The mechanism of resistance to *Xanthomonas vasicola* pv *musacearum* infection in *Musa balbisiana*

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DECLARATION

I Fred Ssekiwoko hereby declare that this thesis for the degree Philosophiae Doctor of University of Pretoria is my original work and has not been previously submitted by me for a degree in any other tertiary institution.

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…… ……

PROF. KARL KUNERT

DEDICATION

To my Grandmother, Kyamulabi Annet and Dad, Kizito Alyzious

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ABSTRACT

Banana Xanthomonas wilt caused by *Xanthomonas vascicola* pv *musacearum* (*Xvm*) devastates banana in East and Central Africa causing total yield loss in diseased plants. Previous germplasm screening experiments has identified *M. balbisiana*, a wild banana type, as an important *Xvm* resistance source but with exact resistance mechanism still to be defined. This PhD study was therefore aimed to evaluate the possible mechanism. For the study, clean *M. balbisiana* plantlets were required that were obtained through a newly developed tissue culture process. In the absence of a suitable banana culture medium, the MS proliferation medium was, in a first step, modified by increasing concentrations of the antioxidants ascorbic acid and thiamine-HCl to 20 mg/l and 0.18 mg/l, respectively. In addition, culturing the explants in total darkness for the first 9 weeks after initiation improved plantlet proliferation. Modifications applied enabled production of up to 40 *M. balbisiana* plantlets per explant. In a further step, multiplication and migration of *Xvm* in *M. balbisiana* plantlets following injection was investigated and compared to susceptible banana EAHB cv '*Nakinyika'*. Treatment of the youngest open leaf with a bacterial suspension delayed symptom development in *M. balbisiana* and symptoms only appeared 10 weeks after inoculation. In addition, wilt incidence and severity were only 33% and 20%, respectively, with only the symptomatic leaves, inoculated and the immediate follower leaves, eventually dying. In addition, a general decline in the population of the bacterium in the plant occurred, though the bacterium initially migrated to the leaves that are immediate followers to the inoculated leaf. In a further molecular dissection of the active defense pathway, a sharp decline in transcription of several genes involved in pathogen resistance was found. After inoculation, transcription of *MbNBS* (an R gene),

MdNPR1 (NPR1 gene) and *PR3* (a typical PR gene) was below basal transcription in both resistant *M balbisiana* and susceptible banana EAHB cv '*Nakinyika'* indicating that *Xvm* can limit the banana's ability to timely detect and set up effective defenses in both resistant and susceptible banana. Gene transcription recovered, however, above basal transcription in later infection stages in resistant *M balbisiana*, although this did not prevent wilt symptoms from eventually developing. Investigation into *M balbisiana* resistance also identified heat stable pre-existing compounds soluble in methanol that can inhibit *Xvm* growth *in-vitro*. Overall, this PhD study has identified resistance gene expression and formation of secondary compounds as potential contributors to *M balbisiana* resistance to *Xvm.*

Thesis composition

CHAPTER ONE introduces this study. Chapter covers the botany of banana, its importance and the challenges to its production including the devastating action of Banana Xanthomonas wilt in East and Central Africa. *CHAPTER TWO* describes a required modification in antioxidant capacity of the basal plant tissue culture medium to allow for proliferation and regeneration of *M. balbisiana* plantlets to be used for studying its mechanism of resistance to *Xvm*. *CHAPTER THREE* provides detailed information of the morphological response of test banana plants to infection with *Xvm.* This includes symptom development, pathogen multiplication and migration within the plant tissues. *CHAPTER FOUR* outlines the molecular changes in the NPR1-PR defense pathway by measuring transcription of selected candidate genes following inoculation of banana with *Xvm*. Chapter also reports how the basal plant defense response initially breaks down in both the resistant and susceptible banana due to *Xvm* infection. Chapter also provides insights into the possible role of pre-existing banana phenolic compounds in the resistance mechanism of banana to *Xvm*. *CHAPTER FIVE* finally is a general discussion about any novel aspects of the PhD study and further provides an overview of possible future research avenues to follow solve remaining scientific problems.

TABLE OF CONTENTS

Index of tables

Index of Figures

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Description and characterization of bananas and plantains

Bananas and plantains are large flowering herbaceous plants whose above-ground parts grow from an underground stem, the corm. The normally tall and fairly sturdy plants could easily be mistaken to be trees because of aggregation of widened bases of the petioles (called sheaths) forming a trunk-like structure, called a pseudostem. The pseudostem (false stem) supports the above ground parts of the plant.

Bananas and plantains belong to the genus *Musa* within the *Musaceae* family and the order *Zingberales*, part of the *commelinid* clade of the monocotyledonous flowering plants. The genus *Musa* is divided into 4 sections: *Callimusa* (2n=20), *Australimusa* (2n=20), *Rhodochlamys* (2n=22) and *Eumusa* (2n=22). The majority of cultivated bananas and the common wild species belong to the Eumusa section. It includes two major species, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome), from which many wild subspecies and the present day cultivated edible varieties arose. Edibility came about as a result of female sterility and pathenocapy, which traits were selected for by humans. Although there are some edible diploids, most of the edible bananas and plantains are polyploids existing as triploids and tetraploids. Polyploidy arose from intra-specific hybridizations among the AAs (resulting in AAAs, AAAAs) and inter-specific hybridizations with BBs resulting in ABs, AABs, ABBs, AABBs, ABBBs and AAABs (Simmonds and Shepherd, 1955; Richardson et al., 1965). Some of these hybridizations were natural following the introduction of AAs by man to areas where BBs were native, while other hybridizations were and continue to be results of human breeding efforts.

1.2 Banana and plantain distribution and production trends

Banana and plantain are mainly produced in Asia, Latin America and Africa. They are tropical crops and require the special climatic conditions of the tropics to grow. These conditions coincide with developing countries, making bananas a crop of the developing countries. Around 98% of world's banana and plantain are produced in developing countries while the developed countries are mainly destinations of those exported from the developing world. By 2007, around 130 countries were producing bananas and plantains, but only 10 were major producers accounting for more than 75% of total production. By 2011, India remained the world's leading banana producer followed by Uganda (Table 1.1).

Table 1.1: Top world's leading producers of bananas and plantains in 2011

Country	Tons (millions)	World total (%)
India	29.7	20
Uganda	11.1	8
China	10.7	7
Philippines	9.2	6
Ecuador	8	6
Brazil	7.3	5
Indonesia	6.1	4
Columbia	5.1	4
Cameroon	4.8	3
Tanzania	3.9	3
Other countries combined	49.6	34

Source: (FAOSTAT, 2013)

World banana production has increased over the years, but increase has not been common to all regions. In the 1970s, America accounted for more than 50% of the world production against 34% for Asia. Over the years, the share of Asia began to increase to 58% by 2007, against 31% for America by the same time. The share of the African continent in the world production relatively declined from 13% in the 1970s through 11% in the 2000s to less than 10% by 2007. In Africa, banana and plantain production is dominated by Uganda, Cameroon and Tanzania. The major varieties grown in the East African region are the East African Highland Bananas (EAHBs).

1.3 Economic importance of banana

Banana and plantain combined is the world's fourth most important crop after rice, wheat and maize. The banana industry is important for: 1) provision of food as a starchy staple in developing countries especially in sub-Saharan Africa, 2) generation of export revenue and 3) provision of employment to hundreds of thousands of people in production and distribution networks in Latin America, the Caribbean, Southeast Asia, and Tropical Africa.

Bananas are the most popular fruits in the world and dominate the international fruit trade. They are the most exported fruits in terms of volume and they rank second after citrus fruit in terms of value. At the international level, bananas are mainly consumed as a fresh fruit (ripe), although in many developing countries different varieties are also a staple commodity occupying an essential component of many cooking dishes. Cooking varieties are an important food security crop as they are available all year round and are a source of income for small holder farmers. In

countries such as Uganda, per-capta consumption is estimated to be 245 kg/year, the highest in the world. It is estimated that the EAHB alone are a staple starchy food for 80 million people in sub-Saharan Africa and are also an important source of income.

Banana is also source of revenue at national and household levels. Banana and plantain export is clearly an important source of earnings to some countries **b**etween the 1970s and today. The international trade in bananas and plantains has also increased and the export trade is dominated by five countries, Ecuador, Colombia, Costa Rica, Guatemala in Latin America and the Philippines. According to FAO statistics, by 2011 the combined export by these five countries was estimated at 11.9 Mt (66.5%) out of a worldwide total of 17.9 Mt, which was valued at US$ 15.79 billion (Table 1.2). The banana crop has many different uses apart form its value as an important food crop. Other products include banana fiber for making handcrafts and paper, use as animal feed, leaves are applied for wrapping food and the crop cover also gives shade to other crops like coffee.

Table 1.2: Top world's leading banana and plantain exporting countries in 2011

Country	Tons (millions)	% of world total
Ecuador	5.2	29
Costa Rica	1.8	10
Columbia	1.8	10
Philippines	1.6	9
Guatemala	1.5	8
Other countries combined	6	34

Source: FAOSTAT 2013

1.4 Major constraints to banana and plantain production

The main constraints to banana and plantain production are pests and diseases, unfavorable climatic conditions, soil infertility and poor cultivation methods. With the exception of pests and diseases, which are universal constraints across all the world's production regions, other constraints are localized and are most pressing to small-holder farmers. The world's major banana pests are the banana weevils (*Cosmopolites sordidus*) and nematodes, especially *Radophorus similis*. The world's major banana diseases include; Sigatoka, especially black Sigatoka caused by *Mycosphaerella fijiensis*, Panama wilt caused by *Fusarium oxysporium* **f.sp.** *cubense* and bacterial wilts caused by *Ralstonia solanacearum* and *Xanthomonas vasicola* pv. *musacearum* (*Xvm*).

1.4.1 Bacterial wilts as major diseases of banana

Since the 1890's, the world has experienced 4 major banana bacterial epidemics. These are: (i) banana *Xanthomonas* wilt, caused by *Xvm* and first observed in 1974 (Yirgou and Bradbury, 1974), (ii) Moko disease caused by *Ralstonia solanacearum. biovar 1 race 2*, and (iii) Bagtok and Blood disease caused by *Ralstonia syzygii* subsp. *celebensis* subsp. Nov (Safni et al., 2014). The details of these diseases including common names, causal agents, distribution and natural hosts are sumarised in Table 1.3.

1.4.2 *Xanthomonas* wilt as a constraint for banana production

Banana *Xanthomonas* wilt is a major constraint to banana production in East and Central Africa. Since its first outbreak on banana in Ethiopia in 1974 (Yirgou and Bradbury, 1974), it has also been reported for Uganda (Tushemereirwe et al., 2004), DR Congo (Ndungo et al., 2006), Rwanda (Reeder et al., 2007), Tanzania, Kenya and Burundi (Carter et al., 2010). The disease causes premature fruit ripening (Figure 1.1b) and leaf wilting in fruit-bearing and non-fruit bearing plants (Figures 1.1d and e) leading to total yield losses. If not well-managed, it spreads from plant to plant very fast, thus potentially threatening the livelihoods of over 80 million people who depend on banana in the region. It can also potentially spread beyond the region to other banana producing regions in the world if not well-managed.

Table 1.3: World's major bacterial wilt diseases of banana, causal agents, distribution and natural hosts

Common name	Causal agent	Distribution and natural hosts
Banana Xanthomonas wilt (BXW) (Enset wilt, Banana Bacterial wilt, BBW) also known as *Kiwotoka* (Uganda) and *Unyanjano wa migomba* (Tanzania)	*Xanthomonas vasicola* pv. *musacearum* (*Xvm*)	Present in Ethiopia, Uganda, D.R. Congo, Rwanda, Tanzania Kenya, Burundi on *Enset* spp. and all cultivated banana types; *Musa balbisiana* less susceptible, Cultivars with persistent bracts and flowers may escape the disease
Moko disease and Bagtok disease	*Ralstonia solanacearum* (Biovar 1 race 2)	In Americas (Mexico, central and south America, southern Caribbean, Jamaica): on all cultivated types and wild *Heliconia* spp. In Philippines: recognized as Moko on AAA types; inflorescence infection on ABB types incompletely systemic and recognized as Bagtok/Tubaglon/Tuprok disease.
Blood disease	*Ralstonia syzygii* subsp. *celebensis* subsp. Nov	In Indonesia; on all cultivated and some wild species

Source: modified from Pillay and Tenkouano, 2011

The disease is caused by a bacterium, *Xanthomonas vascicola* pv. *musacearum* (*Xvm*) (Aritua et al., 2007) formerly *Xanthomonas campestris* pv. *musacearum* (*Xcm*). *Xvm* is transmitted by cutting implements used in banana plantation husbandry and by insects foraging the male part of banana inflorescence. In these cases the bacterium enters through the wound of the knife or from the flower/bract abscission. Cutting implements are contaminated with the bacterium when used to cut an infected banana plant and, when used to cut on a healthy plant, the bacterium passes from the tool surface to the newly created wound. On the other hand, foraging insects in the male part of the banana inflorescence contaminate their body surfaces with oozing bacterium from the small wounds left when the bracts and male flowers fall off from an infected plant. The bacterium subsequently passes from the contaminated body surface of the insect to a fresh wound in a healthy male bud which the insect has visited.

A number of cultural control measures have been developed which involve (a) early removal of the male parts of the banana inflorescence by twisting it to break using a forked stick, (b) disinfection of garden cutting implements every time after using on a plant and (c) rouging out any infected plants in a plantation which are subsequently cut into small pieces and left on the surface to rot or dry up as the bacterium only survives for a short time outside a host plant (Mwebaze et al., 2006). While these cultural control options can be effective in managing this disease, they have not been very effective in controlling the damaging effects and spread of this disease in the region. Since the farming community is a small holder peasantry type, the application of these measures is incomplete. Therefore new approaches have been investigated to find host resistance.

Figure 1.1: Symptoms of banana *Xanthomonas* wilt disease (a) healthy fruit-bearing plant (b) affected fruit-bearing plant (c) healthy non-fruit bearing plant and (d and e) affected non-fruit bearing plant.

1.5 Mechanisms of resistance

Resistance to bacterial wilts is generally rare in *Musa spp*. Some reports, however, have shown that there is a differential response in *Musa sp* to the different bacterial wilt diseases of Moko (Stover, 1972), Blood disease (Supriadi, 2005) and banana *Xanthomonas* wilt (Ssekiwoko et al., 2006; Tripathi and Tripathi, 2009). Differences in disease development and severity of symptom expression have been found after artificial inoculations, with some showing partial recovery and production of new suckers. Some authors have, however, warned that such responses may not represent true resistance and that the symptomless plants may simply serve as reservoirs of inoculum (Pillay and Tenkouano, 2011).

Some banana accesions including 'Pelipita' (ABB), 'Manang' (AA) and *M. balbisiana* (BB) have partial resistance to mechanical inoculation with the Small Fluidal Round (SFR) strain of *Ralstonia solanacearum* (Biovar 1 race 2)(Stover, 1972). In adition, *M. balbisiana* was shown to survive the B strain of *R. solanacearum* (Biovar 1 race 2) and also *Xvm* in East and Central Africa (Ssekiwoko et al., 2006). Further, there are differences in susceptability to inflorescence infection by these bacterial wilt pathogens. Pelipita (ABB) with indehiscent male bracts and flowers was much less susceptible to insect transmited *R. solanacearum* (Biovar 1 race 2). Pelipita was recommended as a replacement for Bluggoe, which was highly susceptible due to its highly dehiscent male bracts and flowers (Stover and Richardson, 1968). The cavendish types, which also tend to retain male flowers and bracts, are also much less susceptible to inflorescence infection with *R. solanacearum* (Biovar 1 race 2) in comparison to Gros Michel where bracts and flowers dehisce. Dwarf cavendish and some East Afican Highland Banana (EAHB-AAA),

especially of the Nakitembe clone set, including Nakitembe and Mbwazirume, which have indehiscent bracts and male flowers (Figure 1.2), were reported to escape the insect transmitted *Xvm* (Addis et al., 2004; Mwangi et al, 2009). In addition, other cultivars have been reported to be less susceptible to inflorescence infection with *Xvm* because their flowers were either less attractive to insects or their scars were less condusive to penetration and survival of *Xvm* (Mwangi et al., 2009). Finally, clones without male buds, such as 'Psang Puju', are not susceptible to infloscence infection by bacterial wilt pathogens (Buddenhagen, 2009).

While such disease escape mechanisms to *Xvm* penetration through the male part of the banana inflorescence exist within *Musa spp,* they do not provide genetic resistance and such varieties remain susceptible to *Xvm* infection from contaminated cutting tools. A more durable and universal form of resistance is thus desired. Efforts are already under way to genetically engineer banana for resistance targeting the hypersensitive response with transgenes from sweet pepper (Namukwaya et al., 2011). In addition, there is an urgent need to find resistance genes within *Musa* sp. itself to supplement these ongoing genetic engineering efforts. Identification of these resistance genes requires, however, an in-depth dissection of known plant defense pathways or mechanisms.

Figure 1.2: Banana inflorescence morphologies (a) of a susceptible cultivar to floral mediated *Xvm* transmission and (b) of a resistant cultivar to floral-mediated *Xvm* transmission.

