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Musa balbisiana resists Xanthomonas wilt disease through interfering with the multiplication of *Xanthomonas vasicola* pv *musacearum* coupled with whole organ (leaf) death

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Abstract

Xanthomonas wilt disease caused by *Xanthomonas vasicola* pv. *musacearum (Xvm)* is the most devastating disease of bananas, plantains and Enset in East and Central Africa. There is no known resistant banana to this disease among the cultivated varieties but here we confirm that one wild type, *Musa balbisiana* is resistant. Plants recovered from wilt symptoms and although it initially migrated beyond the point of inoculation, *Xvm* population did not build up and its presence was restricted to the inoculated and the immediate follower leaves which subsiquently wilted and died in some plants. The pathogen population within plant tissues was observed to drop in a 50 mg tissue from $3.06x10^7$cfu at 18days after inoculation in the inoculated leaf to $1.5x10^4$cfu by the 12th week after inoculation in the immediate follower leaf and later to 0 cfu by the 20th week after inoculation in other follower leaves. In contrust to the same size of tissue in the susceptible cv "Nakinyika" its population increased from $3.41x10^8$ to $1.08x10^{11}$cfu within 3 weeks after inoculation. In addition, bacterial migration within *M. balbisiana* was slow, where it took up to 12 weeks to be detected as far as only the immediate follower leaves. In the susceptible cv "Nakinyika" *Xvm* migration was faster and it had spread to adjacent leaves within 2 weeks after inoculation. Consistent with the decreasing bacterial population and limited migration, initial symptom development was also delayed, only first observed in the inoculated leaf at 10 weeks after inoculation. In addition, the incidence and severity of wilt remained low at 33% and 20% respectively and with the exception of the individual wilted leaves that subsiquently died, the plants were never broken down but continued to grow without anymore symptoms.

Key words: Banana plantlets, resistant, tissue colonization, *Xanthomonas vasicola* pv. *Musacearum*

Introduction

The banana industry world over, generates export earnings and employs millions of people in Latin America, the Caribbean, southeast Asia, tropical Africa and in production, distribution networks and supermarkets worldwide. Bananas and plantains are the main fruit in international trade and the most popular one in the world. In terms of volume they are the first exported fruit, while they rank second after citrus fruit in terms of value (FAO, 2011). They are mainly consumed as a fresh fruit and in many developing countries; some cooking varieties are an

important food security staple and also a source of income for small holder farmers. In Uganda, per-capta consumption is estimated at 245 kg/year, the highest in the world and it is estimated that the East African Highland Bananas (EAHB) alone are a staple starchy food for 80 million people in sub-Saharan Africa.

The production of this important crop is however constrained by diseases including the devastating Banana Xanthomonas Wilt caused by *Xanthomonas vasocola* pv. *musacearum* (*Xvm*) in East and Central Africa (Yirgo and Bradbury, 1974; Ndungo *et al*., 2004; Tushemereirwe *et al*., 2004; Reeder *et al*., 2007; Aritua *et al*., 2008; Carter *et al*., 2010). The disease leads to total yield losses to affected plants (Tushemereirwe *et al*., 2006) and while a number of cultural control technologies have been developed, they have not been very effective. As a result, the disease has continued to spread, threatening the livelihoods of over 80 million people in the region and if not well managed it can spread beyond the region to other banana producing regions in the world. 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 bract and male flower abscission. Some cultivars are however resistant to the male bud mediated *Xvm* penetration because; (a) they lack a male bud, (b) they have indehiscent bracts and male flowers and therefore no wounds are created, (c) their flowers are less atractive to insect vectors, (d) abscission wounds/scars are less condussive to penetration and survival of *Xvm* (Mwangi and Nakato, 2006). While such resistance mechanisms to *Xvm* penetration exist within *Musa spp* they are often broken down when farmers cut the plants using contaminted garden tools such as when pruning and detrushing. A more durable and universal form of resistance is thus desired.

Efforts are already under way to engineer banana for resistance through induced hypersensitive response using transgenes from sweet paper (Namukwaya *et al*., 2011). In addition, the world's banana germplasm has already been accessed and screened in Uganda where only wild *Musa balbisiana* showed promise for resistance when artificially injected with *Xvm* (Ssekiwoko *et al*., 2006). Its mechanism of resistance was however not established yet this would be an important step towards identifying associated genes for exploitation. This work therefore characterized the specific phenotypic response in this wild banana with the ultimate aim of identifying the mechanism of resistance and it is hoped that this will help in identification and isolation of the genes involved to supplement on-going genetic engineering efforts of imparting resistance in susceptible cultivated varieties.

