DEVELOPMENT OF FIELD DIAGNOSTIC TOOLS FOR AND CHARACTERISATION OF *Xanthomonas campestris* pathovar *musacearum*, CAUSAL AGENT OF BANANA XANTHOMONAS WILT

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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ABSTRACT

Xanthomonas campestris pathovar *musacearum (Xcm*) is the causal agent of X*anthomonas* Wilt (XW) of Bananas. It is currently the greatest threat to the existence and production of bananas in Uganda and other neighboring countries. *XW on banana* is able to cause about 80-100% field losses. There are currently no known chemicals, herbicides or resistant banana cultivars that can be used to control this disease. Despite awareness efforts among farmers, this disease continues to bring down the yield of bananas in growing areas. This is also due to the fact that symptoms caused by XW are similar to, and often indistinguishable from those caused by manageable common banana diseases such as *Fusarium* wilt. The key control strategy of XW is immediate destruction of infected plants. There is therefore need for early and accurate detection of *Xcm*: an onsite field detection tool. There are currently PCR (polymerase chain reaction) laboratory based assays and antibody based systems that are used for detection of this bacterium. This study developed another antibody-based system: an ELISA polyclonal antibody assay for *Xcm* formatted in to a lateral flow device (LFD). This study also introduces a new pathovar, *Xanthomonas vasicola* pv *musacearum*, to the species *Xanthomonas vasicola* and therefore carried out large scale comparative pathogenicity testing of the *X.vasicola* pathovars on maize, banana and sugarcane. This study further characterized a new collection of *Xcm* isolates from Western Uganda to determine their genotypes based on a subset of the 86 Single Nucleotide Polymorphisms that divided *Xcm* isolates into two major sub- lineages (I&II) as previously reported (Wasukira *et al*, 2012).

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

STATEMENT OF WORK DONE

I hereby acknowledge that the following work was done by the Plant Health staff at Fera:

- Initial preparation and purification of the polyclonal antibody assay for *Xanthomonas campestris* pv *musacearum*
- Development of the Lateral Flow Device for *Xcm* and initial validation was performed by Foresite Diagnostics, Sand Hutton, UK http://www.forsitediagnostics.com/
- Inoculation and scoring of the sugarcane main pathogenicity trial
- Collection of isolates from Uganda; undertaken by IITA

DEFINITIONS/ABBREVIATIONS

AFB	American Foulbrood Bacteria
BSA	Bovine serum albumin
XW	*Xanthomonas* Wilt
CFU	Colony Forming Unit
CVYV	Cucumber Vein Yellowing virus
DNA	Deoxyribonucleic acid
EFB	uropean Foulbrood Bacteria
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty Acid Methyl Ester
IITA	International Institute of Tropical Agriculture
LAMP	Loop-mediated isothermal amplification
LFD	Lateral Flow Device
NCPPB	National Collection of Plant Pathogenic Bacteria
NCBI	National Collection of Biotechnology Information
OD	Optical Density
PBST	20mM sodium phosphate, 150mM sodium chloride and Tween 20
PCR	Polymerase chain reaction
pNPP	p-Nitrophenyl phosphate
pv	pathovar
Rep-PCR	Repetitive Element Palindromic PCR
Xac	*Xanthomonas arboricola* pv *celebensis*
Xav	*Xanthomonas axonopodis* pv *vasculorum*
Xcp	*Xanthomonas campestris* pv *pelagonii*
Xcc	*Xanthomonas campestris* pv *campestris*
Xcm	*Xanthomonas campestris* pv *musacearum*
Xcv	*Xanthomonas campestris* pv *vesicatoria*
Xvv	*Xanthomonas vasicola* pv *vasculorum*
Xvh	*Xanthomonas vasicola* pv *holcicola*
YDC	Yeast Dextrose Chalk media

1. INTRODUCTION

Importance of bananas in East Africa

Bananas are considered to be the most important staple food in Uganda. Meeting both subsistence and commercial purposes, over 75% of Ugandan farmers cultivate banana on an estimated 1M hectares of the land (FAO, 2010) making Uganda the world's second largest producer of bananas after India (Triptathi *et al*, 2009). At the regional level, the Great Lakes region, covering Uganda, Rwanda, Burundi, Tanzania, Kenya and The Democratic Republic of Congo (DRC), is the largest producer and consumer of banana in Africa (Smale, 2006) with a *per capita* consumption per year estimated at more than 250kg; the highest in the world. In Africa banana provides more than 25% of the carbohydrate requirements for over 70 million people (IITA, 1998).

Both green and table bananas are marketed for food and the crop residues also provide fodder for zero- grazed animals, which in turn provide manure for the farm for the agronomic health of the crop (Spilsbury *et al,* 2002, Komarek *et al*, 2010). Apart from mixed farming, the plant is readily inter-cropped with both perennial and annual crops, especially legumes thus contributing to a balanced diet for producers (Karamura *et al,* 1998).This large plant also reduces soil erosion through driving rain and sheet erosion, by acting as a wind break, especially in highland agro-ecologies. In the urban regions, the manufacture of banana fiber based handicrafts such as mats, baskets, lamp sheds, ropes plus a host of decorations has become an important economic activity.

However, for the past two decades, the steady production of bananas in Uganda and East Africa as a whole has experienced a number of constraints that have affected productivity (yield) and relative preference in cultivation to other crops (Anon, 2007). The major constraints to the production of this major food crop include low soil fertility, pests such as weevils, nematodes and diseases; Black Sigatoka, Banana leaf streak disease and *Fusarium* wilt. *Xanthomonas* wilt (XW) of Bananas otherwise refered to as Banana *Xanthomonas* wilt is the greatest threat to existence of bananas in Uganda.

Up until the discovery of XW, farmers have been able to control all other biotic threats (nematodes, weevils) through cultural practices exploiting differences in cultivar resistances and tolerance. In this way farmers suffered reduced productivity, but maintained reasonable levels of food and income security. However, following the arrival of XW in Uganda, farmers have suffered close to 80-100% field losses.

XW is caused by a bacterium called *Xanthomonas campestris* pathovar (pv) *musacearum (Xcm). Xanthomonas* wilt (XW) was first discovered in Central Uganda in 2001 (Tushemereirwe *et al*, 2004) and subsequently spread to Republic of Congo (Ndungo *et al*, 2006), Rwanda (Reeder *et al*, 2007) and Kenya, Tanzania and Burundi (Carter *et al*, 2010). Prior to this discovery, the disease was only known in Ethiopia on enset plants (*Ensete ventricosum*) (Yirgou *et al*, 1968 & Bradbury *et al*,1974).The pathogen destroys banana plants (*Musa* species), resulting in total loss of production and threatening the livelihoods of millions of people who depend on bananas as a food and income source in the region. There is therefore need for control and containment of *Xcm*, but most especially an onsite field diagnostic tool to detect the bacteria.

2. LITERATURE REVIEW AND BACKGROUND

2.1 Taxonomy of Bananas

Bananas are monocots that belong to the order of Zingiberales and the family of *Musaceae.* Members of this family are large plants 2-9 meters tall with an aerial trunk consisting of compacted leaf sheaths which grow directly from the top of the corm. *Musaceae* contains two genera: *Musa* (Simmonds, 1966) and *Ensete* (Cobley, 1976). Genus *Ensete* is distributed wildly in Africa from the Cameroon throughout East Africa down to the Transvaal in South Africa (Karamura, 1998). *Ensete* differs from *Musa* by being monocarpic, non-suckering with a distinctively swollen base, and having large-sized seeds while *Musa* produces suckers and has small seeds (Cobley, 1976); (Samson, 1992).

The genus *Musa* contains 30-40 species and all wild species are diploids (2n=2x=14, 18) while the cultivated/domesticated species are polyploid. Genus *Musa* is divided into 5 sections, based mainly on the basic chromosome number, orientation and arrangement of flowers in the inflorescences. The sections are *Musa, Rhodochlamps, Callimisa, Australimusa* and *Ingentimusa*. The section *Musa* is the largest with 13-15 species, the most diversified and most ancient (Karamura, 1998). The sections include cooking bananas, dessert bananas and plantains now grown throughout the tropics.
The wild species of *Musa* can reproduce both sexually and asexually (by suckers from a corm). Among the fifteen wild species are the *Musa acuminata* Colla and *Musa balbisiana* Colla, both of which have contributed to the origin of the majority of edible bananas (Purseglove, 1972); (Simmonds, 1987).

2.2 Banana morphology

Bananas are perennial giant plants that grow from an offshoot growing at the base of the plant (the sucker or the corm), that replaces the mother plant. The corm produces aerial roots which arise from the lateral buds which develop into eyes and later suckers. The continuous vegetative growth of the suckers perpetuates the corm's life and hence the perennial status of bananas. The mat, also called stool, is the term used to

designate the mother plant and its suckers. The tightly packed foliage forms the pseudo-stem or the aerial shoot (Anon, 2007). The corm also includes the apical meristem from which the leaves and ultimately the flowers are initiated. When the banana plant has formed an average of 40 leaves(within 8 to 18 months), the terminal bud of the corm develops directly into the inflorescence which is carried up on a long smooth un-branched stem through the centre of the pseudo-stem emerging at the top in the centre of the leaf cluster (Anon, 2007).

The inflorescence consists of male and female flowers arranged in a group. The female inflorescences develop into fingers that constitute the bunch. Banana bunches posses up to 14 hands (clusters), each with up to 10 fingers which varies from cultivar to cultivar. Most cultivated bananas lack pollen while the wild bananas are able to produce both nectar and pollen (Anon, 2007).

2.3 Evolution and introduction of bananas to Africa

Unreduced gamete production at the end of meiosis in diploid edible *M.acuminata* introduced the AAA (acuminata) triploids which by virtue of being more vigorous and for producing bigger fruits (Karamura, 1998), now dominate the world's bananas. There is no clear evidence as to what led to the evolution of edible bananas especially due to wide variability of *M. Acuminata*. There are no natural edible forms of diploid BB *Musa balbisiana* or triploids BBB of this species.

Another important step in the evolution of bananas was the crossing of AA (and perhaps AAA too) cultivated bananas with the wild *M. balbisiana* (BB). Natural hybridization occurred in several genome combinations and the resulting hybrids were AB, AAB and ABB. At present, there are many clones of cultivated bananas belonging to different genome groups, of which AA, AAA, AAB and ABB are the most numerous (Karamura, 1998).

2.4 Banana production and its value in Uganda

2.4.1 Banana cultivars in Uganda

Most of the bananas grown in Uganda are East African bananas. East African bananas are categorized in three groups; dessert bananas (AAA), plantains (AAB) and the East African Highland (AAA-EAHB, cooking and beer) bananas (Karamura, 1998). However the East African Highland bananas (AAA) that grow between 1200 and 1900 meters above sea level are the most widely distributed. Bananas are of diverse types according to end uses: cooking, roasting, dessert and beer bananas. This has been used to group banana varieties - on the basis of their end uses (Karamura, 1998).

2.4.2 Production of banana in Uganda

Bananas are the single most important staple food in Uganda. Uganda ranks second after India in the world banana production with an annual output of 10 million tonnes (FAO, 2010), accounting for more than 11.18% of the world's total production.

The crop has been grown for both commercial and subsistence purposes. Apart from being a key staple food, bananas are increasingly becoming an important source of income for the poor farmers. Excess production is sold in local markets and is the main staple food for urban workers (Karamura, 1998). The bananas in Uganda fall into three categories; cooking bananas which are boiled and eaten, the beer bananas from which juice can be made and the plantains that can be steamed, fried or roasted. Beer production from bananas in Uganda is also on the increase.

Bananas have also become an important component of mixed production systems on farms especially in high population areas where land is limited. Here bananas provide fodder for zero-grazed animals, which in turn provide manure for the farm. Apart from mixed farming, the plant is readily inter-cropped with both perennial and annual crops, especially legumes such as beans thus contributing to a balanced diet for producers (Karamura, 1998). From the dry leaves of the pseudo-stem, mats, lamp shades, ropes

and crafts can be made. Bananas are also used for medicinal purposes to treat abdominal ailments such as ulcers.

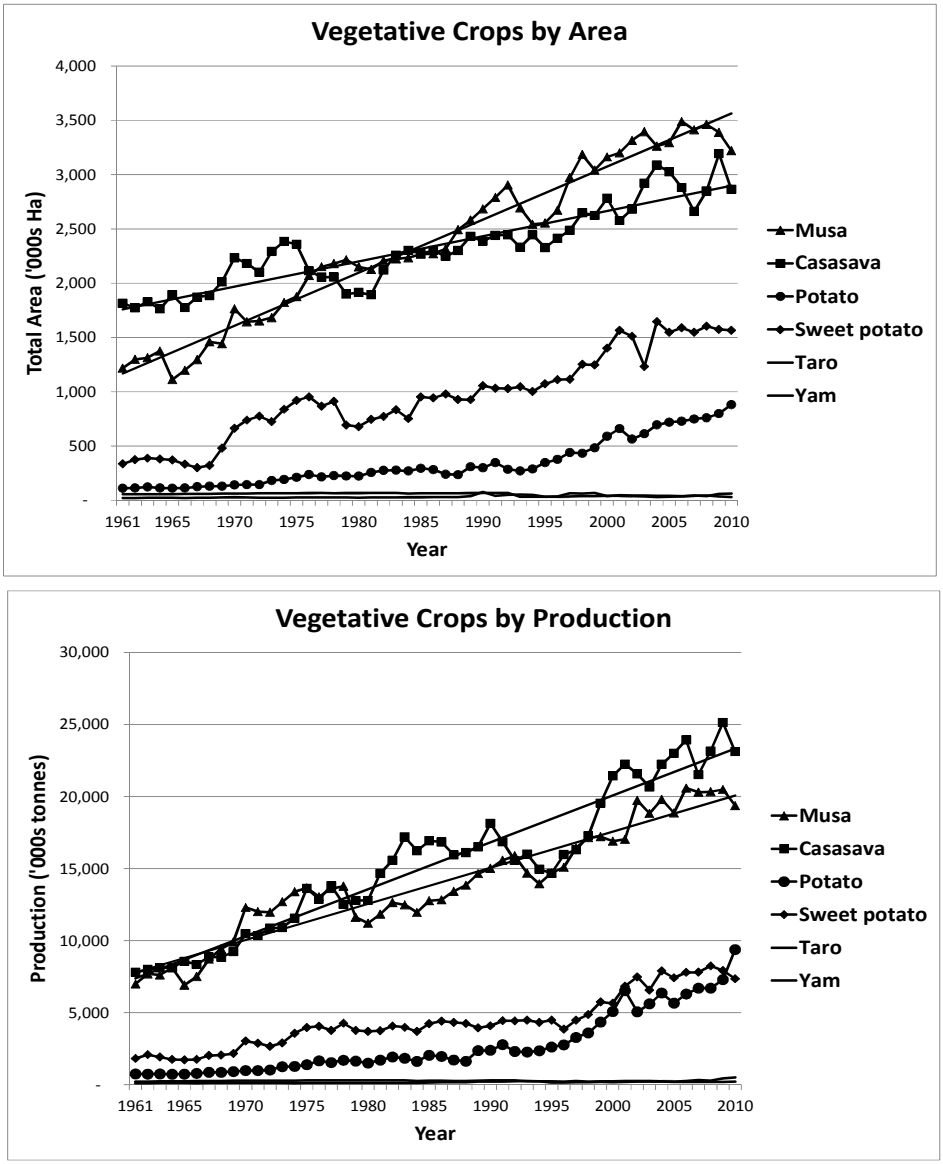


Figure 1: Comparison of VPCs (Vegetative Crops by production) of Eastern Africa (FAO Crop Production data, 2009; Listed East African countries only, excluding non-mainland countries)

2.4.3 Production constraints of Bananas in Uganda

Several factors contribute to the decline of production yields of this important crop.

According to a brief by IFPRI (2005) in terms of associated yield losses and year of plantation cycle, the emerging XW on banana contributes to 80-100% loss in the first year, Black Sigatoka causes about 30-50% in the third year, low soil fertility contributes to about 10-70% loss in the third or fourth year, nematodes cause about 40-60% loss in the fourth year. This makes XW the biggest threat to the production yields of bananas in Uganda. However these constraints do the most damage in already low productivity areas than the high productivity areas.

According to the 2010 FAO statistics report, banana production in Uganda dropped by at least 500,000 tonnes between the years of 2005 and 2008, 3 years after the first report of XW in 2001. Probably with more awareness of XW among farmers, the production of bananas began to increase after 2009 (see Figure 1). The production of cassava, which is the second most grown crop (next to banana) in Uganda, increased after 2001 (since the first report of XW) by about 1,000,000 tonnes compared to the previous years before 2001. This suggests that farmers began to put in more effort in to cropping cassava to combat food losses caused by *Xanthomonas* wilt of bananas.

2.5 Major Banana pests and diseases in Uganda

2.5.1 Fusarium wilt (Panama wilt)

This is caused by a soil-borne fungus, *Fusarium oxysporum* f.sp. *cubense.* This is also known to be among the most destructive diseases and can cause about 100% loss if not controlled (Anon et *al,* 2001). It is a typical vascular disease that causes disruption of translocation and systemic foliage which may lead to the collapse of the pseudo-stem. Fusarium wilt attacks the ABB genome banana cultivars but the indigenous East African Highland AAA cultivars and Cavendish cultivars seem resistant to the disease (Mwangi *et al*, 2007). Fusarium wilt tends to cause similar symptoms as those caused by Banana *Xanthomonas* Wilt, making it difficult for farmers to manage Fusarium wilt.

Symptoms of the disease include yellowing of leaves or premature collapse of the leaves (begins with the older leaves), and dis*colour*ed vascular bundles when you cut up the pseudo-stem. If the disease is severe, the banana plant may fail to produce flowers or bunches (Anon et *al*, 2001). The disease spreads through already infected plant materials, soils and tools with *Fusarium* propagules.

2.5.1a Control of Fusarium wilt

Use of resistant cultivars: Not much can be done once the plant is attacked by *Fusarium* wilt therefore farmers are encouraged to grow the resistant cultivars such as the Cavendish bananas, banana hybrids likes FHIA 17 and FHIA 23(most banana hybrids are tetraploids AAAB or AAAA). If susceptible cultivars are to be planted, it is important that soils are Fusarium free; in some cases soil fumigation can be done (Mwangi *et al*, 2007).

Use of clean planting materials: To be able to use clean banana planting materials, farmers are encouraged to source free tissue culture plantlets (macro-propagation) from the National Agriculture Research Laboratories (Anon et *al*, 2001).

2.5.2 Black Sigatoka

This disease is also known as Black Streak Leaf disease and is caused by a fungus, *Mycosphaerella fijiensis.* The disease reduces and destroys leaves of the banana plant. The leaf necrosis reduces fruit yield of the plant. The disease is also characterized by the appearance of brown spots on the leaf which later enlarge to form black patches which later develop spots with grey centers (Anon et *al,* 2001). High humidity favors the growth of the fungus ad water helps in the spread of its spores to infect other plants.

2.5.2a Control of Black Sigatoka

Cultural practices: Farmers have resorted to cutting off infected leaves of the bananas. This prevents easy spread of the fungus. However this can also lead to the

spread of the disease by continual use of the cutting tools, hence sterilization of the tools is also encouraged.

Good crop husbandry: Proper crop spacing, continuous weeding and good drainage in the fields would help reduce the humidity that would favor the growth of the fungus.

Use of resistant cultivars: This is the most effective method of controlling this disease. However, most of the highland bananas are susceptible to the disease. Most banana hybrids such as FHIA 1, FHIA 3, FHIA 17 and FHIA 23 have been proven to be resistant to Black Sigatoka (Anon *et al,* 2001).

2.5.3 Banana Streak virus

This disease is caused by a plant pararetrovirus belonging to the family of Caulimoviridae. Symptoms include; leaves initially have yellow streaks which later turn to golden necrotic streaks, the plant may have stunted growth and produce smaller bunches or distorted fruits. Symptom expression is temperature dependent and the effect on yield depends on whether flowering and bunch initiation coincide with temperatures that increase virus replication within the plant (Mwangi *et al*, 2007). Spread of the virus is still undetermined.

2.5.3a Control of Banana Streak virus

Use of clean planting materials: Farmers are encouraged to completely destroy already diseased plants and ensure that only healthy suckers (from macro-propagation) are re-planted.

2.5.4 Xanthomonas Wilt (XW) on Banana

The disease is caused by a bacterium *Xanthomonas campestris* pv *musacearum.* It was first reported in Ethiopia causing *Ensete* Bacterial wilt in *Ensete* plants by Yirgou and Bradbury in 1968. It then spread to Uganda and was first reported in 2001. It is emerging as the most deadly disease of bananas. It causes close to 80-100% field

losses. It attacks both young and mature plants and at its advanced stages, the pseudo-stem collapses. Its symptoms are quite similar to those of *Fusarium* wilt and Moko disease. This is the most serious disease that farmers have not been able to control and worse still, there are no known resistant cultivars.

XW will be discussed in more details in subsequent sections.

2.5.5 Banana weevils

The most common of the weevil is the species *Cosmopolites sordidus.* Weevil damage results from larvae (usually from an egg that has been laid near the corm by an adult weevil) that feeds and tunnels through the banana corm and pseudo-stems (Mwangi *et al*, 2007). This causes galleries that weaken the plant and provide entry points for ants and other pests (such as the bacterium *Xanthomonas campestris* pv *musacearum*) and this eventually leads to plants toppling after snapping at the base (Mwangi *et al*, 2007). The damage also affects the uptake of nutrients and water thus weakening the plant. The East African Highland bananas are not easily affected as the weevil prefers lower altitudes about 1000-1200 meters above sea level than the high altitudes of 1500m or more (Anon *et al*, 2001).

2.5.5a Control of the weevil

Use of clean planting materials: Only corms that do not bear symptoms of weevil damage should be planted. Corms should be peeled to expose the white tissue to look for signs of tunneling by the weevils. To be sure that the corms are clean, they should be subjected to hot water or chemical dipping (dursban) to kill any larvae (Anon *et al*, 2001).

Field sanitation: Proper weeding and sucker removal can reduce the niches of the weevils. Discarded corms should be chopped up and destroyed to reduce weevil breeding.

Chemical control: Chemical insecticides can be spread around the banana plants close to the corms to kill weevils. In Uganda, chemicals such as Furadan, Primicid and Dursban are used (Anon *et al*, 2001).

Other potential control methods: Use of resistant cultivars could also be a solution to crop loss by weevils. Two East African Highland banana hybrids M2 and M9 bananas are known to be resistant to weevils (Tinzaara *et al*, 2009).

2.5.6 Nematodes

Nematodes are small worms that cannot be seen with the naked eye. They parasitize the banana root system and can reduce yield by up to 80%. A root corm damaged by nematodes shows reddish brown lesions or necrosis when split or peeled. This results in lower nutrient uptake and increases plant toppling. The most widespread nematodes include *Radopholus similis*, *Pratylenchus goodeyi*, *Pratylenchus coffeae*, *Helicotylenchus multicinctus* and *Meloidogyne* spp. The nematode *R. similis* contributes to most plant toppling (Mwangi *et al*, 2007).

2.5.6a Control of nematodes

Use of clean planting material: Nematodes like weevils can be avoided by planting clean planting materials. Nematodes have limited mobility so most are spread by infected plant material (Tinzaara *et al*, 2009). Corms must be checked for signs of nematode damage. To ensure that clean material is planted, the corms must be dipped in hot water (Anon *et al*, 2001).

Good crop husbandry: Crop rotation of bananas with plants that are not affected by nematodes is also a good way to control nematodes (Tinzaara *et al*, 2009).

Chemical control: Use of nematicides can also be used to eliminate nematodes. They should be spread around the banana corms. The chemical commonly used is Furadan (carbofuran; Anon *et al*, 2001).

2.6 Classification of Xanthomonas campestris pv musacearum

2.6.1 Genus Xanthomonas

Xanthomonas is a genus of the class Gamma-proteobacteria under the order of *Xanthomonadales* under family of *Xanthomonadaceae*. They are Gram-negative bacteria, aerobic and motile by a single polar flagellum. The cells are single, straight rods usually within the range of 0.4 - 0.7um wide and 0.7 - 1.8um long. Most form yellow colonies and are usually chemoorganotrophs. The yellow pigments are mono-dibromo arylpolyenes called xanthomonadins, which are characteristic of the genus (Vauterin *et al*, 1995). The optimum temperature for growth is about 25-30 ◦C. Most *Xanthomonas* are significant plant-pathogens that cause severe crop disease and death. *Xanthomonas* consists of many species.

The classification and taxonomy of *Xanthomonas* has long been based on phenotypic features and host specificity. It was common practice to define a plant-pathogenic Xanthomonad isolated from a new host plant as a new *Xanthomonas* species (Vauterin *et al*, 1995). This 'new host-new species' method increased the number of species within the genus. Later, almost all the *Xanthomonas* species were merged into the one species *X. campestris* by Dye *et al*, (1962) as there was not enough information at the time to classify the species in the genus (Vauterin *et al*, 2000). Young *et al* (1978) then proposed reclassifying the species into pathovars, based on their pathogenicity, and this is the system that is still in use (Vauterin *et al*, 2000). Pathovars are defined by one single feature; distinct pathogenicity on one or more hosts. However, with advances in molecular typing (such as DNA-DNA hybridization or RAPD analysis) methods, it has been noted that most of the former *X. campestris* species or species that had originally been placed in *X. campestris* are indeed heterogeneous. Currently, *Xanthomonas* has over 140 pathovars (Vauterin *et al*, 2000).

Plant pathologists are still trying to find ways of classifying *Xanthomonas* as this will be helpful in combating and controlling the plant diseases they cause. The pathovar classification system that is still in use has limitations; "the host range of most pathogenic *Xanthomonas* species is still unknown, there is significant heterogeneity

within a number of pathovars and this system leaves out non-pathogenic *Xanthomonas"* (Vauterin *et al*, 2000).

Xanthomonas species have been grouped/classified using a number of techniques. These include: comparison of B gyrase sequences within *Xanthomonas* strains (Parkinson *et al,* 2009), Rep-PCR genomic fingerprinting of *Xanthomonas* strains Rademaker *et al*, 2005) and DNA-DNA hybridization technique (Vauterin *et al*, 2000).

The genus seems to comprise 20 or more DNA homology groups of species; *X. fragariae*, *X. hortorum*, *X. populi*, *X. arboricola*, *X. cassavae*, *X. codiaei*, *X. bromi*, *X. cucurbitae*, *X. axonopodis*, *X. oryzae*, *X. vasicola*, *X. pisi*, *X. melonis*, *X. vesicatoria*, *X. campestris*, *X. translucens*, *X. hyacinthi*, *X. theicola*, *X. sacchari* and *X. albilineans* (Vauterin *et al*, 2000). *Xanthomonas campestris* pv *musacearum* strains have until very recently not been included in studies on taxonomy.

Plant pathologists are still trying to find ways of improving the classification of *Xanthomonas* as this will be helpful in combating and controlling the plant diseases they cause. Indeed, the pathovar classification system has limitations.

2.6.2 Classification of Xanthomonas campestris pv musacearum

Recent studies on comparison of B gyrase sequences of *Xanthomonas* strains that included *Xanthomonas campestris* pv *musacearum (Xcm)* showed that the strains fall in the same homology (share genetic similarities) group as *Xanthomonas vasicola (X. vasicola)* strains (Parkinson *et al*, 2009). The *X. vasicola* pathovars are the proposed *X vasicola* pv *holcicola (Xvh)* and *X vasicola* pv *vasculorum (Xvv)(Vauterin et al*, 1995) commonly pathogenic on sorghum and sugarcane respectively. However the reclassification of *Xvv* was considered incomplete in terms of standards for naming pathovars and in turn this invalidated the name of *Xvh* and reducing the pathovar to *X. vasicola* species (Garrity, 2005). It should therefore be noted that *Xvv* and *Xvh* are still proposed names.

However FAME analysis (whole bacteria cell fatty acids converted to methyl esters and analyzed by gas chromatography), Rep-PCR (PCR with primers complementary to naturally occurring conserved repetitive DNA elements within a bacteria genome) and *B gyrase* sequence studies done on the *Xcm* strains showed that they are actually more closely related or genetically similar to strains that commonly attack sugarcane(*Xvv* Aritua *et al*, 2008; Parkinson *et al*, 2009). Pathogenicity tests done in the same study revealed that *Xcm* can cause disease in maize and banana but the *X. vasicola* strains could only cause disease in maize and not in banana. This may suggest that some *X. vasicola* strains, *'Xvv'* in particular may have evolved to include banana as a host as they are able to retain pathogenicity in maize and sugarcane.