1.6 General defense mechanisms in plants

Pathogen defense in plants has been extensively reviewed (Bent and Mackey, 2007). It begins with effective recognition of pathogen structures, or their effectors, by the plants that then initiate an appropriate defense response. Specialized cell-surface pattern recognition receptors (PRRs) in plants, such as flagellin sensing (FLS2), detect the conserved microbe associated molecular patterns (MAMPs) (Jones and Takemoto, 2004). This includes for example the bacterial cold shock protein (CSP), bacterial flagellin (a structural protein in bacterial flagella), bacterial elongation factor Tu (EFTu), bacterial lipopolysaccharides (LPS) and fungal chitin. On perception of a specific MAMP, signal-transduction cascades are activated culminating in establishment of basal defense mechanisms. This includes (i) callose and silicone deposition to reinforce the cell wall, (ii) closure of stomata, (iii) production of reactive oxygen species and (iv) transcriptional induction of pathogenesis-related genes (*PR* genes) (Nicaise et al., 2009).

Some plant bacterial pathogens can, however, suppress or evade these basal defense mechanisms. For example, *Pseudomonas syringe* secretes a virulence factor, termed coronatine (COR), which suppresses hormonal signaling. This phytotoxic molecule mimics the plant hormone jasmonic acid (Melotto et al., 2006) which suppresses stomatal closure via the inhibition of MAMP-induced abscisic acid (ABA) signaling in the guard cell. Coronatine also induces re-opening of stomata and multiplication of bacteria in both local and systemic tissues by inhibiting the accumulation of the key plant immune signaling molecule, salicylic acid (Zheng et al., 2012). Other bacteria, such as *Agrobacterium tumefaciens* and *Xanthomonas campestris* pv *campestris*, modify their flagellin (a MAMP) to avoid detection by FLS2 (a PRR). Other

bacterial pathogens can further suppress basal defense mechanisms by directly injecting their effectors into host cells using a type three secretion system (TTSS; a needle like pilus that injects effectors). For example, the TTSS effector protein, AvrPto from *P. syringae* pv. *tomato* blocks callose deposition in tomato plants. Similarly in *Arabidopsis*, the TTSS effector proteins AvrRpt2 from *P. syringae* pv. *tomato* and AvrRpm1 from *P. syringae* pv. *maculicola*, inhibits defense signaling induced by FLS2 pattern recognition receptors (Kim et al., 2005).

Some plant pathogenic bacteria can evade, suppress or shutdown basal plant defense mechanisms using T3SS effectors, but some plants use phenolic compounds to suppress this secretion system (Li et al., 2009). Other plants have also evolved mechanisms for directly or indirectly recognizing the presence of the effectors inside their cells. They use 'disease-resistance' (R) proteins (Ade et al., 2007; Axtell and Staskawicz, 2003; Deslandes et al., 2003; Kim et al., 2002; Mackey et al., 2002). The R proteins act as specific intracellular receptors for the specific effectors and this sets off a cascade of defense responses rendering the host resistant (Gu et al., 2005a). Programmed and localized death of plant cells surrounding the area of attack, also referred to as the hypersensitive response (HR), is a mechanism of R protein-mediated defense response. This response is also associated with production of reactive oxygen species and nitric oxide. They appear to function both as signaling agents and as direct antimicrobial agents.

1.7 Recognition events in the plant defense response

Following pathogen recognition, there is an increased production of superoxide (O^{-}_{2}) on the apoplastic face of the plasmalemma at the site of infection and later in systemic tissues (Durrant and Dong, 2004). Superoxide dismutase (SOD) in the apoplast rapidly converts O^{-}_{2} to H_2O_2. This may not only affect the pathogen, but also damages the plant cells when in high amounts. H_2O_2 will induce crosslinking at the plant cell wall thereby hindering pathogen penetration. It may also, especially at the site of attack, cause sudden death of plant cells thereby affecting the pathogen therein. This damaging effect must be controlled through detoxifcation of the accumulating H_2O_2. While H_2O_2 detoxification in plants may be achieved through the catalase-based mode, detoxifcation via peroxidases is more important as peroxidases are present in all cells and have a higher affinity for H_2O_2. This, however, requires a reductant. In plants the most important reducing substrate is ascorbate, which detoxifies H_2O_2 through the ascorbate-glutathione cycle (Foyer and Noctor, 2011; Noctor and Foyer, 1998). The enzyme ascorbate peroxidase uses electrons from ascobate to degrade H_2O_2 into water and monodehydroascorbate. Highly unstable monodehydroascorbate quickly dessociates into dehydroascorbate. This is later reduced to ascobate using electrones donated by glutathione, which becomes oxdised into the glutathione disufide form. Glutathione is later regenerated from the oxdised form in a reaction catalysed by glutathione reductase and NADPH. These changes in the redox state of cells causes monomerization of the cytosolic non-expressor of pathogenesis related protein1 (NPR1). This subsequently leads to NPR1 translocation into the nucleus and by interacting with TGA1 transcription factors causes pathogenesis-related (*PR*) gene expression (Foyer and Noctor, 2011;

Mou et al., 2003). Resulting PR proteins, encoded by *PR* genes, are antimicrobial in nature with many of them having chitnase and glucanase activities.

1.8 Justification of study

The importance of banana *Xanthomonas* wilt is indicated by its wide distribution in Eastern Africa, the rapidity of its spread, its ability to cause total yield loss and threatening livelihoods of over 80 million people in the region. This study was aimed to identify a possible mechanism of resistance by comparing the responses of *M. balbisiana,* which is resistant, and the East African Highland Banana (EAHB) cultivar '*Nakinyika*' (AAAEA genome), which is susceptible to *Xvm* infection. The rationale for the selection of '*Nakinyika*' for this study was based on the finding that during the first phase of a current ABSPII project my institute successfully developed embryogenic cell suspensions (ECS) from several EAHB. An *Agrobacterium*-mediated gene transformation protocol was established to genetically transform the EAHB ECS. Successful transformation was achieved for three EAHB cultivars *('Nakinyika', 'Nakasabira' and 'Mpologoma'*). Accordingly, any identified resistance mechanism in *M. balbisiana* could potentially be exploited and supplement ongoing genetic engineering research by my research group to generate resistant (EAHB) banana. Various plant defense pathways were therefore explored in this study both at morphological and molecular level in order to identify any trait that confired *Xvm* resistance to *M. balbisiana*. *'Nakinyika'* is a typical cooking variety cultivar that is an important staple in East and Central Africa, which is often eaten after steaming. While specific information on the overall nutritional content of its edible pulp is scanty, available data shows that it is an important source of vitamin A, at 67.6 µg/100 g retinol equivalent (Bresnahan

et al., 2012). At this level, one needs to consume 4.4 kg of pulp to attain 3000 µg as daily requirement for an average person. This value from *'Nakinyika'* is only inferior to a few varieties among the EAHB including '*Butobe'* (169.2 µg/100 g), '*Nakitembe'* (88.03 µg/100 g), '*Entukura'* (81.86 µg/100 g), '*Nakhaki'* (77.17 µg/100 g) and '*Kibuzi'* (71.62 µg/100 g) (Fungo and Pillay, 2011). Studies in my laboratory have shown that *'Nakinyika'* is also an important source of iron and zinc at 9 mg/kg and 8 mg/kg of dry weight of pulp, respectively. In comparison to other varieties commonly consumed among the EAHB, its iron content is superior to that of *Nakitembe* (7 mg/kg), another popular cooking EAHB variety. Its iron content is also within range of other popular EAHB cooking varieties of '*Mbwazirume'* and '*Mpologoma'* at 10 mg/kg but inferior to that of '*Kisansa'* (11 mg/kg), another popular EAHB variety. The recommended dietary allowance for iron for an average adult male is 8 mg while that of the female is 18 mg. This means that if *'Nakinyika'* was the sole source of iron, an adult male would need to consume one kg while a female would need 2.5 kg per day. The zinc content of *'Nakinyika'* on the other hand is similar to that of the other popular EAHB varieties of '*Kisansa'* and '*Nakitembe'* (7-10 mg/kg), '*Mbwazirume'* and '*Mpologoma'* (8 mg/kg). The recommended dietary allowance for zinc for an average adult male is 11 mg while that of the female is 8 mg. This means that if *'Nakinyika'* was the sole source of zinc, an adult male would need to consume 1.5 kg while a female would need 1kg per day.

In general, *M. balbisiana* is a wild banana native to eastern South Asia, northern Southeast Asia, and southern China. It is characteristically with lush leaves in clumps with a more upright habit than most cultivated bananas. Further, *M. balbisiana* accessions have been previously found to be resistant against *Xvm* in a greenhouse trial (Tripathi and Tripathi, 2009). Also in screen-house

and field trials, this type of banana developed no symptoms over 6 weeks after a single inoculation at dosages that caused the disease on a control (Kumakech et al., 2013). Only after a second inoculation at 6 weeks did any disease symptoms develop.

1.9 Working hypothesis and objectives

In this PhD, it was hypothesized that wild-type *M. balbisiana* provides a reservoir of resistance genes not present in the EAHB cultivar '*Nakinyika*' which ultimately might be used to engineer '*Nakinyika*' plants. This cultivar has lost the resistance trait due to cultivation. *M. balbisiana* could be therefore utilized to engineer the EAHB cultivar '*Nakinyika*' for improved *Xvm resistance*. In general, wild-type plants can carry important complex traits, including resistances to both abiotic (temperature and water) and biotic (disease and pest) stresses, which might be transferable to cultivated banana for improvement. Since previous studies had revealed that *M. balbisiana* is resistant to *Xvm* infection and the EAHB cultivar '*Nakinyika*' (AAAEA genome) is susceptible, this PhD study focused on comparing these two types of plants to identify a possible trait that renders *M. balbisiana* more resistant to *Xvm*. Such comparisons have previously not been done. If successful, outcome of the study would provide a better understanding of *Xvm* resistance in *M. balbisiana*. It would also provide information of possible gene(s) that are responsible for resistance and their transfer into transgenic banana plants to render them *Xvm* resistant.

Since micro-propagated *M. balbisiana* had to be available for the study, the first objective of the PhD study was to have sufficient plant material for subsequent plant analysis. This objective also

included to optimize the propagation process by investigating the effects of cytokinin (BAP) and vitamins to the culture medium and the influence of photoperiod duration on shoot proliferation. A second objective was to determine the morphological response of *M. balbisiana* to *Xvm* in comparison to the *Xvm*-susceptible EAHB cv '*Nakinyika*'. In particular particularly disease development, incidence and severity of wilt and *Xvm* population change within tissues were investigated. A third objective was to better understand the mechanism of resistance of *M balbisiana* to *Xvm* infection by investigating expression changes of selected genes located at specific stages of the defense pathway in plants of *Musa balbisiana* and the EAHB, cv. '*Nakinyika*'. This was carried out following inoculation with *Xvm*. A final fourth objective was to investigate the effect of banana methanolic extract compounds on the *in vitro* growth of *Xanthomonas* to also evaluate a possible passive mode of resistance to *Xvm* infection.

CHAPTER TWO

DEVELOPMENT OF A PROTOCOL FOR *IN-VITRO* GENERATION AND ALSO REGENERATION *M. BALBISIANA* AND EAHB CV. 'NAKINYIKA' PLANTLETS BY TISSUE CULTURE

Ssekiwoko, F., Talengera, D., Kiggundu, A., Namutebi, M.K., Karamura, E and Kunert, K. 2014. In-vitro proliferation of wild *Musa balbisiana* improves with increased vitamin concentration and dark culturing. Journal of Applied Biology and Biotechnology 2: 1-7.

2.1 Abstract

Musa balbisiana is a wild banana genotype with important traits such as drought tolerance and disease resistance. Uniform and disease/pest free *in-vitro* propagated plants of *M. balbisiana* were required to study the mechanism of resistance to banana *Xanthomonas* wilt disease. An optimized protocol for regeneration of *in vitro* plants were established. Anti-oxidant capacity of the *in-vitro* plant proliferation medium was modified through increase of the concentration of ascorbic acid and thiamine-HCl in the medium above that recommended for the basal MS medium. By also subjecting explants in culture to dark culturing conditions multiplication of *M. balbsiana* was improved by over 10-fold which resulted in 40 shoots per initial explant as best result.

2.2 Introduction

The East African Highland Banana (EA-AAA) cultivar '*Nakinyika*' regenerates well on a proliferation medium previously described by Talengera et al. (1994). In contrast, attempts to also regenerate *M. balbisiana* on this medium resulted in no shoot formation. Related studies have further shown that banana genotypes with 'B' genomes have generally not only lower multiplication rates in a tissue culture process but multiplication is also more difficult when the number of B genomes increases (Hirimburegama and Gamage, 1997). Further, the amount of the synthetic cytokinin, 6-benzyladenine (BA) can also limit proliferation as found for the *M. balbisiana* variety 'Kluai Hin' (BBB genome) (Kanchanapoom and Promsorn, 2012). The rate of multiplication is further affected by the degree of browning of shoot tip tissues. Browning is partly attributed to oxidation and tissue death due to oxidative processes either incurs at the cut surfaces of tissues or takes places in the medium due to oxidation of plant phenolic compounds in presence of light (Leng et al., 2009; Abdelwahd et al., 2008; Bhat and Chandel, 1991; Vaughn and Duke, 1984; Mayer and Harel, 1979; Loomis and Battaile, 1966). Phenolic compound oxidation results in generation of toxic derivatives that kill plant tissues due to inhibition of enzymes (Arnaldos et al., 2001) and death of surface tissues further interferes with the tissues' ability to take up nutrients from the media.

Efforts for the regeneration of *M balbisiana* have previously included increase in the cytokinin (BAP) amount, but also reducing light-mediated phenolic oxidation/browning as well as limiting oxidation. Tilt placement and increase in the cytokinin (BA) amount added to a medium has been already found to improve *M. balbisiana* proliferation (Kanchanapoom and Promsorn, 2012).

Also, dark culturing, which generally reduces tissue browning, has been reported to improve plant proliferation (Cassells and Minas, 1983; Hangarter and Stasinopoulos, 1991). In addition, incorporation of antioxidants into the culture medium controlling oxidative tissue browning (Birmeta and Welander, 2004; Ko et al., 2008; Onuoha et al., 2011) and addition of different vitamins, such as thiamine, also enhanced the plants' ability to better tolerate oxidation (Tunc-Ozdemir et al., 2009).

Since the aim of this part of the PhD study was to successfully micro-propagate *M. balbisiana* to have sufficient plant material for subsequent applications, the specific objectives were set to first investigate the effect of addition of various amounts of cytokinin (BAP) and vitamins to the culture medium to reduce oxidative processes and improve shoot formation. Since dark treatment might prevent light-induced medium oxidation, secondly the influence of photoperiod duration on shoot proliferation of *M. balbisiana* was also investigated.

2.3 Materials and methods

2.3.1 Preparation of tissue culture medium

Fresh banana corm tissues with apical meristems were aseptically isolated and then initiated / cultured on a basic proliferation medium as described by Murashige and Skoog (1962) with amendments with constituents as listed in Table 2.1. One liter solutions for a 10X macro-nutrient stock mixture, a 100X micro-nutrient stock mixture and a 100X special iron source stock mixture were prepared by dissolving in warm distilled water facilitated by stirring. Constituents for the special iron stock mixture were separately dissolved and later mixed after they had fully dissolved and kept in amber colored bottles for protection from light. For the vitamin complex, 500 ml of a 100X stock mixture was prepared by dissolving all constituents in sterile distilled water and kept under refrigeration at 4ºC. Also, 250 ml stock solution for ascorbic acid was prepared by dissolving ascorbic acid in water. In addition, 100 ml of BAP stock solution was prepared by adding to the powder drops of 0.1N NaOH until dissolution and then toping up with distilled sterile water. All stock solutions, except BAP and vitamins were autoclaved at 1.05 kg/cm^2, 121°C for 15 min before storage under refrigeration at 4ºC.

Table 2.1: Constituents which were used for preparation of the basal MS proliferation medium

Nutrient	Component (supplier)	Amount (mg/l)
Macro-nutrients	$CaCl_2.2H_2O$ (LOBACHEMIE, No. 2465)	440
	KH_2PO_4 (Duchefa Biochemie, P0574.1000)	170
	KNO_3 (Duchefa Biochemie, P0519.5000)	1900
	$MgSO_4.7H_2O$ (Sigma, No. M7774-500G)	370
	NH_4NO_3 (Duchefa Biochemie, A0501.1000)	1650
Micro-nutrients	KI (HIMEDIA, RM252-250G)	0.83
	H_3BO_3 (Sigma, B-7901)	6.2
	$MnSO_4.H_2O$ (Sigma, M-7899)	22.3
	$ZnSO_4.7H_2O$ (Sigma, Z-1001)	8.6
	$Na_2MoO_4.2H_2O$ (Sigma, M-1651)	0.25
	$CuSO_4.5H_2O$ (Sigma, C-3036)	0.025
	$CoCl_2.6H_2O$ (Sigma, C-2911)	0.025
source of Iron	$FeSO_4.7H_2O$ (Sigma, F-8263)	27.8
	Na_2 EDTA (Duchefa Biochemie, E0511.0500)	37.3
Vitamin complex	Nicotinic acid (Duchefa Biochemie, N0611.0100)	0.5
	Pyridoxine HCl (Duchefa Biochemie, P0612.0250)	0.5
	Thiamine HCl (Duchefa Biochemie, T0614.0100)	1
	Glycine (Duchefa Biochemie, G0709.1000)	2
Ascorbic acid	Ascorbic acid (Duchefa Biochemie, A0602.0250)	10
Growth regulator	BAP (Duchefa Biochemie, B0904.0025)	5
Carbon source	Sucrose	30000
Gelling agent	Gelrite (Duchefa Biochemie, G1101.0500)	2400

One liter of a basal 1X MS proliferation medium was prepared by using appropriate volumes of stock solutions and adjusting the pH to 5.8 using 0.1N NaOH or 0.1N HCl. Gelrite, as a gelling agent, was added at a rate of 2.4 g/l and the medium was autoclaved at 1.05 kg/cm^2, 121°C for 15 min.

