Location of palms and sample numbers

Three hundred coconut palms growing in LD-infected areas were identified and marked for sampling at the beginning of the research in 2008. The palms comprised 100 'WATs; 100 MYD x VTT hybrids and 100 SGD x VTT hybrids. Thirty MYD palms; 40 SGDs and eight VTT palms were also marked for sampling. Some of the SGDs and MYDs had served as mother palms in hybrid production using VTT pollen obtained from Cote d'Ivoire. Twenty WAT palms in the characteristic 'telegraph pole' stage of LD-infection were also marked.

The WAT escapees were located in a diseased, abandoned farmer's field at Fasin in the Shama Ahanta-West district of the Western Region of Ghana. The 20 dead WAT palms were also located in this field. The MYD x VTT hybrids, about seven years old, were planted by a farmer at Daboase in the Shama Ahanta- East district of the Western Region of Ghana using materials produced by the Coconut Sector Development Project (CSDP) of the Ministry of Food and Agriculture (MOFA), Ghana. The SGD x VTT hybrids were collected from two locations in the Western Region of Ghana. The first field, located at Agona, was planted by the CSIR-OPRI, in 1995. This field was previously infected but is now in a state of dormancy. The other plot was a farmer's field, planted in 2007 at Daboase. The VTT palms were also from the Agona field and were planted in 1981. The SGD palms were also collected from two locations;

from a seed-garden established in 1981 and located at Bamiankor in the Nzema-East district and from another seed garden established in 2001 by the CSPD-MOFA located at Bonsaso in the Wassa West district, all in the Western Region of Ghana.

2.2.1.2 Sampling method and periods

Sampling was carried out twice per year, once in the rainy season and once in the dry season. The palms were sampled over a period of three years starting from the rainy season of 2008 (in June) and ending in the dry season of 2011 (January-February). Trunk tissues were collected following the method of Harrison (2002) with slight modifications as follows: a motorised drill fitted with a sterilised drill bit was used to bore a hole of about 10 cm into the stem of the coconut at a height of about 1 m above ground level; in this process the phloem tissues are chipped out in the form of sawdust. About 4-5 g of the dust was collected into clean labelled polythene bags and sent to the laboratory where they were kept in a freezer (-20°C). One sample per palm was collected. However, for palms exhibiting apparent symptoms of the disease, three samples were collected from different levels along the stem of the palm (i.e. just above ground level, 1 m and 1.5 m above ground level), with three different point borings at each level. To avoid cross contamination from palm to palm, a new drill bit was used for each palm. To re-use the bits, they were first thoroughly cleaned with water, after which they were washed in 70% ethanol and flamed to red hot. Samples were also taken from the inflorescence, leaflets and petioles of diseased palms. For the inflorescence, the palm was climbed and the inflorescence cut down, after

which the whole inflorescence was sent to the lab. In the lab a clean section of the inflorescence was cut with a sterilised kitchen knife and chopped into small pieces with a sterilised scalpel blade. For the petiole and leaflets, one frond was cut down and about four leaves pulled from different points along the rachis and a section of the petiole cut. In the lab, the laminas were stripped off the leaflets leaving only the midrib which was chopped into small pieces with a clean sterilised scalpel blade.

2.2.2 Buffers and chemicals used for DNA extraction

1. Cetyl trimethyl ammonium bromide (CTAB) buffer:

   CTAB (3% w/ v) (Sigma)

   100 mM Tris-HCl (pH 8.0)

   1.4 M NaCl

   EDTA 20 mM

2. EDTA 0.5 M solution pH 8.0

   EDTA.$2H_20$ 93.05 g

   Made up to 500 mL with sterile distilled water (NaoH used to adjust to pH 8.0).

3. Tris-HCl pH 8.0

   Tris-HCl 30.25 g

   Tris Base 5.90 g

   Made to 250 mL, using HCl to adjust to pH 8.0

4. Tris EDTA buffer

   10 mM Tris-HCl, pH 8.0

   1 mM EDTA, pH, 8.0

5. Tris Borate buffer 1x

   90 mM Tris-borate

   2 mM EDTA

2.2.3 DNA extraction protocols

A modified CTAB protocol (Daire *et al.*, 1997) was employed for extracting DNA from coconut stem, leaf, petiole, inflorescence and root samples. The DNeasy Plant Mini kit (Qiagen, 2006) was used according to the manufacturer's instructions for extractions involving coconut embryos.

2.2.3.1 CTAB extraction (Daire *et al.* 1997)

A 2 mL tube (Starlab, USA) containing about 6-8 glass beads (Sigma, USA) was half filled with coconut tissues and ground in a fastprep™ machine (Thermo electron corporation, Massachusetts, USA) at 6500 rpm for 45 s. CTAB buffer (700 µL) was added to the tissues and homogenised again at 6500 rpm for 45 s. The tubes were then incubated in a water bath at 65°C for 40 min, mixing the contents about two or three times by inverting the tubes. Phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma, USA) (700 µL) was added, vortexed to mix and

centrifuged at 9500 *g* for 10 min. The aqueous phase was collected into a 1.5 mL tube and an equal volume of ice-cold isopropyl alcohol added to precipitate the DNA. The precipitate was stored in a freezer (-20°C) for at least 3 h to obtain a good yield of DNA after which it was centrifuged at 16060 *g* for 25 min. The supernatant was removed and the pellet washed with 1 mL of 70% ethanol and centrifuged at 16060 *g* for 1 min. The supernatant was removed and the pellet air dried on the lab bench till there was no trace of alcohol, after which the pellet was resuspended in 100 µL of TE buffer.

2.2.3.2 DNA clean-up with polyvinyl polypyrrolidone (PVPP)

To clean the DNA, a Micro Bio-spin Chromatography column (Biorad, USA) was filled with PVPP powder to 2/3 full. Sterile distilled water (400 µL) was added to the PVPP and centrifuged at 855 *g* for 2 min. The filtrate was discarded and another 200 µL of distilled water added to the column and centrifuged at 855 *g* for 2 min. The columns were transferred into sterile labelled 1.5 mL tubes and the DNA extract added. The column was centrifuged for another 2 min at 855 *g* to elute the DNA after which it was stored at –20°C.

2.2.4 PCR analysis

PCRs were performed using 'Illustra PureTaq Ready-To-Go-PCR beads™' (GE lifesciences, UK) or 'MangoMix' (Bioline, UK) (in both cases, all the reaction components with the exception of the primers and template DNA were pre-formulated). A forward and reverse primer with stocks at a concentration of 10 µM was used in all the PCRs. A volume of 0.5 µL of each primer was used in a 25 µL reaction, achieving a final concentration of 0.2 µM. First a master-mix, adequate for the total number of PCRs to be done was made and then dispensed in aliquots of 24 µL into appropriately labelled PCR tubes; before 1 µL of the required template was added. PCR mixtures were subjected to suitable temperature regimes, based on the primers used, in a PTC-100/200 Programmable Thermal Controller (MJ Research Inc. USA.).

Amplification of microsatellite DNA was carried out with an initial denaturation temperature of 94°C for 3 min, followed by 35 cycles of 94°C for 40 s, 54°C for 40 s and 72°C for 1 min 40 s and a final extension of 72°C for 10 min. To amplify gene analogues of cloned resistance genes (RGAs) from coconut, two rounds of PCR were used; a first round PCR run at: 95°C for 2 min; 40 cycles of 95°C for 30 s, 40°C for 30 s, 72°C for 2 min and finally 10 min at 72°C. The second round PCR was run using the following conditions: 95°C for 2 min; 10 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 50 s; 25 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 50 s; 10 min at 72°C. Except for changes in the annealing temperature, PCR amplification of other target sequences was effected

with the same conditions as described for the amplification of microsatellite DNA. Two controls, a positive and water control were included in each batch of PCR.

2.2.5 Agarose gel electrophoresis

PCR products were separated and visualized on either a 1 or 1.5% (w/v) agarose gel depending on the size of fragment to be resolved. Agarose powder was melted in 1 x TBE buffer containing 0.5 μg mL^{-1} of ethidium bromide. PCR products were loaded directly onto the gels in cases where the reactions were carried out with 'MangoMix' which had a loading dye already incorporated; otherwise 6 μL of the PCR product was mixed with 2 μL blue 6 x loading dye (Promega, USA) before loading the gel. A Hyperladder II or Hyperladder V size marker (Bioline, UK), for high and low molecular weight fragments, respectively, was loaded in the first well of each row.

2.2.6 Cloning

Analyses of samples whose resolution were unclear in the diagnostic analyses and isolated RGA fragments were cloned to obtain pure cultures for sequencing. Cloning was carried out using the Promega pGEM®-T Easy Vector system (Promega, USA) cloning protocol with slight modifications made to the manufacturer's instructions as described below:

2.2.6.1 Ligation reactions

Five µl ligation reactions composed of 2.5 µL 2 x ligation buffer, 0.5 µL ligation vector, 0.5 µL ligase and 1.5 µL insert DNA were made. The volume of the insert DNA was adjusted for highly concentrated DNA templates where 1.0 µL was used and the volume of the vector adjusted accordingly. The reactions were incubated at room temperature for 1 h 20 min.

2.2.6.2 Transformation of competent cells

To transform the *E.coli* cells (JM 109), the cells stored at -80°C were thawed on ice and 23 µL of the cells pipetted into each tube. A 2.5 µL aliquot of the ligation mixture was added and mixed by gently flicking the tubes after which they were left on ice for 20 min for transformation (by heat shock). The tubes were transferred to a heated block set at 42°C for 45 s and put back on ice for 2 min. Five hundred µL of SOC medium (composed of: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose) was added and the contents of each tube transferred to separate universal tubes. The tubes were then placed in a shaker at 37°C for 3 h.

To culture the cells, molten Luria Bertani (LB) agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 15 g L^{-1} agar) containing 0.05 mM IPTG (isopropylthio-β-D-galactosidase), 80 µg mL^{-1} X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and 100 µg mL^{-1} ampicillin was poured into plates using standard aseptic techniques. Once set, each cell

culture was spread using a sterilised spreader onto two plates; one with 50 µL and the other with 200 µL. The plates were allowed to dry and incubated overnight at 37°C in an inverted position.

2.2.6.3 PCR assessment of colonies

A number of white colonies presumed to have the insert DNA were screened with PCR using M13F (GTAAAACGACGGCCA) and M13R (CAGGAAACAGCTATGAC) primers to amplify a small section of vector on either side of the insert DNA and the insert DNA at an annealing temperature of 56°C.

2.2.7 Purification of PCR products

PCR products were purified prior to sequencing with the GenElute ™ PCR clean-Up Kit (Sigma, USA), following the manufacturer's instructions. The purified products were eluted with 100 µL of sterilized distilled water and the amount of DNA quantified using a nanodrop.

2.2.8 Melt curve analyses

Melt analysis was carried out using a 2x HRM mix (Sensimix HRM, Bioline, UK) containing reaction buffer, heat activated DNA polymerase, dNTPs, 6 mM $MgCl_2$ and stabilizers) in the presence of EvaGreen™ dye from Bioline, UK. Ten µL reactions were prepared using 5 µL of sensimix buffer, 0.5 µL of Evagreen dye, 0.5 µL each of the forward and reverse primer (stock concentration of 10 µM), 2.5 µL of sterile, double-distilled water and 1 µL of the template DNA. Amplification and melt analyses

were carried out on a Light cycler® (Roche 480 II, USA) with initial polymerase activation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 30 s. Amplification was monitored in real-time by fluorescence detection. This was followed by a melt acquisition phase ramping from 60 to 95°C, rising by 0.25°C each cycle. In the absence of specialised softwares for determining allele size differences, the raw data was exported and the slope of the melt curve calculated and plotted against temperature. Melt temperatures (T_m) were then determined using the 'Max' function of Microsoft Excel (version 2007). The plots were then compared by eye. Inter- and intra-assay variations are unavoidable (Wong and Medrano, 2005) and to minimise intra-assay variation, samples were run in duplicates. Where the melt temperatures of the replicates were different, the average was used. To avoid inter-assay variations, samples which were being compared directly were run on the same plate.

2.2.9 Real-time LAMP

LAMP reactions were performed in real-time on a Genie I machine (Optigene, UK). LAMP reactions were carried out using isothermal master mix (GeneSys Ltd, Surrey, UK) containing dNTPs, $MgCl_2$, ds-DNA binding dye, reaction buffer, DNA polymerase and thermostable inorganic pyrophosphatase. Template DNA (1 µL) was added to 20 µL reactions containing 0.2 µM of F3 and B3, 1 µM of FIP, BIP and (when used for diagnostic work) 1 µM of the loop primers (BL and FL) and 5.75 µL isothermal mix. The reaction was run on the Genie I machine at 63°C for about 30 min (for diagnostic work) and about 1 h when used to detect

SNPs in WRKY gene sequences, measuring fluorescence signals at a ramp rate of 5°C s^{-1}. A melt analyses was performed at the end of the isothermal amplification by heating the amplicons from 63°C to 99°C at a ramp rate of 0.1°C s^{-1}. In the diagnostic work, the identities of the amplified fragments were ascertained by comparing the melting temperatures with that of the positive control.

2.3 RESULTS

2.3.1 Investigating an array of SSR markers for possible linkage to tolerance/resistance or susceptibility in coconut varieties

DNA samples from a subset of the tolerant/resistant varieties identified in the Ghanaian breeding programme (i.e MYD, SGD and VTT), hybrids (SGD x VTT and MYD x VTT) as well as from WAT escapees and dead palms of the susceptible WAT ecotype were analysed with SSR markers with the aim of identifying markers associated with resistance/tolerance or susceptibility. Forty four published SSR oligonucleotides for amplifying SSRs from coconut DNA were analysed using PCR (see Appendix 1 for list of SSR markers). Agarose gel electrophoresis was chosen as a preliminary method for detecting polymorphisms and for a further finer resolution sizing method, melt curve analysis was used. With the exception of markers CAC20 and CnCirA3 which were unsuccessful in the PCR runs, all other primers successfully amplified microsatellite fragments from the coconut samples. Seventeen of the markers which either showed a potentially consistent polymorphism between the varieties by gel analysis, or whose resolution was not clear enough to see consistent differences, were chosen for further analysis using melt curve analyses (see Figures 2.2A & B for examples of the gel analyses). At locus CnCirH4, the analyses showed differences in the banding pattern between the SGDs, MYDs ('dwarfs') and the VTTs ('Tall' palm) (Figure 2.2A). At locus CAC65, the MYDs could be distinguished from the SGDs and the WAT alleles also looked different from the SGDs (Figure 2.2B).

A.

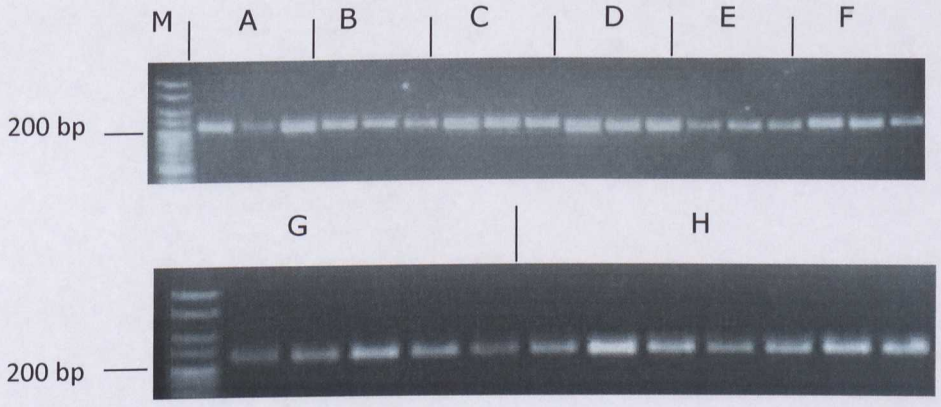


Figure 2.2A Amplification of SSR DNA from coconut at locus CnCirH4. Samples: (A) SGD x VTT; (B) MYD x VTT; (C) MYD; (D) SGD (Bamiankor); (E) SGD (Bonsaso); (F) VTT; (G) WAT (dead palms); (H) WAT (infected palms). M: DNA size marker (Hyperladder II).

B.

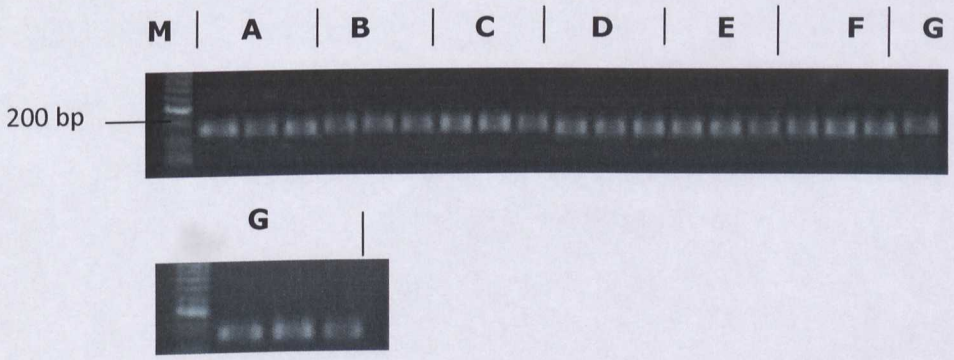


Figure 2.2B Amplification of SSR DNA from coconut at locus CAC65. Samples: (A) SGD x VTT; (B) MYD x VTT; (C) MYD; (D) SGD (Bamiankor); (E) SGD (Bonsaso); (F) VTT; (G) WAT.

2.3.1.1 Improving resolution of allele size differences using melt curve analyses

A. *Preliminary assessment of allele size variation with a small number of samples*

A high throughput melt analysis was adopted to provide finer resolution of allele size differences. Allele size differences were inferred from large differences in the melting temperatures (T_m) of the amplified fragments (Figure 2.3). A preliminary screening was carried out using a subset of three samples of each variety or hybrid type. In these analyses, two markers, CnCirC12 and CAC65 produced results that showed their potential to have alleles associated with particular cultivars. In the analyses with marker CnCirC12, four alleles were observed; the alleles of the MYDs were consistently different from the alleles of the other varieties. The alleles of the WATs were also unique and showed promise of having a cultivar specific allele (Figure 2.4). In the preliminary analyses with marker CAC65, WAT specific alleles were identified as shown by their consistently unique T_m from the alleles of the other varieties (Figure 2.5).

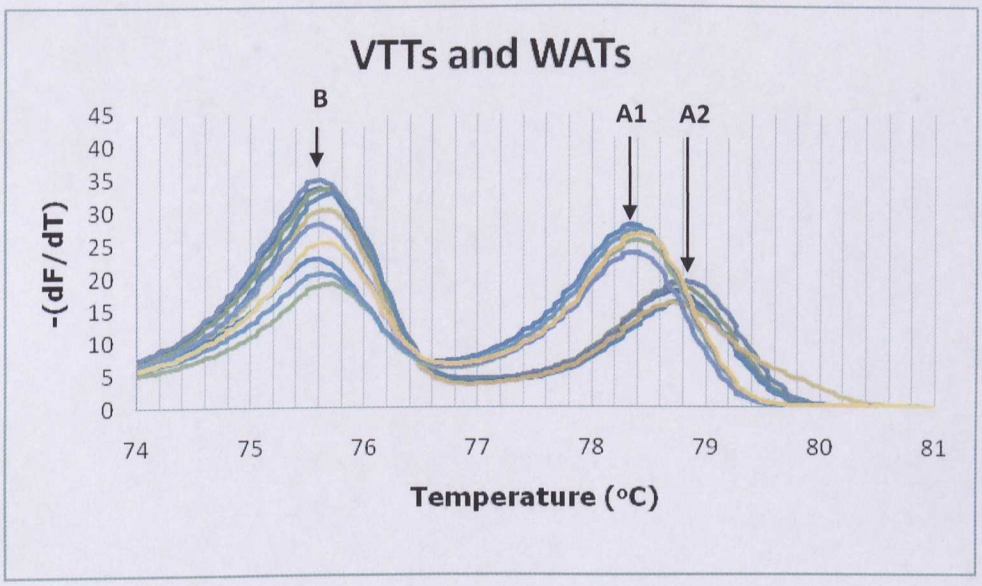


Figure 2.3 Melt analyses of VTTs and WATs at locus CnCirC12. The VTT alleles (A1) are distinguished from the WAT alleles (A2). The first peaks (B) were present in all the analyses and were likely to be non-specific amplifications.

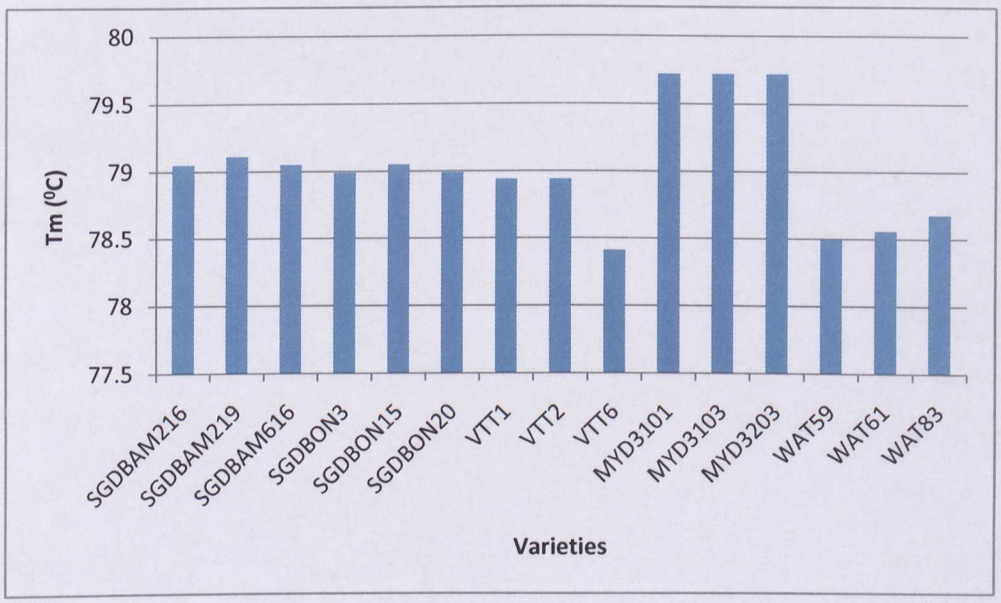


Figure 2.4 Melt analyses of marker CnCirC12 showing clustering around four melt peaks (alleles): the SGDs and VTT1&2; VTT 6; the MYDs and the WATs. (All WATs are probably the same; similar patterns were observed in subsequent analyses).

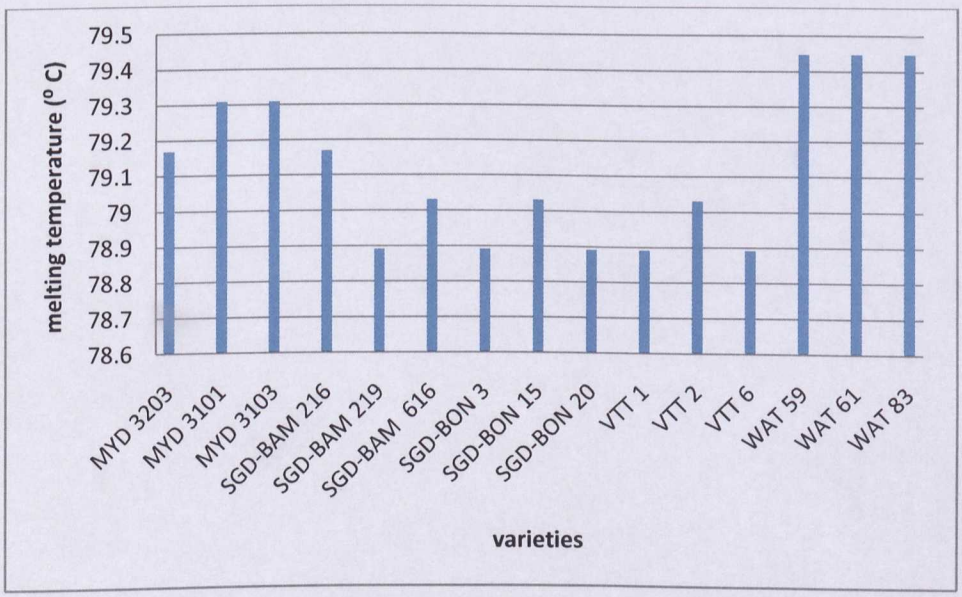


Figure 2.5 Melt analyses of SSR DNA at locus CAC65. Alleles specific to the WATs were observed (in preliminary analyses).

B. *Further investigation of markers CncirC12 and CAC65 to verify possible WAT & MYD specific alleles*

Following the promising results obtained in the initial analyses of SSR loci CnCirC12 and CAC65, further screening with more samples of each variety or hybrid type were undertaken. Five and six samples of each variety type were analysed for loci CAC65 and CnCirC12 respectively. The analyses of locus CAC65 revealed that the allele apparently characteristic of WAT was present in an SGD sample (SGDBAM 503) (Figure 2.6). Analyses of locus CnCirC12 showed that the previously unique allele of the MYDs was shared by two SGDs (SGDBAM 503 & 613), however, the alleles of the WAT seemed specific and were investigated further (Figure 2.7). When more samples of the WAT and SGDs were compared it was observed that the WAT allele was shared by an SGD sample (SGD-BAM 714) (Figure 2.8).

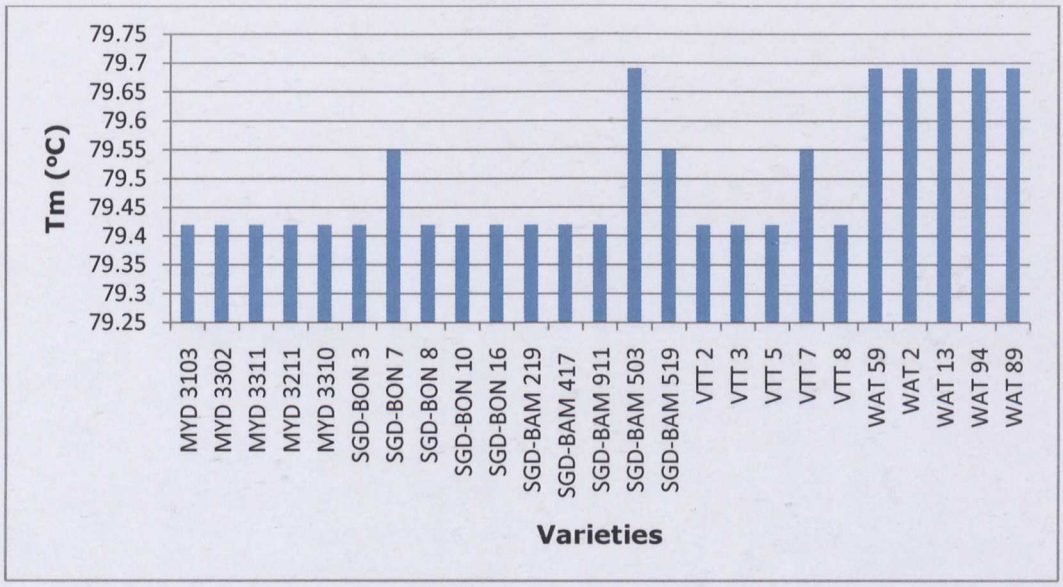


Figure 2.6 Further analyses of locus CAC65 with more samples of each variety type.The WAT allele thought to be unique was found in SGD-BAM 503.

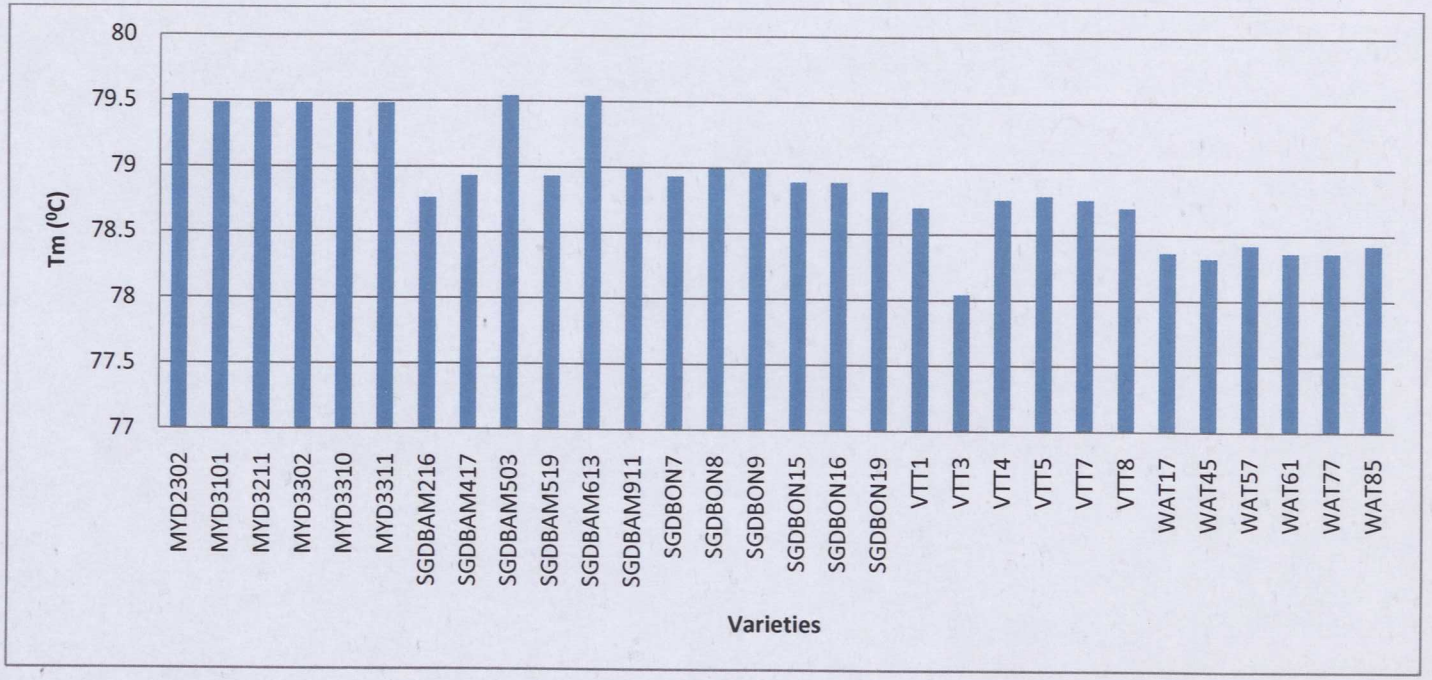


Figure 2.7 Further analyses of locus CnCirC12 with more samples of each variety type. Four alleles seen here (the MYDs and SGDBAM 503 & 613; the WATs; VTT 3; remainder of SGDs and VTTs). Shows WAT allele to be unique.

