

“Survival for the fittest of a species is the total sum effect of accumulated variation produced in each generation better adapted to the environment”

Charles Darwin (1809-1882)

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**Genetic diversity of cotton and bacterial
blight (*Xanthomonas citri* pv. *malvacearum*)
prevalence in Western Kenya**

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Doctor (PhD) of Applied Biological Sciences: Agricultural Sciences

Dutch translation of the title:

Genetische diversiteit van katoen en ras specificiteit van *Xanthomonas citri* pv. *malvacearum* in West Kenia

Illustration on the cover:

Cotton field ready for harvest

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LIST OF ABBREVIATIONS

AFLP – Amplified Fragment Length Polymorphism

ASALs – arid and semi-arid lands

ATC – Agricultural Training Centre

ATP – adenosine triphosphate

Avr – Avirulence

BB – bacterial blight

BC – Before Christ

bp – base pair

BSA – bovine serum albumin

Bt – *Bacillus thuringiensis*

CAN – Calcium Ammonium Nitrate

CDC – Cotton Disease Council

cfu – colony forming units

CODA – Cotton Development Authority

CSIRO – Commonwealth of Scientific, Industrial and Research Organization

ddH₂O – double-distilled water

dHG – desoxyhemigossypol

dpi – days post inoculation

DNA – Deoxyribo Nucleic Acid

dNTPs – deoxynucleoside triphosphates

*EcoR*1 – *Escherichia coli* restriction enzyme 1

ELISA – enzyme-linked immunosorbent assays

ERIC – Enterobacterial repetitive intergenic consensus

EPS – Extracellular polysaccharides

GOT – Ginning outturn

H₂O₂ – hydrogen peroxide

*Hind*III – restriction enzyme (III) obtained from *Haemophilus influenzae*

HVS – Highly virulent strains

GBS – genotyping-by-sequencing

GM – Genetically modified

gyrB – gyrase B

hpi – hours postinoculation

IBPGR – International Board of Plant Genetic Resources

HR –Hypersensitive response

Hrp - Hypersensitive response and pathogenicity

JA – jasmonic acid

KARI – Kenya Agricultural Research Institute

KALRO – Kenya Agriculture Livestock Research Organization

kb – Kilo base

kbp – kilo base pair

KI – KISII

KU – KISUMU

LOX – lipoygenase

LPS – lipopolysacchrides

LR – local resistance

LRR – Leucine rich repeats

MAS – marker assisted selection

Min – minute

mM – Millimolar

MLSA – Multi-locus sequence analysis

*Mse*1 – *Micrococcus* spp.

MLST – Multi locus sequence typing

NCCA – National Cotton Council of America

NCBI – National Centre for Biotechnology Information

NGS – Next generation DNA sequencing

NLS – nuclear localization signals

OD – optical density

PBS – phosphate buffered saline

PCA – principal component analysis

PCR – Polymerase Chain Reaction

PIC – Polymorphic information content

PM – plasma membrane

PR – pathogenesis-related

pv. – Pathovar

QTL – Quantitative Trait Loci

R – Resistance

rep – repetitive extragenic palindromic

RNA – ribonucleic acid

rRNA – ribosomal ribonucleic acid

RAPD – Random Amplified Polymorphic DNA

RFLP – Restriction Fragment Length Polymorphism

ROS – Reactive Oxygen Species

SA – Salicylic Acid

SOD – superoxide dismutase

spp. – species

SAPs – structural adjustment programs

SAR – systemic acquired resistance

SNP – Single nucleotide polymorphism

SSR – Simple sequence repeats

T – TEBERE

TAL – transcriptional activator like

T3SS – type three secretion system

YDC – yeast extract-dextrose- CaCO_3

YEB- Yeast extracts broth (media)

USA – United States of America

USDA-FAS – United States Department of Agriculture – Foreign Agriculture Service

Xcm - *Xanthomonas citri* pv. *malvacearum*

Xcc – *Xanthomonas citri* pv. *citri*

µg – microgram

µL – microlitre

µM – micromolar

°C – degree centigrade

CHAPTER 1

INTRODUCTION, PROBLEM STATEMENT, OBJECTIVES AND OUTLINE



1.1. INTRODUCTION

Upland cotton (*Gossypium hirsutum* L.) is the world leading fibre crop accounting for 90% of commercially grown cotton (Liu et al., 2015). China, India, Pakistan and the USA are the four leading producers and account for over 70% of world production. For 2014/2015, global production was estimated at approximately 119 million (M) bales of cotton (218 kg/bale), obtained from an area of 34.02 M ha globally, giving an average yield of about 762 kg/ha (United States Department of Agriculture-Foreign Agricultural Service [USDA-FAS], 2016).

Cotton is primarily grown for lint which is the unicellular outgrowth of the seed coat. Harvested as seed cotton then ginned to separate seed and lint. Long lint fibres are processed by spinning to produce yarn that is knitted into fabrics. The lint is the driving force behind most textile industries worldwide. By-products such as linters, cotton husks, cotton seed oil, etc. have various uses in many industrial applications. Cotton has played a crucial role in the development of the economy in Kenya. It performed a major role in employment creation through cultivation of the crop and in fabrics manufacture in textile and allied industries during the 1970/80s. These were the years the sector experienced tremendous production growth (Fig. 1.1) with production peaking in the mid-1980s annually exceeding 70,000 bales of lint (Ikitoo, 2008; Ingram, 2005; The Cotton Apex Committee, 2006). In the 1990s, the cotton sector was liberalised with the introduction of structural adjustment programmes (SAPs) by the International Monetary Fund (IMF) and World Bank. This starved the sector of much needed government support through provision of funds for cotton research and development leading to the near collapse of the cotton sector (Cotton Development Authority [CODA], 2009; The Cotton Garment Apex Committee, 2006). Overall, cotton production declined substantially since adoption of SAPs. Figure 1.1 gives production and harvest area of cotton in Kenya from years 1963 to 2014.

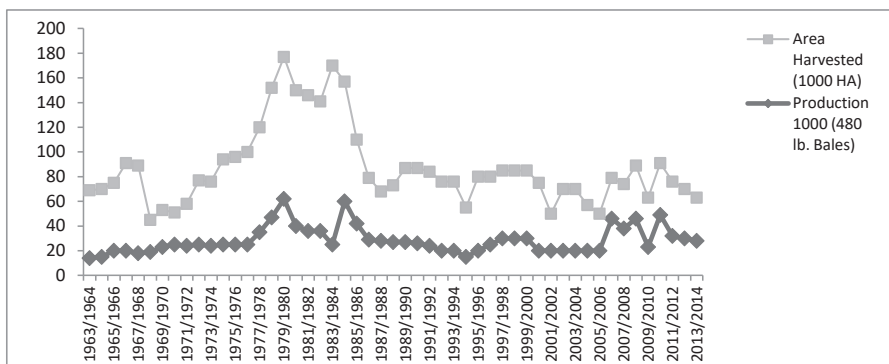


Figure 1.1: Trend in cotton production in Kenya and harvested area since independence (1963 – 2014) (based on data from USDA-FAS, 2014)

The decline of the sector coincided with a rise in the poverty index in the areas that depended on cotton (CODA, 2009; The Cotton Garment Apex Committee, 2006). The government, in an effort to improve the welfare of the people in the subsequent years, realised the need to revive the cotton sector if any meaningful poverty reduction were to be achieved. In 2006, the government started to improve production through the passage of the bill that established the Cotton Development Authority (CODA, 2008). CODA bill was passed to resuscitate the sector by providing structures to ensure farmers are provided with certified cotton seeds and a ready market for their produce. The revival was necessary to ensure increased production and saving the country the cost of importation. The current average annual production (2014/2015) stands at approximately 32,000 bales (USDA-FAS, 2016) implying that close to 168,000 bales of cotton lint have to be imported to meet the country’s requirements for the textile industry which currently stand at 200,000 bales per annum (CODA, 2012; USDA-FAS, 2014).

This revival effort is captured in the government policy strategy “Kenya vision 2030” for addressing poverty (CODA, 2009). The argument behind the policy initiative is that the sector has a crucial role in reduction of poverty and creation of employment with resultant increase in income especially in rural areas. Knowing that approximately 50% of Kenyans live below the poverty line and 40% are unemployed, the need for Kenya to improve its entire cotton supply chain cannot be overemphasised (CODA, 2009). There are still a lot of challenges in cotton that need to be addressed, such as unreliable rains, and poor farmer knowhow, competition from food crops (farmers prefer to plant food crops first, and then they revert to cotton) where cotton is not

planted as a priority crop (it gets less attention during the growing season), restoring the linkages between all stakeholders of the cotton chain, putting up control mechanisms for seed and lint quality, intensifying training and extension services, along with upgrade of research on improved varieties and management of pests and diseases (CODA, 2009; ICAC, 2010; The Cotton to Garment Apex Committee, 2006).

Globally, yield loss in cotton due to biotic stresses can be as high as 80-90% if no interventions such as use of resistant varieties, chemical control of pest and diseases, etc. are put in place (Table 1.1). In Africa, data on crop loss is available for only a few countries and in some cases dates back to 1948 (Table 1.1). Clearly pest and disease management is still a major production constraint in many developing countries especially in Africa and Asia (Dhaliwal & Arora, 1996; Ikitoo et al., 2008). Determination of present status of pest and disease in respective countries would help identify appropriate research, policy as well as cultural practices to minimise yield losses.

A preliminary survey in 2008 by Moi University-VLIR-UOS project team in Western Kenya involving 30 households in the counties of Siaya and Busia showed a major disconnect among key players (farmers, extension officers, ginners, local administration, etc.) in effort to address problems affecting the cotton sector. During the survey, farmers were interviewed and asked to identify what would be the major challenge affecting cotton. Apart from issues of markets, impact of second-hand clothes, extension service, etc. affecting the sector many pointed to the pest and disease concern. Yet, in terms of research to address pests/diseases, very little is being done. Clearly, the need for research to improve current varieties was pointed out by most farmers. Additionally, during the visit typical symptoms of bacterial blight (BB) were observed on many farms, but when farmers were asked to identify it, many could not relate the symptoms with the disease. Thus, great need to investigate this seemingly major challenge cannot be underestimated by stakeholders if real revival of the cotton sector and subsequent poverty reduction is to be achieved in cotton growing regions. Development of disease/pest resistant varieties is the most sustainable approach. The need to breed varieties that are more productive and resistant to major diseases and pests is paramount (Ikitoo et al., 2008). It is on the basis of this background that Moi University-VLIR-UOS project (Agriculture and Biotechnology [AGBIO] project) in synergy with relevant government agencies partnered to carry out research on cotton to boost productivity in Western Kenya in effort to improve the country's cotton sector.

Table 1.1: Percentage cotton yield loss due to pest and disease infestation

Pest/disease	Country	% yield loss	Reference
Bacterial Blight (<i>Xanthomonas citri</i> pv. <i>malvacearum</i>)	India	5-20	Verma & Singh, 1971
		50	Verma 1986; Raghavendra et al., 2009
		23-25	Meshram & Raj, 1992
		19-27	Meshram & Raj, 1988
		30	Ramapandu et al., 1979
	Sudan	20	Last, 1960
		64	Knight, 1948
	Tanzania	49	Arnold, 1960
	Kenya	60	Wickens, 1961
	USA	34-59	Leyendecker, 1950; Bird, 1959
		18.6	Bird, 1952
		3.7	Blasingame, 1990
		9-34	Bird, 1959
		1.5	Hillocks, 1992; El-Zik & Thaxton, 1995
	Asia (China, Russia, Pakistan)	20-30	Tarr, 1959 & 1972.
Verticillium wilt (<i>Verticillium dahlia</i>)	USA	1.46-3.48	Bell, 1992
	Russia	25-30	Bell, 1992
	Uzbekistan	80	Mukhamezhanov, 1966
Grey mildew (<i>Ramularia areola</i>)	India	30	Chidambaram & Kannan, 1989
<i>Alternaria</i> blight (<i>Alternaria macrospora</i>)	India	26	Chattannavar et al., 2006
Pests			
American bollworm (<i>Helicoverpa armigera</i>)	India	20 – 80	Monga & Jeyakumar, 2002
Spotted bollworm (<i>Earias vittella</i>)	India	30 – 40	Panwar, 1995
Pink bollworm (<i>Pectinophora gossypiella</i>)	India	20 – 95	Panwar, 1995
Cotton jassid (<i>Amrasca</i> spp)	India	9.2	Balasubramanian & Chelliah, 1985

NB: Data source: <http://www.plantwise.org/KnowledgeBank/>

1.2. PROBLEM STATEMENT AND JUSTIFICATION

Provided with the above background, this thesis is a research effort to address bacterial blight (BB) disease, a key challenge of cotton production in Kenya. BB caused by *Xanthomonas citri* pv. *malvacearum* (*Xcm*) is a disease with global economic importance especially in hot and humid growing regions including some parts of Kenya (Chapter 3: Fig. 3.1) (Bayles & Varhalen,

2007; Delannoy et al., 2005). Clearly, many farmers in the Western Kenya with similar weather conditions lack information on the symptoms of the BB disease and it could be one of the major factors leading to low yield in cotton.

The western region has continued to grow cotton even during the years that other areas had abandoned it. Reportedly, BB continues to plague cotton production globally with many researches focused on addressing it (Akello & Hillocks, 2002; Ajene et al., 2014; Essenberg et al., 2014; Jalloul et al., 2015; Xiao et al., 2010; Zhai et al., 2013), but in Kenya only a few studies have reported the problem of BB disease in cotton-growing areas since its collapse in 1990s. No information on diversity of *Xcm* present in the country's cotton fields exists. Meanwhile, visits to farmers' fields reveal that this problem is still prevalent despite of the earlier efforts done in the 1980s that led to the development of cultivars considered to be BB-resistant (cultivars KSA 81M and HART 89M grown west and east of the rift valley of Kenya, respectively). The question therefore is: since BB disease still persists in the cotton fields of Western Kenya after commercialisation of cultivars believed to be resistant which were developed in the 1980s, therefore, could the present susceptibility be due to newly introduced pathovars or variants within original races capable of breaking inherent resistance? Hence our aim is to characterise the diversity of *Xcm* present in the fields of Western Kenya. Additionally, it is known that genetic resources that are locally adapted to environmental conditions are desirable for breeding purposes (Xiao et al., 2010). Thus, determination of genetic resources within the country that can withstand *Xcm* races present in the region that can be utilised to improve commercial cultivars would be a valuable input.

In an effort to address these issues in cotton, isolation of the pathogen from the host and determination of prevalent BB races in Kenya is crucial in the development of strategies to address BB disease problem. Pathovars within each *Xanthomonas* species or among xanthomonads cannot reliably be distinguished by their cellular or phenotypic characteristics (Louws et al., 1994; Mhedbi-Hajri et al., 2011). However, polymerase chain reaction (PCR)-based tools give the possibility to distinguish them. For instance, *gyrB* sequences have been utilised to distinguish *Xanthomonas* strains from other bacterial species present in food, feed and diseased crops (Coenye & Lipuma, 2002; Mondal et al., 2013; Parkinson et al., 2007; Slack et al., 2006). Additionally, to avoid potential bias in genetically characterising a pathogen population, neutral markers such RFLP, AFLP, rep-PCR, etc. have been developed. Reliable

characterisation methods are obviously essential in the determination of pathogen diversity (Arshiya et al., 2014; Lee et al., 2008; Versalovic et al., 1991, 1994; Woods et al., 1992).

In addition, sequence analysis of bacterial strains can be used to determine sequence similarities and differences among strains. Sequence-based classification provides a means for identifying both these potential new pathogens as well as established pathogens to improve the monitoring and surveillance (Aritua et al., 2015; Adriko et al., 2014; Parkinson & Elphinstone, 2010). Therefore, sequence-based approaches will play a greater role in the long run in monitoring pathogen diversity. Additionally, sequence-based approaches will help in effort to control bacterial diseases through better targeting of control measures for pathogens invading new regions or to meeting specific threats posed by more aggressive strains (Parkinson & Elphinstone, 2010; Triplett et al., 2015).

Studies to understand the present status of cotton BB in the country in relation to the diversity of cotton germplasm would also help to understand the BB disease challenge and guide on strategies to address it. Screening different cotton lines for their resistance to the pathogen can be very useful in identifying genotypes with potential BB resistance. Several BB resistant genes (termed *R* or *B*) have been identified in cotton, some with major and others with minor effects (Essenberg et al., 2014; Hillocks, 1992; Verma, 1986; Xiao et al., 2010). Identification of some of these *B*-genes among local germplasm will greatly help in prioritising breeding objectives targeting specific strains available in particular regions. Towards this end, this study also focused to establish information on the degree of susceptibility of different Kenyan cotton accessions to BB present in Western Kenya. Bacterial blight disease resistance mechanisms are based on quantitative genetic systems and identification of such resistance genes among local cotton genotypes is essential (Xiao et al., 2010). Molecular tools will definitely play a role in identification of such resistance markers and therefore an attempt is needed to identify possible major/minor *B*-genes associated with BB disease resistance/susceptibility among Kenyan cotton genotypes.

The present commercially grown cotton cultivars in Kenya were developed prior to the near collapse of the cotton sector in the late 1980s using conventional breeding strategies. These conventional methods are based on phenotypic and morphological traits which are prominently influenced by environmental and epistatic factors. As such, there is a need to carry out more evaluations on available accessions to identify those with superior traits that could be incorporated in breeding programmes. This will result in the development of improved varieties

adapted to the local environment to address present production challenges characterised by low yields due to poor cultivars.

In the past, repeated breeding and evaluation on the basis of morphological and agronomical traits have remained the methods of choice in cotton improvement programmes in the country. This approach may have resulted in a narrowing of the cotton gene pool with resultant low genetic variability among existing accessions. Therefore, our second hypothesis was, Kenyan cotton germplasm utilised in breeding programmes exhibit limited diversity. If so, the second question is: can we identify new and more diverse sources of BB resistance among Kenyan germplasm that could be used by researchers to improve the performance of commercial varieties?

The deployment of molecular marker analysis to determine diversity in available germplasm is important to ascertain its variability for crop improvement (Iqbal et al., 2001; Allen & Auld, 2002). This thesis therefore also assessed the diversity of cotton germplasm conserved in Kenyan genebanks using amplified fragment length polymorphism (AFLP) analysis. Morpho-phenotypic evaluation although being influenced by environmental factors can still provide a general overview of the phenotypic variability available in sampled genotypes. To gain insight in actual genetic variability, AFLP was used to generate large numbers of polymorphic DNA fragments, since AFLP analysis has also been applied in cotton to tag genes associated with resistance to fungal wilt diseases. In the same context, an attempt was made to identify AFLP markers related to BB disease to enhance and speed up the screening and breeding of cotton cultivars. The relationship between diversity of cotton germplasm with the earlier study on the present status and prevalent races of *Xcm* pathogens present in the region will enhance the development of strategies for breeding of cotton cultivars with more durable BB resistance. Successful breeding programmes addressing these challenges in cotton in Kenya should improve cotton productivity.

1.3. RESEARCH OBJECTIVES

This study addresses the following objectives:

- Establish the present status of bacterial blight in the cotton fields of Western Kenya.
 - Evaluate cotton genotypes grown under field and greenhouse conditions to establish BB susceptibility/resistance within the Kenyan genotypes; and

- identify the sources of BB resistance in the Kenya cotton germplasm for utilisation in improvement programmes.
- Characterise the BB pathogen *Xanthomonas citri* pv. *malvacearum* (*Xcm*) collected from cotton fields of Western Kenya.
 - Isolate and utilise PCR-based gyrase B (*gyrB*) gene sequence primers to distinguish *Xanthomonas* bacteria isolated from infected cotton leaves;
 - determine the *gyrB* sequence variation among collected isolates from Western Kenya to determine phylogenetic grouping among field strains;
 - determine the diversity of the *Xcm* isolates collected from Western Kenya based on rep-PCR methods of BOX and ERIC analysis; and
 - establish the *Xcm* races present in the fields of Western Kenya using a standard pathogenicity test on cotton differential varieties.
- Genotype the cotton germplasm conserved in genebanks of Kenya based on DNA markers.
 - Establish the genetic diversity of the genotypes using AFLP markers and determine variability of accessions for potential use of local germplasm in cotton improvement programmes in Kenya; and
 - identify AFLP markers associated with bacterial blight disease of cotton.

1.4. THESIS OUTLINE

The thesis starts with **Chapter 1** giving a brief introduction on the subject, problem statement and justification, and specific objectives the study undertakes to address.

Chapter 2: provides a comprehensive literature review giving a historical overview of cotton, domestication, production, importance, and biology along with diversity status of cotton and methods currently utilised in the effort to improve this crop. It also provides a background review of BB disease in cotton with key subjects such as pathogen (*Xanthomonas*) characteristics, recent advances in understanding of *Xanthomonas* characteristics with respect to methods of classification, characterisation and identification; host-pathogen interaction of *Xcm* and its accompanying resistance and avirulence genes responsible for varying resistance and pathovar specificity in cotton. The present advances in *Xcm* studies using molecular tools are highlighted to show the importance of identifying and characterising the xanthomonad species based on these molecular techniques.

Chapter 3: presents field and greenhouse evaluation of a number of Kenyan cotton genotypes. This chapter aims to establish the prevalence of BB in farmers' fields and attempts to identify the potential sources of resistance within Kenyan cotton germplasm that could be utilised for improvement of commercially grown cotton cultivars.

Chapter 4: *Xanthomonas* bacteria are genotyped with PCR-based methods to distinguish them from the other bacterial cultures and determine their diversity. Due to challenges with isolation of xanthomonads using conventional methods such as characteristics of cellular and morphological traits from other bacterial isolates found in cotton, it was important to use PCR approaches based on *gyrB* sequence primers. The isolates confirmed as belonging to *Xanthomonas* by *gyrB* primers were further studied with rep-PCR of BOX and ERIC to determine the diversity of the isolated strains. In addition, the isolates *gyrB* DNA fragments were sequenced to determine the phylogenetic groups based on sequence data. Finally, the strains were evaluated based on standard pathogenicity tests to determine the prevalent pathotypes/races present in the region. A pathovar is a bacterial strain or set of strains with the same or similar characteristics, that is differentiated at infrasubspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant hosts.

Chapter 5: molecular evaluation of cotton germplasm conserved in Kenyan gene banks is reported in this chapter. The AFLP method is utilised because of its suitability to yield high levels of polymorphism. Potential variability in genotypes is explored for improvement of existing commercial cotton cultivars as their performance has been worrying due to their low yield and susceptibility to pests and diseases.

Chapter 6: finally, this chapter summarises the main findings, achievements and the implications of the findings and prospects for further research.

CHAPTER 2: LITERATURE REVIEW

BIOLOGY OF COTTON, GENETIC DIVERSITY AND BACTERIAL BLIGHT DISEASE

2.1 ORIGIN AND PRE-HISTORIC COTTON UTILISATION

Cultivation of cotton (*Gossypium* L.) is very ancient (Kohel & Lewis, 1984). The moment (in time) when humans started using cotton is not known. However, it is a fact that early civilisations in both the eastern and western hemisphere of the world already used cotton. Scientists have excavated cotton fibre and boll fragments from Tehuacan valley in Mexico established to be about 7000 years old (Smith & MacNeish, 1964). In the Indo-Pakistan region, cotton fabric dated back to as early as 3200 BC has been found at Mahenjo-Daro on the banks of the Indus River (Brite & Marston, 2013; Wendel et al., 2010).

There are wild species of *Gossypium* in all continents, except for Europe and Antarctica. The old world cotton probably originated somewhere in the southern half of Africa and then spread eastwards. New world cotton is understood to have originated in Peru, Ecuador and Colombia region and its utilisation in this area is considered to be very ancient (Endrizzi et al., 1985).

2.2 TAXONOMY AND DISTRIBUTION OF COTTON

2.2.1 Taxonomy and evolution of the commercial *Gossypium* species

Cotton belongs to the genus *Gossypium*, order Malvales and family Malvaceae (Smith, 1995). Genus *Gossypium* comprises 50 species with basic chromosome number $n=13$ (Poehlman & Sleper, 1995). Of the known species, 45 are diploid ($2n=2x=26$ chromosomes), grouped into eight genomes A, B, C, D, E, F, G, and K (Percival et al., 1999; Endrizzi et al., 1985). Diploid species with A, B, E, and F genomes are African or Asian in origin, whereas species with C, G and K genomes are Australian in origin. Diploid species containing a “D” genome originated in the western hemisphere (Central and South America) and are referred to as new world species (Endrizzi et al., 1985). There are five allotetraploid species ($2n=4x=52$ chromosome), containing “AADD” genomes, four native to continental America and one to Hawaii. The word “cotton” is used to refer to all species but when information refers to a particular species in this thesis its species name will be mentioned.

According to Wendel et al. (1992), the new world allotetraploid species arose some 1-2 million years ago (MYA) through hybridisation of an old world taxon “A-genome” with a taxon of the “D-genome” (Fig. 2.1).

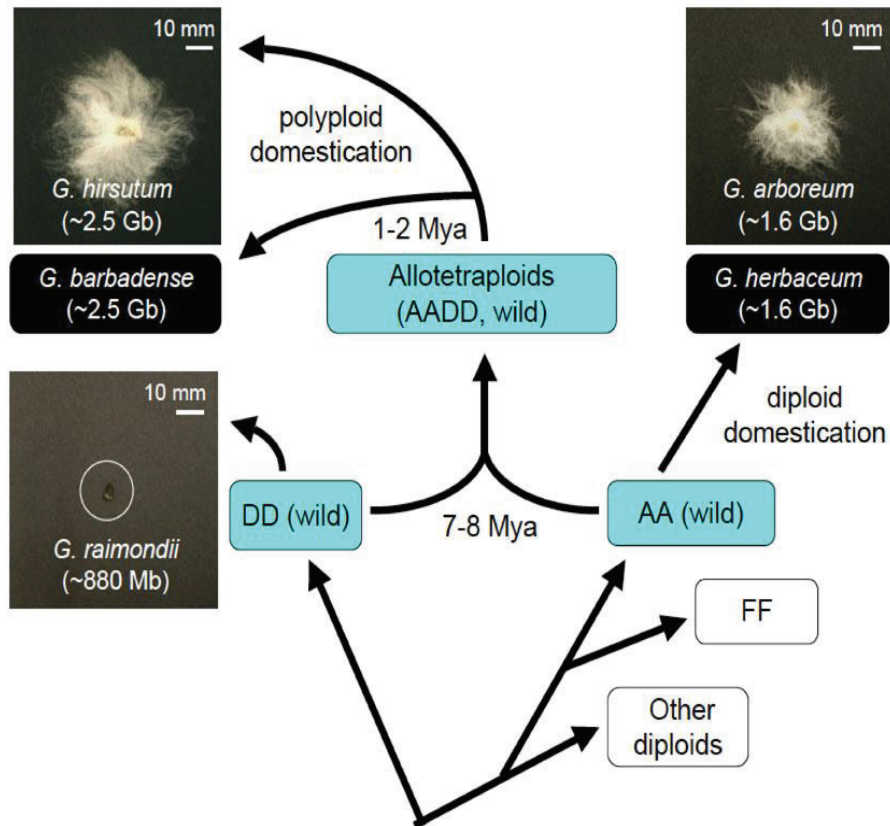


Figure 2.1: Formation of allotetraploid cotton. Extant diploid progenitors diverged 7-8 millions years ago (Mya), and allotetraploidisation occurred naturally 1-2 Mya between a fibre-producing AA-genome extant species and a fibre-poor DD-genome extant species, generating AADD allotetraploid species. Superior fibre yield and quality have been selected in allotetraploid cotton species, as well as in the domesticated diploid *G. arboreum*. The genome sizes in parentheses are cited from Hendrix & Stewart (2005). Mbp: mega base pairs (10^6); Gbp: giga base pairs (10^9); adopted from Cotton – NSF cotton Fibre Genomic Projects (2011), accessed 25 February 2016, > <http://cottongenomics.biosci.utexas.edu/project/cotton.php>>

Smith (1995) reported the origin of allotetraploids to be in the new world, probably Mexico, Peru, Brazil, and American Islands of Hawaii and Galapagos. Among the 45 diploid species, only two (*Gossypium [G] arboreum* L. and *G. herbaceum* L.), and of the five allotetraploid

species, two (*G. hirsutum* L. and *G. barbadense* L.) produce spinnable fibres on their seed coats and are cultivated commercially.

G. barbadense L. originated in Central and South America, and accounts for about 8% of world cotton fibre (Zhang et al., 2008). It produces fibres of the highest quality with superior extra-long, strong and fine cottons. In the USA, it was formerly cultivated along the coast islands of South Carolina and Georgia, so it became known as “Sea Island cotton”. It was introduced to the Nile valley of Egypt, where it became known as “Egyptian cotton”. Nowadays, in USA, *G. barbadense* is widely grown on irrigated fields in Arizona, New Mexico and Western Texas and is generally known as “American Pima”. Other important producers of this species are Egypt, Sudan, and Peru (Todou & Consala, 2011).

G. hirsutum L. originated in southern Mexico and Central America. In the USA, it became known as “American upland cotton” because it was cultivated successfully on high elevations where Sea Island cotton could not mature in time. It is the most important agricultural cotton accounting for about 90% of the global cotton production (Cantrell, 2005; Freeland et al., 2006; Ikitoo, 2008; Peeters et al., 2001; Zhang et al., 2008).

The cultivated diploid species, *G. arboreum* L. originated in the Indo-Pakistan subcontinent and *G. herbaceum* L. originated in southern Africa and both are called “Old World” “Asiatic” or “Desi” cottons. These two species have short-staple fibres with lower commercial value and currently account for about 2% of global production. *G. arboreum* is mainly cultivated in the drier parts of India and Pakistan (Brink, 2011; Jimu, 2011). *G. arboreum* can also be found in tropical Africa; however, it is mainly restricted to gardens and abandoned dwelling places (Jenkins, 2003).

2.2.2 Current production and geographical distribution

Although the cotton plant is native to the tropical agro-ecological zones, cotton production is no longer restricted to the sole tropics. Cotton is cultivated in any region having a sufficiently warm climate of above 15 °C for at least 3-4 months of a specific period of the year (Freeland et al., 2006; Peeters et al., 2001). The present production region stretches from approximately 47° North latitude (China, Bulgaria, Uzbekistan, Ukraine) to 35° South latitude (northern Argentina and Australia), as illustrated in Figure 2.2 (Cantrell, 2005; Peeters et al., 2001). Between these two extremes, cotton is grown in about 80 countries by over twenty million farmers (Cantrell, 2005; Kantartzi & Stewart, 2009; USDA-FAS, 2016). China, India, Pakistan and the USA are

the four leading producers and account for over 70% of global production. For 2015/2016, world production is estimated at approximately 101 million (M) bales of cotton (480 lb or 218 kg/bale (Fig. 2.2) (USDA-FAS, 2016).

2015/2016 Cotton Production

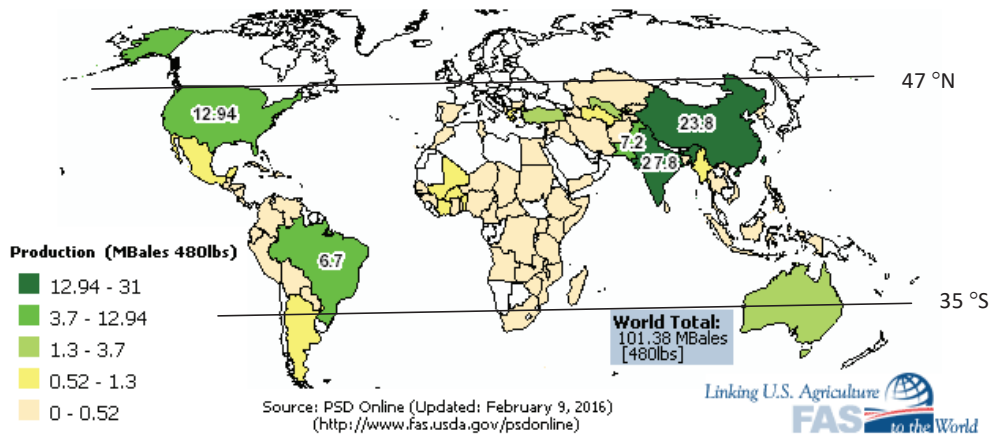


Figure 2.2: Map of world cotton production and distribution which is mainly restricted between latitude 47 °N and 35 °S (USDA-FAS)

2.3 BIOLOGY OF COTTON

Depending on the variety and growing conditions, it takes 4 to 7 months (120-220 days) before cotton is ready to harvest (Ikito, 2011). Three different developmental stages can be distinguished: the first stage lasts from germination until full cotyledon development (duration: 5 to 15 days), the second stage is the period of vegetative growth (50 to 65 days) and the third is the reproductive stage (85 to 140 days) (Peeters et al., 2001).

2.3.1 Cotton growth and development

The cotton plant by nature is a perennial shrub, but is commercially grown as an annual crop (Ikito, 2011; Peeters et al., 2001). The present cultivated species grow to a height of approximately 1.0 – 1.5 m, while in nature, *G. hirsutum* and *G. barbadense* species can reach 1.5 – 2.0 m and 3.0 m, respectively (Australian Government, 2008).

Growth of the cotton plant starts with germination. Germination begins with the entry of moisture into the seed and embryo via the chalazal aperture at the seed's apex (Christiansen & Moore, 1959). The seed/embryo then begins to swell as it absorbs moisture. Under favourable

conditions (temperature $>15^{\circ}\text{C}$), the radicle (root tip) emerges within 2-3 days from the seed and newly germinated seedlings emerge above the soil 5-6 days after emergence of the radicle (Oosterhuis & Jernstedt, 1999). The first true leaf appears 10-12 days after emergence and leaf development reaches its peak about three weeks after the first buds are formed (Ikitoo, 2011; Peeters et al., 2001; Australian Government, 2008). The shoot system of the plant is dimorphic because it forms branches that are either monopodial (with leaves only) or sympodial (with flowers) (Ikitoo, 2011; Peeters et al., 2001; Purseglove, 1974; Ritchie et al., 2007). The first flower/bud appears on the lowest fruiting branch 35-45 days after emergence, depending upon prevailing temperatures. The other flower buds follow at regular intervals until shortly before flowering ceases. The time taken between the appearance of the first flower bud and opening of the flower may be between 25-30 days (Ikitoo, 2011; Peeters et al., 2001; Ritchie et al., 2007). A “pinhead” square is the first stage at which the square (flower bud) can be identified. The next stage of square growth is “match-head” or “one-third grown” square. Prior to the time the flower opens, a candle shape can be seen (Fig. 2.3).

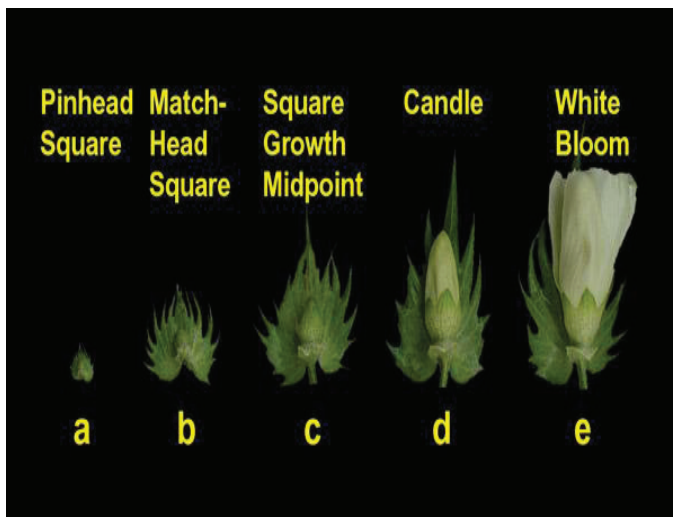


Figure 2.3: Development of the cotton flower bud from match head square (a) to flower (e) involves both a size increase and petal development. Two bracts have been removed from each square, candle and bloom to show this development; accessed 17 February 2016, <http://www.soilcropandmore.info/crops/CottonInformation/B1252/b1252.htm>

Emergence of a large number of flowers is seen for a certain period and thereafter it declines. During the peak period of flowering, vegetative growth is almost negligible and once the rate of flowering declines vegetative growth restarts. This constitutes rank growth commonly observed in cotton. A cotton plant typically blooms or flowers for about 6 weeks (Ikitoo, 2011; Peeters et al., 2001; Kartanзи and Stewart, 2009). The flowering period is reduced by late sowing, strong plant competition and moisture stress. The flowering phase is greatly influenced by environmental factors. Flowering in cotton is sensitive to both thermo- and photoperiods (Dumka et al., 2004) although current hybrids are bred for photoperiod insensitivity. In most varieties, boll bursting begins 140 days after shoot emergence (Peeters et al., 2001). The length of each developmental phase differs, depending upon the species, varieties and weather conditions as well as the cultivation techniques such application of irrigation, etc. implemented (Australian Government, 2008; Ikitoo, 2011; Peeters et al., 2001). Figure 2.4 summarises the developmental stages in cotton which overlap between the stages influenced by environmental factors and management practices.

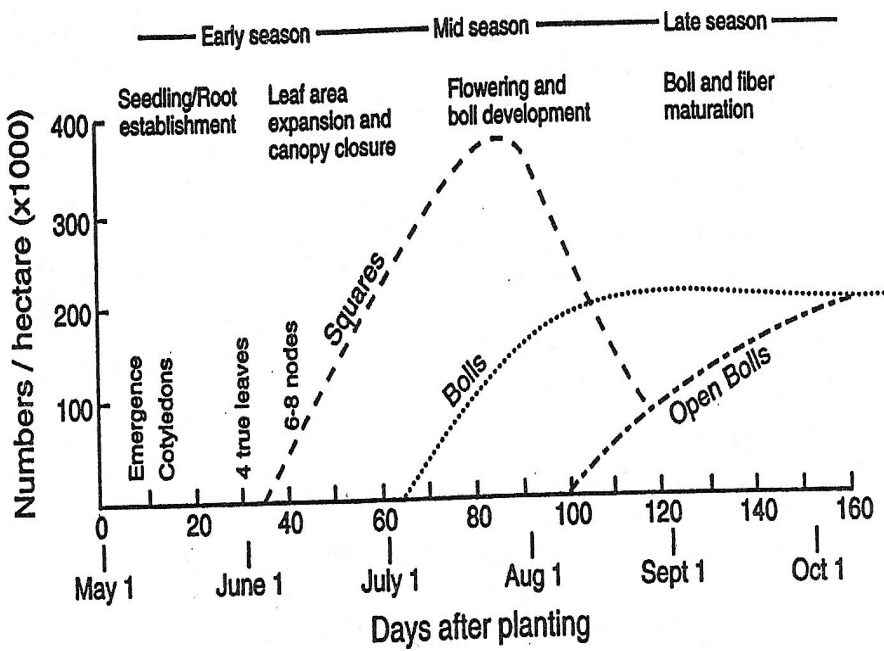


Figure 2.4: Developmental stages in cotton (Oosterhuis & Jernstedt, 1999)

2.3.2 Floral biology

Cotton flowers are extra-axillary, terminal and solitary and are borne on sympodial branches. The cotton plant produces perfect flowers, meaning the flower contains both male and female organs (Fig. 2.5). The flower is subtended by involucre (group of one or more whorls of bracts beneath a flower) of usually three unequal leaf-like bracts. Bracteoles, alternating with the bracts on the inside of the involucre or standing on either side of the small bract, may be present. The calyx, consisting of five undiverged sepals, is persistent and shaped as a shallow cup. The calyx adheres tightly to the base of the boll as it develops. In *G. hirsutum*, the corolla is usually creamy-white coloured, while *G. barbadense* has yellow flowers mostly with a red or purple spot near the base of the petals (Kantartzi & Stewart, 2009; Peeters et al., 2001; Purseglove, 1974). A staminal column surrounds the style and is covered with numerous filaments that terminate in a two-lobed anther (Kantartzi & Stewart, 2009; McGregor, 1976). The stigma of *G. barbadense* is elevated well above the anthers, which is normally not that clearly observed in *G. hirsutum*. The ovary is composed of 3 to 5 locules, each containing about 5 to 15 ovules (Kantartzi & Stewart, 2009; Oosterhuis & Jernstedt, 1999). An average of 8 ovules per locule develops into mature seeds. A cotton flower has 3 floral and 3 extra-floral nectarines located at the base of the bracts, on the inside and the outer side, respectively.