Aritua *et al*, (2008) therefore proposed that *Xcm* be renamed *Xanthomonas vasicola* pv *musacearum*. However comparative pathogenicity studies of the *Xanthomonas vasicola* pathovars has not been done (Garrity, 2005) so the proposal of renaming *Xcm* could not be supported. *Xanthomonas campestris* pv *musacearum* strains did not show much genetic similarity when compared to other *Xanthomonas campestris* pathovars. This is not surprising considering that DNA-DNA homology studies have placed many former *Xanthomonas campestris* species into new other species (Vauterin *et al*, 1995). When *Xanthomonas campestris* pv *musacearum* was discovered in 1968 by Yirgou and Bradbury (Yirgou *et al*, 1968), it was first placed under the *Xanthomonas musacearum* species.

2.6.3 Variation within the isolates of Xanthomonas campestris pv musacearum

Initial studies on *Xcm* strains were based on rep-PCR and showed very limited genetic diversity (Aritua *et al*, 2007, 2008; Odipo *et al*, 2009). Yet with genome wide sequencing, recent studies have shown that there may be two major sub–lineages of the pathogen based on consistent single-nucleotide polymorphisms (SNPs) among the East African isolates (Wasukira *et al*, 2012). This study revealed that *Xcm* isolates from Uganda, Kenya, Tanzania and Burundi (lineage I) are distinct from isolates from Ethiopia, DR Congo and Rwanda (lineage II) based on 86 polymorphic positions. Based on the sequence of the SNPs, primers flanking SNPs that code for enzyme

restriction sites have been designed to allow the two sub-lineages to be resolved by PCR and restriction analysis. However, it was observed that of the two Ethiopian strains (NCPPB 2005 & NCPPB 2251) included in the study these share some similarities with both lineages. As yet the robustness of the lineages has not been established sufficiently and will require study of a more extensive collection of *Xcm* strain from across the region and especially Ethiopia.

As the first reports of XW were from Ethiopia on enset and *Musa* plants and then in Uganda on banana, it has been widely assumed that the origin of the pathogen into Uganda was from Ethiopia and probably as a single event. These findings have provided some challenges to this assumption.

2.6.4 State of current knowledge on Xanthomonas campestris pv musacearum and Xanthomonas vasicola pv vasculorum strains

Draft genomes of *Xanthomonas campestris* pv *musacearum* and *Xanthomonas vasicola* pv *vasculorum* have since been generated by Studholme *et al*, (2010). This study showed that both genomes are highly similar with a few differences. However one of the most striking differences is the difference in the repertoires of their T3SS virulence effectors. T3SS virulence system in bacteria is responsible for colonizing and parasitizing susceptible plant hosts by use of translocation of effectors into the plant (Bretz *et al*, 2004).The effectors, once in the plant, target host defensive cells and cause virulence. However plants have the R genes that can recognize these effectors thereby eliciting defense responses. Disease in a plant happens when the R genes of the plant do not recognize these effectors.

Xcm strains have the YopJ-like C55 cysteine proteases in its T3SS apparatus thought to be able to suppress the defenses of banana while *Xvv* has the Avr*Xv*3 thought to cause avirulence in banana but the R gene in banana is able to recognize it (Studholme *et al*, 2010). Accordingly, it has been speculated that the differences in the T3SS effectors between *Xcm* and *Xvv* strains may be responsible for the differences in host adaptation (Studholme *et al*, 2010).

2.7 *Xanthomonas* Wilt of Bananas (Banana *Xanthomonas* Wilt)

Xanthomonas campestris pv *musacearum*, the etiological agent of *Xanthomonas* wilt of bananas was first reported in Ethiopia in 1968 by Yirgou and Bradbury. It attacks Ensete (*Ensete ventricosum)* plants that are within the same family with banana plants (*Musa spp*). It was first reported affecting bananas within Mukono district in Uganda in 2001 (Tushemereirwe *et al*, 2004). Its symptoms strongly resembled those of Moko disease, a common disease of bananas in Asia, caused by the bacterium *Ralstonia solanacearum*. However after pathogenicity tests were done on healthy plants, the bacterium was confirmed to be *Xanthomonas campestris* pv *musacearum* as referenced from literature (Yirgou *et al*, 1968). Later the disease spread to the neighboring countries of Democratic Republic of Republic of Congo (Ndungo *et al*, 2006), Rwanda (Reeder *et al*, 2007) and Kenya, Tanzania and Burundi (Carter *et al*, 2010).

2.7.1 Diagnosis of Xanthomonas Wilt

Symptoms of *Xanthomonas* wilt on infected banana plants include premature ripening of the fruit, shriveling of male buds, progressive yellowing and wilting of leaves and when the pseudo stem is cut, pockets of yellow bacterial ooze are seen after 15 to 20 minutes, this usually confirms the presence of the disease (Tinzaara *et al,* 2006). Symptoms on floral parts include wilting of bracts, shriveling and rotting of male buds, and yellow–brown flower stalks (Tripathi *et al*, 2009a). Yellow or brown streaks also occur in the vascular tissues of the infected plants. According to Yirgou and Bradbury (1968, 1974), the first symptom of the leaf was the dull green colour of the lamina, which gradually becomes scalded in appearance and folds back on the midrib with the two halves of the leaves touching each other. The scalded leaf eventually withers and the whole plant rots.

2.7.2 Spread and transmission

Xanthomonas campestris pv *musacearum* can be spread through planting already infected plant materials and plant parts and use of contaminated cutting tools

(Karamura *et al*, 2010). The disease can also be spread through contaminated soil, and traded banana products (seedlings, fruits, leaves, and fibers (Tripathi *et al*, 2009a)).

Epidemiological studies have shown that the bacteria may also be carried by insect vectors from infected plants to the male buds of disease-free plants within and between farms (Tinzaara *et al,* 2006). This is in fact is believed to be the primary means of spread. Insects carrying the bacteria will frequently visit the floral parts. Insects that visit the flowers include bumblebees, stingless bees, wasps, hover flies, ants, fruit flies and cockroaches (Tinzaara *et al,* 2006). This correlates with the fact that the first initial symptoms of the disease are usually seen on the male bud (Tushemereirwe *et al*, 2003). Long distance spread of the pathogen is suspected to take place through the transport of rhizomes/suckers that are already infected (Tinzaara *et al,* 2006).

2.7.3 Disease host range, distribution and damage

Other than bananas, *Xanthomonas campestris* pv *musacearum* also attacks *Ensete ventricosum* plants, that belong to the same botanical family as bananas. Host range studies have shown that *Xcm* infect plant families like Musaceae and Cannaceae. Other studies also showed maize to be potential hosts (Aritua *et al*, 2008). Ssekiwoko *et al* (2006) concluded that *Xcm* does perhaps restrict its host range to monocots.

Studies were taken to assess the impact of XW on banana production in Uganda between the years of 2001- 2004. In 2001 (first report of XW in Uganda), areas that had been affected by XW produced relatively similar amount of banana bunches with areas that had not been affected (Karamura *et al*, 2010). But in 2004, the areas that had not been affected by XW produced twice as much as bunches of banana compared to the areas that had previously been hit by XW. The production of bananas among the affected house-holds fell had fallen close to 52% or about 10-17% per annum between the years of 2001-2004 (Karamura *et al*, 2010).

2.7.4 Control measures/strategies for Xanthomonas Wilt of Banana

Strategies have been put in place to combat XW of bananas. Most of the control practices are based on the specific control practices of controlling Moko disease. Moko disease or Bacterial wilt attacks bananas in South East Asia and is caused by the bacterium *Ralstonia solanacearum.*

2.7.4a Cultural practices

Destruction of infected plants/plant material: Once identified, all infected plants or plant materials are either totally uprooted and destroyed or buried. This has been a key control method to stop the spread of the bacteria. However cultural perceptions of the banana plant by people in Uganda make such recommendations of banana mat destruction not easily considered or acceptable (Smith *et al*, 2007). Currently it is recommended that at least 6 months should elapse following the removal of all infected banana material before a new banana stand can be planted in the same field. This is because the bacterium can survive in the soil for up to three months (Mwebaze *et al,* 2006).

De-budding or decapitation of male bud inflorescence: Prompt removal of inflorescence-infected plants and early de-budding of inflorescences (whether the disease is present or not) has been shown to also control *Xanthomonas* Wilt of bananas (Blomme *et al*, 2009).

This reduces the risk of floral infection by insect vector transmission. Studies have shown that suckers do not become infected when pseudo-stems with infected inflorescence were removed at an early stage (Blomme *et al*, 2009).

Cultivars whose male bracts and flowers persist longer also reduce their chances of floral infection. De-budding or bagging immediately after the last hand is formed on the bunch has been shown to be quite effective in the control of floral infections (Blomme *et al*, 2009).

Sterilizing garden tools and equipment: Farmers sterilize tools especially knives used in harvesting bunches to avoid spread of the bacteria from one plant to another. Tools are disinfected using either fire or dipping the tools in sodium hypochlorite solution. However the cost of sodium hypochlorite solution in rural areas makes this way of control difficult.

2.7.4b Chemical and biological control

There is currently no bactericide available to destroy *Xanthomonas campestris* pv *musacearum*. However herbicides such as 2,4-D are available for immediate destruction of infected plants and hence halt the spread of the bacteria (Smith *et al*, 2007).

Host resistance in bananas: Most banana cultivars and genome groups are susceptible to the disease. Studies investigating the susceptibility of the different banana cultivars/genome groups to XW showed that the AAA dessert bananas are most highly susceptible, followed by AAA- East African Highland bananas and BB *Musa balbisiana* (wild type of banana) as the resistant cultivar (Tripathi *et al*, 2009b). Field observations show that the disease appears to be more prevalent on 'Pisang awak' (ABB), a cultivar that has more insect vectors visiting it compared to all other cultivars (Biruma *et al*, 2007).

Awareness of disease among farmers: Raising awareness about the disease is an effective way to combat the spread of the *Xanthomonas campestris* pv *musacearum* (Tripathi *et al*, 2009a). However, despite the fact that there has been awareness of the disease among farmers, XW is still rapidly spreading and destroying banana farms in Uganda and neighboring countries. XW of bananas tends to cause almost similar symptoms to common manageable diseases such as Fusarium wilt is often confused. The confusion of symptoms between the two diseases makes farmers quite reluctant to immediately apply XW control measures (Mwangi *et al*, 2007). There is need for proper eradication and containment measures for XW driven by accurate detection of XW in

infected plants/plant materials; consequently, an on-site/field based diagnostic tool for XW is very much required.

2.7.5 Banana resistance to Xanthomonas Wilt of bananas through genetic engineering

Tripathi *et al* (2010) transformed bananas with the hypersensitive response assisting protein (*Hrap)* gene to enhance the resistance to XW of bananas, by inducing the hypersensitive reaction. The *Hrap* gene was originally isolated from sweet pepper (*Capsicum annum*) and transformed into tobacco plants. The transgenic tobacco plants were able to induce a hypersensitive reaction (HR) to bacteria pathogens. In Tripathi's *invitro* experiment, four of the transgenic lines (a transgenic line consists of 2-6 banana plants) inoculated with *Xcm* cultures remained healthy and four of them showed symptoms 28 days after inoculation instead of the normal 14 days after inoculation (Tripathi *et al*, 2010). The transgenic lines that showed delayed or no symptoms were then potted and put in the glass house. Six of the 8 transgenic plants showed no symptoms until 60 days past inoculation. One showed leaf symptoms but did not spread to other part of the plants. This indicated that banana plants can resist *Xanthomonas* wilt with the help of the *Hrap* gene.

Namukwaya *et al* (2012) also transformed bananas with the *Pflp* gene that conferred resistance to XW of bananas. The *Pflp* gene was also isolated from sweet pepper. The gene is part of the protein Ferredoxin-I that is involved in metabolic pathways such as photosynthesis and lipid reduction. The gene has been able to enhance resistance in tobacco against pathogens such as *Erwinia* and *Pseudomonas* (Namukwaya *et al*, 2012).Of the nine transgenic lines that were successfully transferred to potted plants from *in vitro*, eight of them were resistant to *Xanthomonas* wilt for the duration of the experiment;60 days after inoculation. The control plants became diseased and wilted away. This revealed that the *Pflp* gene enhanced banana resistance to *Xanthomonas* wilt.

2.8 An overview of plant pathogen diagnostics focusing on *Xanthomonas* wilt of bananas

2.8.1 DNA based methods for detection of plant pathogens

2.8.1a Conventional PCR

Polymerase chain reaction (PCR) applications are the most common DNA-based methods used to detect plant pathogenic bacteria. PCR involves making millions of copies of a target DNA sequence, using the enzyme *Taq* polymerase. Two primers (forward and reverse) which are single short DNA strands complementary to the target DNA sequence provide the desired specificity. The two primers are usually 25-30 base pairs. The process involves three steps; denaturation, the separation of the double strands of DNA at high temperatures usually about 94-98◦C, annealing where the primers hybridize through complementary base pairing to the single stranded DNA at lower temperatures approximately 55-65◦C and lastly elongation, the addition of nucleotides to the single strands by the *Taq* enzyme creating a copy of the DNA section at a temperature of 72◦C. The cycle is usually repeated 34- 40 times. The PCR assay typically consisting of the 2 primers, the enzyme Taq polymerase, magnesium chloride, deoxynucleoside triphosphates(dNTPs) and water are usually performed in volumes of 10-50µl. The PCR reactions are usually run by thermal cyclers and the PCR product visualised by gel electrophoresis or staining.

Diagnosis of plant pathogens using PCR depends on two keys; the target sequence chosen to design primers and the extraction method of DNA (Weller *et al*, 2006). The target sequence has to be specific to the DNA of the pathogen and the extraction method must provide clean pure DNA to avoid inhibiting the role of *Taq* polymerase during PCR. The main advantage of conventional PCR is its highly sensitive and can detect even small amounts of DNA. Plant pathologists aim to improve its specificity and sensitivity and this has been done through the use of nested PCR. Nested PCR involves running two consecutive PCR reactions, where by a second set of primers detects a sequence within the initial PCR amplicon produced by the first set of primers (Ward *et al*, 2004).

2.8.1b TaqMan real time PCR

This differs from conventional PCR by using a probe and providing a 'real time' analysis of the reaction. This is achieved by the binding of a probe (single stranded DNA labeled with a fluorescent reporter dye at one end and quencher dye at the other end) to the target DNA sequence between the primers. When in close proximity, the quencher prevents the reporter from fluorescesing. The probe will bind to the complementary part of the target sequence and is degraded when *Taq* polymerase begins extension. This in turn releases the reporter dye to give off fluorescence. The amount of fluorescence released is equal to the amount of DNA generated (PCR amplicon) (Ward *et al*, 2004). The fluorescence is measured throughout the cycle hence providing 'real time' analysis of the reaction kinetics and allowing quantification of specific DNA targets (Weller *et al*, 2006). Real time PCR is also quicker as there is no need to run the PCR amplicons on agarose gels as with conventional PCR.

2.8.2 Conventional PCR assays for detection of Xcm

Lewis-Ivey *et al* (2010) carried out PCR for detecting *Xcm* targeting the conserved sequences of the hypersensitive response (*Hrp*) operon of the hypersensitive response (*Hrp*) gene cluster. The *Hrp* genes that are found in many plant pathogenic bacteria determine pathogenicity in hosts and hypersensitivity in resistant or non-hosts. *HrpB* sequences are highly conserved in *Xanthomonas* strains.

Studies by Leite *et al* (1994) indicated that phytopathogenic *Xcm* strains could be detected and identified by analysis of DNA fragments amplified by use of *hrp* gene specific primers. Cuppels *et al* (2006), later developed primers (RST65/RST69) based on the *hrpB* sequences to detect *Xanthomonas campestris* pv *vesicatoria* that causes bacterial spot in tomatoes. However the primers also hybridized DNA templates from other *Xanthomonas campestris* pathovars including *Xcm*. Based on Cuppels' study, Lewis-Ivey *et al* (2010), designed primers (XW1F and XW3R) specific for *Xcm* that showed the great variability between *Xcm* and other *X. campestris* pathovars. The primers successfully targeted *Xcm* but hybridized non-target DNA templates from *X. axonopodis* pv *vasculorum (Xav)* and *Xvh* strains. Due to the close genetic similarity

within the *Xanthomonas* genus it is challenging to design species-specific primers without the use of the highly conserved sequences such as *B-gyrase* and *hrp* gene clusters. However the chances of non-target reactivities with other pathovars are also high.

Adikini *et al* (2011), also designed primers targeting the intergenic regions of the *Xcm* based on the *Xcm* draft genome sequenced by the University of Oklahoma Genome center. The study used the variability of the intergenic regions of *Xanthomonas* as their basis to design primers specific for *Xcm*. A total of 48 primer pairs were developed and tested for their specificity to *Xcm.* According to Adikini *et al* (2011), the primers *Xcm*12, *Xcm*35, *Xcm*36, *Xcm*38, *Xcm*44, *Xcm*47, and *Xcm*48 were specific for *Xcm*.

Adriko *et al* (2011, also carried out a multiplex polymerase chain reaction; The first set of primers (GspDmFR) target a 265-bp region of the gene encoding the general secretion pathway protein D (GspD) in *Xcm* while the second set of primers (NZ085F/R) target a hypothetical protein in *Xcm*. The primers were designed from *Xcm* sequences found in GenBank and using the NCBI blast tool. Internal controls used were primers targeting the 16S rDNA conserved among bacteria and the 26S mitochondrial rDNA conserved in plants. According to the Adriko's study, primers targeting GspD (GspDmF/R) only amplified DNA templates from *Xcm* strains while the primers targeting the hypothetical protein (NZO85) amplified not only *Xcm* but also DNA templates from *Xav* and *Xvh* strains.

2.8.3 Antibody based assays for detection of Xcm

A polyclonal antibody assay has already been developed for Ugandan *Xcm* strains by IITA (Nakato *et al*, 2011). The polyclonal antibody assay was able to detect *Xcm* from infected banana plant parts such as the corm and pseudo-stem. Further plans are now underway to develop lateral flow devices for use in the field at the border controls.

The DSMZ Plant Virus team in Germany also developed an antiserum for the detection of *Xcm* (DSMZ, 2012) available in a lateral flow device.

2.9 On-site/Field diagnostic tools currently used in plant pathogen detection

On-site detection tools have many advantages, not only are they less costly and require no major laboratory tools but they can be used in rural areas and by non-experts in the field of science.

2.9.1 Enzyme-Linked Immunosorbent Assay and Lateral Flow Devices

ELISA has been the first and most common method plant pathologists have used to diagnose plant disease. This assay makes use of antibody-antigen reactions. The primary antibody binds to the antigen, and the secondary antibody usually attached with an enzyme binds to the primary antibody. In the presence of a substrate, the enzyme reacts to produce a *colour* change which indicates a positive result. Monoclonal and polyclonal antibodies have been developed for detection of many phytopathogenic bacteria in ELISA formats. ELISA assays are quite sensitive to bacterial pathogens from symptomatic plants and fresh cultures.

Despite their sensitivity of ELISA assays they have to be performed in a lab so there is a need for onsite detection and identification of plant pathogens without the use of traditional multi-well ELISA plates. The development of immunochromatographic assays/format such as the lateral flow device has made this possible. This flow test involves two sources of antibody (polyclonal or monoclonal) one of which is immobilized onto a nitrocellulose-based membrane using a sophisticated reagent dispenser while the other is sensitized onto blue-dye latex particles (El-badry, 2005).

The sensitized latex is then airbrushed onto a conjugated release pad and sealed together with the membrane and an absorbent pad into a plastic housing. Specific antigen is ‘sandwiched’ between the immobilized antibody and the antibody-sensitized particles and immune complexes (Danks and Barker, 2000; see Figure 2). This usually gives off a colour change seen as line on the membrane indicating positive results. Results can be achieved in less than 10-15 minutes, and often within 2-3 minutes. Lateral flow devices are not so costly as ELISA and are easy to learn, use and do not

need laboratory equipment which makes them good for field use. One step LFDs have been developed for plant pathogens such as Potato Y potyvirus- PVY, Potato A potyvirus, (Danks and Barker, 2000), Tomato mosaic virus, Pepino mosaic virus.

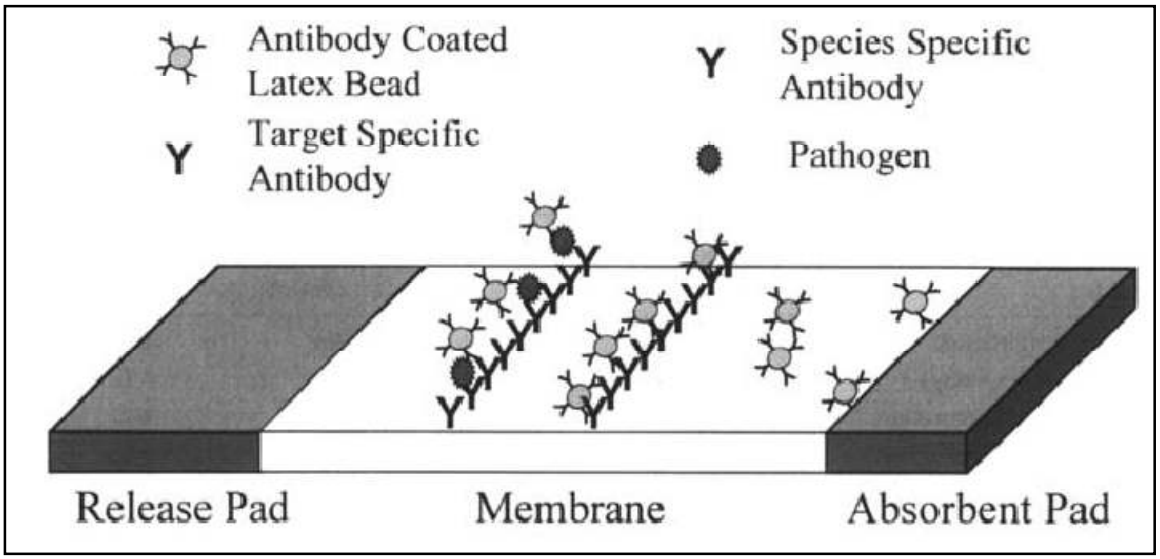


Figure 2: Image of the Lateral flow device (Danks and Barker, 2000)
[Note: The analyteis placed onto the release pad,and the target specific antibody coated with the coloured latex beads will bind to the analyte, and move to the test zone and the complex will bind to the immobilised species specific antibody. The test result will be visualised with two lines; a test line and control line.]

2.9.2 Loop-mediated isothermal amplification (LAMP) assays

Recent studies in plant pathogen detection now involve the use of the LAMP (loop mediated isothermal amplification) a novel technique that amplifies nucleic acid sequences with high specificity, sensitivity and rapidity under isothermal conditions. Unlike PCR, LAMP is characterized by the use of 6 or 4 primers recognizing, respectively 8 or 6 distinct regions of the target DNA and the reactions proceed at a single temperature of around 65°C using *Bst* polymerase. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates the LAMP. The following strand displacement DNA synthesis primed by outer primer releases a single-stranded DNA. This then serves as a template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which

produces a stem loop DNA structure (Notomi *et al*, 2000). LAMP is based on the principle of auto cycling strand displacement DNA synthesis performed by the *Bst* polymerase, for the detection of a specific DNA sequence (Rigano *et al*, 2010). The cycling reaction produces an accumulation of about 10^9 copies of the target DNA in less than an hour (Notomi *et al*, 2000). Amplification efficiency in LAMP is extremely high (Kiatpathomchai *et al,* 2008). LAMP does not require a thermo cycler making it an even less costly technique, with a range of detection options available. For example LAMP assays can also be formatted in to LFDs, which have been successfully used for the detection of some plant pathogens such as Citrus canker (Rigano *et al*, 2010).

2.10 Study Outline

2.10.1 Aim

The aims of the study were:

- to develop field-based diagnostic tools for *Xanthomonas campestris* pv *musacearum* (*Xcm*) detection and
- to improve knowledge of the host-range of *Xcm* and *X vasicola (Xv)* pathovars and genetic diversity of *Xcm* isolates in order to enhance better target control practices.

2.10.2 Rationale

XW of Banana is a relatively new and emerging disease that is a great threat to banana production in Uganda. The current control methods for this disease are largely based on planting clean material and on the early and accurate detection of infected plants. At the time of commissioning this study the diagnostics for *Xcm* were limited to conventional PCR requiring laboratory access. Most banana growing regions are far from the nearest laboratories that are able to diagnose this disease. It was considered therefore that a major advance in progressing timely and effective control options with farmers and multipliers/distributors of planting material would be the development of a diagnostic that was suitable for use in the field and/or in basic facilities.

2.10.3 Objectives

1. Pathogenicity studies/tests performed with strains of *Xcm* and *Xv* pathovars on banana (*Musa* spp), maize, sugarcane and sorghum to support a pathovar designation for *Xcm* with *Xv*.
2. Prepare polyclonal antibodies to *Xcm*, validate efficacy using a standard ELISA format as a Lateral Flow Device.
3. Evaluate existing diagnostic tools for detection of *Xcm.*
4. Assess genetic diversity among populations of *Xcm* from Western Uganda based on SNP markers

3. MATERIALS AND METHODS

3.1 Bacterial collection and testing templates

3.1.1 Bacterial strains

All bacterial strains used in this study are presented in Table 1 and were cultured from the National Collection of Plant Pathogenic Bacteria (NCPPB, The Food and Environment Agency (Fera, York, UK)). The bacterial species and pathovars were selected to provide a good representation of *Xanthomonas* and non-*Xanthomonas* strains for purposes of testing the specificity of the diagnostic tools under evaluation.

3.1.2 DNA extraction

Bacterial strains (Table 1) were streaked and incubated on YDC (Bacto Agar 15g/l, yeast extract 10g/l, $CaCO_3$ 20g/l, D-Glucose 20g/l(Dextrose) and de-ionized water 1l) media at 25°C for 48 hours. Genomic DNA was extracted from a 10µl loop of bacterial growth taken from an area of contiguous colonies using the QiAamp DNA Mini Kit following the manufactures protocol. About 200µl at a concentration of approximately 34ng/µl of DNA was yielded and kept at -20°C in labeled 1.5ml eppendorfs.