2.3.2 Explants preparation

The lower rhizomes (corm) of peeping banana suckers (peepers) of wild *M. balbisiana* were detached from the mother mats by use of a hand hoe, while taking care not to injure the meristematic tissue. With a machete (Panga), all roots were removed and the pseudostem tissue was reduced to 15 cm from the corm base in a mother garden. These corm tissues were returned to the tissue preparation room. Separately, surface tissues of the corm and pseudostem area were sliced away reducing the tissue to about 5-10 cm from the base and to about five pseudostem sheaths for each corm. Each tissue was then suspended in 95% ethanol for 5 min. After this time, the ethanol was discarded and the corms were submerged in warm water (42ºC) containing 10 drops/liter of liquid detergent for 25 min. Water was then discarded and the tissues were further sliced to remove the darkened surface tissue. They were then re-immersed in 95% ethanol for 5 min after which they were immersed in 15% NaOCl (v/v) in sterile water containing 20 drops /liter of Tween 80 for 25 min. This solution was also discarded and the tissues were then rinsed 3-times in an equal volume of sterile water, after which they were ready for culturing on growth medium.

2.3.3 Supplementation of basal MS medium with BAP and vitamins

Tissues of EAHB cv '*Nakinyika*' were cultured on basal MS medium containing 5mg/l BAP, 0.5mg/l nicotinic acid, 0.5mg/l pyridoxine HCl, 0.1 mg/l thiamine-HCl and 10 mg/l ascorbic acid. To determine the effect of BAP on proliferation of *M. balbisiana*, the basal MS proliferation media was supplemented with 5 mg/l, 7 mg/l, 9 mg/l and 10 mg/l of BAP by adding a corresponding volume from the stock. Similarly, the effect of nicotinic acid on *M. balbisiana* proliferation was determined by supplementing the basal MS proliferation medium with 0.5 mg/l, 0.7 mg/l, 0.9 mg/l and 1 mg/l of nicotinic acid. The effect of pyridoxine-HCl on *M. balbisiana* proliferation was determined by supplementing the basal MS proliferation medium with 0.5 mg/l, 0.7 mg/l, 0.9 mg/l and 1 mg/l of pyridoxine-HCl. The effect of thiamine-HCl on *M. balbisiana* proliferation was determined by supplementing the basal MS proliferation medium with 0.1 mg/l, 0.14 mg/l and 0.18 mg/l of thiamine-HCl. The effect of ascorbic acid on *M. balbisiana* proliferation was determined by supplementing the basal MS proliferation medium with 10 mg/l, 20 mg/l, 30 mg/l and 4 0 mg/l of ascorbic acid.

2.3.4 Tissue culturing and sub-culturing regimes

With sterile blades surface tissues for previously sterilized explant material were removed so that the tissue was reduced to about 2cm (transverse diameter at the thickest part of the corm area) having both a corm end and sheaths at the pseudostem end. This tissue was longitudinally sliced into two halves dissecting the meristem into two where each half was placed on proliferation media with the corm tissue end half way seated into the media in a baby jar. The jars were sealed

with cling film and then transferred to growth rooms and incubated at 25ºC illuminated with white-fluorescent lamps at an intensity of 33 µmol $m^{-2}s^{-2}$. Tissues were first cultured for 3 weeks and then transferred to fresh media every after 3 weeks through-out the culturing process up to the end of 18 weeks at varying photoperiod durations.

2.3.5 Variation of the photoperiod duration

During the culturing and sub-culturing regimes, EAHB c '*Nakinyika*' was cultured at 14 hrs lighting and 10 hrs darkness for 18 weeks of culture. For *M. balbisiana*, the duration of illumination was varied at 3 levels: 1) 14 hrs lighting and 10 hrs darkness for 18 weeks of culture, 2) total darkness for the first 9 weeks followed by 14 hrs lighting and 10 hrs darkness for the next 9 weeks, and 3) total darkness for the first 12 weeks followed by normal 14 hrs lighting and 10 hrs darkness for the next 6 weeks. Data were recorded on general appearance of tissues on media and average number of shoots produced after 18 weeks of culturing process. Generally, 4 plants were initiated for each concentration of BAP or vitamins for each photo period duration.

2.3.6 Data collection and analysis

The general appearance of explants throughout the 18 weeks of incubation was described and number of resultant shoots/plantlets after 18 weeks of incubation was recorded. Data were analyzed with the GENSTAT package. ANOVA was conducted and the least significant difference between the mean number of shoots was determined and used to separate the means to

determine those which were significantly different (P = 0.05) at different treatment combinations.

2.4 Results

2.4.1 Proliferation of EAHB cv '*Nakinyika*'

Shoots of the EAHB cv. '*Nakinyika*' grew on basal MS proliferation media without tissue browning from initiation up to the end of 18 weeks of culture under 14 hrs lighting and 10 hrs darkness (Figure 2.1). After 18 weeks, an average of 60 shoots per initial explant of corm tissue had been generated ready for weaning to use as plantlets in subsequent experiments. In *M. balbisiana*, however, explants tissues only turned brown and subsequently died under identical growth condition (Figure 2.2A) and no shoot was regenerated.

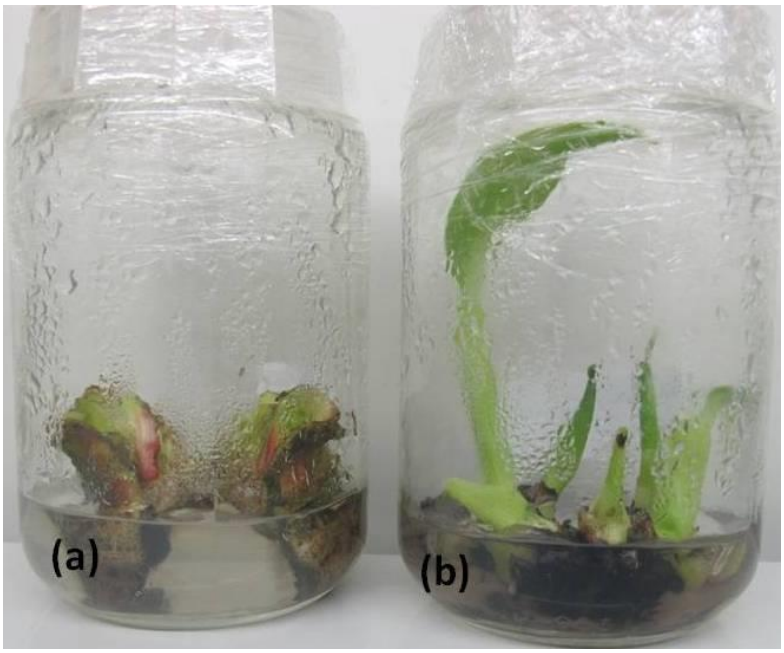

Figure 2.1: Tissues of the EAHB cv. '*Nakinyika*' on basal MS proliferation medium at a 14 hrs photoperiod (a) at 14 days after initiation and (b) at subculture level 2.

However, following media composition modification, proliferation varied (both in appearance and in number of resultant shoots) with type and concentration of vitamins supplied and with various photoperiod duration.

2.4.2 Effects of plant hormones, vitamins and photoperiod duration on proliferation

Explants of *M. balbisiana* cultured under different concentrations of BAP and photoperiod duration often turned black together with the surrounding medium and eventually died (Figure 1a). At best, a single corm tissue (as starting explant material) produced only 1 shoot after 18 weeks of culture (Table 2.2). Variability in the number of shoots was, however, high and the difference in the mean number of shoots was significant (P = 0.017). At a 14 hrs photoperiod, an increase in the mean number of shoots from 0 to 1 was found with an increase in BAP concentration from 5 to 7 mg/l. Any further increase in the BAP concentration to 9 and 10 mg/l under similar photoperiod duration did not improve the number of shoots. Similarly, mean shoot number increased from 0 to 1 at 5 mg/l BAP, when tissues were subjected to dark culturing for the first 9 and 12 weeks followed by a 14 hrs photoperiod in the later stages of culturing. In contrast, increasing the BAP concentration to 7, 9 or 10 mg/l, together with culturing under dark conditions for the first 9 and 12 weeks followed by a 14 hrs photoperiod, prevented tissue proliferation. This suggests that the BAP concentration and incubation of tissues in the dark is not the limiting factor in proliferation of *M. balbisiana* on basal MS medium under the selected conditions.

Table 2.2: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different BAP concentrations for different time intervals (weeks) under various photoperiod/hours of lighting (hrs L).

	Mean number of shoots*		
BAP (mg/l)	**18 weeks/14 hrs L**	**9 weeks/0 hr L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
5	0.00[a]	1.00[ab]	1.25[b]
7	1.25[b]	0.25[ab]	0.00[a]
9	1.25[b]	0.25[ab]	0.25[ab]
10	1.25[b]	0.00[a]	0.25[ab]

Lsd (5%) 1.069
P=0.017
*Means with the same letter are not significantly different

The effect of vitamins and culturing conditions of shoot formation is shown in Figure 2.2 Generally, increase in the concentration of ascorbic acid above 10 mg/l in the basal MS medium significantly improved the proliferation of *M. balbisiana* and resulted in 10 shoots per corm at the best. However, this was only evident when explants were cultured in the dark for the first 9 weeks (Table 2.3). Variability in the number of shoots was, however, high and the difference in the mean number of resultant shoot was significant (P<0.001). At a 14 hrs photoperiod, a single corm proliferated only into 1 plantlet even when the ascorbic acid concentration was increased to 20, 30 and 40 mg/l with most explants often blackening and dying. However, when explants were incubated in the dark for the first 9 weeks, proliferation significantly increased from 1 shoot

per corm at 10 mg/l ascorbic acid to 10 shoots per corm at 20 mg/l ascorbic acid. Under these conditions, despite exudation of compounds that turned the medium to black in the immediate vicinity of the explants, some shoots grew. Further increase in the ascorbic acid concentration to 30 and 40 mg/l significantly reduced tissue proliferation to 7 and 0 shoots per corm, respectively, under similar dark culturing condition. Most explants together with the culture medium turned black and explants died. Prolonged incubation of tissues in dark for the first 12 weeks reduced the mean number of resultant shoots and under these conditions, elevation of the ascorbic acid concentration from 10 mg/l to 20 mg/l only resulted in 6 shoots per corm. Under these conditions, most tissues and the culture medium turned black and tissue died. Increase in ascorbic acid concentration to 30 and 40 mg/l under similar prolonged dark culturing conditions reduced the mean number of resultant shoots to 2 and 1, respectively, and most shoots stopped growing and died. Ascorbic acid seemingly facilitates best proliferation of *M. balbisiana* at a concentration of 20 mg/l only if incubated in the dark for the first 9 weeks after initiation followed by a 14 hrs photoperiod.

Table 2.3: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different concentrations of ascorbic acid for different time intervals under various photoperiod/hours of lighting (hrs L).

Ascorbic acid (mg/l)	Mean number of shoots*		
	18 weeks/14 hrs L	**9 weeks/0 hr L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
10	1.00[a]	1.25[a]	1.00[a]
20	1.00[a]	10.25[c]	5.50[b]
30	1.00[a]	6.75[b]	2.00[a]
40	1.33[a]	0.25[a]	1.00[a]

Lsd (5%) 2.125
P<0.001
*Means with the same letter are not significantly different

Elevation of the concentration of thiamine-HCl above 0.1 mg/l commonly applied in basal MS medium combined with dark culturing improved the general proliferation of *M. balbisiana* in-vitro. This resulted in 27 shoots per corm at the best (Table 2.4). While variation in the mean number of resultant shoots per corm at different concentrations of thiamine-HCl and photoperiod duration was high, the mean difference between respective means was highly significant (P<0.001). At 14 hrs photoperiod, increasing thiamine-HCl up to 0.18 mg/l only resulted in 2 shoots per corm and most tissues blackened and died. Incubation of tissues in the dark for the first 9 weeks significantly increased the number of shoots from 1 at 0.1 mg/l to 7 and 27 shoots/corm at 0.14 mg/l and 0.18 mg/l thiamine-HCl, respectively. However, tissues always

turned pale due to lack of light. At prolonged dark culturing (for the first 12 weeks), there was a non-ignificant drop in the resultant shoots per corm to 6 at 0.14mg/l thiamine-HCl and to 22 at 0.18 mg/l thiamine-HCl. Black staining of the medium was further less intense under dark conditions. This suggests that thiamine-HCl facilitates proliferation of *M. balbisiana* at a high concentration of 0.18 mg/l but that light facilitates tissue blackening which in turn limits proliferation of *M. balbisiana*.

Table 2.4: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different concentrations of thiamine-HCl for different time intervals under various photoperiod/hours of lighting (hrs L).

	Mean number of shoots*		
Thiamine-HCl (mg/l)	**18 weeks/14 hrs L**	**9 weeks/0 hr L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
0.1	1.00^{ab}	0.75^{a}	1.00^{ab}
0.14	2.25^{abc}	7.25^{c}	6.75^{bc}
0.18	2.33^{abc}	27.25^{d}	21.50^{d}

Lsd (5%) 5.934
P <0.001
*Means with the same letter are not significantly different

Increasing the concentration of nicotinic acid above 0.5 mg/l commonly used in basal MS medium to 0.7 mg/l, 0.9 mg/l and 1 mg/l did not improve proliferation of *M. balbisiana* regardless of variation of photoperiod (Table 2.5). While the variation in the mean number of

shoot per corm was high, the difference in their means was non-significant (P=0.749). Only 1 shoot derived from a single corm at best. Most explants as well as the medium turned black under lighting and explants died. This suggests that a low concentration of nicotinic acid combined with tissue exposure to lighting do not limit proliferation of *M. balbisiana* on basal MS medium.

Table 2.5: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different concentrations of nicotinic acid for different time intervals under various photoperiod/hours of lighting (hrs L).

Nicotinic acid (mg/l)	Mean number of shoots*		
	18 weeks/14 hrs L	**9 weeks/0 hrs L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
0.5	1.00[a]	1.00[a]	1.00[a]
0.7	0.75[a]	1.00[a]	1.00[a]
0.9	0.25[a]	0.25[a]	1.00[a]
1	1.00[a]	0.00[a]	0.50[a]

Lsd (5%) 1.312
P=0.749
*Means with the same letter are not significantly different

Increasing the concentration of pyridoxine-HCl above 0.5 mg/l commonly applied in MS medium and additional culturing in the dark improved proliferation of *M. balbisiana* resulting in 9 shoots per corm at best (Table 2.6). Despite high variation in the mean number of resultant shoots per corm at different concentrations of pyridoxine-HCl and photoperiod duration, means were highly significantly different ($P<0.001$). At a 14 hrs photoperiod, there was a significant improvement in proliferation when the concentration of pyridoxine-HCl was elevated from 0.5 mg/l to 0.7 mg/l which resulted in 4 shoots per corm. Further increase in the concentration of pyridoxine-HCl to 0.9 mg/l and 1 mg/l significantly reduced, however, proliferation to 1 shoot per corm. Dark culturing (either for the first 9 or 12 weeks) at 0.5 mg/l pyridoxine-HCl did not

improve proliferation. However, when the concentration of pyridoxine-HCl was increased to 0.7 mg/l under similar dark culturing conditions (for the first 9 and 12 weeks), proliferation significantly improved resulting in 9 and 7 shoots per corm, respectively. Further increase of pyridoxine-HCl concentration to 0.9 mg/l and 1 mg/l under dark condition (either for the first 9 or 12 weeks) significantly reduced, however, proliferation resulting in 1 shoot per corm. Pyridoxine-HCl facilitates best proliferation of *M. balbisiana* at 0.7mg/l and this possibly a limiting factor in *M. balbisiana* shoot production. Shoot proliferation can be further enhanced by dark incubation for 9 weeks after initiation followed by a 14 hrs photoperiod.

Table 2.6: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different concentrations of pyridoxine-HCl for different time intervals under various photoperiod/hours of lighting (hrs L).

Pyridoxine-HCl (mg/l)	Mean number of shoots*		
	18 weeks/14 hrs L	**9 weeks/0 hr L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
0.5	1.00[a]	0.75[a]	1.00[a]
0.7	3.50[b]	9.25[d]	7.00[c]
0.9	0.25[a]	0.25[a]	0.75[a]
1	1.00[a]	0.33[a]	1.00[a]

Lsd (5%) 1.444
P <0.001
*Means with the same letter are not significantly different

2.4.3 Effect of combined vitamins and photoperiod duration

Generally, increasing the concentrations of vitamins (nicotinic acid, pyridoxine-HCl, thiamine-HCl and ascorbic acid) in combination significantly (P<0.001) improved proliferation of *M. balbisiana* and resulted in 39 shoots per corm at best (Table 2.7). At 14 hrs photoperiod, a combination of 0.7 mg/l nicotinic acid, 0.7 mg/l pyridoxine-HCl, 0.14 mg/l thiamine-HCl and 20 mg/l ascorbic acid resulted in 30 shoots per corm. At the same photoperiod, further increase in the concentration of the vitamin combination with 0.9 mg/l nicotinic acid, 0.9 mg/l pyridoxine-HCl, 0.18 mg/l thiamine-HCl and 30 mg/l ascorbic acid did not significantly improve

proliferation and resulted in 32 shoots per corm. Increased concentrations of the vitamin combination to 1 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 0.2 mg/l thiamine-HCl and 4 0 mg/l ascorbic acid significantly reduced proliferation to only 1 shoot per corm.