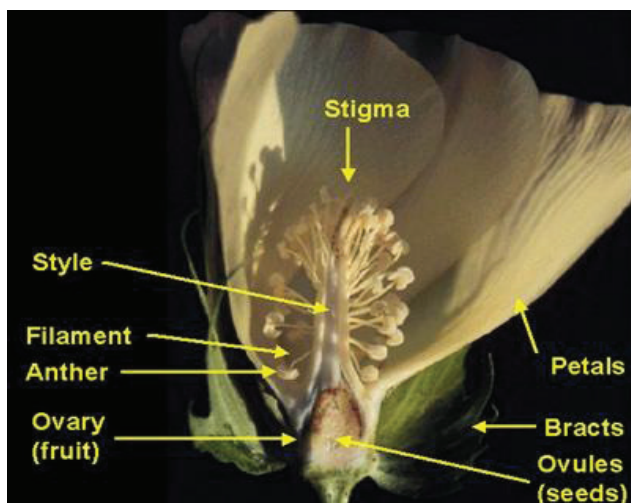


Figure 2.5: A cross-section of a mature cotton flower. The cotton flower contains both male and female parts; accessed 12 February 2016, <<http://www.aganytime.com/Pages/Article.aspx?name=Cotton-Growth-and-Development&fields=article&article=188>>

The stamens are numerous and united to form a tubular sheath which surrounds the pistils except for the exposed portion of style and stigma at the tip. The pistil consists of 3-5 undiverged carpels corresponding to the locular composition of a fully mature dehiscent boll. The ovules are attached to the parietal placenta (placentas are in the ovary wall within a non-sectioned ovary) of each locule. The style varies in length and splits near the apex into three, four or five parts depending on the number of carpels (Kantartzi & Stewart, 2009; Oosterhuis & Jernstedt, 1999). On the first day after anthesis, the corolla changes into pinkish blue and then into red during succeeding days. It withers and falls off on the 3rd day onwards depending on the weather conditions (Fig. 2.6).



Figure 2.6: Development of a cotton bloom. A white flower emerges on day 1 (a), then gradually darkens and takes on a red colour during days 2, 3 and 4 after emergence (b and c). The bloom eventually dries up and either falls off or becomes a bloom tag (d); accessed 15 February 2016, <<http://www.soilcropandmore.info/crops/CottonInformation/B1252/b1252.htm>>

2.3.3 Pollination and fertilisation

Cotton pollen is relatively large, heavy, sticky and watery and thus wind is not a factor in the pollination of cotton (Ikitoo, 2011; Peeters et al., 2001; Kantanzi and Stewart, 2009). Cross-pollination in cotton may vary from zero to more than 20 percent. Many insects, especially

honey bees (*Apis mellifera* L.), are attracted to the cotton flowers and they are active in cross-pollination. Pollination takes place usually in the morning during opening of the flowers and anther dehiscence (Beasley, 1975). Fertilisation takes place between 24-30 hours after pollination. The corolla drops from the fertilised ovary exposing young bolls (Ikitoo, 2011; Kantartzi & Stewart, 2009; Oosterhuis & Jernstedt, 1999; Purseglove, 1974; Ritchie et al., 2007).

2.3.4 Boll development

After pollination the boll begins to develop. Initially, boll development is slow but later growth rate is rapid and steady. Under optimum conditions it requires approximately 45-50 days for a boll to “open” after pollination (Ikiito, 2011). Boll development can be characterised by three phases: enlargement, filling, and maturation (Ikitoo, 2011; Kantartzi & Stewart, 2009; Peeters et al., 2001; Purseglove, 1974; Ritchie et al., 2007).

The enlargement phase in boll development lasts approximately 3 weeks. During this time the fibres produced on the seed are elongating and the maximum volume of the boll and seeds contained therein are attained. Also during this time, the fibre is basically a thin-walled, tubular structure, similar to a straw. Each fibre develops from a single epidermal cell on the seed coat. During boll enlargement and fibre elongation phase, the development of the fibre is very sensitive to adverse environmental conditions. Low water availability, extremes in temperature and nutrient deficiencies (especially potassium) can reduce final fibre length (Ikitoo, 2011; Kantartzi & Stewart, 2009; Peeters et al., 2001; Purseglove, 1974; Ritchie et al., 2007).

The filling phase of boll development begins during the fourth week after flowering. At this time, fibre elongation ceases and secondary wall formation of the fibre begins. This process is also known as fibre filling, or deposition. Cellulose is deposited inside the elongated fibre every 24 hours, filling the void space of the elongated fibre. The deposition of cellulose into the fibre cell is also sensitive to environmental conditions as is the case for fibre elongation. The filling phase of boll development continues into the sixth week after pollination.

The boll maturation phase begins as the boll reaches its full size and maximum weight. During this phase, fibre and seed maturation take place and boll dehiscence occurs. The capsule walls of the boll dry, causing the cells adjacent to the dorsal suture to shrink unevenly. This shrinking causes the suture between the carpel walls to split, and the boll opens. The dry boll usually splits open along the sutures between the locules revealing the fibre-surrounded seeds (Fig. 2.7).



Figure 2.7: Open mature boll (seedcotton) exposing lint fibre; accessed 22 February 2016, <<http://cottonaustralia.com.au/blog/article/mature-fibre-ripe-boll>>

Due to environmental exposure, the fibres dry out and lose their tubular shape; twisted ribbons with convolutions are formed. This dehydration determines the technological properties of the cotton fibre. This is as a result of de-crystallisation, distortion of crystal surfaces, and increased disorder in the intercrystal and interfibril regions, driven by water loss during cell collapse and by the formation of new intermolecular secondary bonding. This, in turn, builds up stress at molecular level, lowering strength and increasing adverse effect and elongation in the dried cotton determining the properties of the fibres (Hu & Hsieh, 2001). Cotton fibres comprise nearly of pure cellulose and represent the main economic value cotton, due to its use in yarn production.

2.4 MORPHOLOGICAL TRAITS IN RELATION TO COTTON IMPROVEMENT

Cotton varieties normally respond differently to different production systems, mainly expressed morphologically as hairy or non-hairy, normal or okra-leaf shaped, normal or frego bract, reddish or green-coloured varieties (Fig. 2.8). These varying phenotypic characteristics contrast with the known genotypic nature of commercial cotton cultivars characterised by a narrow genetic base (Iqbal et al., 2001; Lu & Myers, 2002; Multani & Lyon, 1995; Ullah et al., 2012).



Figure 2.8: Different morphological traits expressed in cotton: leaf shape a) normal leaf, b) okra leaf, c) superokra leaf, boll shape; d) pointed, e) oblong, f) round, plant colour; g) reddish purple, h) green, pollen colour; i) cream (pictures from cotton in the field and greenhouse in Kenya)

Some of these phenotypic characteristics confer certain desirable advantages to varieties so that hairy plant genotypes are resistant to jassids (*Amrasca devastans* (Dist.), and non hairy (glabrous) varieties offer resistance to cotton bollworms (*Helicoverpa armigera*) and pink bollworms (*Pectinophora gossypiella*) due to decreased egg laying opportunities on the trash characteristic of these genotypes (Meredith et al., 1997; Thaxton & El-Zik, 1994). Okra-leaf shaped plants have a characteristic open canopy which permits better light and air circulation. This decreases the population of boll weevil (*Anthonomus grandis*), pink bollworm and boll rot. Okra-leaf is also associated with early maturity and production of fibre with less trash compared to normal leaf cultivars.

The frego bract trait is associated with high level of resistance to boll weevil (Fig. 2.9) (Jones, 1972; Jenkins, 1976), but is also associated with delayed maturity (Jones, 1972; Thaxton & El-Zik, 1985).

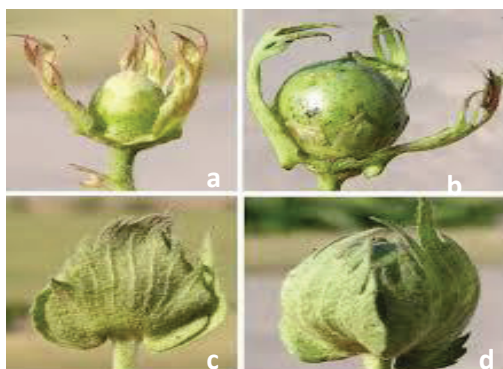


Figure 2.9: Cotton boll characteristics: frego bract (a and b) and normal bract (c and d) at flower bud and complete boll stage, respectively (Rahman et al., 2008).

A red plant colour confers significant degrees of non-preference to boll weevil and cotton aphids (*Aphis gossypii*). Varieties with smooth leaves generally give higher fibre grade than those with normal or densely hairy leaves (Thaxton & El-Zik, 1994). Utilising some of these morphological characteristics in breeding programmes has helped cotton growers to increase yields and good fibre quality with reduced dependence on pesticides.

Apart from morphological traits which can impart specific desirable characteristics, cotton lint yield is the most important breeding objective and cotton improvement primarily focuses on this characteristic. In Africa, cotton yield is extremely variable. The yield is dependent basically on the cultivar grown, soil fertility, climatic conditions and cultural practices such as sowing dates, fertilisers and crop protection that influence both yield and quality of the lint produced (Peeters et al., 2001).

Cotton lint fibres are the most important output of cotton plants. Therefore, improving ginning outturn (GOT), which is the percentage of fibres as compared to total weight of seed cotton, has been a very important breeding objective for many years (El-Feky, 2010; Thaxton & El-Zik, 1994). The higher this percentage, the higher the revenue that accrues to ginners and farmers. Fibre quality is determined by variety, crop pest and disease incidence, weather at harvesting and ginning conditions. Due to pest and disease infestation and their impact on the quality of cotton produced, tremendous cost of production goes into the control of these important biotic factors and therefore form important breeding objectives as well.

2.5 COTTON IMPROVEMENT IN KENYA

Cotton research in Kenya started around 1950 under the Cotton Research Corporation (CRC), by then called the Imperial Cotton Growing Corporation (Anon., 1953). It was transferred to the Ministry of Agriculture's Scientific Research Division in 1975, and in 1988 was moved to the newly established Kenyan Agricultural Research Institute (KARI) (Ikitoo et al., 1997) which was recently renamed Kenya Agricultural and Livestock Research Organisation (KALRO) (Kenya law Act No. 17 of 2013).

Principally, cotton improvement has several main objectives (Peeters et al., 2001; Ikitoo, 2008). The first one is production and focuses on criteria like number and weight of bolls, seed cotton amount, ginning outturn (GOT), resistance to diseases and pests, and adaptation to different climatic conditions. Target quality criteria on the other hand include seed characteristics and fibre properties. Fibre quality is determined by high fibre length and strength with intermediate micronaire, traits required by textile industries. Other objectives are photoperiod insensitivity, early maturity and adaptation to mechanical harvesting.

Previously, these improvements were accomplished through mass selection within available variability based on morphological traits and by creation of intra- or interspecific hybrids followed by family selection (Peeters et al., 2001; Purseglove, 1974).

With the adoption of genetically modified (GM) cotton that started with commercialisation in 1996, focus has shifted to development and adoption of *Bacillus thuringiensis* (*Bt*)-gene incorporated varieties effective against insect pests (Peeters et al., 2001). Only a few African countries, such as Burkina Faso, Sudan and South Africa are growing genetically modified cotton, especially *Bt* varieties (James, 2013). Kenya initiated testing *Bt* cotton with the single-gene insect resistant (Bollgard I) trait in 2004. Trials were conducted in quarantine for three years using deltapine *Bt*-varieties which were tested against local and non-biotech varieties of isogenic origin. Results were encouraging and showed no indications of negative effects on beneficial insects. During the period, Bollgard II became widespread, and Monsanto decided to stop using single gene insect resistant Bollgard I varieties. Kenya has been testing Bollgard II for several years, and the results on beneficial insects are the same as for the earlier Bollgard I varieties. GM cotton was also evaluated for its reaction to seedling rot and wilt diseases, in addition to identifying the pathogens associated with morphological disorders (Ikitoo, 2011). Field trials on GM cotton were completed successfully in 2012. KALRO projected GM cotton

seeds to be available for commercialisation by farmers in 2014 (Muchangi & Kibira, 2012). This never happened since government approval for *Bt* cotton commercialisation is still suspended to date. Considering the controversies surrounding GM crops in many African countries as well as in other parts of the world, conventional non-*Bt* cotton remains central in improvement of cotton sector in the country and GM cotton will only play a supplementary role to increase the cotton productivity since it takes time for farmers to adopt this new technology of GM crops even after approval for commercialisation because of initial costs and seed property rights.

2.6 GENETIC DIVERSITY IN CULTIVATED COTTON

Different studies have indicated that modern cotton cultivars exhibit low levels of genetic diversity (Lacape et al., 2006; Ullah et al., 2012; Wendel et al., 2009). The methods prevailing in characterising the cotton genome include use of isozymes (Wendel et al., 1992) and molecular markers such as restriction fragment length polymorphism (RFLP) (Brubaker & Wendel, 1994), random amplification of polymorphic DNA (RAPD) (Khan et al., 2000), amplified fragment length polymorphism (AFLP) (Abdalla et al., 2001; Pillay & Myers, 1999), microsatellites or simple sequence repeats (SSR) (Gutiérrez et al., 2002; Liu et al., 2000; Rungis et al., 2005) and recently, single nucleotide polymorphism (SNP) (Byers et al., 2012; Elshire et al., 2011; Gore et al., 2014; Islam et al., 2015; Poland et al., 2012; Wang et al., 2015).

The loss of genetic diversity in today's cultivars is a constraint to breeding programmes. This loss of diversity implicates that the introgression of genes from wild cotton species becomes necessary for crop improvement (Wendel et al., 2009). Natural genetic diversity is still conserved in cotton germplasm collections all over the world. The most important collections are with the USDA/ARS in Texas, USA with about 9,000 accessions, Russia (VIR) and Uzbekistan with about 17,000 accessions, China (CAAS) about 8,000 accessions and France (CIRAD) about 3,000 accessions. Other significant collections are found in Australia (CSIRO and ATGGC), Brazil (Embrapa Cenargen), India (CICR and NBPGR), with world estimate of slightly over 50,000 accessions (Campbell et al., 2010; Ikitoo, 2011; Stewart, 2010). Additionally, individual countries store subcollections of these genetic resources for purposes of plant breeding or other agronomic improvement programmes.

With modern science of genomics and molecular technologies there is a need for coordinated interactions between cotton germplasm collection, maintenance and utilisation, along with targeted improvement programmes (Percy, 2009). These genetic resources available for cotton

have been growing rapidly over the recent past (Campbell et al., 2010; Kulkarni et al., 2009; Lubbers & Chee, 2009; Percival et al., 1999; Rahman et al., 2012). Harnessing the potential of these cotton genetic resources will be of great importance to cotton researchers. Recent developments in next generation sequencing (NGS) and RNA-sequencing (RNA-seq) technology have generated high-throughput sequence data that will facilitate the identification of SNPs for effective and highly saturated markers for genetic studies in cotton. Genetic diversity within and between different species of cotton has been characterised by 1000 SNPs and 279 In-Dels from the 270 and 92 loci segregating in *G. barbadense* and *G. hirsutum* to provide mapped molecular markers for crosses within species and introgression of foreign germplasm in cotton (van Deynze et al., 2009).

The International Cotton Genome Initiative was formed in 2001 to co-ordinate cotton genomics research. As a result, more comprehensive genetic maps for the diploid and allotetraploid cotton genomes have been published (Rong et al., 2004), been made available through accessible Internet resources (Blenda et al., 2012). Recently, Wang et al. (2015) constructed the first ultra-dense genetic map composed of approximately 5 million NGS sequence-derived SNP markers in allotetraploid cotton. Public marker discovery is gaining momentum from these new genomic resources and these useful tools for use in marker/genomic-assisted plant breeding which are expected to aid genetic resource utilisation within the available germplasm.

2.7 GERMPLASM UTILISATION AND WAY FORWARD IN COTTON IMPROVEMENT

Cotton is a major source of foreign exchange for many countries around the globe. Cotton improvement is still focused on the enhancement of yield and fibre quality. The increasing knowledge about the cotton genome at the structural, gene expression, and epigenetic levels, as well as the collation of quantitative trait loci (QTLs) linked with fibre quality along with other traits, are contributing to emerging “breeding by design” programmes for cotton improvement especially based on existing SSR and some SNP markers (Chen et al., 2011; Mishra et al., 2010; Rahman et al., 2011). Their exploitation is still not yet widespread in mainstream commercial breeding programmes and certainly not among public cotton breeders. For successful integration in breeding programmes, there needs to be both a drastic change in reliable use and relevance of particular trait-marker associations and a willingness to adopt marker systems by breeders.

Although QTL mapping for various traits such as, fibre yield and quality (Jiang et al., 1998), drought tolerance (Saranga et al., 2001), disease resistance (Niu et al., 2008; Wang et al., 2008), and pest resistance (Niu et al., 2007) has been accomplished in cotton, these may not be helpful to clone causal genes due to lower marker densities. In general, the choice of a molecular marker technique is based on reliability, statistical power, and level of polymorphisms (Constable et al., 2015).

It has been proposed that SNP markers will have enormous influence on marker-assisted selection (MAS) and mapping studies in the future due to their high abundance and easy detection (Koeberner et al., 2003; Wang et al., 2015). Genotyping-by-sequencing (GBS) will clearly become the marker of choice technique for genetic studies in the coming years. So, the development of novel markers based on GBS and SNPs and the accessibility of modern technologies such as DNA chips/microarrays will hasten genome mapping and subsequent gene discovery in cotton for efficient cotton varietal development. The availability of new SNP genotyping technologies based on GBS should begin to address the issues of the low numbers of markers and high costs associated with MAS in cotton genetics and breeding (Wang et al., 2015).

2.8 BACTERIAL BLIGHT OF COTTON

2.8.1 Bacterial blight distribution and production impact

Generally, bacterial blight (BB) caused by *Xanthomonas citri* pv. *malvacearum* (*Xcm*) is widespread in most cotton growing-regions of the world within the tropics and subtropics (Fig. 2.10). Crop yield loss due to BB varies from one region to another and from season to season (Table 1.1). Bird (1959) reported that in the USA, yield reduction caused by BB of cotton was about 9% to 34%. From 1952 to 1993, an average of 1.5% yield losses of the annual cotton crop was due to BB (Hillocks, 1992; El-Zik & Thaxton, 1995). It is one of the most serious diseases in South Asia, Australia and several countries in Africa (Verma, 1986). Under favourable weather conditions and in the absence of resistant cultivars, *Xcm* yield loss can exceed 50% (Verma, 1986; Raghavendra et al., 2009). Although crop losses in Africa and India were 15% on average, complete crop failure also occurred (Verma, 1986). Tarr (1959 & 1972) stated that in China, Pakistan, Russia and surrounding countries, average crop loss was between 20 to 30%. Allen (2011) reported that the only recent source of information is the Compendium of Cotton Diseases that mentions general lint yield losses ranging 5 up to 30%. Indeed, in Africa and Asia

reductions of 10 to 35% have been recorded (Delanoy et al., 2005; Zhai et al., 2010). In the USA and Australia on the contrary, losses are usually less than 1% due to sufficient disease control measures, unless environmental conditions are extremely conducive to the pathogen (Allen, 2011; Bayles & Verhalen, 2007).

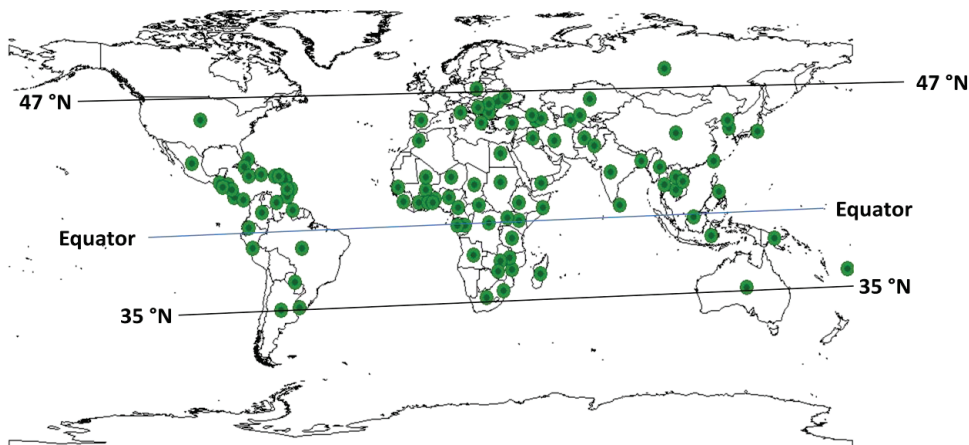


Figure 2.10: Distribution of cotton BB disease within tropical and subtropical regions of the world. Dots represent respective countries where BB has been reported normally located between latitude 47°N and 35°S; accessed 25 February 2016, adopted and modified from <<http://www.plantwise.org/KnowledgeBank/Datasheet.aspx?dsid=56948>>

2.8.2 Symptoms of bacterial blight

In cotton plants susceptible to BB, the pathogen is capable of penetrating and colonising the plant tissue. Normally, as it is observed with common diseases caused by pathogenic bacteria of the genus *Xanthomonas*, symptoms include vascular wilts, cankers, leaf and fruit spots, leaf blights and leaf streak (Mhedbi-Hajri et al., 2011; Schornack et al., 2013).

In cotton, the penetration of a virulent *Xcm* race into susceptible plant tissue does not trigger a rapid cell death. The bacterial multiplication is not blocked at the infection site which allows the pathogen to further invade the plant tissues and cause the typical BB symptoms.

The pathogen can affect all aerial parts of cotton in different stages of its growth cycle: seedling, leaf, stem, and bolls. On seedlings, it causes typical symptoms such as dull-green flaccid areas extending from the periphery of cotyledons and leaves, elongated water-soaked dark brown areas on leaf tissue, yellowing of leaves, tip rotting and vascular infection (Allen & West, 1991;

Sambamurty, 2006). Cotyledons become dry and wither; leaves collapse and die. The disease in seedlings normally has a spreading, systemic nature via the vascular tissues.

In mature plants, several different symptoms of BB, including angular leaf and water-soaked lesions, stem black arm lesions, leaf venial blight, boll rot and gummosis, and plantlet burning has been identified in fields (Allen & West, 1991; Essenberg et al., 2014; Innes, 1983; Sambamurty, 2006). The symptoms are mainly caused by the pathogen-induced enzymatic destruction of plant cells (Schumann & D'Arcy, 2010). Spots on infected leaves may spread along major leaf veins to progress toward leaf petioles and stems, resulting in defoliation (Peeters et al., 2001; Sambamurty, 2006; Verma, 1986). Stems and branches girdled by lesions can easily break under strong winds (Akello & Hillocks, 2002; Innes, 1983). Infection from the corolla may spread to cotton flower buds and young bolls, and cause premature boll shedding (Innes, 1983; Verma, 1986). External boll rot appears as round, oily spots that eventually form round to angular, dark brown or black, sunken lesions. The pathogen and other secondary organisms may further invade the fruit and cause internal boll rot, damaging lint and seed. Typical BB symptoms on cotyledons, leaves, squares and bolls are shown in Figure 2.11.

Normally, the pathogen penetrates leaves through stomata or wounds caused by humans and/or insects (Kay & Bonas, 2009). Systemic invasion of cotton plants occurs after leaf invasion followed by colonisation of intercellular areas. A single infected plant in a cotton field can act as primary inoculum and has the ability to spread the disease within that particular field under favourable environmental conditions.

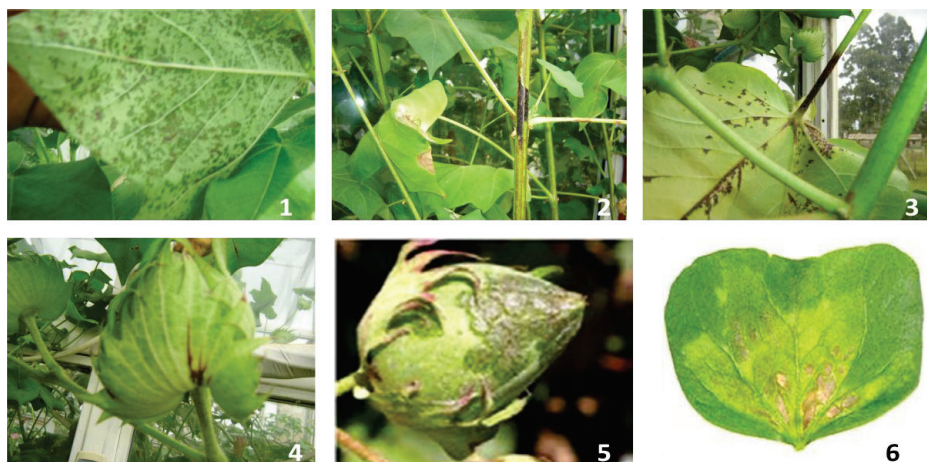


Figure 2.11: Cotton bacterial blight symptoms: angular and water-soaking spots on the leaf (1), stem black arm (2), vein blight and petiole black arm (3), vein blight on squares (4), boll necrosis and gummosis (5), and cotyledon necrosis and gummosis (6). Photos 1-4 author's own greenhouse photos in Kenya and 5-6 are adopted from Jalloul et al. (2015)

2.8.3 Bacterial blight dispersal

Xcm is normally transmitted through contaminated seed similar to other pathogenic *Xanthomonas* species (Schaad et al., 1980). It can be carried externally (on seed/lint) or internally within the seed. Seed is the most important mode of long-distance spread, allowing the introduction of the pathogen in new areas (Cotton CRC, 2012; Nyvall, 1999; Schaad et al., 1980). As with many bacterial pathogens, it is easily spread by splashing water. This in turn creates a huge risk to growers using overhead irrigation as well as to rainfed farmers.

Survival in buds of symptomless plants is also possible (Nyvall, 1999). Bacterial dispersal occurs through wind, splashing water, wind-borne moisture, insects, animals, people, machinery and equipment. High humidity exceeding 85% and temperatures over 25 °C are optimum for its occurrence and spread (Cotton CRC, 2012; Sambamurty, 2006). This therefore suggests that infection tests under glasshouse conditions sometimes will not be effective if temperatures are kept below 25 °C and leaves are too dry. Wind-driven rain, hail and sand-blasting increase severity of disease establishment as a result of injuries on the plant, providing more opportunity for bacteria to enter (Cotton CRC, 2012).

When plants are harvested during or after a period of excessive moist conditions, the pathogen is easily transferred to the seeds of open, blight-infected bolls from other infected parts of the plant

(Cotton CRC, 2012; Nyvall, 1999). On the other hand, many *Xanthomonas* spp. can survive on the soil in association with previously infected dead plant debris to form a source of inoculum for future host crops, which also become infected through wounds in the roots and the cycle repeats itself over and over. Therefore, certain agronomic practices promote/discourage the disease problem such as continuous cropping and crop rotation, respectively.

2.8.4 Classification of *Xcm* pathogen

Xanthomonas bacteria are gram-negative, yellow-pigmented and are associated with plants (Leyns et al., 1984; Mhedbi-Hajri et al., 2013). Genus *Xanthomonas* belongs, together with 29 other genera, to the family of the *Xanthomonadaceae* (National Centre for Biotechnology Information [NCBI, 2016]; Ryan et al., 2011): This family currently comprises 28 genera, including the two plant-pathogenic genera *Xanthomonas* and *Xylella*, and the related genus *Stenotrophomonas* (Mhedbi-Hajri et al., 2011; Ryan et al., 2011). The *Xanthomonadaceae* are further classified under the order of the *Xanthomonadales*, which belongs to the *Gamma* subdivision (class) of the gram-negative *Proteobacteria* (phylum) (Fig. 2.12).

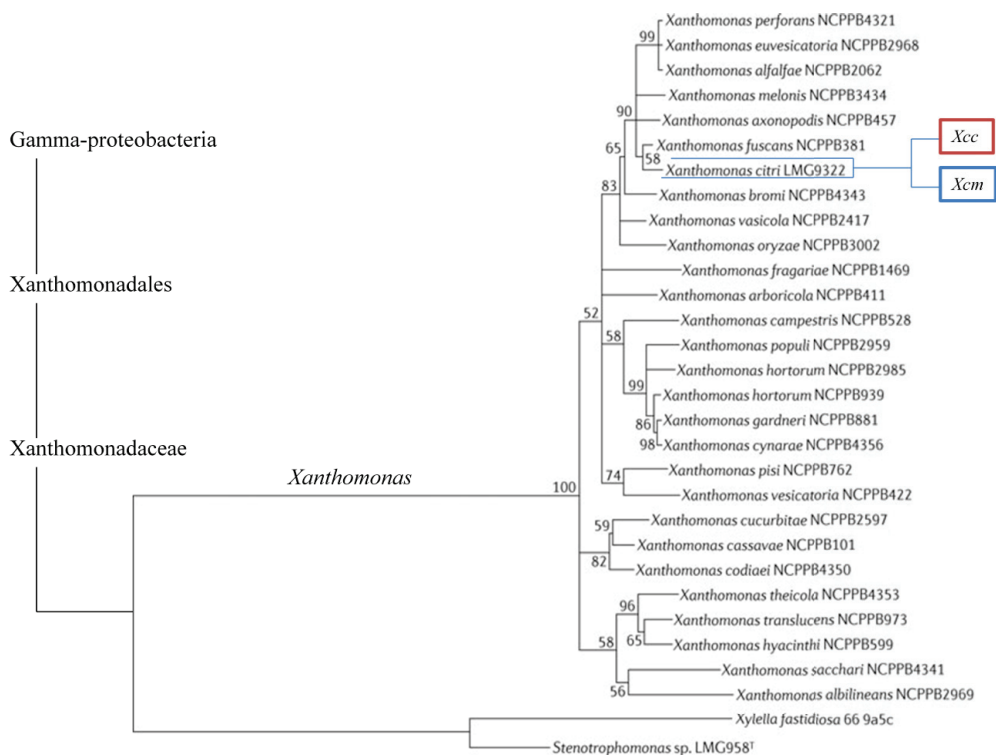


Figure 2.12: General classification of BB pathogen. *Xanthomonas* genus, related genera and two related species pathovars are shown (*Xcc* = *Xanthomonas citri* pv. *citri* and *Xcm* = *Xanthomonas citri* pv. *malvacearum*). The neighbour-joining tree is based on DNA gyrase subunit B (*gyrB*) gene sequences. Adopted and modified from Ryan et al. (2011).

2.8.5 Nomenclature of *Xcm*

Initially, *Xanthomonas* were classified using a “new host-new species” approach in which variants exhibiting a new host range or different disease symptoms were classified as separate species (Starr, 1981). In turn, the genus quickly became very complex. To differentiate between the rapidly growing numbers of species, several phenotypic studies were performed, but the results only further illustrated the uniformity of phenotypic traits of the *Xanthomonas* genus in contrast to their phytopathogenic diversity (Vauterin & Swings, 1997). Modern PCR-based DNA tools sped up the classification and nomenclature of xanthomonads leading to the present structure (Euzéby, 2007; Rademaker et al., 2005; Schaad et al., 2006; Vauterin et al., 2000).

Erwin F. Smith was the first person who described the bacterium that causes bacterial blight in cotton (publication year 1901). Walter Dowson then revised the classification in 1939, by re-classifying plant-pathogenic bacteria mainly into four genera, including the then genus *Xanthomonas*. The bacterium was then cited as *Xanthomonas malvacearum* (ex Smith, 1901) Dowson 1939 (Garrett, 1981). Later, Dye regrouped all the separate species together in the single species *Xanthomonas campestris*, which was followed by a further classification into pathovars (Vauterin et al., 2000). The bacterial species attacking cotton became *X. campestris* pv. *malvacearum* (ex Smith, 1901) Dye 1978.

In the re-classification proposal of Vauterin et al. (1995), the cotton pathovar was transferred to the species *X. axonopodis* (Vauterin et al., 1995, 2000). However, this nomenclature revision could not be accepted since the percentage of DNA-DNA similarity (relatedness) to the new species was too low (Schaad et al., 2000). Thus, *X. campestris* pv. *malvacearum* was retained.

Subsequently, it was proposed to classify *X. campestris* pv. *malvacearum* and the “A” strains of *X. campestris* pv. *citri* (causal agents of citrus canker form “A”) as subspecies of the same species (Schaad et al., 2005). This was based on genetic and phenotypic analysis that revealed a high relatedness between both strains and was recently confirmed with DNA sequence phylogenetic data analysis (Cunnac et al., 2013; Zhai et al., 2013). Before the taxonomic study by Constantin et al. (2016), the 2007 published name *X. citri* subsp. *malvacearum* (ex Smith 1901) Schaad et al. (2007) classification was still the taxonomically correct one in order to refer to the causal agent of bacterial blight in cotton. However, in recent taxonomic revision of the *X. axonopodis* species complex, Constantin et al. (2016) argued that it is inappropriate to create subspecies for groups of strains that are meant to be distinguished on the basis of their pathogenicity on a certain host. And hence for consistency within *Xanthomonas* the subspecies should be lowered in rank to the pathovar level as *X. citri* pv. *malvacearum*. Further, earlier multi-locus sequence analyses of six housekeeping genes (*fusA*, *gap-I*, *gltA*, *gyrB*, *lacF* and *lepA*) for xanthomonads confirmed that BB strains (race 18 and 20, respectively [X18 and X20]) belong to *X. citri* pv. *malvacearum* (2,745 bp with 100% identity) (Almeida et al., 2010), corresponding to DNA-DNA homology group 9.5, which also includes *X. citri* pv. *citri* (Rademaker et al., 2005).

Therefore, in this thesis *X. citri* pv. *malvacearum* will be used because sequence analysis information recently obtained confirms its close relatedness with the citrus *Xanthomonas* bacterium *X. citri* pv. *citri* (Cunnac et al., 2013; NCBI, 2016; Zhai et al., 2013).

2.8.6 Morphological and biochemical characteristics

Genus *Xanthomonas* is among the top ten most important plant pathogenic bacteria, considering its hosts and overall agronomic importance (Boch & Bonas, 2010; Mansfield et al., 2012; Schornack et al., 2013). However, a single strain can only affect a narrow range of hosts, for example a group of genera within a plant family or a group of species within a genus (Brunings & Gabriel, 2003; Mhedbi-Hajri et al., 2011). This host specificity is reflected in the pathovar names. Additionally, all pathogenic and non-pathogenic xanthomonads are always plant-associated, never free-living or soil-borne (Brunings & Gabriel, 2003).

The bacterium is a short motile rod, formed singly or in pairs and equipped with a single polar flagellum. It is gram-negative, aerobic, non-acid producing, and non-spore forming, and the cells measure $1\text{--}1.2 \times 0.7\text{--}0.9 \mu\text{m}$ in culture. *Xcm* produces yellow, convex, slimy colonies on nutrient agar (AbdelRehim, 2005; Bradbury, 1986; Sambamurty, 2006; Verma, 1986).

Useful in the detection are the typical yellow colonies they form on culture media (AbdelRehim, 2005; Chun, 2005). This yellow colour is due to membrane-bound pigments, xanthomonadins, which seem to be unique to the genus. Consequently, examination of the pigments through analysis of chromatographic and spectral absorption properties is a good tool to distinguish xanthomonads from other yellow-pigmented bacteria. Xanthomonadins are a mixture of brominated, water-insoluble, aryl-polyene esters (carotenoid-like); bromination and methylation varies between different xanthomonads. Besides the yellow colour, colonies generally look quite sticky and mucoid. This is due to the production of the extracellular polysaccharide (EPS) xanthan (Büttner & Bonas, 2010; Hanh Thi My Pham, 2009; Schumann & D'Arcy, 2006).

Xanthomonadins are believed to offer protection to *Xanthomonas* against photodamage (Chun, 2005; Poplawsky et al., 2000; Rajagopal et al., 1997; Van den Ackerveken, 2013).

2.8.7 Identification of bacteria by sequencing of the ribosomal DNA or *gyrB* gene

Identification is the feasible use of classification methodology to differentiate an organism from others, to verify authenticity of a strain, or to isolate and identify the organism that incites disease. Nucleic acid-based techniques are widely recognised and powerful plant pathogen identification techniques. Molecular markers such as the non-genic RFLP, RAPD, AFLP, as well as gene-based markers such as the hypersensitive response pathogenicity (*hrp*) genes or pathogenicity (*pth*) genes have been extensively employed to detect and identify pathogenic

bacteria affecting different crops (Bergman et al., 2005; Leite et al., 1994; Opio et al., 1996; William et al., 1990).

Microbial identification based on sequence data is now a commonly used procedure (Young et al., 2008). The sequence of the gene that encodes the 16S ribosomal RNA has become an important standard for definition of many bacterial species. Comparison of the sequences between different species suggests the degree to which they are related to each other. A relatively greater or lesser difference between two species suggests a relatively earlier or later time in which they shared a common ancestor. For closely related organisms, it may be necessary to analyse the entire 16S gene sequence or to look at the Internal Transcribed spacer (ITS) located between 16S and 23S rRNA subunits before sufficient variation in sequence is found to enable their discrimination.

Rapid amplification methods are described for the analysis of DNA from a part of the 16S rRNA genes of bacteria, the 16S–23S intergenic spacer region of bacteria or the gyrase B (*gyrB*) genes from *Pseudomonas* and *Xanthomonas* species (Coenye & Lipuma, 2002; Mondal et al., 2013; Parkinson et al., 2007; Triplett et al., 2015; Young et al., 2008, 2010). These methods are increasingly providing higher levels of discrimination between closely related bacteria. The method involves 3 stages of analysis namely PCR amplification of target sequences using specific primers, purification of the PCR-product, and sequencing of the amplified DNA.

Multi Locus Sequence Typing (MLST) including the *gyrB* gene has been explored as a powerful tool for xanthomonad identification and comparison between species (Brady et al., 2013; Mhedbi-Hajri et al., 2013; Parkinson et al., 2007; Triplett et al., 2015). The *gyrB* is a single-copy gene in all bacterial species and encodes the ATPase domain of DNA gyrase, an enzyme essential for DNA replication (Nitiss, 2009). The *gyrB* gene codes for the B subunit of gyrase, a prokaryotic enzyme belonging to the group of DNA topoisomerases. It is involved in a number of topological inter-conversions of double-stranded DNA molecules, such as negative supercoiling and relaxation of strands (Reece & Maxwell, 1991). *gyrB* is part of the Multi Locus Sequence Analysis (MLSA) housekeeping genes conserved within the *Xanthomonas* genus of which the sequence can be used to analyse taxonomic relationships. *gyrB* presents a higher degree of sequence variance than that of 16S rRNA enabling design of species-specific primers (Almeida et al., 2010; Kasai et al., 1998; Mhedbi-Hajri et al., 2013). Recently, Mondal et al. (2012) demonstrated the power of using *gyrB* primers in detection of *Xanthomonas* bacteria causing BB in pomegranate.

In addition, bacterial genome sequences can now be obtained in-house in many laboratories, in a few hours or days using novel DNA technologies of benchtop sequencers such as illumina MiSeq, Ion Torrent PGM or Roche 454 FLX Junior (Goss, 2015; Loman et al., 2012; Stahl & Lundeberg, 2012; Vinatzer et al., 2014). High throughput sequencing is presently a relatively cheap and fast process requiring no investment in high-tech equipment or software since DNA samples can be sent to advanced laboratories specialising in sequencing. Sequence data for whole genome sequencing or partial sequence of MLST is then supplied back for researchers to analyse using web-based software which is mostly free (Loman et al., 2012; Stahl & Lundeberg, 2012; Triplett et al., 2015). Sequence reads and assemblies from sample strains are downloadable from GenBank® sites and can be used for various analyses (Bielaszewska et al., 2011; Edwards & Holt, 2013). The workflow for analysis normally depends on the user of the data but the analysis flow normally follows a specific five-logical order: assembly of sequence data, ordering of contigs, annotation, genome comparison and typing (Edwards & Holt, 2013). Quality of raw data sequence is important in order to obtain the best results. Analysis based on sequencing technologies can be improved where sequence data quality of raw sequence reads are assessed using FastQC web-based software to determine the quality of raw read sequence sets.