Table 1: Bacteria strains used in the study from the National Collection of Plant Pathogenic Bacteria (NCPPB

Strain name	***NCPPB No.***	***Origin***	***Host***	***PCR***	***ELISA***	***LFD***	***Pathogenicity trial***
Xanthomonas arboricola pv *celebensis*	*1630*	*New Zealand*	*Musa* sp	*X*	*X*	*X*	*X*
Xanthomonas axonopodis pv *vasculorum*	*186*	*Mauritius*	*Saccharum officinarum*	*X*	*X*		
Xanthomonas axonopodis pv *vasculorum*	*899*	*Reunion*	*Saccharum officinarum*	*X*	*X*		*X*
Xanthomonas axonopodis pv *vasculorum*	*796*	*Mauritius*	*Saccharum officinarum*	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*4378*	*Uganda*	*Musa* sp	*X*	*X*	*X*	
Xanthomonas campestris pv *musacearum*	*4389*	*Rwanda*	*Musa* sp	*X*	*X*		*X*
Xanthomonas campestris pv *musacearum*	*4433*	*Burundi*	*Musa* sp	*X*		*X*	*X*
Xanthomonas campestris pv *musacearum*	*4434*	*Kenya*	*Musa* sp	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*2005*	*Ethiopia*	*Enset*	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*2251*	*Ethiopia*	*Musa* sp	*X*	*X*	*X*	
Xanthomonas campestris pv *musacearum*	*4392*	*Tanzania*	*Musa* sp	*X*			
Xanthomonas campestris pv *musacearum*	*4434*	*Kenya*	*Musa* sp	*X*			*X*
Xanthomonas campestris pv *musacearum*	*4379*	*Uganda*	*Musa* sp	*X*	*X*		*X*
Xanthomonas campestris pv *musacearum*	*4380*	*Uganda*	*Musa* sp	*X*	*X*		
Xanthomonas campestris pv	*4381*	*Uganda*	*Musa* sp	*X*	*X*		

Strain name	***NCPPB No.***	***Origin***	***Host***	***PCR***	***ELISA***	***LFD***	***Pathogenicity trial***
musacearum							
Xanthomonas campestris pv *musacearum*	*4383*	*Uganda*	*Musa* sp	*X*	*X*	*X*	
Xanthomonas campestris pv *musacearum*	*4387*	*DRC*	*Musa* sp	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*4388*	*DRC*	*Musa* sp	*X*	*X*		
Xanthomonas campestris pv *musacearum*	*4390*	*Rwanda*	*Musa* sp	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*4391*	*Rwanda*	*Musa* sp	*X*	*X*	*X*	
Xanthomonas campestris pv *musacearum*	*4392*	*Tanzania*	*Musa* sp	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *musacearum*	*4393*	*Tanzania*	*Musa* sp	*X*	*X*		*X*
Xanthomonas campestris pv *musacearum*	*4394*	*Tanzania*	*Musa* sp	*X*			
Xanthomonas campestris pv *campestris*	*529*	*UK*	*Brassica oleracea var. Capitata*	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *perlagonii*	*4031*	*UK*	*Pelargonium x hortorum*	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *perlagonii*	*2985*	*New Zealand*		*X*	*X*		*X*
Xanthomonas campestris pv *vesicatoria*	*422*	*New Zealand*	*Lycopersicon esculentum*	*X*	*X*	*X*	*X*
Xanthomonas campestris pv *vesicatoria*	*701*	*Zimbabwe*	*Lycopersicon esculentum*	*X*	*X*		*X*
Xanthomonas campestris pv *vesicatoria*	*423*	*New Zealand*	*Lycopersicon esculentum*	*X*	*X*		
Xanthomonas vasicola pv *holcicola*	*1060*	*Ethiopia*	*Sorghum vulgare.*	*X*	*X*		*X*
Xanthomonas vasicola pv *holcicola*	*3162*	*India*	*Sorghum sp*	*X*	*X*	*X*	*X*

Strain name	***NCPPB No.***	***Origin***	***Host***	***PCR***	***ELISA***	***LFD***	***Pathogenicity trial***
Xanthomonas vasicola pv *vasculorum*	*795*	*Malagasy Republic*	*Saccharum officinarum*	*X*	*X*		*X*
Xanthomonas vasicola pv *vasculorum*	*895*	*Malagasy Republic*	*Saccharum officinarum*	*X*	*X*	*X*	*X*
Xanthomonas vasicola pv *vasculorum*	*702*	*Zimbabwe*	*Saccharum officinarum*	*X*	*X*		*X*
Xanthomonas vasicola pv *vasculorum*	*206*	*South Africa*	*Zea mays*	*X*			*X*
Xanthomonas vasicola pv *vasculorum*	*890*	*South Africa*	*Saccharum officinarum*	*X*	*X*		*X*
Xanthomonas spp	*1131*	*Eastern Samoa*	*Musa paradisiacal*	*X*	*X*	*X*	*X*
Xanthomonas spp	*1132*	*Western Samoa*	*Musa canksii var. Samoensis*	*X*	*X*	*X*	*X*
Ralstonia solanacearum	*2198*	*Trinidad*	*Musa* sp.	*X*	*X*		*X*
Ralstonia solanacearum	*2315*	*Peru*	*Musa* sp.	*X*	*X*		
Ralstonia solanacearum	*3214*	*India*	*Musa* sp.	*X*	*X*		
Ralstonia solanacearum	*3205*	*Guyana*	*Musa* sp.	*X*	*X*	*X*	
Pseudomonas marginalis pv *marginalis*	*1232*	*Uganda*	*Musa* sp.	*X*	*X*		
Enterobacter	*4168*			*X*	*X*		
Serratia marcescens	*2641*	*USA*	*Medicago sativa*	*X*	*X*		

[Note X means that the particular strain was tested by PCR, ELISA, LFD or the particular strain was used in the pathogenicity trial]

3.2 Evaluating the existing PCR diagnostic tools for *Xanthomonas campestris* pv *musacearum*

Currently available conventional PCR based assays for detection of *Xcm* were assessed against different bacterial strains (Table 1) for their specificity. The primer and amplification conditions are provided in Table 2.

Conventional PCR reactions were composed as follows: each 25µl PCR reaction contained 1µl of DNA template, 12.5µl of 2 x PCR Master Mix (Thermo Reddy Mix), 1µl of each forward and reverse primer from a stock of 10µM and 9.5µl of nuclease–free water. Water was used as a non-template control (in the place of DNA) for each experiment. The PCR reactions were performed in aGeneAmp PCR system 9700, Applied Biosystems. The PCR Assays were done in duplicates to minimize any errors and get accurate results.

PCR amplicons of 5µl were then separated by agarose gel electrophoresis in 1% agarose gels in 1x TBE (Tris/borate/EDTA) at 130V for 60 minutes. Quick load 2 log DNA ladder was used to evaluate the sizes of the PCR amplicons under ethidium bromide staining.

Table 2: Conventional PCR primers for Xanthomonas campestris pv musacearum and cycling conditions

Conventional PCR Primers	**Cycling conditions**	**Expected amplicon size**
XW-1F 5'GTCGTTGGCACCATGCTCA 3' XW-3R 5'TCCGACCGATACGGCT 3' (Lewis Ivey *et al*, 2010)	Denaturation at 95ºC for 5min,then cycle at 95ºC for 30s,annealing at 55ºC for 30s, elongation at 72ºC for 30s for 30 cycles then 72ºC for 5minutes	214bp

GspDm-F 5'GCGGTTACAACACCGTTC 3' GspDm-R 5'AGGTGGAGTTGATCGGA 3' (Adriko *et al*, 2011)	Denaturation at 95ºC for 3min, then cycle at 95ºC for 20s,annealing at 64ºC for 15s, elongation at 72ºC for 60s for 32 cycles then 72ºC for 3minutes	265bp
NZ085F 5'CGTGCCATGTATGCGCTGAT 3' NZ085R 5'GAGCGGCATAGTGCGACAGA 3' (Adriko *et al*, 2011)	Denaturation at 95ºC for 3min,then cycle at95ºC for 20s,annealing at 64ºC for 15s, elongation at 72ºC for 60s for 32 cycles, extension at72ºC for 3minutes	349bp
*Xcm*12F/*Xcm*12R *Xcm*35F/*Xcm*35R *Xcm*36F/*Xcm*36R *Xcm*38F/*Xcm*38R *Xcm*44F/*Xcm*44R *Xcm*47F/*Xcm*47R *Xcm*48F/*Xcm*48R (Adikini *et al*, 2011)	Denaturation at 94ºC for 5min,then cycle at 94ºC for 20s,annealing at 60ºC for 20s, elongation at 72ºC for 60s, for 40 cycles, extension at 72ºC for 10minutes	360bp 480bp 420bp 650bp 350bp 370bp 450bp

3.3 Preparation of ELISA polyclonal antibodies specific for *Xcm*

Polyclonal antibodies for *Xcm* (for NARO-Uganda) were prepared at Fera. Only one rabbit was immunized with a pooled sample of 6 isolates of *Xcm* (see Table 3). To induce different antigenic profiles in the bacterium and potentially antibody production reactions in the inoculated rabbit, the isolates were first grown on a nutrient rich medium (YDC; 24hrs at 24ºC) followed by nutrient poor medium (Nutrient broth; 24 hrs at 24ºC with gentle shaking). Bacterial growth was harvested by centrifugation and re-suspended in 1 x PBS (Phosphate Buffered Saline). This was then mixed with Freunds complete adjuvant and used to immunise the rabbit first. For the following 3 immunisations Freunds incomplete adjuvant was used. This was done over the course of four months. Three types of bleeds were harvested:

- A pre-immunisation - bleed for use as the negative rabbit sera control (the rabbit was bled prior to injection with the *Xcm* cells (the immunogen))

- A post immunisation-bleed for use as the test *Xcm* antibody (the rabbit was injected with the *Xcm* cells to raise the antibody, and then bled)
- A bleed from a rabbit immunized with CVYV as a non-related antibody (raised against CVYV in a different piece of work).

These sera were harvested, purified by HiTrap Protein G column (GE Healthcare) following the manufacturer's protocol and assessed for protein concentrations.

Table 3:Xcm strains used to raise antiserum in rabbit

NCPPB number	Species	Country of Origin	Year isolated
4378	*Xanthomonas campestris* pv *musacearum*	Uganda	2007
4387	*Xanthomonas campestris* pv *musacearum*	D. R. Congo	2007
4389	*Xanthomonas campestris* pv *musacearum*	Rwanda	2007
4392	*Xanthomonas campestris* pv *musacearum*	Tanzania	2007
4433	*Xanthomonas campestris* pv *musacearum*	Burundi	2008
4434	*Xanthomonas campestris* pv *musacearum*	Kenya	2008

3.3.1 Preliminary testing of the non-purified polyclonal antibody

A panel of bacteria was grown to evaluate the specificity of non-purified *Xcm* antibody (see Table 1). The ELISA was performed at room temperature except for the incubations at 33°C for 1 hour. Antigen coated plates were always first washed and dried before use. A 10µl loop of bacteria growth from new cultures of the bacterium under test was first re-suspended in 5mls of PBS buffer. The re-suspension was adjusted to a concentration of 10^9 cfu/ml using a spectrophotometer at 650nM (wavelength). To make a 1 in 100 dilution of the coating buffer(15mM Na_2CO_3, 35mM $NaHCO_3$, ph 3.6) and bacteria, 150µl of the bacterial cell re-suspension was added to 14.85ml of coating buffer to make a concentration of 10^7 cfu/ml (coating buffer + bacteria). The plates were washed with PBST(phosphate buffered saline + tween) three

times in between incubations. Detection was performed using a plate trapped antigen ELISA assay (Indirect ELISA assay), whereby the antigen (test bacteria) is bound to the plastic plate and then incubated with the *Xcm* polyclonal antiserum for 2 hours at 33◦C. Binding of the *Xcm* polyclonal antiserum to the test bacteria is detected using a-goat anti-rabbit polyclonal (secondary antibody) labelled with the enzyme Alkaline Phosphatase (AP). The secondary antibody was diluted in PBST + 0.2% BSA (bovine serum albumin) at concentration of 1:4000. PNPP (*p*-Nitrophenyl Phosphate) was used as the substrate (see Figure 3). The plates were then incubated for 1 hour in the dark at room temperature. The plates were read at 405nm after 1 hour.

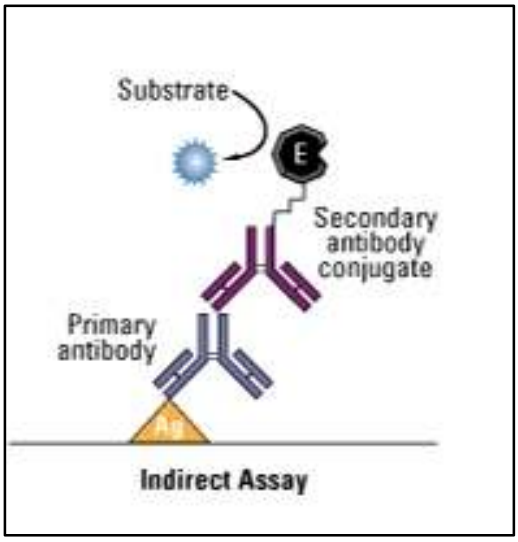


Figure 3: Schematic of indirect ELISA.
[Note: Ag is the test bacterium; primary antibody is the polyclonal antibody raised against Xcm and the secondary antibody is the anti-goat/anti-rabbit antibody (Image is from Thermos scientific website]].

Each plate was divided into three groups of four columns (see Figure 4). Each well was coated with 100µl of the bacteria (10^7 cfu/ml; see Table 1). The columns 1 to 4 were titrated with antiserum raised against *Xcm (*positive sera), columns 5 to 8 were titrated with pre-rabbit bleed and columns 9 to 12 were titrated with post-rabbit bleed against CVYV. The three rabbit bleeds were titrated from the rows A to H with 1:2K as the initial dilution to 1:256K (2-fold dilutions). Standard protocols were used in carrying out this ELISA experiment (see Appendix 1).

	Post-*Xcm* bleed				Pre-*Xcm* bleed				CVYV bleed			
	1	2	3	4	5	6	7	8	9	10	11	12
A 1:2K												
B 1:4K												
C 1:8K												
D 1:16K												
E 1:32K												
F 1:64K												
G 1:128K												
H 1:256K												

Figure 4:Microtitre ELISA plate layout showing the arrangement, wells and dilutions of the three rabbit bleeds in the preliminary testing of the non-purified polyclonal antibody.

3.3.2 Validation of the purified polyclonal antibody assay

The *Xcm* antibody was purified and assessed for sensitivity and specificity compared to the non-purified polyclonal antibody. A comparison was also made to an *Xcm* polyclonal developed by the International Institute of Tropical Agriculture (IITA) (kindly provided by Dr. Lava Kumar, IITA Ibadan, Nigeria).

3.3.2a Sensitivity testing

Initial testing on sensitivity was performed on a known positive (*Xanthomonas campestris* pv *musacearum* NCPPB 4434) and two known negative strains (*Xanthomonas arboricola* pv *celebensis* (*Xac*) (NCPPB 1630) and American Foulbrood Bacteria (AFB). For each strain three ELISA plates were coated with a bacterial suspension of 10^7 cfu/ml and treated as below and as presented in Figure 5A-C:

- Plate 1 was titrated at dilutions of 1:1K to 1:256K (column 2-10) with rabbit pre-bleed (row B to D) and CVYV antiserum (row E to G).
- Plate 2 was a duplicate of Plate 1, but titrated at dilutions of 1:1K to 1:256K (column 2-10) with the IITA *Xcm* polyclonal antibody (row B to D) and the effluent from the purified *Xcm*-polyclonal of Fera (E-G).

- Plate 3 had the same row/column arrangement, but titrated at dilutions of 1:1K to 1:256K (column 2-11) with the non-purified polyclonal antibody (B to D) and at dilutions of 1:4K to 1:1,000K (column 2-10) the purified antibody (E to G).

	1	2	3	4	5	6	7	8	9	10	11	12
		1:1K	1:2K	1:4K	1:8K	1:16K	1:32K	1:64K	1:128K	1:256K	Antibody bleeds	
A												
B Pre *Xcm*												
C Pre *Xcm*												
D Pre *Xcm*												
E CVYV												
F CVYV												
G CVYV												
H												

Figure 5A: Plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
		1:1K	1:2K	1:4K	1:8K	1:16K	1:32K	1:64K	1:128K	1:256K	Antibody bleeds	
A												
B IITA poly												
C IITA poly												
D IITA poly												
E Effluent												
F Effluent												
G Effluent												
H												

Figure 5B: Plate 2.

	1	2	3	4	5	6	7	8	9	10	11	12
		1:1K	1:2K	1:4K	1:8K	1:16K	1:32K	1:64K	1:128K	1:256K	Antibody bleeds	
A												
B NP poly												
C NP poly												
D NP poly												
E P poly												
F P poly												
G P poly												
H												
		1:4K	1:8K	1:16K	1:32K	1:64K	1:128K	1:256K	1:512K	1:1000K		

Figure 5C: Plate 3

Figure 5: Microtitre plate arrangement for sensitivity evaluation of the purified ELISA polyclonal antibody on Xcm NCPPB 4434, Xac NCPPB 1630 and AFB against IITA's polyclonal antibody and Fera's unpurified polyclonal antibody assay.

3.3.2b Specificity testing

Based on the results of the sensitivity screen, specificity was further tested against the strains in Table 1 with the *Xcm* polyclonal of IITA, the purified and non-purified *Xcm* antibody and the CVYV antiserum. For each bacterium at a constant concentration of 10^7 cfu/ml tested in 2-fold-dilutions of antibody from 1:2K to 1:128K for IITA-*Xcm*, non-purified *Xcm* and CVYV antisera and of 1:32K to 1:2,000K for purified *Xcm* (see Figure 6). Each strain and antiserum treatment was replicated once per plate and each plate was duplicated. To determine the threshold between positive and negative results, we used the $3\overline{x}$ (that is 3 times the mean OD values at different dilutions respectively) of the OD values of the known negative plates which were also negative controls (banana sap and EFB). This method is commonly used to determine the positive/negative threshold (Bioreba – Agro diagnostics, Catalina *et a*l,2012, Tomkies *et al*,2008 and Frey *et al,*1998). Other methods for determining the threshold value, that is $2\overline{x}$ were considered but the OD values were still too low (Sutula *et al*,1986). The $3\overline{x}$ method

provides for lower percentage of false positives compared to the $2\overline{x}$ and $\overline{x}$+ 3SD(standard deviation) (Sutula *et al*,1986).

	IITA *Xcm* poly			Non-purified *Xcm*			Purified *Xcm**			CVYV		
A 1:2K												
B 1:4K												
C 1:8K												
D 1:16K												
E 1:32K												
F 1:64K												
G 1:128K												
H 0												

*Purified *Xcm* antibody used at a dilution series of 1:32K to 1:2000K.

Figure 6: Microtitre plate layout showing the arrangement on each ELISA microtitre plate and dilutions of the different polyclonal antibody against the antigen.

3.3.3 Assessment of the ELISA polyclonal Lateral Flow Device

The purified antibody was then passed on to Forsite Diagnostics (Sand Hutton, York, UK) for development as a Lateral Flow Device (LFD). The specificity and sensitivity of the LFD was then assessed by Fera.

3.3.3a Specificity of the LFD

The specificity of the LFDs was tested with the strains indicted in Table 1. 100µl from a dilution of 1:10 solution (20µl of bacterial suspension at concentration of 10^8 cfu/ml and 180µl of LFD extraction buffer that comes with the kit) was added to each LFD, and the results (presence of a blue line) read after 10 minutes.

3.3.3b Sensitivity testing of the LFD

Sensitivity of the LFD was tested with a known positive *Xcm* (strain NCPPB 4434) as described below.

Without banana sap: Sensitivity of the LFD was tested with bacteria suspended in LFD extraction buffer solution (Foresite Diagnostics) at concentrations of 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 cfu/ml for NCPPB 4434. 100µl from each concentration was added to the LFD. Results were read after 10 minutes.

With banana sap: Fresh healthy banana leaf stalk were cut 2.5cm by 5cm and placed for 10 minutes in the LFD extraction buffer to allow the sap to ooze out. After the 10 minutes, 180µl of the banana exudate mixture (sap and buffer) was added to 20µl of a cell suspension of NCPPB 4434 at a concentration of 10^8 cfu/ml, which was then diluted from 10^7 to 10^3 cfu/ml with banana exudate mixture. 100µl of each cell suspension was then added to the LFD. Results were read after 10 minutes.

With crushed leaf: Fresh healthy banana leaf of about 0.25g was cut up and placed in LFD extraction buffer bottle containing small ball bearings (as supplied by Foresite Diagnostics), and shaken for 30 seconds to crushed the leaf material. 180µl of the banana exudate mixture (leaf and buffer) was added to 20ul of a cell suspension of NCPPB 4434 at a concentration of 10^8 Cfu/ml, which was then diluted from 10^7 to 10^3 cfu/ml with exudate mixture. 100µl of each cell suspension was then added to the LFD. Results were read after 10 minutes.

3.4 Pathogenicity trials

To support the proposal of a new pathovar of *Xcm* to *Xanthomonas vasicola* pv *musacearum*, pathogenicity characterisations/comparisons of the *Xanthomonas vasicola* pathovars are needed (George, 2005) on maize, sorghum, sugarcane and banana.

3.4.1 Bacteria and preparation of inoculum

The bacteria species and pathovars included this study included strains from *Xcm* and *Xvh, Xvv*, *Xanthomonas axonopodis* pv *vasculorum* (causes Gumming disease in sugarcane), and other common plant pathovars; the *X. campestris* pathovars *campestris*, *vesicatoria* and *pelagonii* that are not known to cause disease in banana,

maize, sugarcane and sorghum, respectively. Other non-*Xanthomonas* strains included *Paenibacillus larvae*. The strain list is provided in Table 1.

Bacteria were grown on YDA media at 25ºC for 48 hours. Using a 10µl loop, pure colonies were removed from the plate and re-suspended in sterile water. The bacterial suspensions were then adjusted to the required OD using a spectrophotometer (wavelength at 650nm) with either sterile water or addition of bacteria into the suspension.

3.4.2 Plants to be inoculated and growing conditions

Banana (varieties Cavendish and Tropicana (AAA)), sugarcane (variety unknown), maize (variety *cisko*) and sorghum (variety unknown) young plants (almost seedlings) were grown under glasshouse conditions at Fera. A minimum temperature of 25ºC was maintained. See Appendix 2 for full pathogenicity plan used in the study.

3.4.3 Pilot trials

Pilot trials of banana, maize and sorghum were carried out to determine the level of inoculum that would enable pathogenicity to be achieved and for familiarization on what symptoms to expect and the best inoculation method. About 8 young maize and banana plants were inoculated at the base of the stems using a 1ml syringe with 100-200µl of bacterial suspensions of *Xcm* (NCPPB 4434*,* NCPPB 4378*), Xvv* (NCPPB 702,NCPPB 795), *Xvh* (NCPPB 1060) and *Xanthomonas axonopodis* pv *vasculorum* (NCPPB 796, NCPPB 899) at cell concentrations of 10^7 cfu/ml.

The controls were either left untreated or inoculated with sterile water. A pilot on sugarcane and sorghum was not attempted due to a shortage of sugarcane plants. Sedum plants (Umesha *et al*, 2007) were used as a positive plant indicator in the pilot trial. Symptom expression was recorded for 5 weekdays. Photographic evidence of symptoms was taken.

3.4.4 Main pathogenicity trials

Based on the results of pilot trials, a full pathogenicity trial was conducted on maize, sorghum, sugarcane and banana against the bacterial strains, see Appendix 2.

3.4.4a Setting-up of main pathogenicity trials

Four plants of maize, banana and sorghum were used for each of the 3 treatments: untreated, water controls and test bacteria (in total 12 plants). For the sugarcane, due to the number of plants available, three plants were used for each treatment.

Inoculation of water control and test bacteria treatments was as described for the pilot trial. The plants were injected at the base of the stem with about 100-200µl of inoculum standardized across strains under test at 10^7 cfu/ml. The plants were then labeled with numbers 1 to 112 and randomized.

3.4.4b Viability of the bacteria

The viability and cell concentration of the bacterial suspensions used in the inoculation were determined by lawn plating 100µl on to YDC media and incubating at 25ºC for 48 - 72 hours.

3.4.4c Re-isolation of the pathogen and proof of Koch's postulate

Re-isolation of the bacteria was attempted at around 5 weeks post-inoculation irrespective of whether the plants exhibited symptoms or not. One or two leaves and leaf stalks of each treatment were picked and, depending on symptom presence, either part of the leaf bordering a diseased and healthy area or proximate to the inoculation wound taken and cut up in to small pieces (0.1 to 0.3g). The leaf pieces were then crushed and soaked in 1ml of PBS and left to stand for at least 5 to 30 minutes to allow bacteria to ooze out. Single isolate cultures were then obtained by streaking out a few 10µl loops of the crushed leaf-PBS solution on YDC media, incubated at 25ºC for 48 - 72 hours.

For colonies that conformed to *Xanthomonas* morphology, confirmation of re-isolation of the inoculated strain was achieved by PCR using the primers of Adriko *et at* (2011) for *Xcm* and *Xvv* and *Xvh.* Re-isolates from plants that had been inoculated by other *Xanthomonas* and non-*Xanthomonas* strains were identified visually; colonies that looked like *Xanthomonas* were assumed to be those that had been inoculated into that particular plant.

3.5 Characterizing Western Uganda isolates using the SNP primers (Wasukira *et al*, 2012)

3.5.1 Sample collection

Xcm samples from Western Uganda were collected with the help of IITA staff in Uganda. The bacteria were isolated from leaves, pseudo-stems and corms. Samples were from the district of Kabale, Ntugamo and Bushenyi. 45 Isolates from leaf samples were streaked and re-grown on YDC media, incubated at 28ºC for 48 hours.

3.5.2 DNA extraction

Samples (Table 4) were grown in Kings Broth (10ml) overnight, and 1ml of the liquid bacteria culture centrifuged at 4000rpm at 4ºC for 10 minutes to obtain a pellet. The supernatant was poured off and the tube inverted to dry it, the pellet was re-suspended in 2ml of TE buffer (25mM Tri-HCL ph8.0, 10mM EDTA), 300µl of the re-suspension was placed in 1.5ml eppendorfs and the rest frozen. The cells were then lysed with 12 µL of 20mg/ml lysozyme and 1.5µl RNase at 10mg/ml and incubated at room temperature for 10 minutes. Further lysis was done with 17µl of 10% sodium dodecyl sulphate (SDS) and incubated on ice for 5min. Proteins were pelleted with addition of 170µl of 8M ammonium acetate, vortexed for 20s and centrifuged at 4ºC at maximum speed for 15 minutes. The supernatant carefully pipetted in to a clean 1.5ml eppendorfs and DNA precipitated with addition of 0.75 volume isopropanol, centrifuged for 10 minutes. The supernatant was pipetted off and the pellet washed with 100µl of 70% ethanol by centrifuging at maximum speed for 5 minutes. Ethanol was pipetted off and

the pellet dissolved in 200µl of Tris 10mM. This was done to avoid EDTA interfering with PCR.

Table 4: The new collection of Xanthomonas campestris pv musacearum isolates from Western Uganda isolated from leaves of infected banana plants

Sample ID	District	Village	Sample ID	District	Village
2	Kabale	Nyamishaki	43	Ntugamo	Bucence
3	Kabale	Nyamishaki	45	Ntugamo	Bucence
4	Kabale	Nyamishaki	46	Ntugamo	Kitojo
6	Kabale	Rushyebeyal	48	Ntugamo	Kitojo
7	Kabale	Rushyebeyal	49	Ntugamo	Kitojo
8	Kabale	Rushyebeyal	50	Ntugamo	Kitojo
9	Kabale	Rushyebeyal	53	Ntugamo	Kigarama
10	Kabale	Rushyebeyal	54	Ntugamo	Kigarama
11	Kabale	Kanyakutana	55	Ntugamo	Kigarama
12	Kabale	Kanyakutana	59	Ntugamo	Rukoni
13	Kabale	Kanyakutana	62	Bushenyi	Matigi
14	Kabale	Kanyakutana	64	Bushenyi	Matigi
15	Kabale	Kanyakutana	67	Bushenyi	Nyabubare
17	Kabale	Nyamitooma	70	Bushenyi	Nyabubare
21	Kabale	Kateramabareeba	73	Bushenyi	Kizumo
23	Kabale	Kateramabareeba	76	Bushenyi	Nyakabingo
30	Kabale	Kabugarama	77	Bushenyi	Nyakabingo
34	Ntugamo	Ihuriro	81	Bushenyi	Keijengye
36	Ntugamo	Ihuriro	83	Bushenyi	Keijengye
39	Ntugamo	Nyakagongo	84	Bushenyi	Keijengye
40	Ntugamo	Nyakagongo	87	Bushenyi	Kibaare B
42	Ntugamo	Bucence	90	Bushenyi	Kibaare B

[Note: Genomic DNA was extracted from the samples and PCRs run. Only samples that had at least 20ng DNA/µl were used as templates for PCR. PCR digests were done according to the conditions of the enzyme (see Table 6)]

3.5.3 Primers and PCR conditions

PCR gradients had to be done for the primers to determine the best annealing temperature that will give the best product size. PCR reactions were 20µl; 2µl PCR buffer, 0.6µl $MgSO_4$, 1.6µl dNTPS, 1µl forward primer,1µl reverse primer,12µl nuclease free water, 0.8µl of Taq and 1-2µl of DNA template. PCR amplicons were

separated out in 2% agarose gel in 1 x TAE buffer for 45 minutes at 80V. Band sizes were evaluated by the Gene ruler 1kb DNA ladder.

The PCR digest reactions were 25µl; 2.5µl PCR digest Buffer, 0.2-0.5µl depending on the enzyme, 17µl of water and 5µl of PCR amplicon. The reactions were incubated at 37ºC for 2 hours. The products were run in 2% agarose gel in 1 x TAE buffer for 50 minutes at 80V. Band sizes were evaluated with the Gene ruler 1Kb DNA ladder.