Table 2.7: Number of shoots per corm of *M. balbisiana* after 18 weeks of culture on basal proliferation media supplemented with different concentrations of a combination of nicotinic acid (NA), pyridoxin-HCl (PH), thiamine-HCl (TH) and ascorbic acid (AA) for different time intervals under various photoperiod/hours of lighting (hrs L).

	Mean number of shoots*		
Compound mixture	**18 weeks/14 hrs L**	**9 weeks/0 hr L followed by 9 weeks/14 hrs L**	**12 weeks/0 hr L followed by 6 weeks/14 hrs L**
0.5NA+0.5PH+0.1TH+10AA	1.00[a]	0.25[a]	1.00[a]
0.7NA+0.7PH+0.14TH+20AA	30.00[b]	39.00[d]	36.00[c]
0.9NA+0.9PH+0.18TH+30AA	32.00[b]	39.00[d]	37.00[cd]
1NA+1PH+0.2TH+40AA	1.00[a]	1.00[a]	1.00[a]

Lsd (5%) 2.375
P < 0.001
*Means with the same letter are not significantly different

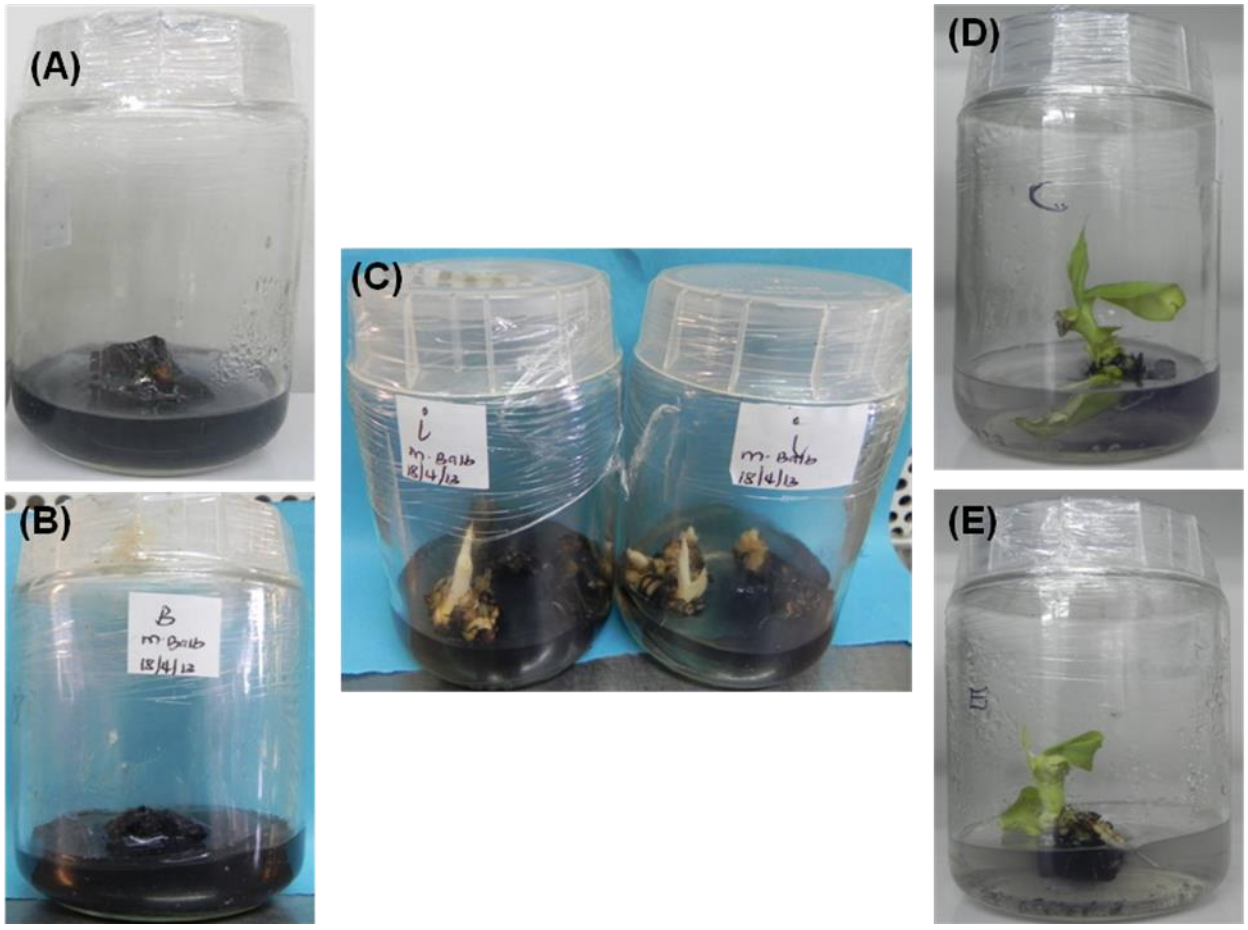

Figure 2.2: Explant tissues of *M. balbisiana* (A) on basal MS proliferation medium at a 14 hrs photoperiod after 6 weeks, (B) on basal MS medium supplemented with 0.18 mg/l thiamine-HCl at 14hrs photoperiod after 9 weeks (C) on basal MS proliferation medium supplemented with 0.18 mg/l thiamine-HCl at 0 hr photoperiod after 9 weeks, (D) on basal MS medium supplemented with 20 mg/l ascorbic acid at a 0 hr photoperiod for 9 weeks followed by a 14 hrs photoperiod for another 9 weeks and (E) on basal MS medium supplemented with 0.18 mg/l thiamine-HCl at a 0 hr photoperiod for 9 weeks followed by a 14 hrs photoperiod for another 9 weeks.

2.5 Discussion

Finding in this part of the study that 6-benzylaminopurine (BAP) even under dark culturing condition does not improve *M. balbisiana* shoot proliferation, is in contrast to the finding of Kanchanapoom and Promsorn (2012) reporting that increasing the cytokinin concentration improves proliferation of the *M. balbisiana* variety '*Kluai Hin*' (BBB genome). BAP, a contact growth regulator, is effective at the site of contact whereby for tissue proliferation the cytokinin binds to specific receptor molecules on the cell surface or within the cytoplasm. This induces cell division and bud formation beginning with an asymmetric division of the target cell which is several cells back from the tip. Further development of the target cell requires the continuous presence of a cytokinin (Saunders and Hepler, 1982). The initial response of cytokinin is mediated by an increase in the cytosolic calcium concentration promoting calcium uptake from the medium with calcium affecting the cytoskeleton, which regulates exocytosis (Hager et al., 1991). Calcium ions further act through the regulating protein calmodulin with each calmodulin possessing four high affinity calcium binding sites. Although calmodulin alone is inactive as a regulator, the calmodulin-calcium complex binds to and activates a number of enzymes. This includes protein-kinase enzymes adding phosphorous to the serine, or tyrosine, hydroxyl group of the protein and enzyme phosphorylation changes their activity. Since the calcium-calmodulin complex acts as a master switch, regulating alternative metabolic pathways within the cell, calcium ions can act as secondary messenger, transforming the hormonal signal into a biochemical switch thereby regulating the initial stages of bud formation (Overvoorde and Grimes, 1994). In this PhD study, an increase in the BAP concentration in the growth medium

did not improve *M. balbisiana* proliferation suggesting possibly due to still unknown limiting factor.

Tissues and surrounding medium continued in this study to brown/blacken and with tissues dying. Such tissue browning is partly attributed to oxidation incurring at the cut surfaces of tissues or to light-dependent oxidation of plant phenolic compounds (Abdelwahd et al., 2008; Bhat and Chandel, 1991; Leng et al., 2009; Loomis and Battaile, 1966; Mayer and Harel, 1979; Vaughn and Duke, 1984). However, dark culturing to reduce on the light mediated oxidation did not improve proliferation in this PhD study. Further, cut plant tissues suffer oxidative stress due to mechanical injury with production of reactive oxygen species (O^{-}_{2} and H_2O_2). In general, anti-oxidative compounds such as ascorbic acid, prevent oxidation. Increasing ascorbic acid concentrations indeed improved *M. balbisiana* proliferation very likely indicating that oxidation limits tissue proliferation. Ascorbic acid is oxidized by reactive oxygen species to monodehydroascorbate (MDHA) radical which, through a series of enzyme driven reactions, subsequently disproportionate to form ascorbic acid and dehydroascorbate (Smirnoff, 2000). While in other banana cultivars (especially the EAHB) 10 mg/l of ascorbic acid is normally sufficient to bring about an antioxidant effect, this PhD study showed that higher ascorbic acid is required for *M. balbisiana* proliferation. Possibly, at tissue injury, the oxidative stress effect in this banana genotype is stronger, requiring a stronger anti-oxidant dose. However, too high amounts of ascorbic acid negatively affected proliferation. Excess of ascorbic acid in the presence of free metal ions can initiate free radical formation causing toxic pro-oxidative reactions.

Addition of higher thiamine and pyridoxine amounts to the medium also improved *M. balbisiana* proliferation Thiamine possibly plays a role in enzyme catalysis and also stress alleviation in various organisms including bacteria, fungi, animals and also plants (Ahn et al., 2005; Sayed and Gadallah, 2002). The mechanism of stress alleviation is, however, not yet well understood but a reduction in reactive oxygen species production has been recently proposed (Tunc-Ozdemir et al., 2009). The difference in number of shoots generated after thiamine treatment further indicates that thiamin might be more potent than ascorbic acid as an addition to stimulate shoot proliferation. This was in contrast to nicotinic acid addition to the medium. Although an increase in embryogenesis has been reported for some plants as a response to nicotinic acid treatment (Barwale et al., 1986) this compound, a precursor for NAD and NADP, has been also found to impact on cellular redox reactions (Demiray and Dereboylu, 2013; Ohlsson et al., 2008), where increasing the concentration in the medium had no positive effect. Similarly, in addition to being a co-factor in enzymatic reactions, pyridoxine has also been reported to have antioxidant properties and modulates active oxygen species in plants (Bilski et al., 2000; Chen and Xiong, 2005; Denslow et al., 2005) as well as protects plants from photo-oxidative stress (Havaux et al., 2009). Compared to Nicotinic acid addition, pyridoxine addition had a more significant positive effect on *M. balbisiana* proliferation possibly contributing to limit the consequences of oxidative stress. However, pyridoxine limited proliferation when it amounts were greatly increased. Reasons for such negative effect on proliferation are, however, unclear and were not investigated in this study in more detail.

Light has previously been shown to aggravate oxidation. Full potential of ascorbic acid and thiamine to possibly alleviate the effect of oxidation/oxidative stress and proliferate shoot

formation was most obvious under dark conditions. Dark culturing greatly reduced blackening of the medium. Such blackening is caused by oxidation of phenolic compounds eventually resulting in generation of toxic derivatives which can kill plant tissues due to inhibition of enzymes (Arnaldos et al., 2001). It was further found that, without any antioxidant addition, shoot proliferation did not improve despite dark culturing. A synergistic effect very likely exists of both dark culturing and increased antioxidant amounts. Prolonged dark culturing, however, negatively affects proliferation resulting only in pale shoots with others dying due to lack of light.

The next chapter outlines the characterization of the morphological response of *M. balbisiana* to banana Xanthomonas wilt infection where banana shoots were used that have been produced on the optimized culture medium.

CHAPTER THREE

CHARACTERIZATION OF THE MORPHOLOGICAL RESPONSE OF *MUSA BALBISIANA*AND EAHB CV '*NAKINYIKA*' TO BANANA XANTHOMONAS WILT INFECTION

F. Ssekiwoko, K. Kunert, A. Kiggundu, W.K. Tushemereirwe and E. Karamura (2013). *Musa balbisiana resists* Xanthomonas wilt disease through interfering with the multiplication of Xanthomonas vasicola pv musacearum coupled with whole organ (leaf) death. *Uganda Journal of Agricultural Sciences,* 14 (2): 13 - 25

3.1 Abstract

Banana Xanthomonas wilt caused by *Xanthomonas vasicola* pv. *musacearum (Xvm)* is the most devastating disease of bananas and plantains in East and Central Africa. Since there is so far no known *Xanthomonas*-resistant banana among cultivated varieties, wild-type *M. balbisiana* was screened for resistance. *M. balbisiana* plants recovered from wilt symptoms with no build up of a *Xvm* population. Pathogen presence was further restricted to the inoculated and the immediate follower leaves, which subsequently wilted and died in some plants. Initial symptom development was further delayed with first symptoms observed in the inoculated leaf only 10 weeks after inoculation. Incidence and severity of wilt also remained low,with the exception of the individual wilted leaves that subsequently died, and plants continued to grow without anymore symptoms. *Xvm* population further dropped almost to a half by 12 weeks after inoculation in the immediate follower leaf and to below detectable limits in other follower leaves by 20 weeks after inoculation. In the susceptible cv '*Nakinyika*' *Xvm* migration was faster than in *M. balbisiana* and the pathogen spread to adjacent leaves already within 2weeks after inoculation. In same size tissue of susceptible cv '*Nakinyika*', the *Xvm* population also increased 300-times within 3 weeks after inoculation.

3.2 Introduction

Banana Xanthomonas wilt caused by *Xanthomonas vasicola* pv. *musacearum* (*Xvm*) causes total yield loss in an affected plant. While a number of cultural Banana Xanthomonas wilt control techniques have been developed, they have so far not been very effective. Finding resistance therefore remains the only viable option. A number of cultivars have been identified that employ physic-chemical barriers to protect themselves from initial natural *Xvm* ingress. Normally, *Xvm* penetrates banana through wounds created during human cutting activities or during male flower and bract abscission. Some cultivars are however resistant to the male bud mediated *Xvm* penetration due to the lack a male bud, indehiscent bracts and male flowers, and therefore no wounds, less attractive flowers for insect vectors, and also due to abscission wounds/scars which are less condussive to penetration and survival of *Xvm*. So far, only wild *M. balbisiana* showed promising resistance (Ssekiwoko et al., 2006). Its mechanism of resistance has, however, not been established yet.

This part of the PhD study was aimed to characterize the specific phenotypic response of resistant *M. balbisiana* to *Xvm* infection in comparison to the *Xvm* susceptible EAHB cv '*Nakinyika*'. Specifically, disease development, incidence and severity of wilt were measured alongside changes in *Xvm* population within tissues.

3.3 Materials and methods

3.3.1 Soil preparation and plant potting

Black top soil from the forest, sand from the shores of Lake Victoria and fully decomposed cow manure from a nearby animal farm/kraal were collected to produce a potted plant growth medium. Individual components were mixed in a ratio of 3:1:1 respectively and then steam-sterilized in a metallic cylindrical drum (45 cm diameter) for 8 hrs. The bottom of the drum was fitted with a rack up to a height of 15 cm and the bottom was filled with water up to 5 cm from the base. The top of the rack was lined with a heat-resistant micro-pore polystyrene mesh on which the mixed soil was placed. The soil was topped up to a height of 1.2 m and covered with same heat-resistant micro-pore polystyrene mesh. Fire was set up below the bottom of the drum to sterilize the soil for 8 hrs. After 5 days, when the soil was cool, 0.25 kg of soil was transferred into individual disposable plastic cups.

3.3.2 Plant weaning and hardening

The cool soil mixture was fully wetted by watering. Rooted banana plantlets in baby jars were drawn from the jars and washed in tap water to clean-off residual laboratory growth medium. Their roots were cut short to about 5cm and the plant corm bases were suspended in a solution of NAA (10 mg/l in ethanol) for 5 min before planting each plantlet into a disposable cup containing soil. The disposable cups, in which plantlets were planted, were then transferred to a make-shift humid chamber which was made of white transparent polyethylene sheeting covering

the surface of a rectangular cage of iron bars. After 2 weeks, the plantlets were put out of the humid chamber into the open in a green-house for 1 week to acclimatize and awaiting transfer to bigger pots.

3.3.3 Pot-layout and plant management

Acclimatized plantlets were transferred to larger pots (3 l capacity) containing 5 kg of steam-sterilized soil. The plantlets were allowed to further establish under screen-house conditions for an additional 2 weeks. Pots were labeled according to banana cultivar/genotype and randomly placed on a table, 1 m above the floor. They were watered once every after 3 days throughout the experimental period.

3.3.4 *Xvm* culture retrieval and inoculum preparation

A bacteriological medium designated YPGA was prepared by weighing 10 g glucose, 5 g yeast extract, 5 g peptone and 14 g agar into a medium preparation bottle, then distilled water added to 900 ml and the pH adjusted to 7 using 0.1 N NaOH or 0.1 N HCl. The volume was then topped up to 1 l, warmed-up to dissolve all ingredients completely in a microwave and then autoclaved at 1.05 kg/cm^2 at 121°C for 15 min before cooling and dispensing 25 ml onto 90 mm disposable and sterile plates. A virulent *Xvm* culture under preservation on 50% glycerol in a -80°C freezer was sub-cultured on YPGA medium by streaking out a thawed 50% glycerol-*Xvm* suspension. Streaked plates were incubated at 25°C for 5 days and the resulting yellow doom-shaped convex circular and shiny colonies typical of *Xvm* were tested for pathogenicity.