The inoculation technique used direct injection of *Xvm* into the tissues of *M. balbisiana* which may have enabled it to overcome the pre-existing barriers and forcing the plant to rely on other induced mechanisms to defend itself. Plants normally recognize pathogens that have jumped the pre-existing barriers using specialized cell-surface pattern recognition receptors (PRRs) which detect specific conserved microbe associated molecular patterns (MAMPs) and trigger 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 genes (PR) and post-transcriptional suppression of the auxin-signalling pathway (Nicaise *et al*., 2009), rendering the plant resistant. *M. balbisiana* could be employing one or more of these mechanisms to resist *Xvm* infection. It is also reported that more virulent plant pathogenic bacteria can often evade and or suppress/shutdown these basal plant defense mechanisms using the T3SS effectors (Abramovitch *et al*., 2006; Jones and Dangl, 2006) and more resistant plants must recognize the presence of these effectors inside the plant cell using R proteins if they are to set up a resistance response (Gu *et al*., 2005). Activation of the R protein by pathogen effectors leads to activation of programmed cell death of plant cells surrounding the pathogen termed hypersensitive response (HR) which affects the pathogen therein. Since *Xvm* had been introduced directly into the tissues of *M. balbisiana* which subsequently showed a resistance response, it could be employing an HR mechanism to resist *Xvm* infection and this needed to be investigated

Whatever the responses, they often manifest themselves as observable morphological or phenotypical changes in the host which can serve as important measures of susceptibility or resistance. Such morphological changes include thickening of the cell wall (through callose and silicone deposition), stomata closure and hypersensitive response (HR) mechanisms. These changes create a barrier to entry, migration and colonization of other tissues by the pathogen and also cause a reduction in pathogen population since they are toxic. In the susceptible hosts on the other hand, there is often a build up in the population of the pathogen within the host plant tissues, colonization of new tissues which are subsequently damaged to varying degrees. The result is retardation of growth, yield loss and in extreme cases there is total plant collapse and death.

In this paper, the therefore, the morphological response of *M. balbisiana* following its interaction with *Xvm* was characterized. Disease development was described, incidence (proportion of infected to total number of plants in a defined area expressed as a percentage) and severity (area of plant damaged relative the total plant area expressed as a percentage) of wilt were measured and the population change of *Xvm* within tissues was determined.

Materials and methods

Preparation of test plant materials

All experiments were conducted at the National Agricultural Research Laboratories (NARL) at Kawanda, 13 kilometers Bombo Road, Kampala, Uganda. Test banana materials of the East African Highland Banana cv 'Nakinyika' and *M. balbisiana* were generated through tissue culture and weaned in sterile soil in a greenhouse. To wean the plantlets, black top soil, sand and decomposed cattle manure were mixed in the ratio of 3:1:1 and then steam sterilized at 100ºC for 8 hours. When the soil was cool, 0.25 kg were transferred into 300 ml capacity disposable plastic cups and fully wetted by watering. Rooted tissue cultured plantlets in baby food jars were drawn from the jars and washed in tap water to clean off residual agar growth medium. Their roots were cut short to

about 5 cm and suspended in a solution of NAA (10 mg/L in ethanol) for 5 minutes before planting in a disposable cup. These disposable cups were transferred to a humid chamber at 100% relative humidity. After 2 weeks, the plantlets were transferred to a green house for 1 week to acclimatize before transferring to bigger pots (5L capacity) containing 3 kg of similar steam sterilized soil. The fully labeled plantlets were transferred to a screen-house and allowed an additional 2 weeks to acclimatize before bacterial inoculation.

Xvm preparation and testing of its pathogenicity

A bacteriological medium designated YPGA and containing 5g/l yeast extract, 5g/l peptone, 10g/l glucose and 14g/l bacteriological agar was prepared at pH 7 on to 90mm disposable plates. A virulent *Xvm* culture under preservation on 50% glycerol at -80p C freezer at the National Biotechnology Center, NARL, was drawn and sub-cultured on YPGA medium by streaking a loopful of thawed 50% glycerol-*Xvm* suspension. Streaked plates were incubated at 25p C for 5 days and the resulting yellow, doom shaped, convex, circular and shinny colonies typical of *Xvm* was tested for pathogenicity prior to challenging the main test plants.