Table 5: SNP Xcm PCR primers and cycling conditions (Wasukira et al, 2012)

Primer set	**PCR conditions**	**Expected amplicon size**
AluACHT010000 43FR	Denaturation at 95ºC for 2min,then cycle at 94ºC for 30s,annealing at 61.7ºC for 30s, elongation at 72ºC for 45s for 35 cycles, extension at 72ºC for 10 minutes	500bp
BglIACHT010002 42FR	Denaturation at 95ºC for 2min,then cycle at 94ºC for 30s,annealing at 61.7ºC for 30s, elongation at 72ºC for 45s for 35 cycles, extension at 72ºC for 10 minutes	500bp
RsaIACHT010000 45FR	Denaturation at 95ºC for 2min,then cycle at 94ºC for 30s, annealing at 53ºC for 30s, elongation at 72ºC for 45s for 35 cycles, extension at72ºC for 10 minutes	500bp
StyIACHT010001 40FR	Denaturation at 95ºC for 2min,then cycle at 94ºC for 30s,annealing at 55ºC for 30s, elongation at 72ºC for 45s for 35 cycles, extension at72ºC for 10 minutes	500bp

Table 6: SNP PCR restriction enzymes conditions (Wasukila et al, 2012)

Primer set	**Enzyme**	**Conditions**	**Expected amplicon size**	***Xcm* SNP characterisation**
AluACHT010000 43FR	AluI	Incubate at 37ºC for 2 hours	250bp	Differentiates at position 10,914 for Ethiopian strains NCPPB 2005 (bp C) and NCPPB 2251 (bp T). Bp C was common to lineage II strains**
BglIACHT010002 42FR	BglI	Incubate at 37ºC for 2	250bp	Differentiates at position 12,462 for

Primer set	**Enzyme**	**Conditions**	**Expected amplicon size**	***Xcm* SNP characterisation**
		hours		Ethiopian strains NCPPB 2005 (bp G) and NCPPB 2251 (bp A). Bp G was common to lineage II strains**
RsaIACHT010000 45FR	RsaI	Incubate at 37ºC for 2 hours	250bp	Differentiates at position 32,848 for Lineage I* (bp G) and Lineage II (bp A) strains
StyIACHT010001 40FR	StyI	Incubate at 37ºC for 2 hours	250bp	differentiates at position 2,515 and 2,530 for lineage I (bps T&C) and lineage II (bp C&T) strains

[Note:

lineage I – Ethiopia: NCPPB 2005, 2251; DR Congo: NCPPB 4387; and Rwanda (NCPPB 4389)

lineage II – Uganda - NCPPB 4379, 4383, 4384, 4380, 4381, Tanzania - NCPPB 4394, 4395, 4381, Kenya - NCPPB 4434 and Burundi - NCPPB 4433]

4. RESULTS

4.1 Comparison and evaluation of existing PCR tools for detection of *Xcm*

Existing PCR tools for detection of *Xcm* were evaluated for their specificity in order to form a baseline for Fera to compare ELISA and LFD formats of polyclonal detection. The most specific primers to *Xcm* strains were GspDmF/R primers from Adriko *et al* (2011), designed from a gene sequence for the secretion pathway D in *Xcm.*

Table 7 summaries the evaluation of the PCR assays evaluated (Adriko *et al*, 2011; Lewis-Ivey *et al,* 2010; Adikini *et al*, 2011) to detect *Xcm* strains. All primers from the three studies were able to amplify DNA from *Xcm* strains. The primers GspDmF/R (Adriko *et al*, 2011) were shown to be specific for *Xcm*, without cross reaction to other strains. Primers NZO85 (Adriko *et al*, 2011) and XW 1F/3R (Lewis-Ivey *et al*, 2010) were specific to *Xcm* and *Xvv* (NCPPB 206, NCPPB 702, NCPPB 890 and NCPPB 895), but failed to detect *Xvh* strains. The 'failed' detection of *Xvh* strains by these primers is at variance with the reported claims made for these primers.

The primers *Xcm*12F/R, *Xcm*35F/R, *Xcm*36F/R, *Xcm*38F/R, *Xcm*44F/R, *Xcm*47F/R, and *Xcm*48F/R) (Adriko *et al*, 2011) exhibited varying levels of specificity. Although all of these primers were shown to amplify *Xcm* strains, no complete specificity to *Xcm* strains was demonstrated and cross reactivety with non-*Xcm* strains was evident. Primers 35F/R were the most specific for *Xcm,* cross-reacting with only one, but not all, *Xvv* strains and no other strains. Primers *Xcm*12F/R exhibited some inconsistent cross reactivity to *Xvv* and *Xvh* strains; whereas primer pair *Xcm* 36F/R cross reacted with only some of the *Xvv* strains. Primers *Xcm*44F/R, *Xcm*47F/R and *Xcm*48F/R exhibited a range of cross reaction capabilities. These results were also not consistent with the results reported in Adikini's study.

In summary,the primers GspDm were shown to be the most specific primers for PCR detection of *Xcm*. Primers XW1F/3R, NZ085F/R and *Xcm* 36F/R are able to distinguish strains of *Xvv* and *Xvh.*

The below figures provide illustration of the results of the three PCR assays against *Xcm* and related strains in gel pictures

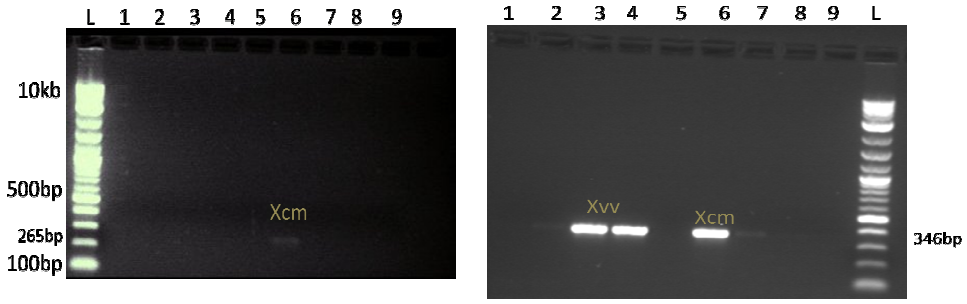


Figure 7: Agarose Gel pictures showing PCR results of GspDm (left) and NZO85 primers (right) (Adriko et al, 2011), respectively, on Xvv, Xcm, and Xav

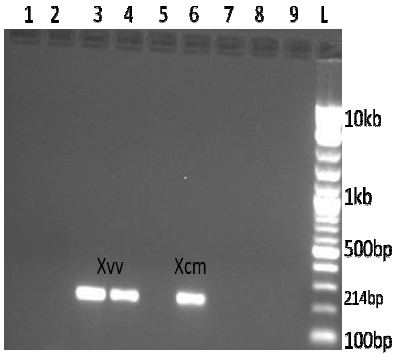
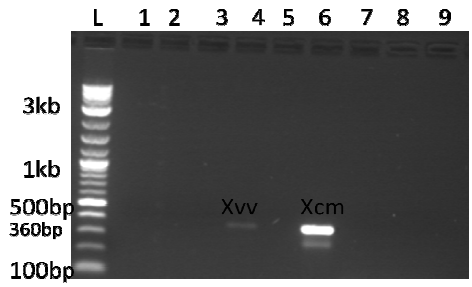
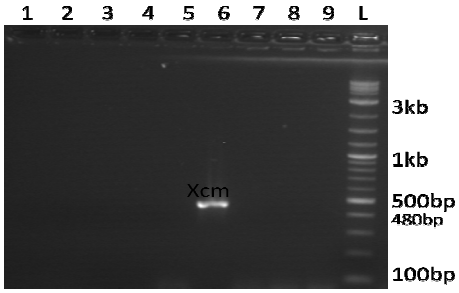


Figure 8: Agarose gels showing PCR results of XW1F/3R primers (Lewis-Ivey et al, 2010) on Xvv, Xav and Xcm strains

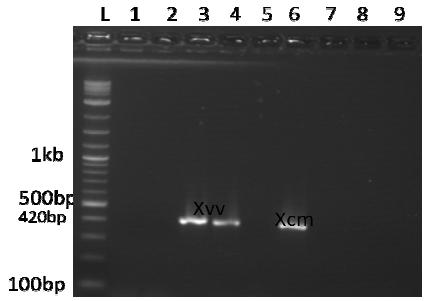
[Note For Figures 7-8: Lanes 1, 2 and 7 - X. axonopodis pv vasculorum (NCPPB 796, 186, 899), Lanes 3 and 4 – X. vasicola pv vasculorum (NCPPB702, 795), Lane 5 and 8 – X. campestris pv vesicatoria (NCPPB 423), Lane 6 – X. campestris pv musacearum (NCPPB 4344), Lane 9 – Water]



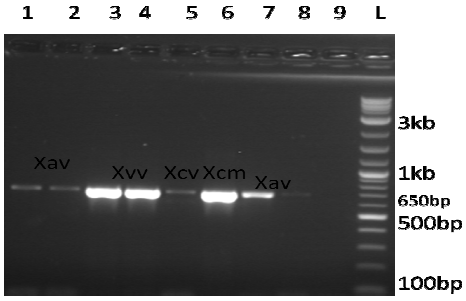
I. *Xcm*12F/R



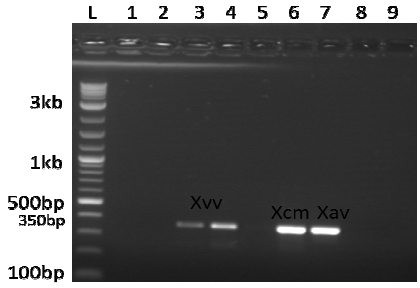
II. *Xcm*35F/R



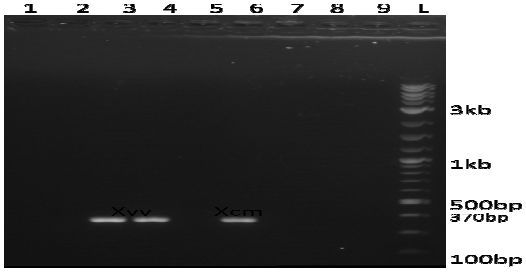
III. *Xcm*36F/R



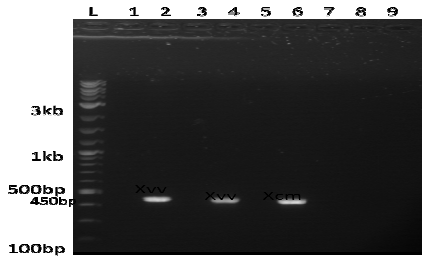
IV. *Xcm*38F/R



V. *Xcm*44F/R



VI. *Xcm*47F/R



VII. *Xcm*48F/R

[Note: Agarose gel to the left;
Lane 2 – X. vasicola pv vasculorum
Lane 3 – X. axonopodis pv vasculorum]

Figure 9: Agarose gels showing PCR results of Primers Xcm12F/R, Xcm35F/R, Xcm36F/R, Xcm38F/R, Xcm44F/R, Xcm47F/R, Xcm48F/R (Adikini et al, 2011), respectively, on Xvv, Xav and Xcm

Table 7: Summary of assessment results of the current conventional PCR assays used to detection of Xcm

Bacterial strain	**NCPPB Number**	**BXW1F/3R**	**GspDmF/R**	**NZO85F/R**	***Xcm*12F/R**	***Xcm*35F/R**	***Xcm*36F/R**	***Xcm*38F/R**	***Xcm*44F/R**	***Xcm*47F/R**	***Xcm*48F/R**
Enterobacter	4168	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Pseudomonas marginalis pv *marginalis*	1232	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Ralstonia solanacearum	2198	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Ralstonia solanacearum	2315	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Ralstonia solanacearum	3205	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Ralstonia solanacearum	3214	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Serratia marcescens	2641	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas arboricola pv *celebensis*	1630	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas axonopodis pv *vasculorum*	186	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas axonopodis pv *vasculorum*	796	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
Xanthomonas axonopodis pv *vasculorum*	899	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Xanthomonas campestris pv *campestris*	529	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas campestris pv *musacearum*	2005	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	2251	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4378	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4379	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

Xanthomonas campestris pv *musacearum*	4380	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4381	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4383	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4387	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4388	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4389	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4390	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4391	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4392	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4392	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4393	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4394	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4433	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *musacearum*	4434	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *perlargonii*	2985	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Xanthomonas campestris pv *perlargonii*	4031	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)

Xanthomonas campestris pv *vesicatoria*	422	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
Xanthomonas campestris pv *vesicatoria*	423	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas campestris pv *vesicatoria*	701	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Xanthomonas sp	1131	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Xanthomonas sp	1132	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Xanthomonas vasicola pv *holcicola*	1060	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(+)	(-)	(+)
Xanthomonas vasicola pv *holcicola*	3129	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)
Xanthomonas vasicola pv *vasculorum*	702	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
Xanthomonas vasicola pv *vasculorum*	795	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
Xanthomonas vasicola pv *vasculorum*	890	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
Xanthomonas vasicola pv *vasculorum*	895	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)

[Note: Green shaded areas represent the results that were similar to those as reported in the previous studies (Adriko et al, 2011; Lewis-Ivey et al, 2010; Adikin et al, 2011) while red represents results that were not as reported in the previous study.]

4.2 ELISA Polyclonal antibody assay

Fera developed an ELISA polyclonal antibody assay (for NARO -Uganda) to detect *Xcm. Xcm* strains (NCPPB 4389, NCPPB 4433, NCPPB 4434, NCPPB 4392, and NCPPB 2251) were inocculated into the rabbit and the antibodies harvested within a period of four months. The unpurified (the serum contains antibodies and other proteins) polyclonal antibody was tested and evaluated against *Xcm* and other *Xanthomonas* strains by ELISA for specificity. It was then purified by HiTrap Protein G columnand re-evaluated against *Xcm* and other *Xanthomonas* and non-*Xanthomonas* strains. The ELISA polyclonal assay was then sent off to Forsite Diagnostics to be formatted into a Lateral Flow Device (LFD). The LFD could in future be used as an on-site tool to detect *Xcm*.

4.2.1 Preliminary testing of the ELISA polyclonal assay

The polyclonal antibody assay (unpurified) was first assessed for its specificity against *Xcm* and other *Xanthomonas* and non-*Xanthomonas* strains. The negative controls were the pre-bleed (the serum before the rabbit was injected with *Xcm* strains) and a CVYV antibody against the CVYV.

The bacterial strains (see Table 1) that were tested had been grown on YDC media for 48 hours at 25ºC. A 10µl loop of the bacteria was then re-suspended in 5ml of PBS solution. The bacteria suspensions were then adjusted to a concentration of 10^7 Cfu/ml using a spectrophotometer at 650nm. ELISA was carried out according the standard protocol (see Appendix 1). The optical density (OD), that is the amount of colour produced from the wells on the ELISA plates after the addition of the substrate (PNPP) to the antibody conjugate (with enzyme Alkaline Phosphatase) was measured by the spectrophotometer specific for reading ELISA plates at 405nm wavelength. High OD value meant the polyclonal antibodies react strongly with the antigen (bacteria tested) and a lower OD meant less binding of the antibody to the antigen. The OD values from wells titrated with the negative controls was very low and consistent compared to the OD values from wells titrated with the *Xcm* polyclonal antibody, thereby confirming the absence of the antigen to which the control antibodies would bind. The unpurified

polyclonal antibody was able to detect and bind to all *Xcm* strains, however they cross-reacted with *Xanthomonas axonopodis* pv *vasculorum (Xav;* causes Gumming disease in sugarcane) but no other *Xanthomonas* strains tested (see Figure 10).

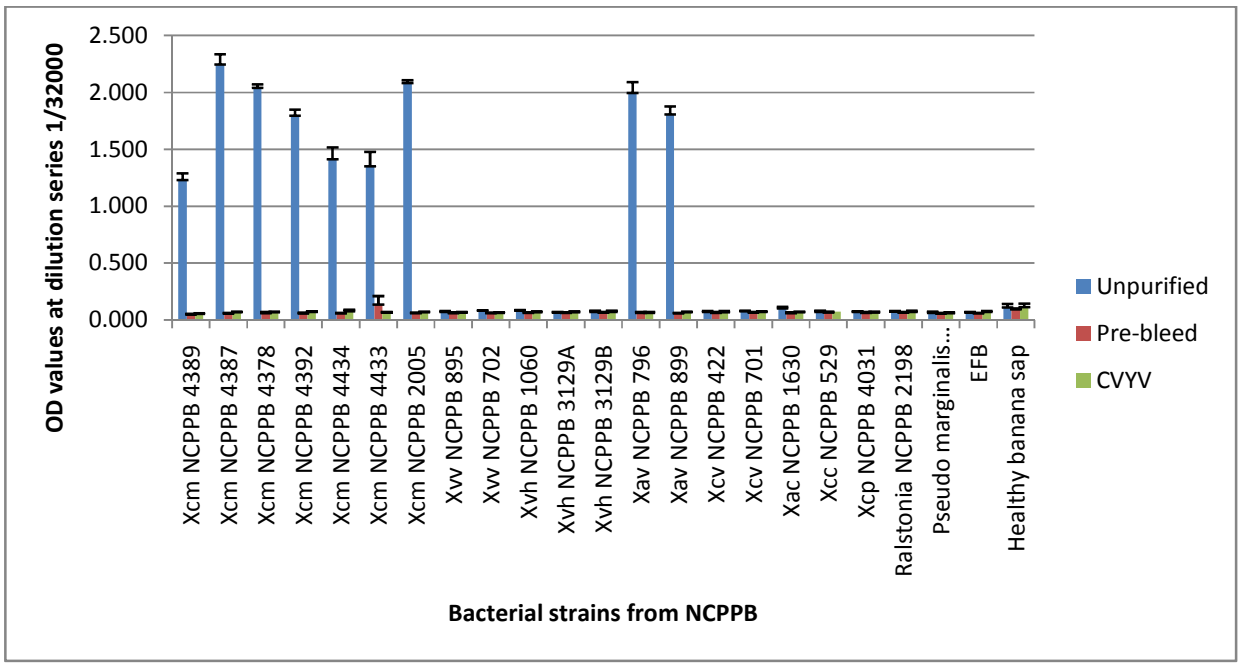


Figure 10: Summary of the ELISA polyclonal antibody assay (unpurified) assessed against Xcm and other Xanthomonas and non- Xanthomonas strains, OD values from 1:32000 dilution of the antibodies (Unpurified polyclonal, Pre-bleed and CVYV) against the antigen. The bars represent the mean OD values of the 4 replicates for each strain at this dilution. The error bars represent the standard deviations

[Note: Unpurified means the unpurified polyclonal antibody to detect Xcm and Pre-bleed is the serum before the rabbit was injected with Xcm strains, used as a control, and CVYV is the antibody used to detect CVYV, this was the second control. Xcm – X. campestris pv musacearum, Xvv – X. vasicola pv vasculorum, Xvh – X. vasciola pv holcicola, Xav – X. axonopodis pv vasculorum, Xcv- X. campestris pv vesicatoria, Xcc- X. campestris pv campestris, Xcp – X. campestris pv perlagonii, Ralstonia solanacearum, Pseudomarginalis pv marginalis, EFB – European Foulbrood

Under a separate assessment the effect of dilution on detection was investigated. The OD values from wells titrated with the unpurified polyclonal antibody (against *Xcm*) from 1:2,000 to 1:256,000 (rows A to E and from columns 1 to 4 (these columns had only the unpurified polyclonal antibody titrated for each plate)) were decreasing with increasing dilution of the antibody. At 1:32,000 dilutions and higher dilutions of the unpurified

antibody, the OD results were still quite high as shown in Figure 11, above an OD of 1. This suggested that the antibody could be further diluted beginning with 1:32,000 dilution of the antibody before we notice loss of colour change or much lower OD than 0.5.

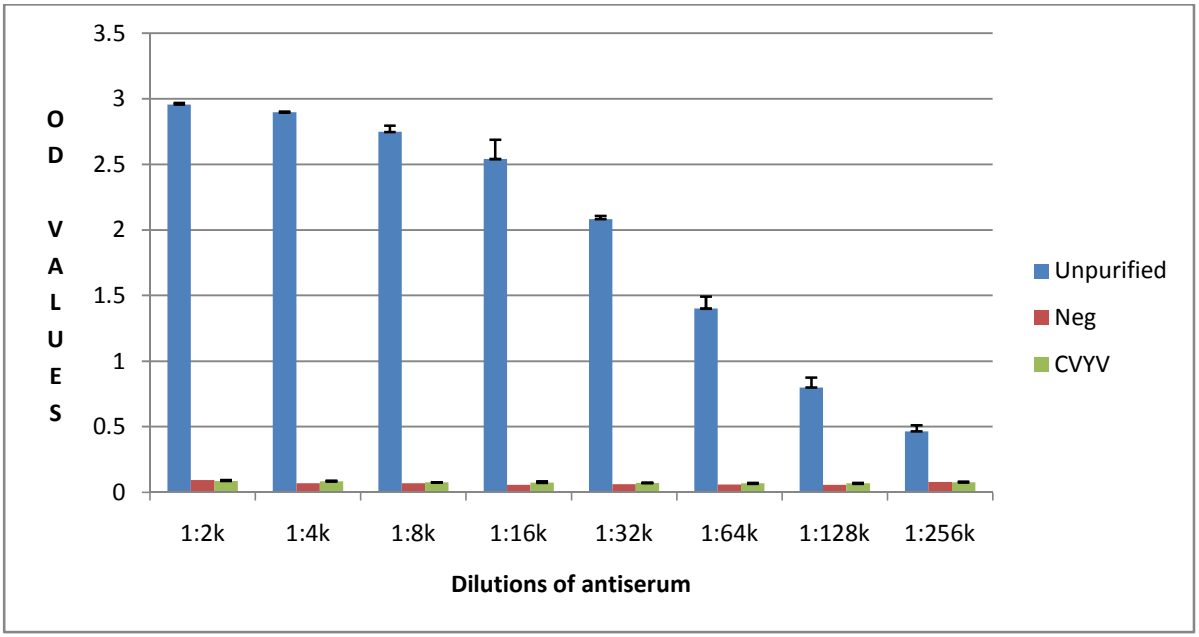


Figure 11: Optical densities at 405nm of the known positive plate 7 (whole cell bacteria strain of Xcm NCPPB 2005) showing the sensitivity of the unpurified polyclonal antibody for XW at increasing dilutions. The bars represent the mean OD values of the 4 replicates of the strain at different dilutions. The error bars represent the standard deviations.The higher the OD, the higher the sensitivity of the antibody.

[Note: Unpurified means the unpurified polyclonal antibody against Xcm, Neg means serum before the rabbit was inoculated used as a control and CVYV means the serum against CVYV used as a control. The colour change is still noticeable at 1:32000 dilution of the unpurified antibody against the antigen (bacteria tested).]

4.2.2 Sensitivity and specificity of the purified polyclonal antibody

The unpurified antibody for *Xcm* was purified to remove contaminant proteins and assessed against bacteria strains of *Xcm* and other *Xanthomonas* and non-*Xanthomonas* strains, as the purification process can alter specificity. The specificity and sensitivity of the purified polyclonal was also compared with that of its unpurified *Xcm* polyclonal antibody of IITA (Nakato *et al*, 2011). We began with a dilution of

1:4,000 for both NARO's purified and IITA unpurified *Xcm* polyclonal antibody and began with a 1:1,000 dilution for polyclonal antibody of IITA in evaluating the sensitivity of the antibodies. Based on the sensitivity results, for specificity testing, we began with a dilution of 1:32,000 for both unpurified and purified *Xcm* polyclonal antibody and 1:2,000 for the polyclonal antibody of IITA.

4.2.2a Sensitivity testing

All three antibodies; NARO's purified and unpurified polyclonal (developed by Fera) and the polyclonal antibody of IITA were able to detect *Xcm*. For purified and unpurified *Xcm* polyclonal antibody, colour changes were still noticeable at higher dilutions (less concentrations of the antibody) of 1:500k and 1:1,000k (See Figure 12) of the antibodies respectively. The purified polyclonal antibody showed more sensitivity than the unpurified polyclonal antibody at the same dilutions as expected.

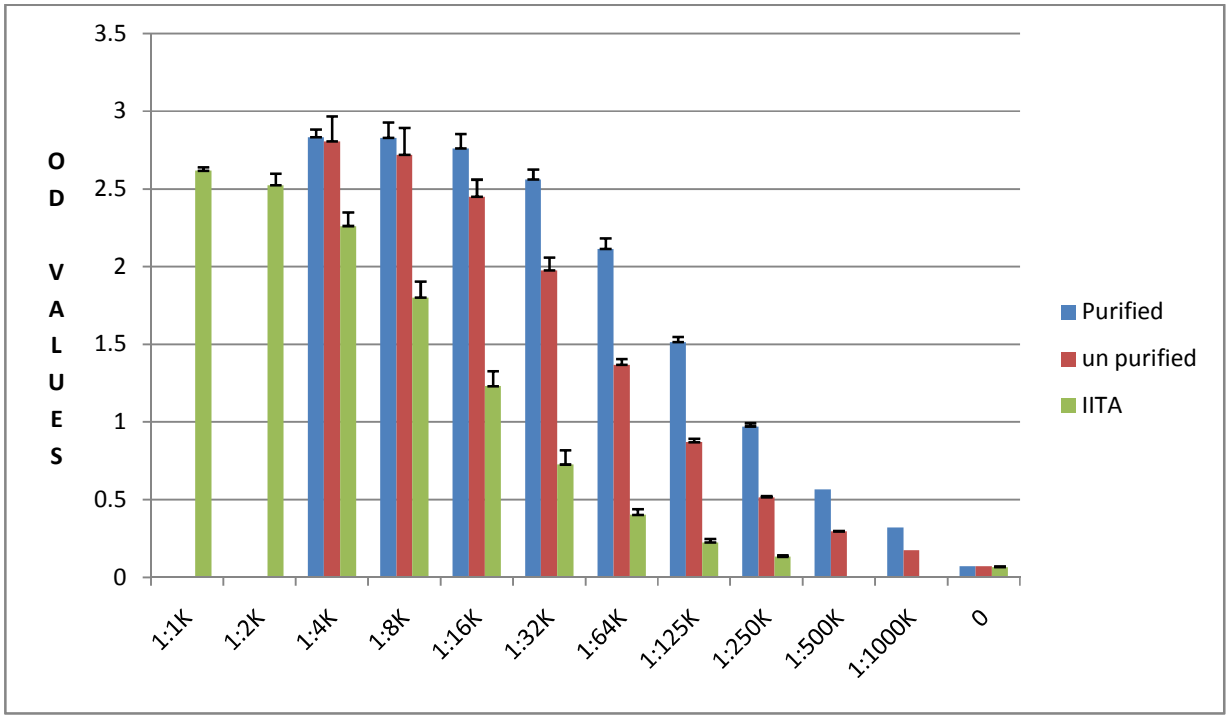


Figure 12: Graph showing the sensitivity of the three polyclonal assays against a known positive (whole bacteria cell of Xcm NCPPB 4434) at different dilutions of the antibodies. The bars represent the mean OD values of the 4 replicates of the strain at different dilutions. The error bars represent standard deviations. High

OD values reveal high sensitivity of the antibody while low OD values reveal less sensitivity of the antibody

[Note: Purified means purified polyclonal antibody, unpurified means unpurified polyclonal antibody and IITA – IITA Xcm unpurified polyclonal. 1:1K means a 1 in 1,000 dilution of the antibody or 1:2K means 1 in 2,000 dilutions of the antibody etc.]

4.2.2b Final specificity testing

For our specificity testing, we compared OD results from ELISA plate readings of purified polyclonal assay and the *Xcm* polyclonal of IITA at the different dilutions. To determine the threshold between positive and negative results, we used the 3x (that is 3 times the mean OD values at different dilutions respectively) of the OD values of the known negative plates which were also negative controls (banana sap and EFB). Threshold values ranged from 0.26 to 0.16 for the purified polyclonal antibody and 0.24 to 0.16 for the *Xcm* polyclonal antibody of IITA (see Figure 13).

Table 8 summaries the results of the assessment of the purified *Xcm* polyclonal antibody assay against different *Xanthomonas* and non-*Xanthomonas* strains. Both the *Xcm* purified polyclonal and the *Xcm* polyclonal of IITA detected all *Xcm* strains, and each cross-reacted with *Xanthomonas axonopodis* pv *vasculorum* strains (NCPPB 796, NCPPB 899). In addition the *Xcm* polyclonal of IITA cross reacted with *Xanthomonas arboricola* pv *celebensis* (NCPPB 1630) strains at lower dilutions (high concentrations) of the antibody at 1:2,000 to 1:8,000 and with 3 of the 4 *Ralstonia solanacearum* species tested (NCPPB 2315, NCPPB 3205, NCPPB 3214) at 1:2,000 dilution.