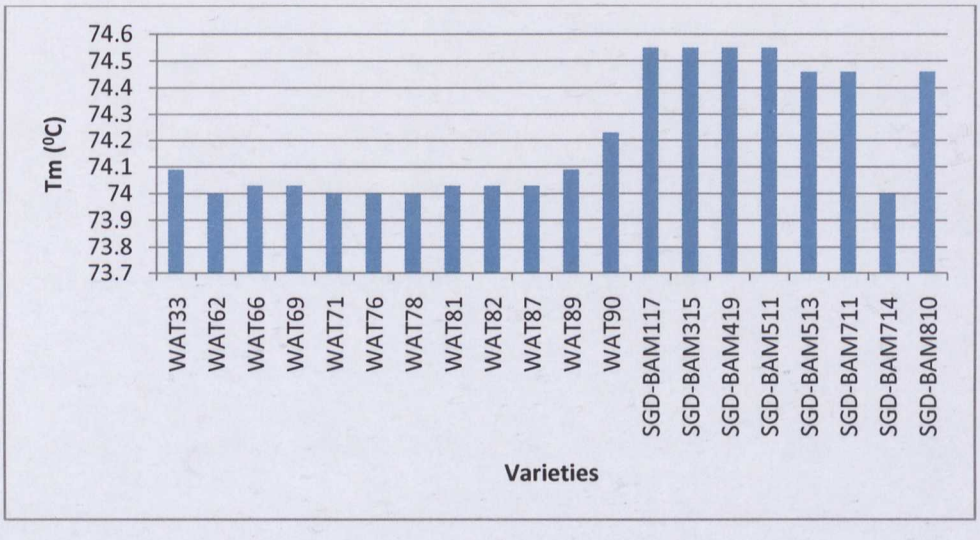


Figure 2.8 Further analyses of WAT and SGD samples for possible WAT specific allele. All WATs are probably the same. The WAT allele was shared by SGD-BAM714.

2.3.2 Developing SNP markers based on genes encoding WRKY transcription factors

SNP markers based on WRKY gene sequences have been developed in coconut by using SSCP analyses (Mauro-Hererra *et al.*, 2006). In this work assays based on the LAMP technique were developed to detect and amplify WRKY gene sequences in an attempt to develop markers based on SNPs that differ consistently between varieties.

2.3.2.1 Development of LAMP assays to detect SNPs in coconut varieties

LAMP primers were designed based on database coconut WRKY sequences containing SNPs (Table 2.1). Under the principles of LAMP, the FIP/BIP primers initiate strand synthesis at their 3' end and therefore the presence of an inappropriate base or a mutation at this position is expected to inhibit strand synthesis. LAMP primers were therefore designed such that the position of the mutation as indicated in the published paper occurred at the 3′ end of either sequence region F2 or B2 and in this way the position of the mutation in the assay was eventually at the 3' end of either FIP or BIP. Two alternative assays were made for each locus with the assays differing only in the mutation at either the 3′ end of FIP or BIP. It was expected that the assay with the genotype that matched will be the only one that would be amplified.

Table 2.1 WRKY SNP containing sequences identified in coconut (Mauro – Hererra *et al.*, 2006)

Locus	GenBank Accession no.	Type of polymorphism	Repeat unit and SNP(s) position	Blast analysis: homology to known WRKY sequences
CnWRKY-01	DQ307149	SSR	$(AG)_{11}$	XP_475778.1 contains WRKY DNA-binding domain (*Oryza sativa*)
		SNP	C/T: 103	NP_197989.2 WRKY50 transcription factor (*Arabidopsis thaliana*)
CnWRKY-02	DQ307150	SNP	A/C: 899	BAB61056.1 WRKY DNA-binding protein (*Nicotiana tabacum*)
				XP_483175.1 WRKY DNA-binding protein (*Oryza sativa*)
CnWRKY-03	DQ307151	SNP	A/G: 100	AAT46067.1 DNA-binding potein WRKY2 (*Vitis vinifera*)
				ABA98690.1 Transcription factor WRKY1 (*Oryza sativa*)
CnWRKY-05	DQ307152	SNP	C/T: 391	AAQ57653.1 WRKY13 (*Theobroma cacao*)
CnWRKY-06	DQ307153	SNP	C/T: 795	AAQ57630.1 WRKY12 (*Theobroma cacao*)
				AAP85545.1 WRKY DNA-binding protein (*Glycine max*)
CnWRKY-10	DQ307154	Probably SNP(s)	Polymorphism detected via SSCP	AAT84156.1 Transcription factor WRKY07 (*Oryza sativa*)
				CAA68326.1 DNA-binding protein (*Avena fatua*)
CnWRKY-13	DQ307155	Probably SNP(s)	Polymorphism detected via SSCP	AAR99334.1 WRKY DNA-binding protein (*Brassica rapa*)
				NP_001031766.1 WRKY18 transcription factor (*Ambidopsis thaliana*)
CnWRKY-16	DQ307156	SNP(s)	A/G: 428	DAA05094.1 WRKY transcription factor 29 (*Oryza sativa*)
			C/G: 525	AAL61859.1 WRKY57; transcription factor (*Arabidopsis thaliana*)
CnWRKY-19	DQ307157	SNP	A/G: 74	NP_568995.2 WRKY51; transcription factor (*Arabidopsis thaliana*)
				AAU44313.1 WRKY transcription factor 67 (*Oryza sativa*)
CnWRKY-21	DQ307158	SNP(s)	C/G: 447	NP_176982.1 WRKY9; transcription factor (*Arabidopsis thaliana*)
			C/T: 486	AAQ57638.1 WRKY5 (*Theobroma cacao*)
			A/G: 552	

LAMP primers were successfully designed for loci CnWRKY-01, CnWRKY-05, CnWRKY-16 and CnWRKY-21(Table 2.2). Primers could not be developed for loci CnWRKY-02; 03; 06 and 19 because either the position of the mutation was too close to the 3' or 5' end of the sequence for design of LAMP primers to be possible. Primers were not designed for loci CnWRKY-10 and 13 because it was not determined that the sequences contained SNPs (Mauro-Herrera *et al.* 2006).

Table 2.2 LAMP primers used in assays developed for amplifying WRKY gene sequences

Locus	**Primers** (5'-3')
CnWRKY-01	CnW1F3a: CCCCCAACAAGTTCATAGG Cn W1B3a: AGCCTTACCAAAAAACTGAC CnW1FIPaT: CCATATTTGCAGGGCAGCAAGCTTTGATTATAGCCAT CnW1FIPaC: CCATATTTGCAGGGCAGCAAGCTTTGATTATAGCCAC CnW1BIPa:TCCTGATTATGAGTAATCCCTTGGTGTAGTGCAAACGAAATTTGAG
CnWRKY-05	CnW5TF3: TGTATGTGCATTGAATGTTTGT CnW5TB3: ACTAGATGATTAAGTGCAACTCT CnW5TFIP: CAGCATGTAGCTGTGGAAATTAAACTATTGTGCATATGTGTACACTGA CnW5TBIP: AATCAACTCTCTGTAAGCAGTAGTGGAAGGAATGCCTCTTCAC CnW5F3: GCAAGCATGAATACGTGTA CnW5B3: CTAGATGATTAAGTGCAACTCT CnW5FIP: CAGCATGTAGCTGTGGAAATTAAACATGTATGTGTATGTGCATTGAATG CnW5VBIP: AATCAACTCTCTGTAAGCAGTAGTGCGAAGGAATGCCTCTTCAG
CnWRKY-16	CnW16F3a: CTCGTTCTGTTAATTTTTTGGAT CnW16F3b: TCAAGCCTAGCTATTATGAACT CnW16B3b: TTGCATCTTGTTTTACTTCGA CnW16FIP: ACGACATGACAGATCTTCATAAAGTTTTTATTATACTGCTACTCGTTCTG CnW16BIPaC: ATTTCGGACCCGCTATTCTTCCGTTTCATTGTTTGGGGAAAGTC CnW16BIPaG: ATTTCGGACCCGCTATTCTTCCGTTTCATTGTTTGGGGAAAGTG
CnWRKY-21	W21F3a: AGGGAGCAAGTTCGCTAG W21B3a : GAGTCCAGCTAGTTTTGTTGAT W21FIPa: ACCCAAAGAAGTGGAACCCTCTATACTGGGTTATCTCCGTGTCC W21BIPaC: TTACGTTTCGTTGCACAAGTCTCATTCATTTTCAATAAATAAGATC W21BIPaG: TTACGTTTCGTTGCACAAGTCTCATTCATTTTCAATAAATAAGATG

2.3.2.2 Real -time LAMP (RT-LAMP) detection of SNPs

Apart from the assays based on CnWRKY-05, all the other sequences analysed failed to identify SNPs between the varieties. Primers initially designed for amplifying locus CnWRKY-05 which differed only in the mutation position at the 3′ of FIP were found to be less efficient due to the formation of primer dimers. An alternative approach was to develop two alternative assays such that each assay had its own F3, B3 and FIP and either form of the mutation was placed at the 3' of BIP. One assay was designed to amplify a 'C' specific SNP and the other a 'G' specific SNP.

The assay designed for the 'C' specific SNP was observed to amplify samples from dwarf varieties and not those from the 'talls' while the assay designed for the 'G' specific SNP amplified samples from tall varieties. In one instance, however, a sample of the WAT was not amplified by this assay (Figure 2.9). PCR and sequence analyses of DNA from several palms confirmed the SNPs at this locus (Figure 2.10). The allele of the WAT sample that was not amplified was, however, not sequenced and so it was not determined whether it contained the correct sequence for a tall palm.

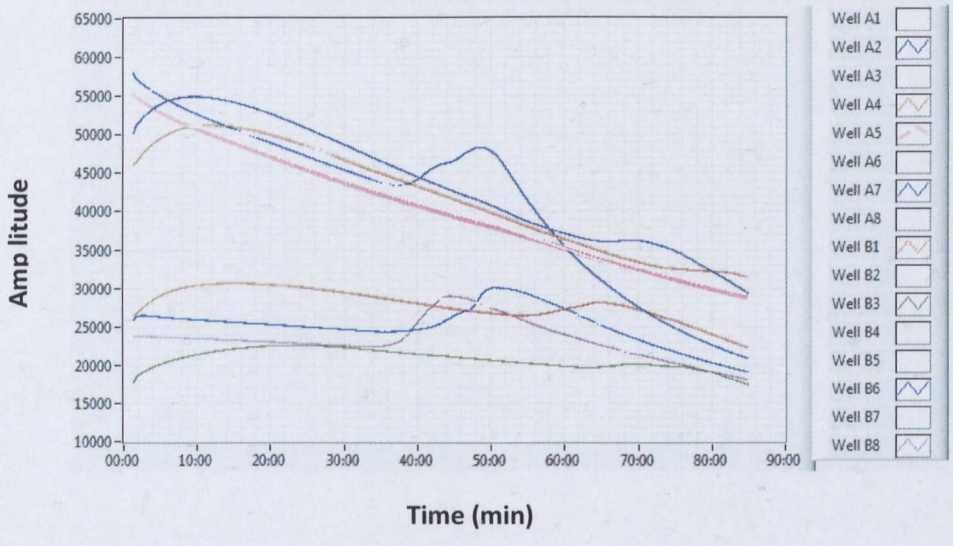


Figure 2.9 RT-LAMP amplification and output for SNP containing sequences using assays based on WRKY-05

Legend:

Wells: (A2) MYDY-3101; (A4) SGD-503; (A5) WAT-96; (A7) VTT-7; (B1) MYD-3101; (B3) SGD-219; (B6) WAT-830; (B8) VTT-1. Assays: C-specific assay (Wells A1-A4; B1-B4); G-specific assay (Well A5-A8; B5-B8). Dwarf palms (MYD & SGD); tall palms (VTT & WAT). The C-specific assay amplified fragments from samples of dwarf palms while the G-specific assay amplified fragments from samples of tall palms. WAT-96, from a tall palm, however, failed to produce an amplicon (bold pink line in figure). NB. Only amplified samples and WAT-96 are shown in figure.

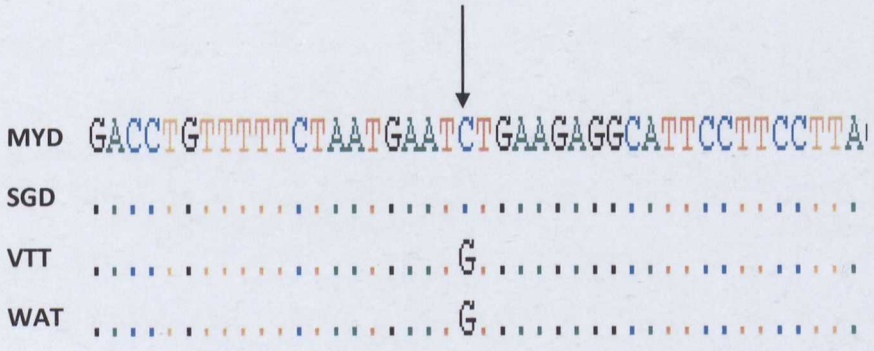


Figure 2.10 Alignment of sequences from tall and dwarf varieties at locus CnWRKY-05; arrow shows position of SNPs ('Talls' have a 'G' specific SNP and 'dwarfs' have a 'C' specific SNP)

2.3.2.3 Testing specificity of assays for amplifying WRKY-05 on mixed DNA samples

An experiment was set up to determine if the two assays designed for locus WRKY-05 (Table 2.2) could selectively amplify a particular genotype in a mixed DNA scenario. The two alternative assays were trialled on samples composed of mixed DNA from different varieties. DNA samples from Tall palms (WAT & VTT) and from a dwarf variety (SGD) as well as from the hybrid SGDxVTT were quantified and their concentrations adjusted to 1 ng μL^{-1} and used in real-time LAMP as described in section 2.2.9. When mixed DNA was used, 0.5 µL of each DNA sample from a variety were mixed and used in the reaction. The mixed samples used are shown in Table 2.3.

The results displayed the specificity of the assays to distinguish between tall and dwarf varieties at this SNP in the reactions involving DNA from the individual ecotypes and hybrid. In the reactions involving mixed DNA samples, the tall-specific assay showed specificity and could selectively amplify the WRKY gene from samples of tall varieties. The dwarf-specific assay, however, failed to amplify the SNP containing gene in samples from dwarf varieties when mixed with DNA from tall varieties. While either assay gave a positive result when used on the hybrid SGDxVTT, the dwarf specific assay gave a negative result when DNA from the hybrid was mixed with DNA from WAT (Table 2.3).

Table 2.3 RT-LAMP output of assays for amplifying CnWRKY-05 using mixed and unmixed DNA samples

Sample	**Dwarf-specific assay**	**Tall-specific assay**
SGD	+	-
VTT	-	+
WAT	-	+
SGDxVTT	+	+
SGD + VTT	-	+
SGD + WAT	-	+
SGDxVTT + WAT	-	+

Legend:

(+) amplification detected; (-) no amplification detected; (SGD+VTT) SGD DNA mixed with VTT DNA; (SGD+WAT) SGD DNA mixed with WAT DNA; (SGDxVTT+ WAT) SGDxVTT mixed with WAT DNA.

2.3.3 Developing markers based on single nucleotide polymorphisms using the candidate gene approach targeting the NBS-LRR Class R-genes

2.3.3.1 Oligonucleotide primers and PCR approach

The objectives of this study were to isolate novel candidate RGAs from the resistant/tolerant and susceptible coconut varieties namely SGD, MYD, VTT and WAT and identify markers based of SNPs that can be used to differentiate between the varieties or between resistant/tolerant varieties and the susceptible WAT genotype.

A nested PCR approach was used successfully to amplify candidate RGAs from DNA samples of the four coconut varieties mentioned above. Oligonucleotide primers based on the P-loop and GLPL amino acid motifs were used as forward and reverse primers respectively in all possible pair-wise combinations in the first round PCR (i.e P-loop1- 8 combined with GLPL1-6 yielding a total of 48 reactions). Primers for amplifying RGAs from coconut are detailed in Appendix 2. The products of the first round PCR generated using a common reverse primer were pooled and diluted 20-fold and used as template in the second round PCR. Primers based on the kinase-2 and GLPL amino acid motifs were used as the forward and reverse primers in all possible pair-wise combinations (kinase-2: 1-4 combined with GLPL1-6, yielding a total of 24 reactions; Table 2.4) to amplify a 300 bp fragment from the coconut samples (Figure 2.11).

Table 2.4 Oligonucleotide combinations used to amplify RGAs from coconut DNA in second round PCR

Primer Code	Template DNA	Forward primer	Reverse primer
A	Pooled 1st round PCR products amplified with GLPL1 as reverse primer	Kinase-2D	GLPL1
B		Kinase-2E	GLPL1
C		Kinase-2F	GLPL1
D		Kinase-2G	GLPL1
E	Pooled 1st round PCR products amplified with GLPL2 as reverse primer	Kinase-2D	GLPL2
F		Kinase-2E	GLPL2
G		Kinase-2F	GLPL2
H		Kinase-2G	GLPL2
I	Pooled 1st round PCR products amplified with GLPL3 as reverse primer	Kinase-2D	GLPL3
J		Kinase-2E	GLPL3
K		Kinase-2F	GLPL3
L		Kinase-2G	GLPL3
M	Pooled 1st round PCR products amplified with GLPL4 as reverse primer	Kinase-2D	GLPL4
N		Kinase-2E	GLPL4
O		Kinase-2F	GLPL4
P		Kinase-2G	GLPL4
Q	Pooled 1st round PCR products amplified with GLPL5 as reverse primer	Kinase-2D	GLPL5
R		Kinase-2E	GLPL5
S		Kinase-2F	GLPL5
T		Kinase-2G	GLPL5
U	Pooled 1st round PCR products amplified with GLPL6 as reverse primer	Kinase-2D	GLPL6
V		Kinase-2E	GLPL6
W		Kinase-2F	GLPL6
X		Kinase-2G	GLPL6
Y	Positive control(cloned coconut RGA fragment)	Kinase-2D	GLPL6
Z	No template control	Kinase-2D	GLPL6

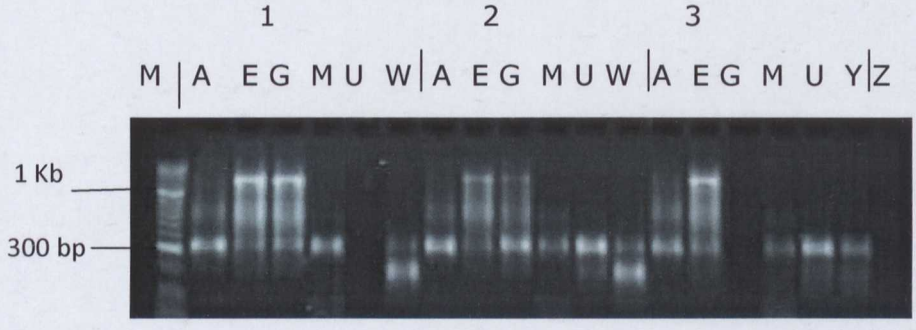


Figure 2.11 Agarose gel showing PCR amplification of RGAs from WAT samples. Lanes: (1) samples from healthy palms; (2) samples from diseased palms; (3) samples from dead palms ;(Y) positive control; (Z) No template control; (M) Hyperladder II. Letters 'AEGMUWYZ' refer to primer combinations used in the reaction (Table 2.4). 300 bp bands represented correct sized fragment.

2.3.3.2 Sequence analyses of amplified fragments

Bands of the expected size (approximately 300 bp) were excised, purified and cloned as described in Section 2.2.6 and clones containing inserts of the correct size were purified (Section 2.2.7) and sequenced by Eurofins mwg (Germany). Sequences were edited to remove vector sequence and a BLAST search carried out at the NCBI GenBank database to determine the closest matches. Thirty one sequences were found to be novel RGAs with sequence homologies to known database RGAs from species such as tobacco, oil palm, *Areca catechu* and wheat (Table 2.5). Phylogenetic analyses were conducted in MEGA 5 and the evolutionary history inferred using Neighbour joining method (Saitou and Nei, 1987; Tamura *et al.*, 2007) (Figure 2.12). Sequences aligned in MEGA 5 were investigated for the presence of SNPs by eye. Consistent SNPs between varieties were, however, not identified among the sequences.

Table 2.5 BLAST searches for closest matches of amplified coconut RGAs

Variety/ primer code	Accession/Description	Query coverage (%)	E-value
WAT-D(A)	AAF24312.1 Resistance protein [Elaeis guineensis]	96	2e-46
WAT-H (A)	AAF24312.1 Resistance protein [Elaeis guineensis]	96	2e-46
MYD (A)	AAF24312.1 : Resistance protein [Elaeis guineensis]	96	2e-46
VTT (A)	AAF24312.1 Resistance protein [Elaeis guineensis]	97	3e-41
MYD (E)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	99	7e-31
VTT (W)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis	99	9e-31
SGD (W)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	99	1e-30
SGD (A)	AAF24312.1 Resistance protein [Elaeis guineensis]	95	1e-30
MYD (G)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	99	2e-30
VTT (G)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis	96	2e-30
MYD (W)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	99	3e-30
WAT- H (E)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	99	1e-29

WAT-DS(G)	XP 002525457.1 : Leucine-rich repeat containing protein, putative [Ricinus communis]	95	7e-29
SGD (A)	XP 002448182.1 Hypothetical protein SORBIDRAFT	97	5e-25
SGD (E)	XP 002448182.1 Hypothetical protein SORBIDRAFT	97	5e-25
MYD (M)	ACO58581.1 : NBS-LRR disease resistance protein RGA4-like protein [Areca catechu]	96	2e-23
SGD (M)	ACO58581.1 : NBS-LRR disease resistance protein RGA4-like protein [Areca catechu]	96	2e-23
SGD (U)	ACO58581.1 : NBS-LRR disease resistance protein RGA4-like protein [Areca catechu]	96	2e-23
MYD (U)	ACO58581.1 : NBS-LRR disease resistance protein RGA4-like protein [Areca catechu]	96	4e-23
WAT-D (U)	ACE79484.1: NBS-coding resistance gene analog [Nicotiana tabacum]	98	3e-18

Legend:

WAT-DS: Infected WAT; WAT-H: Healthy WAT; WAT-D: Dead WAT. All other codes are the same as previously described. Letters in bracket represent primers used (see Table 2.4).

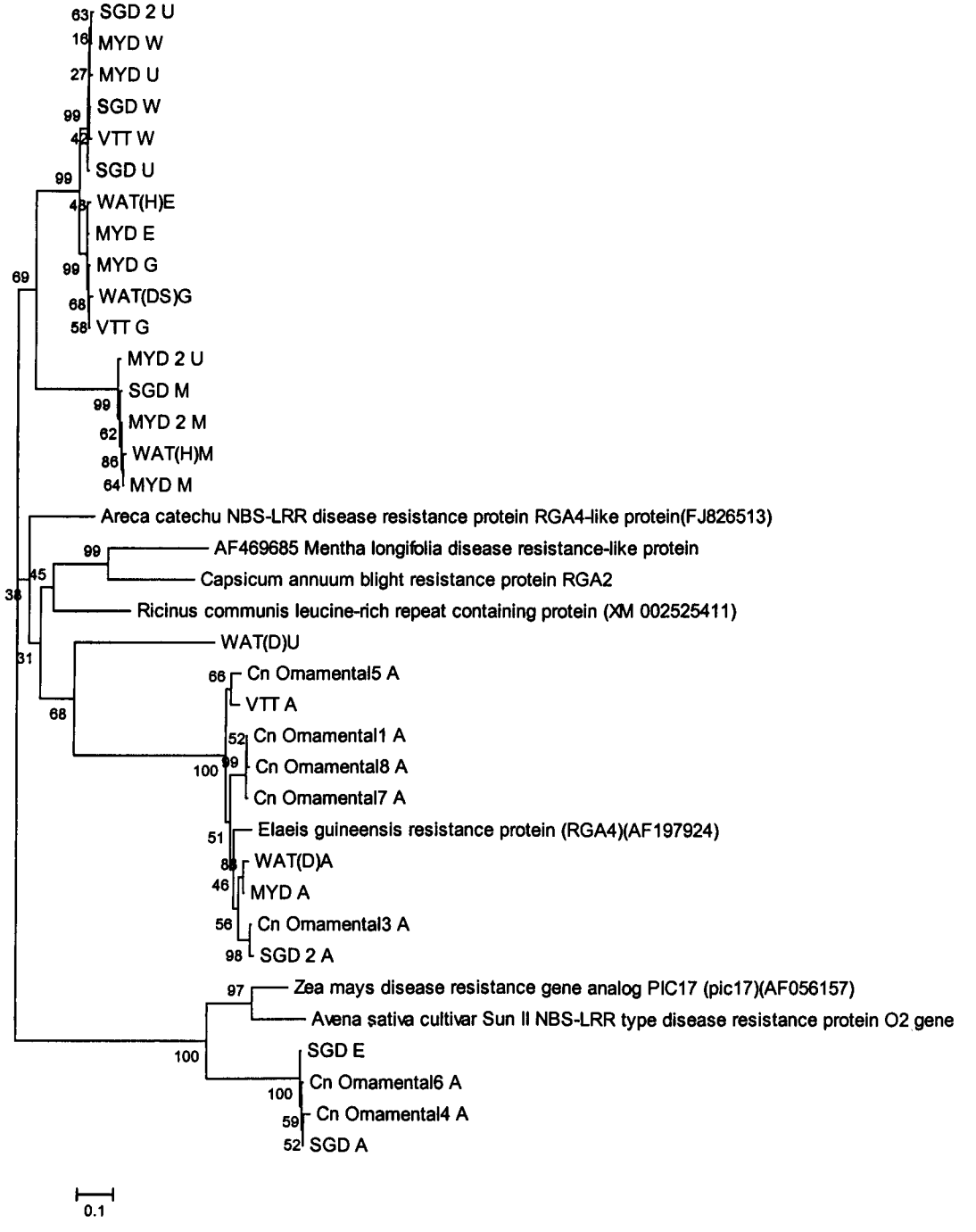


Figure 2.12 Phylogenetic relationships amongst the amplified coconut RGAs and some database RGAs. GenBank accession numbers are shown in brackets. Letters after variety codes represents primers used in amplifying those RGAs. 'Cn ornamental 1-8' were from eight coconut sourced from a UK garden centre. Tree was drawn to scale and bootstrap values were expressed as percentages of 1000 replicates.

2.4 DISCUSSION

Genetic purity of planting materials is essential to the success of any breeding programme; it is necessary for breeding of materials that are true-to-type and for removing duplicate accessions from germplasm conservation fields (Karp *et al.*, 1997). In a disease control context, it is essential that hybrids or supposed resistant/tolerant materials distributed to farmers are exactly what they were claimed to be and this requires characterisation of the breeding materials. The identification of genetic contamination in the MYD parents used in developing the MAYPAN hybrid in Jamaica after the breakdown of resistance in this hybrid to LY speaks volumes to the fact that tools for certifying the identity of cultivars is imperative in coconut breeding programs. In the above mentioned study, the use of 34 SSR markers including the 14 SSR markers of the COGENT microsatellite kit on 69 MYD individuals in comparison with reference MYD DNA from five other countries showed that only 16% of the studied palms were true-to-type (Lebrun *et al.*, 2008).

In Ghana, the materials used in the breeding programmes have not been characterised and it is expedient to develop a reliable set of molecular markers and techniques that can be used to differentiate varieties and ecotypes. The markers trialled in this work were chosen to give a good coverage of the coconut genome: from SSRs which are present in both coding and non-coding regions (Zane *et al.*, 2002) to SNP markers based in introns of WRKY gene sequences (Borrone *et al.*, 2004) and an attempt

to develop potentially functional markers based on RGAs, which would likely be found in coding regions (Collins *et al.*, 1998).

To detect the clearest and easiest SSR markers to score for variety verification, melt curve analysis was adopted for detecting allele size differences at an SSR locus. The technique was found to be simple, using Microscoft Excel functions to undertake the melt plots in the absence of specialised softwares for genotyping using HRM, and the results easy to interpret. The technique showed sensitivity and could distinguish between the expected amplification products and non-specific reaction products differing in their melting temperatures by only about 3°C. The two promising markers initially seemed to have cultivar specific alleles but screening with more samples showed otherwise; this underlies the importance of using larger sample numbers in such studies. Konan *et al.* (2007) using 6-8 individuals of the WAT, SGD and VTT identified a WAT specific allele associated with marker CnCirC12. In this study the WAT allele which seemed to be unique at each of loci CAC65 and CnCirC12 was shared by some SGD palms. The use of more than 20 SGD palms in this work was important in revealing that marker CnCirC12 did not have a WAT specific allele. Allele sharing by the WAT and some SGDs is unlikely to be a result of genetic contamination since the SGD individuals from which those seemingly WAT specific alleles were found were not the same samples in the analyses with both CAC65 and CnCirC12. It is also important to mention that strict isolation distances are maintained between these breeding plots and other palms in the vicinity.

Although none of the SSR markers trialled was found to be variety specific or linked to tolerance/resistance, there is still a chance of finding such markers considering the fact that there are over two hundred known/published SSR markers that were not trialled in this study due to time constraints. In a similar study on oil palm, using polyacrlamide gels, comparisons of SSR profiles of parent plants and their clones were used to verify the fidelity of the clones and to rogue out off-types (Singh *et al.*, 2007).

LAMP assays have been used for the diagnosis of plant diseases including LD (Hodgetts *et al.*, 2011; Yankey *et al.*, 2011). This work sought to broaden the scope of the applications of the technique to cover detection of polymorphisms at marker loci. Although in one case the assay specific for a SNP in a WAT palm failed to amplify that sequence and samples of dwarf varieties could not be amplified in mixed DNA of 'talls' and 'dwarfs', the technique represents a proof of concept that needs to be optimised for improved efficiency. Optimisation steps such as altering the amplification temperature, trialling different concentrations of primers and even designing new primers around the same marker may be considered in future studies. The identification of new SNP markers in coconut would therefore be an opportunity to modify and fine tune the technique. The LAMP technique, because of its amenability to in-field use, is envisaged to be a future field based diagnostic method of choice. The prospects of applying the technique in other areas, as has been demonstrated in this work, will make it more attractive and useful to a

wider range of specialists such as geneticists, breeders and pathologists. LAMP is also less time consuming, less prone to contamination and does not involve the use of potentially toxic chemicals like other detection systems that involve radioactivity.

The mechanisms responsible for the resistance/tolerance in the SGD palms and the relatively high levels of tolerance in the VTT and MYD x VTT hybrids to LD compared to the susceptible WAT in Ghana is yet to be determined. The candidate gene approach provides a means of examining known genes that affect similar processes in other plants (Kuhn *et al.*, 2003). RGAs were isolated with the aim of identifying SNPs that could be mapped to chromosomal regions to determine their possible linkage with resistance to any disease type even if it is not LD. The use of primers in all possible combinations and the identification of those primers that produce successful clones was an important step that cut down on the number of reactions that had to be run to isolate RGAs in subsequent experiments. Although SNPs between varieties were not identified in this work, other resistance gene classes or other gene families could be investigated for possible SNP markers in the future.