Presently, many xanthomonad genomes have been sequenced (Aritua et al., 2015; Indiana et al., 2014; Moreira et al., 2010; da Silva et al., 2002; Qian et al., 2005; Triplett et al., 2015) and many more will be sequenced with advent of next-generation DNA-sequencing platforms. More nucleotide sequences of *Xanthomonas* continue to be deposited and over 300 *Xanthomonas* spp. are available in the NCBI database (Aritua et al., 2015; Indiana et al., 2014; Triplett et al., 2015; Zhai et al., 2013). Meanwhile, only a few drafts of *Xcm* race sequences (race 18 and 20) have been developed, thus further studies of BB in cotton lag behind other *Xanthomonas* species. However, with the development of next-generation sequencing technologies a lot of sequence information on *Xanthomonas* including *Xcm* will be delivered in the years to come (Aritua et al., 2015; Chitsaz et al. 2011, Cunnac et al., 2013; Pareja-Tobes et al., 2012; Zhai et al., 2013) at a faster rate than before and at reduced costs.

2.8.8 Characterisation of *Xanthomonas* based on repetitive DNA-sequence fingerprinting

Characterisation of *Xcm* is important for identification and determination of pathogen diversity in a given region. Pathovars within each species among xanthomonads cannot reliably be distinguished by their cellular or phenotypic characteristics (Louws et al., 1994; Mhedbi-Hajri et

al., 2011). To avoid potential bias in genetically characterising a pathogen population, the population structure must be inferred from a variety of neutral markers. Rep-PCR analysis was developed based on the observed occurrence of specific conserved repetitive sequences [repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements] distributed in the genomes of several bacteria (Versalovic et al., 1991; 1994, Woods et al., 1992; Zhai et al., 2010). However, the term has been extended to include the use of primers for PCR genomic fingerprinting that anneal to any repetitive sequence (George et al., 1997; 1998). Three families of repetitive sequences have been identified, including the 35-40 bp REP sequence, the 124-127 bp ERIC sequence, and the 154 bp BOX elements (Versalovic et al., 1994). Three primer sets are commonly used for rep-PCR genomic fingerprinting analysis, corresponding to REP, ERIC, and BOX sequences. The protocols are referred to as REP-PCR, ERIC-PCR, and BOX-PCR respectively and rep-PCR in general (Priest et al., 1988; Ragni et al., 1996). The primers are designed to amplify intervening DNA sequences between adjacent repetitive elements, although primer annealing to other homologous DNA sequences cannot be discounted. A complex array of 10-30 or more PCR fragments is generated per genome ranging in size from less than 200 bp to more than 6 kb. PCR products of different sizes are resolved by agarose gel electrophoresis, and the resultant DNA fingerprint patterns are used to identify pathogens, to differentiate strains and to assess the genetic diversity of plant pathogens. Each primer set (REP, ERIC, and BOX) is useful to fingerprint diverse bacteria, including both gram-negative and gram-positive phyto bacteria (Alves et al., 2002; Adriko et al., 2014; Arshiya et al. 2014; de Bruijn et al., 1992; Lee et al., 2008; Rodriguez-Barradas et al., 1995; Woods et al., 1992; Zhai et al., 2010).

2.8.9 Cotton *Xcm* race types

The pathovar *Xcm* is further subdivided into pathotypes or races. The presence of different races is the result of resistance breeding in cotton against *Xcm* (Brunings & Gabriel, 2003; Essenberg et al., 2014). Occasionally, after the introduction of resistance genes through breeding, bacteria succeed in breaking that resistance by mutation or loss of the specific avirulence (*Avr*) genes. Consequently, new races emerge as a variant within the pathovar. To date, in the USA cotton belt, the Cotton Disease Council (CDC) has recognised nineteen *Xcm* races of major economic importance. Identification of races is based on their ability/inability to induce disease symptoms on a set of ten cotton host differentials (Table 2.1) (Bird, 1986; Brinkerhoff, 1970; De Feyter et

al., 1998; Delannoy et al., 2005; Essenberg et al., 2014; Wallace & El-Zik, 1990; Wright, 2006; Yang & Gabriel, 1995; Zachowski & Rudolph, 1988). A first set of eight differentials was developed by Hunter in 1968 and supplemented to ten by Hussain and Brinkerhoff a few years later (Bell et al., 2010). These host cultivars each contain specific resistance or *B*-genes which make them to respond differently to infection by a particular race that is carrying a specific *Avr* gene (Bell et al., 2010; Brunings & Gabriel, 2003). These cotton resistance or *B*-genes are listed in Table 2.1. The series of differential lines that display a susceptible response is unique for each separate race, allowing their identification. This method has historically served its purpose well, but is somewhat complementary since it does not use modern advancements in molecular genetics or knowledge about *Xcm* avirulence genes thus limiting development of rapid automation protocols in species/race identification (Hanh Thi My Pham, 2009).

In the USA and Australia, as well as in other countries, race 18 is the most prevalent race (Allen, 2011; Cotton Catchment Communities Cooperative Research Centre, [Cotton CRC], 2012). Although nineteen races have been characterised, several additional highly virulent strains (HVS) have appeared in Central Africa in the 1980s (Delannoy et al., 2005; Huang et al., 2008; Wallace & El-Zik, 1990). HVS are virulent on the entire set of 10 cotton host differentials. In Burkina Faso and Chad, three new isolates were described and named HV1, HV3 and HV7 (Follin, 1981, 1983). Another highly virulent isolate was found in Sudan in 1983. A number of these strains have now been classified as races 20, 21 and 22 (Delannoy et al., 2005; Follin, 1983; Huang et al., 2008). Following the discovery of HVS, a new cotton cultivar S295 was developed in Chad. This cultivar carries the *B*₁₂ gene that confers resistance to all 22 *Xcm* races, including the African isolates which are able to evade the *B*₂*B*₃ and *B*_{9L}*B*_{10L} gene combinations (Girardot et al., 1986; Wallace & El-Zik, 1989). Recently, S295 was added as the eleventh differential to the set of host differentials (Hanh Thi My Pham, 2009; Thaxton, 2006).

Table 2.1: Bacterial blight pathogen responses to host differentials and corresponding B-genes (Hunter et al., 1968; Bell et al., 2010; Hanh Thi My Pham, 2009)

Cotton differential genotypes and their response to <i>Xcm</i> races											
B-Gene	A44	ST2B	ST20	MB1	1-10B	20-3	101-102	Greg	EB4	DP4	S295*
	None	Mp	B_7	B_2	B_{1n}	B_N	B_2B_3	Unknown	B_4	B_4	B_{12}
Race											
1	+	+	-	-	-	-	-	-	-	-	-
2	+	+	+	-	-	-	-	-	-	-	-
3	+	+	-	-	+	-	-	N/A	N/A	N/A	-
4	+	+	-	-	-	+	-	N/A	N/A	N/A	-
5	+	+	-	-	+	+	-	N/A	N/A	N/A	-
6	+	+	-	+	+	-	-	+	-	-	-
7	+	+	-	+	+	+	-	+	-	-	-
8	+	+	+	+	+	-	-	+	-	-	-
9	+	-	+	-	-	+	-	N/A	N/A	N/A	-
10	+	+	+	+	+	+	-	+	-	-	-
11	+	+	-	-	-	-	-	+	-	-	-
12	+	+	+	-	-	-	-	+	-	-	-
13	+	-	-	-	-	-	-	N/A	N/A	N/A	-
14	+	+	+	-	+	+	-	+	+	+	-
15	+	+	+	-	+	-	-	N/A	N/A	N/A	-
16	+	+	+	+	-	+	-	N/A	N/A	N/A	-
17	+	+	+	-	-	+	-	N/A	N/A	N/A	-
18	+	+	+	+	+	+	-	+	+	+	-
19	+	-	-	-	+	+	-	+	-	-	-
20	+	+	+	+	+	+	+	+	+	+	-
21	+	+	+	+	+	+	+	+	+	+	-
22	+	+	+	+	+	+	+	+	+	+	-

*A recent variety (S295) that has shown resistance to all *Xcm* races included in the screening; A44= Acala 44, ST2B= Stoneville 2B-S9, ST20 = Stoneville 20, MB1 = Mebane-B1, 1-10=1-10B, 101-102 = 101-102B, Greg = Gregg, EB4 = Empire B4, DP4 = Deltapine B4, (+) = susceptible reaction of host differential, (-) = resistant reaction, N/A = not available, Mp = Minor polygenes

2.9 INTERACTION BETWEEN *XANTHOMONAS* AND HOST CROPS

2.9.1 Bacterial adhesion through adhesin factors

The adhesion mechanism of the pathogen to the host tissue is widely conserved among gram-negative bacteria because it is an essential step in the early bacterial infection process (Büttner & Bonas, 2003; Mhedbi-Hajri et al., 2011). *Xanthomonas* species produce characteristic extracellular polysaccharides (EPS), xanthan, which give a mucoid appearance to the bacterial colonies. Xanthan also protects bacteria from environmental stresses (e.g. dehydration, toxic compounds) (Chun, 2005; Poplawsky & Chun, 1998; Poplawsky et al., 2000; Van den Ackerveken, 2013).

For vascular *Xanthomonas* pathogens, xanthan causes wilting of host plants due to blockage of water flow in xylem vessels (Chan & Goodwin, 1999; Denny, 1995). Gum genes of several *Xanthomonas* spp. including *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae*, *X. citri* pv. *citri* and *X. axonopodis* pv. *manihotis* have been shown to contribute to epiphytic survival and/ or bacterial in-*planta* growth and disease symptom development (Chou et al., 1997; Das et al. 2009; Dunger et al., 2007; Gottig et al. 2009; Katzen et al., 1998; Kemp et al., 2004; Rigano et al., 2007; Kim et al., 2009; Van den Ackerveken, 2013).

Additional evidence shows that xanthan also suppresses basal plant defence responses such as callose deposition in the plant cell wall, presumably by chelation of divalent calcium ions that are present in the plant apoplast and are required for the activation of plant defence responses (Aslam et al., 2008; Yun et al., 2006). Furthermore, in *X. campestris* pv. *campestris* and *X. citri* pv. *citri*, xanthan has been implicated in the formation of biofilms (Dow et al., 2003; Rigano et al., 2007; Torres et al., 2007). A biofilm is a bacterial population in which bacteria attach to each other or to biotic or abiotic surfaces and are embedded in an extracellular polymeric matrix that mainly consists of EPS, proteins and lipids (Branda et al., 2005; Sutherland, 2001). The formation of a biofilm normally provides protection against antibiotics and host defence responses and is responsible for bacterial epiphytic survival before colonisation of the plant intercellular space or attachment of vascular bacteria to xylem vessels (Stoodley et al., 2002).

Another surface-associated virulence factor of *Xanthomonas* spp. and other plant pathogenic bacteria are the lipopolysaccharides (LPS), which are major components of the bacterial outer membrane and protect the cell from hostile environments. LPS gene clusters of different *Xanthomonas* spp. vary significantly in number and identity of genes that have undergone a

strong diversifying selection (generation of multiple different alleles in different species, pathovars and even strains) (Lu et al., 2008). Variations in the composition of LPS might allow the bacteria to evade recognition by the plant's immune system and supposedly also affect bacterial resistance to phage adsorption and/or infection (Hung et al., 2002; Ojanen et al., 1993). Notably, LPS not only act as virulence factors but also induce plant defence responses such as pathogenesis-related gene expression, oxidative burst and thickening of the plant cell wall (Dow et al., 2000; Meyer et al., 2001). Adhesion of bacteria to biotic surfaces is key for the invasion of the host tissue. Bacterial attachment depends on specific adhesins that are anchored in the bacterial outer membrane and are classified into fimbrial and non-fimbrial adhesins. Fimbrial adhesins are filamentous proteinaceous structures such as type IV pili, which are structurally related to the predicted periplasmic pilus of Type II (T2S) systems (Gerlach & Hensel, 2007). Non-fimbrial adhesins include trimeric auto-transporters of Type V (T5S) systems (e.g. YadA from *Yersinia* spp.) and two partner secretion substrates (e.g. filamentous hemagglutinin from *Bordetella pertussis* and YapH from *Yersinia* spp.) (Gerlach & Hensel, 2007). The complexity of adhesion factors along with the T3S secretion system play a significant role in *Xanthomonas* avirulence. Figure 2.13 shows a general model of adhesion and related pathogenity factors responsible for disease symptom expression or HR depending on resistance or susceptibility of the host plant.

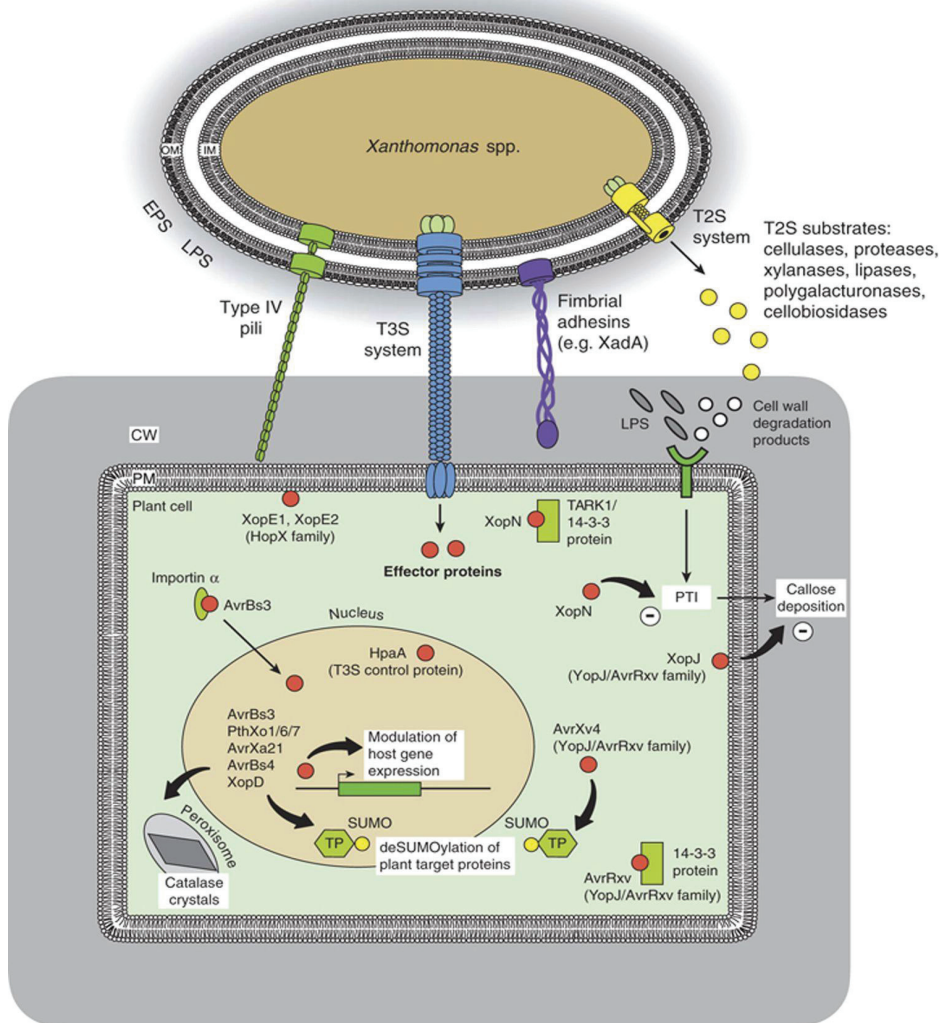


Figure 2.13: Model of known virulence factors from *Xanthomonas* spp. *Xanthomonas* spp. depend on T2S and T3S systems, adhesins, EPS and lipopolysaccharides (LPS) to successfully interact with their host plants. LPS can be released from the bacterial surface and elicit plant defence responses. LPS and other PAMPs are presumably sensed by specific plant receptors that activate plant defence responses [PAMP-triggered immunity (PTI)]. PTI might also be triggered by plant cell wall degradation products that result from the action of degradative enzymes secreted by the T2S system. The T3S system, which translocates effector proteins into the host cell, is essential for bacterial pathogenicity. Members of the *AvrBs3/PthA* family modulate host gene expression. The influence of *AvrBs4* on host gene expression has not yet been analysed, but it was shown that *AvrBs4* induces catalase crystals in peroxisomes when transiently expressed in the plant. The predicted cysteine proteases *XopD* and members of the *YopJ/AvrRxv* family presumably remove SUMO from plant target proteins and/or suppress callose deposition in the plant cell wall as was shown for *XopJ*. IM, inner membrane; OM, outer membrane; CW, cell wall; PM, plasma membrane (Buttner & Bonas, 2010).

2.9.2 Type Three Secretion (T3S) system in *Xanthomonas* pathogenicity

In plant pathogenic bacteria, the T3S system is encoded by the chromosomal *hrp* gene cluster, which contains more than 20 genes that are organised in several transcriptional units (Bhat & Shahnaz, 2014; Buttner & Bonas, 2003, 2010). The T3S system from individual *Xanthomonas* strains transports a cocktail of different effector proteins into the plant cell (Furutani et al., 2009; Roden et al., 2004; Thieme et al., 2005). Plant R proteins activate defence responses upon direct recognition of an effector protein, detection of effector-triggered modifications of plant target molecules or effector-mediated activation of resistance (R) gene expression (Van der Hoorn & Kamoun, 2008). Effector-triggered immunity is often associated with an HR, and effector proteins that elicit the HR in corresponding resistant plants are designated avirulence (*Avr*) proteins (Jones & Dangl, 2006; Marois et al., 2002). However, this observation is misleading because effector proteins supposedly act as virulence factors in susceptible plants for the benefit of the pathogen (Grant et al., 2006; Mudgett, 2005). Despite the fact that a virulence function has been shown only for a few effector proteins from plant pathogenic bacteria, accumulating experimental evidence suggests that individual effector proteins counteract the plant innate immune response that is triggered upon recognition of conserved, pathogen-associated molecular patterns (PAMPs) such as flagellin, cell wall degradation products or LPS (Block et al., 2008; Espinosa & Alfano, 2004; Grant et al., 2006; Jha et al., 2007; Jones & Dangl, 2006; Keshavarzi et al., 2004). Suppression of PAMP-triggered immunity (PTI) by T3S system effectors might therefore be a major requirement for the successful establishment and multiplication of bacteria in the plant tissue.

Recognition of PAMPs by the corresponding pattern recognition receptors (PRR) in plants leads to PTI, a basal immune status effective against a broad spectrum of pathogens. Recognition of PAMPs by extracellular receptor-like kinases (RLKs) promptly triggers basal immunity, which requires signalling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors (Fig. 2.14a). Pathogens have however evolved ways to circumvent this defence by producing effectors to suppress PTI. As a consequence, plants learned to specifically recognise some of these effectors. This leads to effector-triggered immunity, the second layer of plant immunity that is defined as resistance (R-) /avirulence (*Avr*-) protein-dependent gene for gene specific resistance (Boller & He, 2009; Chisholm et al., 2006). Pathogenic bacteria use the T3S system to deliver effector proteins that target multiple host

proteins to suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast (Fig. 2.14b). Plant resistance proteins are the nucleotide binding leucine-rich repeat (NB-LRR) and the extracellular LRR (eLRR) resistance proteins. The NB-LRR class is the most abundant, and members can possess amino-terminal coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) domains. The RRS1-R protein is a member of the NB-LRR class containing a carboxy-terminal nuclear localisation signal (NLS) and a domain with homology to WRKY transcription factors. The CC-NB-LRR and TIR-NB-LRR recognise effector activity and restore resistance through effector-triggered immune responses (Fig. 2.14c).

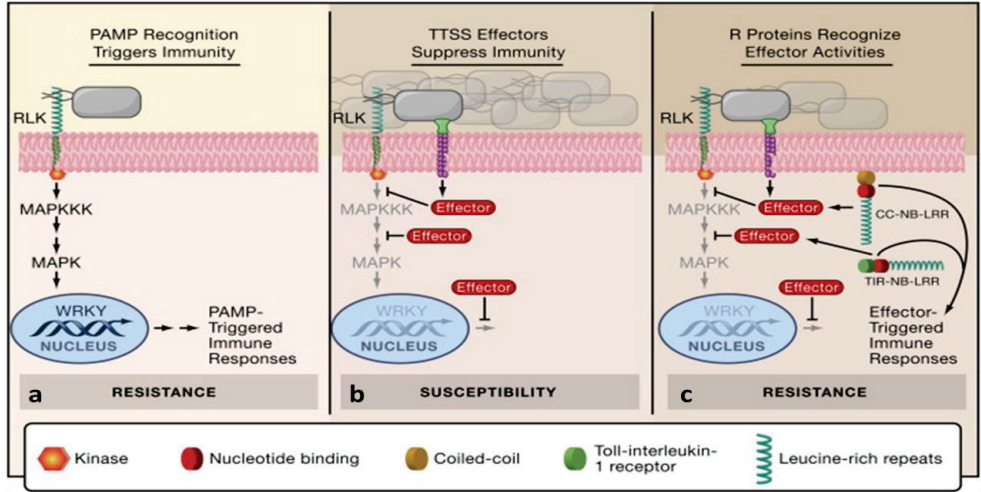


Figure 2.14: Model for the evolution of bacterial resistance in plants a) pathogen associated molecular pattern (PAMP) trigger basal immunity, b) T3S system cocktail of effector proteins suppress host proteins to suppress basal immune response (susceptibility) and c) plant resistance proteins (CC-NB-LRR and TIR-NB-LRR) detect effector activity and restore resistance through effector-triggered immune response) (Chisholm et al., 2006)

Several variants on this model exist (Schornack et al., 2006). They all agree on the fact that detection by R proteins relies more on the virulence activity of the *Avr* proteins than on their structure itself. The exact interaction between cotton and *Xcm* is not yet well explored, but in rice several resistance mechanisms are delineated against the pathogen *X. oryzae* pv. *oryzae* that also provides effectors which are likely to be members of the *AvrBs3* family (Kay & Bonas, 2009; Schornack et al., 2006, 2013).

2.9.3 Transcription activator-like (TAL) effectors

TAL-effectors are *Xanthomonas* proteins that are translocated into the plant cell via T3S system and directed to the nucleus where they seize the cell metabolism by specifically activating plant genes (Bogdanove et al., 2010). In a number of pathovars it has been demonstrated to be a major aggressiveness determinant responsible for symptoms. Additionally, they also act as avirulence factors, i.e., triggering the HR notably when activating “executor” resistance genes (Boch & Bonas, 2010; Zhang et al., 2015). Their mode of action has been detailed and their most outstanding feature is their central repeat domain, which is responsible for their highly specific attachment to DNA in effector binding elements (EBE) regions. This domain contains 1.5–33.5 repeats of 33–35 amino acids. In each repeat the 12th and 13th amino acids are variable (therefore called “Repeat Variable Di-residue” or RVD) and determine the specific interaction with a single nucleotide of the target DNA (Fig. 2.15). Hence the successive RVDs in the protein are involved in specific attachment to a sequence of adjacent nucleotides located in the promoter of the gene to be activated.

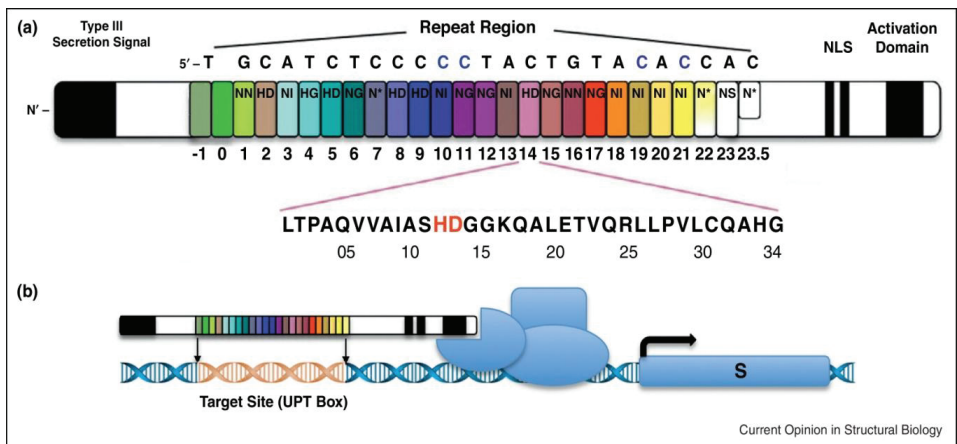


Figure 2.15: TAL effectors domain organisation and activity. (a) TAL effectors contain N-terminal signals for bacterial T3S, variable numbers of tandem repeats that specify the target nucleotide sequence, nuclear localisation signals, and a C-terminal region that is required for transcriptional activation. Blue bases correspond to positions in the target where the match between protein and DNA differs from the optimal match specified by the recognition code. The sequence of a representative repeat (#14) is shown; RVD residues (HD) that recognise cytosine are red. (b) TAL effectors are translocated into the plant nucleus, where they bind to target sites (termed ‘UPregulated by TAL’ or ‘UPT’ boxes, or ‘EBEs’ for ‘Effector Binding Elements’) that are located in the 5’ promoter regions of genes that are subsequently activated (Termed ‘S’ for a gene which confers susceptibility to infection as a result of activation, or ‘R’ for a gene which confers resistance to infection). The C-terminal region of the TAL effector interacts with plant transcriptional machinery as part of the gene activation mechanism. Adopted from Amanda et al. (2013).

The correspondence between RVD and nucleotides, the “TAL code,” has been deciphered and demonstrated experimentally and may be used to predict targets of TAL effectors in plants (Boch et al., 2009; Moscou & Bogdanove, 2009). Researchers are now exposed to a wide variety of potential TAL-effector targets to understand the mechanisms of *Xanthomonas* pathogenicity (through susceptibility targets), as well as some mechanisms underlying plant resistance to *Xanthomonas* (through executor resistance genes). Subsequently, this will help to develop new tools to breed resistant plants, either by escaping susceptibility or by introgressing executor R genes (Boch et al., 2014; Bogdanove et al., 2010). Generally, plants can acquire resistance traits against bacterial infection through at least three separate mechanisms: acquisition of mutations in the EBE that reduce DNA binding affinity, acquisition of mutations in transcription factors that ostensibly inhibit protein-protein association with the TAL-effector acidic activation region, or by integrating the sequence of the EBE box to the promoter region of a resistance gene thereby leading to an avirulence phenotype upon infection (Amanda et al., 2013).

2.9.4 *Xanthomonas citri* pv. *malvacearum* virulence factors

Avirulence/pathogenicity of *Xanthomonas* pathovars is related to the occurrence of *avr* genes in their genomes (De Feyter & Gabriel, 1991; De Feyter et al., 1993; Gabriel, 1999), indicating that the interaction with their hosts is based upon the gene-for-gene concept (De Feyter et al., 1993, 1998). In an incompatible situation, the HR phenotype is characterised by localised necrotic lesions resulting from host cell death (Goodman & Novacky, 1994). Due to variation in the genetic background of *Xcm* races in their hosts (Gabriel, 1999), however, strains are capable of overcoming the R-genes that occur in elite cotton varieties (Schnathorst et al., 1960; Innes, 1983). Differential screening of cotton genotypes with *Xcm* isolates has allowed identification of pathogen races in many regions in the world including Turkey (Zachowski et al., 1990), Syria (Abdo-Hassan et al., 2008), Iran (Razaghi et al., 2012; Saeedi Madani et al., 2010), and in the African countries; Uganda (Akello and Hillocks, 2002), Nigeria (Ajene et al., 2014), and Burkina Faso (Ouedraogo et al., 2009). Evaluation of *Xcm* genetic diversity assessed using rep-PCR suggested that the virulence of *Xcm* was closely related to genotype and/or geoclimatic origin of the strains (Zhai et al., 2010). Twenty-two different races of *Xcm* have been classified on the basis of their capacity to induce resistant or susceptible responses on an established panel of differential cotton genotypes (Section 2.8.4: Table 2.1), including highly virulent strains identified in Central Africa in the 1980s belonging to *Xcm* races 20, 21 and 22 (Follin, 1983;

Follin et al., 1988; Akello and Hillocks, 2002; Delannoy et al., 2005). Structural and functional studies of several *avr* genes cloned from different races of *Xcm* (De Feyter et al., 1993; Chakrabarty et al., 1997) revealed that they belong to the *avrBs3/pthA* gene family, found widely and exclusively in *Xanthomonas* (Leach & White, 1996). Multiple functional and non-functional AvrBs3 homologues in the *Xanthomonas* genome presumably arose by gene duplication and subsequent divergence (Boch & Bonas, 2010). In *Xcm*, the number of members of the *avrBs3* gene family varies from five to ten in the moderately virulent strain *XcmH* (De Feyter & Gabriel, 1991; De Feyter et al., 1998) and the highly virulent strain *XcmN* (Chakrabarty et al., 1997). About twenty *AvrBs3* family genes were cloned from races of *Xcm* (Gabriel, 1999), some of them being able to induce an HR when their products are delivered into resistant cotton plants via T3S system. In *X. citri*, members of the *AvrBs3* gene family provide some selective value for pathogenicity (Essenberg et al., 2014; Gabriel, 1999) associated with release of water-soaking, plant cell hyperplasia, and canker formation in citrus (Yang et al., 1994, 1996).

2.9.5 Hypersensitive response (HR) and disease symptom development

When *Xcm* bacteria penetrate a cotton plant that is not bearing the appropriate R-gene(s), a quick expansion beyond the infection site takes place inducing the typical blight symptoms. A resistant plant on the other hand is able to subdue bacterial invasion by an expeditious localised tissue collapse that causes dry necrotic lesions and the immobilisation of the intruder pathogen (HR) (De Feyter et al., 1993; Essenberg et al., 2014; Klement, 1982). The interaction between *Xcm* and cotton complies with the gene-for-gene concept (De Feyter et al., 1993). The bacterial avirulence (*Avr*) gene cluster (*AvrBs3*) effectors are responsible for the appearance of specific disease symptoms. For instance, *AvrXa7* and other *AvrBs3*-like proteins from *X. oryzae* pv. *oryzae* contribute to lesion development in rice, *AvrBs3* from *X. campestris* pv. *vesicatoria* causes hypertrophy of pepper mesophyll cells, *PthA* from *X. citri* pv. *citri* plays an important role in the formation of citrus canker lesions, and *Avrb6* and other *AvrBs3* homologues from *Xcm* increase disease water-soaking symptom in cotton leaves (Kay & Bonas, 2009).

According to this model, pathogen avirulence or plant disease resistance only manifest itself when the pathogen possesses *Avr* gene and the host plant a corresponding R-gene. The presence of these two complementary genes is required (Flor, 1971; De Feyter et al., 1993). In absence of either of the two, host-pathogen recognition does not occur, which means the reaction is

compatible. Figure 2.16 shows typical interaction events of *Xcm* leading to both virulence and avirulence reaction in a susceptible and resistant host, respectively.

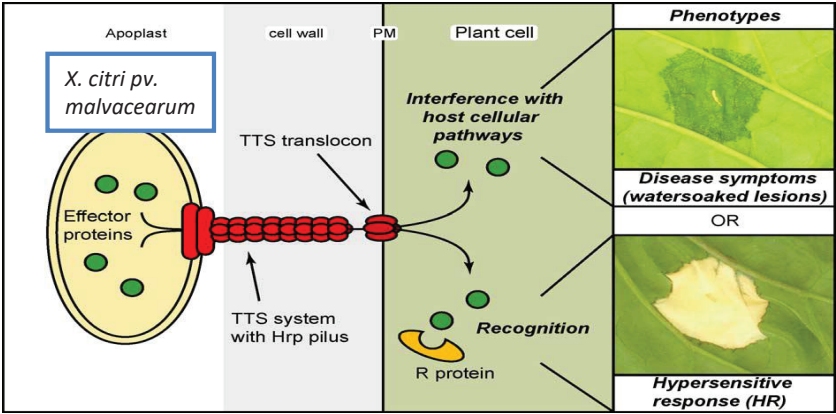


Figure 2.16: The typical interaction of *Xcm* pathovar with a susceptible and a resistant host, modified from Büttner et al. (2003)

Gene-for-gene- resistance implicates an HR that is characterised by a wide range of events mainly occurring within the first 24 hours post infection (hpi) (Delannoy et al., 2003; Marmey et al., 2007). One of the first responses following pathogen invasion is the accumulation of the phytohormone jasmonic acid (JA) at 2 hpi (Marmey et al., 2007). However, the key early event for establishment of the HR is the oxidative burst, consisting of the release of reactive oxygen species (ROS), including superoxide anions ($O_2^{\bullet -}$) at 3 hpi and hydrogen peroxide (H_2O_2) between 4 and 6 hpi (Bell et al., 2010; Delannoy et al., 2003; Martinez et al., 1998, 2000; Torres et al., 2006; Voloudakis et al., 2006). In HR-like resistance, the burst is considered as a key event, which has been investigated in cotton defence to *Xcm*. In cotyledons of the Réba B50 cultivar (containing the B_2B_3 genes) challenged by *Xcm* race 18, the burst dramatically peaked 3 hpi. Several lines of microscopic, biochemical and molecular evidence have suggested that a cationic wall-bound peroxidase (Pod) could be involved in the production of superoxide anions ($O_2^{\bullet -}$) (Martinez et al., 1998; Delannoy et al., 2003). Consequent accumulation of H_2O_2 results from dismutation of $O_2^{\bullet -}$ by a Mn superoxide dismutase (SOD), the transcription of which precedes enzymatic activity and production of $O_2^{\bullet -}$ (Voloudakis et al., 2006). Among these functions further cell wall strengthening via cross-linking of glycoproteins and involvement in a first line of defence occur due to the direct toxic effects of ROS on the pathogen. Furthermore, ROS are responsible for lipid peroxidation of the membrane, leading to cell leakage and death.

This swift collapse of the cells prevents the bacteria from multiplying. The pathogen is trapped at the sites of infection which are characterised by small necrotic lesions. $O_2^{\bullet -}$ and H_2O_2 can also act as signalling molecules or trigger the production of secondary messengers, that will activate defence genes.

An alternative for ROS-dependent enzymatic lipid peroxidation (LOX) has been documented. During the HR, LOX-activity is detected, associated with an increase in activity of LOX anionic isoforms. Massive accumulation of 9S-fatty acid hydroperoxides, drastic water loss of reacting cells leads to appearance of HR lesions at the leaf surfaces (Jalloul et al., 2002). This is then preceded by accumulation of GhLox1 transcripts (Jalloul et al., 2002; Marmey et al., 2007). LOX-dependent lipid peroxidation of the membrane also occurs in compatible interactions, but the difference with the incompatible interaction is a delayed and weaker LOX-activity, with the appearance of leaf chlorosis.

Downstream of the oxidative burst, two peaks of the accumulating defence compound salicylic acid (SA) can be detected. The first one is observed 6 hpi at the HR sites, the second one systemically in the whole plant from 24 hpi (Delannoy et al., 2005; Martinez et al., 2000). Indirect evidence of induced systemic resistance in cotton challenged by *Xcm* is obtained with possible production of ethylene. Ethylene-response transcription factors (ERF) are believed to play crucial roles in the activation of plant defence responses. These elevated levels are probably involved in the induction of local resistance (LR) and systemic acquired resistance (SAR), respectively. The latter includes the transcription and translation of pathogenesis-related (*PR*) genes into PR proteins (Bell et al., 2010).

In addition, flavonoids such as anthocyanins are also involved in the defence strategy of cotton (Bell et al., 2010). Their production is microscopically observed around 9 hpi (Marmey et al., 2007). There is evidence that flavonoids in infected cotton cells provide protection to the mesophyll cells from light-dependent phytoalexin toxicity (Edwards et al., 2008). The final event of the incompatible interaction occurs at 24 hpi and consists of the accumulation of various sesquiterpenoid defence molecules that function as phytoalexins (Abraham et al., 1999; Bell et al., 2010; Delannoy et al., 2005; Marmey et al., 2007). The six antibacterial metabolites observed in hypersensitive cotton plant cells are the 7-hydroxylated cadinanes 2,7-dihydroxycadalene (DHC), lacinilene C (LC), 2-hydroxy 7-methoxycadalene (HMC) and lacinilene C 7-methyl ether (LCME), and the 8-hydroxylated cadinanes hemigossypol (HG) and desoxyhemigossypol

(dHG). The series of events that occur following *Xcm* pathogen invasion in cotton are summarised in Figure 2.17.

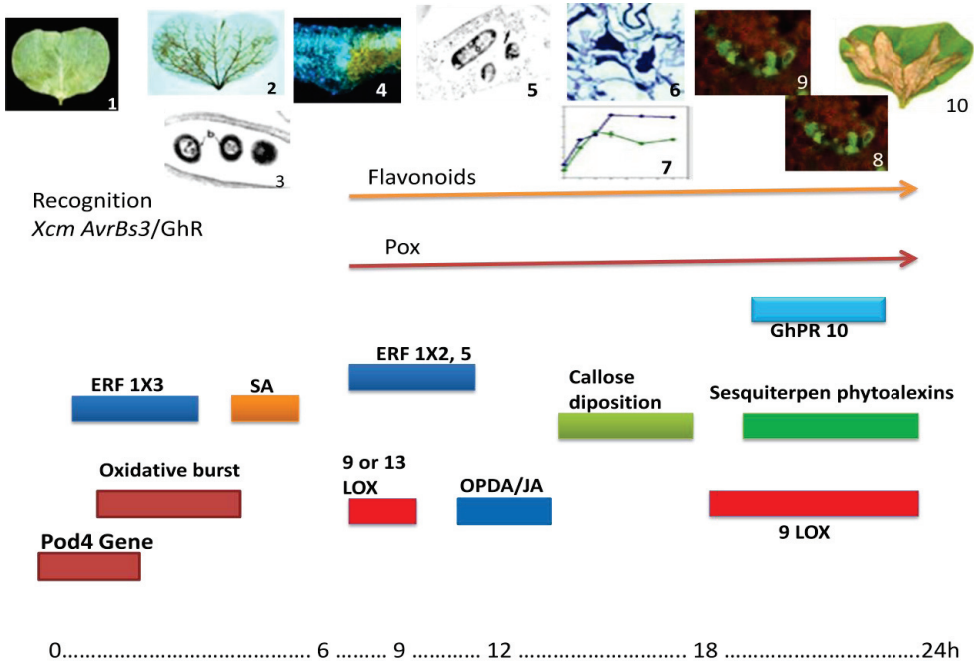


Figure 2.17: Time sequence of physiological events involved in the cotton HR to *Xcm*. Following penetration of leaves by the pathogen (1 and 3), bacterial cells inject the effectors within the host cells. When recognised by host R proteins, several specific mechanisms are activated through signalling pathways. Genes of the ERF IX3 group are transcribed in parallel to the production of ROS (t = 3h; 2: localisation of H₂O₂ in resistant leaves). Accumulation of SA culminates after the burst (t = 6h) and before activation of a 9- or 13-Lox gene (t = 9h), transcription of ERF genes and synthesis of OPDA/JA (t = 12h). Production of flavonoids (orange stained in infected areas; 4) and total peroxidase activity (5: electron microscopic immunolocalisation of peroxidase close to the bacteria) are also detected at the same time. Callose deposition contributes to stoppage of bacterial growth (7: green line) as compared to growth in susceptible plants (7: blue line), and precedes collapse of cells (6: condensation of the cytoplasm of HR cells). Appearance of HR symptoms in infected areas from t = 24h (9) occurs following dramatic increase in 9-Lox activity and strong accumulation of sesquiterpene phytoalexins (8: green fluorescence). Adopted from Jalloul et al. (2015).