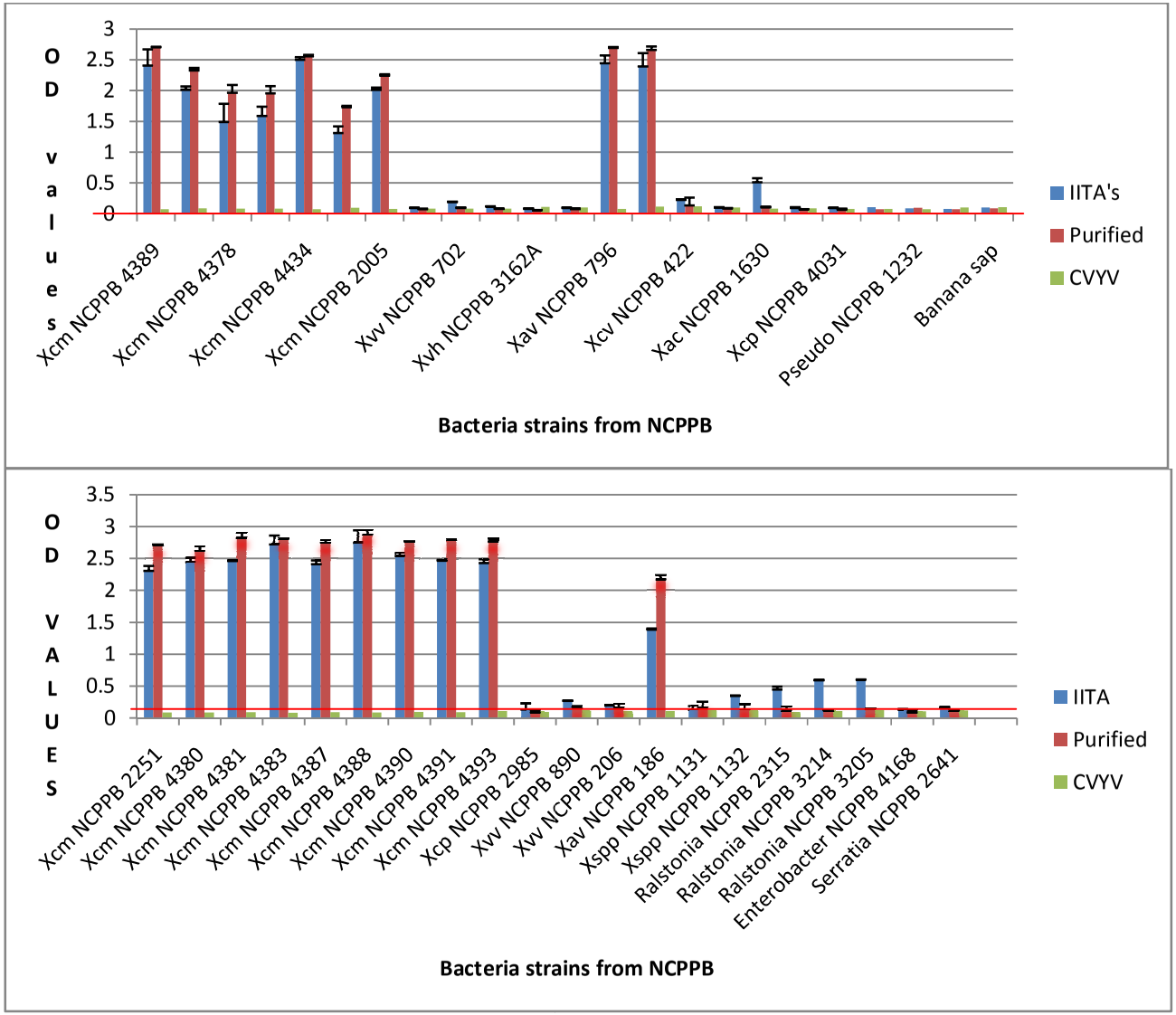


Figure 13: Graphs shows the specificity of purified polyclonal and the unpurified polyclonal of IITA for Xcm, with the bars representing mean OD valuesof the 4 replicates of each strain at 1:2,000 and 1:32,000, respectively, against different Xanthomonas and non-Xanthomonas strains. The error bars represent the standard deviations.

[Note: both polyclonal antibodies cross-react with X. axonopodis pv vasculorum, the pink line represents threshold used to distinguish between negative and positive OD values above the red line were considered positive results and OD values below the line were considered negative results]

Table 8: Summary of ELISA polyclonal antibody assessment against Xanthomonas and non-Xanthomonas strains

Strain	**NCPPB number**	***Xcm* IITA**	**Purified *Xcm***
Banana sap		-	-
Enterobacter spp	4168	-	-
Melissococcus plutonius EFB	6404	-	-
Pseudomonas marginalis pv *marginalis*	1232	-	-
Ralstonia solanacearum	2198	-	-
Ralstonia solanacearum	2315	+	-
Ralstonia solanacearum	3205	+	-
Ralstonia solanacearum	3214	+	-
Serratia marcescens	2641	-	-
Xanthomonas arboricola pv *celebensis*	1630	+	-
Xanthomonas axonopodis pv *vasculorum*	796	+	+
Xanthomonas axonopodis pv *vasculorum*	899	+	+
Xanthomonas axonopodis pv *vasculorum*	186	+	+
Xanthomonas campestris pv *campestris*	529	-	-
Xanthomonas campestris pv *musacearum*	2005	+	+
Xanthomonas campestris pv *musacearum*	2251	+	+
Xanthomonas campestris pv *musacearum*	4378	+	+
Xanthomonas campestris pv *musacearum*	4379	+	+
Xanthomonas campestris pv *musacearum*	4380	+	+
Xanthomonas campestris pv *musacearum*	4381	+	+
Xanthomonas campestris pv *musacearum*	4383	+	+
Xanthomonas campestris pv *musacearum*	4387	+	+
Xanthomonas campestris pv *musacearum*	4387	+	+
Xanthomonas campestris pv *musacearum*	4388	+	+
Xanthomonas campestris pv *musacearum*	4389	+	+
Xanthomonas campestris pv *musacearum*	4390	+	+
Xanthomonas campestris pv *musacearum*	4391	+	+
Xanthomonas campestris pv *musacearum*	4392	+	+
Xanthomonas campestris pv *musacearum*	4393	+	+
Xanthomonas campestris pv *musacearum*	4433	+	+
Xanthomonas campestris pv *musacearum*	4434	+	+
Xanthomonas campestris pv *perlagonii*	2985	-	-
Xanthomonas campestris pv *perlogonii*	529	-	-
Xanthomonas campestris pv *vesicatoria*	422	-	-
Xanthomonas campestris pv *vesicatoria*	701	-	-
Xanthomonas spp	1131	-	-
Xanthomonas spp	1132	-	-

Strain	**NCPPB number**	***Xcm* IITA**	**Purified *Xcm***
Xanthomonas vasicola pv *holcicola*	1060	-	-
Xanthomonas vasicola pv *holcicola*	3162A	-	-
Xanthomonas vasicola pv *holcicola*	3162B	-	-
Xanthomonas vasicola pv *vasculorum*	206	-	-
Xanthomonas vasicola pv *vasculorum*	702	-	-
Xanthomonas vasicola pv *vasculorum*	795	-	-
Xanthomonas vasicola pv *vasculorum*	890	-	-
Xanthomonas vasicola pv *vasculorum*	895	-	-

[Note: Whole cell bacteria strains were used in the plate trap ELISA method, + means its positive result, and – means its negative result. The strains in red were expected to give off negative results but cross-reacted with the particular Xcm polyclonal antibody, while the strains in green gave the positive results as expected.]

4.2.3 Development of the ELISA polyclonal Lateral Flow Device (LFD)

Under this study the *Xcm* polyclonal antibody assay for detection of *Xcm* by ELISA had been successfully demonstrated. The polyclonal antibody assay was assessed against *Xcm*, other *Xanthomonas* strains and non-*Xanthomonas* strains for specificity. Forsite Diagnostics then successfully developed the purified polyclonal antibody in the format of a LFD (see Appendix 3). The LFD involved binding the polyclonal antibody to latex.. When a test sample is added to the sample pad, the bacteria co-migrate with the solute by absorption and ‘cross’ the detection (fixed *Xcm* antibody) zone. In a positive test the *Xcm* bacterium bind with the *Xcm* polyclonal and to the conjugate antibody-coloured particles to give a coloured complex (seen as a line). The conjugate coloured particles also migrate and bind with a second fixed control line. Two coloured lines meant it was a positive result and one coloured line meant it was a negative result. The LFD developed for NARO-Uganda ran successfully (see Figure 14).

Figure 14: The LFDs developed by Forsite Diagnostics.

[Note: The left LFD is with *Xcm* infected *leaf sap (NCPPB 4434) and the right LFD is with healthy leaf sap. The single line furthest from the well and common to both LFD is the control and indicates the test has worked. The second line on the left LFD indicates a positive test for the presence of Xcm. The single line on the right LFD indicates a negative test].*

4.2.3.a Specificity of the LFD

The LFD data was consistent with the ELISA; with detection of all *Xcm* strains evaluated and cross-reaction with *Xanthomonas axonopodis* pv *vasculorum* strains (see Appendix 3).

Table 9: Results for the LFD specificity

Strain	NCPPB number	LFD
Xanthomonas campestris pv *musacearum*	4433	+
Ralstonia solanacearum	3205	-
Xanthomonas arboricola pv *celebensis*	1630	-
Xanthomonas axonopodis pv *vasculorum*	796	+
Xanthomonas campestris pv *campestris*	529	-
Xanthomonas campestris pv *musacearum*	2005	+
Xanthomonas campestris pv *musacearum*	4378	+
Xanthomonas campestris pv *musacearum*	4387	+
Xanthomonas campestris pv *musacearum*	4390	+
Xanthomonas campestris pv *musacearum*	4392	+
Xanthomonas campestris pv *musacearum*	4434	+
Xanthomonas campestris pv perlogonii	4031	-
Xanthomonas campestris pv *vesicatoria*	422	-
Xanthomonas spp	1131	-
Xanthomonas spp	1132	-
Xanthomonas vasicola pv *holcicola*	3129A	-
Xanthomonas vasicola pv *vasculorum*	895	-

[Note:

+ a positive result (ie gave two lines; coloured test and control line)
– a negative result (control line coloured only).
The strains in green were expected to give positive results (the LFD was supposed to detect the strain), while the strains in red were expected to give a negative result, but instead gave positive result.]

4.2.3b Sensitivity of the LFD

The LFD was most sensitive at high *Xcm* cell concentrations of 10^8 to 10^6 cfu/ml (cells suspended in extraction buffer), with positive lines clearly visible after 10 minutes (see appendix 3, D-M). Weak positives were evident at 10^5 cfu/ml and detection was shown to fail at 10^4 cfu/ml and below. These results were then shown to be equivalent for the same concentrations with crushed banana leaf-extraction buffer and banana sap-extraction buffer.

Table 10: Results of the LFD sensitivity assessment against Xcm, Xav and Ralstonia solanacearum strains at different concentrations

Strain	**Isolate origin**	**NCPPB number**	**Bacterial cfu/ml**				
			10^8	**10^7**	**10^6**	**10^5**	**10^4**
Ralstonia solanacearum		3205	--	--	--	--	--
Xanthomonas axonopodis pv *vasculorum*		796	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Ethiopia	2251	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Uganda	4383	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	DR Congo	4387	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Rwanda	4389	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Tanzania	4392	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Burundi	4433	++	++	++	+	--
Xanthomonas campestris pv *musacearum*	Kenya	4434	++	++	++	+	--

[Note:
++ a strong positive result (two coloured lines; coloured test and control line)
+ a faint test line only, hence a weak positive
–a negative result (control coloured line only).

The strains in green were expected to give positive results (the LFD was supposed to detect the strain), while the strains in red were expected to give a negative result (one coloured line) but instead gave positive result.]

4.3 Pathogenicity trials

To further support the introduction of a new pathovar *Xanthomonas vasicola* pv *musacearum* to the *Xanthomonas vasicola* species, there was need for substantial data on the comparative pathogenicity of the *Xanthomonas vasicola* pathovars. The pathogenicity trials began with pilot trials to determine how much of the bacteria was needed to cause virulence within the plants and what symptoms were expected to be seen on the inoculated plants. In the pilot trials, maize, sorghum and banana plants were inoculated with *Xcm, Xvv*, and *Xvh*, and *Xav* on maize and banana. These strains were assumed to be pathogenic to some of the plants. In the large scale trial, other common plant pathogenic *Xanthomonas* pathovars and non-*Xanthomonas* strains were included to be able to bring out the distinct pathogenicity of the *Xv* pathovars and *Xcm* on maize, banana and sugarcane.

4.3.1 Pilot trials

About 200µl to 300µl of bacterial inoculum at 10^7 cfu/ml was enough to cause virulence in maize and banana. All control plants; untreated and those inoculated with sterile water remained healthy.

4.3.1a Pilot trial of pathogenicity on banana

Symptoms on banana appeared 3-week after inoculation for both the Cavendish and Tropicana banana plants that had been inoculated with *Xcm (*NCPPB 4392 and NCPPB 2005) developed necrotic leaf symptoms, wilting and eventually the whole plant rotted by the end of the trial (5-weeks). The banana plants (of both varieties) that were inoculated with the *Xvh* (NCPPB 1060*)* had no visible symptoms and remained healthy for the duration of the pilot trial (5-weeks).

Figure 15: Tropicana bananas that had been inoculated with Xvh NCPPB 1060 (front centre), with Xcm NCPPB 4392 & NCPPB 2005 (back) and the controls (left side) 5-weeks after inoculation the banana pilot pathogenicity trial.

[Note: The four plants that had been inoculated with Xcm are wilted and necrotic.]

Figure 16: Cavendish dwarfs bananas inoculated with Xvh NCPPB 1060 (middle), Xcm NCPPB 2005, NCPPB 4392 (left side) and the controls (right side) 5-weeks after inoculation pilot pathogenicity trial.

[Note: The three plants that had been inoculated with Xcm are wilted and necrotic.]

4.3.1b Pilot trial of pathogenicity on maize

Symptoms on maize appeared 1-week after inoculation for the plants that had been inoculated with *Xv* pathovars and *Xav* strains. Symptoms exhibited included leaf chlorosis, yellow-brown or water soaked streaks or brown lesions on the leaves, usually beginning in the centre of the leaf. The most severe symptoms included deformation of the plant and retarded growth by *Xvv (*NCPPB 702, NCPPB 795*)* and *Xvh* (NCPPB 1060), respectively. Out of the 8 maize plants inoculated with *Xcm (*NCPPB 4434*,* NCPPB 4378*),* only one plant (inoculated with *Xcm* NCPPB 4378) showed identical symptoms, but not as virulent as those seen on plants inoculated with *Xvv* and *Xvh*, the leaves only had yellow-brown streaks. Sedum plants did not show any symptoms and were therefore not used for the rest of the pathogenicity trial.

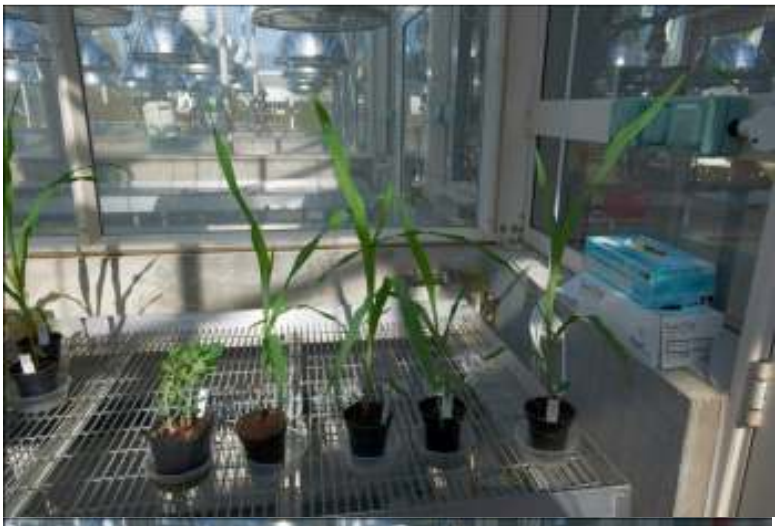


Figure 17: Untreated maize control plants used in the pilot pathogenicity trial and (left) the sedum plant used as a plant indicator; 1-week into the trial

Figure 18: Maize (left) and sedum (right) control plants that had been inoculated with sterile water;1-week after inoculation

Figure 19: Brown lesions leaf symptoms of maize inoculated with Xvv NCPPB 702 and NCPPB 795; 1-week after inoculation

Figure 20: White streak lesions leaf symptoms of maize inoculated with Xvv NCPPB 702 and NCPPB 795; 1-week after inoculation

Figure 21: Yellow streak lesions leaf symptoms of maize inoculated with Xvh NCPPB 1060; 1-week after inoculation

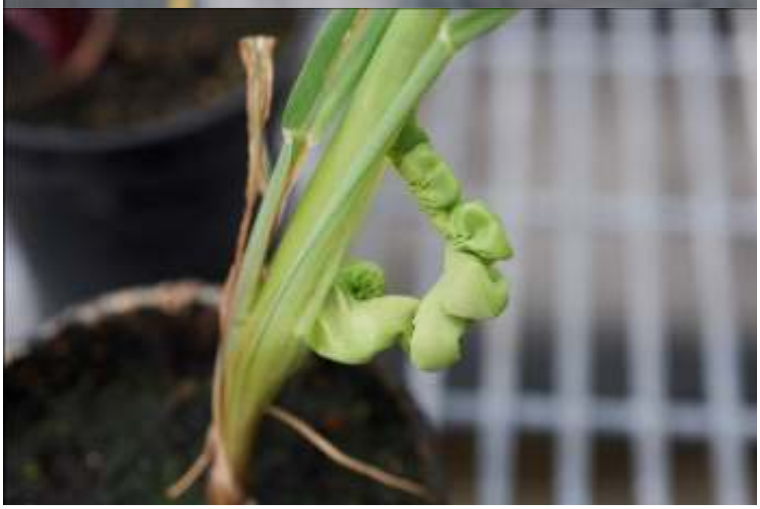


Figure 22: Deformation leaf symptoms of maize inoculated with Xvv NCPPB 702 and 795; 1-week after inoculation

Figure 23: Water soaked leaf symptoms of maize inoculated with Xvh NCPPB 1060; 1-week after inoculation

Figure 24: Stunted growth symptoms of maize inoculated with Xvh NCPPB 1060; 1-week after inoculation

Figure 25: White streak leaf symptom of maize inoculated with Xav NCPPB 796 and NCPPB 899; 1-week after inoculation

Figure 26: Yellow streak leaf symptom of maize inoculated with Xcm NCPPB 4434; 3-weeks after inoculation

4.3.1c Pilot trial of pathogenicity on sorghum

Unexpectedly, all sorghum plants remained healthy including those inoculated with the strains of *Xvh* that is a known host of this pathovar. However an ICRISAT report on Bacterial leaf streak of sorghum in India (Navi *et al*, 2002), also reported unsuccessful attempts at artificially inoculating sorghum seedlings. They concluded that *Xvh* strains could only be isolated from already naturally infected plants.

4.3.1d Sugarcane pilot trial

Unfortunately multiplication of sugarcane plants was slow and we were unable to obtain enough sugarcane plantlets in time for a pilot trial. Therefore the decision was made to maintain the available plants for the full trial only.

4.3.2 Full-scale pathogenicity trial

In the full-scale pathogenicity trial for maize, banana and sugarcane, plants were not only inoculated with the *Xv* pathovars (*Xvv, Xvh, and Xcm*) but also plant pathogenic *Xanthomonas* pathovars: *X. campestris* pv *campestris* and *X. campestris* pv *vesicatoria* etc and some non-*Xanthomonas* species, *Paenibacillus larvae*. The evaluation of this broader set of strains was to bring out the distinct pathogenicity of the *Xv* pathovars on maize, banana and sugarcane. The trial had at least 3 plants inoculated with one pathovar thereby eliminating or minimizing bias and errors due to inoculation.

4.3.2a Viability of bacteria

The viability of the bacterial suspensions used as inoculums was proven; all showed growth on solid media YDC after incubation at 25ºC for 48 hours.

4.3.2b Re-isolation of bacteria from plants

In most cases bacteria were successfully re-isolated after at least 5 – 7 weeks from inoculated plants; especially for *Xcm* and *Xvv* inoculated plants. Confirmation of identity for *Xcm* and *Xvv* strains was achieved by PCR using the primers GspDmFR (Adriko *et al*, 2011) and XW1F/R (Lewis-Ivey *et al*, 2010), respectively. For *Xanthomonas campestris* pv *campestris*, and *X. campestris* pv *pelargonii* where bacterial colonies consistent with a *Xanthomonas* morphology were recovered this was considered diasgnostic for the species. The non-*Xanthomonas* strain of *Paenibacillus* larvae were not recovered at the end of all the trials (usually 7-weeks long).

4.3.2c Full-scale pathogenicity trial of Xanthomonas strains on banana

All banana plants inoculated with *Xcm* strains showed severe symptoms typical of *Xanthomonas* Wilt. From these plants *Xcm* was readily re-isolated. All plants inoculated with the *Xv* pathovars (*Xvv* and *Xvh*) remained healthy, although re-isolation of these strains was successful. Plants that were inoculated with *Xanthomonas arboricola* pv *celebensis* (*Xac*) and *Xanthomonas* species (NCPPB 1131 and 1132), that had been originally isolated from banana, remained healthy and re-isolation of the bacteria was unsuccessful. All banana plants in the trial including the controls (untreated and those inoculated with sterile water) had their older leaves yellowing, with scorched appearance. This was attributed to natural ageing rather than inoculation.

Plants inoculated with *Xcm* showed severe symptoms of XW as described by Yirgou and Brabury (1974) 3-weeks after inoculation (see Figures 27-29). The disease affected the younger leaves first, beginning with dull green colouring of the lamina, folding of the two halves of the midrib until touching each other, yellowing of the leaves, reddish

brown streaks on the leaf, eventually all the leaves wilted and the entire plant rotted. Most of the *Xcm* inoculated plants were dead by the 7th week of the trial.

Symptom severity was scored as follows:

- **0** – no visible symptoms
- **1** – slight wilting/folding of lower leaves
- **2** – pronounced wilting/yellowing of most leaves
- **3** – pronounced necrosis of the whole plant
- **4** – complete death, rotting of the plant

Some *Xcm*-inoculated plants remained healthy and re-isolation of the bacteria from these plants was unsuccessful. Re-isolation of the *Xcm* from most *Xcm*-inoculated plants was successful, but difficult from plants that had already died and had been colonised by faster growing saprophytic microbial organisms that outcompeted *Xcm* on the YDC media plate. Other pathovars (*X. axonopodis* pv *vasculorum*, *X. campestris* pv *pelagonii* (NCPPB 2985, NCPPB 4031), *X.campestris* pv *campestris* (NCPPB 529) and *X. campestris* pv *vesicatoria* (NCPPB 422, NCPPB 701)) did not cause disease in bananas but were successfully re-isolated. This suggests that banana can be a host to other significant *Xanthomonas* plant pathogens other than *Xcm, Xvv* and *Xvh.* Non-*Xanthomonas* strain, *Paenibacillus larvae* was not successfully re-isolated.

Figure 27: Large scale banana pathogenicity trial

[Note: The trial was randomized, that is plants with similar treatments were not adjacent.]

Figure 28: XW symptom score 1; pale colouring of the lamina, folding of the leaves along the mid-rib with the two halves touching; 5-weeks after inoculation

Figure 29: XW symptom score 2; yellowing of the leaves and appearance of reddish-brown streaks on the leaves; 5-weeks after inoculation


Figure 30: XW symptom score 3; wilting of most leaves and entire necrosis of the plant; 5-weeks after inoculation

[Note: For the purposes of the photographs the plants were rearranged by treatment, otherwise they were fully randomized during the trial]

Table 11: Symptom scores for Xcm-inoculated individual banana plants of the main pathogen trial:- 0 – no visible symptoms, 1 – Slight wilting/folding of younger leaves,2 - Pronounced wilting/yellowing of most leaves,3 – Pronounced necrosis of the whole plant ,4 – Complete death, rotting of the whole plant

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 7
Xcm NCPPB 2005	1	0	0	0	0	2	2
	2	0	0	1	1	3	4
	3	0	0	1	1	2	3
	4	0	0	0	0	2	2
Xcm NCPPB 4379	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	1	2	3	4	4
	4	0	0	0	2	2	3
Xcm NCPPB 4387	1	0	0	2	2	4	4
	2	0	0	2	2	3	4
	3	0	0	2	2	3	4
	4	0	0	1	2	3	4
Xcm NCPPB	1	0	0	0	0	0	0
	2	0	0	0	1	1	1

4390	3	0	0	0	1	1	1
	4	0	0	1	3	4	4
Xcm NCPPB 4433	1	0	0	0	2	3	3
	2	0	0	0	0	0	0
	3	0	0	2	4	4	4
	4	0	0	0	0	0	1
Xcm NCPPB 4434	1	0	0	0	1	1	3
	2	0	0	1	2	3	4
	3	0	0	2	2	4	4
	4	0	0	2	2	3	4

4.3.2d Full scale pathogenicity trial of Xanthomonas strains on maize

Control plants that had been inoculated with sterile water and others left untreated remained healthy throughout the trial. Plants inoculated with strains *Xvv, Xvh* and *Xav* displayed symptoms consistent with the pilot studies (see Figures 32-38). Symptoms appeared 6-days after inoculation; these included yellow, brown, white or water soaked streaks as well as brown lesions on the leaves, usually beginning in the centre of the leaf. The most severe symptoms were deformation of the plant and retarded growth by strains of *Xvv* and *Xvh*, respectively, revealing distinct pathogenicity of the two strains on maize.

Symptoms were scored according to the type of symptom, rather the severity:

- **0** – no visible symptoms
- **1** – water-soaked like streaks
- **2** – yellow or brown or white streaks
- **3**- brown lesions
- **4** – deformation of plant or stunted growth

The 28 plants that had been inoculated with *Xcm* did not exhibit any of the symptoms throughout the trial, suggesting that *Xcm* does not affect maize under these conditions. Furthermore, re-isolation from a few of these plants was successful even 5 weeks after inoculation suggesting that *Xcm* can survive in maize without causing any symptoms. Plants inoculated with other common plant pathovars; *X. campestris* pv *campestris*, *X.*

campestris pv *vesicatoria* and *X. campestris* pv *pelargonii* did not show any signs of disease (not re-isolated).

Figure 31: Large scale maize pathogenicity trial (the plant location was randomized)

Figure 32: The control maize plants; untreated and those inoculated with sterile water in the large scale maize pathogenicity trial

Figure 33: Brown streak leaf symptom of maize inoculated with Xav (NCPPB 796 and NCPPB 899)

Figure 34: White streak leaf symptom of maize; 3-weeks after inoculation with Xav (NCPPB 796 and NCPPB 899)

Figure 35: Stunted/ deformed growth symptom (score 4) of maize; 3-weeks after inoculation with Xvh (NCPPB 1060)

Figure 36: Brown streak leaf symptom (score 3) of maize; 3-weeks after inoculation with Xvh (NCPPB 1060)

Figure 37: Stunting/ deformation of plant symptom (score 4) of maize; 3-weeks after inoculation with Xvv (NCPPB 895, NCPPB 702, NCPPB 206, NCPPB 890)

Figure 38: Brown streak leaf symptom (score 2) of maize; 3-weeks after inoculation with Xvv (NCPPB 895, NCPPB 702, NCPPB 206, NCPPB 890)

Figure 39: Maize plants that had been inoculated with Xcm and remained healthy in the pathogenicity trial

The leaf symptoms such as streaks and lesions were not exhibited on leaves that had inoculation sites only thereby confirming the symptoms were not as a result of hypersensitive response. Some of the plants inoculated with *Xvv* and *Xav* that had earlier had their leaves with lesions and streaks (1 to 2-weeks after inoculation) appeared to recover (became healthy), with no more symptoms developing on inoculated leaves or on non-inoculated leaves by the 5th week. Of the plants inoculated with *Xvh* (NCPPB 1060 and 3126) only plants inoculated with NCPPB 1060 exhibited symptoms. Those inoculated with *Xvh* NCPPB 3126 remained healthy throughout the trial even 1-week after inoculation. However, re-isolation of the *Xvh* bacteria was successful for both strains. Results are summarised in Table 12.

Table 12: Symptom scores for individual maize plants that had been inoculated with Xav, Xvh, Xvv and Xcm in the main maize pathogen trial;1-week after inoculation: 0 – no visible symptoms, 1 – Water-soaked streaks, 2 – Yellow brown or white streaks, 3 – Brown lesions, 4 – Deformation of plant or stunted growth.