CHAPTER 3: STUDYING THE GENETIC BASIS OF TOLERANCE/ RESISTANCE OF COCONUT GERMPLASM TO LD AND THE SEASONAL QUANTIFICATION OF PATHOGEN TITRES IN DISEASED PALMS

3.1 INTRODUCTION

3.1.1 Influence of the distribution of phytoplasmas and environmental factors on the development of LY/LD

Many studies have been conducted to understand how phytoplasma-mediated diseases develop within infected plants and although the mechanisms are not yet fully understood, a number of pathogenicity factors have been identified. Some pathogenicity factors considered to be important in phytoplasma-mediated diseases include hormonal imbalance, strain virulence, toxins and concentration and distribution of phytoplasmas within infected plants (Leon *et al.*, 1996; Berges *et al.*, 2000; Tan and Whitlow, 2001; Christensen *et al.*, 2004; Seemuller and Schneider, 2007; Nicolaisen and Horvath, 2008; Oropeza *et al.*, 2011). Knowledge of the build-up and distribution of phytoplasmas in LY/LD-infected coconut palms is important in understanding how colonisation of plant tissues affects plant function and development (Maust *et al.*, 2003; Oropeza *et al.*, 2011). Dery *et al.* (1997) studied the development of LD and made a number of important observations including the fact that the incubation period may range from six months to two years. It was also observed in the above-mentioned study that the longest duration of symptoms occurred in palms in which infection had started between May

and November (i.e mainly the coolest months of the year in Ghana). Seasonality of symptom development has also been suggested in studies on LY in Jamaica, where it was observed that the coolest months favoured the development of disease symptoms. However, in that study it was not determined whether this period corresponded to an earlier period of high infection (Carter and Suah, 1964). Studies conducted on the distribution of phytoplasma in LY infected palms have shown that pathogen titres vary depending on the stage of infection and on tissue type. The highest concentrations of phytoplasmas have been found in sink tissues such as immature leaves, stem and inflorescence, while minimal or no detections have been recorded for source tissues such as mature and intermediate leaves (Thomas and Norris, 1980; Oropeza *et al.*, 2011).

3.1.2 Quantification of nucleic acids by real-time PCR

The current method of choice for nucleic acid quantification is real-time PCR (also known as Q-PCR). Q-PCR provides a reliable means of quantifying phytoplasma in infected palms rather than extrapolating pathogen titres from conventional PCR detections and has been employed for quantifying various phytoplasmas in other plants such as *Euphorbia pulcherrima*, apricots and apple trees (Christensen *et al.*, 2004; Martini *et al.*, 2007; Bisognin *et al.*, 2008). Q-PCR involves the amplification and detection of nucleic acids in real-time using fluorescence detection methods. Various detection chemistries have been developed for detecting amplicons in Q-PCR systems: the commonly used ones include

Taqman probes, SYBR Green I dye and molecular beacons (Ginzinger, 2002). Taqman probes have a reporter and quencher dye at the 5' and 3' ends of the probe respectively and the accumulation of amplicons results in the emission of fluorescence by the reporter dye (as it is separated from the quencher) (Walker, 2002). SYBR Green binds directly to double-stranded DNA and fluorescence increases as the PCR products accumulate. A well-optimized reaction is, however, needed when SYBR Green is used because it binds to all double-stranded DNA which may include non-specific products. A melting curve analysis which enables amplicons to be identified based on their melting temperatures is therefore performed at the end of the reaction to validate the results (Galetto and Marzachi, 2010). Molecular beacons form a stem and loop structure and are equipped with a quencher and a reporter like Taqman probes (Wong and Medrano, 2005).

The PCR process (as detected in Q-PCR) can be broken down into four phases; the linear ground phase; early exponential phase; exponential phase/log linear phase and the plateau phase. During the linear phase amplification products begin to accumulate but are below detectable levels of the Q-PCR instrument. At the early exponential phase, PCR products accumulate sufficiently above background detection to become detectable reliably by the Q-PCR instrument. The cycle at which this occurs is called the cycle threshold (CT). Amplification is optimal at the exponential phase with the products doubling after each cycle until it reaches the plateau phase where due to limitation of reagents or

inhibition of the polymerase or the accumulation of pyrophosphates, the amplicons are no longer generated at an exponential rate (Ginzinger, 2002). The CT is directly proportional to the base 10 logarithm of the starting concentration of the DNA template and therefore the amount of DNA in a material can be extrapolated during the log phase of PCR (Ginzinger, 2002; Wong and Medrano, 2005).

Quantification of nucleic acids by Q-PCR is carried out using two main approaches: 'standard curve quantification' also referred to as absolute quantification and 'relative quantification'. Quantification using the standard curve method involves using serially diluted sets of DNA template of known concentration, referred to as 'standards' to generate a standard curve from which the concentrations of the experimental or test samples ('unknowns') can be determined using their CT values (Giulietti *et al.*, 2001; Christensen *et al.*, 2004). Standard curves are usually generated using genomic DNA or a cloned product; purified plasmid DNA containing an insert of the gene of interest. Relative quantification involves quantification of a gene of interest relative to a control gene within the sample. Using various mathematical models, the difference in the CTs between the control gene and the gene of interest is used to calculate the DNA amount or gene expression levels (using cDNA made from RNA) (Giulietti *et al.*, 2001; Ginzinger, 2002).

Several years of breeding by the Ghanaian research team aimed at finding a resistant/tolerant material to LD has culminated in the development of two hybrids, the MYDxVTT and the SGDxVTT which are being used to replant devastated fields. The SGDxVTT is considered as a promising hybrid in terms of resistance, while the MYDxVTT, which has suffered some losses, performs better than the local WAT. The genetic basis of the superior performance of the SGDxVTT hybrids in relation to LD is, however, not known. It is also not known whether WAT 'escapees' found in LD-devastated farms possess some level of resistance to the disease or whether they are able to harbour phytoplasmas without showing disease symptoms (tolerance). In this work, Q-PCR analyses were used to examine the coconut–phytoplasma interaction and how it impacts on disease development.

3.1.3 Field based diagnostic methods for LD

Routine diagnosis of LD is carried out using PCR with primers designed in both ribosomal and non-ribosomal genes, which is relatively quicker and more reliable as a method of diagnosis compared to older methods such as microscopy and symptomatology. However, current emphasis on nucleic acid amplification techniques is on the use of simpler, quicker and less expensive methods which are just as sensitive and specific as conventional PCR (Notomi *et al.*, 2000). Such methods offer an extra advantage if they can be amended for in-field use. It is against this backdrop that field based diagnostics are considered desirable by plant pathologists because of the potential savings in time spent on diagnosis

once results can be obtained in the field. Field based diagnosis of LD will be a welcome boost for coconut researchers, particularly in Africa, where cost considerations make the routine use of PCR diagnosis unsustainable on a long term basis. They would also be of significant importance to coconut workers in Ghana and other African countries in general since most fields are located in remote areas and therefore there are often time lapses between sample collection and disease diagnosis. One such method which is easily amenable to in-field use is the LAMP technique (Hodgetts *et al.*, 2011). For equipment, the LAMP diagnostic technique at a minimum requires only a heated block for nucleic acid amplification, and assays for detecting plant pathogens including the LD phytoplasma have been developed (Tomlinson *et al.*, 2007; Tomlinson *et al.*, 2010a). LAMP amplicons can be detected by a colour change by the addition of napthol blue or SYBR Green I to the reaction (Reddy *et al.*, 2010; Tomlinson *et al.*, 2010a). Using DNA labels, LAMP amplicons can also be detected with generic lateral flow devices (Tomlinson *et al.*, 2010b). LAMP amplicons can also be detected in real-time using intercalating fluorescent dyes and in this regard specialised real-time LAMP instruments such as the Genie I have been used to detect some pathogens (Bekele *et al.*, 2011). These detection techniques eliminate the need for post amplification methods for amplicon detection such as gel electrophoresis making the technique suitable and amenable to in-field use. One of the essential requirements for a good nucleic acid based in-field diagnostic technique is a quick and simple DNA extraction kit which is not easily prone to cross-over contamination. The development of a lateral flow device (LFD) extraction kit in which the DNA extraction

process can be done in less than 10 min is one such method and has been used successfully in LAMP analyses (Danks and Boonham, 2007; Tomlinson *et al.*, 2010a). In this work a real-time LAMP assay for LD was evaluated for its suitability as both a lab and in-field diagnostic method.

The objectives of this section of the study were therefore to:

1. Develop reliable phytoplasma diagnosis and quantification techniques that could be used to assess phytoplasma titres in diseased palms.
2. Investigate seasonal variation or differences in phytoplasma amounts in organs of the palm.
3. Find out if any of the WAT escapees or SGDxVTT and MYDxVTT hybrids can harbour a level of phytoplasma infection without developing LD.
4. Evaluate a real-time LAMP method for its effectiveness as a diagnostic for LD and particularly as a prospective field based method.

3.2 MATERIALS AND METHODS

Some of the materials and methods described in Chapter 2 were applied in the work described in this chapter and will be referred to when necessary.

3.2.1 Q-PCR primers and preparation of standards

To obtain phytoplasma DNA for use in generating standard curves, oligonucleotide primers RTA (5'-GGCACTAAGAGCCGATGAAG-3') and RTB (5'-TCCTCCCATCTTCCAACAAG-3') based on a segment of the CSPWD phytoplasma 23S rDNA gene (GenBank accession number: EU 168774.1) were used to amplify a 379 bp fragment. This fragment was then cloned using the Promega pGEM®-T Easy Vector system (Promega, USA) as described in Section 2.2.6. A second set of oligonucleotide primers RTribF1 (5'-CGAATGGGGCAACCTACTAC-3') and RTribR1 (5'-CGGATTCGCTCGTCACTAC-3') were designed to amplify a region within the cloned region for use in the Q-PCR assay. Similarly, to obtain coconut DNA to use to normalise the quantification results, a set of oligonucleotide primers, CnA (5'-AACGAGACCTCAGCCTGCTA-3') and CnB (5'-GCGATCCGAACACTTCACC-3') based on a section of the partial sequence of the coconut 18S rDNA (GenBank accession number: AY012393.1) were designed and used to amplify a 365 bp fragment of coconut DNA which was also cloned as described above. A second set of primers: Cn18SF3 (5'-CTGTGATGCCCTTAGATGTTCTG-3') and Cn18SR3 (5'-ACCAACAATTGCAATGATCTATCC-3') was designed to amplify a 136 bp fragment from the cloned region for use in the Q-PCR assay. All Q-PCR

primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) and were synthesized by Eurofins Mwg, Germany. All plasmid gene DNA were extracted using the GenElute™ Plasmid Miniprep kit (Sigma, USA), following the manufacturer's instructions. The DNA extracts were quantified using a nanodrop spectrophotometer (ND-1000 spectrophotometer, NanoDrop Technologies, Inc., Wilmington, USA) and using a starting concentration of 10 ng μL^{-1}, a two-fold serial dilution ranging from 2^{-1} to 2^{-17} was prepared for the two sets of standards.

3.2.2 Q-PCR assay and quantification method

Q-PCR was carried out on a 96 well IQ™ 5 Optical Module or a C1000™ Thermal cycler which uses a CFX 96™ real-time system (Biorad, CA, USA). Twenty five µL reactions comprised 12.5 µL SYBR Green JumpStart *Taq* ReadyMix (Sigma, USA), containing: 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 7 mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), stabilizers, 0.05 unit μL^{-1} *Taq* DNA polymerase, JumpStart *Taq* antibody, and SYBR Green I; 0.75 µL each of the forward and reverse primer (stock concentrations at 10 µM) achieving a final concentration of 0.3 µM and 2.5 µL of the DNA template. All DNA templates (apart from standards) were adjusted to a concentration of approximately 10 ng μL^{-1}. A two step amplification and data acquisition process involving an initial denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min was applied. An image of fluorescence was acquired at the end of the 60°C step using the filter appropriate for SYBR green detection, from which data were extracted by the software (Bio-rad CFX Manager,

version 2.0). Amplification was followed by a melt phase ramping from 55–95°C and rising by 0.5°C per cycle, during which fluorescence was also read.

To quantify the pathogen levels, the CT values were exported to Microsoft Excel (version 2007) and two standard curves, one for the coconut and the other for the phytoplasma standards, were generated by plotting the CT against the $\log_{(10)}$ starting DNA amount (ng) in the reactions amplifying. The starting amounts of the experimental samples ('unknowns') were calculated from the resulting equation of the fit line (i.e (observed CT-y intercept)/slope). Normalisation of pathogen amounts to account for differences in the amount of input DNA, efficacy of extraction and coconut tissue type was done by running a Q-PCR assay for plant 18S rDNA from each sample. The phytoplasma amount was divided by the amount of plant DNA in each sample to obtain the amount of phytoplasma 23S rDNA relative to coconut 18S rDNA. The identity of the amplicons was validated using the melting temperatures generated by the accompanying software of the Q-PCR system.

3.2.3 PCR diagnosis of LD-infection

PCR diagnosis of LD-infection was carried out with three PCR assays involving single round PCRs with the oligonucleotides: CSPWDSecAFor2 (5'-CGAGATGCAGATCGTTTTG-3') and CSPWDSecARev2 (5'-CCATCACCAAATTGACGTCC-3'); P1 (5'AAGAGTTTGATCCTGGCTCAGG AT T-3') (Deng and Hiruki, 1991) and P7 (5'-CGTCCTTCATCGGCTCTT-3')

(Smart *et al.*, 1996) targeting non-ribosomal (*secA* gene) and ribosomal (16S-23S rDNA) sequences respectively. Nested PCR was undertaken by diluting 1 µL of the P1/P7 products into 40 µL of sterile double-distilled water and used as the DNA template in the second round PCR using GH813f (5'-CTAAGTGTCGGGGGTTTCC-3') and AwkaSR (5'-TTGAATAAGA GGAATGTGG-3') (Tymon *et al.*, 1998). To ascertain the presence of inhibitors in the plant materials and to test concurrently the efficiency of extracting DNA from woody coconut tissues, multiplex PCR was carried out using oligonucleotides for amplifying the *secA* gene from phytoplasma DNA and primers for amplifying a microsatellite marker CncirF3 from coconut DNA (Lebrun *et al.*, 2001). The proportion of pathogen DNA was expected to be significantly lower than that of the plant DNA, because of the unusually low titres of phytoplasmas in woody plant tissues (Bertamini *et al.*, 2003; Nejat and Vadamalai, 2010). Accordingly, the volumes of the pathogen primers used were twice as much as those of the plant primers. All PCRs were carried out as described in Section 2.2.4 at annealing temperatures of 55, 56, 53 and 55°C in the CSPWDSecAFor2/Rev2, P1/P7, GH813f/AwkaSR and multiplex PCR assays, respectively.

3.2.4 Detection of LAMP products using lateral flow devices (LFD)

To detect LAMP amplicons on LFDs, labelled primers were used in the LAMP assay. To incorporate the labels, one of the loop primers (Backward Loop (BL)) was labelled with biotin and the other loop primer (Forward Loop (FL)) was labelled with digoxigenin (DIG). The primers and their

sequences used in the LAMP assay are listed in Appendix 3. LAMP reactions were formulated as described in the Section 2.2.9 and incubated in a water bath or on a heated block at 65°C for 60 min and then at 80°C for 5 min to inactivate the *Bst* polymerase. The amplification products were diluted 1 in 500 µL in LFD buffer C (Forsite Diagnostics Ltd, York, UK). Seventy µL of the diluted reaction products were then added to the release pad of the LFD (also obtained from Forsite Diagnostics Ltd, York, UK) and allowed to flow through the membrane. The LFD has a test line which becomes visible when amplicons with incorporated labels are applied; amplification products in which only unincorporated primers are present do not form a test line. The device also has a control line which becomes visible after a successful run; therefore a positive diagnosis results in two lines while a negative one has a single line.

3.2.5 Extraction of DNA using LFD

A rapid DNA extraction method described by Tomlinson *et al.* (2010a) was used to extract DNA using LFD as follows: firstly about 0.3 g of coconut stem tissues was added to 5 mL LFD buffer C in a small screw-cap bottle containing steel ball bearings (5 mm Ø) (Forsite Diagnostics Ltd, York, UK). This was shaken vigorously and/or vortexed to disrupt the plant tissues and to release the DNA. Seventy µL of the buffer was then applied to the release pad of the LFD strip and left at room temperature for about 5 min to allow the buffer to flow along the membrane and to

dry. The devices were put individually into sealed bags and stored in a freezer.

3.3 RESULTS

3.3.1 Assessing the efficacy of the DNA extraction protocol using multiplex PCR

A 380 bp fragment (approximately) from the coconut DNA and a 290 bp amplicon (approximately) from the DNA of LD-infected samples were amplified in the multiplex PCR described in Section 3.2.3. Positive results from infected plant samples appeared either as double bands on the gel representing the plant and pathogen fragments or as single bands with sizes corresponding to the expected pathogen fragment size amplified from plant DNA (Figure 3.1). Single bands amplified with the plant primers indicated that the palms were likely to be uninfected. Absence of bands for both plant and pathogen DNA indicated either a lack of DNA or PCR inhibition.

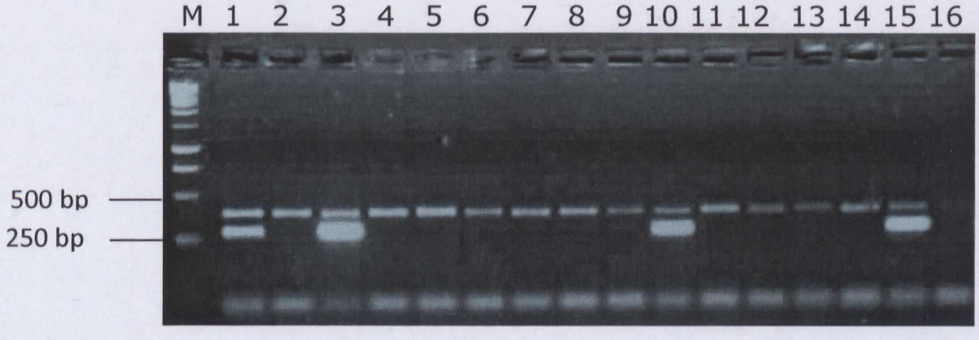


Figure 3.1 PCR amplification of coconut and phytoplasma DNA using CSPWDSecAFor2/Rev2 and CnCirF3 F/R. Lanes: (1, 3, 10 & 15) amplification of plant and pathogen DNA; (2, 4-9, 11-14) amplification of plant DNA only; (16) Water control. The analyses revealed the extraction process to be effective and samples which did not show plant DNA were re-extracted and re-analysed.

3.3.2 Assessing the effectiveness of different PCR assays in detecting the LD phytoplasma

Three PCR assays comprising both ribosomal (P1/P7 and GH813f/Awka SR) and non-ribosomal primers (CSPWDSecAFor2/Rev2) and PCR approaches involving both single round and nested PCR assays were assessed for their effectiveness in detecting the LD phytoplasma in infected palms. The samples analysed comprised DNA extracts from WAT palms of the first three rounds of sampling (one hundred palms at each sampling period). Firstly, the multiplex PCR assay previously described (Section 3.2.3) was used to ascertain the presence of inhibitors or a lack of DNA in the samples before the other assays were applied. DNA samples which failed to amplify were re-extracted and re-analysed. In the analyses of samples from the first season, out of a total of 100 samples, four samples were found to be infected by both the nested PCR (P1/P7 followed by GH813f/AwkaSR) and the *secA* assays while two positive results were recorded for the P1/P7 assay (Table 3.1). Positive results detected by all three assays in the analyses of the second season samples were similar: 19, 20 and 21 positive detections were recorded for the *secA*, P1/P7 and nested PCR assays respectively (Table 3.1). In the third season analyses, the same samples were positively diagnosed by the nested and *secA* PCR assays; both assays produced 39 positive results while the P1/P7 assay detected 33 positive results. All 33 positive results were part of the number diagnosed by the *secA* and nested PCR assays (Table 3.1).

Table 3.1 Comparative performance of different PCR assays at detecting the LD phytoplasma

	Season 1			Season 2			Season 3		
Palm No.	P1	P2	P3	P1	P2	P3	P1	P2	P3
1	-	-	-	-	-	-	-	-	-
2	-	-	-	+	+	+	+	+	+
3	-	-	-	-	-	+	+	+	+
4	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	+	+	+
9	-	-	-	-	-	-	-	-	-
10	-	-	-	+	+	+	+	+	+
11	-	-	-	-	-	-	+	+	+
12	-	-	-	-	-	-	-	-	-
13	-	-	-	+	+	+	+	+	+
14	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
16	-	-	-	+	+	+	+	-	+
17	-	-	-	+	+	+	-	-	-
18	-	-	-	-	-	-	+	+	+
19	-	-	-	-	-	-	+	+	+
20	-	-	-	-	-	-	+	+	+
21	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	+	+	+
26	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	+	+	+
30	-	-	-	-	-	-	+	+	+
31	-	-	-	-	-	-	+	+	+
32	-	-	-	-	-	-	+	+	+
33	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-
35	-	-	-	+	+	+	+	+	+
36	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-
39	-	-	-	-	-	-	+	+	+
40	-	-	-	-	-	-	-	-	-
41	-	-	-	+	+	+	+	+	+
42	-	-	-	-	-	-	+	+	+
43	+	-	+	+	+	+	-	-	-

44	-	-	-	-	-	-	-	-	-
45	-	-	-	+	+	+	+	+	+
46	-	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	-
51	-	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-	-
53	-	-	-	+	+	+	+	+	+
54	-	-	-	-	-	-	-	-	-
55	-	-	-	+	+	+	-	-	-
56	-	-	-	-	-	-	-	-	-
57	-	-	-		+	+	+	-	+
58	-	-	-	+	+	+	+	+	+
59	-	-	-	+	+	+	+	-	+
60	-	-	-	-	-	-	-	-	-
61	+	+	+	+	+	+	-	-	-
62	-	-	-	-	-	-	+	+	+
63	-	-	-	-	-	-	+	+	+
64	-	-	-	+	+	+	+	+	+
65	-	-	-	-	-	-	+	+	+
66	-	-	-	-	-	-	-	-	-
67	-	-	-	-	-	-	-	-	-
68	-	-	-	-	-	-	+	+	+
69	-	-	-	-	-	-	-	-	-
70	-	-	-	-	-	-	-	-	-
71	-	-	-	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-
73	-	-	-	+	+	+	-	-	-
74	-	-	-	-	-	-	-	-	-
75	-	-	-	-	-	-	+	+	+
76	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	+	-	+
78	-	-	-	-	-	-	+	+	+
79	-	-	-	+	+	+	+	-	+
80	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-	-
83	+	-	+	+	+	+	+	-	+
84	-	-	-	-	-	-	+	+	+
85	-	-	-	-	-	-	+	+	+
86	+	+	+	+	+	+	-	-	-
87	-	-	-	-	-	-	+	+	+
88	-	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	+	+	+
91	-	-	-	-	-	-	+	+	+
92	-	-	-	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-	-

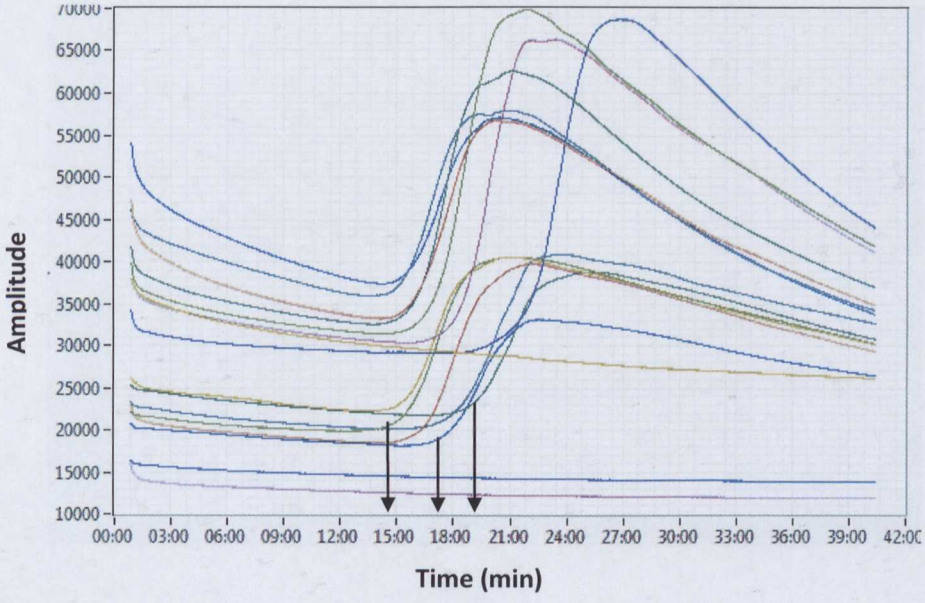
94	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-	-
99	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-
Total	**4**	**2**	**4**	**19**	**20**	**21**	**39**	**33**	**39**

Legend: (+) positive result ;(-) negative result; Primer sets: (P1) CSPWDSecAFor2/Rev2; (P2) P1/P7; (P3) P1/P7 followed by GH 813f/AwkaSR.

3.3.3 Evaluating a real-time LAMP assay as a diagnostic method for LD

In a preliminary experiment to assess the effectiveness of the real-time LAMP (RT-LAMP) assay, DNA samples from both infected and symptomless palms were extracted using CTAB buffer and assayed for phytoplasma infection as described in Section 2.2.9. LAMP amplicons were detected as fluorescent signals and were observed to form as early as within 15 min of the assay starting (Figure 3.2A). Validation of LAMP results by the melting analyses performed at the end of RT-LAMP provided a means of removing false positive results from the analyses (Figure 3.2B).

A



B

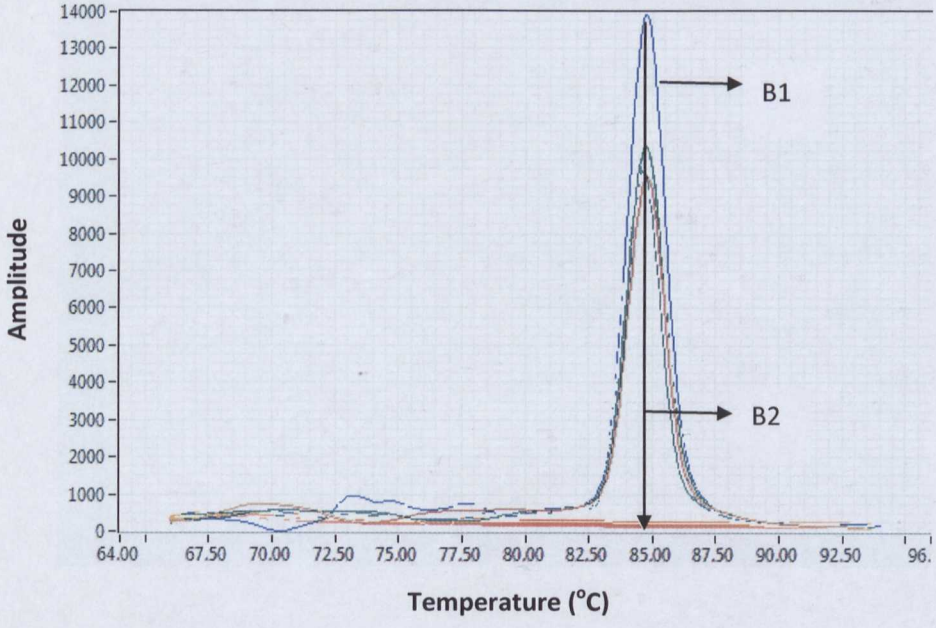


Figure 3.2 RT-LAMP outputs: (**A**) A plot of fluorescence against time (isothermal amplification) showing the time (min) at which amplification starts (indicated by arrows) and (**B**) A melt plot used to validate amplification results by comparing the melting temperature of amplicons to that of a control DNA (B1). The melting temperature is indicated by the arrow (B2).

The assay demonstrated effectiveness and could distinguish between infected and uninfected palms, showing results consistent with recorded symptoms. Results of PCR analyses and LAMP detection (by LFD) of the samples confirmed the effectiveness of the RT-LAMP assay (Table 3.2). In a more comprehensive assessment, the third batch of WAT samples were assessed for LD-infection with the RT-LAMP assay and the results compared with that from both single round and nested PCR assays. Out of 97 samples assessed by both RT-LAMP and PCR, 37 gave positive results in the RT-LAMP analyses and these detections were consistent with the results of PCR analyses involving the *secA* and nested PCR assays (Table 3.3).

In an experiment to determine the efficiency of amplifying DNA extracted on LFD strips in RT-LAMP reactions, four samples (two infected and two uninfected as previously determined by PCR analyses) were extracted using LFDs (Plate 3.1). The LFD DNA templates were run alongside CTAB extracted DNA templates using RT-LAMP. The results of the RT-LAMP analyses agreed with that from the PCR tests; the samples from infected palms were positively diagnosed, while no amplicons were detected from the negative samples (Figure 3.3). Although samples extracted with CTAB buffer were amplified earlier than those with LFD, all the amplicons were formed in less than 30 min (Figure 3.3).

Table 3.2 Comparison of real-time LAMP, LAMP detection by LFDs and PCR assays for LD diagnosis

WAT palm ID	Symptom	RT LAMP	T_A (min)	Melt peak (^{0}C)	PCR	LFD detection
1	Healthy	-	-	-	-	-
2	Infected	+	16.7	85.0	+	+
3	Infected	+	16.2	85.1	+	+
14	Healthy	-			-	-
15	Healthy	-			-	-
25	Infected	+	16.1	84.9	+	+
29	Infected	+	15.6	84.8	+	+
33	Healthy	-			-	-
35	Infected	+	24.0	84.7	+	+
39	Infected	+	20.1	85.1	+	+
Total number of positive detections		**6**			**6**	**6**

Legend: (+) positive result; (-) negative result; (T_A) time of amplification; DNA templates used in all three assays were extracted using CTAB and PCRs were carried out with CSPWDSecAFor2/Rev2 primer sets.

Table 3.3 Further comparison of the RT-LAMP assay with conventional PCR assays (including both single round and nested PCR assays)

	Number of positive results		
		PCR	
Number of samples	RT- LAMP	CSPWDSecA For2/Rev2	P1/P7 +GH813f/AwkaSR
97	37	37	37

All DNA templates used in the analyses were extracted using CTAB buffer. The same samples were positively diagnosed in all three assays.

Coconut wood chippings added directly to the extraction buffer.