2.9.6 Disease control

Currently no curative actions against BB are available, thus preventive control measures are very important. Prevention of the disease can be attained by the use of resistant cultivars, specific cultural practices such as crop rotation, use of clean planting seeds, etc (Cotton CRC, 2012; Koenning, 2004; Nyvall, 1999; Sambamurty, 2006; Thaxton & El-Zik, 2001) and biological control measures. Breeding for multigenic resistance would reduce single gene resistance

deficiencies which pathogens take a short time to overcome. Hence, development of varieties with multiple *R* genes is desirable for stable multigenic resistance.

Cultural practices includes using acid-delinted seed, sowing seed from disease-free plants, destruction of cotton residues, incorporation of plant residues, crop rotation, late sowing, early thinning, good tillage to minimise weeds, early irrigation, destruction of alternative hosts, no cultivation or movement of equipment when foliage is wet, control of plant injurious insects which create entry ports for bacteria, and avoiding rank growth or a dense canopy in order to reduce air humidity. Avoid over-fertilising and the use of growth regulators can help in keeping the canopy as open as possible. In general, good farm hygiene should be pursued.

In addition, research on bio-control agents to protect cotton from BB is currently ongoing, since these agents are known to be environment-friendly. *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Bacillus subtilis* have been collected from cotton soil rhizosphere and tested individually for their efficacy in controlling cotton diseases (Medeiros et al., 2011), including BB (Fallahzadeh-Mamaghani et al., 2009; Fallahzadeh & Ahmadzadeh, 2010; Salaheddin et al., 2010; Jagtap et al., 2012). These bio-agents trigger defence related enzymes involved in the synthesis of phenols. Increased activities of peroxidase, phenylalanine ammonialyase, polyphenol oxidase and β -1,3-glucanase were observed in *P. fluorescens*- and *T. harzianum*-treated cotton plants upon inoculation with *Xcm*. Seed treatment with these bio-agents enhanced seed germination, reduced growth of *Xcm*, and also triggered SAR in plants (Raghavendra et al., 2013). Additionally, medicinal plant extracts (Babu et al., 2007; Satyaa et al., 2007) and a white crystalline solid from the red alga (*Portieria hornemannii*) have also been checked for their anti-bacterial effects on *Xcm* (Sivakumar, 2014). Extracts from plants such as *Allium* spp., *Origanum vulgare*, and *Althea officinalis*, and crude crystalline compounds of *P. hornemannii* have also been tested and they show restrictive effects on *Xanthomonas* growth *in vitro*. These extracts constitute potential candidates for the management of BB. Similarly, plant extracts from *Datura alba*, *Moringa olifera*, *Azadirachta indica* and *Syzygium cumini* have showed notable biological control of BB in greenhouses and in fields (Javed et al., 2013).

Generally, a combination of breeding for increased resistance and appropriate use of cultural and biological control measures will help minimise crop loss due to BB.

2.9.7 Future strategies to achieve resistance against bacterial blight

Breeding for a particular *Xcm* race resistance conferred by a single dominant *R*-gene may be overcome by the pathogen. Breeding for multigenic resistance would help reduce single gene deficiencies which pathogens take a short time to overcome (Dangl et al., 2013). Although creation of pyramided *R* gene-containing lines effective against all races of *Xcm* has not been achieved, the availability of modern molecular and sequencing tools should accelerate incorporation of these BB resistance genes. Hence, development of *R* gene tools capable of incorporating multiple resistance genes offer opportunities to facilitate development of agronomic cultivars possessing stable, multigenic resistance.

Furthermore, with advancement in genome sequencing and genetic engineering, further resources to manipulate plant genomes are now available. Genetic engineering strategies to improve disease resistance are based on the knowledge of the mechanism employed by plants to recognise pathogen proteins. Knowledge of TAL-effector mechanisms offers new interesting opportunities for researchers aiming to develop resistant varieties. Utilising TAL-effector knowledge to generate broad-spectrum and durable resistance is now possible. Based on diversity studies conducted on a particular *Xanthomonas* species, it is now feasible to direct the research for virulence targets from the most dominant TAL-effectors. Further, field surveys allow the collection of strains at a regional, national or even global scale. All or representative strains can be selected for characterisation of the TAL genomes (TALomes), i.e., their whole repertory of TAL-effectors. The analogous susceptibility (*S*) genes in cotton can be identified employing three corresponding strategies. First, the expected major virulence role of a predominant TAL-effector has to be assessed *in planta* (by loss of function mutational analysis or heterologous expression for example). Secondly, different bioinformatics algorithms (TALVEZ, TAL-Effector Nucleotide Targeter, [Talgetter]) based on the TAL-DNA binding specificity code can be employed to predict TAL-effector targets in the host genome (assuming that at least a reference genome is available). Finally, RNA profiling strategies can be employed to identify TAL effector-dependent differentially expressed genes. Any such genes with predictable EBE for the TAL-effector would be strong candidates. Functional characterisation using designer TAL-effectors would be the next step (Boch et al., 2014). Then, bioinformatics and functional analyses can be used to identify *S* hubs. The identification of natural variants in the EBEs from germplasm databases or Eco-tilling or the generation of EBE mutations by

genome editing in specific EBEs will lead to loss-of-function alleles as new plant disease resistance sources. Figure 2.18 summarises the available tools for plant scientists to address *Xanthomonas* disease resistance challenges.

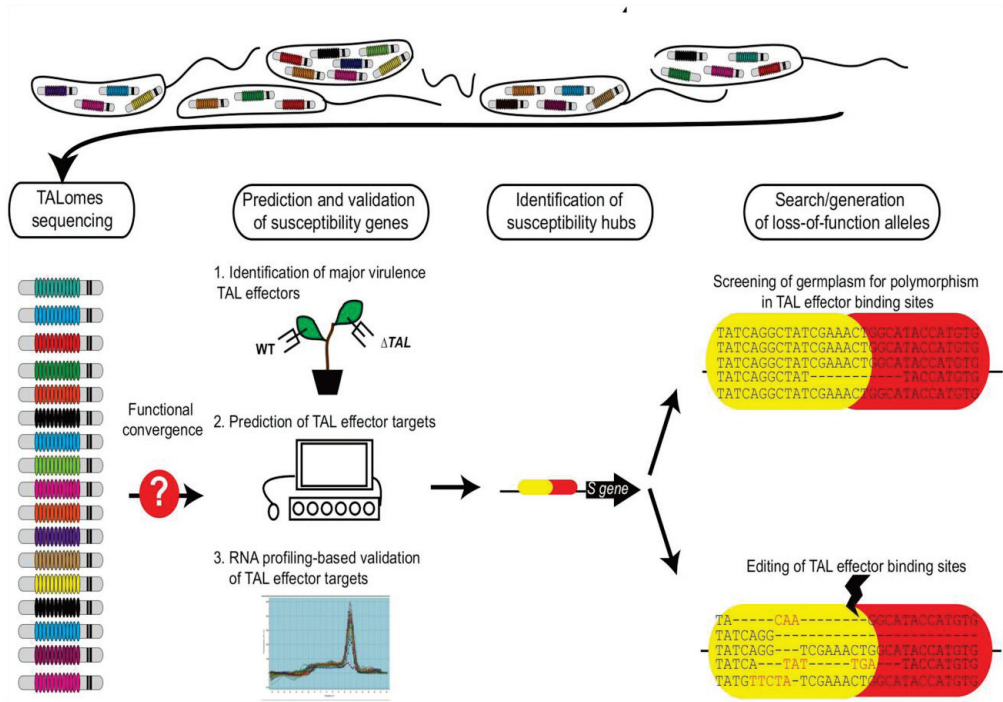


Figure 2.18: Diagrammatic presentation of combined tools currently available to exploit TAL-effector knowledge to identify *S* genes and loss-of-function alleles. Adopted from Hutin et al. (2015)

Therefore, efforts to identify resistance-genes and TAL-effector target genes will continue to play a central role in development of resistance in cotton. Additionally, identification of germplasm with R-genes especially using molecular and sequencing tools will enhance the acquisition of resistant varieties. It is within this context that cotton genotypes were screened using AFLP-associated markers targeted to identify genotypes with possible markers associated with BB resistance/tolerance genes among the Kenyan germplasm (Chapter 5).

CHAPTER 3

Present status of bacterial blight in cotton genotypes evaluated at Busia and Siaya Counties of Western Kenya

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ABSTRACT

Bacterial blight (BB) incited by *Xanthomonas citri* pv. *malvacearum* (*Xcm*) is an important bacterial disease occurring in all cotton growing areas throughout the world, including parts of Western Kenya, that are characterised by a hot and humid climate. The disease causes seedling blight, angular leaf spot, boll rot and black arm on petioles and branches leading to a loss of fruiting branches with yield losses of up to 35% as a result.

Fifty- one Kenyan cotton genotypes (*Gossypium hirsutum* L.) were established in the two counties of Siaya and Busia famous for their cotton cultivation in the western region. BB symptoms caused by natural *Xcm* infection in the field were scored for each cotton accession. In addition, we performed artificial inoculation with the same strains to confirm the status under controlled greenhouse conditions.

Results of BB disease scoring revealed that some accessions (e.g. T 24A and T 24B) possess a reasonable level of resistance. However, most of the Kenyan genotypes included in the survey showed medium to severe symptoms of BB; among them also KSA 81M, the only commercially grown cultivar in the Western Kenya region. Overall, 71% of genotypes appeared to be susceptible and 29% was classified as either resistant or moderately resistant.

There is therefore need to improve the local commercial genotypes by introducing new genetic resources with a more durable BB resistance to ensure a successful revitalisation of the Kenyan cotton sector.

3.1 INTRODUCTION

Cotton (*Gossypium* L.) is the number-one fibre crop in the world. In 2013, it was grown in the tropical and subtropical regions of about 80 countries, China being the top producer with a share of approximately 28% of world cotton (USDA-FAS, 2013). Prior to the 1990s, Kenya had a strong cotton industry until it collapsed in 1991 with liberalisation of the sector (Cotton Development Authority [CODA], 2009; The Cotton to Garment Apex Committee, 2006). The last few years Kenya has started to take measures to resuscitate its once thriving cotton sector. In 2006, CODA was established by the Kenyan government as a regulatory body to revitalise and coordinate the country's cotton industry which has recently been identified as a key subsector within the scope of "Kenya Vision 2030", in order to improve the livelihoods of people living in marginal areas (CODA, 2009).

However, despite the current efforts, several challenges still hamper its revival. One of them is infection of the cotton crop by the bacterium *Xanthomonas citri* pv. *malvacearum* (*Xcm*), formerly referred to as *X. campestris* pv. *malvacearum* or *X. axonopodis* pv. *malvacearum* (Schaad et al., 2006; Vauterin et al., 1995; 2000). Out of the 25 diseases that may occur in cotton (Chattannavar & Hosagondar, 2009), bacterial blight (BB) is the most widespread and destructive causing yield losses ranging from 5 - 35% (Delannoy et al., 2005; Thaxton & El-Zik, 2001). Typical symptoms of the disease on susceptible cotton genotypes are water-soaked lesions, angular leaf spots, stem black arm and boll rot (Nyvall, 1999; Peeters et al., 2001; Sambamurty, 2006).

The presence of BB was first described in the USA by Atkinson (1891). When cotton production all over the world started to rise in the 20th century, control measures were necessary to avert expansion of the disease. The use of resistant cultivars has always been the most effective control method. Resistant varieties evoke a hypersensitive response (HR), due to a rapid localised cell death that counters further bacterial proliferation at infection sites (Klement, 1982). In the 1940s, Knight (1946) discovered the heritable nature of BB resistance and described 10 major resistance or *B* genes. The first breeding programs were set up in Sudan with the development and subsequent introduction of the resistant line Bar XLI as a result (Knight, 1946). In the 1970s, this long staple Lambert-type *G. barbadense* cotton homozygous for the *B₂* gene was replaced by the higher yielding-Barakat cultivar (*B₂B₆*) as commercially grown cultivar in the country (Sidding, 1973). In Uganda, Albar 51 (*B₂B₃*) was selected from the Nigerian Allen for its

resistance to BB and formed the basis for subsequent breeding programmes in Eastern Africa. For Western Kenya, the cultivar KSA 81M was developed in the 1980s as a multiline of UKA59/240 selections. UKA59/240 originated from UKA69 which itself was the result of selections from a cross between Mwanza local and Albar 51. The release of resistant cultivars in the US Cotton Belt started in 1955, after multiple incidences of severe BB damage (Bird, 1986). Breeding for one major resistance appears not to be a durable solution, since bacteria are able to break through the introduced resistance by mutation or loss of their specific avirulence (*Avr*) genes. In this way, new races emerge as a variant within the subspecies. The *Avr* genes from different races of *Xcm* that were isolated up to now generate effector proteins that all belong to the same *AvrBs3/PthA* or transcription activator-like (TAL) family (Boch & Bonas, 2010; De Feyter et al., 1993). These bacterial effector proteins are injected into the plant cell cytoplasm via a type III secretion (T3S) system and cause suppression of plant defense mechanisms and modulation of host physiology (Boch & Bonas, 2010; Gürlebeck et al., 2006; Lahaye & Bonas, 2001; Mudgett, 2005). The central domain of the polypeptide, which is known to play a role in DNA binding within the host cell nucleus, is composed of 13.5 to 25.5 (usually 17.5) nearly identical, tandemly arranged leucine-rich repeats (LRR) of 34 amino acids (aa). Their number and arrangement define recognition-specificity and can therefore vary among *Xanthomonas* races (Gürlebeck et al., 2006; Lahaye & Bonas, 2001; Mudgett, 2005).

In the USA, the Cotton Disease Council has recognised 19 races of *Xcm* up to now. Traditionally, identification of races is based on their ability/inability to induce disease symptoms on a set of 10 cotton host differentials (Bell et al., 2010; Bird, 1986; Brinkerhoff, 1970; De Feyter et al., 1998; Delannoy et al., 2005; Hunter et al., 1968; Wallace & El-Zik, 1990; Yang & Gabriel, 1995; Zachowski & Rudolph, 1988). Recently, the S295 genotype with a *B₁₂* resistance gene has been added to this set to account for some highly virulent strains (HVS) that were detected in Central Africa in the 1980s (races 20, 21 and 22) and was shown to be able to infect all differential lines except S295 (Delannoy et al., 2005; Follin, 1981, 1983; Huang et al., 2008; Thaxton, 2006).

The two recommended cultivars for commercial production in Kenya, HART 89M (grown east of Rift Valley) and KSA 81M (grown west of Rift Valley), both have a yield potential of 2500 kg ha⁻¹ of seed cotton under rainfed conditions (CODA, 2009; The Cotton to Garment Apex Committee, 2006). However, in the year 2011/2012 average yields of only 423 kg ha⁻¹ were obtained, coinciding with as little as 17% of potential yield (USDA-FAS, 2013). This failure can

be attributed to poor agronomic practices, inadequate cotton extension services, low use of farm inputs, and high cost of inputs and poor quality of seed (CODA, 2008). The former also include insufficient pest and disease control. It is against this background that the current study was undertaken. Recent visits to farmers' fields in Western Kenya revealed that BB resistance in commercial cultivars was broken since the disease is again prevailing in the area. Therefore, several Kenyan germplasm were evaluated for their current BB status in the hope to identify potential sources of resistance that can be utilised to improve commercial cultivars in the country. First, a field evaluation of 51 Kenyan cotton accessions was carried out at Siaya and Busia in Western Kenya to determine the natural status of BB and to compare disease expression consistency among genotypes at both sites. In addition artificial inoculation using the raw field inoculum and accessions was undertaken under controlled greenhouse conditions to verify field results based on the consistency of response of accessions to infection by field inoculum.

3.2 MATERIALS AND METHODS

3.2.1 Study sites

The trial was conducted at the experimental fields of the Agricultural Training Centers (ATC) in Siaya and Busia, located in the former provinces of Nyanza and Western, respectively. The two sites both lie northeast of Lake Victoria, in an area with a long history of cotton cultivation since the crop's introduction in Kenya in the 1900s. The fields were formerly used for growing maize. Siaya is geographically situated at 0° 03' 360" N, 34° 17' 102" E and 1231 m altitude while Busia's position is 0° 23' 371" N, 34° 18' 194" E and 1216 m. Soil types can be classified as Acrisol/Luvisol/Lithosol (Siaya) and Acrisol/Cambisol (Busia) (Enserink, 1985). The climate of both regions is defined by a bimodal rainfall. Short rains fall from October to December, while the long rainy season lasts from March to June. However, the pattern can be very unpredictable, varying from year to year.

3.2.2 Cotton genotypes

The Kenyan cotton germplasm used in this research was represented by a number of 50 accessions provided by the Kenya Agricultural Research Institute (KARI) now Kenya Agricultural and Livestock Research Organisation (KALRO) of Mwea-Tebere and Gene-bank Muguga (Central Province). All these accessions are advanced KARI breeding lines being evaluated for various important traits and are well-adapted to the local conditions.

The cotton lines were encoded with an abbreviation referring to their place of collection: KISII (KI), KISUMU (KU) and TEBERE (T) (Table 3.1). Particular accessions were further subdivided (A, B, C) when distinct phenotypes arose within an accession during multiplication of the seed. Beside these accessions from the collection, the commercial cultivar grown in Western Kenya (KSA 81M) was also included in the trial giving a total of 51.

3.2.3 Experimental design and management

3.2.3.1 *Field experiment*

In April 2011, the field was prepared by plowing with a disc-drawn tractor, followed by manual harrowing with hand and fork hoes to remove weed particles and break soil clods prior to planting. The experiment itself was installed on May 19 and 20, 2011 for Busia and Siaya, respectively.

The lay-out of the experiment involved each accession occupying one row of 4 m in length, without repetitions. These rows were placed side-by-side up to a maximum number of 13 and with an inter-row spacing of 1 m. This implies that the experimental field consisted of four parallel blocs, each bloc with 13 rows to include all 51 accessions. Between those blocs of cotton rows, 50-cm-wide paths were provided. Planting holes were dug by means of a hoe and spaced 40 cm apart within the row. A minimum of five seeds was applied to the holes, at a planting depth varying between 3 and 5 cm. After germination, plants were thinned to two seedlings per hill for both Busia and Siaya.

After two months, plants were top-dressed with calcium ammonium nitrate (CAN) fertiliser. Weeds were controlled through regular hand-hoeing; insect monitoring took place throughout the experiment's growth period and sprayings were applied whenever pests were spotted.

3.2.3.2 *Greenhouse experiment*

Every accession planted in the field was also grown in pots in a greenhouse at the campus of University of Eldoret. Unfortunately, the breeder's material used was not always sufficient for both field and greenhouse installation. For that reason, some accessions were only present in the greenhouse and not in the field, or vice versa. Potting substrate consisted of a 1:1 mixture of soil and farm yard manure. Five - eight seeds per pot were used. Since inoculum in the field is probably a mixture of several *Xcm* races, a method based on raw leaf inoculum was used (Innes, 1961). Infected leaves and bolls, coming from different accessions and from both fields, were

soaked together in a beaker with sterile, distilled water. The resulting suspension was sprayed on the entire plants at 5-7 leaf stage, especially targeting the underside of the leaves, with the aid of a plastic hand atomiser. After infection, high humidity was maintained by spraying the walls of the greenhouse with water several times a day. Inside temperatures above 25 °C ensured conditions for a successful infection. Plants were closely monitored for the appearance of BB symptoms.

3.2.4 Bacterial blight assessment

3.2.4.1 Natural field conditions

Natural incidence of BB in Busia and Siaya was recorded according to the scoring system described by Brinkerhoff et al. (1984), and is based on the shape and size of the lesions:

0 (0.0) = immune, no visible lesions; 1 (0.1) = predominantly immune with a few pinpoint lesions; 2 (0.2) = predominantly immune with a few small, round lesions; 3 (1.0) = resistant, dry, small, round to angular lesions; 4 (1.2) = intermediate, predominantly small, angular lesions, but with small, round lesions also present; 5 (2.3) = intermediate, predominantly small, angular lesions but with large, water-soaked, angular lesions also present;

6 (4.0) = fully susceptible, large (1 to 2 mm), water-soaked, angular lesions that turn black on drying plus up to 0.5 cm long water-soaked vein lesions.

These scores can be divided into four disease reaction categories: immune (0), resistant (1, 2, 3), moderately susceptible (4, 5) and fully susceptible (6) (Bayles & Verhalen 2007; Brinkerhoff et al., 1984).

Scoring was performed by randomly selecting three plants per accession during flowering/boll formation stage. From each chosen plant, two leaves were examined individually. This made a total of six scores per accession. In order to facilitate analysis, the Brinkerhoff scores (indicated between brackets) were converted to whole numbers.

3.2.4.2 Greenhouse

Two weeks after artificial inoculation, disease symptoms were scored for the first time. This was repeated after a week and again three weeks later. Each time, only one score per accession was given, based on the general view of the set of plants within one pot. The scoring system differed from the one applied in the field and was based on the size of the visible water-soaked areas (Essenberg et al., 2002; Kangatharalingam et al., 2002): 0.0 = no water-soaking; 1.0 = pinpoint-

sized dots; 1.3 = intermediate between 1.0 and 2.0; 2.0 = small, round speckles; 2.7 = intermediate between 2.0 and 3.0; 3.0 = merged angular patches; 3.3 = intermediate between 3.0 and 4.0= confluent areas.

3.2.5 Statistical analysis

Since the data collected from the field are scores without a normal distribution, they were statistically analysed with non-parametric tests using SPSS software program, version 20. A first analysis was performed to determine whether there was a difference in BB susceptibility between the different accessions at one site. First of all, a Kruskal-Wallis test ($P < 0.05$) was carried out to determine overall differences. When this test was found to be significant, the 51 independent accessions were compared two-by-two using a Mann-Whitney U test ($P < 0.05$) to determine where exactly these differences were situated.

A second analysis aimed at comparing the accessions from Busia with those from Siaya. Therefore, a susceptibility ranking of the accessions was made for each site, based on the average BB score per accession. On those two related rankings, a Friedman test ($P < 0.05$) was performed to see whether there was a statistically significant similarity between the order of susceptibility in Siaya and the one in Busia. Furthermore, this was visualised through a scatter plot with each dot representing an accession whereby the position is determined by the Busia rank on the x-axis and the Siaya rank on the y-axis. The higher the rank, the more susceptible the accession. When correlation between both rankings was found to be significant, the Pearson correlation coefficient was determined ($p < 0.01$).

No statistical analysis was performed on the greenhouse data, since only one score at a time was given per accession.

3.3 RESULTS

3.3.1 Screening cotton accessions for BB under natural field conditions

During the first field screening three months after crop installation, signs of recent BB infections were observed on young plants. Clear, water-soaked, round to angular lesions could be noticed on the abaxial side of the leaves. During the second observation, four months after planting, more symptoms were seen at the two sites since climatic conditions had been conducive for bacterial infection (hot and humid), as shown in Figure 3.1.

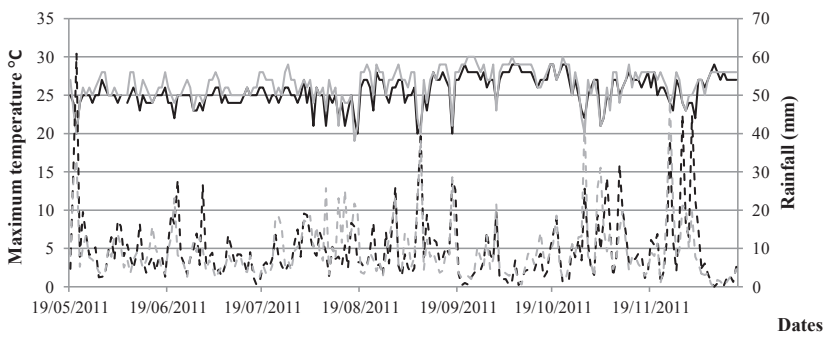


Figure 3.1: Weather data for Busia (light-coloured lines) and Siaya (dark-coloured lines) during field experiment: maximum daily temperature (full lines) and daily rainfall (dotted lines). Maximum humidity was above or close to 90% each day for both sites and therefore not incorporated in the graph. No significant differences between weather patterns of Busia and Siaya can be noted.

Lesions were visible on all aerial parts of the plant: (water-soaked) lesions between or along the veins on the lower side of the leaves, oily spots on the bolls, black areas on the petioles and stems, and (water-soaked) lesions on the squares. For accessions T 09/T 24A/T 24B in Siaya and T 24A/T 24B in Busia it took until the third and final observation, two weeks later, to show water-soaked spots. Actual field scoring was carried out at this moment, using the Brinkerhoff disease scoring system (Brinkerhoff et al., 1984) which is based on shape and size of the lesions (Fig. 3.2)

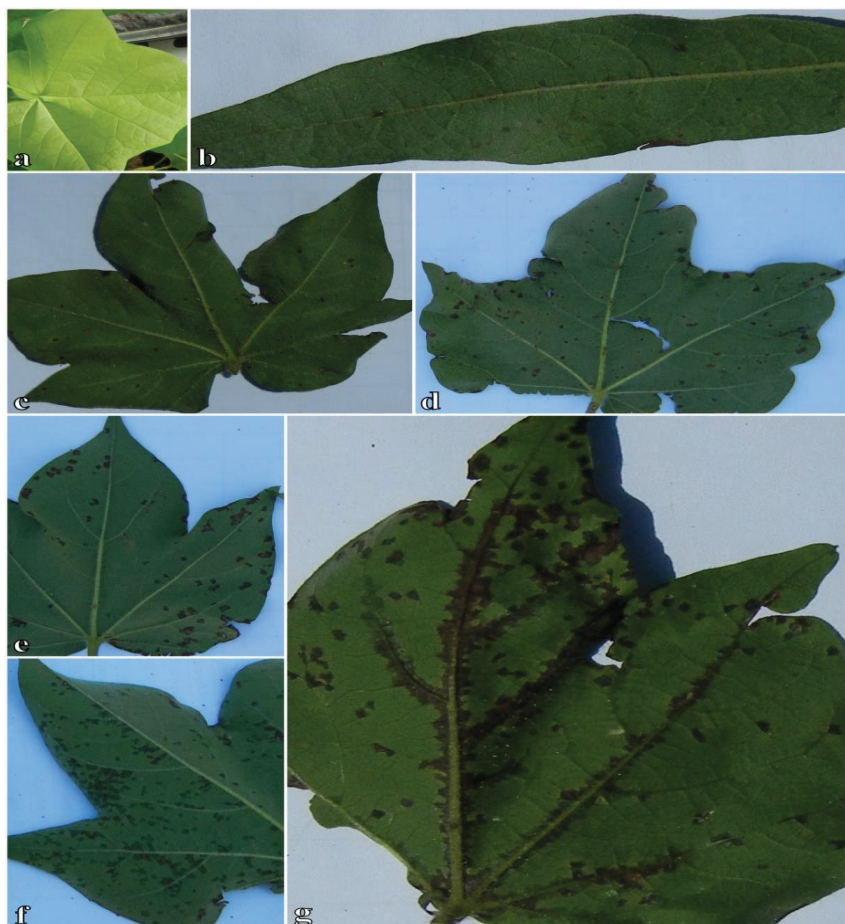


Figure 3.2: Scoring system used in Busia and Siaya for bacterial blight on leaves, based on disease scale of Brinkerhoff et al. (1984): (a) score 0 (picture from non-infected glasshouse plant), (b) score 1, (c) score 2 (d) score 3, (e) score 4, (f) score 5, and (g) score 6.

Scoring results from Busia are visualised through bar graph in Figure 3.3. Accessions are ranked according to their susceptibility, with the bars showing percentage distribution of the given scores within each accession. According to a Kruskal-Wallis test ($p < 0.05$), overall differences are present between accessions.



letter have a similar degree of susceptibility according to Mann-Whitney U analysis ($p < 0.05$).

3.3.2 BB score comparison between Busia and Siaya

A second statistical analysis was performed to compare the findings from Busia with the results from Siaya. A Friedman test on susceptibility rankings of both Busia and Siaya revealed that they are not significantly different from each other ($P < 0.05$). This can also be visualised through a scatter plot, like shown in Figure 3.4. Although at first sight the dots seem to have a scattered position away from the bisector, they do follow a fairly linear pattern, indicated by the two dotted lines. This was confirmed by statistical analysis, indicating a significant correlation ($p < 0.01$) with a Pearson coefficient of 0.362.

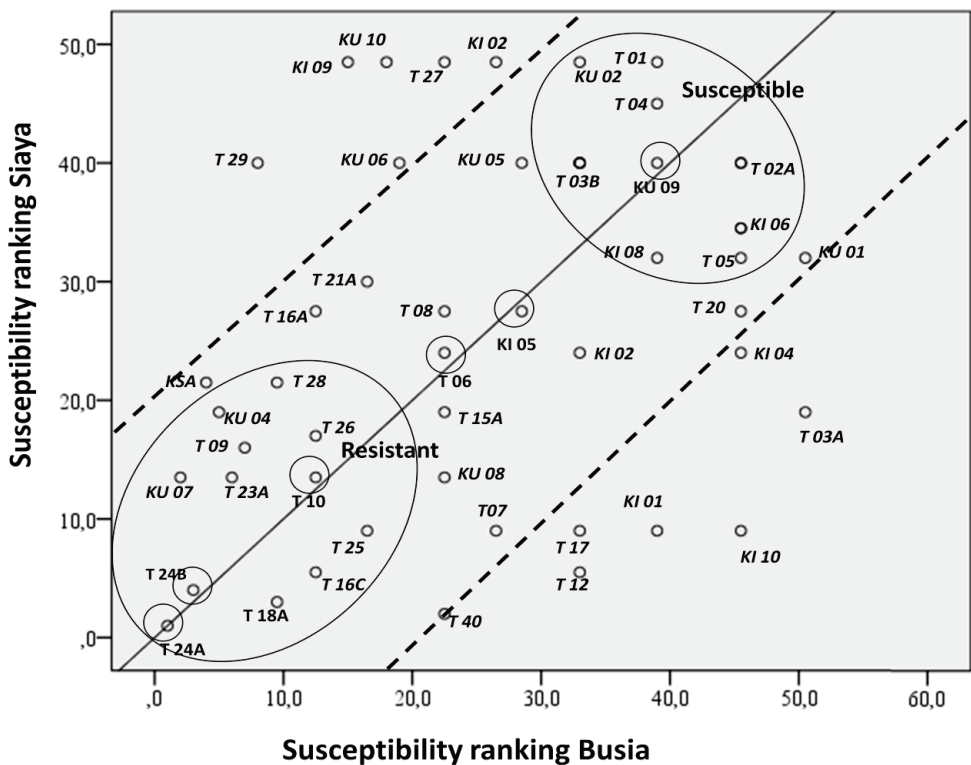


Figure 3.4: Comparison between Busia and Siaya for BB susceptibility of accessions (KI=Kisii, KU=Kisumu, T=Tebera). Every dot represents an accession of which the position is determined by its rank in Busia and Siaya. The higher the rank, the more susceptible the accession. Highly similar ranks at both sites give dots situated on or very close to the first bisector (encircled accessions). Despite the scattered position of the dots, a fairly linear pattern is followed, indicated by the dotted lines on the graph and confirmed by a Pearson correlation coefficient of 0.362. Accessions outside the dotted lines are outliers with non-corresponding scores.

3.3.3 Infection under greenhouse conditions

After spray inoculation in the greenhouse, the first disease symptoms appeared within four days. A first scoring was done 14 days after infection, using a scale based on the size and spreading of the water-soaked lesions like shown in Figure 3.5 (Essenberg et al., 2002; Kangatharalingam et al., 2002).

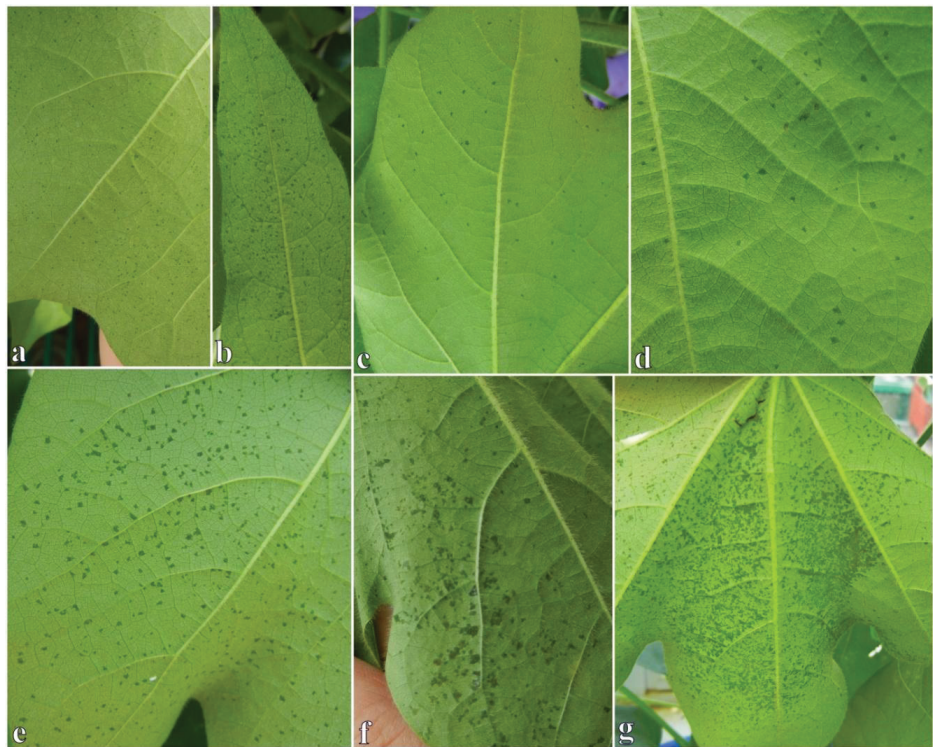


Figure 3.5: Scoring system used in the glasshouse for bacterial blight on leaves, based on the size and distribution of the visible water-soaked lesions (Essenberg et al., 2002; Kangatharalingam et al., 2002): (a) score 1.0, (b) score 1.3, (c) score 2.0, (d) score 2.7, (e) score 3.0, (f) score 3.3, (g) score 4.0. For the immune reaction one is referred to Fig. 3.2 (a).

The results of the first evaluation, together with the other two scorings 21 and 42 days after infection, are shown in Table 3.1. The accessions that appeared to be most resistant to BB are indicated with star and bold.

Table 3.1: Results of scoring for bacterial blight in glasshouse. Scoring system was based on the size and distribution of the visible water-soaked lesions on the leaves (Essenberg et al., 2002; Kangatharalingam et al., 2002; Fig. 3). Each time (14, 21 and 42 dpi^a), only one score per accession was given, based on the general view of the plants within one pot (KI=Kisii, KU=Kisumu, T=Teberre).

Accession	dpi ^a			Accession	dpi ^a		
	14	21	42		14	21	42
KI 01	1.3	1.3	3.0	T 07	4.0	4.0	4.0
KI 02	3.0	4.0	4.0	T 08	2.7	3.0	3.0
KI 03	3.0	4.0	4.0	T 09*	0	0	0
KI 04	2.7	3.0	4.0	T 10	3.3	4.0	4.0
KI 05	4.0	4.0	4.0	T 11*	0	0	0
KI 06	2.7	4.0	4.0	T 12	2.0	3.0	3.3
KI 07	4.0	4.0	4.0	T 15A*	0	0	0
KI 08	3.0	4.0	4.0	T 15C	2.7	3.0	3.0
KI 09	3.0	4.0	4.0	T 16A	2.7	3.0	3.0
KI 10	3.3	4.0	4.0	T 16B*	0	0	1.0
KU 01	2.0	2.0	3.0	T 16C*	0	2.0	2.0
KU 02	2.7	2.7	3.0	T 17	2.7	4.0	4.0
KU 03	2.0	2.0	3.0	T 18A	1.3	3.3	4.0
KU 04	4.0	4.0	4.0	T 18B	2.0	3.0	3.3
KU 05	4.0	4.0	4.0	T 18C	1.3	2.7	3.3
KU 06	2.7	3.0	3.3	T 20	2.7	3.0	3.3
KU 07*	0	0	0	T 21A	2.0	2.7	3.0
KU 08	4.0	4.0	4.0	T 22	1.0	1.3	3.3
KU 09	1.0	2.7	3.3	T 23A	2.7	3.0	4.0
KU 10	3.3	4.0	4.0	T 24A*	1.0	1.3	1.3
T 01	3.3	4.0	4.0	T 24B*	0	0	0
T 2A	3.3	4.0	4.0	T 25A	2.7	3.0	3.3
T 02B	4.0	4.0	4.0	T 26*	0	0	0
T 03A	3.3	4.0	4.0	T 27	3.3	4.0	4.0
T 03B	2.0	3.0	4.0	T 28	2.0	3.3	4.0
T 04	3.0	3.0	3.3	T 29	3.3	4.0	4.0
T 05	4.0	4.0	4.0	T 40	2.7	3.3	4.0
T 06	3.3	3.3	4.0	KSA 81M	1.3	2.7	3.0

^a days post infection

* Accessions with higher level of resistance (no statistics)

3.4 DISCUSSION

3.4.1 Screening cotton accessions for BB under natural field conditions

Figure 3.2 clearly displays what was already obvious during the scoring; most accessions in Busia are fully to moderately susceptible, characterised by higher leaf scores that range from 4 to 6. According to the Mann-Whitney U test, there are almost no differences between those susceptible lines. For example KI 03 and T 03A are fully susceptible as evidenced by six times score 6, but still not statistically different from T 08 which has three 6 scores, one score 3 and two scores 2. From the bars in combination with statistical letters it can be derived that the separation between quite susceptible and more resistant lines can be made somewhere around T 06 or T 15A. From that point, the proportion of high scores (especially 6) starts to decline in favour of the lower scores 4 to 1. This implies that the examined germplasm can be roughly divided into a susceptible part containing approximately 71% of accessions and a more resistant part of about 29%.

Because of the statistical similarity among several accessions, it is difficult to draw solid conclusions from this survey. However, for the last three accessions in the graph (T 24B, T 25A and T 24A), the Mann-Whitney U test clearly shows that they are more resistant than the other lines. It is remarkable that T 24A and T 24B are both subaccessions within T 24 suggesting a possible genetic basis to the disease resistance observed. Furthermore, T 24A is characterised by an okra-leaf shape. It shares this trait only with T 18A, which is also positioned near the resistant end of the graph as well. Several advantageous traits are attributed to okra-leaf cotton, such as early maturity, low susceptibility to some pests and diseases, good sunlight and air penetration, and less tendency for rank growth essential for mechanical harvesting (da Silva et al., 1996; Horrocks et al., 1978; Pettigrew 2003; Soomro et al., 2000). In 1985, Siokra 1-1 cultivar was the first okra-leaf cultivar to be developed and widely grown in the world with good fibre quality and resistance to BB. This breakthrough by “Commonwealth of Scientific, Industrial and Research Organisation” (CSIRO) led to the development of BB-resistant varieties that have dominated the Australian cotton industry to date (CIE, 2002). In addition, okra-leaf cultivars have been reported to be particularly useful for dryland areas (Stiller et al., 2004). Since 87% of the Kenyan land is classified as arid or semi-arid (CODA, 2009), these okra-leaf genotypes may form very useful accessions for these regions to boost cotton productivity. However, despite its advantages, okra-leaf cotton is not widely cultivated since several disadvantages are also

associated with this type of cotton. Although okra-leaf types produce more flowers than normal lines during the first half of the growing season, higher yields are not obtained due to a higher rate of flower abortion later in the season. This is attributed to reduced photosynthetic leaf area that is unable to assimilate enough carbohydrates to meet the demands of a heavy boll load (da Silva et al., 1996; Pettigrew, 2003).

Xiao et al. (2010) noted that resistant germplasm that is elite and locally adapted is most desirable in breeding for disease resistance; therefore identified accessions can form important resources for cotton improvement in the country.