To test for bacterial pathogenicity, a loopful of the growth on the plates was drawn into a 50ml sterile centrifuge tube containing sterile distilled water and the optical density (OD_{600nm}) adjusted to 0.1 (approximately $X10^8$ cells per ml) by reading in a light spectrophotometer and diluting appropriately. One (ml) of the adjusted bacterial suspension was injected using a syringe and needle into the petiole of the youngest fully opened leaf of 3 plantlets of the EAHB-Cv "Nakinyika" and monitored for two weeks for wilt development. Alongside, 2 similar plantlets were similarly injected with sterile distilled water as control. *Xvm* was re-isolated on fresh YPGA plates 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. It was then rinsed three times by wiping with sterile wool soaked in sterile distilled water. Internal tissues were cut out using a sterile surgical blade and suspended in 5 ml of sterile water for bacteria to ooze out into solution to form a bacterial suspension. A loopful of this suspension was streaked on to YPGA plates and incubated for 5 days at 25p C. Yellow doom shaped, convex shiny and circular colonies typical of *Xvm* were increased by sub-culturing on to a new plate of YPGA and incubating at similar conditions.

Inoculation of test plants and disease progression assessment

A completely randomized design was adopted for the study with injection with pure sterile water and a bacterial suspension as treatments. Each plantlet was the experimental unit and a total of 10 plantlets (replicates) for each genotype were inoculated with either *Xvm* or water. Test plants were inoculated by injecting 1 ml of *Xvm* suspension or water using a syringe and needle into the petiole of the youngest fully open leaf.

Plantlets were monitored and the progression of symptoms was noted and described. In addition, wilt incidence and severity index were determined at weekly intervals. 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 test banana type. Wilt severity index was also calculated by counting the number of wilted leaves on every plant and the number of wilted plants for both *Xvm* inoculated and control plants for each genotype. These were then rated on a 1-5 score adopted from Ssekiwoko *et al.* (2006), where; 0= no wilted leaf, 1= one wilted leaf, 2= two or three wilted leaves, 3= four or more wilted leaves, 4= all leaves wilted, 5= dead plant due to *Xvm*. The score was used to calculate the wilt severity indices using;

Wilt Severity Index =

$$\left[\frac{(0\text{^}a)+(1\text{^}b)+(2\text{^}c)+3\text{^}d)+(4\text{^}e)+(5\text{^}f)}{(n\text{^}5)}\right] 100$$

Where:

n = total number of plants injected with *Xvm* for a given genotype and a, b, c, d, e and f were the number of wilted plants at a 0, 1, 2, 3, 4 and 5 scale respectively.

Monitoring *Xvm* population changes in test plants

The relative abundance of *Xvm* in plant tissues at a specific time for the two banana types was recorded at 18, 25, 56, 90 and 150 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 recorded. They were then suspended in sterile water at a rate of 2 mls of water for every gram of tissue. After 15 minutes, this suspension was serially diluted 6 times and then 10 µL of each dilution plated on semi-selective CCA medium containing; 1 g/l D(+) glucose, 1 g/l yeast extract, 1 g/l peptone, 1 g/l ammonium chloride, 3 g/l K_2HPO_4, 14 g/l agar, 1 g/l beef extract, 10 mg/l 5-flourouracil, 10 g/l cellobiose, 40 mg/l cephalexin and 120 mg/l cycloheximide. To make this isolation medium, all components were weighed, made up to 700 mls with distilled water and autoclaved except for glucose, Cellobiose, 5-flourouracil, cephalexin and cycloheximide which were filter sterilized.

The filter sterilized components were only added to media after it cooled to about 40p C, made up to 1 liter with sterile distilled water, mixed and then dispensed into 90 mm plastic petri-dishes awaiting bacterial culturing. After 7 days of incubation at 25ºC following streaking on the same plates, colonies of *Xvm* were counted from that lowest dilution level which gave most well separated-out colonies. Working backwards, the number of bacteria in the original tissues was estimated and was standardized to 50 mg tissue.