To test for bacterial pathogenicity, grown Xanthomonas cells on plates were placed into a sterile Falcon tube containing sterile distilled water. The optical density (OD_{600nm}) was adjusted to 0.1 (approximately $x10^8$ cells/ml) by spectrophotometer reading and appropriate dilution. Adjusted bacterial suspension (1 ml) was injected using a syringe and needle into the petiole of 3 plantlets of the EAHB-cv '*Nakinyika*' monitored for two weeks for symptoms development. Two further plantlets were also injected with sterile distilled water as a control.

After 2 weeks, *Xvm* was re-isolated from symptomatic plantlets. To re-isolate *Xvm*, part of the petiole of the wilted leaf was picked and surface-sterilized by wiping with cotton wool soaked in 1% NaOCl. The leaf was then rinsed 3-times by wiping with sterile wool soaked in sterile distilled water. Internal tissues were cut out using a sterile surgical blade and then suspended in 5 ml of sterile water for bacteria to ooze out into solution to form a bacterial suspension. A sample of the suspension was streaked onto YPGA plates and incubated for 5 days at 25°C. Yellow dome-shaped, convex shiny and circular colonies were increased by sub-culturing on a new YPGA plate and incubation of plates under identical conditions.

3.3.4 Plant inoculation

An inoculum of *Xvm* was prepared as described in section 3.3.4 above and test plants were inoculated by injecting 1 ml of bacterial suspension using a syringe and needle into the petiole of the youngest open leaf. Alongside, similar plantlets were similarly injected with sterile distilled water as controls. A total of 18 plantlets for each genotype EAHB-cv '*Nakinyika*' and *M.*

balbisiana were inoculated with *Xvm* and the same number of plants were inoculated with water as controls. Plantlets were then monitored for symptom development.

3.3.5 Disease progress assessment and description

The progression of symptoms of each plant was monitored. In addition, wilt incidence and severity index were both determined at a weekly interval. Wilt incidence was assessed by counting the number of wilted plants and expressed as a percentage of the total number of *Xvm* inoculated plants for a particular genotype. Wilt severity index was assessed by counting the number of wilted leaves on each plant and the number of wilted plants for both *Xvm* inoculated and control plants. These were rated on a 1-5 score with 0 = no wilted leaf, 1=1 wilted leaf, 2= 2-3wilted leaves, 3= 4or more wilted leaves, 4= all leaves wilted, 5= dead plant due to *Xvm*. The score was applied to calculate the wilt severity indices with the formula:

$$\textbf{Wilt Severity Index} = \left[\frac{(0*a)+(1*b)+(2*c)+(3*d)+(4*e)+(5*f)}{(n*5)} \right] 100$$

Where, n=total number of plants injected with *Xvm* for a given genotype, a-f=number of wilted plants at a corresponding scale.

3.3.6 Relative abundance of *Xvm* in planta over the disease progress time

The relative abundance of *Xvm* in plant tissues at a specific time for the two genotypes was determined at 18, 25, 56 and 90 days after inoculation. To estimate this *Xvm* population in the plants, part of the leaf petiole of the inoculated leaf, 5 cm below point of inoculation and its younger immediate follower leaf at a similar point was cut and returned to the laboratory. Each plant tissue was surface-sterilized by washing in dilute NaOCl (1:5) with water, then rinsed 3-times in sterile water and blotted dry. Using a sterile blade, inner tissues were cut out into a sterile 1.5 ml centrifuge tube and its weight taken. They were then suspended in sterile water at a rate of 2 ml of water for each gram of tissue. After 15 min, this suspension was serially diluted 6-times and then 10 µl of each dilution plated on semi-selective CCA medium (Mwangi et al., 2007). CCA contained (per liter): 1 g D(+) glucose, 1 g yeast extract, 1 g peptone, 1 g ammonium chloride, 3 g K_2HPO_4, 14 g agar, 1 g beef extract, 10 mg 5-flourouracil, 10 g cellobiose, 40mg cephalexin and 120 mg cycloheximide. To make the media, all components, except for glucose, cellobiose, 5-flourouracil, cephalexin and cycloheximide which were filter-sterilized, were weighed, made up to 700 ml with distilled water and autoclaved. The filter-sterilized components were only added to media after cooling to about 40°C. Medium was made up to 1 l with sterile distilled water, mixed and then dispensed into 90 mm plastic petri-dishes for bacterial culturing.

3.4 Results

3.4.1 Symptom development, wilt incidence, and severity changes

Wilt symptoms typical of *Xvm* infection were observed in both the EAHB cv. '*Nakinyika*' and *M. balbisiana*. In the EAHB cv. '*Nakinyika*', first wilting was observed 10 days after inoculation starting with the inoculated leaf, then to the upper younger leaves and finally to the lower older leaves where symptoms last developed in the oldest leaves. In comparison to the normal out-stretched leaf blades (Figure 3.1a), the blades of *Xvm* infected and wilting leaves collapsed along the midrib and eventually touched each other (Figure 3.1b). The blade generally turned pale-green, then yellow and finally brown. The incidence of wilt rose from 0% to 87% within 2 weeks after inoculation and by the 3rd week it had reached 100% (Figure 3.3). Within the same time, wilt severity, as represented by the wilt severity index, rose from 0% to 21% within 2 weeks. By the 4th week at 25 days after inoculation, all leaves and plants of the EAHB cv '*Nakinyika*' were wilted (Figure 3.1d) and by the 8th week, all plants had died and were dried (Figure 3.1f). By this time wilt severity had reached 92% (Figure 3.4).

In *M. balbisiana*, wilt symptoms were only observed 10 weeks after inoculation, also starting with the inoculated leaf. In contrast to the normally green outstretched leaves, *Xvm* infected *M. balbisiana* leaves started yellowing especially at the margins and leaf apex (Figure 3.2b) towards the petiole. Eventually the whole leaf would wilt (Figure 3.2e) and die while in other cases such leaves would only become necrotic and would be restricted to the apex. The incidence of wilt remained at 0% for 8 weeks and by the 12th week, it had only risen to 33 % (Figure 3.3). In the

same period, severity of wilt as expressed by wilt severity index remained at 0% for 8 weeks after which it rose to about 20% by the 12th week (Figure 3.4). In addition, *M. balbisiana* internal necrosis/ tissue browning was noted around the point of inoculation and was more intense at *Xvm* inoculated points in comparison to water-inoculated points.

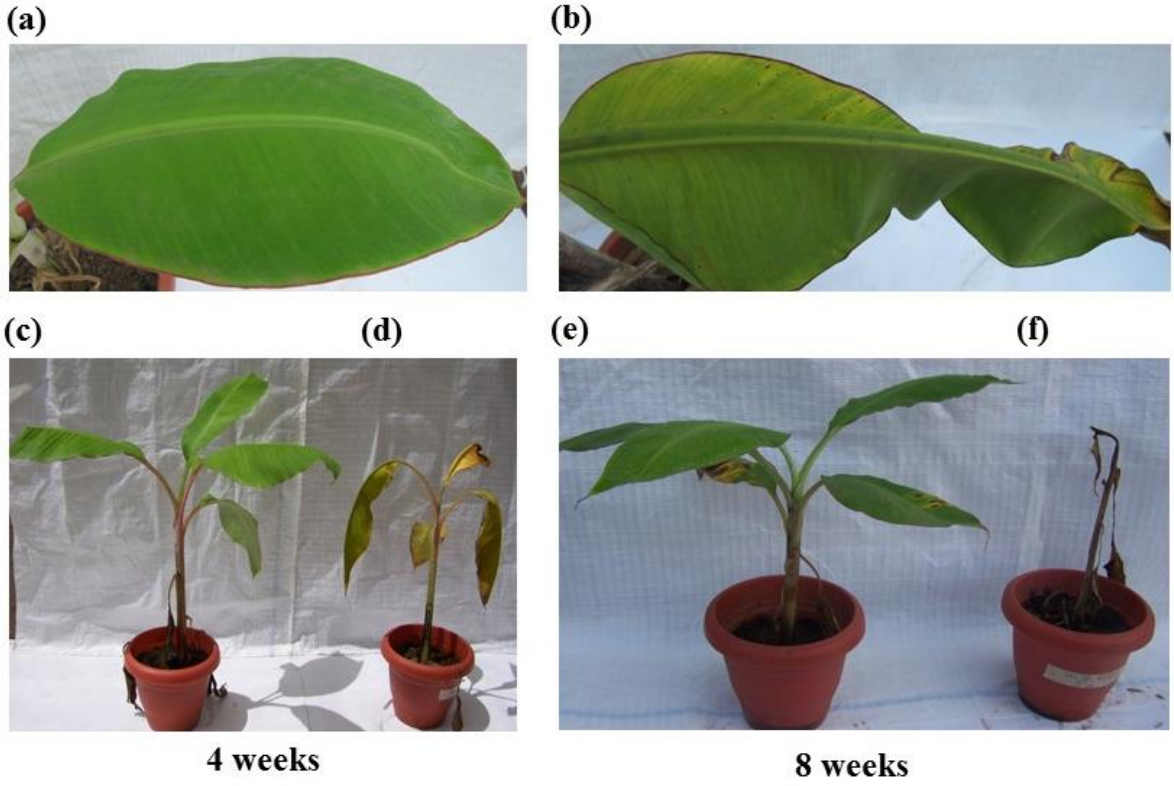

Figure 3.1: The EAHB cv. '*Nakinyika*' (a) leaf of water-inoculated plant, (b) leaf of *Xvm*-inoculated plant after 3 weeks, (c) water-inoculated plant after 4 weeks, (d) *Xvm*-inoculated plant after 4 weeks, (e) water-inoculated plant after 8 weeks and (f) *Xvm*-inoculated plant after 8 weeks.

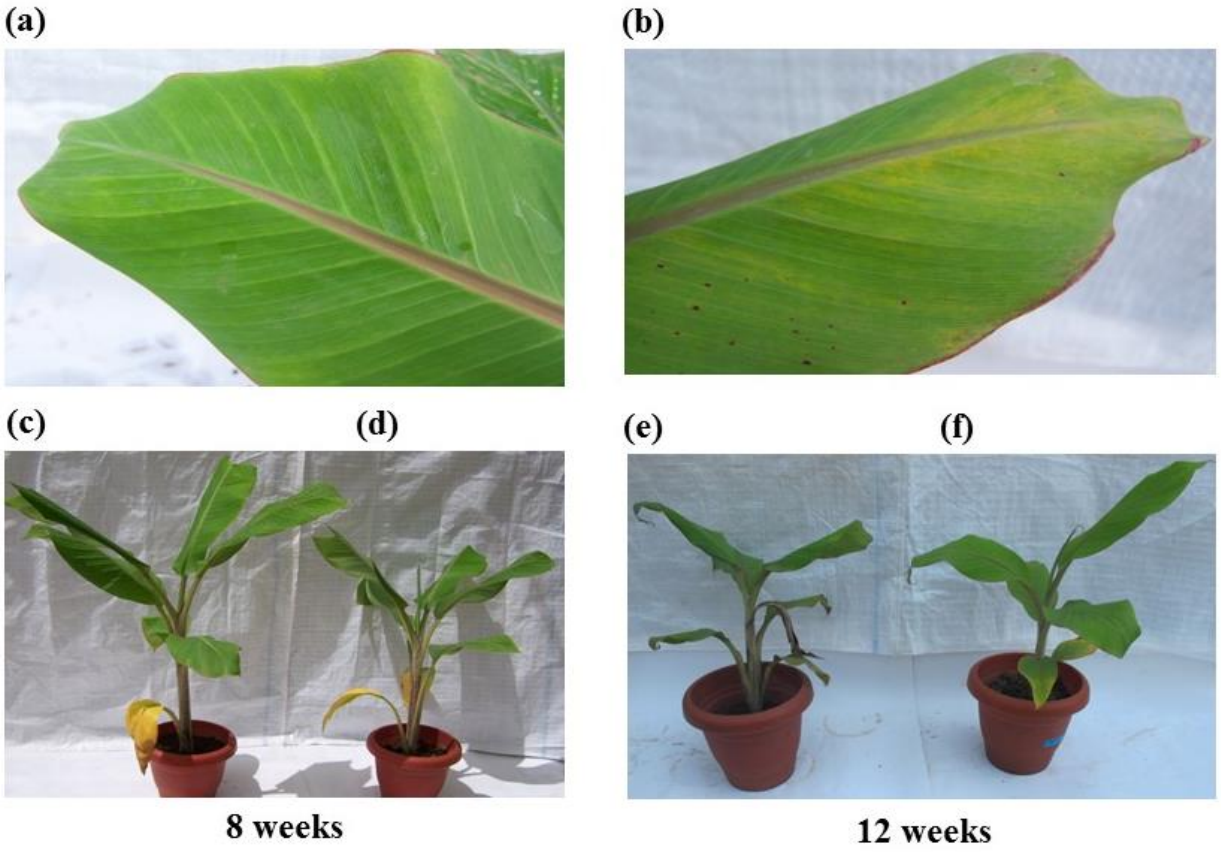

Figure 3.2: *M. balbisiana* (a) leaf of water-inoculated plant, (b) leaf of *Xvm*-inoculated plant after 10 weeks, (c) *Xvm*-inoculated plant after 8 weeks, (d) water-inoculated plant after 8 weeks, (e) *Xvm*-inoculated plant after 12 weeks with a single wilted follower leaf and (f) water-inoculated plant after 12 weeks.

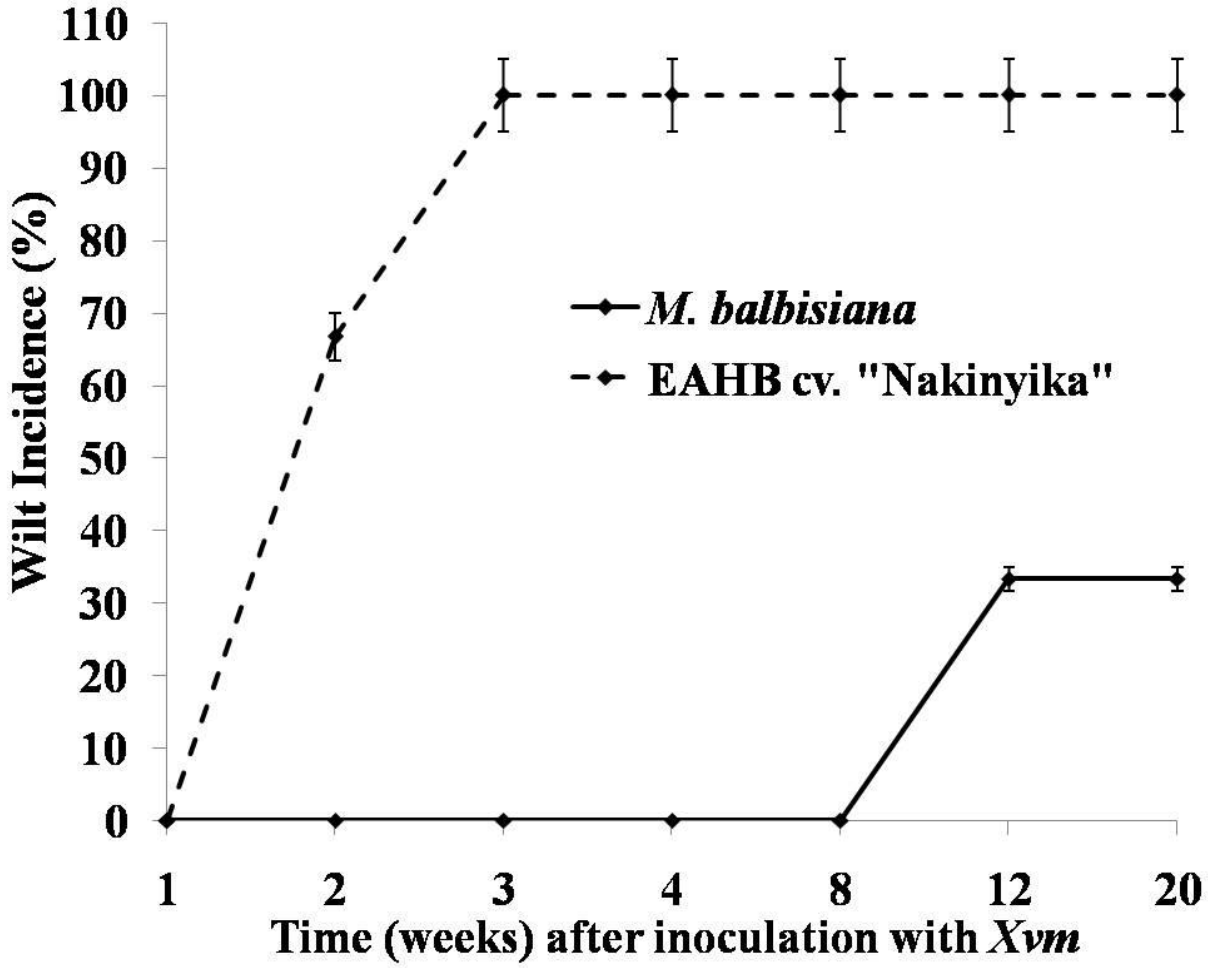

Figure 3.3: Change in wilt incidence over time in EAHB cv. '*Nakinyika*' (dotted line) and *M. balbisiana* (solid line) following inoculation with *Xvm*.