The presence of a varying disease pattern among the 51 genotypes studied clearly indicates that more than one gene and several genetic effects (e.g. additive, dominant, epistatic) are possibly involved in conferring resistance to BB (Bird, 1960; Innes, 1983; Wallace & El-Zik, 1989; Wright et al., 1998). Further molecular analysis of this polygenic nature of BB resistance, is still needed to confirm our observations.

3.4.2 Comparison between Busia and Siaya for BB scores

The fairly linear pattern defined by the dotted lines in Figure 3.4 leads to the assumption that, in general, there is a certain similarity between the order of susceptibility in Siaya and the one in Busia, as was evidenced by the result of the Friedman test and the correlation analysis. Most accessions with a low rank in Busia also appear to have a low rank in Siaya (resistant side of the bisector). The same applies to the susceptible lines. Some accessions, however, are located at a greater distance from the bisector (shown in italics). T 03A is such an outlier, belonging to the highly susceptible lines in Busia, but apparently being less susceptible in Siaya; the opposite can be said of for example T 29. The most important conclusion that can be drawn from this scatter plot is that T 24A is the most resistant accession in both Busia and Siaya although it is not completely immune. The other okra-leaf type, T 18A, also appears to be among the least susceptible lines at both sites. Apart from molecular studies on the accessions, replications of the experiment over several growing seasons and locations still need to be conducted to verify consistency of the present findings. The reason for any outliers, for which the rank in Siaya is completely different from the one in Busia, is definitely not the weather (Fig. 3.1). If environmental conditions at one location would have been more advantageous for the development of the disease, the BB scores would have been systematically higher for each accession, resulting in the same susceptibility ranking as that of the other location. The most

probable explanation left is the likely disease escape by some accessions at one site or variation in BB pathogen population between both fields. Since susceptibility of cotton to BB depends on the resistance genes possessed by a cultivar and the races of *Xcm* present in the field, it is necessary to examine both pathogen diversity present in the region as well as the molecular basis of BB resistance genes in the accessions, which is not presented in this chapter.

3.4.3 Infection under greenhouse conditions

The findings of the greenhouse scoring (Table 3.1) are not surprising since the rather resistant accessions are also among the least susceptible ones in Busia and Siaya. Genotypes T 11 and T 16B seem to be quite immune in the glasshouse, but unfortunately they were not included in the field experiment due to a limited amount of breeder's seeds provided. The accessions that were present in the glasshouse but not in the field will therefore form part of future field evaluations and molecular studies (Chapter 5).

A special remark concerns T 09. Six weeks after infection, the accession still showed an immune response. It is also this line that did not show signs of infection in Siaya until final scoring. In Busia, recent symptoms were observed earlier but the severity remained low, leading to the position of fourth most resistant line. Even though parental information was not provided for the cotton line, results show that the line requires further studies on observed susceptibility/resistance. A similar observation could be made for T 24A and T 24B. Water-soaked symptoms in the field appeared relatively late with disease symptoms not severe at both sites. This low degree of susceptibility is again reflected in the glasshouse results. The second okra-leaf type, T 18A, reacted more susceptible to artificial infection.

3.5 CONCLUSION

Bacterial blight caused by *Xcm* is a serious disease present in all cotton-growing areas throughout the world. Overall, results of this study clearly prove that BB is a problem in Kenyan cotton fields as well, currently contributing to low yields. The most widely grown cultivar in Western Kenya, KSA 81M, was introduced 30 years ago as a BB-resistant line but is nowadays showing susceptibility to the disease, as evidenced by our research findings and farm visits. More likely than loss of BB resistance in the cultivar itself, KSA 81M may have lost most of its resistance to BB disease as a result of changes in the pathogen population that could have mutated or lost its specific *Avr* genes as discussed earlier in this chapter. Therefore, this research

aimed at identifying potential sources of BB resistance within the Kenyan cotton germplasm that could be useful to breeders for introducing BB resistance into future cultivars. However, most of the 51 cotton lines that were evaluated for disease symptoms in the field and/or greenhouse appeared to be susceptible to some extent, varying from fully susceptible to more resistant. In general, about 29% of the lines in the field (Busia) were considered to be less susceptible than the other 71%. Although accessions with complete immunity to the disease were not found, T 09, T 24A and T 24B were identified as among the most resistant accessions to both natural and artificial infection. Further molecular analysis is needed to determine the genetic background of this higher resistance. The fact that these lines are adapted to the Kenyan environment could be an advantage in improving BB resistance in Kenyan cotton. However, it is already a disadvantageous that bacteria can infect the genotypes, although at rather limited scale. When taking the latter into consideration, it would be necessary to introduce new sources of durable disease resistance. To enable the development or introduction of resistant varieties, it should be known which *Xcm* races are present in Kenya. When this information is available, it would be possible to use these varieties, or develop them through breeding, that carry *B* genes corresponding to the *Avr* genes of the present *Xcm* races. Kenya borders both Uganda and Sudan from where highly virulent strains that are capable of breaking all resistance genes except *B₁₂*, have been reported, it is important that steps are taken to manage the menace that has/is likely to be posed by presence of new virulent races of the disease. Therefore, more durable resistance needs to be developed. Since BB is a serious constraint to yield, this issue needs to be addressed within the current attempts to revive the cotton industry in Kenya. The present study findings will therefore add valuable insight to the efforts needed to address the challenges facing both the research on cotton improvement as well as the farmers' effort to increase cotton yield.

CHAPTER 4

Prevalence of bacterial blight (*Xanthomonas citri* pv. *malvacearum*) race 18 in cotton leaves isolated from Western Kenya revealed by PCR approaches, sequence analysis and pathogenicity test

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In preparation

ABSTRACT

Xanthomonas citri pv. *malvacearum* (*Xcm*) causes bacterial blight (BB) of cotton and is the most significant bacterial disease which infects all aerial parts of the host. Damage due to this disease is estimated at about 5-35% of yield loss. During 2011, 2012 and 2013 cotton growing seasons in Western Kenya, typical symptomatic plants were collected from farms in Siaya, Busia and Amagoro to survey the causal agent of the disease. Leaves of infected plants were utilised for bacterial isolation. Several strains were obtained and partial *gyrB* sequences were determined to distinguish xanthomonad from non-xanthomonad species. Identified *Xanthomonas* strains were then characterised by rep-PCR (BOX and ERIC) to determine the diversity of xanthomonad bacteria obtained from infected cotton tissue. From thirty-two field strains (2011/12 season) and seven reference strains *gyrB*-DNA fragments were also sequenced and sequence analysis was used to develop phylogenetic tree grouping for field isolates. Finally, field strains identified as *Xcm* were inoculated on cotton differential lines to determine their race. *gyrB* primers positively detected all laboratory xanthomonad strains isolated from infected tissues. Results of diversity analysis for field isolates based on rep-PCR analysis revealed tremendous variability among xanthomonads present on cotton plants; some pathogenic and others non-pathogenic. Results of phylogenetic analysis and pathogenicity tests confirmed the prevalence of *Xcm* pathovar race 18. This pathotype/race is a strongly virulent strain with global occurrence and capable of overcoming all resistance genes except *B₁₂*. This finding is the first report on the presence of *Xcm* race 18 on cotton in Kenya and will help cotton breeders to develop breeding programmes that will enable development of cotton cultivars with more durable BB resistance.

Key words: Cotton; Bacterial blight; race 18; *gyrB*, rep-PCR (ERIC and BOX); DNA sequencing; pathogenicity test.

4.1 INTRODUCTION

Cotton (*Gossypium* spp.) is the world's leading fibre and an important oil and livestock feed crop. Even though cotton no longer stands among Kenya's leading cash crops since its collapse in 1990s, it was once an important source of income for rural communities in semi-arid areas with low agricultural potential, as well as an important source of raw material for the national textile industry. Before the collapse more than two decades ago, the sector comprised 52 textile mills and employed over 42,000 people compared to the present where only 15 mills operate below capacity (Kapchanga, 2013). Despite the sector's decline in recent years, cotton is still considered one of the few cash crops with real potential for increasing employment opportunities and food security through income generation in the arid and semi-arid lands (ASALs) of Kenya (CODA, 2009; Pkania et al., 2014). Therefore, revitalising the cotton sector is one of the government's key development and industrialisation initiatives to be implemented mainly in the ASAL regions, and also in other high-potential areas where this crop is grown, as captured under Kenya's "Vision 2030" (CODA, 2009).

Despite this potential, cotton production is constrained by infestation with many pests and diseases (Ikiito, 2008). Bacterial blight (BB), incited by *Xanthomonas citri* pv. *malvacearum* (*Xcm*), is an important disease with economic significance worldwide especially in areas with hot and humid climatic conditions such as those experienced in Western Kenya (Bayles & Verhalen, 2007; Delannoy et al., 2005; Pkania et al., 2014; Sambamurty, 2006). Infection from *Xcm* can cause reduced stand because of premature leaf defoliation, stem blighting, blackarm/girdling, weakened stem breaking, boll shedding, boll rotting and reduced grade of cotton due to lint staining leading to severe yield and quality loss (Ray, 1946; Verma, 1986). Lint yield loss due to BB is estimated at about 5 up to 35% or even more (Allen, 2011; Bayles & Verhalen, 2007; Delannoy et al., 2005).

According to Akello and Hillocks (2002), BB was among the main factors that limited commercialisation of cotton after being introduced in the 1900s in Africa and remains a major threat to production to date. Earlier screening of the status of BB in Western Kenya revealed susceptibility of most Kenya cotton germplasm including the commercial cultivar (KSA 81M) initially bred for BB resistance. The BB disease survey established the need to collect data on the incidence and diversity and hence to determine prevalent races of *Xcm* in the cotton-growing

areas in Kenya (Pkania et al., 2014; Chapter 3) and in order to help design strategies for disease control.

To determine the prevalent races of *Xcm* present in farmers' fields, it is important to conduct a survey and do isolations. *Xcm* races vary in their aggressiveness to different cotton genotypes based on inherent resistance genes possessed therefore their varying susceptibility to BB. Recent years has reported tremendous advancement in methods for identification and characterisation of plant pathogenic bacteria. Simple, fast and reliable polymerase chain reaction (PCR)-based methods are desirable and have found practical application in modern pathology and microbiology studies (Adriko et al., 2014; Louws et al., 1999; Narayanasamy, 2011). PCR is widely used for diagnosis of several bacterial plant diseases (Hartung et al., 1993; Coletta-Filho et al., 2005). DNA fingerprinting for some PCR-based methods does not require isolation of pure bacterial cultures from infected tissues (Lewis-Ivey et al., 2009) and in most cases PCR-based techniques are accurate, sensitive, rapid, less labour intensive and more economical than conventional diagnostics (Adriko et al., 2014; Degefu, 2008). Several genomic regions have been characterised in plant pathogenic bacteria, using intergenic regions of 16S and 23S rRNA of bacteria, hypersensitive response and pathogenicity (*hrp*) genes and housekeeping gene clusters for PCR-based detection methods (Leite et al., 1994; Louws et al., 1999; Mondal et al., 2012, 2013; Parkinson et al., 2007; Trindade et al., 2007; Young et al., 2008). The rep-PCR polymorphisms of bacterial cultures have been used to detect diversity and differentiate bacterial species including *Xanthomonas* (Arshiya et al., 2014; Asgarani et al., 2015; Pan et al., 1997). The sequence of the gyrase B (*gyrB*) has been used for species identification within the genus *Xanthomonas* (Mondal et al., 2012, 2013; Parkinson et al., 2007). Its reliability in identifying *Xanthomonas* species has been proven useful for phylogenetic studies (Mhedbi-Hajri et al., 2013; Parkinson et al., 2007; Triplett et al., 2015).

Since the introduction of cotton in Kenya, few researches have been devoted to the BB disease problem despite its increasing prevalence. This is usually attributed to low interest in cotton since its collapse in the 1990s. Perhaps a better explanation for this is the unavailability of data on *Xcm* races in Kenya and the near denial by research centres and farmers of the presence of BB disease in the cotton fields (farmers' personal communication). The scientific community in the country is probably unaware of the disease incidence or tends to ignore the BB disease problem and its impact, because more priority goes to important food and cash crops such as maize, wheat, rice, beans, sugarcane, coffee, and tea, etc. The problem affecting many of the farmers on

the other hand is ignorance of the disease, where they observe the symptoms but mistakenly think it is related to natural leaf senescence (farmers' personal communication).

The main objective of this study was to isolate, establish diversity and determine the prevalent *Xcm* races present in cotton fields of Western Kenya based on DNA-based protocols that are simple, fast and reliable.

4.2 MATERIALS AND METHODS

4.2.1 Collection of plant material

Cotton leaves with typical BB symptoms of infection according to literature (Brinkerhoff et al., 1984) were collected from fields in Siaya, Busia and Amagoro of Western Kenya (Fig. 4.1). The three regions in Western Kenya were selected on the basis of having a long history of cotton cultivation where farmers have continued cultivation even after other regions abandoned the crop due to the challenges the sector continues to experience since the near collapse in 1990s. Samples were randomly taken from farmers' fields in the named locations. At least three fields per location were selected for sampling based on plants showing visible infection symptoms. In each field at least three plants were sampled per location. In 2011, sampling and isolation was done for Busia and Siaya with the aim to determine the races present in the region. However, isolates collected in 2011 failed the pathogenicity test for race identification. Additional isolates were needed to establish whether symptoms observed were genuinely due to *Xcm*. Therefore, in 2012 more isolates were collected from Busia in addition to strains collected in 2011. Strains were subjected to PCR of *gyrB* and rep-PCR analysis along with a pathogenicity test and sequencing to determine whether *Xcm* strains were actually present. Further, additional strains were collected in 2013 from Busia and Amagoro.

The sampled leaves were dried by pressing them between newspapers and then stored in paper bags for transport to Ghent University Belgium where further laboratory research was carried out.

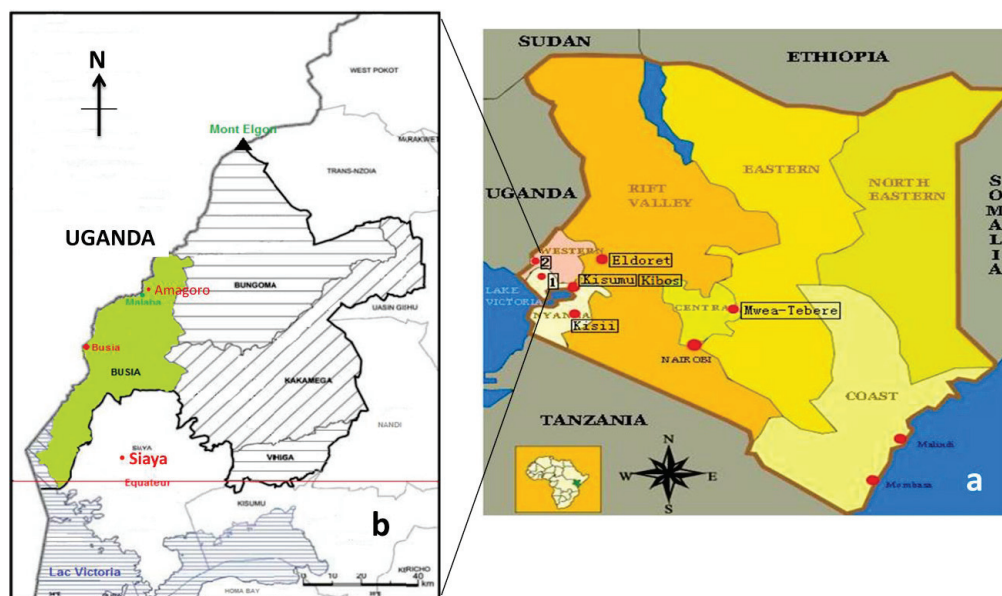


Figure 4.1: Map of Kenya: a) showing the former provinces and the two counties sampled indicated (1= Siaya; 2= Busia), b) the two counties zoomed; green colour (Busia) and white (Siaya)

4.2.2 Isolation of the bacteria

1.0 -1.5 cm² necrotic parts of the collected cotton leaf tissues collected were excised from the infected leaves and surface sterilised in NaOCl₂ and rinsed in distilled water for 3-4 times. The sterilised leaf lesions were then placed on yeast extract-dextrose-CaCO₃ (YDC) plates and incubated at 27 °C for 24-48 hours (hr). Yellow colonies around the lesions, were taken and streaked on separate YDC plates. Subculturing was carried out 3-4 times until pure colonies were obtained. Two freeze-dried *Xcm* strains (LMG 7430 isolated from Thailand and LMG 10422 from Nigaragua, respectively) provided by BCCMTM/LMG were included as reference strains in the identification analysis for 2011 isolates. Additional *Xcm* BCCMTM/LMG reference strains: {(R1 [LMG 9570] and R10 [LMG 9571] both collected from USA), (R6 [LMG 9574] and R7 [LMG 9573] both collected from Turkey), and (R18 [LMG 9572 collected from Nigaragua)]} were also included for the analysis of the 2012 and 2013 strains. Table 4.1 presents the isolates collected over the three years and used in this study.

Table 4.1: Table showing location, the *gyrB* positive strains and total bacterial isolates collected.

Year	Location	<i>gyrB</i> positive isolates for <i>Xanthomonas</i>		Total isolates collected
2011	Siaya	1	69	76
		14	75	
		15	82	
		16	84	
		29	85	
		38	86	
		41	88	
		44	122	
		54	130	
		60	134	
		61	167	
		64	172	
		67	173	
	Busia	19	104	51
		30	121	
		92	137	
		97	144	
		98	147	
		101	148	
2012	Busia	102	149	36
		B-11	B-26	
		B-12	B-27	
		B-20	B-29	
		B-21	B-ET	
		B-22	B-20-3	
2013	Amagoro	B-25		29
		A-1	A-6	
		A-2	A-7	
		A-3	A-8	
		A-4	A-9	
	Busia	A-5		21
		B-1 (B09)	B-4 (B23)	
		B-2 (B20)	B-5 (B24)	
		B-3 (B22)	B-6 (B25)	

NB: Total isolates collected each year were subjected to *gyrB*-PCR-analysis to identify those that are xanthomononads

4.2.3 DNA extraction

To prepare the bacteria for DNA extraction, single strain colonies obtained from overnight grown cultures were suspended in 10 mL Oxoid nutrient broth and incubated for 24 hr on a

shaker in a growth chamber with temperature set at 27 °C. After 24 hr the entire suspension was vortexed and transferred to a 15 mL falcon tube. Bacterial cells were harvested by centrifuging for 10 min at 7,500 rpm in a Sorvall RC-26 Plus floor centrifuge (Kendro Laboratory Products, Newtown, Connecticut). The bacterial pellet was suspended in phosphate buffered saline (PBS) solution and transferred to a 1.5 mL tube and centrifuged for 5 min at 13,000 rpm in a microcentrifuge (Eppendorf 5415 D, Hamburg, Germany). Subsequently, total DNA was prepared from the pellet using the Qiagen DNeasy 96 Blood & Tissue Kit (Venlo, The Netherlands) according to the manufacturer's protocol for extracting DNA from gram-negative bacteria and animal tissues.

The dsDNA concentration was quantified with an Eppendorf BioPhotometer and high concentrations were adjusted to 100-200 ng μL^{-1} for the PCR. The purity of the DNA was also assessed, using the ratio absorbance at 260 and 280 nm (A260/A280). The purified DNA was kept at -20 °C for further analysis.

4.2.4 PCR identification of *Xanthomonas* isolates

To detect *Xanthomonas* strains among the isolates, a specific PCR was needed. The BCCMTM/LMG *Xcm* strains were used as a reference, by comparing their DNA profiles with those of the field isolates.

In order to identify *Xanthomonas* strains among the field isolates, two primer pairs were used based on the conserved sequence of xanthomonad *gyrB* (Parkinson et al., 2007). The forward primer *gyrB3* (5'-CACCCAGGTGCTGCCGAT-3') was picked along nucleotide (nt)-positions 297-314 and the reverse primer *gyrB4* (5'-TTCATTTGCGCCAGGCCCT-3') was chosen at nt-positions 497-515 with an amplicon size of 200 bp.

Total DNA amplification volume was 25 μL , comprising 1 μL of DNA (100 ng) and 24 μL reaction mixtures consisting: 5 μL of 5X Green GoTaq, 1 μL (5 μM) of each primer pair, 1.25 μL dNTPs (4 mM for each dNTP), 0.125 μL Go Taq polymerase (5 U μL^{-1}) and 16 μL mQH₂O. The PCR cycling conditions were set at initial denaturation for 2 min at 94 °C; then 38 cycles each consisting of: denaturation for 45 sec at 94 °C, annealing for 40 sec at 59 °C, extension at 40 sec at 72 °C; and final extension for 7 min at 72 °C.

After amplification, DNA fragments were subjected to electrophoresis on a 1.5% agarose gel in TAE buffer. The bands were then stained with ethidium bromide and visualised on a UV trans-illuminator.

To determine identity of xanthomonad strains collected from the field, the *gyrB*-DNA amplicon fragments of size 200 bp for selected 27 isolates and 7 reference strains (2011 and 2012 season) were sent to South Korea for sequencing. The nucleotide gene sequences of each individual isolate were aligned using the ClustalW algorithm and afterward trimmed in frame to obtain equal lengths. Sequence alignment, trimming, nucleotide translation into amino acid sequences, and phylogenetic analysis was performed in MEGA 6 (Tamura et al., 2011). Phylogenetic trees for each independent gene alignment were constructed using the maximum composite likelihood method for calculating distances and the neighbour joining (NJ) algorithm for clustering. Phylogenetic analysis for the corresponding amino acid sequences was performed using the p-distance method and NJ clustering. For all phylogenetic trees, bootstrap analysis was performed using 1,000 bootstrap replicates.

4.2.5 rep-PCR analysis of *Xanthomonas* isolates

Isolates that were detected as *Xanthomonas* with PCR-based *gyrB* gene primers were further analysed by running a rep-PCR according to Louws et al. (1999) and applied by Zhai et al. (2010). For the 2011 season, 40 isolates and for the 2012 season eleven isolates identified as xanthomonads by *gyrB* primers were subjected to rep-PCR analysis.

The primers used were BOX (5'-CTACGGCAAG-GCGACGCTGACG-3'), ERIC1 (5'-ATGTAAGCTCCTGGGGATTAC3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGA- GCG-3'). They are complementary to repetitive sequences that are present throughout the bacterial genome. Total reaction volume was 25 μ L, including 2 μ L of DNA (100 ng μ L⁻¹) supplemented with 23 μ L of a prepared mixture of the components (12.5 μ L reaction mixture containing Taq DNA polymerase and dNTPs, 2 μ L BOX primer or 1 μ L ERIC1 and 1 μ L ERIC2 primer couple, 8.5 μ L mQH₂O).

The thermocycler conditions were set to run at: initial denaturation for 5 min at 94 °C, then 35 cycles each comprising of denaturation for 30 sec at 94 °C, annealing (BOX) for 1 min at 53 °C and (ERIC1 & ERIC2) for 1 min at 52 °C, extension for 4 min at 72 °C and a final extension for 6 min at 72 °C.

Amplification of different sized DNA fragments were separated by electrophoresis on a 2% agarose gel in TAE buffer. To enable a more precise estimation of the band sizes, a mixture of two promega markers was used (100 bp and λ DNA/*Hind*III). This combined ladder was loaded several times, for example every six samples. The gel had to run for 16 hr at a temperature of 4 °C (to prevent overheating). The gel was visualised using a UV trans-illuminator after staining in ethidium bromide for 30-45 minutes. The bands for each strain were scored as present or absent and a binary dataset was generated which was used for principal coordinate analysis (PCA) in GENALEX version 6.4 statistical software.

4.2.6 Pathogenicity test on cotton differential cultivars

To determine the races found in cotton fields in Western Kenya, the eleven cotton differential lines were inoculated with distinct *Xanthomonas* strains (identified by *gyrB* primers). *Xanthomonas* isolates were revived from -80°C by streaking on YDC media and cultured for 24-48 hr at 27°C. They were subcultured in liquid broth and harvested by centrifugation after 12-15 hr. The pellet of bacterial cells was suspended in PBS solution. The suspension was adjusted to an optical density (OD) at 620 nm of 0.1 using a Multiskan Ascent photometer (Thermo Fisher Scientific Inc. Waltham Massachusetts), corresponding with a concentration of 10^8 cfu mL⁻¹, and used to inoculate the cotton differentials.

The infection of cotton differential genotypes was conducted in a tropical greenhouse located at the UGent-ILVO campus (Melle, Belgium). The greenhouse is designed to simulate tropical conditions with a temperature set above 26°C, relative humidity >85% and a 16 hr photoperiod. Cotton differential lines were planted in individual pots with 4-6 plants of a single differential line. There were several pots per differential cultivar corresponding to the number of strains to be tested for all differential lines. The individual bacterial strains were used to inoculate the differential lines using the leaf infiltration method without a syringe (Fig. 4.2). Differential genotypes were inoculated when plants had 5-7 leaf stage. As control, differential plants were also inoculated with distilled water. Plants for each set of differential lines inoculated by an individual *Xcm* strain were grouped together. Bacterial blight typical water-soaking symptoms were monitored and recorded three times (3, 5 and 7 dpi) during the period as susceptible (+) or resistant (-). Plants with water-soaking symptoms were recognised as BB susceptible. Any differential pot that showed HR symptoms during the study period was considered as BB resistant.



Figure 4.2: Syringe inoculation using infiltration method

4.3 RESULTS

4.3.1 Bacterial isolation and purification

The bacteria isolation from cotton leaves in Kenya was done in three years (2011-2013). After incubation of the surface sterilised lesions on growth medium, bacterial growth could be observed around the leaf parts (Fig. 4.3). Most colonies seemed to be yellow or creamy coloured, sporadically an orange culture was seen.

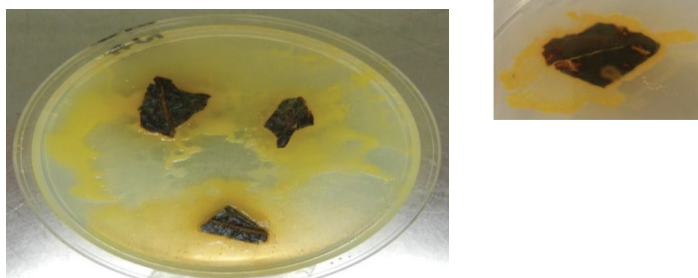


Figure 4.3: Bacterial growth around pieces of infected cotton leaves

In 2011, 2012 and 2013; 127, 36 and 50 isolates were retained for DNA extraction and storage on porous beads within a Pro-Lab Diagnostics Microbank™ cryovial at -80 °C, respectively (Table 4.1). The differential colours of the cultures obtained indicated the presence of more than one strain of bacterium from a single leaf lesions. The purpose of subculturing was to obtain pure and single colonies that had arisen from a single cell. Based on the colour, some cultures seemed to be quite uniform from the beginning (Fig. 4.4a & b), while others clearly showed to be mixed

cultures (Fig. 4.4c). As already noticed with the bacterial growth around the leaf pieces, the predominant colours were yellow (Fig. 4.4a) and white/creamy (Fig. 4.4b).



Figure 4.4: Cultures obtained during subculturing: (a) and (b) quite uniform, (c) more heterogeneous

4.3.2 PCR analysis of xanthomonad strains

4.3.2.1 Amplification of partial *gyrB* gene sequences from the 2011 season isolates

The result of the PCR amplification based on *gyrB* gene primers in distinguishing *Xanthomonas* species from the 2011 season is shown in Figure 4.5. The banding profile of the first 94 isolates can be seen in Figure 4.5a, the last two samples are the LMG strains. Since these latter strains are certainly *Xanthomonas*, their bands were used as a reference: all isolates with a single band at the same height as the reference strains were designated as *Xanthomonas*. Figure 4.5c shows the two LMG strains and two isolates that were positively identified as belonging to the *Xanthomonas* genus. Samples with no visible bands, bands at the different position compared to reference strains or multiple bands were excluded from further research. Figure 4.5b is a zoom of possible *Xanthomonas* strains showing a single band similar to reference strains, and also a few negative ones with (multiple) bands. Eventually, for 2011 only 40 from the 127 isolates were considered to be *Xanthomonas* and retained for diversity analysis. Among those samples, 14 came from Busia and the other 26 from Siaya (Table 4.1).

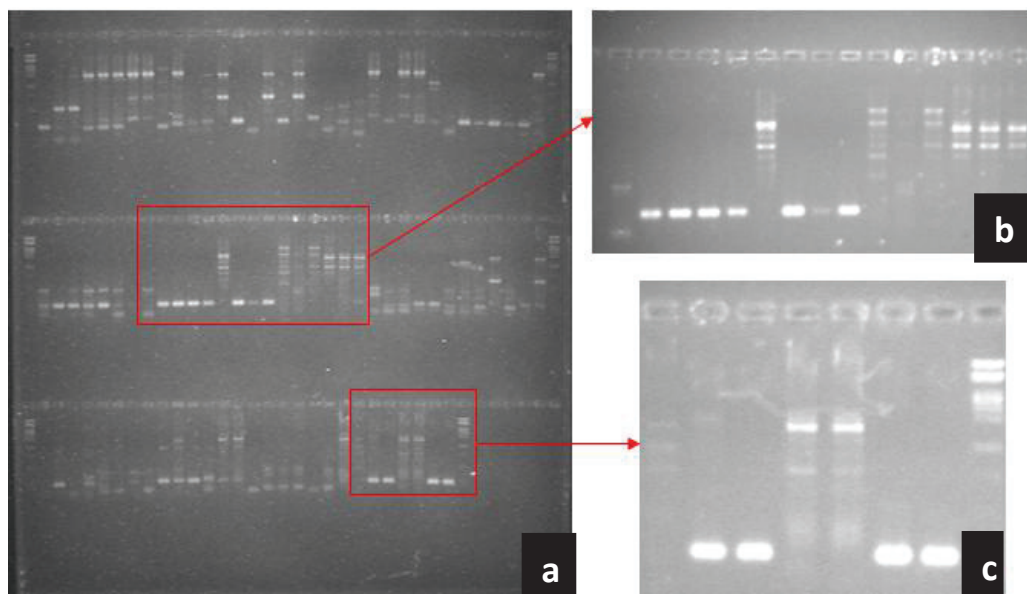


Figure 4.5: Banding profile of the first 94 isolates after *gyrB*-PCR analysis: (a) overview of 94 isolates band profiles, others with single amplified bands and others with multiple bands, (b) zoom on *Xanthomonas* (single bands) and non-*Xanthomonas* isolates, (c) zoom on the two LMG strains (right) and two *Xanthomonas* isolates (left)

4.3.2.2 Amplification of partial *gyrB* gene sequences for the 2012 isolates

Since selected isolates collected in 2011 failed for the pathogenicity test on cotton differential lines, 36 additional strains (2012) were collected from Busia to confirm earlier results (Table 4.1). The results of *gyrB* amplification for 25 selected field strains collected in 2012 and four reference strains are shown Figure 4.6. Noticeable is the single 200 bp band in some strains and multiple bands for others. Eleven field isolates were positively amplified by *gyrB* primers. This was very similar to amplification observed for strains collected during the 2011 cropping season (Fig. 4.5). As expected the reference strains (races: R1, R7, R10 and R18) amplified a single band. For each *Xanthomonas* strain (both isolates and reference strains) the *gyrB* primer was able to amplify a single 200 bp band suggesting the sensitivity of these primers to distinguish *Xanthomonas* strains from other non-xanthomonad isolates. Fifteen strains with multiple bands or single band strains but distinct compared to LMG reference strains were excluded from further analysis and were presumed non-xanthomonads.

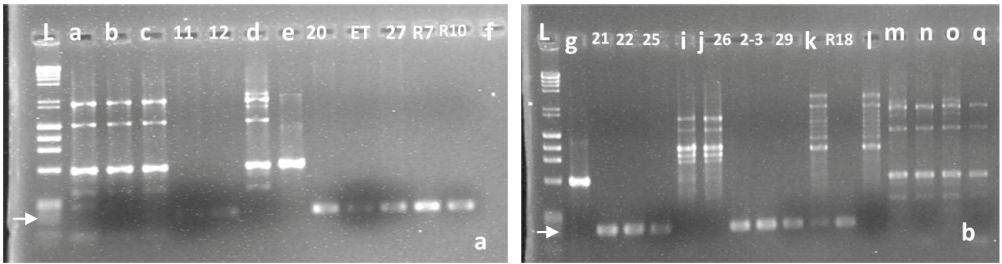


Figure 4.6: DNA profile of selected strains isolated in 2012 from infected cotton leaf samples amplified by *gyrB* primer pair. The field strains (coded 11, 12, 20, ET, 27, 21, 22, 25, 26, 2-3 and 29) amplified a single band similar to the reference strains from BCCMTM/LMG collection (R7, R10 and R18) in gel (a & b). The arrows show 200 bp markers for *Xanthomonas* strains. Samples with multiple bands or single bands with distinct bands (in alphabetical a-q) to reference strain bands were considered non-*Xanthomonas*. DNA ladder is in the first well for each gel labeled (L).

4.3.2.3 Amplification by *gyrB* for 2013 isolates

Fifty more isolates were collected in 2013 (Table 4.1) from Busia (21 isolates) and Amagoro (29 isolates) to further verify whether the results of isolation in 2012 could be replicated and therefore genuinely *Xanthomonas* capable to incite pathogenicity in cotton. As expected *gyrB* gene primers amplified a single 200 bp band for *Xanthomonas* strains (5 strains from Busia and 8 from Amagoro) and multiple bands for non-xanthomonads (Fig. 4.7). Similar results were observed for earlier strains collected in 2011 and 2012. This was therefore a clear confirmation of the presence of *Xanthomonas* in cotton.

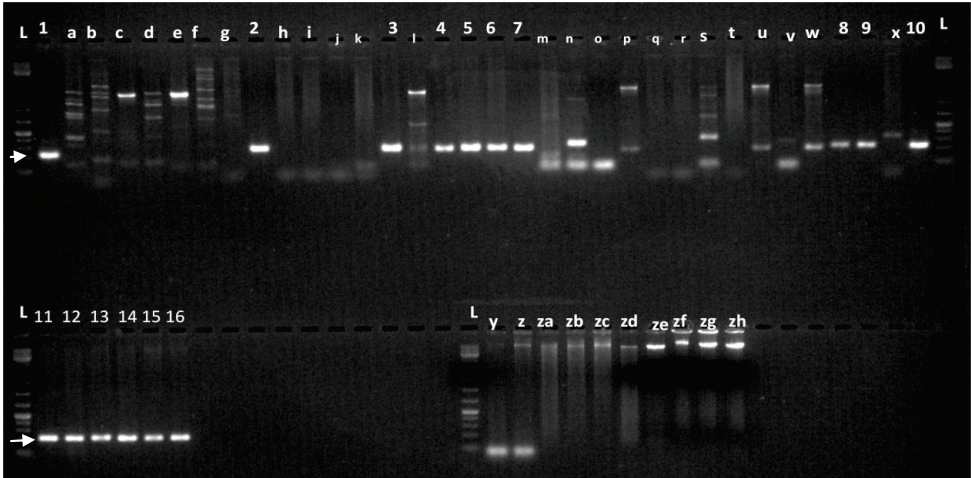


Figure 4.7: *gyrB* amplified products for 2013 field strains: Strains in numerals are positive for *gyrB* amplification (strains 2-7 [B09, B20, B22, B23, B24, and B25] were collected from Busia and 8-15 [A1, A2, A3, A4, A5, A6, A7, and A8] collected at Amagoro (Table 4.1), strain 1= reference strain race [R18] and 16 = reference strain [R7]). All strains labelled in alphabet are negative for *gyrB* amplification. Arrows show the 200 bp band marker.

4.3.2.4 Diversity analysis of the *gyrB* positive isolates

BOX-PCR analysis for the 40 identified *Xanthomonas* isolates yielded complex fingerprint patterns, with 29 different polymorphic markers that could be detected among the strains. The ERIC-PCR was performed to further evaluate the diversity since its primers target other repetitive sequences in the bacterial genome than the BOX-primers. ERIC-PCR yielded a total of 28 polymorphic markers (data not given).

In Figure 4.8, the genomic fingerprint of the BOX-PCR is shown. The isolate number is indicated to facilitate the link with further statistical analysis. In between every six samples, a DNA ladder was inserted. The DNA profiles indicate there is quite some diversity among the field xanthomonad isolates collected on cotton and between the isolates and reference strains. But some strains share many common bands or have nearly identical patterns, like for example the strains 30 and 38 or 172 and 173.

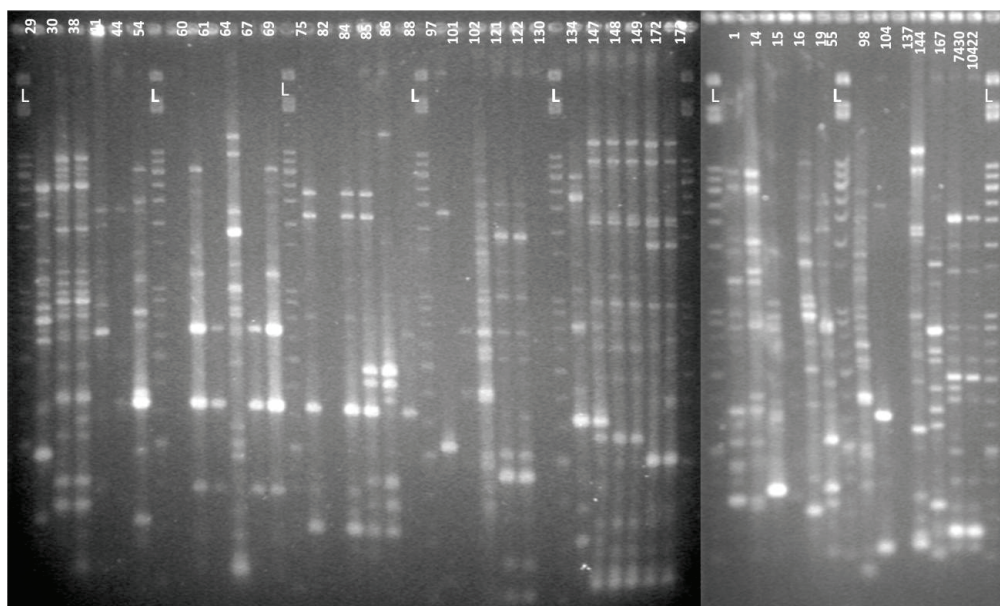


Figure 4.8: Patterns of BOX-PCR fingerprint after agarose gel electrophoresis of 40 identified *Xanthomonas* isolates numbered including reference (LMG strains: 7430 & 10422) and between every six samples a ladder (L) used to assist in analysis.

With the aid of the DNA ladder that was inserted several times, the position of the bands could be normalised and gave a total of 57 polymorphic markers for the combined analysis. The bands for each strain were scored as present or absent and a binary dataset was generated which was

used for principal coordinate analysis (PCA) in GENALEX version 6.4 statistical software. Isolates with an insufficient number of bands were left out. The first two principal components resulting from the PCA were plotted in a two-dimensional graph (Fig. 4.9). For the BOX-PCR, 27.69% of the variation is explained by the x-axis and 19.94% by the y-axis. The molecular variance between the population from Busia and the one from Siaya was only 1.4% ($\Phi_{IPT}=0.014$) with a P-value of 0.273, which means the two populations were not significantly different ($P \leq 0.05$) from each other (Fig. 4.9a). For the ERIC-PCR, the x-axis accounted for 23.14% of the variation and the y-axis for 21.09%. The variance among both populations was 1.7% with a P-value of 0.201, which is again not significant (Fig. 4.9b). For a final analysis, the results of both PCRs were combined, leading to the plot shown in (Fig. 4.9c). The x-axis explains 25.85% of the variation and the y-axis 18.07%. A variance among the populations of 0.7% with a P-value of 0.318 proves once more that there is no difference between the Busia and Siaya bacterial populations. The low percentage of molecular variance between the two populations is probably due to the relatively short distance of approximately 60 km in between the two locations and the same cotton cultivar that is commercially grown in the entire area of Western Kenya (KSA 81M). Farmers are also supplied cotton seed by one main supplier (CODA). In addition, it must be noted that the two counties of Siaya and Busia share a common border and they also lie on shores of Lake Victoria to the north-east with similar weather conditions (Chapter 3: Fig. 3.1). These many shared similarities between the two regions may therefore explain the low population variability between the two populations. For future research, it could be interesting to examine the *Xanthomonas* populations in Eastern Kenya, on the other side of the Rift Valley, where climatic conditions are completely different (a lot drier) and another commercial cultivar (HART 89M) is cultivated.