Treatment	Plant no.	Week 1	Week2	Week 6
Xav NCPPB 796	1	1,2	2,3	0
	2	1,2	3	0
	3	1,2	2,3	2
	4	1,2	2	2
Xav NCPPB 899	1	1	3	0
	2	0	3	2
	3	0	3	2
	4	0	2	2
Xvh NCPPB1060	1	2	4	2
	2	1	3	2,3
	3	1	1,2	2
	4	1,2	2,3	2,3
Xvh NCPPB 3162	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xvv NCPPB 895	1	1	2	0
	2	1	1,2	2,3
	3	1,2	2,3	2,3
	4	1	2,3	2,3
Xvv NCPPB 702	1	1,2,3	2,3	0
	2	1,2	2,3	0
	3	1,2	3	1,2
	4	2	3	0

Xvv NCPPB 890	1	1	1,2	0
	2	1,2	2	0
	3	1,2	3	0
	4	1,2	2,3	0
Xvv NCPPB 206	1	2,4	4	4
	2	1,3	2,3	3
	3	2,3	2,3	2
	4	1	2,4	4
Xcm NCPPB 4379	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4387	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4390	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 2005	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4434	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4433	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0

[Note: All plants inoculated with Xcm remained healthy throughout the trial. Plants inoculated with Xav NCPPB796 and NCPPB 899 and Xvv NCPPB 890 and NCPPB 702 exhibited recovery from initial symptoms]

4.3.2e Full scale pathogenicity trial of Xanthomonas strains on sugarcane

All control plants; those inoculated with sterile water and the untreated remained healthy throughout the trial. *X. campestris* pv *pelargonii*, *X. campestris* pv *vesicatoria* and the *Xanthomonas* species (NCPPB 1131), caused white lesions around the inoculation site

on the leaves suspected to be a hypersensitive reaction (HR) to the pathogens in sugarcane,1-week after inoculation (see Figure 40).

Xav, Xcm, Xvh and *Xvv* caused foliar symptoms such as reddish-brown streaks and spots. *Xcm* also caused white and yellow streaks on the leaves. Similarly, the *Xav* strain caused white streaks on the leaves. These symptoms appeared 1-week after inoculation. Out of the 6 plants inoculated with *Xvh*, only those inoculated with NCPPB 1060 gave symptoms (those inoculated with *Xvh* NCPPB 3126 remained healthy). Out of the 6 plants inoculated with *Xav,* 2 of them remained health. Out of the 12 plants inoculated with *Xvv,* 2 of them remained health. Out of 21 plants inoculated with *Xcm,* 7 remained healthy throughout the trial. All other common plant *Xanthomonas* pathovars as well as *Paenibacillus* larvae did not affect sugarcane.

As with maize, symptoms were scored according to the type of symptom, rather the severity:

- **0** – no visible symptoms
- **1**- white streaks or lesions
- **2** – reddish-brown streaks or lesions
- **3** – yellow streaks

The results are summarised in Table 13.

Figure 40: Main sugarcane full scale pathogenicity trial in the glasshouse

Figure 41: Left, white hypersensitive response patches seen about points of inoculation on the leaves of sugarcane inoculated with X.campestris pv pelargonii (NCPPB 2985); 1-week after inoculation in the main sugarcane pathogenicity trial

Figure 42: Below, reddish/brown leaf symptoms caused by Xvv (NCPPB 895, NCPPB 206); 1-week after inoculation in the main sugarcane pathogenicity trial

Figure 43: Left, reddish/brown leaf symptoms (score 2) caused by Xvh NCPPB 1060; 1-week after inoculation in the main sugarcane pathogenicity trial

Figure 44: Reddish/brown leaf symptoms (score 2) caused by Xcm; 1-week after inoculation in the main sugarcane pathogenicity trial

Figure 45: White streak leaf symptoms (score 1) caused by Xav (NCPPB 796); 1-week after inoculation in the main sugarcane pathogenicity trial

Figure 46: Reddish/brown leaf symptoms (score 2) caused by Xav; 1-week after inoculation in the main sugarcane pathogenicity trial

Table 13: Symptom scores of individual plants inoculated with Xvv, Xvh, Xav and Xcm in the main sugarcane pathogen trial: 0 – no visible symptoms, 1- white streaks or lesions, 2 – reddish-brown streaks or lesions, 3 – yellow streaks.

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4
Xav NCPPB 796	1	0	2	2++	2
	2	0	0	0	0
	3	0	0	0	0
Xav NCPPB 899	1	2	2++	2,3++	2,3
	2	2	2	2,3++	2,3
	3	0	0	1++	1
Xvh NCPPB 1060	1	2	2,3++	2,3++	2,3
	2	2,3	2,3++	2,3++	2,3
	3	2	2+	2+	2
Xvh NCPPB 3162	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
Xvv NCPPB 895	1	2	2,3	2,3+++	2,3
	2	0	0	0	0
	3	2	2	2	2
Xvv NCPPB 702	1	0	2	2	2
	2	2	2	2,3	2,3
	3	0	2	2	2
Xvv NCPPB 890	1	0	2	2	2
	2	0	0	0	0
	3	3	2,3	2,3	2,3
Xvv NCPPB 206	1	2	2	2,3+++	3
	2	2	2	2,3+++	2,3
	3	2	2	2++	2

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4
Xcm NCPPB 4379	1	0	0	0	0
	2	0	0	0	0
	3	1	1	0	0
Xcm NCPPB 4387	1	0	0	0	0
	2	0	0	0	0
	3	1	1,2++	1,2++	1,2
Xcm NCPPB 4390	1	0	0	0	0
	2	0	1	1	1
	3	0	0	0	1
Xcm NCPPB 2005	1	0	0	0	0
	2	0	0	2+	2
	3	2	1,2	1,2++	1,2++
Xcm NCPPB 4434	1	2	2+	2+	2
	2	0	2+	2+	2
	3	2	2+	2+	2
Xcm NCPPB 4433	1	1	1+	1	1
	2	1	1+	1	1

[Note: Xvh NCPPB 3162 did not cause any symptoms on sugarcane plants in the trial, + means severe, ++ means very severe]

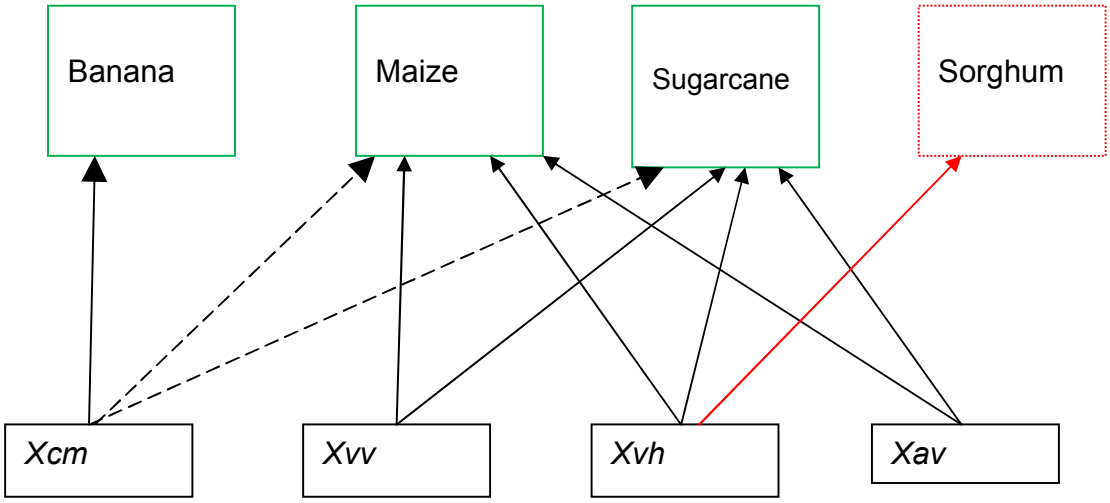
Table 14: Summary of results of comparative pathogenicity testing of the X. vasicola pathovars

Strain	NCPPB no.	Banana			Maize			Sugarcane		
		No. of plants inoculated		Re-isolation	No. of plants inoculated		Re-isolation	No. of plants inoculated		Re-isolation
Paenibacillus larvae		4	0	-	4	0	-	3	0	
Xanthomonas arboricola pv *celebensis*	1630	4	0	+	4	0	-	3	0	
Xanthomonas axonopodis pv *vasculorum*	796	4	0	+	4	4	+	3	1	
Xanthomonas axonopodis pv *vasculorum*	899	4	0	+	4	4	+	3	3	
Xanthomonas campestris pv *campestris*	529	4	0	+	4	0	-	3	1	
Xanthomonas campestris pv *musacearum*	2005	4	4	+	4	0	+	3	2	
Xanthomonas campestris pv *musacearum*	4379	4	3	-	4	0	-	3	1	
Xanthomonas campestris pv *musacearum*	4387	4	4	-	4	0	-	3	1	
Xanthomonas campestris pv *musacearum*	4390	4	3	-	4	0	-	3	1	
Xanthomonas campestris pv *musacearum*	4433	4	3	+	4	0	+	2	2	
Xanthomonas campestris pv *musacearum*	4434	4	3	+	4	0	+	3	3	

Strain	NCPPB no.	Banana			Maize			Sugarcane		
		No. of plants inoculated		Re-isolation	No. of plants inoculated		Re-isolation	No. of plants inoculated		Re-isolation
Xanthomonas campestris pv *pelagonii*	2985	4	0	+	4	0	+	0	0	
Xanthomonas campestris pv *pelagonii*	4031	4	0	+	4	0	-	0	0	
Xanthomonas campestris pv *vesicatoria*	422	4	0	-	4	0	-	0	0	
Xanthomonas campestris pv *vesicatoria*	701	4	0	+	4	0	-	0	0	
Xanthomonas spp.	1131	4	0	+	4	0	-	0	0	
Xanthomonas spp.	1132	4	0	+	4	0	-	0	0	
Xanthomonas vasicola pv *holcicola*	3162	4	0	+	4	0	+	3	0	
Xanthomonas vasicola pv *holcicola*	1060	4	0	+	4	4	+	3	3	
Xanthomonas vasicola pv *vasculorum*	702	4	0	+	4	4	+	3	3	
Xanthomonas vasicola pv *vasculorum*	890	4	0	+	4	4	+	3	2	
Xanthomonas vasicola pv *vasculorum*	895	4	0	+	4	4	+	3	2	

[Note: + indicates the re-isolation of that pathogen was successful after the pathogenicity trial whilst – indicates that the re-isolation of that pathogen was unsuccessful. The boxes in green inicates that the correlating pathogens were expected to cause disease in the particular crop (maize, sugarcane and banana). Re-isolations from sugarcane were not undertaken.]

Figure 47: Schematic of host range of Xcm and Xav and Xv pathvars



[Note:

Means bacteria strain is consistently pathogenic on host

Means bacteria strain can sometimes be pathogenic on host

Pathogenicity on sorghum was not successfully demonstrated for any strain under this study; however, Xvh is reported in the literature as a pathogen of sorghum and is indicated as a pathogen of sorghum]

4.4 Genotyping of *X.campestris* pv *musacearum* isolates from Western Uganda

Previous studies (Aritua *et al*, 2008, Aritua *et al*, 2007, Odipio *et al*, 2009 and Lewis-Ivey *et al*, 2010) that conducted FAME analysis, REP-PCR and ERIC-PCR had shown populations of *Xcm* to be genetically homogenous with very limited genetic variability. The assays described above are suitable, with various levels of success, to detect and identify *Xcm* and distinguish between *Xcm* and its close relatives that are not banana-pathogens. However recent genome-wide sequence analyses of *Xcm* have revealed some (limited) genetic diversity within *Xcm* (Wasukira *et al*, 2012). The study revealed that there was genetic variations within *Xcm* based on Single nucleotide polymorphisms (SNPs) and that these 86 SNPs divided the *Xcm* populations between two major sub lineages (I&II) or genotypes. These two lineages appear, based on a limited number of isolates, to be associated with specific geographical locations: lineage I were *Xcm* strains from DR Congo, Rwanda and Ethiopia whilst lineage II are *Xcm* strains from

Uganda, Kenya and Burundi. Primers designed based on these SNPs will distinguish between different genotypes of *Xcm* and may be informative for tracking spread of the pathogen over space and time.

A new collection of *Xcm* strains from Western Uganda were analyzed based on the SNPs primers to validate some of the genetic variation reported by Wasukira *et al* (2012) and to explore the genetic diversity within this single geographical region, Western Uganda, a region whose *Xcm* genotypes had not previously been investigated and which lies geographically between two regions (Eastern DRC and central Uganda) that were genetically distinct from each other.

The SNPs primers amplified the target DNA around the SNPs (at different loci) as reported by Wasukira *et al* (2012) and the 500bp PCR amplicon was digested by respective restriction enzymes to detect these SNPs that fell within the restriction sites. The four sets of primers(see Table 6) chosen were used to run PCR reactions on the *Xcm* samples and only the reactions from which clear products were got were further digested by the specific restriction enzymes. The controls of the PCR digests were three *Xcm* strains: *Xcm* NCPPB 2005 and *Xcm* NCPPB 2251 from Ethiopia (Lineage I) and *Xcm* NCPPB 4380 from Uganda (Lineage II).

4.4.1 RsaACHT01000045FR primer PCR and RsaI restriction digestion of Xcm isolates from Western Uganda

The primers RsaACHT01000045FR distinguished between the two sub-lineages (lineage I & II) of *Xcm* populations. *Xcm* strains in lineage I have the nucleotide G at position 32848 while those in lineage II have the nucleotide A in the same position in the draft *Xcm* genome (Wasukira *et al*, 2012). They amplified the DNA target region around the SNPs and the 500bp product was digested by the enzyme RsaI. The RsaI enzyme recognizes and digests the restriction site-sequence 5'-GT↑AC-3' or 3'-CA↑TG-5'.

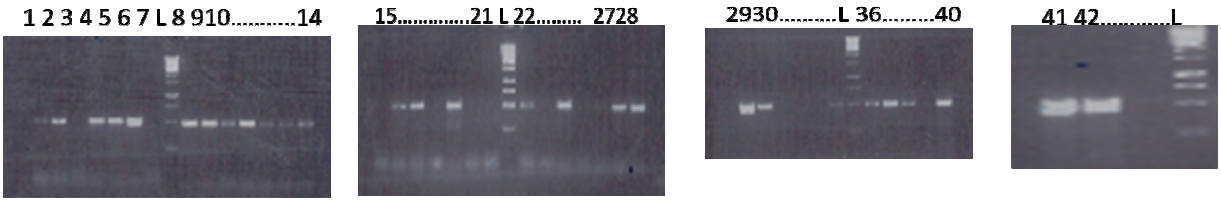


Figure 48:RsaACHT01000045FR PCR amplicons (500bp) of the Xcm samples (1-42) from Western Uganda (refer to Table 15), the PCR amplicon was 500bp

[Note: L 36...40 means the next gel wells are sample 36,sample 37,sample 38,sample 39 till sample 40]

4.4.1a RsaI digest of the products

Refer to Table 15 for summary of the restriction digest results. The PCR digest product was 250bp. *Xcm* isolates 2, 12, 15, 16, 17, 67 and 73 were left out in this assessment. Only one control *Xcm* (NCPPB 4380; from Uganda) was digested by the RsaI enzyme, while both Ethiopian *Xcm* strains *(*NCPPB 2005 and *Xcm* NCPPB 2251) were included. Not all PCR amplicons were digested, suggesting that the *Xcm* populations from Western Ugandan not only include *Xcm* strains from Uganda (lineage II) but also *Xcm* strains from lineage I and hence share a similar genotype with those from Rwanda, DR Congo and Ethiopia. Samples that digested with *Xcm* NCPPB 4380 (isolate from Uganda, Wasukira *et al*, 2012) were assumed to share a similar genotype with *Xcm* NCPPB 4380.

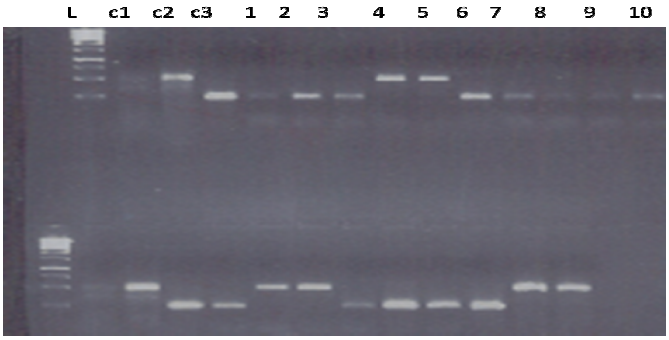


Figure 49: RsaI digests of the isolates from Western Uganda give a 250bp product. The PCR digest separates the two major sub-lineages (I &II) of Xcm.

[Note: c1, c2, c3 are the controls Xcm NCPPB 2251(Ethiopia, lineage I), Xcm NCPPB 2005 (Ethiopia, lineage I) and Xcm NCPPB 4380 (Uganda, lineage II), respectively, the

digest product is 250bp. Only control Xcm NCPPB 4380 together with other samples were digested.]

4.4.2 StyACHT01000140FR primer PCR and StyI restriction digestion of Xcm isolates from Western Uganda

The primers StyIACHT01000140FR also distinguished between the two sub-lineages (lineage I & II) of *Xcm* populations. *Xcm* strains in lineage I have the nucleotide T at position 2515 and the nucleotide C at position 2530 while those in lineage II have the nucleotide C at position 2515 and the nucleotide T at position 2530 in the draft genome of *Xcm* (Wasukira *et al*, 2012). The StyI enzyme recognizes and digests the restriction site-sequence CCT↑AGG or CCA↑TGG) and CCT↑TGG or CCA↑AGG. The target DNA from *Xcm* isolates was amplified around the SNPs. The PCR amplicon was 500bp.

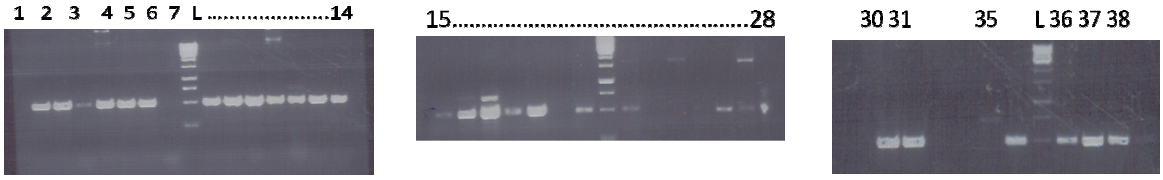


Figure 50: StyACHT01000140FR PCR amplicons of the new collection of Xcm isolates from Western Uganda, the PCR amplicon was 500bp (see Table 15 for summary of results).

[Note: L...14, means the next gel wells are the ladder, sample 8,9,10,11,12 till sample 14 while 15....28,means samples 15,16,17,18,19,20,21,22,23,24,25,26,27 till sample 28.]

4.4.2a StyI digest of the products

Refer to table 15 for summary of the restriction digest results. The product size of the digestion was 250bp.Only one control *Xcm* NCPPB 4380 (from Uganda) was digested by the StyI enzyme further confirming that some of the *Xcm* populations from Western Uganda fell within sub lineage I and were of similar genotype as *Xcm* NCPPB 4380 (Wasukira *et al*, 2012). Not all PCR amplicons of other *Xcm* isolates were digested further confirming that the *Xcm* populations from Western Ugandan not only include *Xcm* strains from Uganda (lineage II) but also *Xcm* strains from lineage I and hence share a similar genotype with those from Rwanda, DR Congo and Ethiopia.

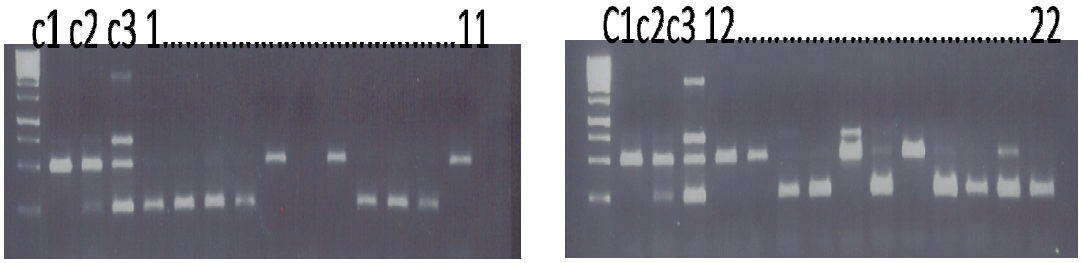


Figure 51: Styl digests of the Xcm isolates from Western Uganda by the enzyme Styl, the PCR digest separates the two sub lineages I&II of Xcm.

[Note: c1, c2, c3 are the controls Xcm NCPPB 2251(Ethiopia, lineage I), Xcm NCPPB 2005(Ethiopia, lineage I) and Xcm NCPPB 4380 (Uganda, lineage II), respectively, the digest product was 250bp. Only control Xcm NCPPB 4380, together with other isolates were digested. 1.....11,means the next gel wells are samples 1,2,3,4,5,6,7,8,9,10 till sample 11 while 12...22 means sample 12,13,14,15,16,17,18,19,20,21 till sample 22.]

4.4.3 AluACHT010000 43FR primer PCR and AluI restriction digestion of Xcm samples from Western Uganda

The primers AluACHT01000043FR distinguished between the two Ethiopian strains *Xcm* NCPPB 2005 and *Xcm* NCPPB 2251 at position 10914 with the nucleotide C in *Xcm* NCPPB 2005 and the nucleotide T in *Xcm* NCPPB 2251. However the nucleotide C is also found at the same position (10914) within the lineage II strains from Uganda – NCPPB 4379, 4383, 4384, 4380, 4381, Tanzania- NCPPB 4394, NCPPB 4395, NCPPB 4381, Kenya- NCPPB 4434 and Burundi- NCPPB 4433 (Wasukira *et al*, 2012). The target DNA of the *Xcm* isolates was amplified around the SNPs. The PCR amplicon was 500bp and was digested by the enzyme AluI. This enzyme recognizes and cuts the site sequence AG↑CT or TC↑GA.

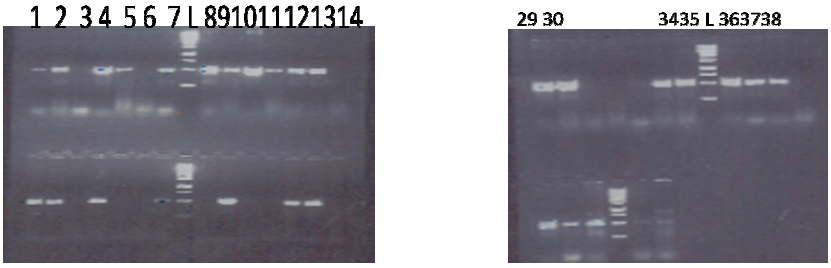


Figure 52: AluACHT010000 43FR PCR amplicons from the new collection of Xcm isolates from Western Uganda, the PCR amplicon was 500bp (see Table 15 for summary of results).

4.4.3a AluI digest of the products

The above products were set up for digestion by the restriction enzyme AluI. Of the three controls used in the digests, *Xcm* NCPPB 2215 (Enset Ethiopia, Lineage I), *Xcm* NCPPB 2005 (Ethiopia, Lineage I) and *Xcm* NCPPB 4380 (Uganda, lineage II), only *Xcm* NCPPB 2005 and *Xcm* NCPPB 4380 were digested, producing one band of 250bp. This revealed that some of *Xcm* populations within the Western Uganda have similar genotype to the Ethiopian stain *Xcm* NCPPB 2005 and the Ugandan strain *Xcm* NCPPB 4380. However not all PCR amplicons were digested also suggesting that some of *Xcm* populations from Western Uganda may also share a similar genotype with the Ethiopian isolate *Xcm* NCPPB 2251.

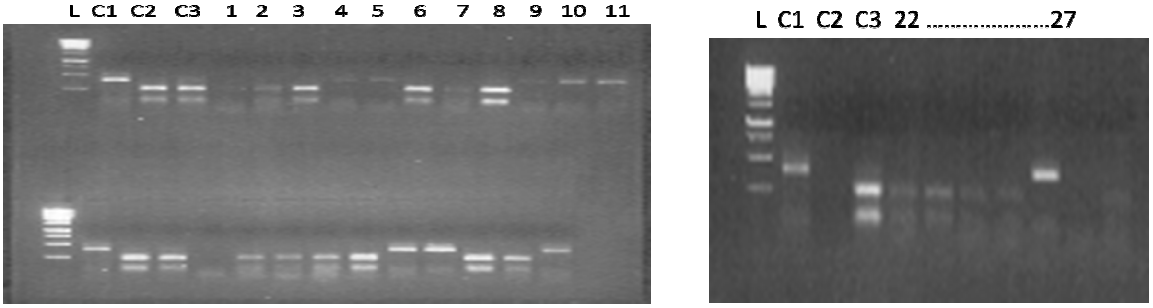


Figure 53: ALuI digests of the AluACHT0100043FR PCR amplicons

Note: c1, c2, c3 are the controls Xcm NCPPB 2251(Ethiopia, lineage I), Xcm [NCPPB 2005(Ethiopia, lineage I) and Xcm NCPPB 4380(Uganda, lineage II) respectively, the digest product is 250bp. The controls Xcm NCPPB 4380 and Xcm NCPPB 2005 together with other samples were digested. 22.....27 means the next gel wells are samples 23,24,25,26 till sample 27.]

4.4.4 BgCHT01000242FR primer PCR and BgII restriction digestion of Xcm isolates from Western Uganda

The BgII enzyme also differentiates between the two Ethiopian strains *Xcm* NCPPB2005 and *Xcm* NCPPB 2251 at position 12462 with the nucleotide G in *Xcm* NCPPB 2005 and the nucleotide A in *Xcm* NCPPB 2251. However, the nucleotide G is also found in the same position within the lineage II strains: Uganda –NCPPB 4379, NCPPB 4383, NCPPB 4384, NCPPB 4380, NCPPB 4381, Tanzania- NCPPB 4394, NCPB 4395, NCPPB 4381, Kenya- NCPPB 4434 and Burundi- NCPPB 4433 (Wasukira *et al*, 2012)). The target DNA of the *Xcm* isolates was amplified around the SNPs. The PCR amplicon was 500bp and was digested by the enzyme BgII.

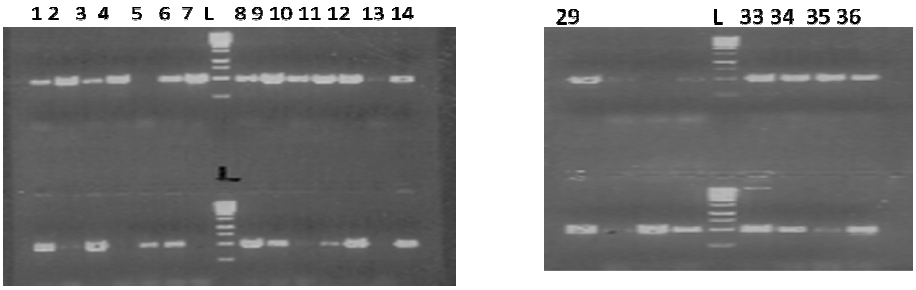


Figure 54: BgACHT01000242FR PCR amplicons from the new collection of Xcm isolates from Western Uganda, the PCR amplicon was 500bp (see Table 15 for summary of results).
[Note: 29.....L means the next gel wells are samples 30,31,32 till the ladder.]

4.4.4a BgII digestion of the PCR amplicons

The products were digested with the restriction enzyme BgII and the product size was 250bp. Two controls: the Ethiopian strain *Xcm* NCPPB 2005 (isolated from Enset, Lineage I) and the Ugandan strain *Xcm* NCPPB 4380 (lineage II) were digested while the other Ethiopian control *Xcm* NCPPB 2251 was not. This suggests that some of the *Xcm* populations within Western Uganda share the same genotype as that of the Ugandan *Xcm* NCPPB 4380 and the Ethiopian *Xcm* strain NCPPB 2005. Not all products were digested further which suggested that some of the *Xcm* populations of

Western Uganda may also share a similar genotype with *Xcm* NCPPB 2251 from Ethiopia.