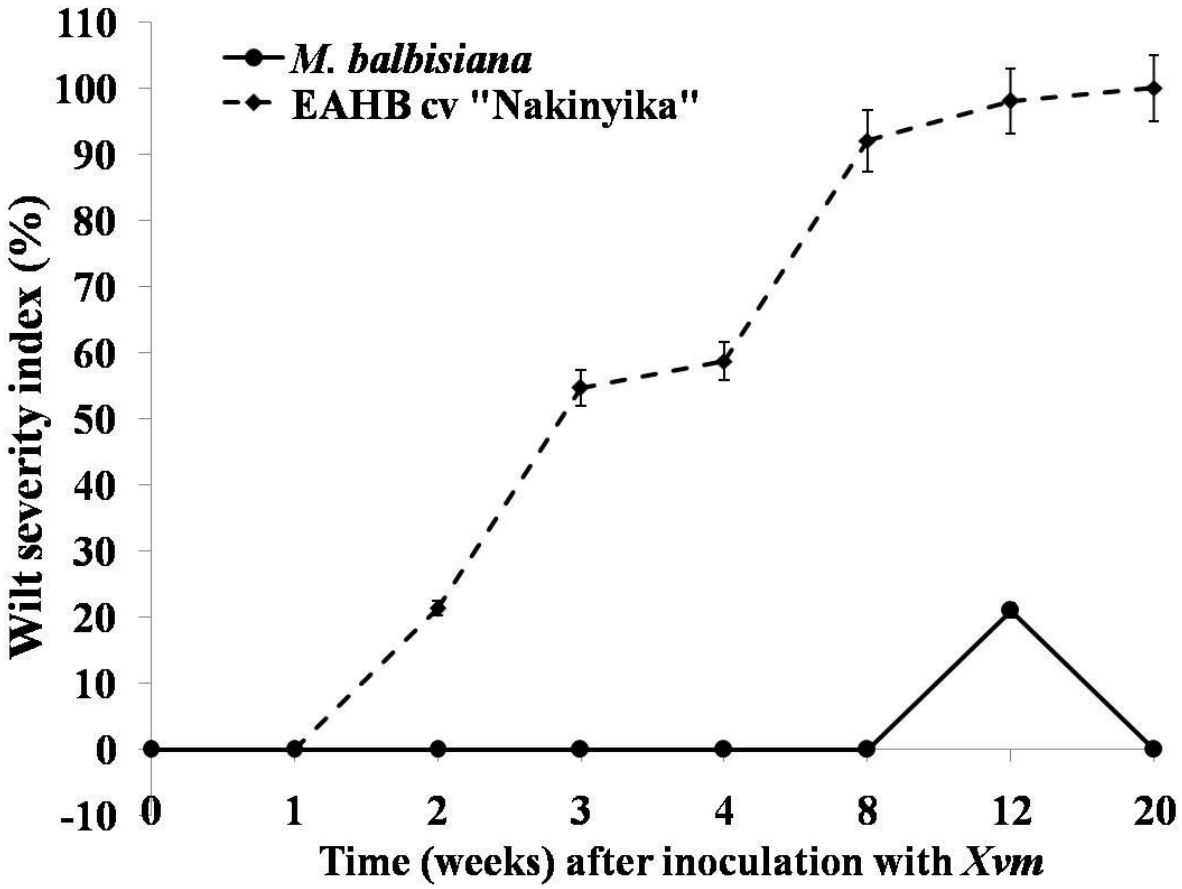

Figure 3.4: Change in wilt severity index over time in EAHB cv. '*Nakinyika*' (dotted line) and *M. balbisiana* (solid line) following inoculation with *Xvm*.

3.4.2 Migration and multiplication of *Xvm* within banana

In the EAHB cv. '*Nakinyika*', *Xvm* multiplied and migrated from the inoculated leaf to follower leaves. At 18 days after inoculation, *Xvm* was detected in the leaf immediately following the inoculated one (Table 3.1). In addition, the population of *Xvm* in both inoculated and follower leaf tissues continuously built up until 25 days after inoculation and the population in follower leaves was higher than that in inoculated leaves (Table 3.1). After 56 days, plants had died and *Xvm* could not be recovered. In *M. balbisiana*, *Xvm* could initially also be detected in inoculated leaves at 18 days. *Xvm* did, however, not migrate to follower leaves yet and the population in inoculated leaves was reduced (Table 3.1). *Xvm* had migrated by 90 days after inoculation and was detectable in the follower leaves. However, the population was low when compared to the population in susceptible '*Nakinyika*'.

Table 3.1: Relative average number of *Xvm* cells recovered from petiole tissue

	M. balbsiana		*Nakinyika*	
DAI	Inoculated leaf	Follower leaf	Inoculated leaf	Follower leaf
18	$3.06x10^{7}$	0	$3.41x10^{8}$	$3.55x10^{8}$
25	$2.23x10^{6}$	0	$1x10^{10}$	$1.08x10^{11}$
56	$8.7x10^{5}$	0	*	*
90	*	$1.5x10^{4}$	*	*

Inoculation of petiole tissue (50 mg)
*=Dead and rotten part due to *Xvm* infection
(DAI=days after inoculation with *Xvm*.

3.5 Discussion

In this part of the PhD study it was found that *M. balbisiana* and the EAHB cv. '*Nakinyika*' responded differently to infection with *Xvm* with *M. balbisiana* strongly resistant to Xanthomonas. While plants can use physic-chemical barriers, such as the waxy cuticle, a thick cell wall and stomata closure (Melotto et al., 2008; Melotto et al., 2006) to limit/defend themselves against invading pathogenic microbes, the mode of inoculation used in this study bleached such barriers and created wounds through which *Xvm* could penetrate. Any defense response that would eventually be effective in limiting *Xvm* effects would be therefore those that are normally active when the pathogenic bacteria are inside the host plant tissues. Leaf wilting, which is the major external symptom of *Xvm* infection in non-flowered banana plants, was less severe in *M. balbsiana* (20%) than in *Nakinyika* (92%) 12 weeks after inoculation with *Xvm*. This confirms a previous report that cultivars within the East African Highland bananas, which also includes *Nakinyika*, are highly susceptible to *Xvm* (Ssekiwoko et al., 2006). *Nakinyika* could not resist *Xvm* infection and the pathogen successfully multiplied, migrated through the whole plant ultimately causing total wilting and breakdown of the plant. This migration of *Xvm* was further found consistent with observations by (Ocimati et al., 2013) that from inoculated leaves *Xvm* moves to younger leaves before moving to the older leaves. However, even susceptible hosts can detect invading pathogens and develop some degree of defense for limiting pathogen multiplication and migration except that defense responses either breakdown or are evaded by the pathogens which results in disease development (Kim et al., 2005b; Zheng et al., 2012).

In *M. balbisiana* wilt symptoms delayed to develop. This is, therefore, the first report of delayed symptom expression in *M. balbisiana* although long latent periods of *Xvm* infection in cultivated

banana have already been reported (Ocimati et al., 2013). Long latent periods of bacterial infections in plants have been associated with a low bacterial population density that cannot enable bacteria to express pathogenesis traits in plants (Loh et al., 2002; Von Bodman et al., 2003). Plant defensive compounds, such as phenolics, phytoalexins and plant resistance proteins, can thereby delay disease incubation and cause long latent periods either through interference with virulence factors (Li et al., 2009) or through direct killing of pathogens (Maddox et al., 2010). Low wilt severity, coupled with limited multiplication and migration of *Xvm* in *M balbisiana* was a unique response not reported before for banana. Bacteria, as short generation organisms, build up their populations quickly in a matter of hours. Recovery of *Xvm*, though in low number 8 weeks after inoculation, indicates some multiplication of *Xvm* in *M. balbisiana*. Hosts that greatly limit the build-up of pathogenic bacteria populations are considered resistant which has already been found in many other resistant host plants. This includes resistance; in rice to *Xanthomonas campestris* pv. *oryze* (Barton-Willis et al., 1989), tomato to *Ralstonia solanacerum* (Nakaho et al., 2004) and beans to *Xanthomonas campestris* pv. *phaseoli* (Goodwin et al., 1995).

In addition to limited multiplication, it was also shown that *Xvm* did not quickly migrate to proximate parts in *M. balbisiana*. Restrictions of bacterial multiplication and migrations beyond points of inoculation has been previously attributed to either the hypersensitive response (HR), especially to biotrophic bacteria (Iakimova et al., 2005; Khurana et al., 2005; Morel and Dangl, 1997) but also to thickening of the pit membranes, accumulation of electron-dense materials around pits, and apposition layers in parenchyma cells next to vessels, as reported for *Ralstonia solanacearum* infections in resistant tomato (Nakaho et al., 2004). HR is observed as rapid

programmed death of plant tissue/cells around the point of infection followed by death of pathogens there in. These typical HR reactions, localized leaf necrosis, did not develop in *M. balbisiana*. Tissue death alone would also have not blocked *Xvm* migration to other parts as it is not an obligate biotrophic. To a limited extent, *Xvm* multiplied and migrated beyond the point of infection even in the resistant type. Plants can also defend themselves against certain pests by initiation of abscission leading to dropping infested parts to substantially lower the pest load (Williams and Whitham, 1986). Observed death of individual leaves in *Xvm*-challenged *M. balbisiana* could, therefore, also be a result of programmed leaf abscission eliminating the affected leaf and preventing further pathogen migration. Such leaf abscission is further mediated by elevated production of ethylene (Reid, 1985). However, ongoing studies in my research group have recently shown that ethylene synthesis genes are repressed in *Xvm*-challenged *M. balbisiana,* but strongly expressed in Xanthomonas-susceptible bananas. Plant phenolic compounds, including salicylic acid, are known to inhibit synthesis of ethylene but are also naturally antimicrobial (Maddox et al., 2010) which might be of relevance to *M. balbisiana* being Xanthomonas resistant.

The response of *M. balbisiana* to the *Xvm* including PR proteins expression and accumulation of phenolic compounds is presented in the next chapter.

CHAPTER FOUR

MOLECULAR RESPONSE AND PHENOLICS PRODUCTION OF *MUSA BALBISIANA*

F. Ssekiwoko, A. Kiggundu, W.K. Tushemereirwe, E. Karamura, K. Kunert (2015). *Xanthomonas vasicola* pv. *musacearum* down-regulates selected defense genes during its interaction with both resistant and susceptible banana. *Physiological and Molecular Plant Pathology,* 90: 21-26.

4.1 Abstract

A comparative analysis of expression of a disease resistance gene *MbNBS* (*R* gene), the nonexpressor of pathogenesis-related protein1 (*NPR1*genes) and the pathogenesis-related gene PR3 was carried out after *Xvm* infection. An initial decline in the expression of all tested genes was found in both resistant *M. balbisiana* and susceptible banana EAHB cv '*Nakinyika'* possibly indicating that both are initially disabled in their ability to actively defend themselves against *Xvm* infection to allow pathogen infestation and disease development. However, expression significantly increased in the later stages of infection particularly in resistant banana *M. balbisiana* though, plants continued to develop symptoms. To further investigate the reason why *Xvm* did not sustainably establish in *M. balbisiana* with multiplication and migration slowed down and subsequently cleared from host tissues, a passive mode of resistance to *Xvm* infection was also investigated in *M. balbisiana* and the measured plant-derived phenolics significantly inhibited *Xanthomonas* growth.

4.2 Introduction

Pathogen infection induces expression of a range of genes to overcome pathogen infection (Pieterse and Van Loon, 2004; Wally et al., 2009). The hypersensitive response (HR) with cell death of resistant plants is thereby a prominent protection process against invading pathogens. HR can be further associated with an increase in salicylic acid production, transcription of pathogenesis-related (*PR*) genes, and the establishment of a long-lasting systemic response known as systemic acquired resistance (Hammond-Kosack and Jones, 1996). A central component of this process is the nonexpressor of pathogenesis-related proteins1 (NPR1), which interacts with transcription factors resulting in activation of various forms of antimicrobial pathogenesis-related (PR) genes (Cao et al., 1994; Foyer and Noctor, 2011; Mou et al., 2003; Shi et al., 2010). *NPR1* genes have already been isolated from banana (*MNPR1A* and *MNPR1B*; Endah et al., 2008 and *MdNPR1*; Zhao et al., 2008) in response to infection with *Fusarium oxysporium* f.sp. *cubense* and *Xvm*. While *MNPR1A* and *MNPR1B* differ in their sequence, they have been shown to confir similar protection in transgenic *Arabidopsis* to various pathogens (Endah et al., 2012). Their expression in non transgenic banana was also reported to be sequencial, first *MNPR1A* and later *MNPR1B* following infection with *Xvm* where their upregulation also corralated with upregulation of *PR1* and *PR3* (Endah et al., 2008) in banana normally less sensitive to pathogens. While such responses were noted both in *Arabidopsis* and banana, there is no evidence that these genes can actually confir resistance to *Xvm* in banana especially in the now confirmed resistant wild *M. balbisiana*. Other than active defense responses due to resistance gene expression, plants use pre-existing passive barriers and antagonists to resist pathogen. Many plant secondary metabolites, including phenolics, present

the first line of chemical defense against plant pathogens where in-vitro studies have shown them to have antimicrobial activity (Maddox et al., 2010). A steroidal saponin was, for example, reported to inhibit growth of the banana pathogenic fungus; *Mycospherella fujiensis* (Cruz-Cruz et al., 2009), while another phenolic compound, p-coumaric acid, was shown to represses the Type Three Secretion System of *Dickeya dadantii* (Li et al., 2009).

In this part of the PhD study, an attempt was made to better understand the mechanism of resistance of *M. balbisiana* to *Xvm* infection by first investigating expression changes of selected genes located at specific stages of the defense pathway in plants of *M. balbisiana* and the East African Highland banana, cv. '*Nakinyika*' following inoculation with *Xvm*. Secondly, the effect of banana methanolic extract compounds on the *in-vitro* growth of *Xanthomonas* was determined to also evaluate a passive mode of resistance to *Xvm* infection.

4.3 Materials and methods

4.3.1 Gene expression

4.3.1.1 *Plant materials, inoculation and sampling*

A screen-house pot experiment was set up with tissue cultured banana plantlets of *M. balbisiana*. Alongside these, plantlets of an East African Highland banana, cv. '*Nakinyika*' which is highly susceptible to *Xvm* infection was also included. Fourty two plants of each banana type were weaned in plastic pots and allowed to establish for 8 weeks. A completely randomized design was adopted for the trial in which the two treatments, injection with either water or *Xvm*, were randomly applied to 21 plants of each genotype.The *Xvm* inoculum of approximately 10^8 cells per ml was prepared using one of the vilulent preserved cultures at the National Biotechnology center Kawanda, Kampala Uganda. Water, or *Xvm* (100 µl each), as inoculum was drawn into a hypodermic syringe and then injected into the second youngest open leaf petiole of the test plants. A random sample of 3 plants for each treatment was drawn at intervals of 0 hr, 6 hr, 72 hr, and 14 days post inoculation for RNA extraction. To collect a plant sample, the petiole of the follower leaf to the inoculated leaf was cut at once with a sterile surgical blade, wrapped in a labeled aluminum foil sheet and immediately frozen in liquid nitrogen for RNA extraction. Later, another random sample of three plants for each treatment was drawn at 18, 25, 56, 90 and 150 days after inoculation for *Xvm* isolation. For assessment of bacterial population, changes within plant tissues part of the leaf petiole of the inoculated leaf at 5 cm below point of inoculation and its younger immediate follower leaf was cut, surface-sterilized by washing in NaOCl, diluted 1:5

with water, then rinsed 3-times in sterile water and blotted dry. Tissues were aseptically cut, weighed and suspended in sterile water (0.5 g/ml of water). After suspending for 15 min, this suspension was serially diluted 6-times, 10 µl of each dilution were plated on semi-selective CCA medium (Mawangi et al., 2007) and incubated at 25^{o}C for 5 days.

4.3.1.2 *RNA extraction*

To extract RNA from each sample, the CTAB nucleic acid extraction protocol was used. Generally, 0.1 g of each frozen plant tissue was crushed in a separate sterile and frozen motor into a powder and 1.5 ml of pre-warmed (65ºC) extraction buffer containing: CTAB (20 g/l), NaCl (81.76 g/l), PVP-40 (20 g/l), Tris-HCl (15.7 g/l), EDTA (18.6 g/l) and 2% ß-mercapto-ethanol was added. The mixture was incubated at 65°C for 15 min while mixing every 2 min by inversion after which it was spun at 13000 rpm for 5min. In a new sterile tube, 700 µl of supernatant were transferred and cooled on ice for 2 min after which 700 µl of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. It was then spun at 13000 rpm for 5 min at 4°C. The aqueous phase (600 µl) was transferred to a new tube and an equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1) added, mixed by inversion, then spun at 13000 rpm for 5 min at 4°C. Finally, 500 µl of the aqueous phase was transferred to a new tube, and RNA precipitated by adding 10 M lithium chloride to a final molarity of 2.25 M then incubated at -20ºC for 8 hr. The precipitate was then collected by spinning at 13000 rpm for 45 min after which the supernatant was discarded. The pellet was reconstituted in water to 450 µl, then 1/9 volume of 3 M sodium acetate, pH 5.2 added followed by an equal volume of cold iso-propanol (-20°C) and mixed by inversion. The mixture was kept at -20°C overnight and then spun at

13000 rpm for 2min at 4°C. Iso-propanol was decanted and the pellet washed twice in 500 µl of 70% ethanol by tapping and spinning at 13000 rpm for 2min at 4°C. The pellet was air-dried at room temperature for 40 min after which it was re-suspended in 30 µl of sterile nuclease free water and stored at -20ºC awaiting genomic DNA removal.