The appearance of the dot plots in general reveals the fairly scattered position of the dots indicating the presence of molecular diversity within the collected strains, which was already clear from the DNA banding profiles. No particular clustering specific for a particular region is observed, with the exception of the isolates 147, 148, 149, 172 and 173. In each graph they cluster together, away from the large point mass that contains the other strains. These five isolates all appeared to have white colonies. However, more white strains were included in the diversity analysis, but no link with colour can be made. Moreover, it was clear that colour was not a very stable characteristic since during subculturing and storage changes in colour, intensity were observed.

Concerning the two LMG collection strains, they appear to show some degree of relatedness, being located at the margin of the point mass in the BOX and BOX+ERIC graph. However, all PhiPT- and associated P-values indicate there is a significant percentage of variance between the reference collection strains and the populations in Busia and Siaya, possibly suggesting that the isolates are not *Xcm*.

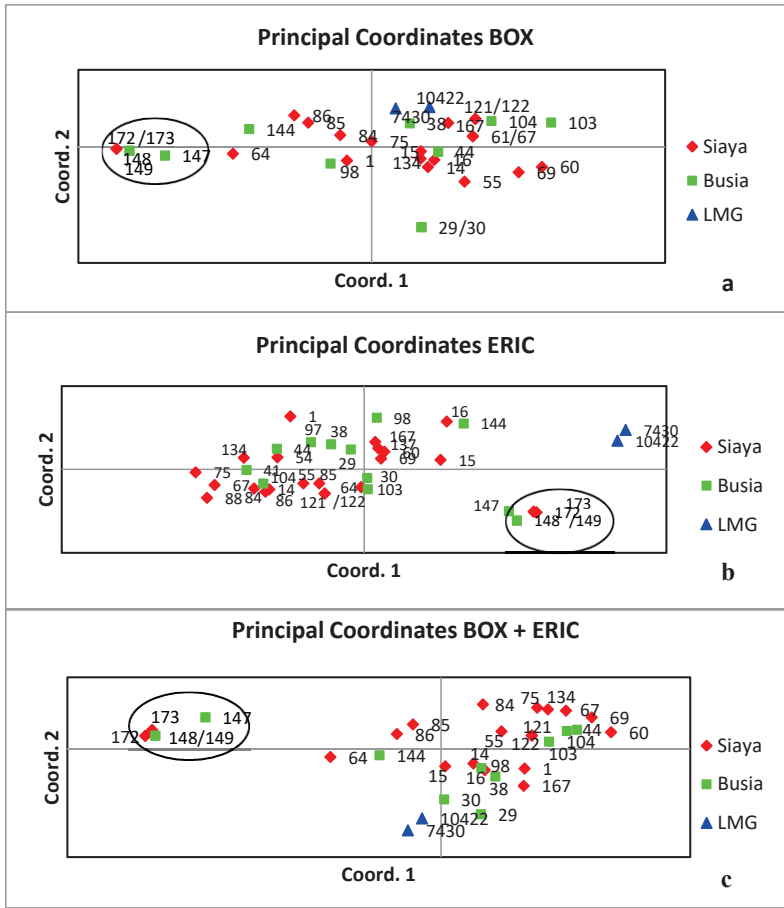


Figure 4.9: Diversity analysis (PCA) of *Xanthomonas* isolates: (a) PCA based on BOX-PCR, (b) PCA based on ERIC-PCR, (c) PCA based on combination of BOX and ERIC PCR; unique clustering of strains 147, 148, 149, 172 and 173 indicated with black circle. The dispersion of strains in disregard to location can clearly be seen showing no unique strains were associated with a particular location.

4.3.2.5 *rep-PCR characterisation of 2012 Xanthomonas strains*

The 2012 strains were genotyped with ERIC and BOX-PCR analysis and the latter one is shown in Figure 4.10a. All isolates collected from the infected leaf samples of cotton in Busia and positively identified by *gyrB* amplification were coded as: 11, 12, 20, 21, 22, 25, 26, 27, 29, B-ET (ET in gel), 20-3 (23 in gel). rep-PCR analysis based on BOX for all 2012 isolated strains gave similar fingerprinting patterns (Fig. 4.10a). The banding profile is similar to the bands of the LMG reference strain race 18 (R18). All additional LMG reference races R1, R6, R7 and R10 gave similar DNA banding profiles to the strains collected from fields except for 1, 2 or 3 variable band(s) indicated by arrows (Fig. 4.10a), suggesting a very close relationship between the reference strains with the field isolates. Among the eleven isolates, 18 polymorphic bands were identified with the BOX PCR. PCA analysis grouped all field strains to the right of the vertical bisector of the graph along with reference strain race 18 while the other reference strains were scattered to the left of the vertical bisector (Fig. 4.10b).

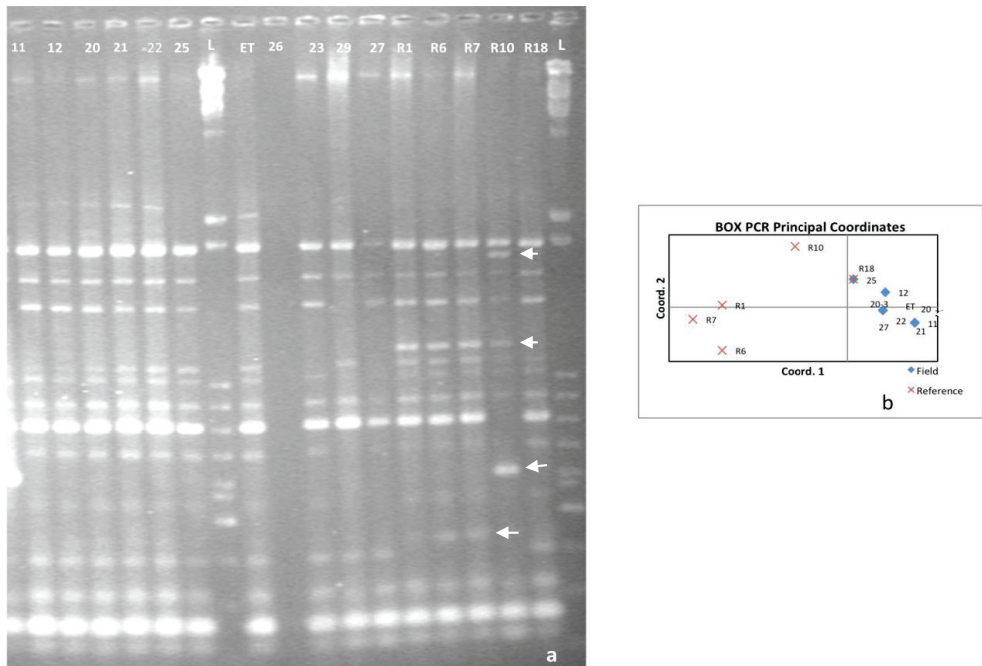


Figure 4.10: Diversity of 2012 xanthomonad isolates: a) gel picture of the isolates showing uniformity of the band pattern between field strains and reference race 18 (R18); b) BOX PCA analysis of field isolates showing all isolates on the right of the vertical bisector are supposedly *Xcm* race 18 while other reference strains lie to the left of the bisector. The arrows show band variation with other reference strains used (R1, R6, R7, and R10). Between every 11th sample a DNA ladder (100 bp and λ DNA/*Hind*III) was inserted to aid in analysis.

The BOX-PCR based analyses revealed its likely ability to differentiate the different pathotypes (races) of *Xcm* of cotton. The strains that were collected from the field were probably all *Xcm* race 18 as shown by their similarity in DNA profile and therefore BOX-PCR fingerprinting proved its usefulness in determination of diversity as well as ability to differentiate some strains of *Xanthomonas*. No rep-PCR analysis was performed for the 2013 isolates.

4.3.3 Pathogenicity test

Strains isolated and determined by *gyrB* analysis as xanthomonads were subjected to pathogenicity tests on cotton differential varieties as the ultimate determinant of *Xcm* pathotypes. The pathogenicity test for all the strains collected in 2011 resulted in an HR on all accessions whereas the typical BB symptom of water-soaking was obtained with the reference

strains on the susceptible accessions (Table 4.2). This was unexpected since the strains were selected based on phenotypic and molecular traits typical for *Xanthomonas*.

The additional isolates collected in 2012 and 2013 season were also subjected to a pathogenicity test along with five isolates obtained in 2011 that were re-tested. Eleven strains were used for the 2012 season and the 13 strains were used for 2013 season, along with five reference strains (Table 4.2). The susceptible differential lines showed water-soaking symptoms first, followed by chlorotic halos around the inoculation site (Fig. 4.11a). The resistant differential plants on the other hand expressed an HR at the inoculation site and no water-soaking symptom was observed (Fig. 4.11b). The control infection with distilled water showed no sign of water-soaking or HR and normally within a few hours of infection, the leaf lost the “water spot” initially observed upon inoculation and remained so throughout the experimental period (Fig. 4.2).

The pathogenicity test results of infection on cotton differential cultivars confirmed that the bacterial isolates collected from Western Kenya were all race 18 (Table 4.2). The isolates collected in the 2011 season (1, 64, 67, 84, and 85) again used to infect the cotton differential cultivars in 2012 and all of them resulted in an HR on all differential cultivars (negative pathogenicity test) confirming the earlier results obtained in 2011 that they were non-*Xcm* strains and therefore unable to cause BB in cotton.

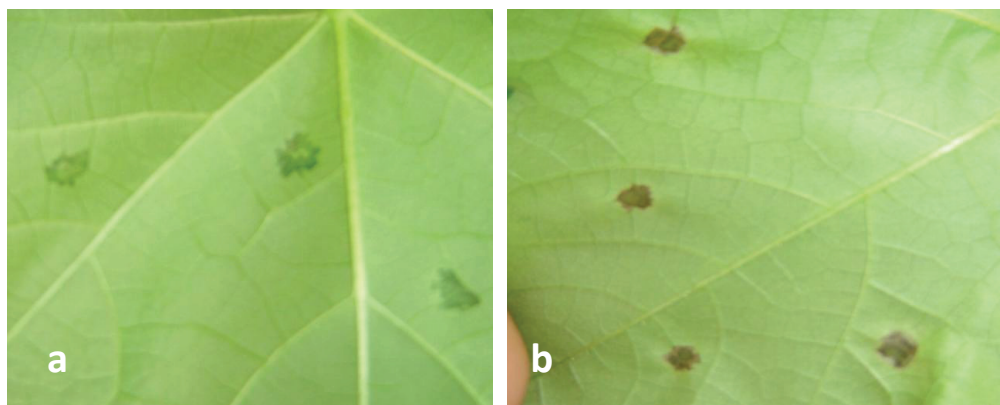


Figure 4.11: Pathogenicity reaction of field strain isolates on cotton differential cultivars 3-10 dpi: (a) susceptible water-soaking reaction and (b) hypersensitive reaction (HR)

Table 4.2: Reaction of cotton differential cultivars to infection with field and reference strains

Cotton differential genotypes											
	a	b	c	d	d	e	f	g	h	i	j*
Isolates (2011)											
1	-	-	-	-	-	-	-	-	-	-	-
64	-	-	-	-	-	-	-	-	-	-	-
69	-	-	-	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-	-	-	-
R18	+	+	+	+	+	+	-	+	+	+	-
Isolates (2012)											
B-11	+	+	+	+	+	+	-	+	+	+	-
B-12	+	+	+	+	+	+	-	+	+	+	-
B-20	+	+	+	+	+	+	-	+	+	+	-
B-21	+	+	+	+	+	+	-	+	+	+	-
B-22	+	+	+	+	+	+	-	+	+	+	-
B-25	+	+	+	+	+	+	-	+	+	+	-
B-26	+	+	+	+	+	+	-	+	+	+	-
B-27	+	+	+	+	+	+	-	+	+	+	-
B-29	+	+	+	+	+	+	-	+	+	+	-
B-ET	+	+	+	+	+	+	-	+	+	+	-
B-20-3	+	+	+	+	+	+	-	+	+	+	-
R18	+	+	+	+	+	+	-	+	+	+	-
R1	+	+	+	-	-	-	-	-	-	-	-
R6	+	+	-	+	+	-	-	+	-	-	-
R7	+	+	-	+	+	+	-	+	-	-	-
R10	+	+	+	+	+	+	-	+	-	-	-
Isolates (2013)											
A-1	+	+	+	+	+	+	-	+	+	+	-
A-2	+	+	+	+	+	+	-	+	+	+	-
A-3	+	+	+	+	+	+	-	+	+	+	-
A-4	+	+	+	+	+	+	-	+	+	+	-
A-5	+	+	+	+	+	+	-	+	+	+	-
A6	+	+	+	+	+	+	-	+	+	+	-
A7	+	+	+	+	+	+	-	+	+	+	-
A8	+	+	+	+	+	+	-	+	+	+	-
B-09	+	+	+	+	+	+	-	+	+	+	-
B-20	+	+	+	+	+	+	-	+	+	+	-
B-23	+	+	+	+	+	+	-	+	+	+	-
B-24	+	+	+	+	+	+	-	+	+	+	-
B-25	+	+	+	+	+	+	-	+	+	+	-
R18	+	+	+	+	+	+	-	+	+	+	-

Isolates collected from Amogoro (A1-8) and from Busia (B-). Differential cultivars; a=Acala 44, b=Stoneville 2B – S9, c=Stoneville 20, d=Mebane-1, e=1-10B, f=101-102B, g=Gregg, h=Empire B4, i=Deltapine B4, j*=S295, R=reference race strains (R1, R6, R7, R10 and **R18** from LMG collection; (+) = susceptible reaction of host differential; (-) = resistant (HR) reaction; 2011 strains = 1, 64, 69, 84 and 85 are non-*Xcm* strains

4.3.4 Partial *gyrB* gene sequence analysis of selected *Xanthomonas* strains from diseased cotton

To further determine the percentage identity of strains collected from the field for both 2011 and 2012 cropping seasons, purified *gyrB*-amplicons of individual isolates were sent to South Korea for sequencing. Eleven 200 bp *gyrB*-DNA fragments for 2012 strains and 16 from 2011 along with 7 from reference strains were sequenced. The 2011 strains were selected based on rep-PCR profiles most closely related to that of the reference strains. Since all 2011 strains were negative for pathogenicity, only a few strains were retained to check their relation to *Xcm*. A phylogenetic tree was generated using the neighbour-joining method using MEGA6 software for the field strains collected during the two seasons (2011 and 2012). Figure 4.12 shows the twenty five strains sequenced from the field grouped into two major clusters; group **I** consisting of all 2012 isolates clustering with the reference strains (LMG; 10422, 11726, 7430, 9572, 9571, 9574 and 9570) as indicated with the red brace (RG 9.5) and group **II** consisting of the 2011 isolates. The sequence results show that the 2011 isolates were composed of xanthomonad group (**II** [SLC1]) (Fig. 4.12) whose identity is still obscure. The 2011 isolates form a subgroup with GenBank® *Xanthomonas* LMG736^T (*X. vasicola* pv. *holcicola*) and LMG 9042^{PT} (*X. campestris* pv. *cannabis*). On the other hand, the 2012 isolates formed a cluster with the *Xcm* LMG reference strains and are clearly the *Xcm* strains since they form a single group with LMG reference strains with 99% identity (Fig. 4.12). Group **I** forms a subcluster with LMG 2797^T (*X. euvesicatoria*), LMG 982^T (*X. axonopodis* pv. *axonopodis*) and LMG 29033^T (*X. phaseoli*). The other group (**III**) consists of sample reference *Xanthomonas* strains from the GenBank collection which were included to further determine how they cluster with field isolates. The group **III** strains are known pathogens for different plants LMG 5047^T (*X. oryza* pv. *oryzae* [pathogen in rice]), LMG 747^T (*X. arbiricola* pv *juglandis* [walnuts]) LMG 568^T (*X. campestris* pv. *campestris* [brassica]) LMG 508^T (*X. fragariae* [strawberry]) and LMG 911^T (*X. vesicatoria* [peppers and tomatoes]) and clearly the phylogenetic tree cluster separates them from the *Xcm* field isolates and LMG *Xcm* reference strains.

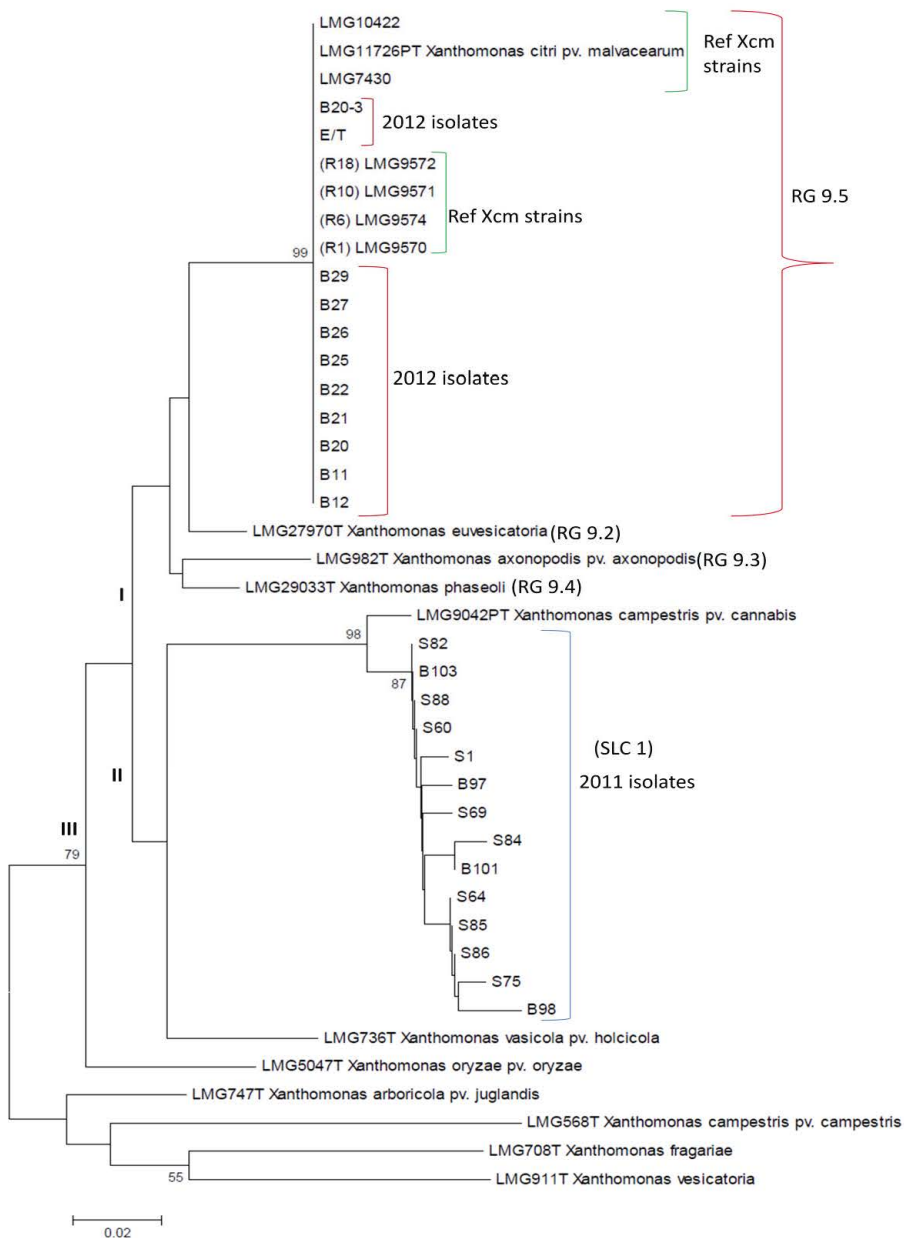


Figure 4.12: Phylogenetic relationship of Kenyan *Xanthomonas* isolates from cotton among type strains of selected *Xanthomonas* sp. based on partial *gyrB* sequences. The tree was constructed with MEGA 6 software using a neighbour-joining algorithm. Bootstrap values greater than 50% are shown for 1,000 replicates. Horizontal scale bar (0.02) at the bottom represents number of nucleotide substitutions. SLC 1= Species Like Clade 1 as reported by Parkinson et al., 2009; RG= refers to the *Xanthomonas axonopodis* subgroups defined by Rademaker et al., 2005; T= type strain; PT= pathotype strain.

4.4 DISCUSSION

In recent years, there has been a steady progress in methods used for the identification and detection of bacteria. At the end of the previous century immunological methods, such as fluorescent antibody procedures and enzyme-linked immunosorbent assays (ELISA), followed by DNA probe-based methods (Enne & Rijkelt, 1999; Feng, 1997) became very popular tools for a rapid and reliable identification of bacteria. Nowadays, sequence-based methods have revolutionised the identification and characterisation of microbes due to their reliability, sensitivity and higher resolution making them the current methods of choice (Brady et al., 2013; Meng et al., 2015; Martens et al., 2008; Triplett et al., 2015; Young et al., 2008, 2010). The strength of these methods was applied in this study to reliably identify xanthomonads isolated from infected cotton leaves in Western Kenya.

Altogether, the results of the *gyrB* phylogenetic analysis (Fig. 4.12) and the rep-PCR analysis for the 2011 and 2012 strains (Fig. 4.8, 4.10, respectively) confirmed that the strains collected in 2011 are non-*Xcm* strains. This is also in agreement with the results of the pathogenicity tests for the 2011 strains being non-*Xcm* therefore unable to infect cotton (Table 4.2). All strains collected in 2012 formed a single phylogenetic cluster (Fig. 12 group I) and are clearly *Xcm* strains since they form a single cluster with LMG reference strains R1, R6, R10 and R18 confirming rep-PCR fingerprinting and PCA analysis (Fig. 4.10).

The results of the *gyrB* sequence analysis therefore support the presence of xanthomonad non-*Xcm* and *Xcm* species of bacteria which naturally co-existing on cotton. Sometimes these bacteria have similar morphological and physiological characteristics, albeit that they are genetically different. In addition, the results show that some of these xanthomonad species are not pathogenic on cotton (Fig. 4.12 group II; Table 4.2 [isolate 1, 64, 69, 84 and 85]). *Xanthomonas* bacteria are known to exhibit host specificity and the results of the pathogenicity tests are in consensus that these saprophytic xanthomonads though present they are not pathogenic to cotton. The *gyrB* phylogenetic tree analysis of the field *Xcm* strains and reference strains (Fig. 4.12) agrees with pathogenicity test results observed on cotton differential lines (Table 4.2). A possible variation in a few base pairs on the sequences for different BB races is responsible for differential specificity to cotton differential cultivars but this is not clearly discernible among the field strains and the reference strains (R1, R6, R10 and R18). The results of *gyrB* phylogenetic

analysis of the field and reference strains along with rep-PCR analysis and pathogenicity test results therefore confirm race 18 presence in Western Kenya.

4.4.1 Identification of *Xanthomonas* strains by partial *gyrB* sequence analysis

Xanthomonas phenotypic and physiological characteristics are very homogenous (Mhebdi Hajri, 2011). Detection of pathogens in both symptomatic and asymptomatic plant tissues has become of importance in certification programmes for both domestic and international quarantine (Adriko et al., 2014). In this study, *gyrB* primers were used to amplify a distinct amplicon that can differentiate *Xanthomonas* species from non-*Xanthomonas* species (Mondal et al., 2012, 2013; Parkinson et al., 2007).

The comparison of *gyrB* sequences has proven a valuable and routine tool to identify *Xanthomonas* species collected from the field before proceeding to pathogenicity tests and race determination, especially when a large sample size is involved. Parkinson et al. (2007, 2011) reported that the *gyrB* locus provides the capability for identification to species and sequevar (strains characterised by a specific DNA sequence) level of plant pathogens within *Xanthomonas* and *Pseudomonas syringae*, respectively. Diagnostic protocols are currently being re-evaluated for inclusion of sequence-based diagnosis and identification. The molecular evolution of the *gyrB* gene has been determined to be faster than that of the 16S rRNA (Hauben et al., 1997; Mondal et al., 2013). Sequence analysis of *gyrB* further provides additional verification both for potential new pathogens as well as established pathogens because a blast search to GenBank can reveal true identity based on similarity/dissimilarity in their sequences. Phylogenetic grouping of field isolates clearly separated 2012 isolates corresponding to RG 9.5 according to Rademaker et al. (2005) (Fig. 4.12). The 2011 isolates form their own xanthomonad group (SLC1) according to Parkinson et al. (2009) and their role in cotton pathogenicity is still unknown (Fig. 4.12).

In the present study the number of isolated strains for example (2011 season) was reduced substantially based on amplification of a distinct *gyrB* fragment indicative for *Xanthomonas* (out of 127 strains only 40 strains turned out to be xanthomonads). In general, the *gyrB* sequence-based method proved very useful to identify xanthomonads isolated from cotton, enabling efficient use of resources and time.

4.4.2 Diversity of *Xanthomonas* strains isolated from diseased cotton in Western Kenya

The rep-PCR is a proven tool for the determination of genetic diversity of bacterial plant pathogens and also for epidemiological studies (Arshaya et al., 2014; Asgarani et al., 2015; de Bruijn 1992, Louws et al., 1998). The rep-PCR technique has effectively been used to profile gram-negative bacteria, such as *Xanthomonas*, *Pseudomonas*, *Ralstonia*, and *Agrobacterium* (Arshaya et al., 2014; Asgarani et al., 2015; Çepni & Gürel, 2012; de Bruijn 1992; Prasannakumar et al., 2012; Saïdi et al., 2011).

Here, we explored the genetic diversity of *Xanthomonas* strains collected from cotton farms in Western Kenya counties of Siaya and Busia and characterised them using rep-PCR (ERIC and BOX). For 2011 isolates BOX and ERIC-PCR revealed 29 and 28 polymorphic bands, respectively while for 2012 isolates, BOX analysis revealed 18 polymorphic bands size ranging from 100bp to 3kb; similar results were recently reported for other *Xanthomonas* studies (Arshaya et al., 2014; Asgarani et al., 2015). The large difference in polymorphic bands between 2011 and 2012 isolates is clearly the difference in strains; 2011 isolates consist of a variety of xanthomonad spp. strains including LMG *Xcm* reference races used in rep-PCR analysis while 2012 strains consist mainly of *Xcm* race 18 strains collected from the field along with LMG reference race strains which share many bands (Fig. 4.10).

In our study the combined analysis of the PCR patterns obtained by ERIC and BOX primers revealed a higher degree of genetic diversity among the non-pathogenic *Xanthomonas* strains than among the *Xcm* strains. The results further revealed a lack of molecular differentiation between Busia and Siaya populations which could be due to the close proximity of the two regions as well as the fact that farmers use one cotton cultivar (KSA 81M) and the seed is supplied by one main government agency (CODA). Since transmission of *Xanthomonas* is frequently occurring via contaminated seed, we suppose the main reason for lack of molecular differentiation between Busia and Siaya populations is the single source of infected cotton seed. Most bacterial strains over-winter in seed or crop residues and as the crop plants emerge during germination BB pathogen infect the plants, while other bacterial strains also regenerate, living a saprophytic lifestyle along with the BB pathogen. With isolation of *Xcm* strains during the 2012 and 2013 cropping seasons, we confirm the presence of the BB pathogen in Western Kenya.

Worldwide, the biggest threat to biosecurity of crop plants is the threat from bacterial plant pathogens (CRCNPB, 2012). Difficulties in early and accurate detection and identification of

pathogenic bacteria to the subspecific or pathovar level could seriously delay management of a disease during major outbreaks on significant crops, including cotton (Adriko et al., 2014; Louws et al., 1999; Narayanasamy, 2011). With advancement of PCR-based protocols these approaches need to be developed and routinely utilised to monitor bacterial diseases (Adriko et al., 2014; CRCNPB, 2012). A good example is the way some countries like the USA and Australia have a continuous scouting and monitoring of the cotton belts as part of national priority and routine procedure in the control of BB. As such, it is possible to detect an emerging pathogen threat and possibly arrest it early enough (quarantine) before it spreads to other regions within the cotton belt. This likely explains why these countries experience quite limited yield loss due to BB (Hillocks 1992; El-Zik & Thaxton 1992; Xiao et al., 2010). In conclusion, reliable and sensitive identification methods will enable rapid detection of *Xanthomonas citri* pv. *malvacearum* and help relevant authorities to devise appropriate strategies to limit the disease spread/damage.

4.4.3 Bacterial blight pathovars present in Western Kenya

By using rep-PCR, the genetic diversity among strains of plant pathogenic *Xanthomonas* can be demonstrated as has been proven in several publications (Massomo et al., 2003; Zhai et al., 2010). For instance, combined analysis of the PCR patterns obtained with ERIC and BOX primers showed polymorphism among Brazilian *Xanthomonas campestris* pv. *viticola* strains and allowed their distinction into subgroups (Trindade et al., 2005). Therefore, rep-PCR fingerprinting remains an important tool for distinguishing and tracking the diversity of a pathogen in a given affected region.

In our study, we explored the diversity of *Xanthomonas* strains collected from cotton farms of Western Kenyan region. Races of *Xcm* were determined by inoculation on a set of differential cultivars with isogenic background. Eleven *Xcm* strains were obtained from the 2012 season and 13 from the 2013 season. The pathogenicity test on cotton differential cultivars (Table 4.2) indicated that all strains belong to *Xcm* pathovar race 18. Also, BOX and ERIC DNA fingerprinting profiles agreed to a certain extent with the differentiation into races.

In breeding for disease resistance, it is important to understand the natural diversity of the pathogen population present in farmers' fields in a specific region (Delanoy et al., 2005; Fourie & Herselman, 2011; Xiao et al., 2010). Interestingly, all eleven *Xcm* strains isolated in 2012 from Western Kenya turned out to be race 18. The same result was found for the 13 isolates collected from Busia and Amagoro in 2013 (pathogenicity test [Table 4.2]). Since BB is a serious

constraint to cotton yield, this issue needs to be addressed within the current attempts to revive the cotton industry in the country. Allen and West (1991) observed the dominance of race 18 in Australia and speculated that prevalence of this race in many regions is the result of its aggressiveness and efficient dispersal by a single source of inoculum. The ability of this race to disperse easily compared to other races remains obscure and needs further investigation. Our results confirm the presence of this aggressive race 18 in Kenya, which would support it being the most frequently encountered race worldwide (Allen & West, 1991; Allen, 2011; Hussian, 1984; Thaxton et al., 2001; Verma & Singh, 1975). Race 18 is also capable to overcome most of the known resistance genes (B_1 , B_2 , B_{nb} , B_7 , and B_6); therefore proper strategies are needed to develop more resistant varieties. Since cotton differential cultivar 101-102B exhibited a resistant reaction in the pathogenicity screening on differential cultivars, we therefore rule out the presence of the so called “highly virulent strains” (HVS) reported in the neighbouring countries of Uganda and Sudan (Akello & Hillocks, 2002; Huang et al., 2008; Zhai et al., 2010). Our results, however, do not rule out the potential future emergence of HVS in Kenya due to unregulated cross border movement of cotton genetic material between these countries (Kameri-Mbote, 2005). Considering that HVS are capable of breaking all resistance genes except B_{12} , it is the ideal time for durable resistance breeding to incorporate especially resistance gene B_{12} . Therefore, breeding based on B_{12} resistance in a polygenic nature needs to be considered in the breeding programmes because there are high chances of HVS being introduced from neighbouring countries.

This is the first report on the presence of BB, caused by *Xcm* race 18, in Kenya. Our results therefore add crucial knowledge needed for breeding BB resistant cotton to improve overall productivity of the cotton sector.

4.5 CONCLUSION

The study presented used a *gyrB* sequence-based method to distinguish *Xanthomonas* isolates from other bacterial isolates found on infected cotton leaves. The rep-PCR DNA fingerprinting analysis of isolated bacteria for 2011 revealed diverse fingerprints indicating that there is a high genetic diversity among those isolates that, moreover, were negative for pathogenicity on cotton. Analysis of molecular variance between the populations from the two regions revealed limited variability between Busia and Siaya. This may be attributed to similar agricultural practices, environmental factors and government policy on cotton seed distribution shared by the two

regions. Phylogenetic analysis based on partial *gyrB* gene sequence analysis identified the strains as belonging to an unknown species within the genus *Xanthomonas*.

The field surveys made in subsequent years (2012 and 2013 season) yielded a collection of *Xcm* strains responsible for BB disease infection. Race determination on a differential set of 11 isogenic cultivars revealed the prevalence of race 18 in the region. Based on the present results HVS strains reported in the two neighbouring countries of Uganda and Sudan (Akello & Hillock 2002; Huang et al., 2008) are absent in Western Kenya. Since race 18 is closely related to HVS (Zhai et al., 2013), the present and future breeding strategies need to incorporate more sustainable resistance especially based on polygenic nature of resistance.

This being the first report on BB disease and molecular evaluation of diversity of *Xanthomonas* spp. in cotton in Kenya, the findings will therefore open opportunities for further studies on breeding for durable BB resistance in cotton. In conclusion, comparison of partial *gyrB* gene sequences from *Xanthomonas* strains isolated from diseased cotton and their pathogenicity testing on cotton cultivars, showed the presence of *Xcm* race 18 in Kenya but also the occurrence on cotton of saprophytic xanthomonads, whose role is still obscure.

CHAPTER 5

Genetic diversity of Kenyan cotton germplasm (*G. hirsutum* L.) determined by Amplified Fragment Length Polymorphism analysis.

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In preparation

ABSTRACT

Cotton (*Gossypium* spp.) is one of the most important and widely produced agricultural and industrial fibre crops in the world. Cotton is also a heavily traded agricultural commodity with over 100 countries involved in the export and import of cotton and its by-products. Morphological and molecular markers can play a critical role in determination of genetic diversity in cotton. Morphological markers are strongly influenced by environmental factors and are quantitatively inherited. Meanwhile molecular markers such as AFLP have high multiplex ability and provide a high level of resolution for organisms with a known limited diversity. Previously, morphological markers have been utilised to determine genetic diversity among cotton genotypes in Kenya.

In the current study, the genetic variability of Kenyan cotton accessions was evaluated using morphological and AFLP analysis. Genetic similarity among *G. hirsutum* genotypes based on AFLP analysis ranged from 0.890-0.975 and between the two populations of Tebere and Genebank Muguga ranged from 0.993-0.996 indicating quite limited genetic diversity in Kenyan cotton. Furthermore based on AFLP analysis, we can partially detect possible seed mixing among genotypes, a major impediment to cotton breeding in the country. In general more diverse germplasm of *G. hirsutum* L. and other tetraploid species need to be introduced to boost genetic variability to gain from breeding and selection.

Keywords: Cotton germplasm; Genetic diversity; AFLP analysis; Kenya.

5.1 INTRODUCTION

Cotton (*Gossypium* spp.) is one of the most important and widely produced agricultural and industrial fibre crops in the world. It is grown in more than 80 countries on about 2.4% of the world's arable land (Blaise, 2006), making it one of the most significant crops in terms of land use after food grains and soybeans. Cotton is also a heavily traded agricultural commodity with over 100 countries involved in the export and import of cotton and its by-products. China, India, Pakistan and the USA are the four leading producers accounting for over 70% of the world production (USDA-FAS, 2016).

The genus *Gossypium* comprises 50 known species with a basic chromosome number of $n=13$ (Poehlman & Sleper, 1995). Of those species, 45 are diploid ($2n=2x=26$ chromosomes), grouped into eight genomes A, B, C, D, E, F, G, and K while five species are tetraploid (Endrizzi et al., 1985; Percival et al., 1999). The narrow genetic base of the modern tetraploid cultivars is a result of human domestication and breeding. In the beginning, species were restricted to the areas of origin. After domestication, the specific ranges expanded across the world so that they eventually overlapped. The consequence was a blend of the genetic composition through interspecific gene flows (Wendel et al., 2009). In addition, a large part of the available genetic diversity was left unused due to lack of information on traits of interest by human selection and breeding programmes as the focus remained with few accessions with known useful traits, leading to genetic narrowing (Wendel et al., 2009).

Commercially grown tetraploid cultivars (*G. hirsutum* L. and *G. barbadense* L.) are known to have been developed from a few genotypes resulting in a quite limited genetic variability (Iqbal et al., 2001; Lu & Myers, 2002; Multani & Lyon, 1995). Nevertheless, many cotton genotypes express substantially diverse phenotypic characteristics in a variety of traits: leaf colour, shape, hairiness, pollen colour, stigma position, plant height, number of bolls, etc. This contradiction has created a major challenge to cotton breeders in selection of breeding parents for their breeding programmes based on phenotypic traits to gain from selection and hybridisation. Understanding of genetic relationships among plant genotypes is significant to know the complexity of the available germplasm, to enable the discovery of differences in available genotypes, and to build up useful conservation strategies (Dahab et al., 2013). Until recently, breeders have been improving both qualitative and quantitative inherited traits by conventional breeding based on phenotypic characteristics which is resource and time consuming. This

approach still persists in some developing countries with limited funding for research and development, among them Kenya. Characterising genetic diversity and the degree of association between and within varieties is the first step towards utilising crop germplasm and cultivars. Successful crop improvement depends on genetic diversity (Rana & Bhat, 2004; Russel et al., 1997). A lack of genetic diversity may limit breeding and gain from selection leading to poor cultivars characterised by low yields and susceptibility to pests and diseases.

In chapter 3, BB disease evaluation revealed that the degree of susceptibility among the Kenyan cotton genotypes differed significantly ranging from highly susceptible to resistant but none of the genotypes was totally immune (Pkania et al., 2014). Additionally, BB pathogen diversity evaluation revealed the predominance of race 18 in the pathovars collected from fields of Western Kenya (Chapter 4). To get an idea if the varying BB susceptibility in Kenyan cotton genotypes was due to genetic factors or not, an attempt to tag markers associated with BB disease resistance is necessary. The focus of this study therefore was to determine the molecular diversity of Kenyan cotton genotypes by screening cotton germplasm conserved in genebanks to identify possible sources of BB resistance.

Molecular markers have proved to be valuable tools in the characterisation and evaluation of genetic diversity within and between species and populations. Marker systems differ in their information content, which depends on polymorphisms. Several DNA fingerprinting techniques are currently routinely available. DNA-based markers possess many advantages, which make them superior to morphological markers. With the advancement in molecular techniques, traditional DNA markers such as RFLP, RAPD, ISSR, AFLP, SSR, etc used in genetic studies are losing appeal with the development of genome-wide association techniques. In association mapping, non-structured (bulk) populations are phenotyped and genotyped to tag the trait associated with the marker (Myles et al., 2009). This leads to capturing of wider recombination and higher resolution mapping as compared to traditional linkage mapping (Zhu et al., 2008). The applications of association mapping for cotton helps in extensive deployment of natural genetic diversity conserved within the global collections of cotton germplasm (Abdurakhmonov, 2007). In addition, with advances in sequencing technologies, recent next generation DNA sequencing (NGS) has enabled researchers to rapidly develop large numbers of single nucleotide polymorphism (SNP) markers at relatively low cost (Maughan et al., 2009; van Deynze et al., 2009; Wang et al., 2015). Low cost NGS technologies have been successful for whole genome sequencing (Li et al., 2014; Wang et al., 2011, 2015; Xu et al., 2011). SNP discovery for both

small and large genome sized organisms including complex polyploid organisms such as cotton with a narrow genetic base is now possible (Byers et al., 2012; Gore et al., 2014; Wang et al., 2015).