Buffer-coconut chippings mixture shaken or vortexed into a suspension

The buffer is applied to the release pad of the strip. A section of the strip is then added to the RT-LAMP reaction

Plate 3.1 Extraction of coconut DNA using LFDs

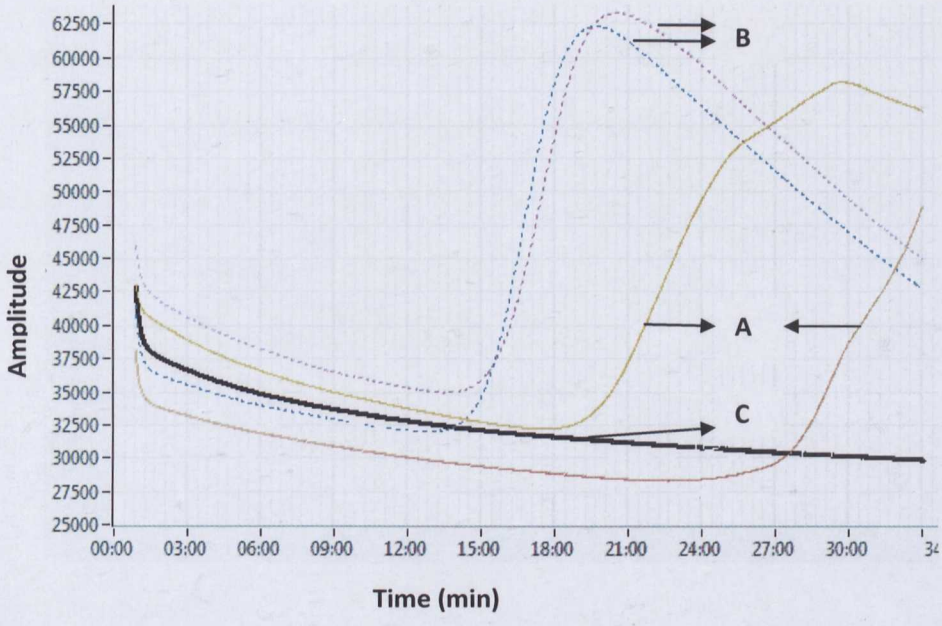


Figure 3.3 RT-LAMP output of analyses using DNA extracted with LFD. Lines: (A) amplicons of DNA extracted on LFDs; (B) amplicons of DNA extracted using CTAB buffer; (C) negative (water) control.

3.3.4 Assessment of LD-infection and disease development characteristics among the selected coconut varieties

Monitoring of disease symptoms, PCR diagnosis and quantification of pathogen load in infected palms were undertaken every six months with the aim of identifying palms that can sustain a level of infection without showing disease symptoms.

At the start of the study, palms not exhibiting disease symptoms were selected to ensure that disease incidence during the period of the study would be obvious. None of the SGDxVTT palms at both Agona and Daboase was diagnosed positively for LD phytoplasma infection and no symptoms of infection were recorded during the period of the study. Although about three MYDxVTTs had presumably died from LD-infection and were destroyed by the farmer, samples taken from some of the palms which had some remains left did not show phytoplasma infection, according to PCR analyses. As was expected disease incidence among the WAT palms located in a heavily diseased field was high and so the WAT population was used to gain information about the development of the disease. Results of PCR diagnosis and symptom observation showed that from seasons one to three, there were palms that had not started developing symptoms of LD-infection but could be positively diagnosed by PCR. Four, 19 and 39 palms were positively diagnosed by PCR while zero, 18 and 36 palms had developed symptoms of LD-infection from seasons one to three, respectively (Table 3.4). From season three onwards, the palms began to die, and PCR diagnosis of LD phytoplasma infection of tissues from dead palms was found to be technically difficult. Out of 20 dead palms sampled in an initial sampling experiment, only five

gave positive results in nested PCR analyses. Consequently, while 49, 56 and 56 palms were observed to be infected from seasons four to six, respectively, only 29, 20 and five palms were positively diagnosed by PCR, respectively, during the same period (Table 3.4).

Table 3.4 Summary of disease incidence among the selected palms

	No. of Palms	Season 1		Season 2		Season 3		Season 4		Season 5		Season 6	
		PCR	Sym	PCR	Sym	PCR	sym	PCR	Sym	PCR	Sym	PCR	Sym
WAT	100	4	-	19	18	39	36	29	49	20	56	5	56
MYDxVTT	100	-	-	-	-	-	-	-	-	-	-	-	-
SGDxVTT (Agona)	60	-	-	-	-	-	-	-	-	-	-	-	-
SGDxVTT (Daboase)	40	-	-	-	-	-	-	-	-	-	-	-	-

Legend: (-) absence of LD-infection; (sym) symptoms of LD-infection; PCR was carried out using CSPWDSecAFor2/Rev2. Symptoms include palms which had died from LD-infection.

Between six monthly sampling, none of the palms was observed to have fully recovered from infection. PCR analyses confirmed these observations as previously positively diagnosed palms continued to remain infected in subsequent sampling periods, apart from palms which had died from the disease and did not have detectable levels of the pathogen. Disease incidence increased with time during the study, with all infected palms eventually dying from the disease. All four palms determined to be infected at the first sampling period were observed to have died from infection by the fourth sampling period. Out of 19 palms that had been infected by the second sampling period, only four survived through the next two sampling periods and by the fifth sampling period, all 19 palms had died from infection. By the fifth sampling period, 56 palms had been infected cumulatively, none of which was alive at the sixth sampling period (Table 3.5).

Table 3.5 Survival rate of infected WAT palms

Sampling Period	No Infected (cumulative)	Number of palms infected in previous season but surviving in present sampling				
		S2	*S3*	*S4*	*S5*	*S6*
S1	4	4	1	0		
S2	19		4	4	0	
S3	39			13	0	
S4	49				3	0
S5	56					0

Legend: S1-S6 refers to 1st to 6th sampling periods. The number of infected palms is a cumulative record of disease and includes palms showing disease symptoms, dead palms and symptomless palms positively diagnosed by PCR (CSPWDSecA For2/Rev2).

3.3.5 Seasonal quantification and distribution of phytoplasma levels in infected palms

Observation of disease symptoms among the WAT palms revealed that symptoms develop all year, in both rainy and dry seasons (Table 3.4). Seasonal quantification of pathogen load in infected palms was carried out with the objective of determining phytoplasma titres in palms identified by PCR diagnosis and symptom monitoring to be LD-infected. The Q-PCR assay was also used to gain useful information on the dynamics of the coconut-phytoplasma interaction within the infected palm.

3.3.5.1 Performance characteristics of the real-time PCR assay

The performance of the Q-PCR primers was assessed by running a conventional PCR on a number of serially diluted plant and pathogen standards. These were viewed on agarose to verify the presence of only one amplicon (Figure 3.4). To simulate conditions that would be applied for Q-PCR, the number of cycles used was reduced from 35 cycles to 28 cycles and the annealing temperature was raised to 60^0C as used in the Q-PCR. Fragments of 140 bp and 136 bp represented the expected fragment sizes, were amplified from the phytoplasma and coconut DNA, respectively. A single band was also detected in all the dilution series ranging from 2^1 to 2^{17} in both sets of standards (Figure 3.4).

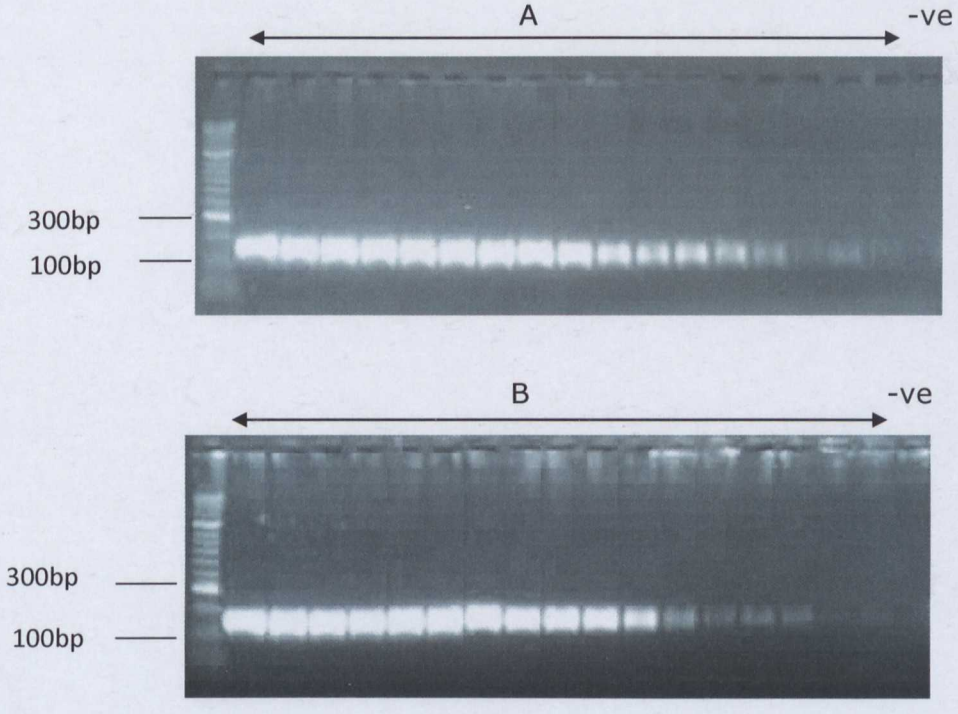
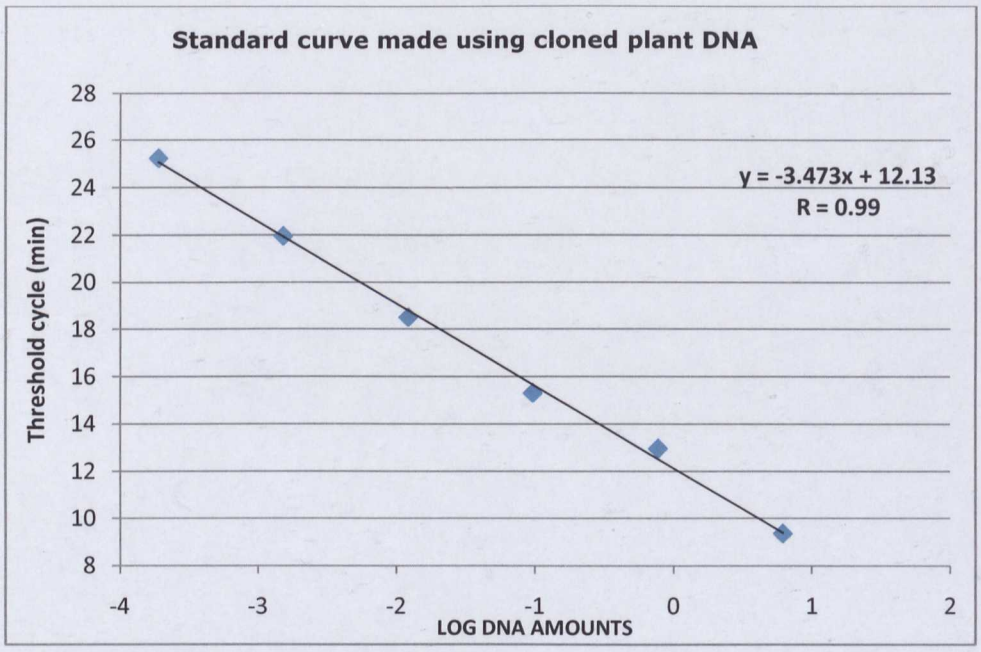


Figure 3.4 Agarose gel analyses of PCR amplified plant and pathogen standards. Lanes: (A) serial dilutions of plant standard (2^{-1}–2^{-17}); (B) serial dilutions of pathogen standard (2^{-1}–2^{-17});-ve: water control. A signal was seen in all the dilution series; although 2^{-16} and 2^{-17} were quite faint.

The Q-PCR assay was also used on a range of standards to generate standard curves for primers amplifying the plant and pathogen genes, which, showed that in both curves the 2^{12} (ng) to 2^{17} (ng) dilutions were in the dynamic range (i.e the range of concentrations for which CT values are in linear relationship with logarithms of concentrations) (Figure 3.5). The specificity of the Q-PCR assay was demonstrated by agarose gel analyses of the amplification products of the Q-PCR assay, which produced the expected fragment size of 140 bp and 136 bp from the phytoplasma and coconut DNA, respectively. The unique melting temperatures of the phytoplasma and coconut DNA fragments confirmed the specificity of the assay (Figure 3.6). The assay also showed sensitivity as a signal indicated by clear bands was seen in the dilutions series up to 2^{16} (Figure 3.6).



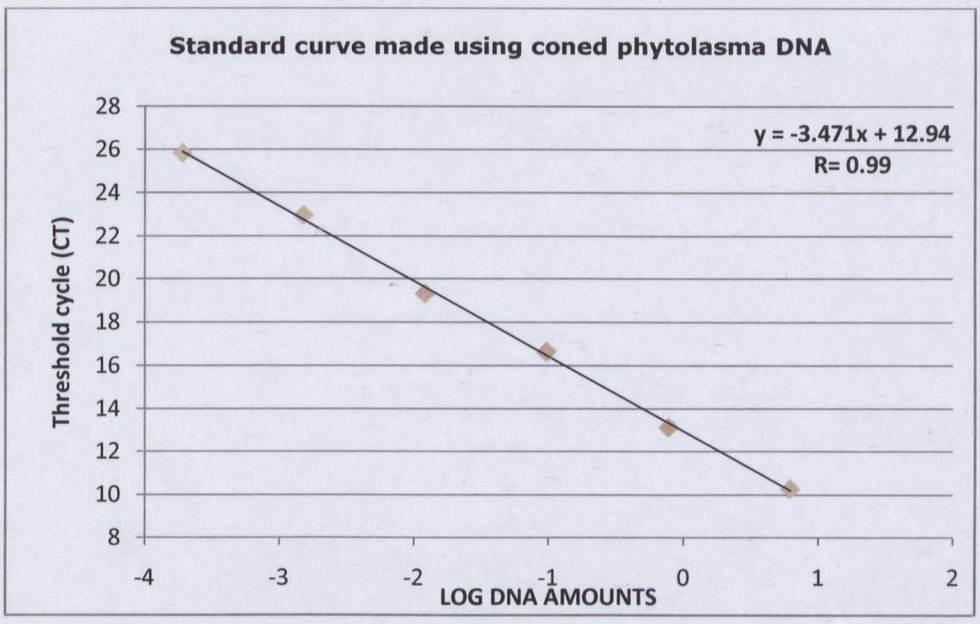


Figure 3.5 Standard curves of plant (A) and (B) pathogen standards. The threshold number of PCR cycles (CT: mean of duplicates) are plotted against the LOG DNA amount. Curves were generated in both cases using the dilutions 2^{-2}; 2^{-5}; 2^{-8}; 2^{-11}; 2^{-14}; 2^{-17}. The equations of the fit lines and the linear regression coefficients (R) are shown in the graph.

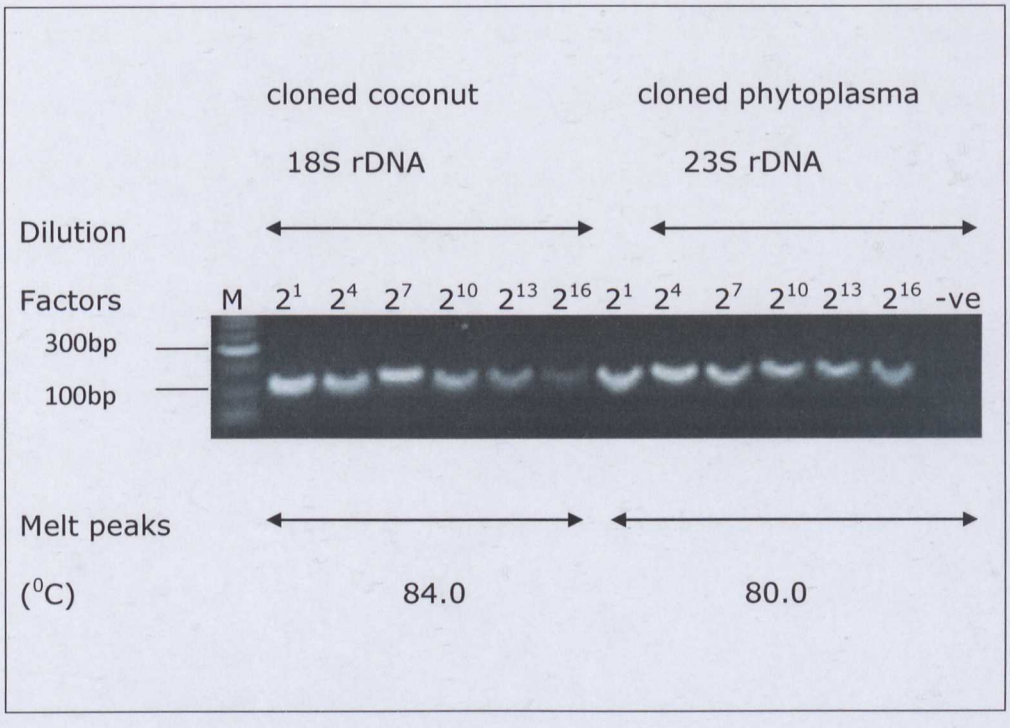


Figure 3.6 Gel analyses of Q-PCR products. A replicate of PCR using each of the serial dilutions (prepared for use in Q-PCR) was run on a 1.5% agarose gel. Melt peak temperatures used to identify the amplified fragments are also indicated. Marker (Hyperladder II) shows band sizes are approximately 100 bp (expected band size was 140 bp). NB. Reactions had completed 40 cycles.

3.3.5.2 Assessing the levels of phytoplasmas in infected palms and their seasonal fluctuations

Phytoplasma levels in infected palms were quantified six monthly for three years. Analyses of representative samples taken from the same palms in both rainy and dry seasons (seasons one & two, respectively) revealed that there was no pattern in pathogen titres between seasons: the amount could be higher or lower in any of the seasons within a single palm (Figure 3.7). Season one samples were from symptomless palms but detectable levels of phytoplasmas were found. At season two, all four palms had begun to develop symptoms of LD-infection.

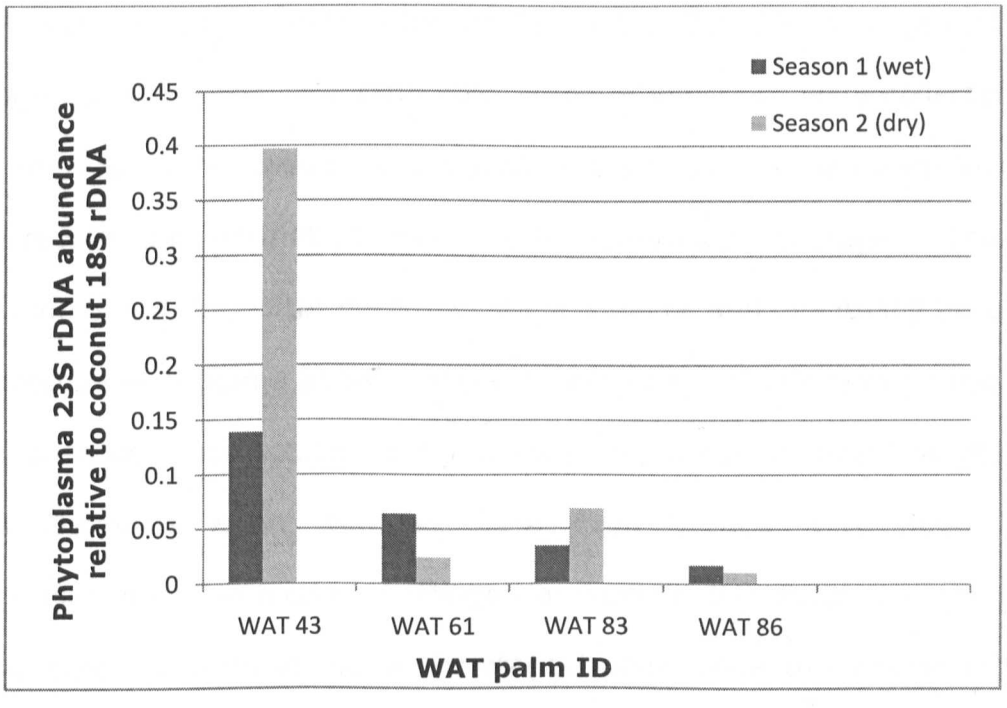


Figure 3.7 Seasonal variation of phytoplasma titres in infected palms. Samples analysed were obtained from palms found to be infected at the first and second sampling periods. No pattern in pathogen amounts between the rainy and dry seasons was identified.

To assess the variation of pathogen levels at different sections of the coconut trunk, multiple samples collected from three different heights along the stem of infected palms (i.e. the base of the trunk, 1 & 1.5 metres above ground level) were assayed for pathogen titres. The analyses showed that the titres could be greater or lower at any of the levels, and differences of up to five fold between these sections in an individual palm were found (Figure 3.8). The phytoplasma titres in the petioles, leaflets and roots could be higher or lower than those found in the trunk of the same palm (Figures 3.9).

To determine the phytoplasma titres in palms at different stages of LD-infection, samples from palms at stages 1 to 3 and from stages 2 to 5 (stage five being the 'telegraph pole' stage of infection) were collected in the rainy and dry seasons respectively and assayed for pathogen levels. The results showed that pathogen titres increased from stage 1 through to stage 3 and thereafter decline in stage 4 palms and eventually become undetectable in palms at the telegraph pole stage of infection (stage 5). The absence of detectable levels in stage 5 palms confirmed results of PCR diagnosis where samples from dead tissues were negatively diagnosed for LD-infection. Pathogen amount in the stage 2 palm was about twice as much as that in the stage 1 palm, while the amount in the stage three palm was as much as three-fold greater than the amounts in the stage 2 palm (3.10).

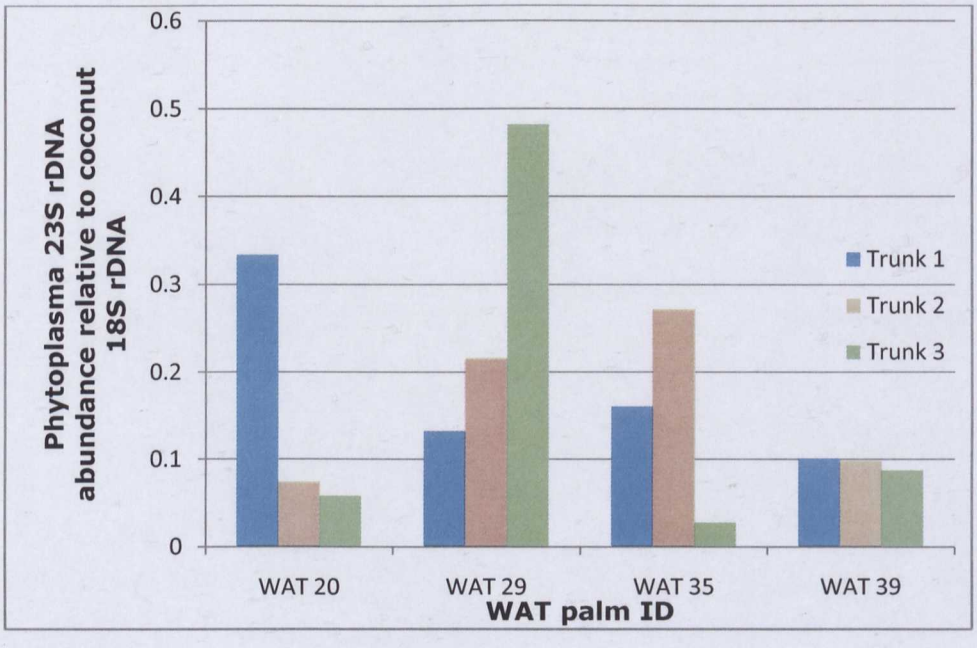
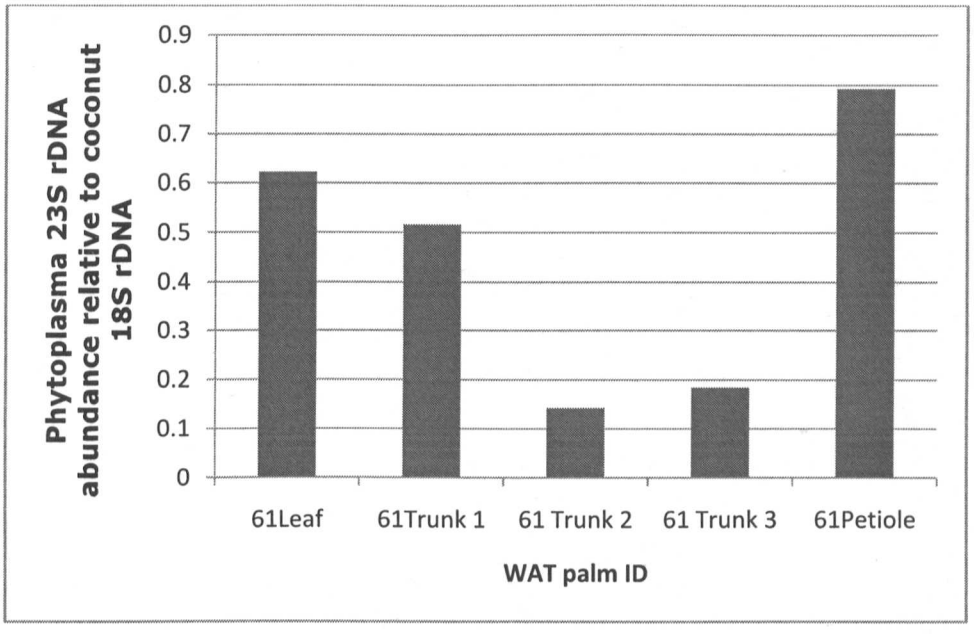


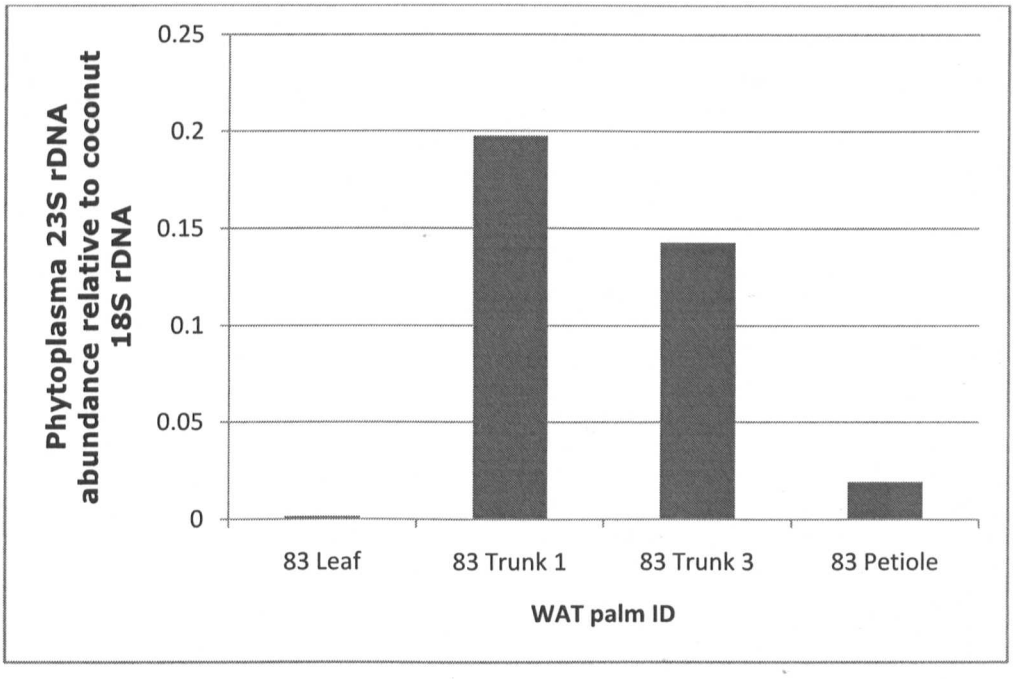
Figure 3.8 Distribution of phytoplasmas in the trunk of infected palms

Legend: Trunk 1: samples taken from base of stem; Trunk 2: samples from 1 metre above ground level and Trunk 3: samples from 1.5 metres above ground level (these designations applies throughout the remaining figures). At each level, DNA was extracted from bulked samples taken from three different points around the stem to account for any possible cross sectional variation in phytoplasma levels.

A.


Phytoplasma 23S rDNA abundance relative to coconut 18S rDNA
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
61Leaf
61Trunk 1
61 Trunk 2
61 Trunk 3
61Petiole
WAT palm ID

B.


Phytoplasma 23S rDNA abundance relative to coconut 18S rDNA
0.25
0.2
0.15
0.1
0.05
0
83 Leaf
83 Trunk 1
83 Trunk 3
83 Petiole
WAT palm ID

C.

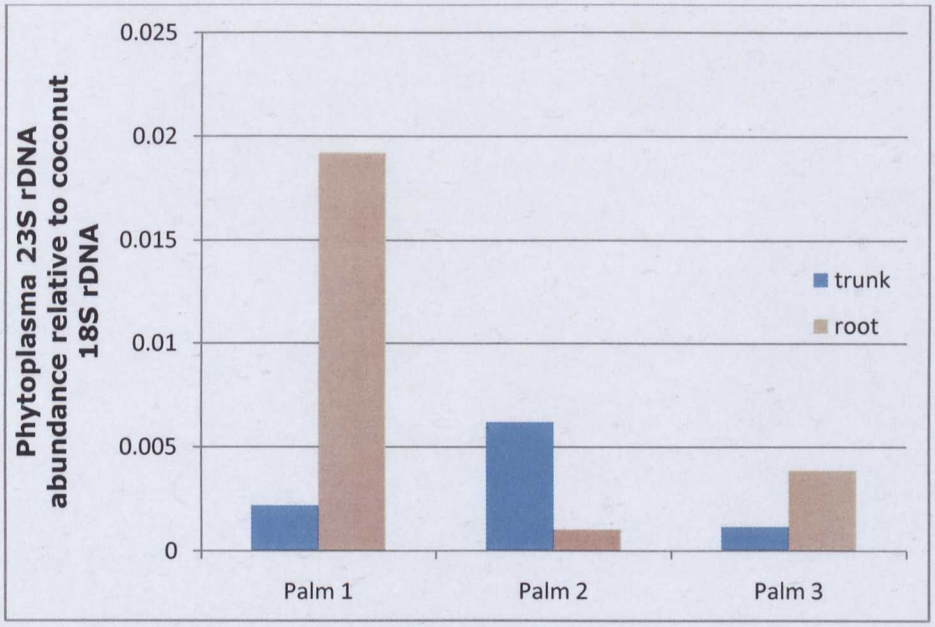
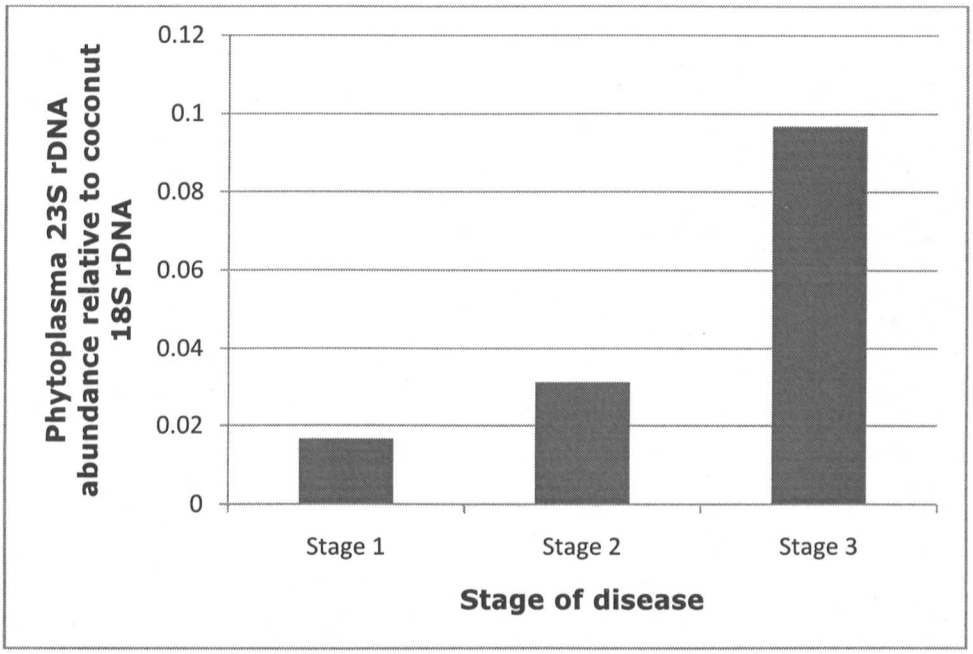


Figure 3.9 Variation of phytoplasma levels in different tissues of the palm. Leaf and petiole samples in (A) were sampled from young leaves (inner canopy) and those in (B) were from matured leaves (mid-canopy). The pathogen amounts in the trunks (C) were averaged from titres occurring in the three levels previously described.