Results

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 later to the lower older leaves (symptoms last developed in the oldest leaves). In comparison to the normal out-stretched leaf blades (Fig. 1a), the blades of *Xvm* infected and wilting leaves

collapsed along the midrib and eventually touched each other (Fig. 1b). The blade generally turned pale green, then yellow and finally brown. The incidence of wilt rose from 0% to 68% within 2 weeks after inoculation and by the 3rd week it had reached 100% (Fig. 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 (Fig 1d) and by the 8th week, all plants had died and were dried (Fig. 1f). By this time wilt severity had reached 92% (Fig. 4).

In *M. balbisiana*, wilt symptoms delayed and 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 (Fig. 2b) towards the petiole. Eventually the whole leaf would wilt (Fig. 2e) and die while in other cases such leaves would only develop necrotic patches which would be restricted to the apex. The incidence of wilt remained at 0% for eight weeks and by the 12th week it had only risen to 33 % at which it remained even up to 20 weeks after inoculation (Fig. 3).

In the same period, severity of wilt as expressed by wilt severity index remained at 0% for eight weeks after which it rose to about 20% by the 12th week before dropping to 0% by the 20th week (Fig. 4) In addition, internal necrosis or tissue browning was noted around the point of inoculation in *M. balbisiana* and was more intense at *Xvm* inoculated points in comparison to water inoculated points.

Migration and multiplication of *Xvm* within banana

In the EAHB cv "Nakinyika", *Xvm* multiplied and migrated from the inoculated leaf to follower leaves. By 18 days after inoculation, *Xvm* could be detected in the leaf immediately following the inoculated

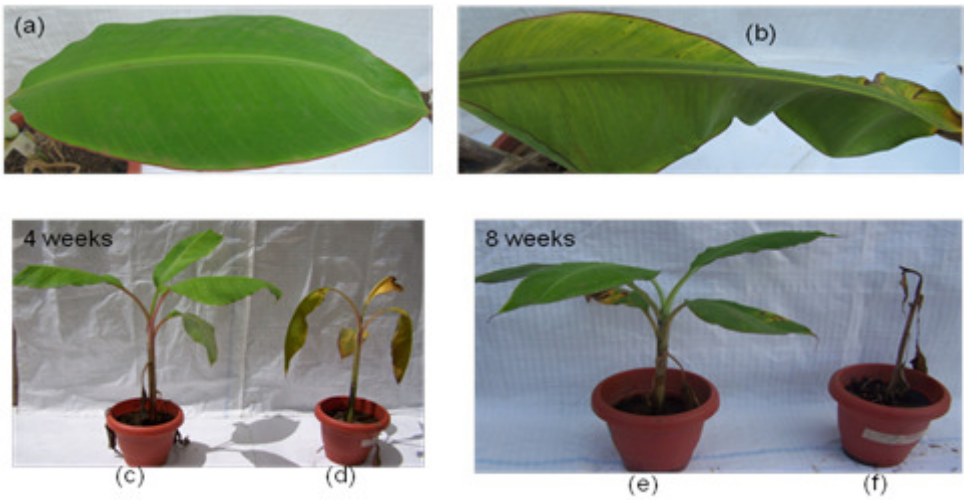

Figure 1. The East African Highland Banana cv "Nakinyika" (a) leaf of water inoculated plant, (b) leaf of *Xvm* inoculated plant after 3 weeks, (c) water inoculated plant after 4 weeks, (d) *Xvm* inoculated plant after 4 weeks, (e) water inoculated plant after 8 weeks and (f) *Xvm* inoculated plant after 8 weeks.