4.3.1.3 *Removal of genomic DNA*

To remove any trace of genomic DNA, a DNase treatment kit by Thermo-scientific (No. EN0521) was used following the manufacturer's protocol. To 6 µl of nuclease-free water was added 1 µl of 10X buffer (400 mM Tris-HCl, pH8, 100 mM $MgSO_4$, 10 mM $CaCl_2$), then 2 µl of RNA (approximately 0.5 µg) and 1 µl of DNase I (1 U/µl). The resultant 10 µl reaction mixture was incubated at 37°C for 30 min after which 1 µl of 50 mM EDTA was added and incubated at 65ºC for 15 min to inhibit any further DNase I reaction. The content was immediately chilled on ice for 1 min, collected by brief spinning and then stored at -20°C awaiting cDNA synthesis.

4.3.1.4 *Synthesis of complimentary DNA (cDNA)*

cDNA was synthesized using RevertAid™ first-strand cDNA synthesis Kit (Fermentas Life Sciences, UK) according to the manufacturer's protocol. To 8 µl of nuclease-free water 3 µl of pure RNA sample was added and 1 µl of random hexamer primers (0.2 µg/µl). This mixture was incubated at 65°C for 5 min, then immediately chilled on ice for 5 min and the content was collected by brief spinning. During reverse transcription, to the primer annealed reaction mixture 4 µl of 5X reaction buffer, 1 µl Ribolock RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix,

and 1 µl of reverse transcriptase (200 U/µl) were added. This mixture was incubated at 25°C for 5 min after which it was further incubated at 42°C for 1 hr followed by heating at 70°C for 5 min to inactivate reverse transcriptase. The content (now cDNA) were then chilled on ice, collected by spinning and the cDNA was quantified using a nanodrop 2000 instrument for all samples taken and was standardized to 0.08 µg/µl by dilution in sterile nuclease-free water before storing at -20°C awaiting PCR reaction.

4.3.1.5 *Primer design and semiquatitative PCR*

Primers used in the study are shown in Table 4.1. MbNBS primers for amplification of genes with NBS-LRR binding sites that are typical of detecting pathogen effectors were designed from banana sequences with the NBS-LRR domains of similar banana sequences deposited in the NCBI data base. MdNPR1 primers were as used by Zhao et al. (2008). For amplification of a banana NPR1, PR3 and the 25 s rRNA (a house-keeping gene) were as designed and applied by Endah et al. (2010).

Table 4.1: Primer sequences optimum working conditions and expected band sizes.

Primer	Primer sequence	Working concentration			Annealing temparature	Band size (bp)	Reference
		Primer	dNTPs	$MgCl_2$			
25s	F: ACATTGTCAGGTGGGGAGTT R: CCTTTTGTTCCACACGAGATT	0.15µM	0.2mM	1.5mM	57ºC	106	Endah et al., 2010
MbNBS	F: TGCTGGTGTTGGATGATGTT R: GGCAGGTCTTGGTAGCTCAG	0.3µM	0.2mM	1.5mM	62ºC	433	Self designed
MdNPR1	F: GATAAGGCTATGGTGGAAGA R: CGCCCTAGTTAGTCTCCTACAT	0.1µM	0.4mM	4mM	55ºC	555	Zhao et al., 2008
PR3	F: GGCTCTGTGGTTCTGGATGA R: CCAACCCTCCATTGATGATG	0.1µM	0.4mM	4mM	60ºC	149	Endah et al., 2010

For the same set of plants at a specific sampling point, cDNA was pooled prior to running a PCR. A water-diluted PCR mixture (10 µl) containing 0.05 µl Taq polymerase, 1X Taq buffer, dNTP mix, $MgCl_2$, all ingredients from Qiagen, Germany, 1 µl cDNA(30 ng/µl), and each of the forward and reverse primers (Table 4.1) was set up and run in a thermos-cycler at an initial activation temperature of 95^{o}C for 5 min, followed by cycle denaturation at 94^{o}C for 5 sec for *25s rRNA* and *PR3* and 15 sec for *MdNPR1* and *MbNBS*. Primer annealing was set for 5 sec for PR3, 10sec for *MdNPR1* and 15 sec for *MbNBS* and cycle extension was set at 72^{o}C for 10 sec for *25s rRNA* and PR3 and 15 sec for *MdNPR1* and *MbNBS*. All PCR reactions for the different primers were run for 25 cycles and a final extension at 72^{o}C for 20 sec. The amplified DNA was subjected to electrophoresis in a 2% gel at 110 V for 40 min, stained in ethidium bromide, visualized under UV and finally photographed.

4.3.2 *Xvm* growth on plant extracts

4.3.2.1 *Extraction of freely bound soluble phenolics*

One half (0.5) kg of corm and leaf tissue of each of *M. balbisiana* and the EAHB cv '*Nakinyika*' was quickly crushed in 2 liters of water-diluted (80%) methanol in a juice blender. 25 ml aliquots of the suspension were centrifuged at 6000 rpm for 10 min and the supernatant saved in an amber-colored bottle. This was then evaporated to a gelatinous paste in a rotary-evaporator at 60°C. Part of the resultant 3 g paste was used to determine its effect on the growth and multiplication of *Xvm in-vitro*.

4.3.2.2 *Column chromatographic separation of extract into fractions*

To separate the extract into fractions, a 50 ml burette was filled with 50 ml of water-diluted methanol as solvent (50% v/v) and then packed with a 5 ml volume of silica gel for chromatography. The solvent was drained out until it was just above the packed silica. Then 1 g of gelatinous extract from the corm tissue, dissolved in 2 ml of solvent, was introduced. The burette was then refilled with solvent and the tap was opened to allow flow over the stationary phase (silica gel). The extract run down the silica and was collected in 1.5 ml vials. After all the extract had entered the stationary phase (silica gel), 5 ml of water-diluted (80%) methanol was added. When the meniscus was just above the stationary phase, another 5 ml of un-diluted (100%) methanol was added, each time collecting 1.5 ml of elute that corresponded to a change in sample darkness intensity. Five fractions were obtained and concentrated in a 'speed-vac'

concentrator to 0.5 ml. Then 200 µl of each fraction was used to determine its effect on the growth and multiplication of *Xvm in-vitro*.

4.3.2.3 *In vitro growth and multiplication of Xvm on extracts/fractions*

Xvm (20 µl at $X10^6$ CFU/ ml of suspension) were surface-spread on a 9 cm plastic disposable plate containing 25 ml of YPGA. Three 0.7 cm diameter holes were bored through the media on each plate using a sterile cork-borer. The holes were filled with 200 µl banana extracts including the one separated into various fractions. Nine holes in total were used for each sample and all plates incubated for 5 days at 25°C.

4.3.3 Data collection and analysis

Data on expression of specific genes was captured as band intensity differences on an agarose gel. This band intensity difference was converted into numerical data applying GelQuantNETsoftware. Band intensities of the housekeeping 25s rRNA were standardized and factorial differences were used to estimate intensity values for *PR3, MdNPR1* and *MbNBS*. The corrected band intensity values for the target genes were log transformed and analyzed with SAS version 9. Mean band intensity due to *Xvm* relative to basal intensity was expressed as a ratio of band intensity due to water relative to basal intensity. The resultant ratios at a particular sampling point were plotted as Log10 ratio of relative expression against time after inoculation.

Data on action of phenolics on *Xvm* growth was collected on the inhibition diameter and was subjected to statistical analysis using Statistical Analysis System (version 9). ANOVA was conducted and the difference in mean inhibition diameters compared for significance (at P = 0.05) for the different samples.

4.4 Results

4.4.1 Expression of resistance genes

Almost equal amplification of the 25s RNA gene, used a house-keeping gene, was found when identical amounts of cDNA derived from the different *Xvm* and water inoculated samples were tested. When expression of the *MbNBS, MdNPR1* and *PR3* genes were measured over a period of 14 days, transcription decreased in both *Xvm*-resistant plants of *M. balbisiana* and the *Xvm*-sensitive East African Highland banana cultivar '*Nakinyika*' during the first 6 hr after *Xvm* treatment (Figures 4.1 and 4.2). However, only in *Xvm*-resistant *M. balbisiana* transcription of all three genes increased between 6 hr and 72 hr. This was followed by a general expression decrease of all the three genes and a still a higher transcription of genes in *M. balbisiana* after 14 days than in *Nakinyika* (Figures 4.1 and 4.2).

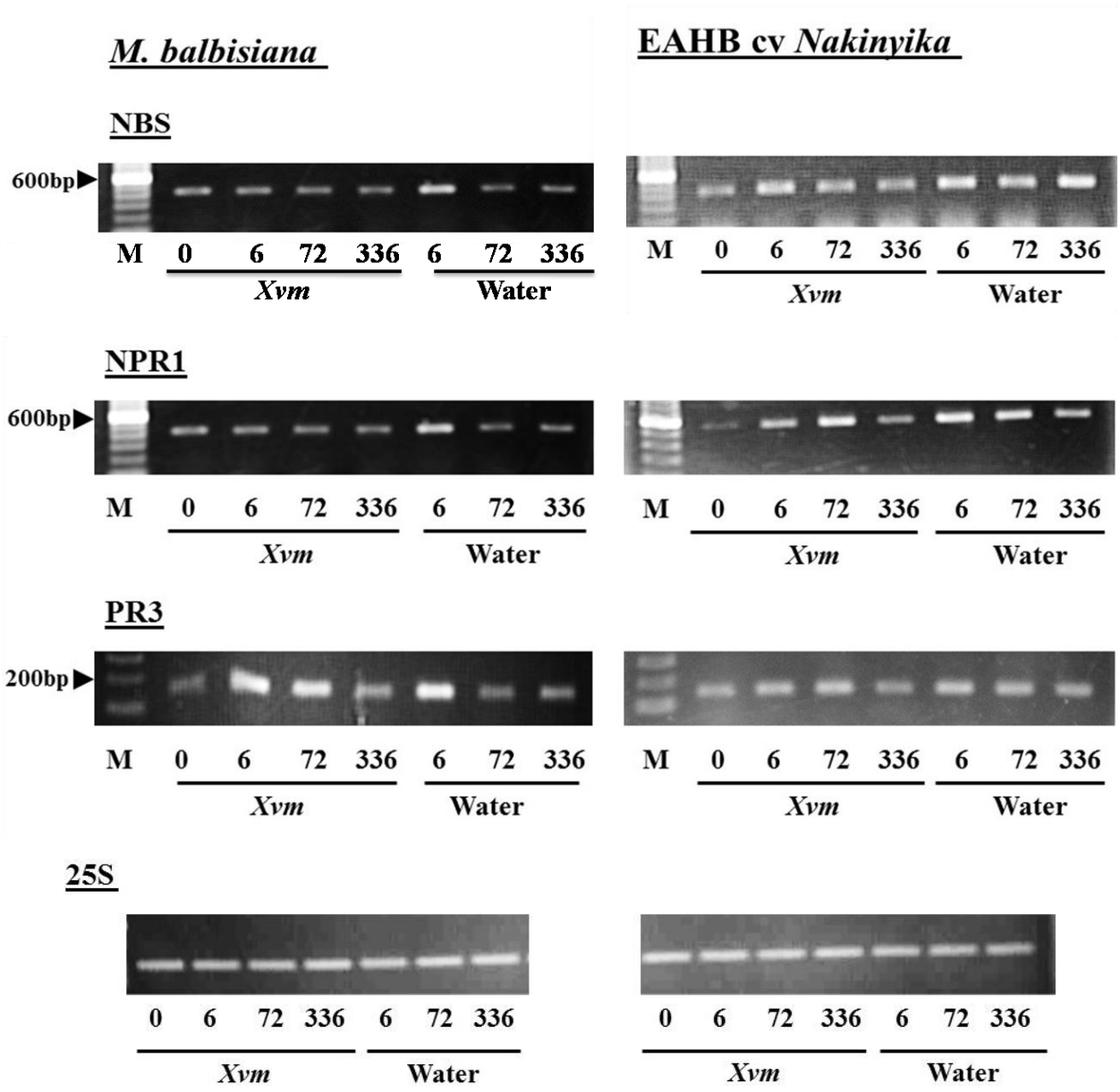

Figure 4.1: Expression profile of *MbNBS*, *MdNPR1*, *PR3* and *25S rRNA* in *M. balbisiana* and EAHB cv '*Nakinyika*' inoculated with *Xvm* and water. Measurements were made at 0, 6, 72, and 336 hr post inoculation. M indicates DNA size marker.

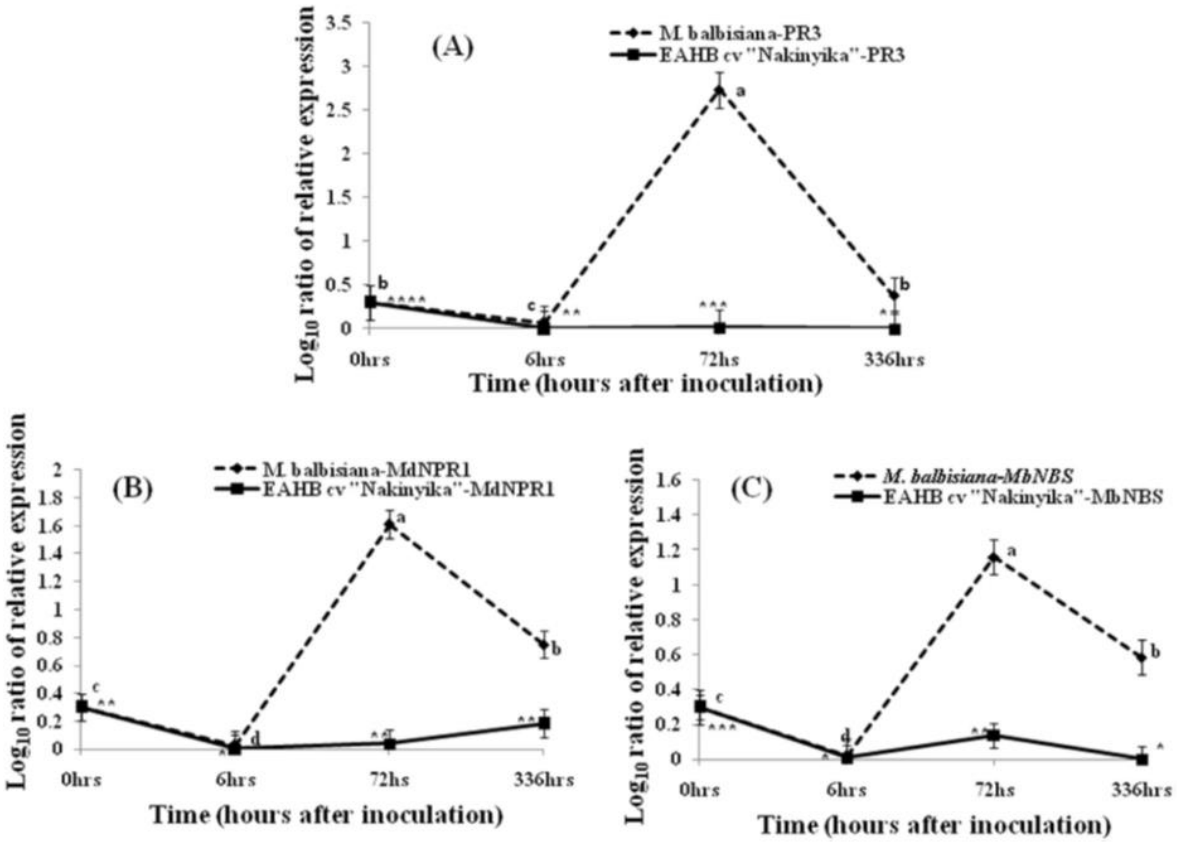

Figure 4.2: Relative expression of selected genes (A) *PR3*, (B) *MdNPR1* and (C) *MbNBS* in the banana defense pathway following inoculation of *M. balbisiana* and EAHB cv '*Nakinyika*' with the banana Xanthomonas wilt pathogen (*Xvm*).

4.4.2 Extract effects on growth and multiplication of *Xvm in vitro*

Since *Xvm* multiplication, migration and disease development were greatly reduced in *M. balbisiana* when compared to EAHB cv '*Nakinyika'*, the potential of plant extracts as a further cause for *Xvm* growth inhibition was investigated. An extract from the corm of *Xvm*-resistant *M. balbisiana* significantly inhibited the growth of *Xvm in-vitro* when compared to an extract from susceptible *Nakinyika* (Table 4.2). Further, controls (water or 80% methanol) did not inhibit *Xvm* growth (Table 4.2). Five fractions of the *M. balbisiana* extracts, designated A-E, from early (A) to late elution (E) variously affected the *in vitro* growth of *Xvm* on an artificial growth media. The fractions eluting last and being more dark-colored (D and E) significantly inhibited *Xvm* growth when compared to earlier lightly colored fractions (A, B and C) or the methanol and water controls (Table 4.3). The inhibition of growth of *Xvm* on artificial growth media by extract from *M. balbisiana* spread over an average distance of 1 cm from the point of application (Figure 4.3).