A.



B.

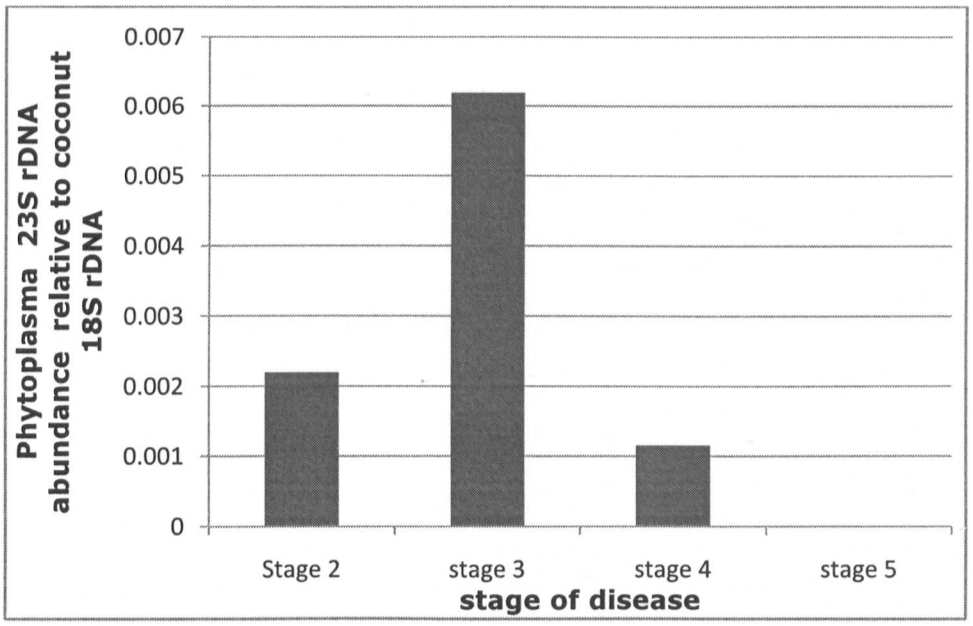


Figure 3.10 Pathogen titres in palms at different stages of infection. Samples used in the analysis for plot (A) were sampled in the dry season and those for plot (B) sampled in the rainy season. The pathogen titres represent the amounts in the trunk of the palms and were calculated from averages of the amounts at three levels of the palms.

3.4 DISCUSSION

Early and accurate diagnosis of LD is an important trigger for the initiation of containment measures which includes felling of infected palms in order to reduce disease spread (Nkansah-Poku *et al.*, 2005). Accurate diagnosis requires the use of good quality DNA template and reliable assays and procedures that will reflect the health status of the palms. Many of the assays available for the diagnosis of LD do not incorporate an internal control to check for the presence of PCR inhibitors which can cause false negative results. The use of a multiplex PCR test with an in-built internal control proved to be a simple technique that helps to identify false negative results arising from the presence of PCR inhibitors or an inefficient DNA extraction protocol.

The performance of the suite of PCR assays assessed in this work offers diagnostic options to the coconut research community. The comparable performance of the *secA* primers to the nested PCR assay is particularly encouraging. Although nested PCR is known to be highly sensitive, the numerous processes and handling steps involved makes them time consuming and highly prone to contamination. It is in this light that the possibility of replacing such assays with equally efficient single round PCR assays as demonstrated in this work is welcoming.

The LAMP diagnostic assays and detection techniques previously developed for detecting the LD phytoplasma have demonstrated the potential of the method to be an in-field diagnostic technique (Tomlinson

et al., 2010a). The essential requirements for a good in-field diagnostic method, including an easy and quick method of obtaining nucleic acids for amplification, have been addressed by the development of the LFD DNA extraction kit (Danks and Boonham, 2007; Tomlinson *et al.*, 2010a). The evaluation of a real-time LAMP detection system as discussed in this work has shown a further simplification of the technique in terms of the time spent on diagnosis as well as a reduction in the handling steps. Detection of LAMP amplicons by a colour change or on an LFD typically involves running the reaction for about 1 h and some downstream processes if using an LFD detection system (Njiru *et al.*, 2008; Tomlinson *et al.*, 2010b). Amplicons of RT-LAMP on the other hand could be detected within 15 min when DNA extracted with CTAB buffer was used and within 30 min when DNA extracted on LFD strips was used. The use of LFDs for DNA extraction makes the method suitable for in-field use. Real-time LAMP platforms that can be run on a rechargeable battery have now been developed (Hodgetts *et al.*, 2011) and the combination of speed and portability is ideal for an in-field diagnostic method. The comparable performance of the RT-LAMP assay to nested PCR means that sensitivity would not be compromised for speed and ease of using this method in-field. Added to this, the redundancy of downstream processes in RT-LAMP and the closed tube system in which the whole reaction takes place leaves little room for cross-over contamination.

In terms of disease resistance rating, the WAT ecotype is considered highly susceptible, the MYDxVTT hybrid is described as 'less susceptible'

while the SGDxVTT hybrid, based on a genetic model is predicted to be appreciably less susceptible than the MYDxVTT hybrid (Dery *et al.*, 2008; Quaicoe *et al.*, 2009). In this study, the WAT largely followed its predicted resistance rating but none of the hybrids died from LD-infection. It is worth mentioning that the disease has been dormant for almost 10 years at Agona and none of the palms in this plot (which has been under screening since 1995 has ever been infected). Although there is an active disease focus at Daboase, none of the hybrids became infected; the palms selected were, however, quite young (approximately one year old) and this may have affected their susceptibility since LD-infection rate among young palms is known to be minimal (Eden-Green, 1997). These factors may have contributed to the 0% infection rate observed among the SGDxVTTs in the two locations or the results may simply be due to a lack of infection in these fields. Although none of the MYDxVTT hybrids became infected during the course of this study, they are widely known to suffer losses from LD-infection (Dery *et al.*, 2008). The results of this study seem to suggest that there is no subpopulation of the WAT which possess some resistance or tolerance to the disease since all infected palms eventually succumbed to the disease. These results buttress the observations made from the molecular marker work described in Chapter 2 in which no useful SNPs could be identified between WATs that were diseased or had died from infection and those that were healthy. The SGDxVTT and MYDxVTT hybrids, however, still need to be monitored for LD phytoplasma infection (including other field sites with disease foci) and any resistance/tolerant populations identified

could be investigated using the techniques and methods developed in this study.

A novel sensitive and specific Q-PCR assay for quantifying the LD phytoplasma was developed and used to assess phytoplasma levels in infected palms and their seasonal fluctuations. Most Q-PCR assays developed for phytoplasma detection employ a primer-probe combination to enhance the specificity of the assays. In this work, SYBR Green I dye detection was used instead of a probe and a melting curve analyses performed at the end of the PCR used to validate the results and thereby identify any non-specific amplicons. As highlighted by Wong and Medrano (2005), DNA binding dyes such as SYBR Green are the least costly of all the Q-PCR detection chemistries and the success in using a SYBR Green detection system to accurately quantify phytoplasmas as has been demonstrated in this work is a welcome cost saving measure. The specificity of the assay was further enhanced by using primers with a high annealing temperature compared to those used in conventional PCR. The 18S rDNA which was used to normalise the quantification data also acted as an in-built internal control which was used to check for PCR inhibition. The sensitivity of the assay was confirmed by its capacity to detect phytoplasmas in symptomless palms. Although phytoplasma levels have long been known to be unevenly distributed in LY/LD-infected palms (Thomas, 1979; Thomas and Norris, 1980), the exact fold differences between different tissues or between palms at different stages of infection have not been determined. The distribution of phytoplasmas in

LY-infected palms was investigated by Oropeza *et al.* (2011); however, pathogen levels in that study were inferred from percentages of positive detections in various plant tissues using conventional PCR rather than quantitative techniques. This work, the first quantitative determination of the relative phytoplasma titres in tissues of LD-infected palms or their levels in palms at different stages of infection sheds some light on the development of LD. Phytoplasmas were detected all year round in the aerial parts of the palm (trunk) and this reveals that unlike some deciduous trees such as apples and pear in which phytoplasmas overwinter in the roots and are undetectable in the aerial parts during cold periods (Schaper and Seemuller, 1982), this phenomenon is absent in coconut. It stands to reason that the significant build up of phytoplasma titres in palms at stages 2 and 3 of infection speeds up the disease development process and this may explain the relatively shorter duration of stages 2 to 5 compared to stage 1 as observed by Dery *et al.* (1997). The detection of high levels of phytoplasmas in other tissues of the palm provides options on tissues of the palm to sample from for disease diagnosis. While it may not be convenient to sample from tissues of the canopy of the palm, the roots which showed high phytoplasma levels of could be sampled in the absence of a motorised drill in remote field locations. The presence of high levels of phytoplasmas in young leaves and the barely detectable levels in matured leaves is consistent with the observation that higher levels of phytoplasmas are present in actively growing tissues than in mature ones (Thomas and Norris, 1980; Oropeza *et al.*, 2011).

CHAPTER 4: SEARCHING FOR ALTERNATE HOSTS OF THE LD PHYTOPLASMA

4.1 INTRODUCTION

The LY phytoplasma is known to have other hosts apart from coconut in the Americas, where it is reported to infect about 38 palm species (Harrison *et al.*, 1999). Although some of the palms identified as alternate hosts of the LY phytoplasma in the Americas such as *Veitchia* and *Phoenix* species have been monitored in Ghana for several decades, none has been found to develop LD (Quaicoe *et al.*, 2009). Weeds are considered as important agents for the incidence and spread of disease pathogens; they may serve as obligate or passive alternate hosts of plant pathogens and their vectors or even act as vectors themselves in the case of parasitic weeds (Wisler and Noris, 2005). Weeds have been reported to host several phytoplasmas including the LY phytoplasma (Marcone *et al.*, 1997; Brown *et al.*, 2008; Meneguzzi *et al.*, 2008). In Ghana, no alternate hosts of the LD phytoplasma are known and recent efforts to find such plants did not produce any positive results (Yankey *et al.*, 2009). To further investigate the existence of alternate hosts of the LD phytoplasma, more plant species were investigated. The identification of alternate hosts would increase our understanding of the biology of the pathogen and the epidemiology of the disease. The identification of alternate hosts may also lead to the identification of the vector of LD by investigating insects that visit such plants. Such plants can also serve as lab plants for storing the LD phytoplasma *in vivo* if found to harbour significant titres of the pathogen. This will make the LD phytoplasma

source more accessible to researchers and would not have to always obtain it from the coconut palm. The objective of this aspect of the study was therefore to screen an array of plant species for their ability to host the LD phytoplasma.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials, locations and sampling method

Selected plant species growing within and around coconut farms heavily infected with LD were the test plants for analyses. The criteria used for the selection of the plant species sampled included sampling plants which are reported to host any type of phytoplasma and other members of the families to which they belong. Plants which showed any of the general symptoms of phytoplasma infection such as stunting, yellowing and withering were also sampled. Plants exhibiting other potential symptoms of phytoplasma infection such as 'little leaves', leaf curl, chlorosis and aberrations of flowers and fruits as well as some symptomless plants were also considered.

The test plants were sampled from four locations; Cape Three Points, Fasin and Aluku in the Western Region (WR) and Asebu in the Central Region (CR) of Ghana. At Cape Three Points, sampling was carried out from a disease screening field of the CSIR-OPRI, as well as from some farmers' fields surrounding this plot. The Fasin and Aluku collections were carried out in abandoned LD-infected coconut farms. The Asebu sampling was carried out in a 'vector transmission studies' field belonging to the CSIR-OPRI. All four sites were disease foci. Sampling was carried out both in the rainy and dry seasons from November 2008 to May 2009. The Asebu and Cape Three Points collections were carried out in the dry

season while the Fasin and Aluku collections were undertaken in the rainy season.

The tender parts of the plants which were usually from the stem upwards were cut with a sterilized kitchen knife. For creeping plants, the vines and the leaves were the parts that were cut. For every species, the plants were collected from all over the sampling site. Samples of a species were put in a labelled polythene bag before being transported to the lab. For identification of the plants, a botanist from the Cape Coast University was sent to the field to identify the plants before sampling was done.

4.2.2 Sample preparation

Firstly, each plant sample was swabbed with a paper towel soaked in 70% ethanol, to remove dust particles, insects and microorganism that might be on the plant surfaces. Much of the lamina was then removed, leaving only a small strip around the midribs and veinlets. Two individual plants of each species which were pooled together to form a sample were cut into small pieces (about 0.2 x 1 cm) into plastic bags and stored at -18°C until ready for use.

4.2.3 DNA extraction

As a result of the toughness of some of the plant tissues, the Daire *et al.* (1997) CTAB DNA extraction protocol described in Section 2.2.3.1 was slightly modified to extract DNA from the plant species. The method involved grinding 1 g of plant tissue in 5 mL of CTAB buffer using a sterilized lab mortar and pestle. The remainder of the processes were as previously described.

4.3 RESULTS

4.3.1 Assessing the diversity of the sampled flora

Based on the criteria set for sampling, species were mainly herbaceous weeds and a few shrubs. In all, 41 plant species belonging to 25 botanical families were sampled (Table 4.1). These included plants belonging to families such as the Asteraceae, Euphorbiaceae, Curbitaceae Cyperaceae and Solanaceae which are known to have members that host phytoplasmas (Table 4.2). Chlorosis of leaves, stunting and leaf spots were the main defining symptoms of infection (Plate 4.1).

Table 4.1 List of plant species, their families and number of samples analysed

Plant species/Sampling location	**Plant family**	**Number of samples analysed**
Fasin		
1. *Acroceras zizanioides*	Poaceae	20
2. *Securinega virosa*	Euphorbiaceae	20
3. *Selaginella mysorus*	Sellaginellaceae	20
4. *Stachytarpheta cayennensis*	Verbanaceae	20
5. *Scleria naumanniana*	Cyperaceae	20
6. *Asystasia gangética*	Acanthaceae	20
7. *Costus afer*	Zingiberaceae	20
8. *Anthocleista nobilis*	Loganiaceae	20
9. *Unknown*		20
10. *Mimosa púdica*	Fabaceae	20
11. *Paspalum conjugatum*	Poaceae	20
12. *Dissotis rotundifolia*	Melastomataceae	20
ASEBU		
1. *Vernonia cinérea*	Asteraceae	20
2. *Schwenkia americana*	Solanaceae	20
3. *Dioscorea* sp.	Dioscoreaceae	20
4. *Caricaca papaya*	Caricaceae	12
5. *Portulaca olerecea*	Portulacaceae	20
6. *Citrullus lanatus*	Cucurbitaceae	3
7. *Tridax procumbens*	Asteraceae	20
8. *Priva lappulacea*	Verbanaceae	20
9. *Ipomoea involúcrate*	Convolvulaceae	20
10. *Manihot esculenta*	Euphorbiaceae	20
11. *Cyperus difformis*	Cyperaceae	20
12. *Justicia flava*	Acanthaceae	20

Table 4.1 continued.

Plant species/Sampling location	**Plant family**	**Number of samples analysed**
Cape Three Points		
1. *Croton lobatus*	Euphorbiaceae	20
2. *Stachytarpheta indica*	Verbanaceae	20
3. *Waltheria indica*	Sterculiaceae	20
4. *Spigelia anthelmia*	Loganiaceae	20
5. *Cassytha filiformis*	Lauraceae	20
6. *Lycopersicon esculentum*	Solanaceae	20
7. *Cissampelos mucronata*	Menispermaceae	20
8. *Cyperus* sp.	Cyperaceae	20
9. *Polygonum* sp.	Polygonaceae	20
10. *Torenia thoursii*	Scrophulariaceae	20
11. *Saccharum officinale*	Poaceae	20
12. *Vernonia cineria*	Asteraceae	20
Aluku		
1. *Psidium guajava*	Myrtaceae	20
2. *Hoslundia opposite*	Lamiaceae	20
3. *Euphorbia hirta*	Euphorbiaceae	20
4. *Vernonia* sp.	Asteraceae	18
5. *Pteridium aquilinum*	Denstaedtiaceae	16

Table 4.2 Plant families known to host phytoplasmas and whose members were sampled

Plant family	Reference
Poaceae	Jung *et al.*, 2003; Lee *et al.*, 2000
Euphorbiaceae	Lee *et al.*, 1997
Asteraceae	Samuitiene *et al.*, 2007
Cucurbitaceae	Montano *et al.*, 2007
Solanaceae	Lee *et al.*,1998; Gungoosingh-Bunwaree *et al.*, 2007
Scrophulariaceae	Samuitiene *et al.*, 2007

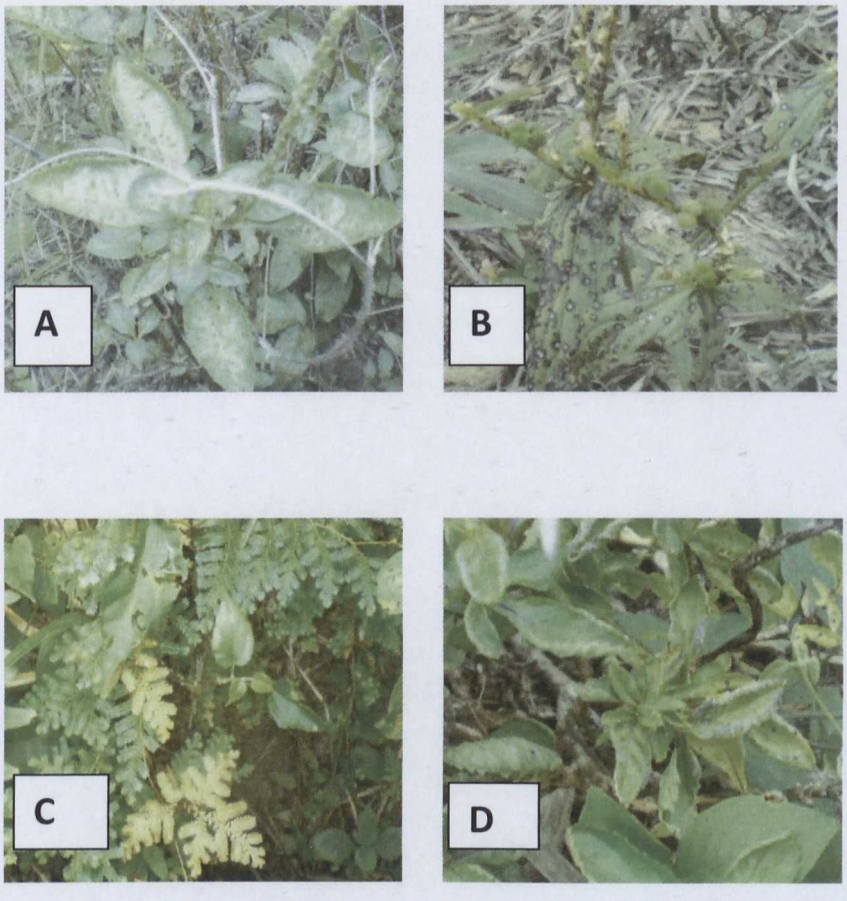


Plate 4.1 Plants showing symptoms of phytoplasma infection and 'tell tale' symptoms of disease. Leaf spots (A), Stunting and leaf spots (B) Chlorosis (C) and curling of leaves (D).

4.3.2 Assessing the effectiveness of the extraction process

To assess the effectiveness of extracting DNA from the diverse plant materials using the CTAB extraction protocol, the genomic DNA from a random selection of samples was run on an agarose gel to ascertain the quality of the DNA. The analyses showed clear discrete bands of about 2 kb in nearly all samples indicating the presence of clean and good quality DNA (Figure 4.1).

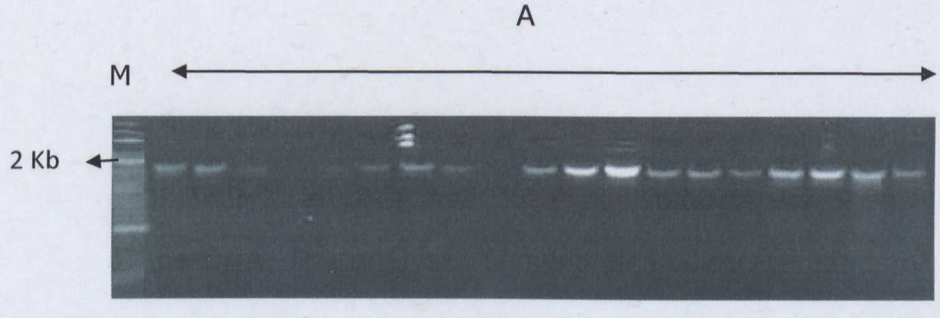


Figure 4.1 Genomic DNA extracted by the modified Daire *et al.* (1997) CTAB protocol. (A): random selection of samples from each plant species of the Cape Three Points collection; (M): DNA size marker (Hyperladder II).

4.3.3 Assessment of plant samples for LD phytoplasma infection

To detect the LD phytoplasma in the sampled plant species, the DNA samples were screened with conventional PCR using the LD phytoplasma-specific *secA* oligonucleotide primers (CSPWDSecAFor2/Rev2). The results of these analyses showed that none of the plant species was infected with the LD phytoplasma. Following the failure to detect the LD phytoplasma with the *secA* primers, a nested PCR assay (P1/P7 followed by GH813f/AwkaSR) was applied to the samples collected from Cape Three Points to determine if the lack of amplification was due to presence of very low titres of the pathogen. The results were, however, not different from that of the *secA* assay; none of the plant species was found to be infected.

4.4 DISCUSSION

A clear understanding of the epidemiology of LD is important for the development of integrated control measures against the disease. Management of phytoplasma diseases involves the use of resistant varieties if available, control of the vectors involved if identified and the elimination of alternate hosts if present (Charles *et al.*, 2002). This work sought to broaden the scope of the search for alternate hosts of the LD phytoplasma by screening four areas of active LD-infection, two of which were not considered in previous investigations by Yankey *et al.* (2009). Sampling of plant species was done in the rainy and dry seasons to take into account any influences of environmental conditions on the population dynamics of the pathogen, vectors and the hosts plants involved. Due to this possible seasonality, some potential hosts of the pathogen can be missed if sampling is not done in the two seasons. Although, phytoplasmas are known to infect plant such as tomato, cassava and sugarcane (Alvarez *et al.*, 2007; Gungoosingh-Bunwaree, 2007; Rao *et al.*, 2007), the phytoplasmas involved are different from the LD phytoplasma. It was, however, decided that given the limited time of the study, the most likely candidates should be prioritized and so these plants and other member of the plant families to which they belong were sampled.

The LD specific *secA* primers which had demonstrated similar performance as the nested PCR assay (as discussed in Chapter 3) were used in the analyses with the aim of reducing the possibility for contaminations to avoid any false positive results and subsequently any false conclusions. Although the variety of plant species that can be found

in LD-infected fields is large, the difficulty of identifying alternate hosts in the past (Yankey *et al.*, 2009) and present efforts seem to suggest that there are no alternate hosts of the LD phytoplasma. Clearly, though, further sampling and studies would be required to verify that this is the case. One of the limitations to the numbers of plant species that were analysed in this work was the amount of time spent on sampling and on diagnosis; the availability of in-field diagnostic assays such as the LAMP method will greatly facilitate the screening of larger sample numbers in future studies.

CHAPTER 5: INVESTIGATING THE POSSIBILITY OF PHYTOPLASMA TRANSMISSION FROM SEEDS OF LD-INFECTED PALMS TO PROGENY PLANTS

5.1 INTRODUCTION

Plant pathogens including viruses, fungi and bacteria are known to infect seeds and cause diseases in the resulting seedlings; they may enter the seed during the formative stage, during storage or through injury to the seed (Baker and Smith, 1966; Stace-Smith and Hamilton, 1988; Johansen *et al.*, 1994; Iwai *et al.*, 2001; Basak and Lee, 2002). It is important to mention that the presence of a pathogen on or in the seed does not necessarily constitute a diseased condition or that the seed will develop into an infected seedling (Rodriguez and Menezes, 2005).

Seed transmission of phytoplasmas has not been demonstrated (Cordova *et al.*, 2003), although reports of the presence of phytoplasma DNA in embryos have been made in plants such as tomatoes, winter oilseed rape and lime (Botti and Bertaccini, 2006) and in coconut (Harrison and Oropeza, 1997; Harrison *et al.*, 1999; Cordova *et al.*, 2003; Nipah *et al.*, 2007; Oropeza *et al.*, 2011). It is therefore necessary to conduct further research on the possible transmission of phytoplasma infection into progeny plants through the embryo. In coconut, Cordova *et al.* (2003) found 18% of seeds analysed to contain phytoplasma DNA (13 out of 72) while Nipah *et al.* (2007) found 17% (nine out of 52) seeds to contain phytoplasma DNA, showing that the presence of phytoplasma

DNA in coconut embryos was not a rare occurrence. Nipah *et al.* (2007) also observed that seeds derived from infected palms had a higher germination percentage than those from healthy palms. Seedlings, as well as embryo cultures derived from seeds of infected palms, however, did not show phytoplasma infection in the study by Nipah *et al.* (2007).

Cordova *et al.* (2003), using the DNA stain DAPI, observed that in coconut embryos, phytoplasmas were mainly found in the plumules and some adjacent cells. Work on somatic embryognesis in coconut has also shown that plumules are the explants that readily form callus and produce shoots (Chan *et al.*, 1998; Saenz *et al.*, 2006). This means it will be possible to culture the plumule *in vitro* while the remainder of the embryo can be tested for the presence of phytoplasma DNA. However, the size of plumules that will successfully grow into plantlets *in vitro* would have to be determined experimentally.

Embryo culture involves the *in vitro* cultivation of embryos into whole plantlets in artificial media containing all the essential nutrients required for the embryo's growth and development. Embryo culture in coconut is quite established and protocols for collecting embryos *in vitro* have been developed (e.g. Assy Bah *et al.*, 1987). The Y3 media developed for *in vitro* cultivation of coconut has been variously modified by different research organisations for growing coconut embryos (Eeuwens, 1976; Batugal and Engelmann, 1998). Nipah (2007) evaluated three Y3 media for their effectiveness at supporting the rapid growth of embryo cultures

to enable the resulting plantlets to be tested for phytoplasma infection in the shortest possible time. These comprised media used by three organisations namely, Philippine Coconut Authority (PCA), Central Plantation Crop Research Institute, India (CPCRI) and the French Institute of Scientific Research for Development in Cooperation (ORSTOM), now called the Institute de Recherche pour le Développment (IRD). The study concluded that PCA medium amended with 1 mg L^{-1} of 6-benzylaminopurine (BAP) and excluding activated charcoal, was the best medium for rapidly generating plantlets from embryo cultures. Accordingly, in this study embryo cultures were grown on PCA Y3 medium.

If demonstrated, seed transmission of LD will have serious implications for the spread of the disease in plantations since infected seedlings planted at different locations in a field will serve as disease foci for the rapid spread of the disease. There would also be phytosanitary issues; it will be easy to spread the disease to non-diseased parts of the country as well as limit the prospect of sharing research material with other countries (e.g Mozambique which has the same strain of the Ghanaian LD phytoplasma). The objectives of this section of the study were therefore to:

- Determine if tissue cultures derived from seeds of infected palms contain phytoplasmas.
- Determine if seeds of infected palms develop into seedlings infected by LD.

5.2 MATERIALS AND METHODS

5.2.1 Sampling of coconut seeds

Two categories of seeds were collected for this section of the study: seeds of infected palms investigated for their ability to grow into diseased seedlings and seeds used for embryo culture studies. For the first category, two batches of seed collection were undertaken from several farmers' fields in the Western Region of Ghana. In the first collection, eight palms, between stages 1 and 2 of LD-infection located in Asanta, Kikam and Ampain were marked and a total of 118 seeds collected. In the second sampling, a total of 108 seeds were harvested from five infected palms at Fasin. Seeds harvested were from palms between stages 1 and 3 of LD-infection. A total of 51 seeds used as healthy controls were harvested from three palms located at Takinta, a disease-free area, in the Western Region of Ghana.

Coconut seeds used for embryo culture studies were sampled from Kikam and Aluku. A total of 60 seeds from nine infected palms and 332 seeds from 18 infected palms were collected from Kikam and Aluku respectively. Twenty-nine seeds used as healthy controls were collected from palms at Takinta. All seeds were appropriately labelled and transported in jute sags for storage in a cool, dry place until they were ready for use. The collections were carried out between August and November 2008.

5.2.2 Establishment of seed nursery

Seeds set apart to be nursed were planted on two seedbeds at the office premises of the CSIR-OPRI at Sekondi (Table 5.1). The part of the seed containing the embryo was pared slightly using a machete prior to planting to allow the shoots to emerge easily. The nuts were planted close to each other with the pared section facing upward. The nursery was maintained following the recommendations of the STANTECH manual (Santos *et al.*, 1994). To prevent potential insect vectors from coming into contact with the seeds and to ensure disease-free conditions, sieved cages were erected over the seedbeds and insecticides applied to the nursery beds fortnightly. Seeds were monitored for germination weekly over a seven month period. The upper part of the apical leaves was sectioned as close as possible to the growing point without killing the seedlings for DNA extraction.