Since Kenyan cotton parental selection for hybridisation has been based on morphological and other agro-phenotypic characteristics there is a need for molecular marker analysis of available genetic material for cotton improvement. Despite the availability of modern NGS technologies, traditional molecular marker technologies such as AFLP still remain valuable for diversity analysis. One of the advantages of the AFLP technology is that genome-wide screening of genetic diversity is possible without a priori knowledge of genome sequences (Schlötterer, 2004; Vuylsteke et al., 2007). AFLP is also useful for organisms with known low genetic divergence (Bonin et al., 2007; Meudt & Clarke, 2007) as is the case for cotton. It is capable of detecting the presence of point mutations, insertions, deletions and other genetic reorganisations and is very reliable and reproducible for genetic diversity evaluation. Capillary electrophoresis allows for rapid and high throughput fluorescent or infrared detection (Meudt & Clarke, 2007; Vuylsteke et al., 2007). Furthermore, analyses of capillary profiles with currently available automated scoring software allows the users to control several parameters that influence the resulting data matrix with often objective, repeatable and far less time consuming analysis.

The main aim of this study was therefore to evaluate cotton accessions in Kenyan genebanks to determine the level of genetic diversity using AFLP analysis that would help identify potential breeding parents for essential agronomic traits such as BB resistance which can be utilised in breeding programmes to improve commercially grown cultivars in the country.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Besides the 51 cotton genotypes that were evaluated for BB (Chapter 3 [Fig. 3.1]), additional accessions T11, T18C (not in the field), one *Gossypium barbadense* L. (Samburu[SA]) genotype and S295 (cotton BB differential line) were included in this study. The Kenyan breeding accessions (Tebere) are under evaluation for various morpho-agronomic characteristics and fibre quality, while the Kisii/Kisumu accessions are genotypes collected from KALRO Genebank Muguga where they are conserved as genetic resources which researchers can access and utilise for different breeding objectives.

Table 5.1: list of Kenyan cotton genotypes

Cotton genotype	Genotype code	Cotton genotype	Genotype code	Cotton genotype	Genotype code
Kisii 01	K 01	Tebere 01	T 01	Tebere 18B	T 18B*
Kisii 02	K 02	Tebere 02A	T 2A*	Tebere 18C	T 18C*
Kisii 03	K 03	Tebere 02B	T 02B*	Tebere 20	T 20
Kisii 04	K 04	Tebere 03A	T 03A*	Tebere 21A	T 21A*
Kisii 05	K 05	Tebere 03B	T 03B*	Tebere 21C	T 21C*
Kisii 06	K 06	Tebere 04	T 04	Tebere 22	T22
Kisii 07	K 07	Tebere 05	T 05	Tebere 23A	T 23A
Kisii 08	K 08	Tebere 06	T 06	Tebere 24A	T 24A*
Kisii 09	K 09	Tebere 07	T 07	Tebere 24B	T 24B*
Kisii 10	K 10	Tebere 08	T 08	Tebere 25	T 25
Kisumu 01	KU 01	Tebere 09	T 09	Tebere 26	T 26
Kisumu 02	KU 02	Tebere 10	T 10	Tebere 29	T 29
Kisumu 03	KU 03	Tebere 11	T 11	Tebere 40	T 40
Kisumu 04	KU 04	Tebere 12	T 12	Commercial Cultivar	KSA 81M
Kisumu 06	KU 06	Tebere 15A	T 15A*	Samburu	SA
Kisumu 07	KU 07	Tebere 16B	T 16B*	S295	S295
Kisumu 08	KU 08	Tebere 16C	T 16C*		
Kisumu 09	KU 09	Tebere 17	T 17		
Kisumu 10	KU 10	Tebere 18A	T 18A*		

NB: All accessions are *G. hirsutum* L. except Samburu (SA) (*G. barbadense*). Genotypes in bold with asterisk are accessions provided as a single genotype but upon multiplication individual plants exhibited different morphological characteristics and were harvested separate and differentiated as either A, B or C.

5.2.2 Morphological trait evaluation of Kenyan cotton accessions

In effort to study Kenyan cotton, the 51 accessions established for BB disease evaluation (Table 5.1) were also utilised for morphological characterisation studies. The morphological properties consisted of leaf shape, colour and hairs, stem colour and hairs, petal and pollen colour and stigma position. Evaluation of morphological traits was based on the Descriptor List for cotton (revised 1985) according to International Board for Plant Genetic resources (IBPGR). In addition to the eight traits from the field morphological characterisation, two extra properties were included, being stem colour of the seedlings and 100-seed weight (or seed index).

5.2.3 AFLP analysis of Kenyan cotton genotypes

For the purpose of AFLP analysis three plants per accession were grown in individual pots in the growth chamber of Ghent University, Belgium for DNA extraction (Table 5.1). The Peltracom substrate containing a mixture of organic and inorganic fertiliser suitable for plant growth was utilised to grow the seedlings. Conducive germination and growing temperatures were provided in the growth chamber with day temperatures of 26 °C and 18 °C at night.

5.2.3.1 DNA extraction

Extraction of the DNA was performed by using an economical and rapid method for extracting cotton genomic DNA, which is a modification of the traditional CTAB method as modified by Zhang and Stewart (2000). The concentration and purity was then determined with a nanodrop spectrophotometer at dilution 50 by taking 1.5 µL of DNA to display the absorbance spectrum (Blignaut et al., 2013).

5.2.3.2 AFLP analysis

AFLP analysis was performed based on the technique of Vos et al. (1995) with some modification. The 2.0 µL (250 ng µL⁻¹) DNA was digested with *EcoR1* (hexa-cutter) and *Mse1* (tetra-cutter) at 37 °C for 2 hrs in a final volume of 25 µL. The digested samples were then incubated at 70 °C for 15 min to inactivate the restriction enzymes.

EcoR1 adapter (5 µmol) and *Mse1* adapter (50 µmol) were ligated to the restricted DNA fragments in ligation buffer (10x T4-DNA ligase buffer, 0.2 U of T4-DNA ligase) and incubated at 16 °C overnight.

Pre-amplification of ligated DNA was carried out with primers (complimentary to *EcoR1* and *Mse1* adaptors, with one selective nucleotide adenine and cytosine, respectively) in a total of 20.0 µL consisting of 4.0 µL of restriction/ligation product as DNA template, 4.0 µL PCR buffer (5x [1.5 mM MgCl₂]), 8.9 µL mQH₂O, 0.8 µL dNTP (5 µM each), 1.1 µL primer (5 µM [*Mse1*, *EcoR1* each]) and 0.1 µL Taq polymerase (0.5U µL⁻¹). The amplification reaction was performed in a PCR thermocycler (GeneAmp PCR System 9700, Applied Biosystems Inc, Foster City, California) with the following programme: 2 min 72 °C, (20 sec 94 °C , 30 sec 56 °C , 2 min 72 °C) ×20, 30 min 60 °C, 4 °C overnight.

The diluted (20-fold), pre-amplified products were used as template for selective amplification. The selective amplification was carried out with 3 different primer combinations of *EcoRI*-adapter primer (fluorescently labeled) and *MseI*-adapter primer (non-labeled) with three selective nucleotides (Table 5.2) in a total volume of 20 μL comprising of 5.0 μL of pre-amplified DNA product, 4.0 μL PCR buffer (5x [1.5mM MgCl_2]), 5.7 μL ddH₂O, 0.2 μL BSA, 0.8 μL dNTP (5 mM each), 1.1 μL *MseI* (5 μM), 1.0 μL *EcoRI* primer (1 μM [fluorescently labeled]), 0.1 μL Taq polymerase (5 U μL^{-1}) and 2.1 μL ddH₂O. Touchdown PCR amplification was used to reduce non-specific amplification by optimising the optimal annealing temperature. The touchdown PCR programme used was as follows: 2 min 94 °C, (30 sec 94 °C, 30 sec (65 °C decreased by 0.7 °C during 13 cycles), 2 min 72 °C), (30 sec 94°C, 30 sec 56 °C, 2 min 72 °C)×23, 4 °C if the thermal cycler runs overnight.

Table 5.2: Adapter and primer sequences for AFLP Analysis of Kenyan cotton accessions

<i>MseI</i>-adapter	<i>EcoRI</i>-adapter
5'-GACGATGAGTCCTGAG-3'	5'-CTCGTAGACTGCGTACC-3'
3'-TACTCAGGACTCAT-5'	3'-CATCTGACGCATGGTTAA-5'
<i>MseI</i>-primer + 1	<i>EcoRI</i>-primer + 1
(5'-GATGAGTCCTGAGTAAAC-3')	5' GACTGCGTACCAATTCA-3'
<i>MseI</i>-primers + 3	<i>EcoRI</i>-primers + 3
(5'-GATGAGTCCTGAGTAA-NNN -3')	(5'-GATGCGTACCAATTC-NNN -3')
<i>MseI</i> +CTC	<i>EcoRI</i> +ACT/FAM
<i>MseI</i> +CTC	<i>EcoRI</i> +ACC/NED
<i>MseI</i> +CTA	<i>EcoRI</i> +ACG/JOE

PCR products were prepared for capillary electrophoresis by mixing loading buffer containing 24.0 μL deionised formamide and 1.0 μL of GENESCAN-500 (ROX) sized standard sufficient for each sample. 25.0 μL of loading buffer mix was added to a sample tube and finally 0.1 μL of selective amplification product was then added to each tube. Samples were then denatured at 95 °C for 5 min followed by a quick cooling in ice. AFLP fragments were resolved in an ABI PRISM 310 automated capillary sequencer. AFLP primer combinations and adapters used for screening the cotton accessions were selected based on those showing higher polymorphism (Adawy et al., 2006) and are shown in Table 5.2. The ABI PRISM sequencer results were then collected and recorded in genescan software. The presence or absence of all fragments was confirmed manually since intensity differences between samples might result in false absences. The resulting AFLP fragments displayed as peaks by electropherogram were coded using a

binary unit character (1 for presence and 0 for absence) in the GeneMapper program. Data was summarised in a data matrix in excel for varieties based on both unique and shared fragments.

5.2.3.3 *Genetic diversity*

Locus-specific variability was measured with the polymorphic information content (PIC) for dominant markers (De Riek et al., 2001). For each genotype we generated a binary presence-absence data matrix. From this we calculated the total number of loci generated per primer pair as well as the percentage of polymorphic loci for each population in GENALEX version 6.4 (Peakall & Smouse, 2006). Expected heterozygosity (H_E), under the assumption of Hardy-Weinberg equilibrium, was also calculated in GENALEX version 6.4 (Lynch & Milligan, 1994). The resulting character matrix of 0's and 1's was also exported for hierarchical clustering using GENSTAT (version 12 edition). Fragment peaks smaller than 50 bp were excluded from the matrix.

5.3 RESULTS

5.3.1 Morphological diversity analysis

The morphological scores developed based on the IBPGR system were transformed into binary data that could be used in the program GENALEX version 6.4 to run a Principal Coordinate Analysis (PCA). To visualise diversity, the different accessions were plotted in a two-dimensional principal coordinate graph. In addition, an AMOVA analysis (999 permutations) was performed to examine the degree of variance between the Kisii/Kisumu and Tebere accessions. When the PhiPT-value was accompanied by a P-value ≤ 0.05 , the variance was considered statistically significant.

Evaluation of morphological characteristics was done at both sites. However, in Siaya most plants had no flowers because the plants were still recovering from hail stone damage through new vegetative growth. The consequences of the hail-storms meant smaller plants as a result of the main stems being severed by the hail. In effect, the height of the plants was not determined. Furthermore, it was decided not to determine factors like number of bolls and yield since the BB disease incidence and hail stones had damaged the plants so severely, which would affect the results leading to non-representative statistics for respective accessions. Since flower-associated traits could not be determined in Siaya, diversity analysis was performed using the data from the Busia scoring only, which are listed in Table 5.3.

In general, most accessions seemed to share similar phenotypic traits, the exceptions are indicated in bold/red in Table 5.3. The leaf shape was mostly lobed (normal leaf shape), with only T 18A and T 24A having okra-leafed phenotypes. T 18A was among the first lines to show boll formation, confirming the early maturity characteristic that is typically observed for okra-leaf genotypes. Leaves were mostly green in colour, with the exception of T 15A, T 16A, T 16C and T 23A, which were characterised by a red leaf colour. Furthermore, only T 08 and T 16C were characterised with long and dense hairs on the leaf surface while the others were mainly short haired. The stem hairs, on the other hand, were generally long. For the stem colour, green, red and greenish purple were observed. Concerning the flower traits, petal colour was mostly creamy and pollen colour usually yellow. However, K 07, KU 04, T 08 and T 20 formed creamy coloured pollen and with T 15A, T 16A and T 23A purple pollen was seen. The stigma was mostly positioned above the anthers. In some accessions (K 07, T 03B, T 04, T 10, T 18B, T 22, T 29 & KSA 81M) stigma and anthers were at the same level, while only T 20 had a stigma position below the anthers. In T 08, all three possibilities were observed at the same time even for flowers from the same plant. In addition, the stem colour of the seedlings and the seed index were also included in the scoring. The seedlings usually had a green stem colour, however red or a mixture of both colours was also observed. These observations changed as plants grew older.

Table 5.3: Morphological characteristics studied for all accessions in Busia

Characteristic	K 01	K 02	K 03	K 04	K 05	K 06	K 07	K 08	K 09	K 10	KU 01	KU 02	KU 03	KU 04	KU 05	KU 06	KU 07	KU 08	KU 09	KU 10	T 01	T 02A	T 03A	T 03B	T 04	T 05
Leaf shape (1 = entire; 2 = lobed; 3 = subokra; 4 = okra)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Leaf colour (1 = green; 2 = greenish purple; 3 = red)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Leaf hairs (0 = glabrous; 3 = short hairs; 7 = long hairs)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Stem colour (1 = green; 2 = greenish purple; 3 = red)	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	2	2	3	3
Stem hairs (0 = glabrous; 3 = short hairs; 7 = long hairs)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Petal colour (1 = white; 2 = cream; 3 = light yellow; 4 = yellow; 5 = lavender)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Pollen colour (1 = cream; 2 = yellow; 3 = purple)	2	2	2	2	2	2	1	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2
Stigma position (1 = below anthers; 2 = same level as anthers; 3 = above anthers)	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	3
Stem colour seedlings (g = green; r = red)	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g
Seed index (1 = ≤ 9.9 g; 2 = 10-11.9 g; 3 = 12-13.9 g; 4 = ≥ 14 g)	1	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	2	3	2	3	3

Characteristic	T 06	T 07	T 08	T 09	T 10	T 12	T 15A	T 16A	T 16C	T 17	T 18A	T 18B	T 20	T 21A	T 22	T 23A	T 24A	T 24B	T 25A	T 26	T 27	T 28	T 29	T 40	KSA
Leaf shape (1 = entire; 2 = lobed; 3 = subokra; 4 = okra)	2	2	2	2	2	2	2	2	2	2	4	2	2	2	2	2	4	2	2	2	2	2	2	2	2
Leaf colour (1 = green; 2 = greenish purple; 3 = red)	1	1	1	1	1	1	3	3	2	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1
Leaf hairs (0 = glabrous; 3 = short hairs; 7 = long hairs)	3	3	7	3	3	3	3	3	7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Stem colour (1 = green; 2 = greenish purple; 3 = red)	2	2	2	2	2	3	3	3	3	2	3	3	2	2	2	3	3	3	3	2	3	2	3	3	3
Stem hairs (0 = glabrous; 3 = short hairs; 7 = long hairs)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Petal colour (1 = white; 2 = cream; 3 = light yellow; 4 = yellow; 5 = lavender)	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Pollen colour (1 = cream; 2 = yellow; 3 = purple)	2	2	1	2	2	2	3	3	2	2	2	2	1	2	2	3	2	2	2	2	2	2	2	2	2
Stigma position (1 = below anthers; 2 = same level as anthers; 3 = above anthers)	3	3	1-3	3	2	3	3	3	3	3	3	2	1	3	2	3	3	3	3	3	3	3	2	3	2
Stem colour seedlings (g = green; r = red)	r	g	g	g	g	g/	r	r	g	g	g	g	g	g	g	g	r	g	g	g	g	g	g	g	g
Seed index (1 = ≤ 9.9 g; 2 = 10-11.9 g; 3 = 12-13.9 g; 4 = ≥ 14 g)	2	3	3	3	3	3	2	2	2	2	2	2	3	3	4	2	2	1	2	2	2	2	3	2	?

Samples highlighted in (red) differed in trait characteristic to the rest of the samples

With regard to the seed index, a clear distinction could be made between the Kisii/Kisumu (K/KU) and Tebere (T) seeds. Nearly all K/KU (Genebank accession) seeds could be identified with a 100-seed weight below 10 g. The values of the Tebere- seeds accessions ranged between 10 and 14 g. Only T 22 exceeded this range with a mean weight of 14.2 g. An overview of some of the selected traits is given in Figure 5.1.



Figure 5.1: Unique morphological trait diversity among sampled accessions: (a) okra-leaf type T 24A (left) & red-leaved line T 23A (right), (b) long haired accession T 08, (c) purple pollen of T 15A, (d) stigma position same level as anthers (KSA 81M), (e) seedlings of TEB 15A with red stems

It is these unique morphological properties in the studied genotypes that contribute to the diversity. This is represented in the two-dimensional principal coordinate graph resulting from the PCA (Fig. 5.2). The first two principal components account for 58.56% of the variation. The accessions were divided into two populations, based on their origin. Population 1 comprises the K/KU lines and population 2 consists of the Tebere (T) accessions. A statistically significant variance of 30% ($\Phi_{IPT}=0.3$) was found between the two populations. This was partly explained by the lower 100-seed weight of the K/KU accessions. The difference in seed index is probably due to the different origin of the seeds. The K/KU (Genebank accessions) genotype seeds had been stored for a long time in the seed bank which may have facilitated the loss of vigour, while the Tebere accessions (breeders seed) came from the KALRO Mwea-Tebere research station where the seeds are under active regeneration for evaluation for specific traits of interest, perhaps

suggesting a concerted effort in KALRO (Mwea-Tebere) to focus on selection of cotton with a greater number of large and heavy seeds as an important breeding objective in cotton.

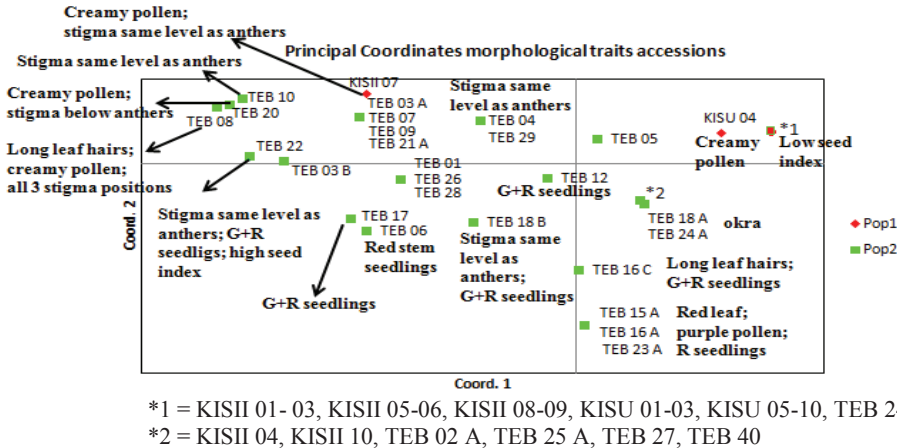


Figure 5.2: Two-dimensional principal coordinate graph showing the diversity among the cotton accessions, with unique traits indicated; population 1 = Kisii & Kisumu accessions, pop 2 = Tebere accessions; G = green, R = red

Based on the ten scored morphological traits, the principal coordinate graph shows there exist sufficient diversity between the accessions evaluated. Furthermore some traits expressed variability within the same genotype such as stigma position, stem colour, pollen colour, etc (Table 5.3). This poses a challenge to distinctly characterise particular accessions based on morphological characters. In the present study, it is not clear whether this variability is due to seed mixing or due to segregation resulting from cross pollination within a given genotype because the breeding materials provided were coded by the supplier. No parental information was provided but the results show the limitation of morpho-phenotypic traits in characterisation of cotton accessions. In addition, whether this phenotypic/morphological variability translates into gain in hybridisation process requires evaluation of the progeny of the cross between any selected accessions which requires a long time to achieve. It is clear that a number of environmental factors ranging from vagaries of weather to genetic-environmental interaction affect the phenotype trait characteristic. Meanwhile due to damage by hailstones and BB disease, few traits were scored, if it would have been possible to include more traits like plant height, number of bolls, seed cotton yield and fibre quality, this would have given a better representation based on morphological diversity. Finally, no link seems to be found between a particular morphological trait and the susceptibility to BB (Chapter 3; Fig. 3.4). However, the most tolerant

line (T 24A) is characterised by an okra leaf shape, a trait that is shared by only two accessions (T 18A and T24A).

5.3.2 Genetic diversity and AFLP analysis of cotton genotypes in Kenya

To determine differentiation based on AFLP analysis, accessions were also grouped into two populations based on the seed bank collected from (Tebere and Genebank Muguga). Polymorphic information content (PIC) between populations and across markers ranged between 0.31 – 0.49. Overall, AFLP analysis revealed mean PIC of 0.30, 0.44 and 0.28 for FAM, JOE and NED primer pairs, respectively (Table 5.4). The fragment range for all primer pairs ranged between 52 – 497 bp (Table 5.4).

Table 5.4: Percentage of polymorphic informative loci of cotton accessions collected for the two populations in Kenya

Population		Primer pair		
	Pop size	FAM	JOE	NED
Genebank	20	30.71%	47.88%	22.66%
Tebere	33	34.65%	49.70%	29.69%
Reference	2	25.20%	33.33%	31.25%
Mean		30.18%	43.64%	27.86%
SE		2.74%	5.18%	2.64%
Fragment range		52-487 bp	62-497 bp	57-451 bp

The total number of loci for the three primer pairs amplified was 420. FAM primer pair amplified 127 loci with 89 polymorphic loci representing 70% polymorphism. JOE and NED primer pair amplified 165 and 128 loci with 114 and 86 polymorphic loci representing 69% and 67%, respectively. The total of unique bands (private bands) revealed by FAM, JOE and NED primer pairs was 23, 43 and 24, respectively (Table 5.5). The expected Heterozygosity was calculated based on the Nei diversity index of the GENALEX 6.4 program and the number of unique bands per population was determined for the different primer combinations. Expected heterozygosity (H_E) among the populations studied and different primer pairs ranged from 0.073 – 0.138. All primer pairs expressed an H_E lower than 0.150 illustrating limited heterozygosity present in the Kenyan genotypes (Table 5.5). Overall, each primer pair revealed a limited expected heterozygosity within Kenyan cotton accessions (Tebere and Genebank). This is expected since Euclidean distance treats all individuals as completely inbred as expressed by all primer pairs (<0.150 expected heterozygosity).

Table 5.5: Total band patterns for polymorphic data for populations and primer pairs

Primer Pair	FAM			JOE			NED		
Population	Genebank	Tebere	Ref	Genebank	Tebere	Ref	Genebank	Tebere	Ref
No. Bands	88	89	79	113	114	85	80	86	83
No. Bands Freq. $\geq 5\%$	88	79	79	113	103	85	80	76	83
No. Private Bands	5	6	12	12	15	16	0	6	18
Mean H_E	0.073	0.080	0.104	0.107	0.110	0.138	0.058	0.069	0.129
SE of Mean H_E	0.013	0.013	0.016	0.012	0.013	0.015	0.012	0.013	0.017

NB: Ref = reference genotypes, No. Private bands = unique bands determined, H_E =Expected heterozygosity, SE=Standard error

The pairwise Nei genetic identity between cotton accessions for the two populations of Genebank and Tebere according to GENALEX 6.4 program ranged from 0.993-0.996 for all primer pairs (Table 5.6).

Table 5.6: Pairwise population matrix of Nei genetic identity for the three primer pairs

Population	Genebank	Tebere	Reference	Primer Pair
Genebank	1.000			NED
Tebere	0.995	1.000		
Reference	0.922	0.919	1.000	
Genebank	1.000			JOE
Tebere	0.996	1.000		
Reference	0.919	0.920	1.000	
Genebank	1.000			FAM
Tebere	0.993	1.000		
Reference	0.922	0.919	1.000	

The limited heterozygosity observed suggests limited variability among Kenyan genotypes. Clearly, more heterozygosity was observed between reference genotypes comprising of only two accessions (Samburu [SA] and S295) expressing higher unique bands (36 unique band for the three primer pairs compared to Genebank 17 and Tebere 27) (Table 5.5). Since only two reference genotypes were used, results based on this should be interpreted with caution due to limited samples used. The limited heterozygosity observed requires further analysis with more samples and markers to verify the findings.

5.3.3 AMOVA analysis among cotton accessions

The first two principal coordinates resulting from the PCA were plotted in GENALEX 6.4 in a two dimensional graph (Fig. 5.3). For the FAM primer pair, 24.27% of the variation was explained by the x-axis and 18.08% by the y-axis. The molecular variance between the populations from Genebank and Tebere is 9.6% ($\Phi_{PT}=0.096$) with P value greater than 0.05 implying the two populations are not significantly different from each other. AMOVA for the JOE primer set was explained 27.49% by the x-axis and 21.18% by the y-axis ($\Phi_{PT}=0.075$) while for NED 28.01% was explained by the x-axis and 22.67% by the y-axis with ($\Phi_{PT}=0.065$) both p value greater the 0.05 showing that both primer pairs revealed the populations are not significantly different from each other. The PCA analysis for all primer pairs grouped accessions close together with no specific grouping associated with a particular population (Fig. 5.3). The accessions from the two populations tended to cluster together which can be associated with the fact that there is exchange of genotypes among the populations i.e. some accessions in Tebere may actually be duplicate accessions in Genebank and vice versa. The Genebank accessions are duplicate accessions stored for purpose of long term storage while Tebere accessions are breeders' genetic material. The PCA evaluation enables the partitioning of existing variation among the genotypes and also among populations. Based on PCA analysis unique genotypes can be determined providing opportunity for these genotypes to be evaluated for further research. In this particular case no unique genotype could be observed among all the genotypes except for SA (*G. barbadense*) which appears distinct from the other genotypes revealing lack of genetic variability within the Kenyan collection of *G. hirsutum*.

Considering the ability of AFLP to explore a high number of loci, it was expected that the two populations would be significantly different and unique genotypes would be identified that could be explored more. Unfortunately, for the current study, the two populations were not significantly different. However, AFLP analysis was able to distinguish the *Gossypium hirsutum* genotypes from *G. barbadense* (SA) since in all primer pairs accession SA was distinct from the other genotypes (Fig. 5.3) validating the ability of AFLP analysis to distinguish cotton species. Due to the lack of parental information of the accessions used it was not possible to explain the ability of AFLP to distinguish different genotypes. Another genotype *G. hirsutum* (S295), used as a reference for determining the susceptibility to bacterial blight, was also included but it clustered with the rest of the *G. hirsutum* genotypes (Fig. 5.3).

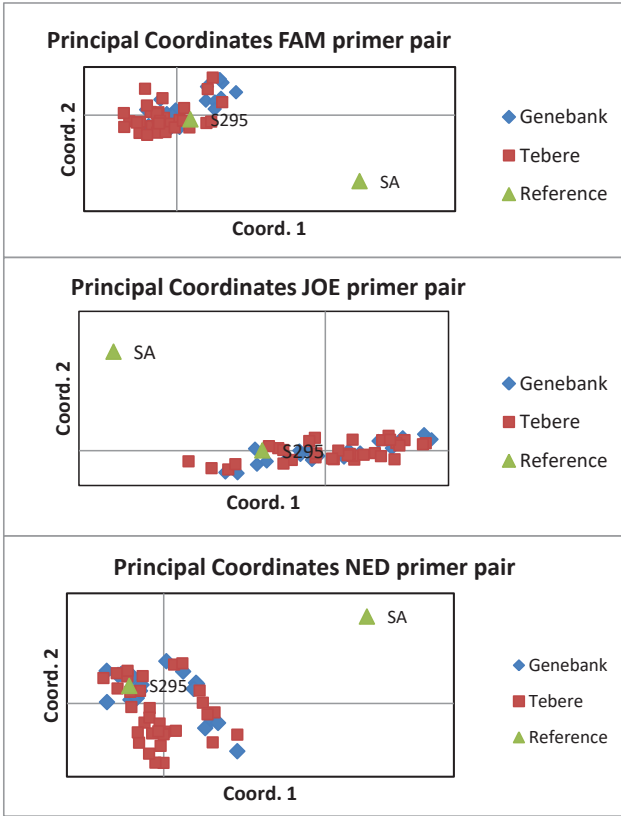


Figure 5.3: PCA of the Kenyan genotypes based on AFLP analysis and grouping based on the two populations and reference genotypes. Individuals from different populations are labelled with different symbols. The reference individuals are indicated. Horizontal and vertical scales represent the first and second principal axes of variation. The two cotton populations collected from Tebere and Genebank grouped together in disregard to the seed bank obtained.

5.3.4 Hierarchical cluster analysis

Since PCA evaluation of the Kenyan genotypes could not group accessions according to population, a further analysis to determine relationship between accessions and/or the site of collection was done with the hierarchical clustering method. The differences among accessions at the DNA level were determined by comparing the genotypes based on presence or absence of variation in the loci. Clustering of genotypes was determined using the dice coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using GENSTAT version 12 to establish if there is any link between genotypes and the two populations. As earlier observed with PCA, hierarchical clustering; all genotypes also clustered together in disregard of the

population (Fig. 5.4). This suggested the consistency upon which AFLP is able to genotype and group accessions based on their similarity or dissimilarity in the allelic loci. Clearly, two groups were observed: the single genotype A-cluster consisting *G. barbadense* genotype and the B-cluster with the *G. hirsutum* accessions (Fig. 5.4). The clustering based on the dice similarity coefficient of the FAM primer pair (SA genotype [*G. barbadense*]) clustered with *G. hirsutum* accessions at co-efficient of 0.735. For the *G. hirsutum* genotypes the least coefficient clustering was observed at 0.890. The most similar accessions are T07 and T22 with coefficient clustering of 0.975. Initially during seed bulking, some accessions were visually evaluated and selected from a given genotype, but upon multiplication expressed variable morphological characteristics (T02A & T02B, T03A & T03B, T18A, T18B & T18C, T15B & T15C, T23A & T23B, T24A & T24B; Table 5.1). The seeds were provided as single genotypes but when grown, the plants expressed varied morphological characteristics and therefore the next generation seeds were harvested separately as different accessions. The AFLP hierarchical grouping separated the particular accessions in different clusters suggesting the possibility of the accessions being different genotypes rather than half-sib products of segregation. This may have resulted from seed mixing during the process of bulking (Fig. 5.4). Similar results were also observed for NED and JOE primer pairs for all cotton accessions collected (data not given).

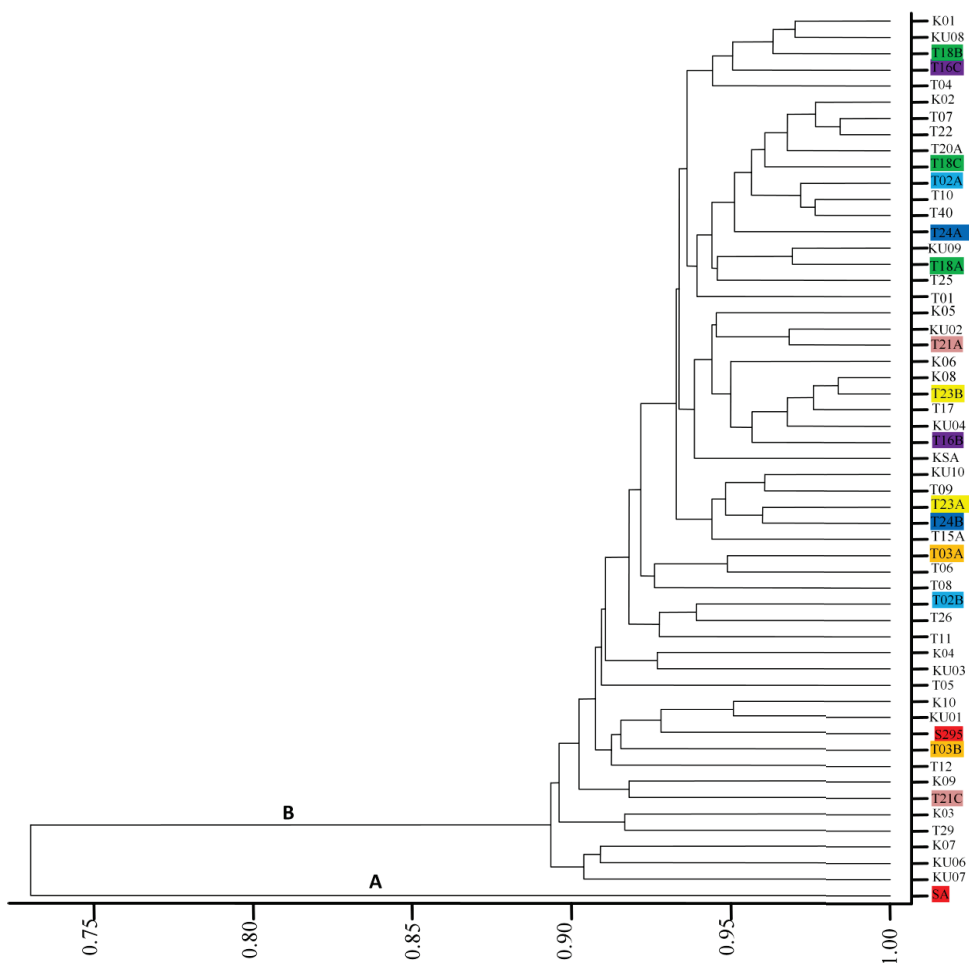


Figure 5.4: Phenogram cluster of Kenyan cotton accessions and reference genotypes based on the FAM primer pair based on the dice coefficient and the UPGMA of GENSTAT software version 12 edition. Subaccessions of the same genotype (highlighted in different colours for example green for T18A, T18B and T18C) clustered separately in the subclusters suggesting possible seed mixing during bulking rather than being half-sibs. Accessions highlighted in red are the reference genotypes (S295 and *G. barbandense* [SA]).

5.4 DISCUSSION

Among the cotton accessions evaluated, distinct phenotypic and morphological traits were observed for particular accessions that varied between accessions in the form of plant/stem colour, leaf shape, leaf and flower colour, stigma position, etc. The results of the morphological

characterisation revealed sufficient diversity among the genotypes evaluated. The first two principal components accounted for 58.56% of the variation, a significant level of variation based on phenotypic traits. However, the difference between cultivars at the DNA level based on AFLP analysis was quite limited with least coefficient of 0.890 for *G. hirsutum* accessions. This suggests the complexity of the cotton genome and the possible strong effect of the environment and epigenetic factors along the evolution time in determining phenotypic traits in cotton. Constable et al. (2015) reported that epigenetic changes could be contributing to variation in plant phenotypes, not accounted for by any sequence variation between individuals in cotton. The limited DNA variability is attributed to the fact that many of the cotton cultivars were developed from few genotypes or hybrids of established cotton cultivars (Iqbal et al., 2001; Lu & Myers, 2002; Multani & Lyon, 1995) but its genome evolution and organisation leading to a particular phenotype could be more complex (Constable et al., 2015).

The usefulness of genetic markers in studying populations is measured by how informative they are (polymorphism information content (PIC) *sensu* (Blignaut et al., 2013; Da et al., 1999; Mateescu et al., 2005)). We determined that the PIC of each primer pair was both comparable between populations and between markers. Overall, AFLP analysis revealed mean PIC of 0.30, 0.44 and 0.28 for FAM, JOE and NED primer pairs, respectively (Table 5.3) where PIC value of ≥ 0.3 is considered of high discriminatory value (Blignaut et al., 2013; De Riek et al., 2001). Therefore our results with similar PIC ranges show that AFLP analysis of Kenyan cotton accessions could be used to discriminate accessions effectively based on the observed polymorphisms.

We also determined expected heterozygosity (H_E), which is a measure of within-population gene diversity and is equivalent to Nei's unbiased gene diversity (H_S), as adapted for dominant markers under the assumptions of Hardy-Weinberg equilibrium and the Lynch-Milligan model (1994). Here, the overall expected heterozygosity ($H_E < 0.150$) for cotton which is lower than 0.39 reported by Adawy et al. (2006). This is attributed to the fact that H_E treats all accessions as inbred so expected heterozygosity is low.

The genetic diversity for examined cotton genotypes was generally small for all primer pairs utilised. The principal coordinate analysis evaluation of the two populations collected from two seed banks generally grouped accessions together in disregard to their populations. Based on hierarchical analysis, one major cluster was formed representing *G. hirsutum* and another single accession cluster of *G. barbadense*. The high degree of genetic similarity between the Tebere

and Genebank populations was estimated 0.993-0.996. Among *G. hirsutum* accessions the genetic similarity ranged between 0.890-0.997 which agrees with results of others: 0.925-0.975 (Lukonge et al., 2007), 0.797-0.973 (Adawy et al., 2006). Multani and Lyon (1995) found in Australian cotton that the genetic similarity ranged from 0.921 – 0.989 among nine cultivars of *G. hirsutum*. The low level of genetic variation between *G. hirsutum* accessions poses a challenge for cultivar identification based on DNA markers. Under this circumstance, multiple samples of each cultivar are certainly necessary to assess the genetic diversity within a given cultivar to ascertain the uniformity for a given cultivar and therefore establish the genetic relationship for *G. hirsutum* accessions under evaluation for purpose of breeding.

Although genetic diversity between *G. hirsutum* and between the Tebere and Genebank populations was limited, sufficient DNA polymorphism was found among *G. hirsutum* accessions to allow differentiation. Polymorphic information content (PIC) between populations and across markers for our study ranged between 0.25 – 0.49. De Riek et al. (2001) reported that a PIC value of ≥ 0.30 has reasonable ability to discriminate accessions. Similarly, Blignaut et al. (2013) while evaluating four invasive and four native angiosperm species (*Pinus pinaster* (Pinaceae), *Pennisetum setaceum* and *Poa annua* (Poaceae), *Lantana camara* (Verbenaceae), *Bassia diffusa* (Chenopodiaceae), *Salvia lanceolata*, *Salvia africana-lutea*, and *Salvia africanacaerulea* (Lamiaceae) made similar observations in relation to PIC ranges. In our study, accessions presumed to be the progeny of the same genotype were determined to be actually unrelated since they clustered separately from each other (Fig. 5.4: T02A & T02B, T03A & T03B, T16B & T16C, T18A, T18B & T18C, T23A & T23B, T24A & T24B). Since no molecular characterisation has been done for Kenyan cotton accessions, the results reveal that there is need for genotype cleaning to purify genotypes for purposes of breeding within the Kenyan germplasm. Cleaning would involve selfing genotypes for a number of generations to minimise the effect of natural cross pollination that normally occurs in cotton, this would then be followed by molecular characterisation. Currently, most accessions used for breeding have been contaminated by natural cross-pollination known to occur in cotton and seed mixing during physical seed bulking, making it difficult to differentiate genotypes. The purification along with characterisation of all genotypes in the Genebanks with molecular markers is necessary. This would be followed by development of a core collection for these accessions based on the similarity and differences. The core collection would include a given percentage to represent all accessions and therefore form a working group/collection which breeders can approach for their

respective breeding objectives. Such an effort would save time and money and where distinct genotypes would be stored with clear progeny information. It will also enhance development of cultivars with superior characteristics. At present many accessions are actively maintained at different seed banks in the country's research stations yet they may well be just duplicates of the same accession.