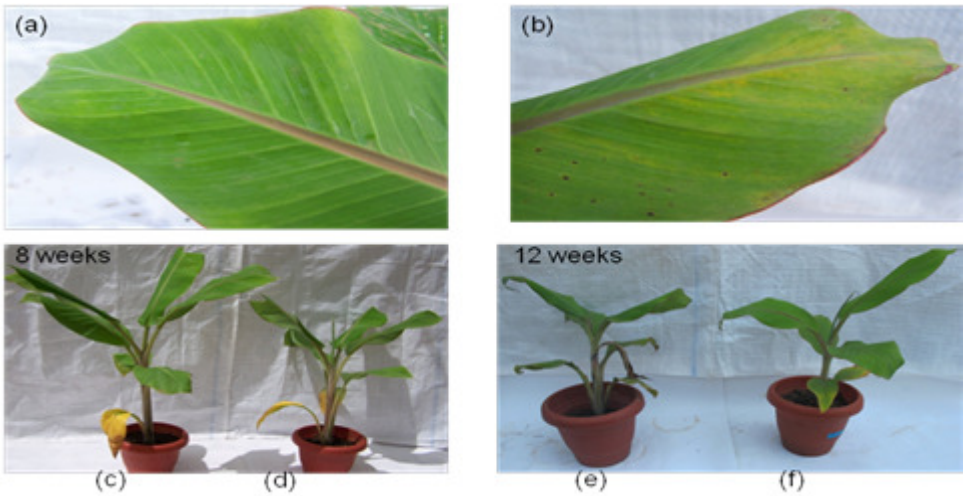

Figure 2. ***M. balbisiana*** **(a) leaf of water inoculated plant, (b) leaf of *Xvm* inoculated plant after 10 weeks, (c) *Xvm* inoculated plant after 8 weeks, (d) water inoculated plant after 8 weeks, (e) *Xvm* inoculated plant after 12 weeks with a single wilted follower leaf and (f) water inoculated plant after 12 weeks.**

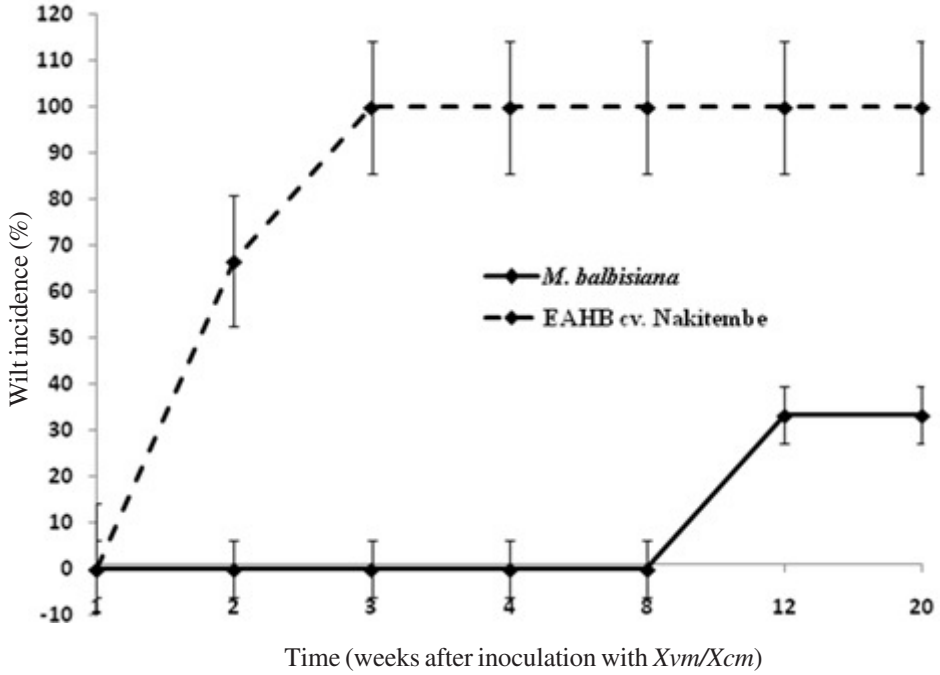

Figure 3. Change in wilt incidence over time in EAHB cv "Nakinyika" and *M. balbisiana* following inoculation with *Xvm*.

one (Table 1). In addition, the population of *Xvm* in both inoculated and follower leaf tissues continually built up till 25 days after inoculation when its population in follower leaves was higher than that in inoculated leaves (Table 1). By 56 days, the plants had died and too rotten to recover any *Xvm*. In *M. balbisiana*, *Xvm* was also detectable in inoculated leaves before they died away after 56 days. By this time, *Xvm* had not migrated to follower leaves yet its population in inoculated leaves had reduced (Table 1). By 90 days after inoculation, *Xvm* had migrated to the follower leaves though its population was lower than that in susceptible cv "Nakinyika" and by 20 weeks after inoculation, it was not detectable anymore in *M. balbisiana* tissues.