Table 5.1 Seeds harvested for planting and for assessing the presence of phytoplasma DNA in coconut embryos

First collection	**Numbers**
Number of seeds from diseased palms:	118
Number nursed:	84
Number of embryos tested for phytoplasma:	34
Second collection	
Number of seeds from diseased palms:	108
Number nursed:	94
Number of embryos tested for phytoplasma:	14
Healthy controls	
Number of seeds collected:	51
Number nursed:	45
Number of embryos tested for phytoplasma:	6

5.2.3 Extraction of solid endosperm cylinders containing the embryos

Protocols developed by Assy-Bah *et al.* (1987) for collecting coconut embryos *in vitro* were modified to suit local conditions. To ensure sterile conditions, all seeds were carted to the lab premises in Takoradi where the nuts were de-husked and split open into two with a machete. Subsequent procedures were carried out in the preparation room of the

lab. Solid endosperm cylinders were cut out from the half containing the embryos using a cork borer (Ø 20 mm) with the aid of a wooden hammer (Plate 5.1). The solid endosperms were pushed out with the aid of forceps and batches of 20 cylinders washed with sterile distilled water before transferring them into another bowl containing 500 mL of 100% bleach (containing less than 5% sodium hypochlorite; less than 5% anionic detergent; less than 5% limescale detergent) for 20 min. The endosperm cylinders were then washed in three changes of distilled water for 5 min each. Embryos which were assessed for phytoplasma infection ahead of germination studies were excised under sterile conditions in the Takoradi lab. To obtain the embryos, sterile scalpel blades were used to whittle away the endosperm till the whole embryo could be taken out from the endosperm. The embryos were washed in distilled water before storing them in a fridge in zip-lock bags. All DNA extractions from embryos were carried out within 48 h of their excision. All forceps and cork borers were surface sterilized by first washing them in distilled water after which they were dried with a paper towel. They were then dipped in 70% ethanol before flaming to red hot on a gas burner. They were then allowed to cool before use.

A

De-husked nut showing position of the embryo

Position of the embryo

B

Nut split into two along central region using a machete

C

Cork borer and a wooden hammer are used to cut out embryo cylinder

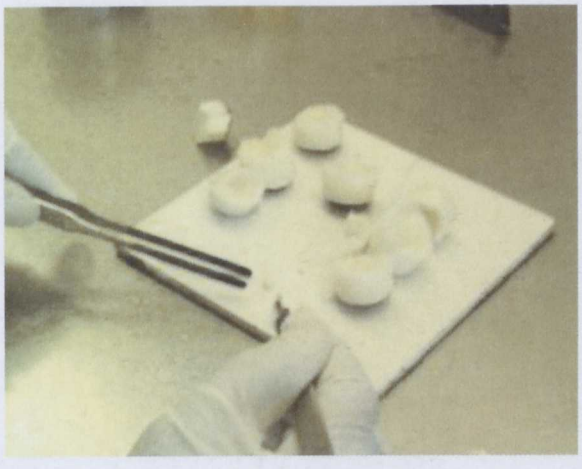
D

Embryo cylinder with intact embryo

Plate 5.1 Extraction of endosperm cylinders from coconut seeds

5.2.4 Surface sterilisation of embryo cylinders and excision of embryos used for plumule culturing

Plumule culturing was carried out in the labs of the Biotechnology and Nuclear Research Institute (BNARI) in Accra. All nuts were dehusked in Takoradi and transported to Accra for plumule culturing. The nuts were cut open and the embryo cylinders excised with sterilized knives and cut into shape (about 3 cm Ø). The cylinders were rinsed under tap water for 3 min before taking them under a flow hood for sterilisation. The cylinders were first surface sterilized in 70% ethanol for 5 min and rinsed in sterile distilled water. A second surface sterilisation was carried out with 2% mercuric chloride for 5 min and then rinsed in sterile distilled water. Embryos were then individually excised using a scalpel (Plate 5.2).



A scalpel is used to whittle away the endosperm to obtain the embryo

Whole embryos successfully excised from embryo cylinders

Plate 5.2 Excision of embryos from endosperms for plumule culture

5.2.5 Preparation of PCA Y3 culture medium

Excised plumules were cultured on Y3 medium developed by the Philippine Coconut Authority (PCA) (Batugal and Engelmann, 1998) with slight modifications as described in Nipah *et al.* (2007). Firstly, 500 mL of three stock solutions: Stock 1 (20 x solution of macro nutrients); Stock 2 (200 x solution of micro nutrients) and Stock 3 (200 x solution of iron source) were prepared using the amounts detailed in Table 5.2. A 50 mL solution of each of the chemicals was prepared before mixing them one after the other and the volumes made up to 500 mL. Stocks 1 and 3 were stored in dark bottles. The required volumes of the stock solutions needed to obtain the final concentrations detailed in Table 5.2 were determined and used in preparing a 1 L medium: 50 mL of stock 1 and 5 mL each of stocks 2 and 3 were used to obtain a final 1 x concentration.

The vitamin myo-inositol was added directly to the medium, while 1 mg mL^{-1} stocks were prepared for all the other vitamins. Except for biotin (which had to be dissolved in a small amount of KOH before adding distilled water), all other vitamins easily dissolved in water. Stocks of the growth regulators naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) were also prepared at 1 mg mL^{-1}. These are insoluble in water so for NAA, 100 mg of the chemical was dissolved in 70 mL of absolute alcohol and topped up with 30 mL of hot water to prevent evaporation in storage. BAP (100 mg) was also mixed with 1 mL HCl and heated to dissolve before topping up with distilled water to 100 mL.

Table 5.2 Composition of PCA Y3 medium

CHEMICAL	Amt in stock (g/500 mL)	Final conc (mg/L)
Stock 1- Macro nutrients (20 x stock solution)		
Ammonium chloride (NH_4Cl)	5.35	535
Potassium nitrate (KNO_3)	20.2	2020
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	2.47	247
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	2.94	294
Potassium chloride (KCl)	14.92	1492
Sodium di-hydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)	3.13	312
Stock 2- Micro nutrients (200 x solution)		
Potassium iodide (KI)	0.83	8.3
Boric acid (H_3BO_3)	0.31	3.1
Manganese sulphate tetrahydrate ($MnSO_4.4H_2O$)	1.12	11.2
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	0.72	7.2
Copper sulphate pentahydrate ($CuSO_4.5H_2O$)	0.025	0.25
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	0.024	0.24
Sodium molydate monohydrate ($NaMoO_4.H_2O$)	0.024	0.24
Nickel chloride hexahydrate ($NiCl.6H_2O$)	0.0024	0.024
Stock 3 – Iron source (200 x stock solution)		
Iron sulphate heptahydrate ($Fe_2SO_4.7H_2O$)	1.39	13.9
Disodium EDTA (Na_2EDTA)	3.73	37.3
Vitamins, Growth regulators & others		
Myo-inositol		100
Pyridoxine HCl		0.05
Thiamine HCl		0.05
Nicotinic Acid		0.05
Ca-D-pantothenate		0.05
Biotin		0.05
BAP		1.0
NAA		0.5
Sucrose		45.0 g L^{-1}
Ph		5.8

Source: Batugal and Engelmann, 1998 with slight modifications by Nipah *et al.* (2007).

To obtain the working concentrations of the vitamins and growth regulators detailed in Table 5.2, for vitamins needed at a final concentration of 0.05 mg L^{-1}, 50 µL of the stocks were used; 500 µL and 1 mL of NAA and BAP stocks were used to obtain the final concentrations of 0.5 and 1 mg L^{-1} respectively. Sucrose (45 g) was added to the medium. The medium was made up to 1L and the pH adjusted to 5.8 using 0.1-5 M NaOH or 0.1-5 M HCl. The medium (80 mL) was dispensed into 350 mL honey jars and autoclaved at 121°C and at 15 psi for 15 min.

5.3 RESULTS

5.3.1 Assessment of seedlings derived from diseased palms for the presence of phytoplasma DNA

This study was conducted to determine whether seeds derived from infected palms grow into phytoplasma infected seedlings. To achieve this objective, a proportion of the seeds sampled from infected and uninfected palms as detailed in Table 5.1, was nursed on two seedbeds in a disease-free environment and assessed six monthly for LD phytoplasma infection. Whole embryos excised from the remainder of the seeds were tested for phytoplasma infection ahead of germination studies.

5.3.1.1 Confirmation of LD-infection in sampled palms and assessment of embryos for the presence of phytoplasma DNA ahead of germination tests

To confirm LD phytoplasma infection in palms from which seeds were collected, DNA samples extracted from stem borings of these palms were assessed for phytoplasma infection. The healthy controls were also assessed in the same way to ensure that they were free from infection. DNA extracted from embryos of seeds that were set aside as detailed in Table 5.1, were assessed for phytoplasma infection. PCR analyses (P1/P7) confirmed phytoplasma infection in all eight palms of the first collection and analysis of 34 embryo samples from this collection for phytoplasma infection gave three positive results in nested PCR (P1/P7 followed by GH813f/AwkaSR) (Figure 5.1).

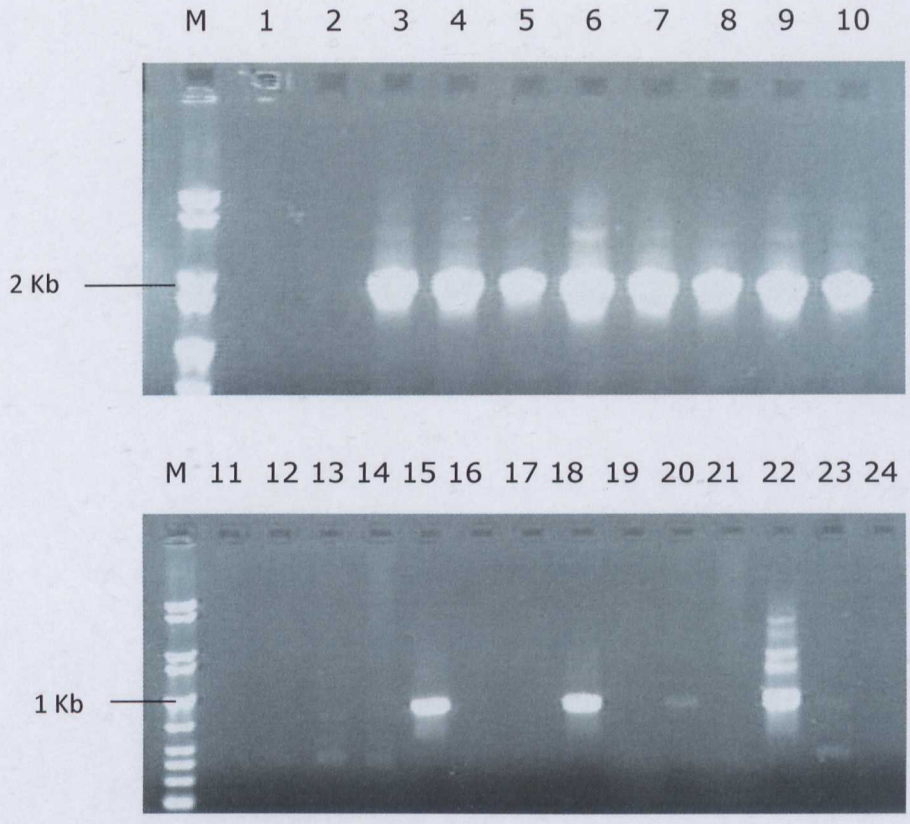


Figure 5.1 P1/P7 PCR amplification of phytoplasma DNA from trunk samples and nested PCR (P1/P7 followed by GH813f/AwkaSR) detection of phytoplasma DNA in coconut embryos (from first collection). Lanes: (1-2) trunk samples from uninfected palms; (3-10) trunk samples from eight infected palms; (11-21) DNA samples from coconut embryos; (22 & 23) positive controls; (24) water. Lanes 15, 18 and 20 (positive detections in embryos), all samples were from the same palm.

A trunk sample each from the second collection which had been grouped according to the stage of LD-infection showed phytoplasma infection and out of 14 embryos assessed for infection, four were found to be infected (Figure 5.2). Phytoplasma DNA was detected in seven out of 48 embryo samples (i.e 14.6%). No phytoplasmas were detected in trunk and embryo samples of uninfected palms.

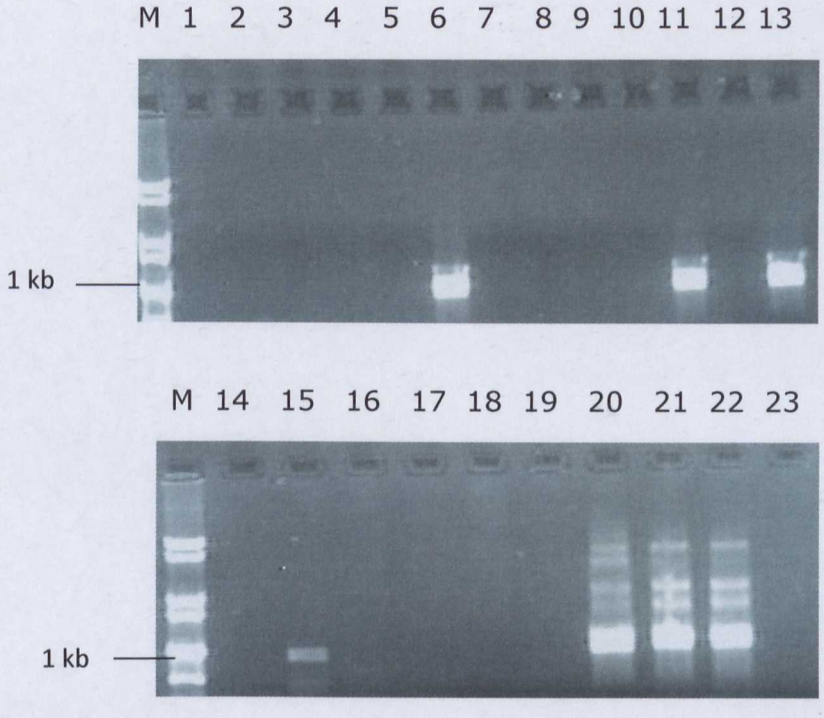


Figure 5.2 Nested PCR (P1/P7 followed by GH813f/AwkaSR) amplification of phytoplasma DNA from trunk and embryo DNA samples (second collection). Lanes: (1-19) embryo samples; (6) stage 1 sample; (11 & 13) stage 2 samples; (15) stage 3 sample; (20-22) trunk samples from palms at stages 1-3 of LD-infection; (23) water.

5.3.1.2 Determining the germination rates of seeds from infected versus healthy palms and assessment of seedlings for phytoplasma DNA

Out of 45 seeds from healthy palms, 37 germinated (i.e. 82.2%) and, out of 174 seeds from infected palms, 105 germinated (i.e. 60.3%). In both cages higher germination percentages were recorded in the healthy seeds than in the seeds from diseased palms; 84% as against 57.8% in cage 1 and 80% against 63.1% in cage 2 (Table 5.3). The differences were statistically significant at 5% level (F pr =0.023; CV = 4.7%; s.e =3.32).

Analysis of leaf samples from the seedlings using nested PCR (P1/P7 followed by GH813f/AwkaSR) at six, 12 and 18 months failed to detect phytoplasma DNA in any of the samples. None of the seedlings also developed symptoms of LD-infection over a two year period.

Table 5.3 Germination percentages of seeds of healthy and infected palms

Cage 1			
	Nursed	**Germ**	**%**
Diseased	90	52	57.8
Healthy	25	21	84
Cage 2			
	Nursed	**Germ**	**%**
Diseased	84	53	63.1
Healthy	20	16	80

F Test of % germination for healthy and disease seeds: *F pr = 0.023; CV = 4.7%; s.e =3.32*

5.3.2 Assessment of embryo sections for the presence of phytoplasma DNA ahead of embryo culturing

The experiments described in section 5.3.1 rely on the assumption that infected seedlings are from phytoplasma infected seeds, since it is not possible to test the seed for phytoplasma infection and plant the same seed. This section of the study was therefore aimed at culturing part of the embryo *in vitro* and testing the remaining portion for phytoplasma infection. A preliminary experiment was set up to determine the portions of the embryo that could be successfully grown *in vitro* and still have a remainder to test for phytoplasma infection. Ten plumules, 10 'full

embryos', 10 'half embryos', 10 'quarter embryos' and 10 tips of the embryo from the plumule side were excised and cultured on PCA Y3 medium to assess their growth rate. The cultures were kept in the dark and after six weeks, four of the full embryos, three each of the half and quarter embryo were observed to have swollen indicating signs of growth. One of the plumules also showed signs of slight swelling while none of the 'embryo tips' showed any sign of growth. Analyses of the remaining portions of the embryos using nested PCR showed phytoplasma infection in four of the samples; three of the samples were from the remainder of the embryos in which only the plumules had been sectioned and one from the remainder of the 'quarter embryo' group. In order to have a considerable portion of the remaining part of the embryo to test for phytoplasma infection and based on the fact that phytoplasma DNA could still be detected in the remains of the 'quarter embryo' section, this portion was chosen to be used for subsequent culturing. The developing cultures in this aspect of the study, however, suffered serious bacterial or fungal contamination and could not be assessed for phytoplasma infection.

In a larger experiment, 319 quarter embryos from infected palms and 27 embryos from healthy palms were sectioned and inoculated on PCA Y3 medium. All cultures were incubated in the dark for four weeks after which developing embryos were transferred to fresh medium and maintained in 16 h photoperiod and at 22°C. Out of the total 319 remaining portions of the embryo assessed for phytoplasma infection,

only three samples gave positive results in PCR using the LD-specific *secA* oligonucleotide primers described in Section 3.3.2. Although extra care had been taken to ensure sterile conditions in the culturing processes, heavy contamination of fungal and bacteria (about 80%) was observed among the plumule cultures and had to be discarded. Only five of the remaining cultures differentiated leaves (Plate 5.3). None of these cultures was found to be infected when assessed in nested PCR.

Plate 5.3 Plumule cultures showing differentiating leaves

5.4 DISCUSSION

Seed transmission of phytoplasmas has been considered unlikely because the phloem sieve elements in which phytoplasmas reside lack direct connection with seeds (Cordova *et al.*, 2003). Although phytoplasma DNA have been detected in the embryos of some plants including coconut, the mechanism by which they enter the seed is not known. Oropeza *et al.* (2011) analysed batches of embryos from fruits at different maturity levels and observed that the percentage phytoplasma DNA in the fruits ranged from 14–45 %. Nipah *et al.* (2007) also found 17% of coconut embryos analysed to contain phytoplasma DNA. In this work, the percentage of phytoplasma DNA in the embryos analysed was comparable to that observed in previous works; seven out of 48 whole embryos gave positive results in nested PCR (i.e. 14.6%; Section 5.3.1.2) and showed that phytoplasma presence in coconut embryos was not a rare occurence. The number of positive detections in the remainder of the embryos tested for the presence of phytoplasma DNA after excising the plumules for culturing was, however, low (three out of 319). This could be due to the occurence of low phytoplasma titres in the sections that were tested after the excision of the plumules. The plumules and the surrounding cells are reported to contain the highest concentration of phytoplasma DNA (Cordova *et al.* 2003). Nipah *et al.* (2007) observed that seeds derived from phytoplasma infected palms had significantly higher germination percentages than those derived from healthy palms. These results were, however, not confirmed in a similar work by Oropeza *et al.* (2011) in which the opposite scenario was observed. In the work of the former authors, the diseased and infected

palms were located in areas about a 100 km apart, unlike in the work of the latter authors, in which both infected and healthy palms were at the same site. Oropeza *et al.* (2011) therefore suggested that differences in local conditions could have impacted the germination rates observed by Nipah *et al.* (2007). This study, a follow-up on the work of Nipah *et al.* observed a higher germination rate in seeds from healthy palms (82.2%) compared to those from infected palms (60.9%) although sampling was done in the same locations where Nipah *et al.* did their sampling. It therefore seems to suggest that other factors other than the impact of phytoplasma infection of palms determine germination rates of seeds from infected and uninfected palms. This may be related to the relative maturity levels of the seeds planted. Although a moot point, some studies suggest that the level of seed ripeness is important for the seed's capacity to germinate; very mature seeds are thought to have poor germination rates (Silva and George, 1971, see Ohler, 1984, pp 125). Such factors will therefore have to be taken into account in future studies. None of the resulting seedlings of both infected and uninfected seeds gave a positive result in PCR using both single round and nested PCR in six monthly analyses over an 18 month period and none of the seedlings also developed symptoms of infection after two years of monitoring. Similarly both Nipah *et al.* (2007) and Oropeza *et al.* (2011) failed to detect phytoplasma DNA in seedlings derived from seeds of infected palms.

Plumules are known to give the best response in clonal propagation of coconut (Chan *et al.*, 1998) and are also largely the section of the embryo where most phytoplasma DNA are found (Cordova *et al.*, 2003).

Although three samples of the remainder of the 'plumule only' sections gave positive result in PCR, their excision was difficult and a number of embryos were destroyed in the process. Growth of their cultures was also poor. In order to reduce contamination, measures such as culturing embryos on the same day they were excised and using good surface sterilisation agents were taken, nevertheless, massive contamination of the cultures still occurred. Time constraints did not permit thorough investigations into the source of the contamination.

This study and previous studies failed to identify the presence of phytoplasma DNA in progenies of seeds of infected palms and in tissue cultures of embryos derived from infected palms. Thus, it seems likely that phytoplasma are not seed transmitted and that the DNA fragments detected in the embryos are not associated with a viable organism that can cause disease in progeny plants.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

Coconut is a subsistence crop in Ghana and is cultivated mostly by rural dwellers who are usually poor and do not have the skills or means to engage in other sources of employment. Coconut is cultivated as a source of income, food and drink in Ghana. The crop is reported to support the livelihood of about 4.2% of Ghana's population (Adam *et al.*, 1996). The fortunes of the coconut industry, however, began to decline from 1932 with the incidence of the lethal disease (LD) caused by a phytoplasma (Chona and Andoh, 1970; Ofori and Nkansah-Poku, 1997). The disease has destroyed several thousand hectares of coconut palms over the years and continues to pose a threat to the survival of the coconut industry (Dery *et al.*, 1997; Ofori and Nkansah-Poku, 1997).

The disease was able to wreak havoc because of the lack of varieties and hybrids that were tolerant or resistant to infection. To address this deficit, many varietal resistant trials have been conducted using materials imported from outside the country (Dery *et al.*, 1997; Quaicoe *et al.*, 2009). These efforts have led to the identification of some ecotypes and hybrids which have showed varying levels of tolerance/resistance to infection (Dery *et al.*, 2008; Dollet *et al.*, 2009; Quaicoe *et al.*, 2009). In Jamaica, resistance to LY (a similar disease to LD) by the MAYPAN hybrid used to replant devastated farms broke down and this demonstrated the need to properly manage breeding programmes (Broschat *et al.*, 2002; Baudouin *et al.*, 2008; Lebrun *et al.*, 2008). An important lesson learnt from the Jamaican experience was the need to use materials of certified

identities in breeding programmes (Lebrun *et al.*, 2008). In Ghana, the materials used in the breeding programmes are yet to be characterised. The need to understand the genetic basis of resistance/tolerance in the coconut varieties, as well as the necessity to develop molecular markers to underpin the sustainable breeding of high value varieties, have formed the basis of this thesis.

The varieties and hybrids used in the Ghanaian breeding programme include: SGD and VTT which have shown some resistance to LD-infection; the hybrid MYD x VTT, which although it has suffered some losses to the disease is less susceptible than the local WAT ecotype and the SGD x VTT hybrid which has also demonstrated to have some resistance to LD (Dery *et al.*, 1997; Dery *et al.*, 2008; Dollet *et al.*, 2009). The local WAT ecotype is highly susceptible to LD and thousands of palms have died from infection (Ofori and Nkansah-Poku, 1997). Nevertheless in LD-infected fields, there are often palms which escape infection (Dery *et al.*, 2005); it is not known whether such palms possess some resistance/tolerance to LD and this study aimed to investigate this phenomenon. To understand the genetic bases of resistance (palms which are not infected by the phytoplasma) or tolerance (palms which are infected but do not develop LD or palms which remit disease symptoms), a sample of 100 palms each of the SGD x VTT, MYD x VTT and WAT escapees located in diseased areas were selected and monitored for disease development for three years. Symptomless infections as well as resistance to phytoplasma infection have been reported in some plant

species (Lee *et al.*, 2000). Six monthly observations of disease symptoms and diagnosis by PCR were carried out. A novel real-time quantitative PCR (Q-PCR) assay targeting the 23S rDNA was developed for detecting and quantifying the LD phytoplasma in infected palms with the aim of determining pathogen titres in palms identified to be tolerant to infection (Section 3.2.1). The assay was found to be sensitive and specific (Section 3.3.5.1). It is important to normalise Q-PCR data (Giulietti *et al.*, 2001) and normalisation was achieved by running a Q-PCR assay of coconut 18S rDNA for each sample. The 18S rDNA assay also served as an internal control to check for PCR inhibition. Q-PCR is considered the method of choice for quantifying nucleic acids and several phytoplasmas have been quantified using the method (Christensen *et al.*, 2004; Martini *et al.*, 2007; Bisognin *et al.*, 2008).

None of the MYD x VTT and SGD x VTT hybrids was found to be infected throughout the period of this study. This was probably due to a lack of infection in these fields. The absence of infection therefore hampered investigations into the bases of resistance/tolerance in the SGD x VTT and MYD x VTT hybrids. This situation highlights the difficulty of working with phytoplasmas which cannot be cultured *in vitro* and cannot be manually inoculated into host plants. Resistance/tolerance screening trials therefore have to rely on the natural incidence of disease which is unpredictable. The study therefore failed to establish whether the relatively less susceptible status of the MYD x VTT hybrid compared to the WAT ecotype is due to a capacity of some of the MYD x VTT hybrids

to harbour a level of infection without developing LD. The SGD x VTT hybrid is only recently being distributed to farmers and the establishment of the palms in diseased areas would permit further investigations into the genetic basis of its resistance to LD. This aspect of the study on the hybrids was therefore also inconclusive. Nevertheless, the reliable diagnostic and quantification methods developed in this study can be used in future studies if the disease becomes established in these fields or when other sites are employed for further analyses of these hybrids.

Six monthly monitoring of symptoms of infection and confirmation with PCR diagnosis over a three year period did not show remission of symptoms or symptomless infections in the WAT palms; all infected palms eventually died from infection (Section 3.3.4). Dery and Philippe (1997) monitored WAT palms monthly for disease development and reported symptom remission in three palms out of a sample of 47 palms. The authors suggested that, there may be subpopulations of the WAT ecotype that are tolerant to LD-infection. Remission of symptoms for 22-32 weeks was observed for these palms, however, the eventual fate of these palms after the observation period is not known. Reports of temporal remission of symptoms have also been made (Quaicoe, R.N. personal communication). In this study, the palms were monitored six monthly and it is possible to have missed any temporal remission of symptoms which might have occurred in-between the sampling periods. This study therefore contends that even if symptoms are temporarily remitted, the WAT palms eventually die from infection and that WAT

escapees observed in LD-devastated farms do not represent resistant/tolerant subpopulations, rather difference in timings of infection is likely to account for the apparent escape from LD-infection.

Seasonal variation of phytoplasma levels in some deciduous plant hosts have been reported (Schaper and Seemuller, 1982). Symptom development in phytoplasma-mediated diseases is influenced by the distribution and concentration of phytoplasmas (Berges *et al.*, 2000; Christensen *et al.*, 2004; Marcone, 2010). Seasonality of symptoms and fluctuation in phytoplasms titres in the wet and dry seasons were investigated. Observation of symptoms and PCR diagnosis revealed that symptoms develop all year round and that new detection of phytoplasmas could be made in samples of infected palms collected in both rainy and dry seasons. Quantification of pathogen titres in diseased palms sampled from the rainy and dry seasons did not establish a relationship between pathogen titres and the period of year (Section 3.3.4.2). This study therefore concludes that the season (wet or dry) has no effect on phytoplasma titres in the coconut population studied. However, in apple and pear trees infected by apple proliferation and pear decline diseases respectively, phytoplasma levels in infected plants are almost undetectable in the aerial parts of the plants but can be detected in the roots during winter (Marcone, 2010). Phytoplasma levels are known to be unevenly distributed in tissues of infected palms; the highest concentrations have been found in sink tissues such as immature leaves, stem and inflorescence, while minimal or no detections have been

recorded for source tissues such as mature and intermediate leaves (Thomas and Norris, 1980; Oropeza *et al.*, 2011). The comparative amounts have, however, not been quantitatively determined and this was investigated. Results of quantification of phytoplasma titres in samples collected from young leaves and mature leaves agreed with the above-mentioned findings. Time constraints, due largely to technical problems with the Q-PCR machine, including an unusual high running temperature, did not permit a more detailed study into the nature of the pathogen-host interaction. The machine was eventually replaced but at a rather late period of the study. Further analysis of a larger dataset might be valuable.

Critical to the success of any breeding programme is the use of certified materials that are true-to-type and this requires the use of reliable methods to validate the identities of supposed resistant/tolerant materials. Characterisation using molecular markers is considered a better approach for cultivar identification than the use of morphological or biochemical methods (Karp *et al.*, 1997; Jones *et al.*, 1997; Lebrun *et al.*, 2005; Agarwal *et al.*, 2008). Chapter 2 was therefore aimed at finding markers with cultivar specific alleles that can be used to certify the identity of a variety before distribution to farmers and to ensure genetic purity in breeding programmes. A successful molecular marker system requires the identification of markers that are polymorphic between different varieties and a suitable method to detect these differences (Orita *et al.*, 1989; Argawal *et al.*, 2008; Modini *et al.*, 2009).

The use of molecular markers for diversity studies in coconut is well documented and many markers, particularly SSR markers, have been developed for such purposes (Rivera *et al.* 1999; Perera *et al.* 2000; 2003; Lebrun *et al.*, 2005; Mauro-Herrera *et al.*, 2006). An array of SSR markers (44) was assessed for its usefulness in differentiating between the Ghanaian breeding materials. Polymorphisms between SSR fragments are usually detected using different forms of gel electrophoresis; these require staining with hazardous or radioactive dyes and size differences are determined by eye which can be subjective. To provide improved methods, a novel rapid and high throughput method of detecting polymorphisms at a SSR locus using melt curve analysis was developed (Section 2.2.8). The method involved incorporating a DNA binding dye in the PCR reaction which allows the detection of amplicons as fluorescent signals. After the PCR, amplicons incorporating SSR regions were melted and the temperature at which the double stranded DNA separate, the melting temperature (T_m) determined. Allelic size differences were then inferred from the melting profiles of amplicons. Out of 44 SSR markers initially assessed with agarose gel analyses, 17 of them which showed interesting differences or whose resolution were not clear enough to see consistent differences were selected for a finer size resolution using the melt curve method. Two of these markers CnCir12 and CAC65 initially appeared to be having a WAT specific allele (Section 2.3.1.1). Konan *et al.* (2007) using a limited number of samples identified a WAT specific allele which was the same one identified in this work. Analyses with further samples, however, showed that the allele was shared by a few samples of other varieties. The study found that, the varieties studied did

not show consistent genotypes for variety-specific SSR markers to be identified. An F2 population of SGD x VTT has been established in a diseased area and it is hoped that as infection sets in, the use of the diagnostic methods and marker detection techniques would allow the identification of markers associated with resistant/tolerant genotypes that might be found. Single nucleotide polymorphic markers (SNPs) based on sequences encoding WRKY transcription factors have been developed in coconut (Mauro-Hererra *et al.*, 2006). The published SNPs were used on the Ghanaian materials in an attempt to develop SNP markers that could be used to determine the genotype of a variety. A method based on the LAMP technique was developed to detect different alleles of the SNP at each locus. The method involved using alternative LAMP assays targeting either form of a mutation at each SNP locus to determine the genotype of a variety. Assays were successfully developed for four out of the eight SNP loci (Table 2.2). Only one of the assays (CnWRKY-05) was largely successful in distinguishing between tall palms (VTT and WAT) and dwarf palms (MYD and SGD). A test of the ability of either form of the assay to selectively amplify a specific genotype in a mixed DNA scenario was, however, not successful. The technique represented a proof of concept which would be optimized as new SNP markers become available. The prospect of using LAMP assays for genotyping coconut varieties is a particularly encouraging one since such methods can be easily transferred to Ghana and Africa at large. Although a reliable set of markers that could provide a means of certifying the identity of the materials used in the breeding programme were not identified within the time span of this study, the methods and procedures developed in this

study will serve as a valuable resource for identifying markers in future studies.