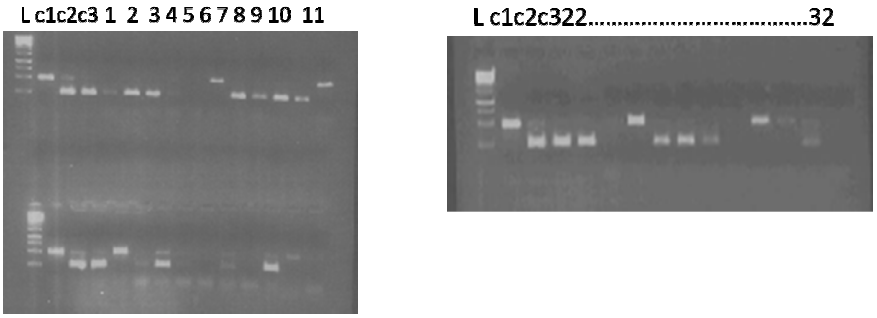


Figure 55: BgII digests of BgACHT01000242FR PCR amplicons

[Note: c1, c2, c3 are the controls Xcm NCPPB 2251(Ethiopia, lineage I), Xcm NCPPB 2005(Ethiopia, lineage I) and Xcm NCPPB 4380(Uganda, lineage II) respectively, the digest product is 250bp. The controls Xcm NCPPB 4380 and Xcm NCPPB 2005 together with other samples got digested. 22.....32 means the next gel wells are samples 23,24,25,26,27,28,29,30,31 till sample 32.]

4.4.5 Summary of SNP genotyping of Western Ugandan Xcm samples

Table 15 summaries the results of the genotyping of the *Xcm* isolates from Western Uganda based on the SNP primers used. Out of the 86 SNPs that distinguish between lineage I and II (Wasukira *et al*, 2012), only 2 SNP primers were assayed. The *Xcm* isolates (from the three districts; Bushenyi, Kabale and Ntungamo in Western Uganda) consist of strains that fall either within the sub-lineage I and sub-lineage II, but also revealed isolates that were new recombinants between lineage I and II. The presence of new recombinants suggests that the two sub-lineages have been in close proximity for a long time. Those that fall within sub-lineage II consistent with Wasukira *et al* (2012) are more genetically similar to the Ugandan strain *Xcm* NCPPB 4380 (originally from Kampala district, central region). However, some of these Western Ugandan isolates also share similar SNP positions with the Ethiopian strain *Xcm* NCPPB 2005 isolated from enset in Ethiopia. The samples within sub-lineage I have the same genotype as the Ethiopian strain *Xcm* NCPPB 2251 (also similar to those from DR Congo NCPPB 4378, and Rwanda NCPPB 4387). There is evidence of DNA recombination between *Xcm* strains from sub-lineage I and sub-lineage II, forming a new 'hybrid' genotype; for

example sample 14 and 54. Both sub-lineages of *Xcm* were present in all districts and even within a village.

Table 15: Summary of the SNP analysis of Xcm samples from Western Uganda, revealing their genotypes and sub-lineages

Sample ID	Alu45FR AluI	BglI242FR BglI	Rsa45FR RsaI	Sty140FR StyI	Similar Genotype	Sub-lineage	District	Village
NCPPB 2215	0	0	0	0		I	n/a	n/a
NCPPB 2005	1	1	0	0		I		
NCPPB 4380	1	1	1	1		II		
2	1	1	Nd	1	NCPPB 4380	II	Kabale	Nyamishaki
3	1	1	1	1	NCPPB 4380	II	Kabale	Nyamishaki
4	Nd	1	Nd	Nd	NCPPB 4380/2005	not known	Kabale	Nyamishaki
6	1	1	1	1	NCPPB 4380	II	Kabale	Rushyebeya
7	Nd	Nd	1	1	NCPPB 4380	II	Kabala	Rushyebeya
8	0	0	Nd	0	NCPPB 2215	I	Kabale	Rushyebeya
9	Nd	0	Nd	Nd	NCPPB 2215	I	Kabale	Rushyebeya
10	0	0	0	0	NCPPB 2215	I	Kabale	Rushyebeya
11	1	1	1	1	NCPPB 4380	II	Kabale	Kanyakutana
12	1	1	1	1	NCPPB 4380	II	Kabale	Kanyakutana
13	1	1	1	1	NCPPB 4380	II	Kabale	Kanyakutana
14	0	1	Nd	0	NCPPB 2005/2251	I	Kabale	Kanyakutana
15	0	0	Nd	0	NCPPB 2215	I	Kabale	Kanyakutana
17	0	Nd	Nd	0	NCPPB 2215	I	Kabale	Nyamitooma
21	Nd	0	Nd	Nd	NCPPB 2215	I	Kabale	Kataramabareeba
23	1	1	1	1	NCPPB 4380	II	Kabale	Kataramabareeba
30	1	Nd	1	Nd	NCPPB 4380	II	Kabale	Kabugarama
34	Nd	1	Nd	Nd	NCPPB 4380/2005	not known	Ntungamo	Ihuriro

36	1	Nd	1	1	NCPPB 4380	II	Ntungamo	Nyankagongo
39	Nd	1	Nd	Nd	NCPPB 4380/2005	not known	Ntungamo	Nyankagongo
40	Nd	1	Nd	Nd	NCPPB 4380/2005	not known	Ntungamo	Nyankagongo
43	Nd	0	Nd	Nd	NCPPB 2215	I	Ntungamo	Bucence
44	1	0	1	Nd	NCPPB 4380	II	Ntungamo	Bucence
46	0	0	Nd	Nd	NCPPB 2215	I	Ntungamo	Kitojo
49	Nd	Nd	0	0	NCPPB 2215	I	Ntungamo	Kitojo
53	1	1	1	1	NCPPB 4380	II	Ntungamo	Kigarama
54	1	Nd	1	0	Recombinant	Recombinant	Ntungamo	Kigarama
62	Nd	0	Nd	Nd	NCPPB 2215	I	Bushenyi	Matigi
64	1	0	Nd	1	NCPPB 4380	II	Bushenyi	Matigi
67	1	1	Nd	1	NCPPB 4380	II	Bushenyi	Nyabubare
70	1	1	1	1	NCPPB 4380	II	Bushenyi	Nyabubare
73	1	1	Nd	1	NCPPB 4380	II	Bushenyi	Kizumo
76	1	Nd	1	Nd	NCPPB 4380	II	Bushenyi	Nyakabingo
77	0	1	0	Nd	NCPPB 2005	I	Bushenyi	Nyakabingo
81	0	0	0	Nd	NCPPB 2215	I	Bushenyi	Keijengye
83	0	0	Nd	Nd	NCPPB 2215	I	Bushenyi	Keijengye
84	Nd	0	Nd	Nd	NCPPB 2215	I	Bushenyi	Keijengye
90	Nd	1	Nd	Nd	NCPPB 4380/2005	Unknown	Bushenyi	Kibaare B

[Note: 1 indicates sample was digested by enzyme, 0 indicates it was not digested by enzyme, nd indicates sample was not assessed, the controls were Xcm NCPPB 2251 and NCPPB 2005 from Ethiopia (Lineage I) and Xcm NCPPB 4380 from Uganda (Lineage II). The strains that fell in neither lineage I or II were considered to be recombinants (in red) or new genotypes.]

5. DISCUSSION

This study has been able to provide more substantial data on the comparative pathogenicity for strains identfied as *Xanthomonas vasicola* (*Xv*) pathovars and *Xanthomonas campestris* pv *musacearum* (*Xcm*). Strains of *Xv* pv *vasculorum (Xvv)* and *holcicola (Xvh)* are able to cause disease in maize and sugarcane through artificial inoculation. Even though two pathogens may cause similar symptoms such as lesions or streaks on leaves of maize and sugarcane, they caused distinct symptoms in maize: *Xvv* causes deformation of the plant while *Xvh* causes stunted growth. Unfortunately, we were not able to conduct pathogenicity tests on sorghum, to further confirm the difference between *Xvv* and *Xvh*. The *Xv* pathovars are divided into two groups (Vauterin *et al*, 1995) *holcicola* and *vasculorum* for sorghum and sugarcane respectively. *Xvv* caused deformation/distortion of the plant on maize while *Xvh* causes stunted growth on maize, revealing distinct pathogenicity on the same host. However, some of the maize plants that had been affected seemed to recover and showed no more signs of disease suggesting that maize may be less susceptible to both pathogens. Symptoms caused by both *Xv* pathovars including *Xcm* on sugarcane were similar, that is reddish-brown streaks or lesions on the leaves. Though one strain of *Xvh* NCPPB 3162 used in all the main pathogenicity trials was successfully re-isolated, it did not cause any symptoms in both maize and sugarcane. This may be an indication that there could also be variation in virulence among strains within this pathovar and that under some circumstances some strains can act as endophytes. The study has also shown *Xv* pathovars to be non-pathogenic on banana, but able to survive within the plant asymptomatically. This suggests banana can be a host to other *Xanthomonas* strains (apart from *Xcm*) including *Xvv* and *Xvh* strains. *Xvv* and *Xvh* showed distinct pathogenicity on maize: *Xvv* caused deformation of the plant while *Xvh* causes stunted growth. *X. axonopodis* pv *vasculorum (Xav)* is a pathovar that though does not fall within the *X. vasicola* species, but causes similar symptoms (apart from deformation and stunted growth) on maize and sugarcane. It was demonstrated that *Xav* is pathogenic on maize and sugarcane, but not banana. This suggests that *Xav* may share a few genetic similarities with the *X. vasicola* pathovars in terms of its ability to cause disease both in maize and sugarcane.

Previous studies have shown *Xcm* to be able to cause disease in maize (Aritua *et al*, 2008)but these findings have been weakly supported in our study. Possible reasons as to why maize plants in the main trial that had been inoculated with *Xcm* remained healthy include differences in maize cultivars, growth stage or growth conditions of the two trials. The ability of a pathogen to cause disease at one time and not another usually occurs due to changes in the host that alters the host-microbe interaction. This suggests that most likely the conditions of the green house or the maize varieties used in both studies were critical factors. However, as seen from the pathogenicity trials where by the maize plants and some banana plants inoculated with *Xcm* remained healthy further supports that fact that at times disease is the exception rather than the rule (Guest and Brown, 1997). It is not always clear cut that disease will depend on the resistance or susceptibility of the host plant. The host-parasite-environment interaction may be mediated by other complex interchange of signals (Guest and Brown, 1997).

The successful re-isolation of *Xcm* from the healthy maize plants also further suggests that maize can be a reservoir for *Xcm* strains. This is highly significant and should contribute to the control methods of *Xanthomonas* wilt (XW) of bananas especially since maize are among the crops that usually intercropped with bananas in Uganda. *Xcm* was also clearly pathogenic on sugarcane and the symptoms on sugarcane were very similar to those caused by the *Xv* pathovars. Only *Xcm* was distinctly pathogenic on banana while the *Xv* pathovars did not affect banana. The probable cause for the healthy *Xcm*-inoculated banana plants may have been due to an inoculation error or low concentrations of the bacteria in the inoculums. *Xanthomonas* species NCPPB 1131 and 1132 originally isolated from bananas (Studholme *et al*, 2011) were shown to be non-pathogenic on banana. Previous studies (Aritua *et al*, 2008, Parkinson *et al*, 2009) have shown *Xcm* to fall within the *Xv* species and the pathogenicity trials have revealed that there are clearly interspecific pathovar differences within the species. Of all the pathovars, *Xvv* is the most virulent and it is therefore significant that it is not pathogenic on banana. The pathogenicity trials support the hypothesis that *Xcm* is distinct from *Xvv.*

The ELISA polyclonal antibody assay, formatted into an LFD, was able to detect *Xcm*. However, it cross-reacted with *Xav,* the strain that causes Gumming disease in sugarcane*. Xav* may not be a significant strain in this study but it causes similar symptoms of leaf lesions and streaks as the *Xv* pathovars as seen in the pathogenicity trials. Though DNA-DNA homology studies and B-gyrase analysis have shown this strain to belong to the *X. axonopodis* species, it may share most of its virulence proteins with *Xvv* and *Xcm.* This hypothesis is supported by the fact that *Xav* was not only pathogenic on sugarcane but also maize consistent with *Xv* pathovars. Furthermore, XW PCR primers (Adikini *et al*, 2011) that were designed from intergenic regions of *Xcm* also amplified DNA templates from *Xav* strains.

The ELISA cross-reaction also confirms that *Xcm* and *Xav* may be sharing distinctive proteins that are not conserved in *Xvv.* To test this hypothesis we ran a protein blast on the NCBI website between *Xcm* NCPPB 4381, *Xav* NCPPB 702 and *X.axonopodis* pv *citri* (the closest available whole genome to *Xav*). However, the percentage similarity between *Xcm* and *Xvv* or *Xcm* and *X.axonopodis* pv *citri* was at least 95% and this made it difficult to pinpoint which proteins may be differentiating.

The cross-reaction may also be due to the fact that the lipo-polysaccharide locus in *Xcm* closely matches that of *Xanthomonas axonopodis* (93% nucleotide sequence identity; Studholme *et al*, 2010). Although lipo-polysaccharides are not proteins, theoretically the polyclonal antibodies could interact with the cell walls (made of lipo-polysaccharides) of the antigen (live bacteria) first.

This cross-reaction of the polyclonal, formatted for ELISA or LFDs, does not lessen the usefulness of the diagnostic as *Xav* is not a known pathogen on banana and therefore unlikely to be found in banana at high concentrations. IITA's polyclonal antibody for *Xanthomonas* wilt of bananas not only cross-reacted with *Xav* but with *Ralstonia solanacearum* and *Xanthomonas arboricola* pv *celebensis*. This also implies that some proteins in *Xcm* may be similar to those in *R. solanacearum* and *Xanthomonas*

arboricola pv *celebensis.* We blasted proteins from *R. solanacearum* Po82, one of the available genomes on the NCBI website against *Xcm* NCPPB 4381 to determine any proteins shared. Only few proteins (such as putative secreted proteins 44% or phusphoglycosamine mutase 52%) were similar between the two strains but were less than 55% similar, which does not sufficiently explain why the IITA's XW polyclonal cross-reacted with *R. solanacearum.* Potentially the cross-reactivity associated with the IITA polyclonal may present a diagnostic constraint as both *R. solanacearum* and *Xac* are pathogens of banana.

The LFD was able to detect bacteria at concentrations as low as 10^4 cfu/ml. It can be assumed that an infected banana plants (most likely the East African Highland bananas) in Uganda will tend to have more than 10^{10} cfu/ml concentration of bacteria and therefore be easily detected.

Under this study the most effective PCR diagnostic assays for detecting *Xcm* was shown to be the GspDmFR and NZO85FR primers from Adriko *et al* (2011) and XW1F/3R primers from Lewi-Ivey *et al* (2010). Despite the fact that they (apart from GspDmFR), hybridize DNA templates from the closely related *Xvv* they still can be effective tools for confirming *Xcm* as the *Xv* pathovars are not pathogenic on bananas and any positive amplification can therefore be assumed to be *Xcm*. With whole genome wide sequencing studies on *X.campestris* pv *musacearum* and *Xvv*, it is now possible to design more specific primers for *Xcm* (Wasukira *et al*, 2012).

SNP analysis of the Western Ugandan *Xcm* isolates revealed multiple genotypes within the samples. Most of the samples fall within the sub-lineage II and have the same genotype as *Xcm* NCPPB 4380 originally from Kampala (Central region of Uganda, where XW of banana was first reported in 2001). This suggests that there may be 'movement' of these strains from the Central region where *Xcm* was first reported in 2001 to the Western part of the country. The districts of Kabale and Ntungamo are also close to the borders of Rwanda and DR Congo making it possible that Western Uganda isolates within sub-lineage I could have been carried into Uganda across the two

borders perhaps through already infected suckers or plant material (see map in Figure 56).

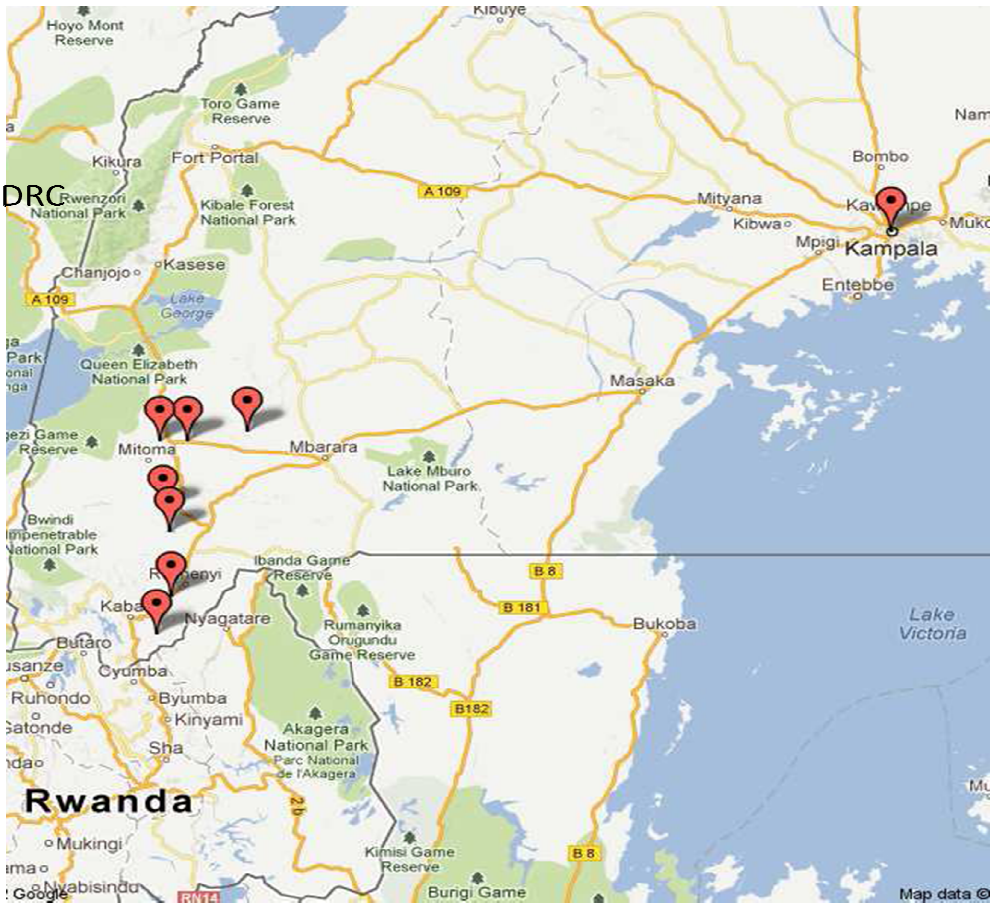


Figure 56: Google Map showing the towns from which the Western Ugandan Xcm samples came from and their close proximity to Kampala (Central region), DR Congo (DRC) and Rwanda

[Note: The red dots are some of the sub-counties (left side) (Kaharo, Bwongyera, Nyabubare, Maziba, Nyabihoko, Kigarama from which the Western Uganda samples were isolated.]

SNP genotyping of these isolates also revealed new 'hybrid' genotypes between bacteria under sub-lineage I and bacteria under sub-lineage II most probably through recombination, suggesting that these two sub-lineages have been in close proximity for a long period of time.

6. CONCLUSION AND FUTURE WORK

The pathogenicity trials have added substantial data to the comparative pathogenicity studies of the *Xv* pathovars which due may be sufficient to reassign *Xcm* to *X.vasicola* pv *musacearum.* However, the failure under this study to cause disease on sorghum may yet still frustrate this change. Pathogenicity tests of the *Xv* pathovars on sorghum is important to further confirm the difference between *Xvv* and *Xvh.* Genome wide sequencing of *Xav* would reveal the relationship between *Xav* and the *Xv* pathovars. It would be significant as well to isolate *Xanthomonas* strains from naturally growing maize in the fields to determine if *Xcm* naturally resides in maize as well.

The development of the LFDs based on the ELISA polyclonal antibody should be able to help plant inspectors contain the spread of *Xcm* pathogen across borders through its early detection as an on-site field tool. To complement the polyclonal diganostics, especially the LFD, Fera is developing a LAMP assay for *Xcm*, designing primers from the gene sequence of the general secretion pathway D (GspD) of *Xcm* (Adriko *et al*, 2011). This will work hand in hand with the LFD to confirm presence of the pathogen through PCR- based and antibody based systems.

Further SNP analysis of more isolates from other countries such as DR Congo and Ethiopia (as the probable centre of origin for *Xcm*) will continue to establish more genotypes of *Xcm,* which in turn would assist in determining the patterns of spread of the pathogen within the different regions of East Africa. Such knowledge will motivate new methods of possible containment and control of the pathogen.

7. APPENDIX

Appendix 1: Full Standard protocol for the Plate trapped antigen ELISA assays

1. Prepare dilution of antigen in coating buffer
2. Coat appropriate number of plates with 100µl per well
3. Wrap plates in cling film and incubate at 4◦C overnight
4. Wash plates three times with PBST,250µl per well
5. Prepare blocking buffer (3% BSA/PBST) and block plates with 200µl per well
6. Wrap plates with cling film and incubate for 1hour at 33◦C
7. Wash plates three times with PBST,250µl per well
8. Dilute control antibody if available in 1:1000
9. Add antibody to relevant wells,100µl per well
10. Wrap plates with cling film and incubate for 1hour at 33◦C
11. Wash plates three times with PBST,250µl per well
12. Prepare dilution of anti-species polyclonal antibody in dilution buffer (PBST/ 0.2% BSA)
13. Add anti-species polyclonal antibody used, 100µl per well
14. Wrap plates with cling film and incubate for 1hour at 33◦C
15. Wash plates three times with PBST,250µl per well
16. Add appropriate substrate
17. If the anti-species polyclonal antibody used was alkaline Phosphatase conjugated, prepare a solution of pNPP at 1mg/ml in substrate buffer
18. Add 100µl per well
19. Incubate in the dark for 1 hour at room temperature
20. Read plates at 405nm

Appendix 2: Full pathogenicity trial plan for banana, maize and sugarcane

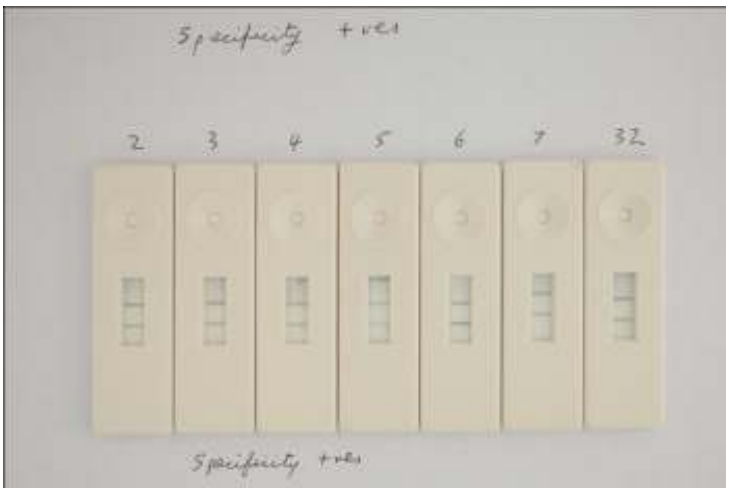
Plant number	NCPPB no.	Species name
1	---	Untreated
2		
3		
4		
5	---	Dummy inoculated
6		
7		
8		
9	2985	*Xanthomonas campestris* pv *perlagonii*
10		
11		
12		
13	2198	*Xanthomonas arboricola* pv *celebensis*
14		
15		
16		
17	796	*Xanthomonas axonopodis* pv *vasculorum*
18		
19		
20		
21	899	*Xanthomonas axonopodis* pv *vasculorum*
22		
23		
24		
25	1060	*Xanthomonas vasicola* pv *holcicola*
26		
27		
28		
29	3129	*Xanthomonas vasicola* pv *holcicola*
30		
31		
32		
33	895	*Xanthomonas vasicola* pv *holcicola*
34		
35		
36		
37	702	*Xanthomonas vasicola* pv *holcicola*

Plant number	NCPPB no.	Species name
38		
39		
40		
41	890	*Xanthomonas vasicola* pv *holcicola*
42		
43		
44		
45	422	*Xanthomonas campestris* pv *vesicatoria*
46		
47		
48		
49	701	*Xanthomonas campestris* pv *vesicatoria*
50		
51		
52		
53	4379	*Xanthomonas campestris* pv *musacearum*
54		
55		
56		
57	4387	*Xanthomonas campestris* pv *musacearum*
58		
59		
60		
61	4390	*Xanthomonas campestris* pv *musacearum*
62		
63		
64		
65	206	*Xanthomonas vasicola* pv *vasculorum*
66		
67		
68		
69	2005	*Xanthomonas campestris* pv *musacearum*
70		
71		
72		
73	4434	*Xanthomonas campestris* pv *musacearum*
74		
75		
76		

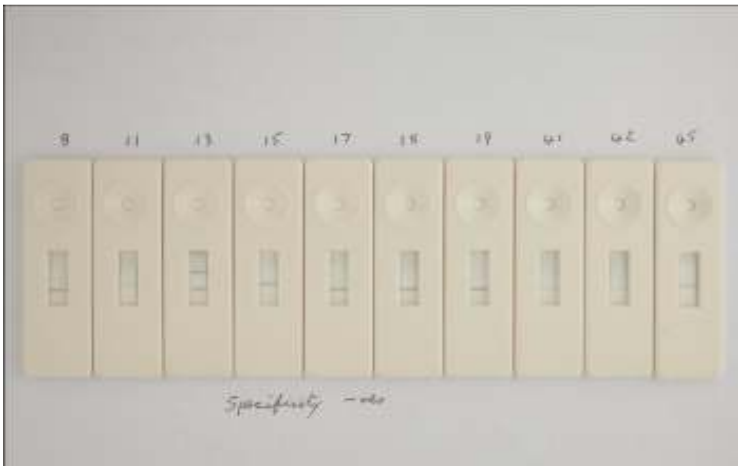
Plant number	NCPPB no.	Species name
77	4433	*Xanthomonas campestris* pv *musacearum*
78		
79		
80		
81	529	*Xanthomonas campestris* pv *campestris*
82		
83		
84		
85	4031	*Xanthomonas campestris* pv *perlargonii*
86		
87		
88		
89	1131	*Xanthomonas* spp.
90		
91		
92		
93	1132	*Xanthomonas* spp.
94		
95		
96		
97	4393	*Xanthomonas* spp.
98		
99		
100		
101	P	Paenibacillus larvae
102		
103		
104		
105	---	Untreated
106		
107		
108		
109	---	Dummy inoculated
110		
111		
112		

Appendix 3: Photos of Lateral Flow Devices showing specificity and sensitivity at different concentrations of bacteria

A) Infected leaf sap (left; Xcm NCPPB 4434) and healthy leaf sap control (right)



B) Specificity of the LFD, 2 – 7, 32 Xcm strains (refer to Table 3)

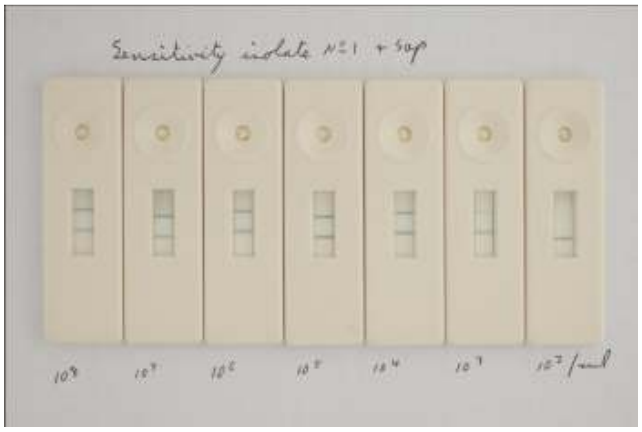


C) Specificity of LFD; 8 – Xvv NCPPB 896, 11-Xvh NCPPB 796, 13-Xav NCPPB 796,15-Xcv NCPPB 422,17-Xac NCPPB 1630,18-Xcc NCPPB 529,19-Xcp NCPPB 4031, 41 & 42-Xanthomonas spp NCPPB 1131 & 1132, 45 –Ralstonia solanacearum NCPPB 3205