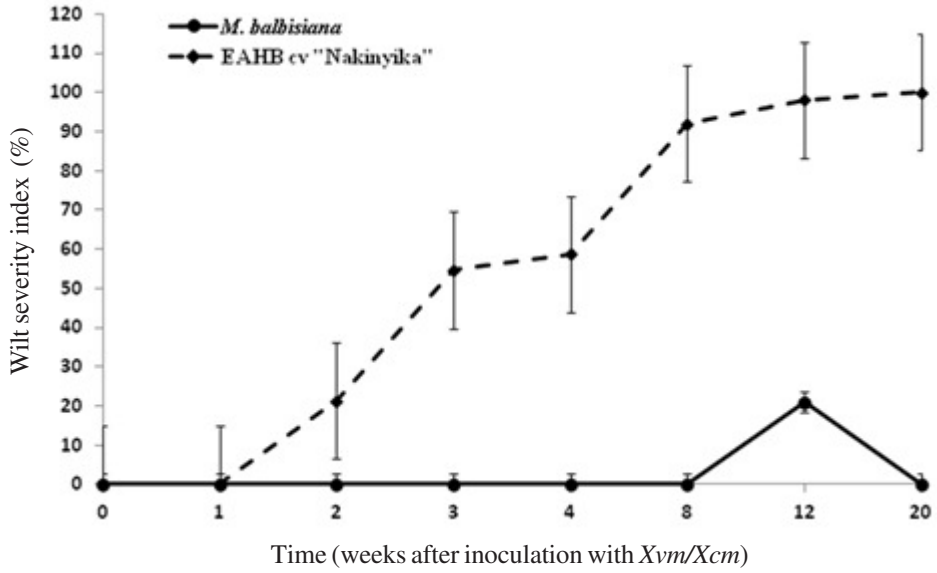

Figure 4. Change in wilt severity index over time in EAHB cv "Nakinyika" and *M. balbisiana* following inoculation with *Xvm*.

Table 1. Relative average No. of *Xvm* cells recovered from the petiole tissue (50mg) of Inoculated and follower leaves of *M. balbisiana* and cv "Nakinyika" at various days after inoculation (DAI) with *Xvm*.

DAI	*M. balbsiana*		Cv "Nakinyika"	
	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	*	$1.5x10^4$	*	*
150	*	0	*	*

* = Dead and rotten plant part due to *Xvm* infection

Discussion

While plants can use physco-chemical barriers such as the waxy cuticle, a thick cell wall and stomata closure (Melotto *et al*., 2008) to limit or defend themselves against invading pathogenic microbes, our mode of inoculation bleached such barriers, where *Xvm* was injected directly into host tissues. It thus follows that any defense responses that would eventually be effective in limiting the damaging effects of *Xvm* in our study plants would be those that are normally at play when pathogenic bacteria are inside the host plant tissues. Indeed there were notable responses in the banana plantlets following inoculation with *Xvm* as revealed by the disease assessment parameters used.

Leaf wilting which is the major external symptom of *Xvm* infection in non flowered banana plants was very severe in cv "Nakinyika" reaching 92% by the 12th week after inoculation. While it is generally true that even susceptible hosts detect invading pathogens and put up some degree of defense that would limit pathogen multiplication and migration to some extent (Kim *et al*., 2005; Zheng *et al*., 2012), Cv "Nakinyika" could not resist *Xvm* infection as the pathogen quickly multiplied and migrated through the whole plant causing total wilting and death of the plants. These observations suggest that this cultivar is highly susceptible as rightly previously predicted for all cultivars within the East African Highland bananas (Ssekiwoko *et al*., 2006).

On the other hand, *Xvm* infection was less severe in *M. balbsiana* only climaxing at 20% before dropping back to 0%. While *Xvm* was present in tissues of the inoculated leaf of this genotype even 8 weeks after inoculation, wilt symptoms delayed up to 10 weeks and its population had reduced when compared at 18 days when it was first quantified. In addition to limited multiplication, *Xvm* did not quickly migrate to distant parts, only detectable in the immediate follower leaf by the 12th week (Table 1) and never migrated to distant follower leaves as examined by the 20th week. This is the first report of delayed symptom expression in *M. balbisiana*, though long latent periods of *Xvm* infection in susceptible cultivated banana have already been observed (Ocimati *et al*., 2013). This therefore seems to suggest that the factors responsible for this response are generally present in *Musa spp* and are only super expressed in *M. balbisiana*.