Table 4.2: Effect of crude banana extracts on *Xvm* growth *in vitro*

Part and cultivar	n	Mean inhibition diameter (cm)	% Inhibition
Corm of *Musa balbisiana*	24	2.1±0.53a	26.8±6.71a
Corm of *Nakinyika*	24	0b	0b
80% methanol	6	0b	0b
Water	6	0b	0b
Blank	12	0b	0b

Means with different letters in column are significantly different (P = 0.05)

Table 4.3: Effect of *M. balbisiana* extract fractions on *Xvm* growth *in-vitro*

Fraction	n	Mean inhibition diameter (cm)	% Inhibition
Water	3	0c	0c
50% methanol	3	0c	0c
A	3	0c	0c
B	3	0.1±0.1c	1.9±1.85c
C	3	0.1±0.06c	2.5±1.10c
D	3	0.4±0.12b	8±2.13b
E	6	0.9±0.25a	17.9±4.78a

Fraction E had the highest percentage inhibition, averaging at a radius of 0.5cm from the point of application.

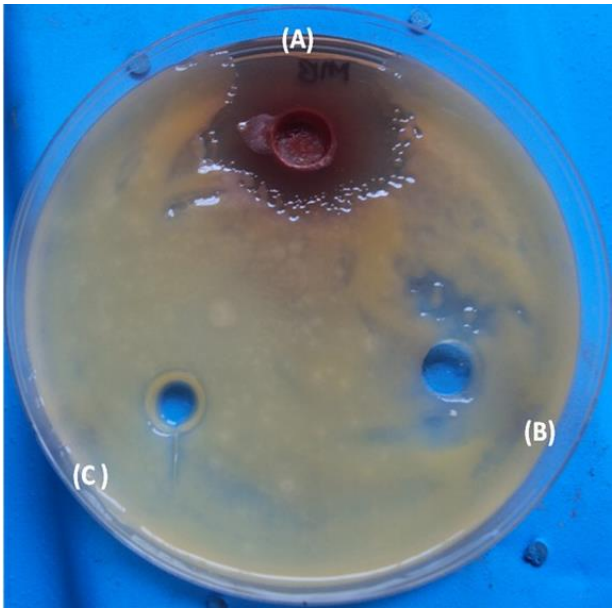

Figure 4.3: Growth of *Xvm* on yeast peptone glucose agar medium in presence of extracts from *M. balbisiana* (A), EAHB cv. '*Nakinyika*' (B) and water (C).

4.4 Discussion

A molecular dissection of the NPR1-PR defense pathway in this part of the PhD study revealed that expression of selected candidate genes varied over time after inoculation with *Xvm* in the *M. balbisiana* and the susceptible EAHB cv. '*Nakinyika*' genotype. There was a general increase over time in the expression of all three genes tested in the resistant genotype *M. balbisiana*. However, when compared to water-treated leaves, *Xvm*-treated leaves of both the resistant *M. balbisiana* and the susceptible EAHB cv. '*Nakinyika*' had an initial reduction in the expression of all tested genes. This result possibly indicates that both are initially disabled in their ability to actively defend themselves against *Xvm* infection to allow pathogen infestation and disease development. Further, gene expression was induced in the subsequent period up to 72 hr post inoculation, especially in the resistant banana leading to induction of *MdNPR1* and subsequently *PR3* expression. This is, however, rather late and consequently plants develop wilt symptoms in *M. balbisiana* much later. A similar result and an even longer delay was also recently reported by Kumakech et al. (2013). The expression of the *NPR1* gene (*MdNPR1*) is also consistent with the expression of *PR3* since *NPR1* is a known molecular switch that activates various forms of antimicrobial pathogenesis-related (PR) genes (Shi et al., 2010) leading to establishment of systemic acquired resistance and induced systemic resistance (Pieterse and Van Loon, 2004; Wally et al., 2009). *PR3* is normally induced in response to different abiotic and biotic stress factors. Further, since *PR3* gene product is an antimicrobial chitinase, finding in this study might also indicate that this protein could affect the pathogen establishing a resistance phenotype in *M. balbisiana*.

In a typical basal resistance response, host plants use specific pattern recognition receptors (PRRs), such as Flagelin sensing 2 and Elongation factor Tu receptor, on their cell-surfaces to detect the conserved microbe associated molecular patterns (MAMPs), such as bacterial flagellins, lipopolysaccharides, elongation factors, fungal chitin and hepta-glucosides of the invading microbes (Jones and Takemoto, 2004). This recognition triggers activation of signal-transduction cascades that turn on basal defense mechanisms, such as callose and silicone deposition to reinforce the cell wall, closure of stomata, production of reactive oxygen species, transcriptional induction of pathogenesis-related (PR) genes and post-transcriptional suppression of the auxin-signaling pathway (Nicaise et al., 2009).

The NBS-LRR domains of *MbNBS*, also measured in this PhD study, are characteristic of R genes that recognize and bind specific pathogen effectors inducing HR and SAR (Marone et al., 2013; Wang et al., 2012; Yue et al., 2012). Genome wide sequence data for *Xvm* further predicted that this pathogen uses 'type three secretion' effectors during host infection (Studholme et al., 2010), in which a bacterial needle-like structure delivers pathogens effectors into the hosts during infection. Work in my laboratory has already shown that *Xvm* expresses HrpF, which is an indispensable gene for establishment of the type three secretion system, during banana infection. Pathogen effectors are known to suppress host defense mechanisms (Cheong et al., 2013; Üstün et al., 2013). Repression of *MbNBS* below basal levels in the early hours following inoculation with *Xvm*, as found in this study, suggests that *Xvm* possibly first down-regulates *MbNBS* to evade detection (Gu et al., 2005b). Some pathogens can, indeed evade, and or suppress/shutdown, basal plant defense mechanisms by directly introducing their effectors into the cells using the type three secretion system (TTSS). Only plants that can recognize the

presence of these effectors inside the plant cell sets in a chain of reactions which eventually arrests the pathogen and the cell becomes resistant (Gu et al., 2005b).

Despite the success in disabling of the host banana detection system and related downstream reaction with also expected responses to a biotrophs, it is still unclear why *Xvm* did not sustainably establish in *M. balbisiana* with multiplication and migration slowed down and subsequently cleared from host tissues. A passive mode of resistance to *Xvm* infection was therefore also investigated in this banana genotype. Other than the HR-mediated disease resistance response and the action of pathogenesis-related proteins, various phenolic compounds have been reported to be antimicrobial affecting the multiplication and migration of bacteria within plants (Maddox et al., 2010). Many of such plant secondary metabolites, including phenolics, can present a first line of chemical defense with antimicrobial activity against plant pathogens (Maddox et al., 2010). A steroidal saponin was, for example, found to inhibit growth of the banana pathogenic fungus *Mycospherella fujiensis* (Cruz-Cruz et al., 2009) while *p*-coumaric acid repressed the Type Three Secretion System of *Dickeya dadantii* (Li et al., 2009). In this PhD study, constitutively produced plant-derived compound/s in *M. balbisiana* had also antibacterial activity against *Xvm in-vitro*. It is, therefore, likely that one or more of these phenolic compounds is also responsible for the resistance response in *M. balbisiana*. However, isolation of the specific compound and its chemical analysis are beyond the scope of this study.

CHAPTER FIVE

GENERAL DISCUSSION

This PhD study was motivated by the devastating effect of Banana *Xanthomonas* wilt on all cultivated EAHB banana cultivars including *'Nakinyika'* and the lack of resistance against the pathogen among these cultivars. These EAHB cultivars are an important staple in East and Central Africa, of which *Nakinyika* is a typical cooking variety, typically eaten after steaming. The interaction between pathogen-resistant *M. balbiasiana* and *Xvm* was therefore investigated with the aim to ultimately improve the important, but susceptible, EAHB banana genotypes exploiting a possible *M. balbisiana* resistance mechanism. *M. balbisiana* is a wild highly seeded banana species native to Southeast Asia where it grows up to 1,100 m above sea level. In East and Central Africa, it is restricted to germplasm collection centers including those maintained by Bioversity International. It exhibits disease resistance (Ssekiwoko et al., 2006) and drought tolerance (Ravishankar and Rekha, 2011), traits that are major limitations in most cultivated bananas. It could thus be used in conventional breeding to improve popular cultivars. The only challenge here however is that sequences of one of the major banana diseases, banana streak virus (BSV, a plant para-retrovirus, genus *Badnavirus* from the Caulimoviridae family, to which all EAHB are susceptible) are integrated in its nuclear genome (Harper et al., 1999). When inter-specific hybridization occurs, this virus becomes activated (Jain and Priyadarshan, 2009) affecting the new hybrid. Clones without this integrated viral genome have been developed to enhance breeding efforts.

Since availability of clean *M. balbisiana* plantlets was a necessity for the success of this study to investigate the interaction between pathogen-resistant *M. balbiasiana* and *Xvm*, a tissue culture process was first developed to allow production of sufficient clean *M. balbisiana* plantlets. Since phenolic compounds produced by *M. balbisiana* tissues prevented tissue proliferation,

establishment of an optimized *M. balbisiana* tissue culture process was achieved by supplementing the culture medium with a correct amount of anti-oxidative compounds to protect against phenolic action had to be achieved first. Damage to *M. balbisiana* tissues, including dissection-related damage, very likely causes oxidative stress and production of toxic phenolic compounds, particularly in the presence of light. This can result in the death of plant tissues due to inhibition of enzyme activity ultimately causing total tissue death (Abdelwahd et al., 2008; Bhat and Chandel, 1991; Leng et al., 2009; Loomis and Battaile, 1966; López Arnaldos et al., 2001; Mayer and Harel, 1979; Vaughn and Duke, 1984). In this study, *M. balbisiana* plantlets proliferated well when the medium contained optimized ascorbic acid and thiamine-HCl amounts as antioxidants to prevent oxidation. Also, culturing explants for a certain period in total darkness after initiation had a further positive effect on plantlet proliferation. Overall, modifications applied to the tissue culture process enabled the production of up to 40 *M. balbisiana* plantlets per explants and prevented any plantlet death. This plantlet number was, however, still lower than the number normally obtained on a proliferation medium with other banana cultivars under optimized culture conditions.

In particular methanol-soluble compound with along elution time in a column chromatography pre-existed in *Xvm*-resistant *M. balbisiana*, but not in the susceptible EAHB banana *'Nakinyika*'. Such phenolic compounds can very likely interfer with *Xvm* multiplication. Such low-molecular weight secondary metabolites are generally essential for plant disease resistance and accumulation of plant secondary metabolites is induced upon pathogen attack. Phenolic compounds with defensive functions are a class of plant secondary metabolites that contain one or more hydroxyl-derivatives of benzene rings and they are mainly synthesized from the phenyl-

propanoid pathway (Boudet, 2007; Maddox et al., 2010). Li et al. (2009) also reported that such phenolic compounds interfere with delivery of pathogen effectors into their hosts which is most likely also true for the *Xvm-M. balbisiana* interaction. In contrast, lack of such *Xvm* suppressing compounds in the susceptible EAHB banana *'Nakinyika'* allowed to sustain wilt symptoms and eventually caused plant death.

Production of such phenolic compounds might, however, not be the sole process to obtain *Xvm* resistance in *M. balbisiana*. The resistance mechanism in *M. balbisiana* might be more complex and also involves expression of resistance genes. An interesting finding in this study was that any defense gene expression was intially suppressed after *Xvm* infection in both types of banana investigated. This included expression of *MbNBS*, a typical *R* gene with the conserved NBS-LRR domain, as well as expresion of *PR3* and *NPR1*. *MbNBS* generally plays a role in pathogen detection and to set in a defense response whereas NPR1 represents a molecular switch. After monomerization and translocation of the NPR1 protein into the nucleus, the protein binds to transcription factors leading to transcription of pathogenesis-related genes such as *PR3*. Initial reduced expression levels below basal levels after inoculation found in this study suggests that *Xvm* might initially suppress *MbNBS* expression to avoid any detection before establishing infection and disease development. However, despite the success in initially suppressing the active defense response in both types of banana, expression of all tested three genes recovered in the later stages of pathogen infection. But only in *M. balbisiana* expression of these genes recovered above original basal levels with *Xvm* not sustaining infection and finally being cleared from the host tissue. Although Xanthomonas initially migrated to the leaves that are immediate followers to the inoculated one, the population of the bacterium declined in the plant tissue. In

addition, any symptom development was delayed with both wilt incidence and severity relatively low, and only the symptomatic leaves, the inoculated and the immediate follower leaves, eventually dying. The delayed symptom development was a unique resistance response. At the time when symptoms were observed, the bacterial density in the plant tissue was also much lower than before symptoms had appeared. Such delayed symptoms expression had previously been attributed to a possible lack of a bacterial quorum (Loh et al., 2002; Von Bodman et al., 2003).

Based on findings with *M. balbisiana*, this study has provided strong indication about the possible processes which might be targeted in EAHB banana cultivars to improve *Xvm* resistance when molecular tools, such as banana engineering, will be applied. Engineering of EAHB banana cultivars is currently a well-established procedure at NARO/Uganda. An interesting idea might also be that EAHB banana cultivars have lost an efficient natural response to stress, in particular to *Xvm*, through selection and they cannot respond any longer in a similar way like *M. balbisiana* to *Xvm* infection. To test this idea, future research might include experimental establishment of a possible correlation between *Xvm* resistance and resistance gene expression/phenolic compound production by screening any existing banana crossings in which *M. balbisiana* was a parent. Also screening of existing EAHB banana cultivars for variability in resistance gene expression/phenolic compound production might be a future action. Furthermore, this PhD study has already clearly indicated that any resistance response to *Xvm* should also include both induced expression of resistance genes and production of specific phenolic compounds not found in the susceptible EAHB banana *Nakinyika*. Unfortunatley, isolation and exact chemical analysis of phenolic compounds were beyond the scope of this PhD study due to

the lack of required infrastructure in Uganda. However, any strategy to enhance production of phenolic compounds might also negatively affect any tissue culture process involved in transgenic banana production. Finding a balance between an effective tissue culture process and improvement of resistance would therefore be a vital necessity.

Since EAHB banana cultivars possibly lack both sufficient resistance gene expression and production of phenolic compounds, over expressing the oxidative metabolic pathway EAHB by genetic engineering and production of phenolic compounds could present a worthwhile and viable strategy to obtain *Xvm* resistance. The oxidative burst is one of the most rapid plant defense responses to pathogen attack. It includes production of superoxide and hydrogen peroxide at the invasion site by various oxidases and peroxidases sources, such as NADPH oxidase (Torres et al., 2006; Nühse et al., 2007; Nicaise et al., 2009). Already, the action of the Plant Ferredoxin-Like Protein (PFLP) and the Hypersensitive Response Assisting Protein (HRAP), as defense proteins, cloned from sweet pepper, have been recently exploited to stimulate the oxidative state in transgenic EAHB banana cultivars to obtain resistance to *Xvm* (Tripathi et al., 2010; Namukwaya et al., 2012). PFLP encodes a 12.6 kDa protein (Dayakar et al., 2003) and in response to bacterial harpins, its casein kinase II phosphorylation (CK2P) site at the C-terminal induces the expression of the NADPH oxidase gene, *AtrbohD*, whose enzymatic product caytalyses production of reactive oxygen species (Lin *et al.*, 2011) which in turn sets in a cascade of reactions that culminate in Hypersenstive Response. HRAP on the other hand encodes a 29 kDa amphipathic protein and also intensifies the harpin-mediated hypersensitive response (Chen et al., 1998) . Harpins are microbial elicitors that interact with plant cell surface proteins thereby activating the plant defense response (Lee et al., 2001). In an *in-vitro* assay,

recombinant PFLP has previously been found to have anti-microbial activity against *Escherichia coli, Pectobacterium carotovorum* subsp.*carotovorum,, Xanthomonas campestris* pv. *vesicatoria, Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringae* (Huang et al., 2006). First results at NARO have further shown that over expressing the oxidative state in an EAHB banana cultivar greatly enhanced resistance to *Xvm* (Tripathi et al., 2010; Namukwaya et al., 2012).

Finally, a more detailed exploitation of the *M. balbisiana* resistance mechanism might also include searching for other genes, either stimulating the defense response or involved in phenolic compound production, which are not expressed in EAHB banana cultivars. Such genes could be then applied in banana engineering approaches. In this regard, an excellent example of a specific gene action is the resistance of grape phenolic stilbenes to fungal colonization due to ectopic expression of a grape stilbene synthase gene. Expression in the model plant tobacco led to increased resistance of transgenic tobacco to infection by *Botrytis cinerea* (Hain et al., 1993) Such search for genes might for example include transcriptome analysis in combination with Next Generation Sequencing as well as metabolome analysis to identify unique phenolic compounds produced. In particular, identification of genes differentially expressed in *M. balbisiana* and EAHB banana cultivars by RNASeq as a Next Generation Sequencing tool will be a useful strategy. The specific application of the RNASeq technology is thereby a viable option since the banana genome has been already sequenced and will be available as a reference genome. However, in the meantime investigating morphological modifications within the male inflorescence including total absence of male inflorescence, persistence of bracts and flowers, dehiscence of bracts and flower after complete healing of the abscission scars and screening banana varieties that limit the spread *of Xvm* through the male inflorescence are still the most

promising conventional options. Also, as a further interim option, cultural practices that include; breaking off male buds, sterilization of garden tools between plant operations, rouging of infected plants and planting only certified plantlets should still be a major activity for the crop protectionists and farmers.

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