Phytoplasmas have not been cultured in cell-free media and so their diagnoses have been carried using molecular biology techniques; assays are mainly based on the PCR technique. The PCR technique, while very sensitive and accurate, is not easily amenable to in-field use. The use of methods that can accurately and rapidly diagnose LD-infection in-field was investigated. Assays based on the LAMP technique have been used to diagnose a number of plant pathogens and methods for in-field use have also been developed (Tomlinson *et al.*, 2007; Tomlinson *et al.*, 2010a). LAMP, because it is carried out at a single temperature, can be run on any suitable equipment that can maintain a constant temperature, such as in a water bath or on a simple heated block. LAMP amplicons can be detected by a range of methods including real-time methods (Reddy *et al.*, 2010; Bekele *et al.*, 2011). This study evaluated a real-time LAMP assay run on a Genie I instrument for its effectiveness at detecting the LD phytoplasma. The method involves the detection of amplicons as fluorescent signals. The specialised machine can also perform a melt curve analysis which provides a means of validating the results. Infected samples positively diagnosed by nested PCR were also detected by the LAMP assay (Section 3.3.3). LAMP primers are easy to design and the qualitative performance of the assays detailed in this report would serve as a basis to develop assays for other plant pathogens in Ghana. The simplicity of the method and the many detection techniques available will

make it easy for extension agents to use the method in-field with minimal training. The author therefore hopes to take a lead in popularising the technique in Ghana.

Although phytoplasmas are phloem restricted pathogens (and usually transmitted by insect vectors), reports of the detection of phytoplasma DNA in the embryo of plants including coconut have raised concerns about the possibility of seed transmission of phytoplasmas. Nipah *et al.* (2007) investigated the possibility of seed transmission of LD but did not find any evidence to this effect. Seed transmission of LD would greatly restrict the movement of coconut within and between countries since this would amount to introducing the disease to new areas. These implications necessitated the need for further investigations to firmly clarify this subject. Although the study confirmed the presence of phytoplasma DNA in the embryo of coconut seeds, it failed to establish that phytoplasmas are transmitted through the seeds. Out of 105 seedlings raised from seeds of infected palms, none was found to be infected after six monthly diagnoses by PCR analyses over an 18 month period (Section 5.3.1.2). Although the plumule cultures suffered heavy losses due to contaminations, no infection was detected in all five plantlets that differentiated leaves. A recent study by Oropeza *et al.* (2011) also failed to establish seed transmission for LY. These results suggest that phytoplasmas are not seed transmitted. These results suggest that the DNA fragment detected in the embryo is not a viable organism. The apparent non-transmissibility of the pathogen via the seed

may also be related to the time the pathogen enters the embryo if it is a viable organism. Baker and Smith, (1966) suggested that some viral infections of embryos do not result in transmission because the viruses tardily infect the meristematic tissues of the embryo.

Several weed species have been identified as alternate hosts of the LY phytoplasma in Jamaica (Brown *et al.*, 2007; Brown and McLaughlin, 2011). A search for alternate hosts of the LD phytoplasma yielded no positive results (Yankey *et al.*, 2009). To expand the search for alternate hosts of the LD phytoplasma, 41 plant species belonging to 24 botanical families and sampled from four locations were investigated for phytopasma infection. None of the plant species was found to be infected with the phytoplasma. Given the number of plant species that have been studied in the present and previous studies, it is apparent that alternative hosts of the LD phytoplasma are likely to be rare if they exist at all. The rapid in-field diagnostic assays developed in this study will facilitate the screening of further species in future studies.

LD continues to pose a serious threat to coconut plantations in Ghana. The use of sensitive methods for the timely and accurate diagnosis of LD phytoplasma infection and the development of tolerant/resistant varieties will provide an integrated approach to manage the disease. The methods and techniques developed in this study have provided an invaluable resource to the coconut research community for the development of holistic approaches to manage the disease.

The main conclusions from this study therefore are:

1. Palms within a variety of the Ghanaian breeding materials do not show consistent genotypes and it is therefore difficult to develop SSR-based markers associated with individual varieties.
2. LD-infected WAT palms eventually die from infection and that escapees do not represent tolerant or resistant subpopulations. Difference in timings of infection is likely to account for the apparent escape from LD-infection.
3. Phytoplasmas can be detected all year round in LD-infected tissues and pathogen titres in infected palms are not influenced by the season of the year.
4. LAMP assays are quick and as sensitive as nested PCR in detecting the LD phytoplasma. Real-time detection of LAMP amplicons using a Genie I instrument can be done in less than 30 min and this makes the method suitable for in-field use.
5. Coconut seeds derived from LD-infected palms do not appear to germinate into LD-infected seedlings.
6. There is still no indication of the presence of alternate hosts of the LD phytoplasma in Ghana.

Future work:

Based on the results of this work, it will be important to monitor MYDxVTT hybrids planted in diseased farms for infection. The SGDxVTT hybrids will also have to be planted in other diseased areas; this will permit further studies to understand the nature of the

tolerance/resistance in these hybrids. Given that the marker CnCirC12 revealed some heterogeneity in the SGD palms, which is a self-pollinating variety, the level of heterogeneity in this variety needs to be established. This can be done with marker CnCirC12 which revealed polymorphic alleles in the SGD or with further SSR markers that may be trialled. Once LD becomes incident in the SGDxVTT F_2 population, established in a diseased field, segregation of resistance will be studied and hopefully markers linked to resistance/tolerance identified. The LAMP approach of genotyping based on SNPs is a very simple method that will be pursued as new SNP containing genes in coconut become available. The method can, however, be extended to other plants for which such markers are available. Large portions of the genome of cocoa, for example, has been sequenced (about 76% of the estimated genome size) (Argout *et al.*, 2010) and such markers can easily be developed to enhance the breeding of the crop.

REFERENCES

Adam, M., Arthur, R., Duhamel, G., Ghartey, N., Overfield, D. and Willougby, N. (1996) Analysis of natural resource utilization, livelihoods systems, coping strategies and natural resource research needs in coconut growing areas of the coastal zone ecosystems in Ghana. *NRI Project CO834, Vol 1, Main Report.*

Addison, E.A. (1980) Varietal resistance trials with some dwarf coconut palms against the Cape Saint Paul wilt disease in Ghana. *African Journal of Plant Protection 1, 129-141.*

Agarwal, M., Shrivastava, N. and Padh, H. (2008) Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Reports 27, 617-631.*

Alvarez, E., Juan, F.M., Llano, G.A. and Loke, J.B. (2007) Detection and characterisation of a phytoplasma associated with frog skin disease in cassava. *Bulletin of Insectology 60, 273-274.*

Angelini, E., Bianchi, G.L., Filippin, L., Morassutti, C. and Borgo, M. (2007) A new Taqman method for the identification of phytoplasmas associated with grapevine yellows by real-time PCR assay. *Journal of Microbiological Methods 68, 613-622.*

Argout, X., Salse, J., Aury, J-M., Guiltinan, M.J., Droc, G., Gouzy, J., Mathilde Allegre, M., Chaparro, C., Legavre, T., Maximova, S.N., Abrouk, M., Florent Murat, F., Fouet, O., Poulain, J., Ruiz, M., Roguet, Y., Rodier-Goud, M., Barbosa-Neto, J.F., Sabot, F., Kudrna, D., Ammiraju, J.S.S., Schuster, S.C., Carlson, J.E., Sallet, E., Schiex, T., Dievart, A., Kramer, M., Gelley, L., Shi, Z., Bérard, A., Viot, C., Boccara, M., Risterucci, A.M., Guignon, V., Sabau, X., Axtell, M.J., Ma, Z., Zhang, Y., Brown, S., Bourge, M., Golser, W., Song, X., Clement, D., Rivallan, R., Tahi, M., Akaza, J.M., Pitollat, B., Gramacho, K., D'Hont1, A., Brunel, D., Infante, D., Kebe, I., Costet, P., Wing, R., McCombie, W.R., Guiderdoni, E., Quetier, F., Panaud, O., Wincker, P., Bocs, S. and Lanaud, C. (2011) The genome of *Theobroma cacoa*. *Nature Genetics 43, 101-109.*

Ashburner, G.R., Thompson, W.K., Halloran, G.M. and Foale, M. (1997) Fruit component analysis of South Pacific coconut palm populations. *Genetic Resources and Crop Evolution 44, 327-335.*

Assy-Bah, B., Durand-Gasselin, T. and Pannetier, C. (1987) Use of zygotic embryo culture to collect germplasm of coconut. *FAO/IBPGR Plant Genetic Resources Newsletter, 71, 4-10.*

Bai, X., Zhang, J., Ewing, A., Miller, S.A., Radek, A.J., Shevchenko, D.V., Tsukerman, K., Walunas, T., Lapidus, A., Campbell, J.W. and Hogenhout, S.A. (2006) Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. *Journal of Bacteriology 188, 3682-3696.*

Baker, K.F. and Smith, S.H. (1966) Dynamics of seed transmission of plant pathogen. *Annual Review of Phytopathology 4, 311-332.*

Basak, A.B. and Lee, M.W. (2002) Prevalence and transmission of seed-borne fungi of maize grown in a farm in Korea. *Mycobiology 30, 47-50.*

Batugal, P.A. and Engelmann, F. (1998) *Coconut Embryo In Vitro Culture.* P.A. Batugal and F. Engelmann (Eds.) Papers presented at a workshop on Embryo Culture, 27-31 October 1997, Banao, Guinobatan, Albay, Philippines, IPGRI-APO, Serdang. Pp 164.

Batugal, P., Rao, V.R. and Oliver, J (Eds.) (2005) *Coconut Genetic Resources.* International Plant Genetic Resources Institute– Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Pp XII.

Baudouin, L., Lebrun, P., Berger, A., Myrie, W., Been, B. and Dollet, M. (2008) The Panama Tall and the Maypan hybrid coconut in Jamaica: did genetic contamination cause a loss of resistance to Lethal Yellowing? *Euphytica 161, 353-360.*

Baudouin, L., Lebrun, P., Rognon, F. And Rtter, E. (2005) Use of molecular markers for coconut improvement: Status and prospects. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources*. International Plant Genetic Resources Institute– Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. *Pp 268-281*.

Bekele, B., Hodgetts J., Tomlinson, J., Boonham, N., Nikolic, P., Swarbrick, P., Dickinson, M. (2011) Use of a real-time isothermal assay for detecting 16SrII and XII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley. *Plant Pathology 60, 345-355*.

Berges, R., Rott, M. and Seemuller, E. (2000) Range of phytoplasma concentrations in various plant hosts as determined by competitive polymerase chain reaction. *Phytopathology 90, 1145-1152*.

Bertaccini, A. (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontiers in Bioscience 12, 673-689*.

Bertaccini, A. and Duduk, B. (2009) Phytoplasma and Phytoplasma disease: A review of recent research. *Phytopathologia Mediterrania 48, 355-378*.

Bertamini, M., Grando, M.S. and Nedunchezhian, N. (2003) Effects of phytoplasma infection on pigments, chlorophyll-protein complex and photosynthetic activities in field grown apple leaves. *Biologia Plantarum 47, 237-242*.

Bertamini, M. and Nedunchezhian, N. (2001) Effect of phytoplasma [stolbur-subgroup (Boir noir-BN)] on photosynthetic pigments, saccharides, ribulose 1,5-biphosphate carboxylase, nitrate and nitrite reductases and photosynthetic activities in field –grown grapevine (*vitis vinifera* L. cv. Chardonnay) leaves. *Photosynthetica 39, 119-122*.

Bisognin, C., Schneider, B., Salm, H., Grando, M.S., Jarausch, W., Moll, E. and Seemuller, E. (2008) Apple proliferation resistance in apomitic rootstocks and its relationship to phytoplasma concentration and simple sequence genotypes. *Phytopathology 98, 153-158*.

Borrone, J.W., Kuhn, D.N. and Schnell, R.J. (2004) Isolation, characterisation, and development of WRKY genes as useful genetic markers in *Theobroma cacao*. *Theoretical and Applied Genetics 109, 495-507.*

Botti, S. and Bertaccini, A. (2006) Phytoplasma infection through seed transmission: further observations. (Abstract). In: Ayling, R.D., Citti, C., Nicholas, R.A.J. (Eds.) *Proceedings of the 16th International congress of the International Organisation for Mycoplasmology*. Cambridge, UK.

Bourdeix, R., Konan, J.L. and N'cho, Y.P. (2005) *Coconut, A Guide To Traditional and Improved Varieties*. Collection catalys, Editions Diversiflora. Novoprint SA, Barcelona, Spain. Pp 3-16.

Broschat, T.K., Harrison, N.A. and Donselman, H. (2002) Losses to lethal yellowing cast doubt on coconut cultivar resistance. *Palms 46, 185-189.*

Brown, S.E., Been, B.O. and McLaughlini, W.A. (2008) First report of the presence of the lethal yellowing group (16SrIV) of phytoplasma in the weeds *Emelia fosbergii* and *Synedrella nodiflora* in Jamaica. *Plant Pathology 57, 770.*

Brown, S.E. and McLaughlin, W.A. (2011) Identification of lethal yellowing group (16SrIV) of phytoplasmas in the weeds *Stachytarpheta jamaicensi*, *Macroptilium lathyroides* and *Cleome rutidosperma* in Jamaica. *Phytopathogenic Mollicutes 1, 27-34.*

Cardena, R., Ashburner, G.R. and Oropeza, C. (2003) Identification of RAPDs associated with resistance to lethal yellowing of the coconut (*Cocos nucifera* L.) palm. *Scientia Horticulturae 98, 257-263.*

Cardena, R., Oropeza, C. and Zizumbo, D. (1998) Leaf proteins as markers useful in the genetic improvement of coconut palm. *Euphytica 102, 81-86.*

Carter, W. and Suah, J.R.R. (1964) Studies on the spread of lethal yellowing disease of the coconut palm. *FAO Plant Protection Bulletin 12, 73-78.*

Chan, J.L., Saenz, L., Talavera, C., Hornung, R., Robert, M. and Oropeza, C. (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryognesis. *Plant Cell Reports 17, 515-521.*

Chang, C-J. (1998) Pathogenicity of aster yellows phytoplasma and *Spiroplasma Citri* on periwinkle. *Phytopathology 88, 1347-1350.*

Charles, J.G., Allan, D.J., Andersen, M.T., Langford, G. and Mossop, D. (2002) The search for a vector of strawberry lethal yellows (SLY) in New Zealand. *New Zealand Plant Protection 55, 385-389.*

Choi, Y.T., Tapis, E.C., Kim, H.K., Lefeber, A.W.M., Erekelens, C., Verhoeven, J.Th.J., Brzin, J., Zel, J. and Verpoorte, R. (2004) Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using ^{1}H-NMR spectroscopy and multivariate data analysis. *Plant Physiology 135, 2398-2410.*

Chona, B.L. and Adansi, M.A. (1970) Coconut in Ghana. *Crops Research Institute Bulletin* No. 3. Crops Research Institute, Council for Scientific and Industrial Research (CSIR), Ghana.

Chona, B.L. and Andoh, P.G. (1970) Cape St Paul wilt: the present position. In: Coconut in Ghana. *Crops Research Institute Bulletin* No. 3. Crops Research Institute, Council for Scientific and Industrial Research (CSIR), Ghana.

Christensen, M., Sunde, L., Bolund, L. and Ørntoft, T.F. (1999) Comparison of three methods of microsatellite detection. *Scandinavian Journal of Clinical Laboratory Investigation 59, 167-178.*

Christensen, N.M., Nicolaisen, M., Hansen, M. and Alexander, S. (2004) Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions 17, 1175-1184.*

Christensen, N.M., Axelsen, K.B., Nicolaisen, M. and Schulz, A. (2005) Phytoplasmas and their interactions with hosts. *Trends in Plant Science 10, 526-535.*

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B. and Pang, E.C.K. (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica 142, 169-196.*

Collins, N.C., Webb, C.A., Seah, S., Ellis, J.G., Hulbert, S.H. and Pryor. (1998) The isolation and mapping of disease resistance gene analogs in maize. *Molecular Plant-Microbe Interactions 11, 968-978.*

Cordova, I., Jones, P., Harrison, N.A. and Oropeza, C. (2003) *In situ* detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. *Molecular Plant Pathology 4, 99-108.*

Corinne, C.R. and Hachler, H. (2001) Real-time PCR and melting curve analysis for reliable and rapid detection of SHV extended-spectrum b-Lactamases. *Antimicrobial Agents and Chemotherapy 45, 1730-1736.*

Creste, S., Tulmann, N. and Figueira, A. (2001) Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Reporter 19, 229-306.*

Daire, X., Claire, D., Reinert, W. and Boudon, E. (1997) Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. *European Journal of Plant Pathology 103,507-514.*

Danks, C. and Boonham, N. (2007) Purification method and kits. *Patent WO/2007/104962.*

Davey, J.E. and Staden, V. (1981) Endogenous cytokinin levels and development of flower virescence in *Catharanthus roseus* infected with mycoplasmas. *Physiological Plant Pathology 19, 193-200.*

Deng, S. and Hiruki, C. (1991) Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of Microbiological Methods 14, 53-61.*

Dery, S.K., N'Cho, Y.P., Sangare, A. and Arkhurst, E.D. (1997) Cape St Paul wilt disease: resistance screening and prospects for rehabilitating the coconut industry in Ghana. In: Eden- Green S.J. and Ofori F. (Eds.) *Proceedings of an International workshop on lethal yellowing-like diseases of coconut*, Elmina, Ghana, November 1995. Pp 147-151.

Dery, S.K., Nipah, J.O. and Ofori, F. (2005) Status of coconut genetic resources research in Ghana. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources*. International Plant Genetic Resources Institute- Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. *Pp 661-666.*

Dery, S.K. and Philippe, R. (1997) Preliminary study on the Epidemiology of Cape St Paul wilt disease of Coconut in Ghana. In: Eden- Green S.J. and Ofori F. (Eds.) *Proceedings of an International workshop on lethal yellowing-like diseases of coconut*, Elmina, Ghana, November 1995. Pp 255-260.

Dery S.K., Philippe, R., Baudoin, L., Quaicoe, R.N., Nkansah-Poku, J., Owusu-Nipah, J., Arthur, R., Dare, D., Yankey, N., Dollet, M. (2008) Genetic diversity among coconut varieties for susceptibility to Cape St Paul wilt disease. *Euphytica 164, 1-11.*

Dickinson, M. (2003) *Molecular Plant Pathology*. Bios Scientific Publishers, Taylor and Francis Group, London and New York. Pp 159-173, 197.

Doi, Y.M., Teranaka, M., Yora, K. and Asuyama, H. (1967) Mycoplasma or PLT-group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or paulownia witches' broom .(Abstract). *Annals of Phytopathological Society of Japan 33, 259-266.*

Dollet, M., Quaicoe, R. and Pilet, F. (2009) Review of coconut "lethal yellowing" type diseases: diversity, variability and diagnosis. *Oleagineux 16, 97-101.*

Eden-Green, S. (1997) History, distribution and research on coconut lethal yellowing-like diseases of palms. In Eden- Green S.J. and Ofori F. (Eds.) *Proceedings of an International workshop on lethal yellowing-like diseases of coconut*, Elmina, Ghana November, 1995. Pp 9 - 25.

Eeuwens, C.J. (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro. Plant Physiology 36, 23-28.*

Egea-Gilbert, C., Dickinson, M.J., Bilotti, G. and Candela, M.E. (2003) Isolation of resistance gene analogs in pepper using modified AFLPs. *Biologia Plantarum 47, 27-32.*

Firrao, G., Garcia-Chapa, M. and Marzachi, C. (2007) Phytoplasmas: genetics, diagnosis and relationship with the plant and insect host. *Frontiers in Bioscience 12, 1353-1375.*

Foale, M. (2005) An introduction to the coconut palm. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources*. International Plant Genetic Resources Institute–Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Pp 1-8.

Galetto, L. and Marzachi, C. (2010) Real-time PCR diagnosis and quantification of phytoplasmas. In: Weintraub, P.G. and Jones, P. (Eds.) *Phytoplasmas: Genomes, Plant Hosts and Vectors*. Pp 1-14.

Garcia-Martinez, J., Acinas, S.G., Anton, A.I. and Rodriguez-Valera, F. (1999). Use of the 16-23S ribosomal genes spacer region in studies of prokaryotic diversity. *Journal of Microbiological Methods 36, 55-64.*

Gasparich, G.E. (2010). Spiroplasmas and phytoplasmas: Microbes associated with plant hosts. *Biologicals 38, 193-203.*

Ginzinger, D.G. (2002) Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology 30, 503-512.*

Giuliette, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods 25, 386-401.*

Guatelli, J.C., Gingeras, T.R. and Richman, D.D. (1989) Nucleic acid amplification *in vitro*: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infecton. *Clinical Microbiology Reviews 2, 217-226.*

Gungoosingh-Bunwaree, A., Bertaccini, A. and Benimadhu, S.P. (2007) Presence of phytoplasma infections in tomato plants in Mauritius. *Bulletin of Insectology 60, 151-152.*

Gungoosingh-Bunwaree, A., Contaldo, N., Paltrinieri, S., Benimadhu, S.P. and Bertaccini, A. (2011) Detection of phyotplasmsa infection in okra in Mauritius. *Bulletin of Inssectology 64, S103-S104.*

Haggis, G.H. and Sinha, R.C. (1978) Scanning Electron Microscopy of mycoplasmalike organisms after freeze fracture of plant tissues affected with clover phyllody and aster yellows. *Phytopathology 68, 677-680.*

Harries, H.C. (1978) Lethal yellowing disease of coconuts in global perspective. *Philippines Journal of Coconut Science 3, 1- 4.*

Harries, H.C., Baudouin, L. and Cardena, R. (2004) Floating, boating and introgression: molecular techniques and the ancestry of coconut palm population on Pacific Islands. *Ethnobotany Research and Applications 2, 37-53.*

Harrison, N.A., Bourne, C.M., Cox, R.L., Tsai, J.H. and Richardson, P.A. (1992) DNA probes for detection of mycoplasmalike organisms associated with lethal yellowing disease of palms in Florida. *Phytopathology 82, 216-224.*

Harrison, N.A., Cordova,I., Richardson, P. and Dibonto, R. (1999) Detection and diagnosis of lethal yellowing. In: Oropeza. C., Verdeil,J.L, Ashburner, G.R., Cardena, R., and Santamaria, J.M. (Eds.) *Current Advances in Coconut Biotechnology*. Kluwer Academic Publishers, Dordrecht. The Netherlands. Pp 183 – 193.

Harrison, N.A. and Oropeza, C. (1997) Recent studies on detection of lethal yellowing disease phytoplasmas in the Americas. In: Eden- Green S.J. and Ofori F. (Eds.) *Proceedings of an International workshop on lethal yellowing-like diseases of coconut*, Elmina, Ghana, November 1995. Pp 221-234.

Harrison, N.A., Womack, M. and Carpio, M.L. (2002) Detection and characterisation of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. *Plant Disease 86, 676-681.*

Heinrich, M., Botti, S., Caprara, L., Arthofer, Strommer, S., Hanzer, V., Katinger, H., Bertaccini, A. and Machado, M.L.D.C. (2001) Improved detection methods for fruit tree phytoplasmas. *Plant Molecular Biology Reporter 19, 169-179.*

Hodgetts, J., Ball, T., Boonham, N., Mumford, R. and Dickinson, M. (2007) Use of terminal restriction fragment length polymorphism (T-RFLP) for identification of phytoplasmas in plants. *Plant Pathology 56,357-365.*

Hodgetts, J., Boonham, N., Mumford, R. and Dickinson, M. (2009) Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas. *Applied and Environmental Microbiology 75, 2945-2950.*

Hodgetts, J., Boonham, N., Mumford, R., Harrison, N. and Dickinson, M. (2008) Phytoplasma phylogenetics based on analysis of *sec A* and 23S rRNA gene sequences for improved resolution of candidate species of '*Candidatus* Phytoplasma'. *International Journal of Systematic and Evolutionary Microbiology 58, 1826-1837.*

Hodgetts, J. and Dickinson, M. (2010) Phytoplasma phylogeny and detection based on genes other than 16S rRNA. In: Weintraub, P.G. and Jones, P. (Eds.) *Phytoplasmas: Genomes, Plant Hosts and Vectors*. Pp 93-113.

Hodgetts, J., Tomlinson, J., Boonham, N., Gonzalez-Martin, I., Nikolic, P., Swarbrick, P., Yankey, E.N. and Dickinnson, M. (2011) Development of rapid in-field loop-mediated isothermal amplification (LAMP) assays for phytoplasmas. *Bulletin of Insectology 64, S41-S42.*

Hogenhout, S.A., Oshima, K., Ammar, E., Kakizawa, S., Kingdom, H.N. and Namba, S. (2008) Phytoplasmas: bacteria that manipulate plants and insects. *Molecular Plant Pathology 9, 403-423.*

Howard, F.W., Norris, R.C., Thomas, D.C. (1983) Evidence of transmission of palm lethal yellowing agent by a planthopper, *Myndus crudus* (Homoptera: cixiidae). *Tropical Agriculture (Trinidad) 60, 168-171.*

Hren, M., Boben, J., Rotter, A., Kralj, P., Gruden, K. and Ravnikar, M. (2007) Real-time PCR detection systems for flavescence doree and bois noir phytoplasmas in grapevine: comparison with conventional PCR detecton and application in diagnosis. *Plant Pathology 56, 785-796.*

International Committee on Systematic Bacteriology (ICSB), subcommittee on the taxonomy of mollicutes (1993) Minutes of the interim meetings, 1 and 2 August, 1992, Ames, Iowa. *International Journal of Systematic Bacteriology* 43, 394-397.

International Committee on Systematic Bacteriology (ICSB), subcommittee on the taxonomy of mollicutes. (1997) Minutes of the interim meetings, 12 and 18 July, 1996, Orlando, Florida, USA. *International Journal of Systematic Bacteriology 47, 911-914.*

(IRPCM) Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group. (2004) 'Candidatus Phytoplasma', a taxon for wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology 54, 1243-1255.*

Ishikawa, T., Kamei, Y., Otozaiz, S., Kim, J., Sato, A., Kuwahara, Y., Tanaka, M., Deguchi, T., Inoharaz, H., Tsujimura, T., Takeshi, T. (2010) High-resolution melting curve analsis for rapid detection of mutations in a Medaka TILLING library. *BMC Molecular Biology 11, 70.*

Iwai, T., Kaku, H., Honkura, R. Nakamura, S. Ochiai, H., Sasaki, T. and Ohashi, Y. (2001) Ehanced resistance to seed-transmitted bacterial diseases in transgenic rice plants overproducing an oat cell-wall-bound thionin. *Molecular Plant-Microbe Interactions 15, 515-521.*

Johansen, E., Edwards, M.C. and Hampton, R.O. (1994) Seed transmission of viruses: current perspectives. *Annual Review of Phytopathology 32, 363-368.*

Jomantiene, R. and Davies, R.E. (2006) Clusters of diverse genes existing as multiple sequence–variable mosaics in a phytoplasma genome. *FEMS Microbiology Letters 255, 59-65.*

Jones, N., Ougham, H. and Thomas, H. (1997) Markers and mapping: we are all geneticists now. *New Phytologist 137, 165-177.*

Jung, H-Y., Sawayanagi, T., Wongkaew, P., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S., Ugaki, M., Hibi, T. and Namba, Shigetou (2003) 'Candidatus Phytoplasma oryzae' a novel phytoplasma taxon associated with rice yellow dwarf disease. *International Journal of Systematic and Evolutionary Microbiology 53, 1925-1929.*

Kanazin, V., Marek, L.F., Shoemaker, R.C. (1996) Resistance gene analogs are conserved and clustered in soybean. *Proceedings of the National Academy of Science – USA 93, 11746-11750.*

Karp, A., Kresovich, S., Bhat, K.V., Ayad, W.G. and Hodgkin, T. (1997) Molecular tools in plant genetic resources conservation: a guide to the technologies. *IPGRI Technical Bulletin* No. 2.

Karp, A., Seberg, O. and Buiatti, M. (1996) Molecular techniques in the assessment of botanical diversity. *Annals of Botany 78, 143-149.*

Kirkpatrick, B.C. (1992) Mycoplasma-like organisms – plant and invertebrate pathogens. In: *The Prokaryotes.* Ballows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. (Eds.) 2nd Edition, 4, 4050-4067.

Kirkpatrick, B.C., Stenger, D.C., Morris, T.J. and Purcell, A.H. (1987) Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science 238, 197-200.*

Konan, K.J.N., Koffi, K.E., Konan, J.L., Lebrun, P., Dery, S.K. and Sangare, A. (2007) Microsatellite gene diversity in coconut (*Cocos nucifera* L.) accessions resistant to lethal yellowing disease. *African Journal of Biotechnology 6, 341-347.*

Kuhn, D.N., Heath, M., Wisser, R.J., Meerow, A., Brown, J.S., Lopes, U. and Schnell, R.J. (2003) Resistance gene homologues in Theobroma cacao as useful genetic markers. *Theoretical and Applied Genetics 107, 191-202.*

Kuske, C.R. and Kirkpatrick, B.C. (1992) Distribution and multiplication of western aster yellows mycoplasmalike organisms in *Catharanthus roseus* as determined by DNA hybridization analysis. *Phytopathology 82, 457-462.*

Lammerts van Bueren, E.T., Backes, G., Vriend, H. E. and Ostergard, H. (2010) The role of molecular markers and marker assisted selection in breeding for organic agriculture. *Euphytica 175, 51-64.*

Lebrun, P., Baudouin, L., Bourdeix, R., Konan, J.L., Baker, J.H.A., Aldam, C., Herran, A. and Ritter, E. (2001) Construction of a linkage map of the Rennel Island Tall coconut type (*Cocos nucifera* L.) and QTL analysis for yield characters. *Genome 44, 962-970.*

Lebrun, P., Baudouin, L., Myrie, W., Berger, A. and Dollet, M. (2008) Recent lethal yellowing outbreak: why is the Malayan Yellow Dwarf coconut no longer resistant in Jamaica. *Tree Genetics and Genomes 4, 125-131.*

Lebrun, P., Berger, A., Hodgkin, T. and Baudouin, L. (2005) Biochemical and molecular methods for characterizing coconut diversity. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources.* International Plant Genetic Resources Institute– Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Pp 225-246.

