Spread of *Xanthomonas campestris* pv. *musacearum* in Banana (*Musa* spp.) Plants Following Infection of the Male Inflorescence

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Abstract

***Xanthomonas campestris* pv. *musacearum* (Xcm) causes *Xanthomonas* wilt of banana (*Musa* spp.) and enset (*Ensete ventricosum)* in East and Central Africa. The disease is spread by insects that visit the male inflorescence, through the use of infected planting materials and by contaminated garden tools. To evaluate the most appropriate control options, the spread of bacteria within the plant following natural flower infection was studied in Luwero and Mpigi districts of central Uganda. Banana tissue samples were collected from the corm, true stem and leaf sheaths of 'Pisang Awak' (ABB genome) and 'Matooke' (AAA genome) mother plants, showing four progressive stages of disease development: stage 1 – male bud wilting; stage 2 – decaying rachis; stage 3 – premature fruit ripening; and stage 4 – rotting of fruit bunches. Thirty plants were sampled per stage and per cultivar. Additional samples were taken from attached suckers. Bacteria were isolated from surface-sterilized plant samples and identified by colony characteristics on a semi-selective medium. Following inflorescence infection, Xcm moved along the true stem, into the youngest leaf sheaths inserted on the true stem, down into the corm and into the older leaf sheaths. At early stages of inflorescence infection (stage 1), bacteria were restricted to the upper parts of the true stem in 'Pisang Awak', but had moved further down the stem in 'Matooke'. Therefore, cutting down mother plants at stage 1 could stop Xcm from reaching the corm and eventually crossing to the suckers of 'Pisang Awak' but this was less likely to be effective for 'Matooke' plants. The bacteria were recovered from suckers of both cultivars showing symptoms at stage 4, but at stage 3 only from 'Pisang Awak'. It is recommended that whole mats should be completely uprooted or killed by herbicides in case mother plants show symptoms beyond stage 1 for 'Pisang Awak' and at all disease symptom stages for 'Matooke'.**

INTRODUCTION

Xanthomonas wilt (XW), caused by *Xanthomonas campestris* pv. *musacearum* (Xcm), was first described in Ethiopia in 1968 (Yirgou and Bradbury, 1968, 1974) and has more recently been reported in Uganda (Tushemereirwe et al., 2003), the Democratic Republic of Congo (Ndungo et al., 2004), Rwanda, Tanzania (Mgenzi et al., 2006) and Kenya. The first visible symptom of inflorescence infection on banana is wilting of male buds, followed by decaying of the rachis, premature fruit ripening and finally bunch rotting, followed by wilting and yellowing/browning of leaves. The disease also affects pre-flowering plants that can become infected directly from diseased mother plants, or from contaminated tools. These pre-flowering plants develop yellow/brown wilting leaves. Internally, cross-sections of the pseudostems show yellow bacterial ooze, while cross-sections of the banana fingers show rusty brown stains. These internal symptoms can be considered definitive for XW of enset (*Ensete ventricosum*) and banana (*Musa* spp.) in East and Central Africa (ECA), because other bacterial wilts causing similar symptoms in these hosts do not exist in this region Tushemereirwe et al. (2003).

The epidemiology of banana *Xanthomonas* wilt (BXW) in ECA is similar to that of other banana bacterial wilts such as Moko disease (or bugtok), caused by *Ralstonia*

solanacearum race 2 and Blood disease, caused by *Ralstonia solanacearum* phylotype IV (Davis and Liberato, 2006), which is present in Latin America and Asia (Buddenhagen and Elsasser, 1962; Eden-Green, 1994; Soguilon et al., 1995). In common with Moko disease, BXW is primarily spread by insects from oozing male buds to healthy inflorescences (Buddenhagen and Elsasser, 1962; Tinzaara et al., 2006) and also by contaminated cutting tools (Yirgou and Bradbury, 1974). However, Xcm rarely persists in the soil for more than 3 months (Mwebaze et al., 2006a), in contrast to longer survival in soil of *R. solanacearum*.

The similarity of the BXW epidemiology to other banana bacterial wilts led to the provisional adoption in ECA of control measures used for these diseases, as an emergency action pending clearer recommendations based on problem-specific research. These measures included timely removal of male buds with a forked stick, routine disinfection of garden tools using household bleach (NaOCl) or gently heating the blade by fire, and cutting down and burying whole mats with disease symptoms. The destruction option was laborious and met with poor adoption; farmers tended to remove only the visibly diseased plants within the mat. This posed a risk of incomplete control that would allow the disease to survive in corms and attached suckers. Previous systemic studies on Blood disease have shown an incomplete systemicity (Eden-Green, 1994). Some suckers obtained from severely infected mats were observed to grow uninfected up to bunch harvest.