Previously, long latent periods of bacterial infections in plants have been attributed to low bacterial population density which phenomenon limits them from expressing certain pathogenesis traits in plants (Loh *et al.*, 2002; Von Bodman *et al*., 2003). However this cannot explain the delayed symptom expression in *M. balbisiana* as at the time when symptoms were observed, the bacterial density in plant tissues was much lower than before.

Plant defensive compounds such as phenolics, phytolexins and plant resistance proteins are reported to delay disease incubation and latent periods through interference of the expression system of virulence factors (Li *et al*., 2008) or direct killing of pathogens (Christina *et al*., 2010).

An investigation into the contribution of phenolics and pathogenesis related (PR) proteins in the resistance response of *M. balbisiana* to *Xvm* infection is the subject of on-going work.

The low wilt severity attained, limited multiplication and migration and the subsequent disappearance of *Xvm* in *M. balbisiana* was a very unique response.

This is the first report of such a response to *Xvm* in banana. Bacteria are short generation organisms and their populations normally build up so quickly in a matter of hours. Recovery of *Xvm*, though in lower numbers at 8 weeks after inoculation, implies that there is indeed an initial multiplication of *Xvm* within *M. balbisiana* except that there seems to be elevated rates of bacterial cell death such that it subsequently disappears. Hosts that limit build up of pathogenic bacteria populations are considered resistant and this phenomenon has already been observed in many resistant host plant species including; resistant rice to *Xanthomonas campestris* pv. *oryze* (Barton-Willis *et al*., 1989), resistant tomatoes to *Ralstonia solanacerum* (Nakaho *et al*., 2004) and resistant beans to *Xanthomonas campestris* pv. *phaseoli* (Godwin *et al*., 1995).

This limitation in bacterial multiplication in such plants was attributed to activation of host plant defenses.

Restrictions of bacterial multiplication and migrations beyond points of inoculation has also previously been attributed to; hypersensitive response (HR) especially to the biotrophic bacteria (Morel and Dangl, 1997; Khurana *et al*., 2005; Iakimova *et al*., 2005) and also to thickening of the pit membranes and apposition layers in parenchyma cells next to vessels in *Ralstonia solanacearum* infections in resistant tomatoes (Nakaho *et al*., 2004). Hypersensitive response is normally observed as rapid programmed death of plant tissue or cells around the point of infection and this is followed by death of pathogens there in. In this study, typical HR reaction did not develop and therefore not observed in *M. balbsiana*, instead whole organs (some individual leaves) died. Leaf wilting and death of plants is typical of Banana Xanthomonas wilt disease and in susceptible plants, it is usually brought about in part by blockage of the transport system and by the time such happens, the bacteria has already wildly multiplied and migrated through the entire plant.

This was clearly not the case in *M. balbisiana* where whole organs (leaves) wilted before *Xvm* had widely migrated to distant leaves.

Xanthomonas vasicola pv. *musacearum* is hemi-biotrophic (Van Loon *et al.,* 2006) where tissue death alone would not have limited its migration to other parts. However under decay conditions *Xvm* is heavily outcompeted by saprophytes (Mwebaze *et al*., 2006) which factor could have contributed to the reduction in its population. It has also previously been observed that plants can defend themselves against certain pests by initiation of abscission which leads to dropping of the infested parts and this substantially lowers the pest load (Williams and Thomas, 1986). In other instances, plants have been reported to bloke transport vessels (Sun *et al*., 2007) cutting off water supply to specific organs which subsequently wilt and die. This observation would make us postulate that death of individual leaves in *Xvm* challenged *M. balbisiana* was a result of programmed leaf abscission to eliminate the affected leaf and stop further pathogen migration.

Leaf abscission is mediated by elevated production of ethylene (Reid, 1985) and its role in *Xvm* challenged *M. balbisiana* is the subject of ongoing work. This current study therefore finds that *M. balbisiana* resists banana Xanthomonas wilt disease by slowing the multiplication and migration of *Xvm* followed by subsequent death of the affected organs

(Leaves) together with *Xvm* there in resulting first in delayed symptom development, low disease severity and later in complete plant recovery.

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