Lebrun, P., N'cho, Y.P., Seguin, M., Grivet, L. and Baudouin, L. (1998) Genetic diversity in coconut (*Cocos nucifera* L.) revealed by restriction fragment length polymorphism (RFLP) markers. *Euphytica 101, 103-109.*

Lee, I-M., Davis, R.E. and Gundersen-Rindal, D.E. (2000) Phytoplasmas: phytopathogenic mollicutes. *Annual Review of Microbiology 54, 221-255.*

Lee, I-M., Dawn, E., Gundersen, D.E. and Bertaccini, A. (1998) Phytoplasma: ecology and genomic diversity. *Phytopathology 88, 1359-1366.*

Lee, I-M., Gundersen, E., Hammond, R.W. and Davies, R.E. (1994) Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology 84, 559-567.*

Lee, I-M., Hammond, R.W., Davies, R.E. and Gundersen, D.E. (1993) Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology 83, 834-842.*

Lee, I-M., Klopmeyer, M., Bartoszyk, I.M., Gundersen-Rindal, D.E., Chou, T-S., Thomson, K.L. and Eisenreich. (1997) Phytoplasma induced free-branching in commercial poinsettia cultivars. *Nature Biotechnology 15, 178-182.*

Lee, I-M., Zhao, Y., and Davies, R. (2010) Prospect of multiple gene-based systems for differentiation and classification of phytoplasmas. In: Weintraub, P.G. and Jones, P. (Eds.) *Phytoplasmas: Genomes, Plant Hosts and Vectors.* Pp 51-63.

Legoupil, J. and Courbet, P. (2008) Worldwide coconut production – Importance and impact of the lethal yellowing disease. In: Legoupil, J. (Ed) *Proceedings of an International workshop on coconut lethal yellowing diseases,* Accra, Ghana, June 2008. Pp 19–24.

Leon, R., Santamaria, J.M., Alpizar, L., Escamilla, J.A. and Oropeza, C. (1996) Physiological and biochemical changes in shoots of coconut palms affected by lethal yellowing. *New Phytologist 134, 227-234.*

Lepka, P., Stitt, M., Moll, E. and Seemuller, E. (1999) Effect of phytoplasmal infection on concentration and translocation of carbohydrates and amino acids in periwinkle and tobacco. *Physiological and Molecular Plant Pathology 55, 59-68.*

Li, Y.C., Korol, A.B., Fahima, T., Beiles, A. and Nevo, E. (2002) Microsatellites: genomic distribution, putative functions, and mutational mechanisms: a review. *Molecular Ecology 11, 2453-2465.*

Liu, Y., Wang, Z., Qian, Y., Mu, J., Shen, L., Wang, F. and Yang, J. (2010) Rapid detection of tobacco mosaic virus using the reverse transcription loop-mediated isothermal amplification method. *Archives of Virology 155, 1681-1685.*

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002) Survey and summary: real-time PCR in virology. *Nucleic Acids Research 30, 1292-1305.*

Marcone, C. (2010) Movement of phytoplasmas and the development of disease in the plant. In: Weintraub, P.G. and Jones, P. (Eds.) *Phytoplasmas: Genomes, Plant Hosts and Vectors.* Pp 115-131.

Marcone, C., Neimark, A., Ragozzino, A., Lauer, U. and Seemuller, E. (1999) Chromosome sizes of phytoplasmas composing major phylogenetic groups and subgroups. *Phytopathology 89, 805-810.*

Marcone, C., Ragozzino, A. and Seemuller, E. (1997) Detection and identification of phytoplasmas in yellows-diseased weeds in Italy. *Plant Pathology 46, 530-537.*

Martini, M., Loi, N., Ermacora, P., Carraro, L. and Pastore, M. (2007) A real-time PCR method for detection and quantification of '*Candidatus* Phytoplasma prunorum' in its natural hosts. *Bulletin of Insectology 60, 251-252.*

Mauro-Herrera, M., Meerow, A.W., Borrone, J.W., Kuhn, D.N. and Schnell, R.J. (2006) Ten informative markers developed from WRKY sequences in coconut (*Cocos nucifera* L.). *Molecular Ecology Notes 6, 904-906.*

Maust, B.E., Espadas, F., Talavera, C., Aguilar, M., Santamaria, J.M. and Oropeza, C. (2003) Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. *Phytopathology 93, 976-981.*

Meerow, A.W., Wisser, R.J., Brown, J.S., Kuhn, D.N., Schnell, R.J. and Broschat, T.K. (2003) Analysis of genetic diversity and population structure within Florida coconut (*Cocos nucifera* L.) germplasm using microsatellite DNA, with special emphasis on the Fiji Dwarf cultivar. *Theoretical and Applied Genetics 106, 715-726.*

Meneguzzi, N.G., Torres, L.E., Galdeano, E., Guzman, F.A., Nome, S.F. and Conci, L.R. (2008) Molecular characterisation of a phytoplasma of the ash yellows group (16SrVII-B) occurring in *Artemisia annua* and *Conyza bonariensis* weeds. *Agriscientia 25, 7-15.*

Modini, L., Noorani, A. and Pagnotta, M.A. (2009) Assessing plant genetic diversity by molecular tools. *Diversity 1, 19-35.*

Montano, H.G., Brioso, P.S.T., Pereira, R.C., Pimentel, J.P. (2007) Sicana odorifera (Cucurbitaceae) a new phytoplasma host. *Bulletin of Insectology 60, 287-288.*

Mpunami, A. A., Tymon, A., Jones, P. and Dickinson, M.J. (1999) Genetic diversity in the coconut lethal yellowing disease phytoplasma of East Africa. *Plant Pathology 48, 109-114.*

Nagamine, K., Hase, T. and Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes 16, 223-229.*

Nejat, N. and Vadamalai, G. (2010) Phytoplasma detection in coconut palm and other tropical crops. *Plant Pathology Journal 9, 112-121.*

Nicolaisen, M. and Bertaccini, A. (2007) An oligonucleotide microarray-based assay for identification of phytoplasma 16S ribosomal groups. *Plant Pathology 56, 332-336.*

Nicolaisen, M. and Horvath, D.P. (2008) A branch-inducing phytoplasma in *Euphorbia pulcherrima* is associated with changes in expression of host genes. *Journal of Phytopathology 156, 403-407.*

Nipah, J.O. (2007) Improving breeding methods for legitimate seed production in Cape Saint Paul wilt disease-affected coconut plantations. *Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy*, pp 70-88.

Nipah, J.O., Jones, P. and Dickinson, M.J. (2007) Detection of lethal yellowing phytoplasma in embryos from coconut palms infected with Cape St Paul wilt disease in Ghana. *Plant Pathology 56, 777-784.*

Njiru, Z.K., Mikosza, A.S.J., Amstrong, T., Enyaru, J.C., Ndung'u, J.M. and Thompson, A.R.C. (2008) Loop-mediated isothermal amplification (LAMP) method for rapid detection of *Trypanosoma brucei rhodesiense. PLoS Neglected Tropical Diseases 2, e147.*

Nkansah-Poku, J., Dery, S.K. and Philippe, R. (2005) Reduction of spread of CSPWD of coconut by insecticidal hot-fogging and removal of diseased palms. *Ghana Journal of Agricultural Science 1, 193-198.*

Nkansah-Poku, J., Philippe, R., Quaicoe, R.N., Dery, S.K. and Arthur, R. (2009) Cape Saint Paul wilt disease of coconut in Ghana: surveillance and management of disease spread. *Oleagineux 16, 111-115.*

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research 28, e63.*

Ofori, F. and Nkansah-Poku, J. (1997) Cape Saint Paul Wilt Disease of coconut in Ghana: History of its occurrence and spread. In: Eden- Green, S.J., and Ofori, F. (Eds.) *Proceedings of an International workshop on lethal yellowing-like diseases of coconut*, Elmina, Ghana, November 1995. Pp 27-32.

Ohler, J.G. (1984). Coconut, tree of life. *FAO Plant Production and Protection paper* 57, Rome. Pp 14-30, 125.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-stranded conformation polymorphisms. *Proceedings of the National Academy of Science- USA 86, 2766-2770.*

Oropeza, C., Cordova, I., Chumba, A., Narvaez, M., Saenz, L., Ashburner, R. and Harrison, N. (2011) Phytoplasma distribution in coconut palms affected by lethal yellowing disease. *Annals of Applied Biology 159, 109-117.*

Oropeza, C., Escamilla, J.A., Mora, G., Zizumbo, D. and Harrison, N.A. (2005) Coconut lethal yellowing. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources*. International Plant Genetic Resources Institute – Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Pp 349-363.

Oshima, K., Kakizawa, S., Nishigawa, H., Jung, H-Y., Wei, W., Suzuki, S., Arashid, A.R., Nakata, D., Miyata, S., Ugaki, M. and Namba, S. (2004) Reductive evolution suggested from the complete genome sequence of a plant pathogenic phytoplasma. *Nature Genetics 36, 27-29.*

Perera, L., Russell, J.R., Provan, J. and Powel, W. (2000) Use of microsatellite DNA markers to investigate the level of genetic diversity and population genetic structure of coconut (*Cocos nucifera* L.). *Genome 43, 15-21.*

Perera, L., Russell, J.R., Provan, J. and Powel, W. (2003) Studying genetic relationships among coconut varieties/populations using microsatellite markers. *Euphytica 132, 121-128.*

Perera, P.I.P., Perera, L., Hocher, V., Verdeil, J.-L., Yakandawala, D.M.D. and Weerakoon, E.L.K. (2008) Use of SSR markers to determine the anther-derived homozygous lines in coconut. *Plant Cell Reports 27, 1697 – 1704.*

Philippe, R., Nkansah-Poku, J., Fabre, S., Quaicoe, R., Pilet, F. and Dollet, M. (2007) Search for the vector of Cape Saint Paul wilt (coconut lethal yellowing) in Ghana. *Bulletin of Insectology 60, 179-180.*

Philippe, R., Simon, R., Deschamp, S., Nkansah-Poku, J., Quaiceo, R.N., Pilet, F., Fabre, S. and Dollet, M. (2009) Study on the transmission of lethal yellowing in Ghana. *Oleagineux 16, 102-106.*

Quaicoe, R.N., Dery, S.K., Philippe, R., Baudouin, L., Nipah, J.O., Arthur, R., Dare, D., Yankey, E.N., Pilet, F. and Dollet, M. (2009) Resistance screening trials on coconut varieties to Cape Saint Paul wilt disease in Ghana. *Oleagineux 16, 132-136.*

Qiagen (2006) Protocol: Isolation of Total DNA from Plant Tissue using the DNeasy Plant Mini Kit. *DNeasy® Plant Mini and Plant Maxi Handbook: For isolation of DNA from plant tissue.* Pp 24-27.

Rajesh, M.K., Nagarajan, P., Jerard, B.A., Arunachalam, V. And Dhanapal, R. (2008) Microsatellite variability of coconut accessions (*cocos nucifera* L.) from Andaman and Nicobar Islands. *Current Science 94, 1627-1631.*

Rao, V.R., Hodgkins, T. and Bourdeix, R. (2005) Locating coconut genetic diversity. In: Batugal, P., Rao, V.R., Oliver, J (Eds.) *Coconut Genetic Resources.* International Plant Genetic Resources Institute – Regional office for Asia, the Pacific and Oceania (IPGRI-APO), Serdang, Selangor DE, Malaysia. Pp 13-31.

Rao, G.P., Sangeeta, S., Maneesha, S. and Marcone, C. (2007) phylogenetic relationships of sugarcane grassy shoot phytoplasma with closely related agents. *Bulletin of Insectology 60, 347-348*

Razin, S. (1985) Molecular biology and genetics of mycoplasmas (mollicutes). *Microbiological Reviews 49, 419-455.*

Razin, S. (2007) Molecular biology and genomics of mollicutes. *Bulletin of Insectology 60, 101-103.*

Razin, S., Yogev, D. and Naot, Y. (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular biology Reviews 62, 1094-1156.*

Reddy, A.K., Balne, Reddy, R.K., Mathai, A. and Kaur, I. (2010) Loop-mediated isothermal amplification assay for the diagnosis of retinis caused by herpes simplex virus-1. *Clinical Microbiology and Infection 17, 210-213.*

Rivera, R., Edwards, K.J., Barker, J.H.A., Arnold, G.M., Ayad, G., Hodgkin, T. and Karp, A. (1999) Isolation and characterisation of polymorphic microsatellites in *Cocos nucifera* L. *Genome 42, 668-675.*

Rodriguez, A.A.C. and Menezes, M. (2005) Identification and pathogenic characterisation of endophytic *Fusarium* species from cowpea seeds. *Mycopathologia 159, 79-85.*

Rozen, S. and Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S. and Misener, S. (Eds.) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ. Pp 365-386.

Saenz, L., Azppeitia, A., Chuc-Armendarizi, B., Chan, J.L., Verdeil, J.L., Hocher, V. And Oropeza, C. (2006) Morphological and histopathological changes during somatic embryo formation from coconut plumule explants. *In Vitro Cellular and Developmental Biology - Plant 42, 19-25.*

Saitou, N. and Nei, M. (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution 4, 406-425.*

Samuitiene, M., Jomantiene, R., Valiunas, D., Navalinskiene, M. and Davis, R.E. (2007) Phytoplasma strains detected in ornamental plants in Lithuania. *Bulletin of Insectology 60, 137-138.*

Santos, G.A., Batugal, P.A., Othman, A. Baudouin, L. and Labouisse, J.P. (1994) *Manual on Standardised Research Techniques in Coconut breeding*. International Plant Genetic Resources Institute and Coconut Genetic Resources Network. Pp 13-14.

Schaper, U. and Seemuller, E. (1982) Condition of the phloem and persistence of mycoplasmalike organisms associated with apple proliferation and pear decline. *Phytopathology 72, 736-742.*

Schlotterer, C. and Tautz, D. (1992) Slippage synthesis of simple sequence DNA. *Nucleic Acids Research 20, 211-215.*

Schuiling, M., Kaiza, D.A. and Mpunami, A. (1992) Lethal disease of coconut palm in Tanzania. II- History, distribution and epidemiology. *Oleagineux* 47, *516-522.*

Semagn, K., Bjornstad, A. and Ndjiondjop, M.N. (2006) An overview of molecular marker methods for plants. *African Journal of Biotechnology 5, 2540-2568.*

Seemuller, E. and Schneider, B. (2007) Differences in virulence and genomic features of strains of Candidatus Phytoplasma mali', the apple proliferation agent. *Phytopathology 97, 964-970.*

Selkoe, K.A. and Toonen, R.J. (2006) Microsatellites for ecologists: a practical guide for using and evaluating microsatellite markers. *Ecology Letters 9, 615-629.*

Shen, K.A., Meyers, B.C., Islam-Faridi, M.N., Chin, D.B., Stelly, D.M. and Michelmore, R.W. (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in Lettuce. *Molecular Plant-Microbe Interactions 11, 815-823.*

Siddique, A.B.M., Guthrie, J.N., Walsh, K.B., White, D.T. and Scott, P.T. (1998) Histopathology and within-plant distribution of the phytoplasma associated with Australian papaya dieback. *Plant Disease 82, 1112-1120.*

Singh, R., Nagappan, J., Tan, S-G., Panandam, M. and Cheah, S-C. (2007) Development of simple sequence repeat (SSR) markers for oil palm and their application in genetic mapping and fingerprinting of tissue culture clones. *Asia Pacific Journal of Molecular Biology and Biotechnology 15, 121-131.*

Smart, C., Schneider, B., Blomquist, C.L., Guerra L. J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemuller, E. and Kirkpatrick, B.C. (1996) Phytoplasma-specific PCR primers based on sequences of the 16-23S rRNA spacer region. *Applied and Environmental Microbiology, 62:2988-2993.*

Stace-Smith, R. and Hamilton, R.I. (1998) Inoculum threshold of seedborne pathogens: viruses. *Phytopathology 78, 875-880.*

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolution Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution 24, 1596-1599.*

Tan, P.Y. and Whitlow, T. (2001) Physiological response of *Catharanthus roseus* (periwinkle) to ash yellows phytoplasmal infection. *New Phytologist 150, 757-769.*

Teulat, B., Aldam, C., Trehin. R., Lebrun, P. Barker, J.H.A., Arnold, G.M., Karp, A., Baudouin, L. and Rognon, F. (2000) Analysis of genetic diversity in coconut populations from across the geographic range using sequence-tagged microsatellites (SSR_s) and $AFLP_s$. *Theoretical and Applied Genetics 100, 764-771.*

Thomas, D.L. (1979) Mycoplasmalike bodies associated with lethal declines of palms in Florida. *Phytopathology 69, 928-934.*

Thomas, D.L. and Norris, R.C. (1980) The use of electron microscope for lethal yellowing diagnosis. *Proceedings of the Horticultural Society of Florida 93, 196-199.*

Tomlinson, J.A., Barker, I. and Boonham, N. (2007) Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. *Applied and Environmental Microbiology 73, 4040-4047.*

Tomlinson, J.A., Boonham, N. and Dickinson, M. (2010a) Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathology 59, 465-471.*

Tomlinson, J.A., Dickinson, M.J. and Boonham, N. (2010b) Rapid detection of *Phytophthora ramorum* and *P. Kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology 100, 143-149.*

Trkulja, N., Ivanovic, Z., Dolovac, E.P., Dolovac, N., Zivkovic, S., Jovic, J. and Mitrovic, M. (2011) Stolbur phytoplasma infection of kale crops (Brassica oleracea var. Gemmifera L.) in Serbia. *Bulletin of Insectology 64, S81-S82.*

Tymon, A.M., Jones, P. and Harrison, N.A. (1998) Phylogenetic relationships of coconut phytoplasmas and the development of specific oligonucleotide PCR primers. *Annals of Applied Biology132, 437-452.*

Walker, N.J. (2002) A technique whose time has come. *Science 296, 557-559.*

Wei, W., Davis, R.E., Lee, I-M. and Zhao, Y. (2007) Computer-simulated RFLP analysis of 16S rRNA genes, identification of ten new phytoplasma groups. *International Journal of Systematic and Evolutionary Microbiology 57, 1855-1867.*

Wei, W., Lee, I-M., Davies, R.E., Suo, X. And Zhao, Y. (2008) Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. *International Journal of Systematic and Evolutionary Microbiology 58, 2368-2377.*

Wei, W., Shigeyuki, K., Suzuki, S., Jung, H-Y., Nishigawa, H., Oshima, S.M.K., Ugaki, M., Hibi, T. and Namba, S. (2004) In Planta dynamic analysis of onion yellows phytoplasma using localized inoculation by insect transmission. *Phytopathology 94, 244-250.*

Wisler, G.C. and Noris, F. (2005) Interaction between weeds and cultivated plants as related to management of plant pathogens. *Weed Science 53, 914-917.*

Wong, M.L. and Medrano, J.F. (2005) Real-time PCR for mRNA quantitation. *BioTechniques 39, 1-11.*

Xu, Y., Zhu, J., Huang, N. and McCough, S.R. (1997) Chromosomal regions associated with segregation distortion of molecular markers in F_2, backcross, double haploid and recombinant inbred populations in rice (*Oryza sativa* L.). *Molecular and General Genetics 253, 535-545.*

Yankey, E.N., Pilet, F., Quaicoe, R.N., Dery, S.K., Dollet, M. and Dzogbefia, V.P. (2009) Search for alternate hosts of the coconut Cape Saint Paul wilt disease pathogen. *Oleagineux 16, 123-126.*

Yankey, E.N., Swarbrick, P., Dickinson, M., Tomlinson, J., Boonham, N., Nipah, J.O. and Quaicoe, R.N. (2011) Improving molecular diagnostics for the detection of lethal disease phytoplasma of coconut in Ghana. *Bulletin of Insectology 64, S47-S48.*

Zane, L., Bargelloni, L. and Patarnello, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology 11, 1-16.*

Zhao, Y., Wei, W., Davies, E. and Lee, I-M. (2010) Recent advances in 16S rRNA gene-based phytoplasma differentiation, classification and taxonomy. In: Weintraub, P.G. and Jones, P. (Eds.) *Phytoplasmas: Genomes, Plant Hosts and Vectors*. Pp 64-92.

Zhao, Y., Wei, W., Lee, I-M., Shao, J., Suo, X. and Davis, R.E. (2009) Construction of an classification tool, iPhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). *International Journal of Systemic and Evolutionary Microbiology Microbiology 59, 2582-2593.*

Zizumbo-Villarreal., D. and Pinero, D. (1998) Pattern of morphological variation and diversity of *Cocos nucifera* (Arecaceae) in Mexico. *American Journal of Botany 85, 855-865.*

APPENDICES

Appendix 1: Primers used to amplify coconut microsatellite DNA

A. Perrera *et al.* (2000)		
Locus	**Forward primer (5′ – 3′)**	**Reverse primer (5′- 3′)**
CAC2	AGCTTTTTCATTGCTGGAAT	CCCCTCCAATACATTTTTCC
CAC3	GGCTCTCCAGCAGAGGCTTAC	GGGACACCAGAAAAAGCC
CAC4	CCCCTATGCATCAAAACAAG	CTCAGTGTCCGTCTTTGTCC
CAC6	TGTACATGTTTTTTGCCCAA	CGATGTAGCTACCTTCCCC
CAC8	ATCACCCCAATACAAGGACA	AATTCTATGGTCCACCCACA
CAC10	GGAACCTCTTTTGGGTCATT	GATGGAAGGTGGTAATGCG
CAC11	GATCTTCGGCGTTCCTCA	TCTCCTCAACAATCTGAAGC
CAC13	GGGTTTTTTAGATCTTCGGC	CTCAACAATCTGAAGCATCG
B. Perrera *et al.* (2003)		
Locus	**Forward primer (5′- 3′)**	**Reverse primer (5′- 3′)**
CAC20	CTCATGAACCAAACGTTAGA	CATCATATACATACATGCAAA
CAC21	AATTGTGTGACACGTAGCC	GCATAACTCTTTCATAAGGGA
CAC23	TGAAAACAAAAGATAGATGTCAG	GAAGATGCTTTGATATGGAC
CAC39	AATTGAGATAAGCAGATCAGTG	GTCGGTCTTTATTCAGAAGG
CAC65	GAAAAGGATGTAATAAGCTGG	TTTGTCCCCAAATATAGGTAG
CAC68	AATTATTTTCTTGTTACATGCATC	AACAGCCTCTAGCAATCATAG
CAC71	ATAGCTCAAGTTGTTGCTAGG	ATATTGTCATGATTGAGCCTC
CAC72	TCACATTATCAAATAAGTCTCACA	GCTCTCTTTCTCATGCACA
CAC84	TTGGTTTTTGTATGGAACTCT	AAATGCTAACATCTCAACAGC
C. COGENT microsatellite kit (Lebrun *et al.*, 2005)		
CnCirA3	AATCTAAATCTACGAAAGCA	AATAATGTGAAAAAGCAAAG
CnCirA9	AATGTTTGTGTCTTTGTGCGTGTT	TCCTTATTTTTCTTCCCCTTCC TCA
CnCirB6	GAGTGTGTGAGCCAGCAT	ATTGTTCACAGTCCTTCCA
CnCirB12	GCTCTTCAGTCTTTCTCAA	CTGTATGCCAATTTTTCTA
CnCirC3′	AGAAAGCTGAGAGGGAGATT	GTGGGGCATGAAAAGTAAC
CnCirC7	ATAGCATATGGTTTTCCT	TGCTCCAGCGTTCATCTA
CnCirC12	ATACCACAGGCTAACAT	AACCAGAGACATTTGAA
CnCirE2	TCGCTGATGAATGCTTGCT	GGGGCTGAGGGATAAACC
CnCirE10	TTGGGTTCCATTTCTTCTCTCATC	GCTCTTTAGGGTTCGCTTTCT TAG
CnCirE12	TCACGCAAAAGATAAAACC	ATGGAGATGGAAAGAAAGG
CnCirF2	GGTCTCCTCTCCCTCCTTATCTA	CGACGACCCAAAACTGAACC
CnCirG11	AATATCTCCAAAAATCATCGAAAG	TCATCCCACACCCTCCTCT
CnCirH4′	TTAGATCTCCTCCCAAAG	ATCGAAAGAACAGTCACG
CnCirH7	GAGATGGCATAACACCTA	TGCTGAAGCAAAAGAGTA

Appendix 1 cont'd

A. Lebrun *et al.* (2001)		
CnCirA4	ATATAGATGTTGTTGGTTACTGGA	ACCAGCCTTTTCCTTCG
CnCirB3	CATCTTGCTTTTCACCATCC	AATACTGTGCGGTTTTGCTT
CnCirB11	TCTGCATCCCTTCTTTATTA	TTGTCTTTCTTTATTCTATTGG
CnCirC5	ACCAACAAAGCCAGAGC	GCAGCCACTACCTAAAAAG
CnCirC9	CAGAAAGGAGAAAGGAAAT	CTACGATAGAGGAATGAGC
CnCirC11	TGTTATTTTGTTATTTCAGG	TCACCATCTTCTCAGTTTC
CnCirD1	GGGAGGGGAGCGAGACTATG	AATTCAGGCCAACACCAGACC
CnCirD8	GCTCTTGATGTGGCTGCT	AGGCGTGTTGAGATTGTGA
CnCirE1	CTTGTTATGTCGTTTGTTG	CTGAGACCCTGTTGATGT
CnCirF3	TGCCCTACTCCCTCAT	TACCACATAACAGAAACAAGATAA
B. Rivera *et al.* (1999)		
CNZ 24	TCCTAAGCTCAATACTCACCA	CGCATTGATAAATACAAGCTT
CNZ 43	TCTTCATTTGATGAGAATGCT	ACCGTATTCACCATTCTAACA
CNZ 44	CATCAGTTCCACTCTCATTTC	CAACAAAAGACATAGGTGGTC

Appendix 2: Oligonucleotide primers used to amplify RGAs from coconut DNA (Collins *et al.*, 1998).

Conserved amino acid motif	primer name	primer sequence (5'-3')
GVGKTT (P-loop)	P-loop1	AAGAATTCGGNGTNGGNAAAACAAC
	P-loop2	AAGAATTCGGNGTNGGNAAAACTAC
	P-loop3	AAGAATTCGGNGTNGGNAAAACCAC
	P-loop4	AAGAATTCGGNGTNGGNAAAACGAC
	P-loop5	AAGAATTCGGNGTNGGNAAGACAAC
	P-loop6	AAGAATTCGGNGTNGGNAAGACTAC
	P-loop7	AAGAATTCGGNGTNGGNAAGACCAC
	P-loop8	AAGAATTCGGNGTNGGNAAGACGAC
L(I/V/L)VLDDV (Kinase-2)	Kinase-2D	CTACTGNTNCTNGACGACGT
	Kinase-2E	CTACTGNTNCTNGACGATGT
	Kinase-2F	CTACTGNTNCTNGATGACGT
	Kinase-2G	CTACTGNTNCTNGATGATGT
GLPL	GLPL1	AACTCGAGAGNGCNAGNGGNAGGCC
	GLPL2	AACTCGAGAGNGCNAGNGGNAGACC
	GLPL3	AACTCGAGAGNGCNAGNGGNAGTCC
	GLPL4	AACTCGAGAGNGCNAGNGGNAGCCC
	GLPL5	AACTCGAGAGNGCNAGNGGCAATCC
	GLPL6	AACTCGAGAGNGCNAGNGGCAAACC

Appendix 3 LAMP Primers (5'-3') for LD assay (Tomlinson *et al.*, 2010a)

CSPWDF3: TAGAGGAAGGGCCTATAGCTCAGT

CSPWDB3:GTATCGCCGTTAATTGCGTC

CSPWDFIP:TGAATAAGAGGAATATGGTATGGGTGTGGTAGAGCACAC

GCTTGATAAG

CSPWDBIP:TCTCTAATGACACACCAATGAAGGACATCGGCTCTTAGTG

CCAAG

CSPWDFL[A]: GGACTTGAAACCATTGACCG

CSPWDBL[B]:AAGGGCGTACAGTGGATGC

[A] 5'- Labelled with digoxigenin when used with LFD detection of LAMP products

[B] 5'- Labelled with biotin when used with LFD detection of LAMP products

Appendix 4 Photographic documentation of plant species sampled for alternate hosts studies

A. Plant species sampled from Fasin

1. *Acroceras zizanioides*

2. *Securinega virosa*

3. *Selaginella mysorus*

4. *Stachytarpheta cayennensis*

5. *Scleria naumanniana*

6. Asystasia gangetica

7. *Costus afer*

8. *Anthocleista nobilis*

9. *Unknown*

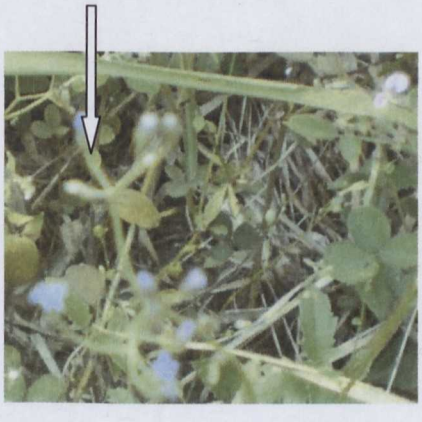
10. *Mimosa pudica*

11. *Paspalum conjugatum*

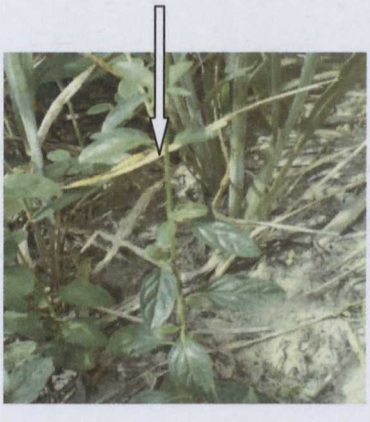
12. *Dissotis rotundifolia*

2. Plant species sampled from Asebu

1. *Vernonia cinérea*



2. *Schwenkia americana*



3. Dioscorea spp

4. *Caricaca papaya*

5. *Portulaca oleracea*

6. *Citrullus lanatus*

7. *Tridax procumbens*

8. *Priva lappulacea*

9. *Ipomoea involúcrate*

10. *Manihot esculenta*

11. *Cyperus difformis*

12. *Justica flava*

C. Plant species sampled from Cape Three Points

1. *Croton lobatus*

2. *Stachytarpheta indica*

3. *Waltheria indica*

4. *Spigelia anthelmia*

5. *Cassytha filiformis*

6. *Lycopersicon esculentum*

7. *Cissampelos mucronata*

8. *Cyperus spp*

9. *Polygonum spp*

10. *Torenia thoursii*

11. *Saccharum officinale*

12. *Vernonia cinérea*

D. Plant species sampled from Aluku

1. *Psidium guajava*

2. *Hoslundia opposite*

3. *Euphorbia hirta*

4. *Vernonia spp.*

5. *Pteridium aquilinium*