The present study was undertaken to relate visible disease symptoms to the spread of bacteria to different plant organs following inflorescence infection in order to establish whether timely removal of single inflorescence-infected plants from the mat can be an effective control measure for BXW.

MATERIALS AND METHODS

This study was carried out in Luwero and Mpigi districts, Central Uganda, on farmers' fields using two cultivars, e.g. 'Pisang Awak' (syn. 'Kayinja', ABB genome) and 'Matooke' (AAA genome, East African Highland Banana subgroup). The study areas have an average daily temperature of 25°C and a maximum temperature of 29°C. The climate is moist to sub-humid, with a mean annual rainfall of 1,100 mm that has a bi-modal distribution (March-May and September-November). Plants with clear symptoms of inflorescence infection by BXW, and according to the farmer, without a previous history of infection within the same mat, were selected at different stages of symptom development. In addition, plants were selected in fields where de-budding and green leaf pruning were not practiced. Thirty inflorescence-infected plants were selected at each of the four stages of disease symptoms (e.g., total of 120 plants per cultivar): wilting of male bud (stage 1); decay of rachis (stage 2); premature fruit ripening (stage 3); and rotting fruit bunches (stage 4). The study period covered 16 months from January 2007 till April 2008. Plant height (i.e., from soil level to the point of emergence of the inflorescence stalk from the leaf sheaths) was measured for each plant in order to calculate the percentage of inner inflorescence stalk (i.e., true stem) length already colonized by Xcm.

The selected mother plant was uprooted and samples were taken from the corm, leaf sheaths, and true stem. In addition, leaf sheaths and corm samples of one randomly selected lateral shoot (not exceeding a height of 1 m) were also collected. The leaf sheaths from the five innermost, and hence youngest, leaves including the flag leaf (i.e., small leaf attached to the uppermost part of the real stem, in close proximity to the site of infection) were sampled. The leaf sheaths were carefully removed from the true stem and numbered starting with the innermost. Scalpels used to remove the samples were sterilized by flaming each time a new cut was made or a different sheath was cut. The inner inflorescence stalk was sampled by cutting small sections at 30 cm intervals across its whole length. The corms were cut with machetes sterilized by flaming. All samples were labeled, put into sampling bags and taken to the laboratory for bacterial isolation. In the laboratory, each section of the true stem was surface sterilized by wiping with cotton wool soaked in 95% ethanol. A 1 mm thick (approximately 10 g) transverse section was

cut from the middle of each section, chopped into small pieces and suspended in distilled sterile water to obtain a bacterial suspension. Leaf sheaths were similarly surface sterilized and a 1 mm thick section was cut at the base of each sheath and suspended in sterile distilled water to obtain a bacterial suspension. The corms were carefully cleaned to remove the soil and were then surface sterilized by soaking for three minutes in 0.9% NaOCl solution (1 part of 5.3% NaOCl in 5 parts of water), followed by five rinses with sterile water. Specific corm samples (approximately 10 g) were taken from the outer cortex, the inner cylinder and the layer of Mangin (i.e., meristematic or cambium-like layer), at a point halfway between the insertion point of the true stem and the bottom part of the corm. These were suspended for five minutes in sterile distilled water to obtain a bacterial suspension. Efforts were taken to ensure that the samples represented the whole cross section of the corm. For all samples, five 10-fold dilutions were prepared in sterile distilled water.). In order to limit contamination with soil, following Mwebaze et al. (2006b), a drop of each dilution (approximately10 µL) was spread on a plate with a semi-selective isolation medium, 5-fluorouracil-Cephalexin Agar (FCA) that contained a yeast extract (1 g L^{-1}), glucose (1 g L^{-1}), peptone (1 g L^{-1}), NH_4Cl (1 g L^{-1}), $MgSO_4.7H_2O$ (1 g L^{-1}), K_2HPO_4 (3 g L^{-1}), Agar (14 g L^{-1}), Cephalexin (40 mg L^{-1}), 5-fluorouracil (10 mg L^{-1}) and cycloheximide (120 mg L^{-1}). The plates were incubated for five days at 25–28^{o}C and observed for presence of predominant shiny, dome-shaped, circular, smooth, mucoid and yellow colonies characteristic of Xcm.

The percentage of samples that were positive or negative for presence of Xcm in the different plant parts were analyzed using the Statistical Analysis System (SAS) computer software (SAS institute, 1999). The percentage of true stem length (measured from soil level) free of Xcm was calculated from the data obtained from samples taken at 30 cm intervals along the entire true stem length.