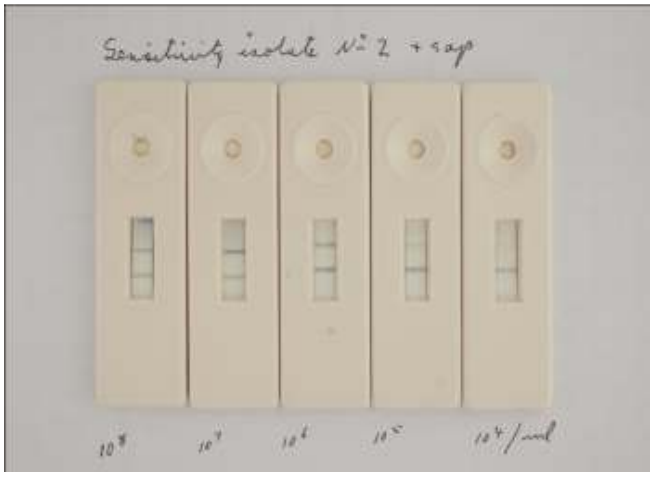
D) Sensitivity of the LFD with Xcm NCPPB 4378 strain in buffer A (extraction buffer) at different concentrations left to right; 10^7, 10^6, 10^5, 10^4, 10^3 cfu/ml

E) Sensitivity of the LFD with negative strain Xvh NCPPB 3162 in buffer A(extraction buffer) at different dilutions, left to right; 10^7, 10^6, 10^5, 10^4, 10^3 cfu/ml

F) Sensitivity of the LFD with Xcm NCPPB 4433 strain in crushed leaf extract at different dilutions left to right; 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 cfu/ml



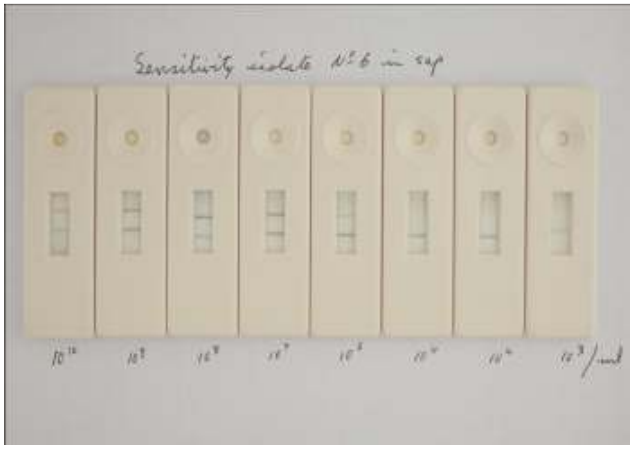
G) Sensitivity of the LFD with Xcm NCPPB 4389 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cfu/ml



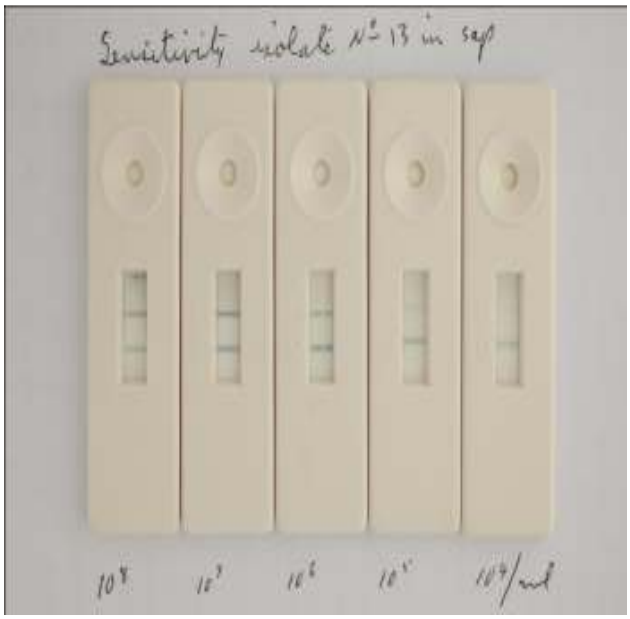
H) Sensitivity of the LFD with Xcm NCPPB 4387 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml

I) Sensitivity of the LFD with Xcm NCPPB 4392 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml

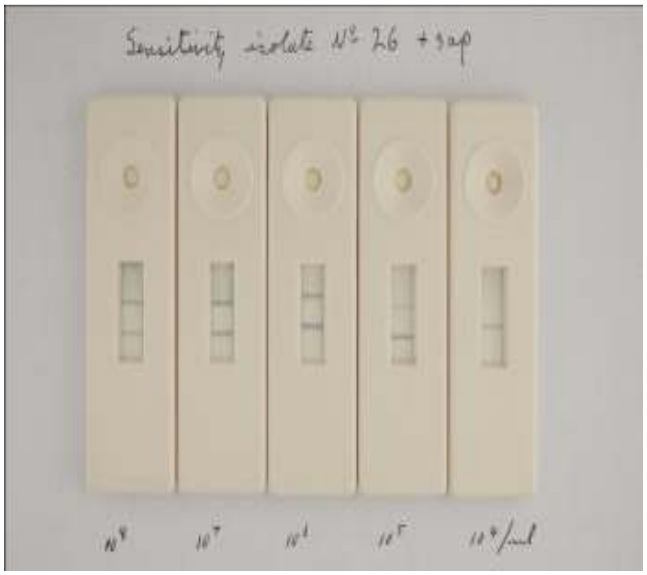
J) Sensitivity of the LFD with Xcm NCPPB 4434 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml



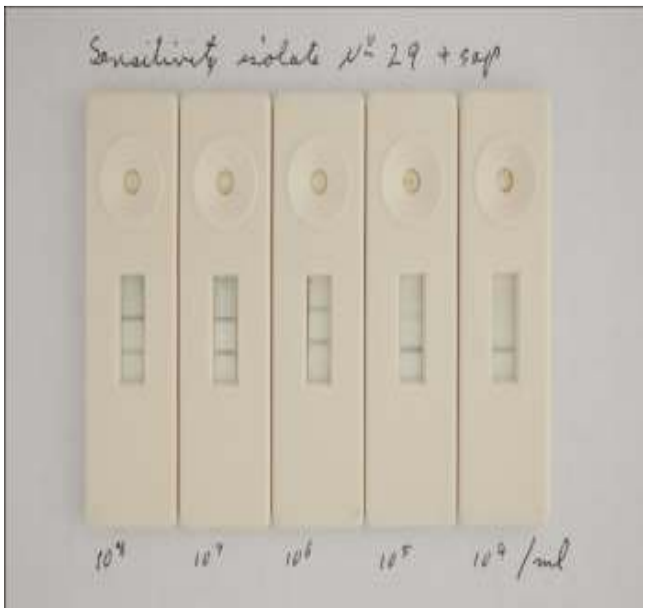
K) Sensitivity of the LFD with Xcm NCPPB 4433 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 cfu/ml



L) Sensitivity of the LFD with Xav NCPPB 796 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml

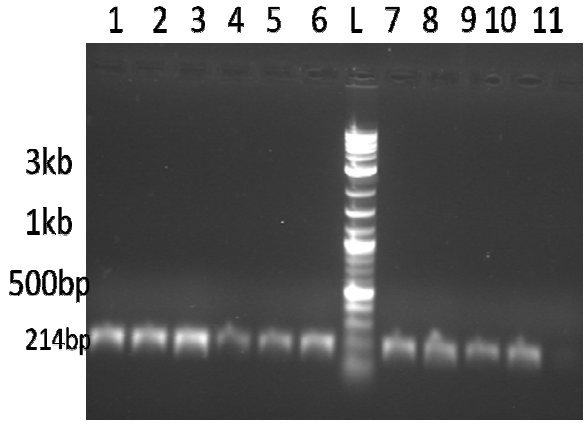


M) Sensitivity of the LFD with Xcm NCPPB 2251 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml

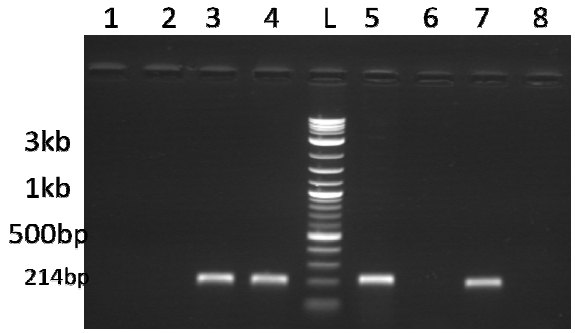


N) Sensitivity of the LFD with Xcm NCPPB 4383 in banana sap at different dilutions, left to right; 10^8, 10^7, 10^6, 10^5, 10^4 cfu/ml

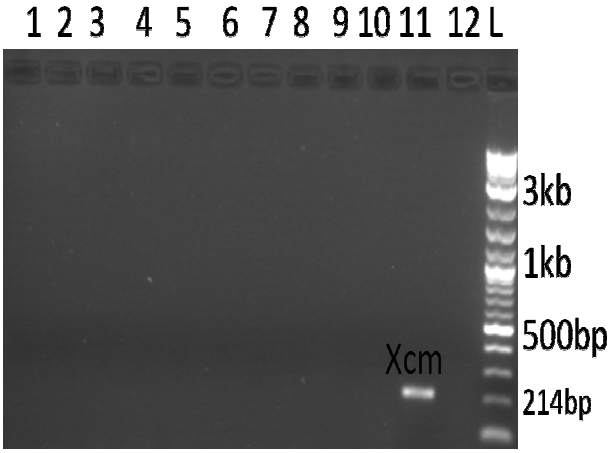
Appendix 4: PCR results of XW1F/3R on *X.campestris* pv *musacearum* strains and other bacteria strains from NCPPB



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm*
11	Water	



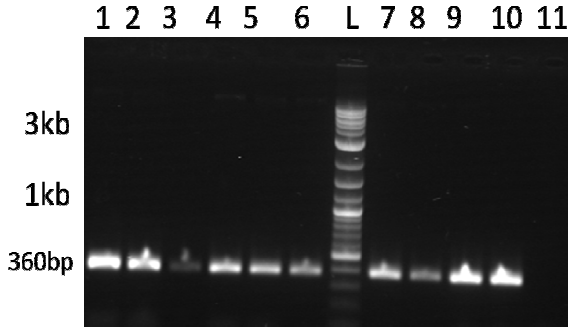
Lane	NCPPB	Strain
1	4102	*Xvh*
2	4459	*Xcv*
3	4379	*Xcm*
4	4391	*Xcm*
5	4394	*Xcm*
6	5898	*Xcm*
7	6235	*Xcm*
8	water	



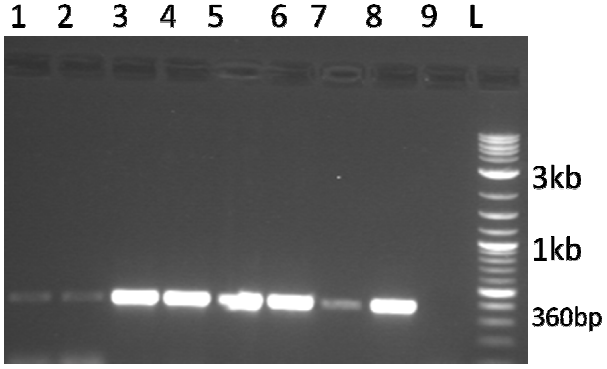
Lane	NCPPB	Strain
1	8022	Xac
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis pv marginalis*
8	8029	*Enterobacter* sp.
9	8030	*S. marcescens*
10		Healthy banana DNA
11	5959	*Xcm* (+ve control)
12	Water	

Appendix 5: PCR results of *Xcm*12FR, *Xcm*35FR, *Xcm*36FR, *Xcm*38FR, *Xcm*44FR, *Xcm*47FR, and *Xcm*48FR primers (Adikini *et al*, 2010) on *X.campestris* pv *musacearum* strains and other bacteria strains from NCPPB

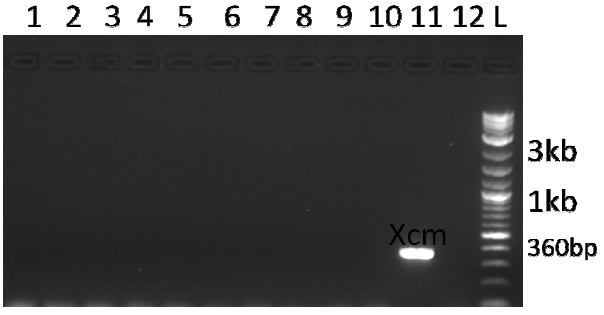
Primers Xcm12FR



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	

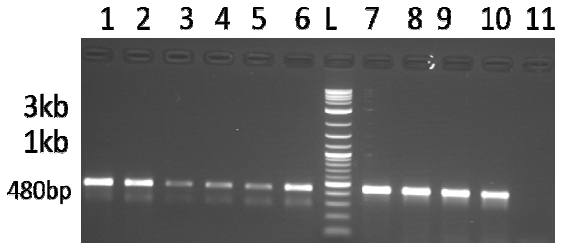


Lane	NCPPB	Strain
1	4102	*Xvh*
2	4459	*Xcv*
3	4379	*Xcm*
4	4391	*Xcm*
5	4394	*Xcm*
6	5898	*Xcm*
7	6235	*Xcm* (+ve control)
8	Water	

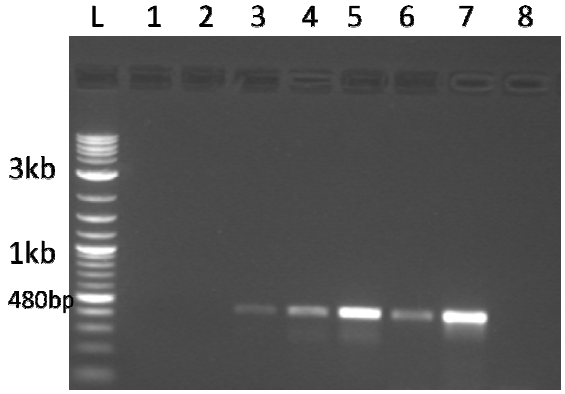


Lane	NCPPB	Strain
1	8022	Xac
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	Enterobacter sp.
9	8030	S. marcescens
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

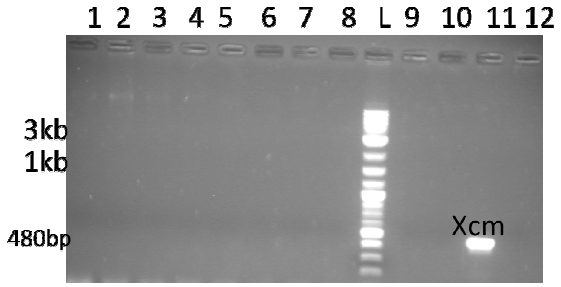
Primers Xcm35FR (Adikini et al, 2010)



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	
Lane	NCPPB	Strain

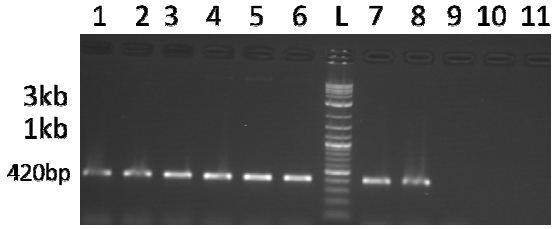


Lane	NCPPB	strain
1	4102	*Xvh*
2	4459	*Xcv*
3	4379	*Xcm*
4	4391	*Xcm*
5	4394	*Xcm*
6	5898	*Xcm*
7	6235	*Xcm* (+ve control)
8	water	

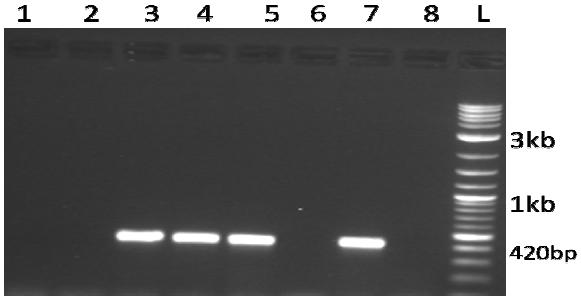


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter sp*
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	water	

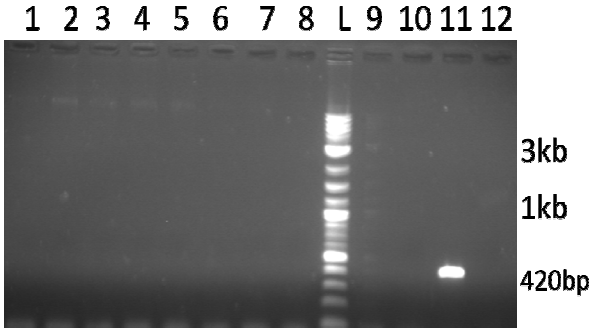
Primers Xcm36FR (Adikini et al, 2010)



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	

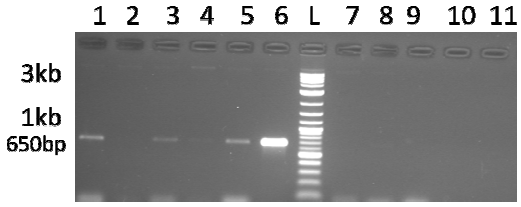


Lane	NCPPB	Strain
1	4102	*Xvh*
2	4459	*Xcv*
3	4379	*Xcm*
4	4391	*Xcm*
5	4394	*Xcm*
6	5898	*Xcm*
7	6235	*Xcm* (+ve control)
8	Water	

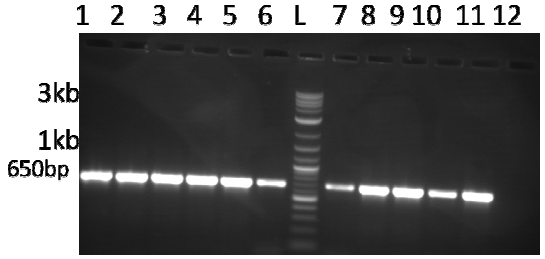


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R.solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp.
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

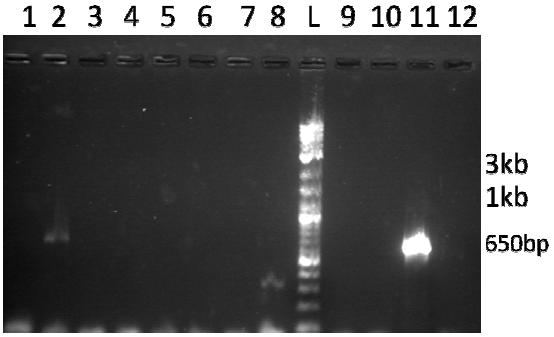
Primers 38FR (Adikini et al, 2010)



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	

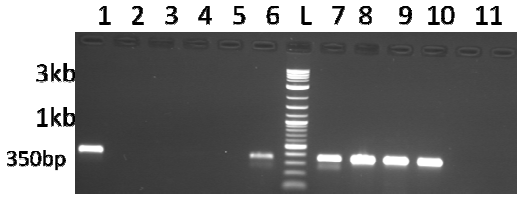


Lane	NCPPB	strain
1	4392	*Xcm*
2	4381	*Xcm*
3	4388	*Xcm*
4	4390	*Xcm*
5	4393	*Xcm*
6	4102	*Xvh*
7	4459	*Xcv*
8	4391	*Xcm*
9	4394	*Xcm*
10	5898	*Xvv*
11	6235	*Xcm* (+ve control)
12	Water	

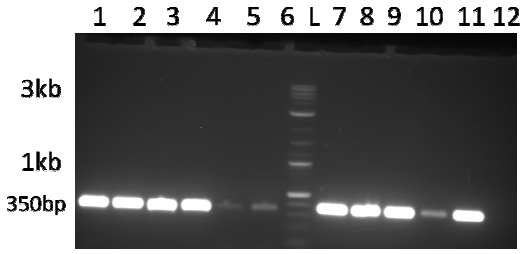


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	water	

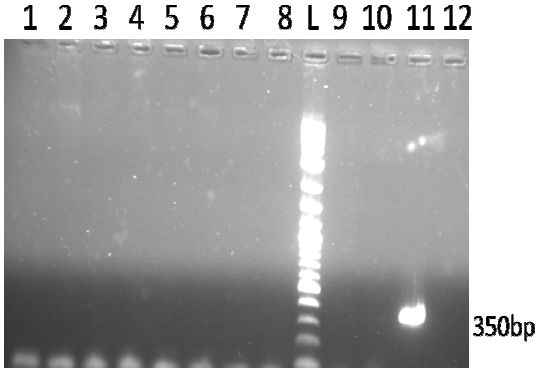
Primers 44FR (Adikini et al, 2010)



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm*(+ve control)
11	Water	

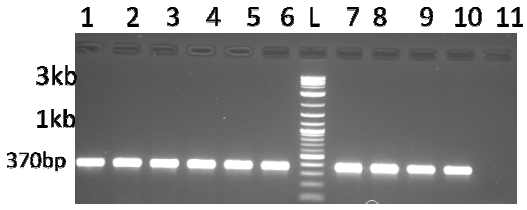


Lane	NCPPB	Strain
1	4392	*Xcm*
2	4380	*Xcm*
3	4381	*Xcm*
4	4383	*Xcm*
5	4102	*Xvh*
6	4459	*Xcv*
7	4379	*Xcm*
8	4391	*Xcm*
9	4394	*Xcm*
10	5898	*Xvv*
11	6235	*Xcm* (+ve control)
12	Water	

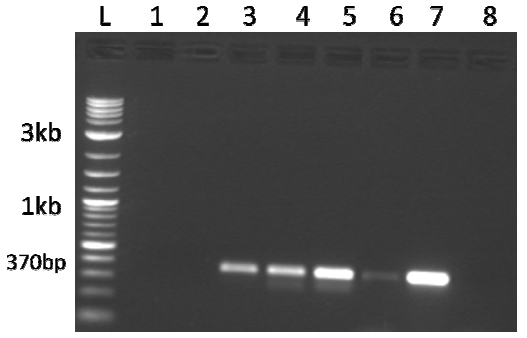


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

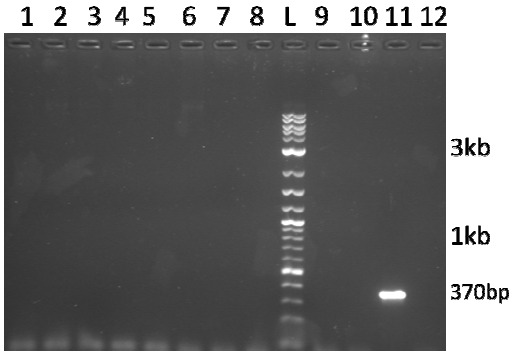
Primers 47FR (Adikini et al, 2010)



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	

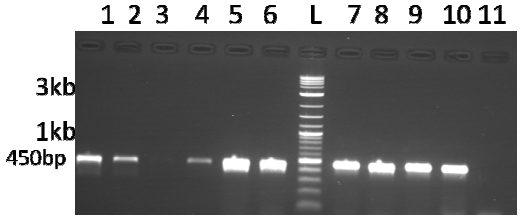


Lane	NCPPB	Strain
1	4102	*Xvh*
2	4459	*Xcv*
3	4379	*Xcm*
4	4391	*Xcm*
5	4394	*Xcm*
6	5898	*Xcm*
7	6235	*Xcm* (+ve control)
8	Water	

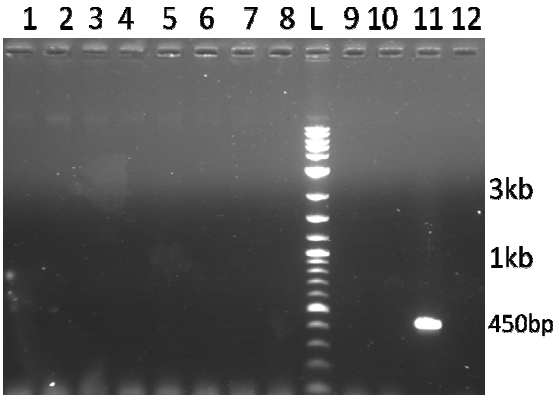


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

Primers 48FR (Adikini et al, 2010)



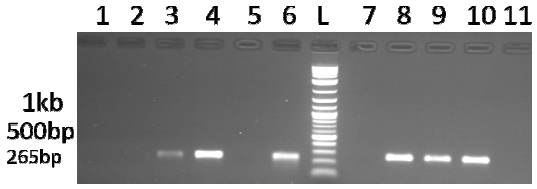
Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	



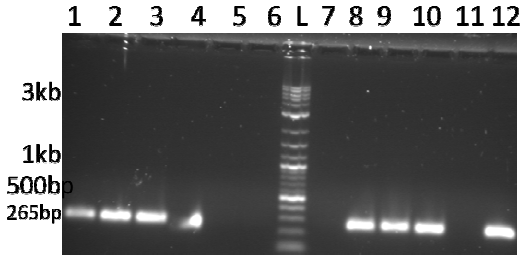
Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

Appendix 6: PCR results of GspDmFR and NZO85FR primers (Adriko *et al*, 2011) on *X.campestris* pv *musacearum* strains and other bacteria strains

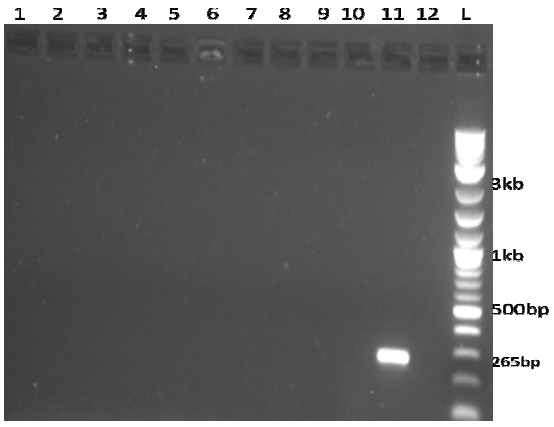
GspDmFR



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	

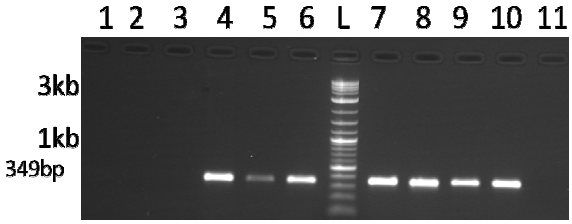


Lane	NCPPB	Strain
1	4392	*Xcm*
2	4380	*Xcm*
3	4381	*Xcm*
4	4383	*Xcm*
5	4102	*Xvh*
6	5898	*Xvv*
7	4379	*Xcm*
8	4394	*Xcm*
9	4391	*Xcm*
10	5898	*Xvv*
11	Water	
12	6235	*Xcm* (+ve control)

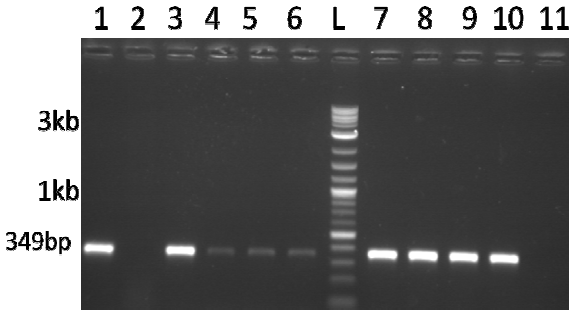


Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis* pv *marginalis*
8	8029	*Enterobacter* sp
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

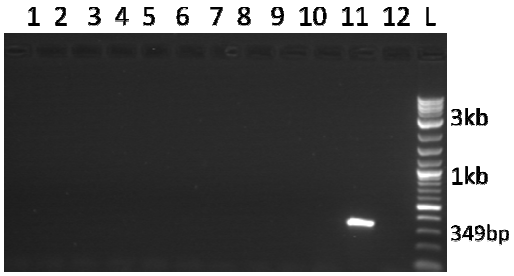
NZO85FR



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4381	*Xcm*
5	4383	*Xcm*
6	4387	*Xcm*
7	4388	*Xcm*
8	4390	*Xcm*
9	4393	*Xcm*
10	6235	*Xcm* (+ve control)
11	Water	



Lane	NCPPB	Strain
1	2005	*Xcm*
2	4392	*Xcm*
3	4380	*Xcm*
4	4102	*Xvh*
5	5898	*Xvv*
6	4459	*Xcv*
7	4379	*Xcm*
8	4394	*Xcm*
9	4391	*Xcm*
10	5898	*Xvv*
11	6235	*Xcm* (+ve control)
12	Water	



Lane	NCPPB	Strain
1	8022	*Xac*
2	8023	*R. solanacearum*
3	8024	*R. solanacearum*
4	8025	*R. solanacearum*
5	8026	*R. solanacearum*
6	8027	*R. solanacearum*
7	8028	*P. marginalis pv marginalis*
8	8029	*Enterobacter sp*
9	8030	*S. marcescens*
10		Healthy banana
11	5959	*Xcm* (+ve control)
12	Water	

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