Based on AFLP analysis no link was established between BB disease susceptibility and specific AFLP markers. The *G. hirsutum* genotypes clustered together with S295 accession used as reference in BB studies based on PCA and hierarchical clustering. Clearly, accessions that showed reasonable resistance to BB (T24A, T 24B) disease (Chapter 3) did not group together with the resistant reference line S295, in fact among the most susceptible accessions KU01 and K10 clustered with S295 (Fig. 5.4). Therefore, no clear link can be established between BB resistance/susceptibility with a particular cotton genotype based on AFLP analysis. This is due to the fact that no AFLP marker associated with BB resistance could be determined. Future screening should focus on screening based on identified SSR and SNP markers located in chromosome 14 that are tightly associated with the B₁₂, B_{2B3}, B_{9L}B_{10L} and other genes that confer resistance to BB (Silva et al., 2014; Xiao et al., 2010). These markers could be more useful for marker-assisted selection and germplasm screening for bacterial blight resistance.

5.5 CONCLUSIONS AND RECOMMENDATIONS

The limited genetic diversity of *G. hirsutum* as observed among the Kenyan genotypes studied emphasises the need to introduce more diverse cotton varieties from other countries into Kenya. This should be followed by characterisation with both morphological and molecular markers and development of a core collection for the Kenyan cotton germplasm. The core collection would form a working collection for breeders to use and manipulate for respective breeding objectives. Introduction of germplasm resources should further include other tetraploid species (*G. barbadense*) to enable improvement of the available material through introgression via wide hybridisation to increase variability within the collection.

Our results indicate that AFLP analysis is a sensitive technique for detecting molecular polymorphism in cotton even among closely related genotypes (Iqbal et al., 2001; Pillay & Myers, 1999). Results obtained in this study based on genetic hierarchical analysis revealed a genetic similarity ranging from 0.890-0.997 among *G. hirsutum* genotypes. Meanwhile,

population genetic similarity between Tebere and genebank accessions range from 0.993-0.996 indicating a narrow genetic diversity within the two populations hindering identification of polymorphism essential for cotton improvement. AFLP analysis also managed to detect possible genotype seed contamination requiring the need for purification of the Kenyan accessions. Hierarchical clusters grouped the supposedly progenies of the same accessions separately suggesting possible seed mixing during seed bulking or effects of natural cross-pollination during seed multiplication.

AFLP is a useful molecular marker system to study genetic diversity essential for cotton breeding programmes. However, based on reports from literature, SSR analysis can improve the effectiveness of marker systems to reveal cotton genetic diversity and thus enhance breeding (Hussein et al., 2005). Furthermore, with advancements in molecular technology, better approaches based on next generation sequencing (NGS) are now available. Such technologies enable whole genome sequencing at limited cost (Li et al., 2014; Wang et al., 2011, 2015; Xu et al., 2011). These methods can reveal the genetic basis for certain traits based on discovery of SNP markers associated with useful traits such as fibre quality, disease and pest resistance, etc. This would be useful especially for a complex polyploid crop such as cotton with a known narrow genetic base (Byers et al., 2012; Gore et al., 2014) that can be exploited for improvement. This NGS will speed development of superior cultivars that farmers can commercialise to improve yield, disease and pest resistance and fibre quality. Future research is needed to identify molecular markers linked to important traits based on NGS such as genotyping by sequencing (GBS) which facilitates the detection of a wide range of SNPs associated with particular traits using many accessions currently lying idle in many genebanks. This will enhance better utilisation of many genotypes available across the world.

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVES OF BB MANAGEMENT IN COTTON

6.1 RECALLING THE OBJECTIVES

The first objective was to establish the present status of BB disease in the cotton fields of Western Kenya (Chapter 3). The second objective aimed to identify and characterise the field *Xanthomonas* strains and determine the prevalent races of *Xcm* in the region (Chapter 4). The 3rd objective was to evaluate the genetic diversity of Kenyan cotton germplasm (Chapter 5). To address the hypothesis and achieve these objectives an approach utilising a combined research strategy targeting the host (cotton germplasm) disease resistance potential and the pathogen *Xanthomonas citri* pv. *malvacearum* (*Xcm*) diversity analysis was pursued in this study. The study of diversity of BB pathovar race-types available in cotton in Western Kenya and genetic variability among genotypes provides crucial background information on the present status of Kenyan germplasm BB susceptibility and therefore possible strategies that can be used to improve commercial cultivars.

6.2 BACTERIAL BLIGHT DISEASE OF COTTON

Field evaluation of Kenyan germplasm is important to determine natural disease incidence on available cotton genotypes grown in the region (Chapter 3). Commercially grown cultivars in Kenya were developed in the 1980s with inherent BB resistance. The commercial cultivar KSA 81M was bred as a multiline with resistance to BB and was recommended for cultivation in Western Kenya. At present, several challenges have been attributed to the low cotton productivity in the region. The current observed BB symptoms on the commercial cultivar suggest that the disease remains one of the major challenges leading to reduced productivity in cotton.

In addition, the resurgence of BB disease raises several challenges on the durability of inherent resistance in the cultivars developed and commercialised in the 1980s in the country. Previous studies on cotton BB disease resistance globally have revealed that permanent resistance is a major challenge (Innes, 1983) due to the BB pathogen ability to mutate through loss of avirulence genes or due to the introduction of new strains in the region capable of overcoming

the existing resistance. One of the major options to boost the durability of newly introduced host resistance genes is to screen the pathogen population for virulence prior to and after the cultivar commercial release in a given region. This enables a region to track changes in pathogen population over time. The cotton industries in USA and Australia have constant BB monitoring teams in their cotton belts during the cropping season. Monitoring allows early detection of novel pathogen strains or adaptation within the pathovar population. This enables the possibility of developing strategies to prevent or slow down the spread during disease outbreaks. The results of such effort have seen the two countries normally report less than 1% yield loss due to BB disease (Allen, 2011; Hillocks, 1992; El-Zik & Thaxton, 1995) whenever it occurs. Unfortunately, this approach is very expensive and requires intensive surveillance networks (CRCNPB, 2012) which may not be practical for a country like Kenya with limited financial allocation for research and development. Therefore, periodic surveillance of the cotton belts after a number of years may be a more realistic approach.

McDonald and Linde (2002) suggested that breeding efforts should concentrate on quantitative resistance, which might need to be renewed regularly to stay ahead of the pathogen. During periodic surveillance, inherent resistance against present BB pathovars may require pyramiding of additional genes to bolster inherent resistance in case of disease breakdown or pre-emptive breeding to remain ahead of the pathogen. Quantitative resistance, controlled by multiple genes each with small effect is often more durable than race-specific dominant resistance because pathogens often adapt more slowly to it if at all (Brown & Tellier, 2011; Trough, 2013). The cotton cultivar (KSA 81M) developed in the 1980s may have lost its resistance due to mutation within existing pathovars or introduction of new more virulent strains.

Traditionally, the source of disease resistance is provided by the germplasm material stored in genebanks. Evaluation of a number of available genotypes is essential to identify diversity and potential sources that can be commercialised or utilised to improve the current cultivars. Based on the accessions evaluated, most Kenyan accessions are susceptible (with 71% considered susceptible and 29% fairly resistant to *Xcm* strains available in the region). Among the genotypes evaluated only a few (T 24A, T 24B, and T 25A) were classified as resistant or moderately resistant. T 24A & B are subaccessions within accession T 24 suggesting a possible common genetic basis of their disease resistance. Other genotypes also exhibited reasonable resistance in the field and under greenhouse conditions (T 9) while T 11 and T 16B remained immune under greenhouse conditions (Table 3.1). These accessions suggest that there is potential among local

germplasm to improve commercial cultivars. Whereas there's promise, these genotypes already showed some susceptibility under both field and greenhouse condition casting doubts on their ability to sustain disease resistance. These results suggest the need for additional studies to determine the nature of the resistance in these accessions.

6.3 PATHOGEN DIVERSITY

While identification of sources of BB resistance is important, the diversity of the pathogen in the field is also paramount. To address this question, a second objective to identify and characterise the field inoculum was pursued (Chapter 4). This involved evaluation of xanthomonad diversity in strains isolated from diseased cotton plants and eventual determination of prevalent BB pathovar races. No report, past or present has documented the nature/diversity of strains present in Kenya's cotton fields. Knowledge about the population structure of the pathogen can aid in prioritising the resistance breeding objectives. Results on the occurrence of multiple races of the pathovar are important in understanding of resistance in plant genotypes, as well as interpret disease resistance screening. Infected leaves collected from different regions of Western Kenya were utilised to isolate *Xcm* strains. Conventional isolation procedures based on single colony approach resulted in the collection of a large number of bacterial strains. Conclusive identification of *Xcm* pathogens is normally based on a pathogenicity test on cotton differential cultivars which requires the establishment of many hundreds of pots with those cultivars to analyse for instance 100 bacterial strains. Research has revealed that it is difficult to distinguish yellow pigmented bacteria especially *Xanthomonas* that infect crop plants based on the phenotypic characteristics (Mhebdi Hajri et al., 2011). Since several bacterial species can invade the cotton plant as either pathogens or just surviving epiphytically as saprophytic strains it was necessary to utilise approaches that are more efficient, based on PCR and with the ability to distinguish the strains conclusively.

For rapid identification of the genus *Xanthomonas* among the samples, PCR was performed based on the *gyrB* sequence. This method has been utilised in identification of other *Xanthomonas* species isolated from food, feed, and diseased plants (Kasai et al., 1998; Mondal et al., 2012, 2013; Slack et al., 2006) but has not been tried on cotton BB pathogen. The *gyrB* primers were developed based on conserved sequences specific for the genus *Xanthomonas*. The result of this study on identification was not only fast with single bands amplified for *Xanthomonas* strains but also sensitive enough with drastic reduction of the number of strains

collected from original 127 to 40 believed to be xanthomonads in 2011. The PCR approach based on *gyrB* showed the resolving power of this method in distinguishing the different strains as either xanthomonads or non-xanthomonads, therefore saving time and resources compared to conventional approaches. Subsequently, the strains confirmed as *Xanthomonas* were characterised based on rep-PCR primers of BOX and ERIC. These primers amplify different sequences in the strains enabling distinction between closely related strains. ERIC and BOX primers have exhibited substantial potential in diversity studies for both gram positive and negative bacterial species (Arshiya et al., 2014; Asgarani et al., 2015; de Bruijn et al., 1992; Katara et al., 2012; Louws et al., 1994; Zhai et al., 2010). Among the xanthomonad strains identified, some isolated strains significantly differed from the reference strains indicating them being possibly non-*Xcm*. The others gave a DNA profile very similar to reference strains suggesting a strong relationship with *Xcm*, the causal agent of BB.

The DNA of isolated xanthomonad strains was also sequenced to determine phylogenetic grouping among the field isolates. *gyrB* sequence analysis further corroborated the rep-PCR results showing a strong relationship of the collected field *Xcm* strains with the reference strains while non-xanthomonads displayed very distinct sequences from the reference *Xcm* strains.

To finally confirm the strains identity, the pathogenicity test based on eleven cotton differential cultivars was carried out on all strains confirmed as *Xanthomonas citri* pv. *Malvacearum* (eleven strains from 2012 and 13 from 2013 seasons, respectively). The pathogenicity tests for all twenty four *Xcm* strains identified the predominant strain as the strongly virulent race 18. Allen and West (1991) reported the existence of race 18 in many regions of the world and it has been established as the most encountered pathotype. Race 18 is among the strongly virulent strains closely related to HVS strains (Cunnac et al., 2013; Zhai et al., 2013) but according to the present study results, the findings confirm absence of HVS in Western Kenya. Though HVS strains are not reported in the current study, there is need for breeding based on durable resistance since neighbouring countries have already reported its presence. Generally, breeding for durable resistance can be improved by utilising molecular approaches to analyse both the host and the pathogen (Chapter 4 and 5).

In conclusion, this study is the first report on BB races present in any part of Kenya. The study revealed a diversity of *Xanthomonas* (genus) strains available on cotton some saprophytic or non-pathogenic. In addition, the presence of *Xcm* pathogenic bacteria was confirmed in the

region of Western Kenya. Of significance is the fact that one of the most virulent strains (race 18) capable of overcoming most resistance genes is confirmed to be present in the region.

The molecular approaches utilised to identify and characterise the pathogen were not only reliable and fast but also sensitive in distinguishing *Xanthomonas* from other microbial cultures thus saving time and resources. Further, evaluation of more *Xcm* strains from other regions of the country need to be undertaken to obtain a wholesale status of *Xcm* pathovar diversity in the country to enable design of strategies for disease management that target not only part of the country (Western Kenya) but the whole nation or even the whole of the East African region that share similar climatic conditions and *Xcm* strains.

6.4 DIVERSITY OF KENYAN COTTON GERMPLASM

Molecular marker methods are presently routine tools available to plant breeders and pathologists. The 3rd objective was centred on the premise to analyse the diversity of Kenyan germplasm (Chapter 5). Previous studies of cotton genotypes in the country for improvement of commercial cultivars have relied on morpho-agronomic marker traits which are slow, environmentally influenced and not conclusive. In the present study genotypes were evaluated using AFLP based on the ability to reveal sufficient polymorphisms among the genotypes thereby enabling determination of the genotypic variability. 51 accessions were utilised in this study composed of advanced breeding lines, genebank accessions and some cotton differential cultivars as check varieties. The results of AFLP analysis revealed limited genetic variability between the sampled genotypes with a coefficient similarity range of 0.890-0.997. This low level of genetic variability agrees with earlier observation of the need to introduce more novel genotypes (Chapter 3) to improve genetic diversity and eventual gain from selection.

Evaluation results with AFLP envisaged identifying a marker associated with BB resistance. Unfortunately, no marker associated with BB was identified among the screened genotypes. Additional markers need to be surveyed especially based of genome-wide association markers to identify markers associated to BB to enhance the identification of local germplasm with BB resistance genes to speed the development of resistant cultivars.

6.5 MAJOR ACHIEVEMENT AND FUTURE PERSPECTIVE OF THE STUDY

First and foremost the study reports the presence of BB disease in farmers' fields of Western Kenya. No research finding in the recent past has documented the status of BB in cotton in Kenya since the release of BB varieties of the 1980s (KSA 81M and HART 89M). Meanwhile most Kenyan cotton germplasm accessions available for purposes of cotton improvement are susceptible to *Xcm* races present in the region. Among all accessions evaluated including the commercial cultivar grown by farmers (KSA 81M) and only a few accessions can form possible accessions for cotton BB disease resistance breeding programme (Chapter 3). In addition, the findings show that the cotton germplasm available for cotton improvement lacks sufficient genetic diversity essential for improvement of commercial cultivars. Therefore, there is need to acquire new exotic genotypes that would boost the diversity of available accessions to gain from selection and breeding for important traits in cotton (Chapter 3 & 5).

Secondly, this study, reports for the first time prevalence of race 18 on cotton grown in Western Kenya (Chapter 4). No report past or present has reported major BB races in Kenya which is crucial in addressing breeding for durable BB disease resistance. In the context of the present finding, future research perspective to address BB in cotton in Kenya should focus to further: a) survey all regions of Kenya to determine the major races present in the country, b) determine the genetic basis of supposedly BB resistant commercial cultivars (KSA 81M and HART 89M initially developed and grown in west and east of rift valley, respectively) to enable identify appropriate R-genes to be incorporated in polygenic environment to increase BB disease resistance durability and c) characterise selected cotton genotypes in the country and develop a cotton core collection based on both phenotypic and molecular marker traits. This will enhance utilisation of available germplasm and breeding for useful traits in cotton. This will in the long run lead to improved cotton production and eventually revival of the currently subdued sector for the benefit of rural communities that rely on this crop.

6.6 CONCLUSION AND RECOMMENDATIONS

The development of disease resistant cultivars is the most effective and sustainable method to control BB in cotton (Brinkerhoff et al., 1984; Kirkpatrick & Rothrock, 2001; Wallace & El-Zik, 1989; Xiao et al., 2010). Planting resistant cultivars to control BB has been thought to be a permanent control and a replacement to all other physical, chemical and biological control

management options for BB disease (Innes, 1983). Monitoring the population of *Xcm* is important because new virulent races have evolved putting at risk the deployment of resistant cultivars (Brinkerhoff et al., 1984; Xiao et al., 2010). The uncertainty of the durability of the plant resistance genes and the variability of the pathogen race abolished the proposition that a permanent control can be reached through only using resistant varieties. Meanwhile, a two prong approach targeting both a resistant crop plant and the pathogen must be monitored at all times to management of any changes expressed by the pathogen as well as the plant disease resistance response within the existing cultivars. Figure 6.1 provides a diagrammatical model approach to BB disease management in cotton that breeders and pathologists can approach to monitor the disease during their breeding cycles.

The use of molecular markers to identify accessions with BB resistance when incorporated in breeding programme will hasten the development of cultivars with sustainable resistance in a less time and therefore help reduce losses due to this disease. With advancement in molecular technology better approaches based on next generation sequencing (NGS) are now available at limited cost (Li et al., 2014; Wang et al., 2011, 2015; Xu et al., 2011). Therefore, the problem of traditional markers will be overcome with the use of SNP markers associated with useful traits such as fibre quality, disease and pest resistance, etc. especially for a complex polyploid crop such as cotton with known narrow genetic base (Byers et al., 2012; Gore et al., 2014).

The present findings provide a substantial body of knowledge required by the researchers to come up with cotton cultivars with a durable BB resistance to control the disease and improve productivity. These findings will form a valuable body of knowledge that will be shared with the scientific community as well as policy makers to come up with strategies to address the presently low productivity in the cotton sector for the benefit of the farmers and country. In general both classical breeding and a molecular marker approach will play a critical role in this process enhancing sustainability of the breeding programmes and commercial cultivars leading to a more sustained revival of the once vibrant cotton sector.

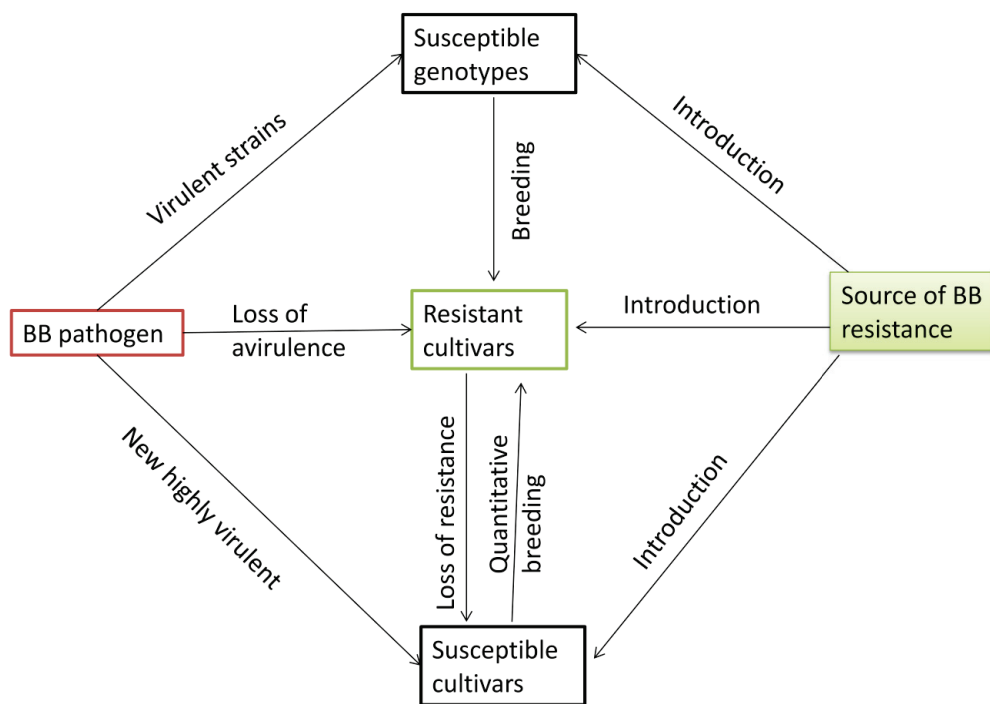


Figure 6.1: Diagrammatic representation of the interaction of cotton (host) and pathogen (*Xcm*) requiring constant monitoring to manage breakdown of BB resistance. Monitoring of the diversity of the pathogen and the resistance of commercial cultivars using classical and molecular tools will enhance BB management.

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SUMMARY

Cotton (*Gossypium* spp.) is a very important fibre crop in the world. In Kenya, the importance has been recognised in the recent years with the worsening poverty levels in areas that previously depended on the crop since the collapse of the sector in the 1990s. In an effort to reduce poverty levels in the areas, the government has taken initiatives to revive the sector under the blueprint “vision 2030”. Unfortunately, the crop suffers attacks from several pests and diseases with serious impacts on its productivity. The effort to address pest problem globally has partially been addressed with development of genetically modified (*Bt*) cultivars with commercialisation of cotton varieties with *Bt* gene incorporated in many countries. Among diseases, bacterial blight incited by *Xanthomonas citri* pv. *malavacearum* is an important disease worldwide. The damage due to BB has been estimated at 5-35% or even more in some areas. To control the damage due to the BB, breeding for durable resistance is the most recommended method. Unfortunately, *Xcm* the causal agent of BB has a way of overcoming inherent resistance by mutation or loss of avirulence especially the one due to single gene resistance. The main source of resistance is the germplasm available in genebanks which can be approached to provide this important resource. In Kenya, limited research on BB has been done since the near collapse of the industry in the 1990s. In fact information on BB races in farmer’s fields of Kenya is non-existent. Proper breeding strategies to control the disease will only be addressed if sufficient information in the country is provided, unfortunately currently unavailable. Information on the prevalence of BB disease and race types available in the cotton fields along with available sources of resistance need to be studied to design strategies that can be adopted to reduce yield losses due to this disease. The results can then be formulated into breeding strategies and extension approaches that will minimise the impact of the disease leading to increased productivity. This will in the long run lead to more sustainable revival of the cotton subsector with meaningful impact on the population that depends on this crop.

In the first part of this dissertation, a field evaluation of the cotton genotypes was carried out in two fields at different locations of Western Kenya. The two sites have a long history of cotton cultivation but with favourable conditions for BB occurrence. The genotypes were evaluated for disease incidence under natural infestation. A correlation analysis of the disease severity was done to compare genotype response to natural infection between the two locations. To further confirm the field results, raw field inoculum was infected on the same genotypes under

controlled conditions in a greenhouse experiment. The evaluation results in the field and greenhouse confirmed prevalence of BB in the fields of Western Kenya. The findings of genotype evaluation also revealed that most Kenyan genotypes including the commercial variety KSA 81M previously bred for BB resistance is susceptible to the BB pathovars currently present in the region. The results also identified a few genotypes with reasonable resistance though none showed total immunity to current *Xcm* pathovars. These genotypes can form valuable resources for breeding for BB disease resistance. Overall, further evaluation is needed based on molecular approaches to verify the higher BB resistance in identified genotypes. In addition, acquisitions of more genotypes with known durable resistance need to be incorporated to improve commercial cultivars. Cultivar KSA 81M was developed in the 1980s and it seems the inherent resistance has been lost either through *Xcm* strain mutation, introduction of more virulent strains or loss of avirulence and therefore future resistance need to be utilised based on more durable resistance by introducing from exotic cultivars with known resistance and incorporating onto the local commercial varieties in a background with polygenic nature of disease resistance which offer more sustained resistance.

In the second part, we considered to determine the prevalent races of *Xcm* in the cotton fields of Western Kenya. To undertake this work, infected leaf samples were collected from different regions of Western Kenya. Leaves were dried and utilised to isolate strains of *Xcm* from infected leaf tissue based on single colony approach. Several strains of bacteria were collected and to enable faster identification of *Xanthomonas*; modern faster, reliable and sensitive PCR methods were used to identify xanthomonad species among the isolates. The *gyrB* gene sequence is known to be present in single copies in bacteria and was explored as an appropriate method to distinguish *Xanthomonas* bacteria from other inherent bacteria present on cotton. *gyrB* is part of MLSA housekeeping genes conserved in bacteria which can be sequenced and used to study taxonomic relationship among strains. *gyrB* gene also presents a higher degree of sequence variation enabling design of species specific primers. This study developed *gyrB* primers based on *Xanthomonas* sequences and used to amplify specific fragments on strains obtained from BB infected cotton leaves. *gyrB* primers identified the strains in the genus *Xanthomonas* and these strains were further characterised with rep-PCR primers of BOX and ERIC to determine the *Xcm* diversity in the fields of Western Kenya. BOX and ERIC primers amplify specific but different DNA sequences between repetitive/adjacent repetitive elements revealing arrays of 10-30 PCR

fragments per genome ranging from about 200bp to over 6kb. PCR products of different strains can be separated by electrophoresis and the resultant DNA fingerprint used to characterise and identify the isolated strains. DNA of identified *Xcm* strains was also sequenced to determine strain phylogenetic relationships based on partial *gyrB* sequence information. The sequence data revealed that the strains collected from the fields of Western Kenya belong to the genus *Xanthomonas* and *Xcm* the causal agent of BB was among the strains isolated. In addition, most strains isolated were xanthomonad bacterial species mainly saprophytic on cotton since they had no effect on infection on their own but their function on BB infection is not very clear.

To distinguish xanthomonad bacteria from *Xcm* pathovars the collected strains were subjected to pathogenicity test on eleven differential genotypes. The strains were inoculated onto host cotton differential cultivars under controlled greenhouse conditions with respective collected strains. The results confirmed presence of predominantly race 18 of *Xcm* pathovar. Race 18 is strongly virulent strain capable of breaking all resistance genes except *B₁₂* resistance. In the recent past, neighbouring countries of Uganda and Sudan have reported presence of HVS but our finding rule out its presence in Western Kenya. This however does not rule out future presence of these strains due to uncontrolled movement of cotton seeds between these sisters' countries. Since the most common means of the pathogen transfer from one region to another is basically seed and considering most of cotton growing areas in Western Kenya borders Uganda then presence of these highly virulent strains are highly likely in the near future.

The last part of this work we evaluated the Kenyan cotton genotypes using AFLP markers to determine its diversity. Evaluation of cotton in Kenya has previously been based on morphological and agronomic characteristics which are significantly influenced by environmental factors. Therefore AFLP marker analysis which reveals tremendous polymorphism was utilised to establish the variation present in Kenyan cotton germplasm. Benefit due to selection and breeding is a product of variation. Cotton is known to have resulted from limited selection of a few lines resulting to the present limited variability among commercial varieties grown worldwide. This has led to limited variability among cotton varieties and reduced benefits from selection. As a result more evaluation needs to be carried out to identify more genotypes from cotton genebanks that can be exploited to gain from breeding. This study evaluated Kenyan genotypes with results revealing quite limited variability and close

genetic relationships among accessions. This implies that more exotic lines are needed to widen the present Kenyan breeding stock to gain in the breeding process currently being undertaken.

Overall, the knowledge gathered in this dissertation offer valuable insight on the present status of cotton BB and diversity among Kenyan accessions. In addition, the study findings provide the information on the true genetic basis that exists in both the host and pathogen and the required strategies to achieve maximum breeding objective for BB resistance. For a successful breeding process for disease resistance a combined strategy targeting both the host and the pathogen is always desirable. Monitoring the pathogen population is an important function because new virulent races may have emerged putting at risk the deployment of perceived resistant cultivars. The uncertainty of the durability of the plant resistance genes and the variability of the pathogen race abolished the proposition that a permanent control can be reached through using resistant varieties. Meanwhile, a two prong approach targeting both the resistant crop plants and the pathogen must be followed at all times to management of any changes expressed by the pathogen as well as the plants. This is in agreement with Darwin's theory of "**natural selection**" which states that evolutionary change comes through the production of variation in each generation and differential survival of individuals with better combinations of these variable characters over time. *Xcm* pathovar the causal agent of BB seems to have perfected this art naturally and breeders and pathologists have to be ahead of this competition to survive this race of nature with pathogens.

This thesis tried to explore both strategies to identify resistant genotypes within the country's germplasm collection and also determine the variability within the *Xcm* present in the farmers' fields. The findings will help develop strong strategies for improvement of commercial varieties of cotton through development of BB resistant cultivars reducing disease production constraints which will eventually lead to increased yield and overall revival of the presently depressed cotton sector.

SAMENVATTING

Katoen (*Gossipium* spp.) is het belangrijkste vezelgewas ter wereld. De ineenstorting van de sector onder invloed van wereldwijde overproductie in de jaren 1990, heeft in Kenia gezorgd voor toenemende armoede in regio's die afhankelijk waren van de katoenproductie. Om dit te verhelpen werkte de regering een relance plan uit om de katoensector te doen heropleven. De gevoeligheid van katoen voor diverse ziekten en plagen zijn echter een hinderpaal. Ook de introductie wereldwijd van Bt-katoen biedt hiervoor slechts een gedeeltelijke oplossing.

Xanthomonas citri pv. *malvacearum* (*Xcm*) is wereldwijd een belangrijke ziekte voor katoen. De opbrengstderving wordt geschat tussen de 5 en 33% in sommige regio's kan dit zelfs hoger oplopen. De meest economische manier om *Xcm* te beheersen is het selecteren van resistente genotypes. Genenbanken zijn hierbij de meest aangewezen bron voor het vinden van resistentiegenen. Helaas zijn de meeste resistenties gesteund op gen-om-gen reacties die door mutaties ter hoogte van het avirulentiegen snel doorbroken worden.

Het onderzoek naar *Xcm* in Kenia is zeer beperkt vooral sedert de terugval van de teelt in de jaren '90. Zo ontbreekt elke informatie omtrent de pathotypes van *Xcm* die aanwezig zijn in Kenia. Deze informatie is essentieel voor het veredelen naar resistentierassen. Onderzoek hiernaar in combinatie met opsporen van de gepaste resistentiegenen is essentieel voor veredelingsprogramma's en het terug aantrekkelijk maken van de katoenteelt in Kenia. Het verhogen van de inkomsten uit de katoenteelt zal een betekenisvolle impact hebben op het inkomensniveau van de rurale bevolking.

In het eerste gedeelte van het doctoraatswerk worden de resultaten van een veldevaluatie op gevoeligheid voor *Xcm* van diverse katoen genotypes weergegeven. De evaluatie werd uitgevoerd in twee regio's die van oudsher vertrouwd zijn met de katoenteelt doch ook een hoge ziektedruk van *Xcm* kennen. Van de verschillende genotypes werd de aantastingsgraad ingeschat en een vergelijking gemaakt van de resultaten bekomen op de verschillende locaties. Daarnaast werden dezelfde genotypes getoetst op hun *Xcm* gevoeligheid onder gecontroleerde omstandigheden en dit om de veldresultaten te bevestigen. Deze infectieproef werd uitgevoerd met een mengsel van diverse isolaten. Uit de resultaten blijkt dat de meeste geteste genotypes gevoelig zijn voor de huidige *Xcm* populatie, ook de variëteit KSA81M die eerder veredeld werd op resistentie tegen *Xcm*. Een beperkt aantal genotypes vertoonde echter een goede weerstand

tegen *Xcm*. Deze genotypes kunnen te samen met andere buitenlandse variëteiten met gekende resistentiegenen, een waardevolle input zijn voor bestaande Keniaanse veredelingsprogramma's. Ook het zoeken naar een polygeen bepaalde resistentie is nuttig om te komen tot een meer duurzame resistentie. De resultaten van de infectieproef bevestigen de veldresultaten.

In een tweede gedeelte werd de diversiteit van *Xcm* in katoenvelden in het Westen van Kenia bestudeerd. Daartoe werden bladeren met duidelijke symptomen van verschillende katoenvelden verzameld. Vanuit de geïnfecteerde spots werden via verdunningsreeksen verschillende stammen geïsoleerd. Met behulp van het *gyrB* gen werd een onderscheid gemaakt tussen *Xcm* en andere aanwezige *Xanthomonas* stammen. *GyrB* behoort tot de MLSA 'housekeeping' genen en kan na sequentie gebruikt worden voor taxonomische studies. Tijdens het doctoraatswerk werden specifieke *GypB* primers ontwikkeld op basis van *Xanthomonas* sequenties. Ook *hrp* primers werden aangewend om *Xanthomonas* stammen te onderscheiden; verdere analyse werden uitgevoerd met BOX en ERIC primers en dit om de diversiteit binnen *Xcm* te onderscheiden. Uit de resultaten van het eerste jaar bleek dat vooral saprofytische *Xanthomonas* stammen aanwezig waren die katoen niet konden infecteren. Door het opzuiveren moet dus blijkbaar een selectie zijn gebeurd naar niet pathogene stammen. Een beperktere bemonstering en isolatie bracht nadien wel aan het licht dat het meest voorkomende pathotype van *Xcm* pathovar 18 was. Dit werd bevestigd door het uitvoeren van een infectieproef met een gekende 'differential set' van genotypes. Het pathotype 18 is zeer virulent en is met uitzondering van *B12* door alle resistentiegenen gebroken. De aanwezigheid van HVS in West-Kenia kon niet worden vastgesteld. Import van zaai zaad vanuit Oeganda en Soedan waar HVS reeds is vastgesteld verhoogd de kans op insleep van dit pathotype in Kenia.

In het laatste gedeelte van het doctoraatswerk werd de genetische diversiteit van Keniaanse katoen genotypes bepaald op basis van AFLP. Tevens werd de fenotypische diversiteit nagegaan op basis van morfologische kenmerken doch dit wordt sterk beïnvloed door de groeiomstandigheden. Intensieve veredeling heeft wellicht geleid tot genetische erosie en een vernauwing van de genetische basis. In de studie werden 51 Keniaanse genotypes geanalyseerd. De resultaten maken duidelijk dat de genetische basis van de Keniaanse genotypes weinig variatie vertoont. Input van niet-inheemse genotypes is derhalve nodig om nieuwe waardevolle variëteiten te ontwikkelen.

De uitgevoerde studie leverde waardevolle informatie op over de *Xcm* status in Westelijk Kenia. De studie maakte duidelijk dat er slechts een beperkt resistentieniveau aanwezig is binnen de

Keniaanse genenpool t.a.v. de aanwezige pathotypes. De Keniaanse genenpool is tevens te weinig divers om een oplossing te bieden zodat moet gezocht worden naar nieuw resistent genetisch materiaal. De bekomen inzichten kunnen lokale veredelingsbedrijven helpen bij het zoeken naar duurzame resistenties en op die manier bijdragen tot een heropleving van de katoenteelt in Kenia.

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Permanent Address:	C/O MIMA Academy P.O Box 3939-30200 Kitale
Email Address:	kenpkania@gmail.com , or KennedyChepkurui.Pkania@Ugent.be
Mobile:	+254735588816 or +254728081396
Date of birth:	27 th June 1973
Languages:	English and Swahili (proficiency in written & spoken) Chinese (proficient in spoken)
Marital Status:	Married
Sex:	Male
Religion:	Christian
Nationality:	Kenyan

Career Objective:

To work as Lecturer, Researcher and Administrator

Education

March 1993 to Nov 1998:	Egerton University Bachelor of Science (Agricultural Education & Extension) Second class Honors upper division
Sept 2002 to July 2005:	Zhejiang University P.R. China Master of Science (Genetics and Plant breeding). ‘Unlocking rice germplasm genetic potential using genotypic value to develop quality core collections’ Promoters: Prof. Dr. Chunhai Shi; Supervisor; Dr. Jian-Guo Wu
April 2010 -2016:	PhD research, Faculty of Applied Bioscience Engineering Ghent University ‘Genetic diversity of Kenyan cotton and bacterial blight (<i>Xanthomonas citri</i> pv. <i>malvacearum</i>) prevalence in Western Kenya’. Promoters: Prof. Dr. ir. Geert Haesaert and Prof. Dr. Godelieve Gheysen

Working Experience

August 2009- Date: Tutorial Fellow University of Eldoret- Kenya

June 2008-August 2009: Assistant Lecturer Masinde Muliro University of Science and Technology

January 2008- July 2008: Coordinator CFC/ICO collaborative project between five countries titled; “Increasing resilience of coffee production to leaf rust and other diseases in India, Kenya, Uganda, Rwanda and Zimbabwe”

Responsibilities:

- Manage the day to day operation of the project activities and ensure implementation schedule.
- Responsible with the overall reporting and administration of the project.
- Coordinate with the project implementing agency on the progress of the project

April 2007 to July 2008: Head of Coffee Breeding Section/ Researcher Officer Coffee Research Foundation (CRF)

Responsibilities:

- To formulate and develop research projects in line with CRF strategic plan
- Compile all approved projects in CRF annual program of research
- Implement all approved projects and record necessary data and prepare annual reports of the research activities
- Prepare scientific papers for publication in scientific journals
- Prepare annual sectional budgets and procurement plan according to approved budget
- Keep all records of the section in liaison with other complementary sections

2005- 2007: Lecturer Kilifi Institute of Agriculture, lecturing Undergraduate and Diploma in crop sciences, Genetics, Plant breeding and Plant biotechnology; certificate students, in crop science

Publications

Participation in International Journals

1. Pkania Chepkurui Kennedy, Jian-Guo Wu, Hai-Ming Xu, Chang-Tao Li, and Chun-Hai Shi: **Addressing rice germplasm genetic potential using genotypic value to develop quality core collections.** Journal science of food and Agriculture (2007) 87: 326-333.
2. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Gheysen Godelieve, Haesaert Geert: **Rapid identification of bacterial blight strains isolated from cotton in Western Kenya using *gyrB* gene sequence.** Communications in agricultural and applied biological science (2013) Vol. 78(3): 443-446
3. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Gheysen Godelieve, Haesaert Geert: **Present status of bacterial blight in cotton genotypes evaluated at Busia and Siaya Counties of Western Kenya.** European journal of plant pathology (2014) 139: 863-874.
4. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Haesert Geert, Gheysen Godelieve: **Diversity studies of *Xanthomonas campestris* pv *malvacearum* strains isolated from cotton in Western Kenya based on rep-PCR analysis.** African Journal of Education, Science and Technology (2014) Vol 1(4): 142-149.

Participation to International Scientific Conferences

1. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Haesert Geert, Gheysen Godelieve: Rapid identification of bacterial blight strains isolated from cotton in Western Kenya using *gyrB* gene sequence. 65th International Symposium on Crop Protection, May 21, 2013, Ghent, Belgium. **Oral presentation**
2. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Haesert Geert, Gheysen Godelieve: Diversity studies of *Xanthomonas campestris* pv *malvacearum* strains isolated from cotton in Western Kenya based on rep PCR analysis. 1st Interdisciplinary International Conference (IIC- 2013) on Knowledge and Technological Innovation for Global Competitiveness, 3rd to 5th September, 2013, Eldoret – Kenya. **Oral presentation**
3. Pkania Chepkurui Kennedy, Venneman Jolien, Audenaert Kris, Kiplagat Oliver, Haesert Geert, Gheysen Godelieve: Diversity studies of *Xanthomonas citri* pv. *malvacearum* (*Xcm*) strains isolated from cotton in Western Kenya based on rep-PCR analysis. 7th Gent Africa Platform symposium (GAPSYM7), 6th December 2013, Ghent Belgium. **Poster presentation**

Supervision of Master Students

Jolien Venneman (2011-2012). Evaluation of different Kenyan cotton genotypes for disease resistance to *Xanthomonas campestris* pv. *malvacearum*. Promotor: Prof. Dr. ir. Geert Haesaert

Supervision of Undergraduate Students

Henry Lepakiyo (2011-2012). Evaluation of Kenya Bread Wheat (*Triticum aestivum* L) Pure Lines and Recombinant Inbred Lines for Aluminium Tolerance Using Phenotypic and Microsatellite Markers (SSRs).

Mwangi David Wairire (2011-2012). Elimination of sweet potato feathery mottle virus in sweet potato (*Ipomea batata*) through tissue culture.

Kivindu Francis Ngonyo (2011-2012). Elimination of bacterial wilt in tomatoes (*Lycopersicon esculentum*) through tissue culture.

Chelimo Sylvia Jepkoech (2012-2013). Effects of plant growth regulators on *in vitro* culture of *Warbugia ugandensis*.

Joseph Sire (2012-2013). Evaluation of tea clones for resistance to Nyambene weevils (*Sprigodes mixtous*).