RESULTS AND DISCUSSION

There was no significant difference in plant height between disease stages and cultivars (data not shown). The presence of Xcm within the true stem for plants at different disease symptom stages is presented in Table 1. The distance bacteria had moved down the true stem differed significantly (P<0.05) for different symptom stages in ‘Pisang Awak’ plants. ‘Pisang Awak’ plants with wilting male buds (stage 1) did not have Xcm at the base of the true stem, and 62% of true stem length (from the base upwards) was free of Xcm for ‘Pisang Awak’ plants at this stage. This was significantly (P<0.05) higher than for plants with more advanced symptoms, where the percentage of the true stem length free of Xcm did not significantly differ between stages 2–4 (Table 1). This suggests that if ‘Pisang Awak’ plants with bract wilting (stage 1) symptoms are carefully cut at the base, transmission of the bacteria from the mother plants to the suckers can be prevented, but this may not always be effective for plants with more advanced symptoms.

In contrast, the spread of Xcm along the true stem in inflorescence-infected ‘Matooke’ plants was not significantly related to the stage of disease symptoms. Bacteria could be detected at the base of the pseudostem even at the very early stage of male bud wilting (Table 1), suggesting that removing infected plants at any stage of disease symptoms is not likely to be effective in preventing the disease from affecting the attached lateral shoots. At stage 1, 33% of the corms from ‘Matooke’ mother plants were already invaded by Xcm, compared to none for ‘Pisang Awak’ mother plants (Table 1). It is not clear whether Xcm spreads faster in Matooke or if there is simply a delayed expression of symptoms, such that the progress of infection is wrongly judged from external appearance of symptoms.

At stage 4 (rotting fruit bunches), the percentage of plants with bacteria in the leaf sheaths was significantly lower for ‘Matooke’ than for ‘Pisang Awak’ (Table 1). It is often observed in the field that, by this stage, ‘Pisang Awak’ plants have green functional leaves, while leaves of ‘Matooke’ have wilted and dried. In this experiment, most of the leaf sheaths sampled at this stage from ‘Matooke’ were already decaying. The comparatively rapid decay of ‘Matooke’ leaf sheaths may have reduced the survival and

detection of Xcm, due to competition with saprophytic bacteria, which colonized many of the isolation plates.

For all stages of disease development, more 'Matooke' than 'Pisang Awak' plants were positive for Xcm in the corm (Tables 1 and 2), with bacteria in a significantly (P<0.05) higher percentage of samples from the layer of Mangin compared to the inner cylinder and the cortex (Table 2). These data suggest that when Xcm invades the corm, bacteria first colonize the layer of Mangin, which consists mainly of vascular bundles, followed by the inner cylinder and outer cortex, which are largely a mass of starchy parenchyma (Stover and Simmonds, 1987). This is consistent with the view that Xcm colonizes the plant via the vascular tissues.

Independent of cultivar, more plants were positive for Xcm in leaf sheaths compared to corms (Table 1), and generally Xcm was recovered more frequently from the younger, innermost leaf sheaths than from the older, outer ones (Table 3). This suggests that Xcm first colonizes some of the youngest leaf sheaths before it invades the corms. This is to be expected as the flag leaf plus the nine youngest leaf sheaths of both cultivars are directly inserted on the true stem (Stover and Simmonds, 1987) (Table 4). Older leaves originate from the corm and will be the last to be invaded following inflorescence infection. Xcm did not invade the lateral shoots of 'Pisang Awak' plants until fruits of the infected mother plants had started ripening prematurely (stage 3), while in 'Matooke', Xcm could only be detected in the lateral shoots at bunch rotting (stage 4; Table 1).

As with many observations in farmers' fields, it was difficult to be certain that all of the observed disease cases were derived only from natural infections via inflorescences, and that no infection was introduced via older leaves or roots damaged during cultivation operations. However, these other pathways of infection would have resulted in invasion of the corm, leading most probably to the development of yellowing or wilting symptoms in older leaves before inflorescence symptoms became advanced. Although this pattern of symptom development was not observed in the plants sampled and is not consistent with the results of this study, the possibility that some of the plants sampled here may have been affected by other modes of infection cannot be ruled out.

CONCLUSIONS AND RECOMMENDATIONS

Cutting down mother plants at stage 1 may stop the bacteria from reaching the corm and eventually crossing to the suckers of 'Pisang Awak', but this is less likely to be effective for 'Matooke' plants. It is, therefore, recommended that whole mats should be completely uprooted or killed by herbicides in case mother plants show symptoms beyond stage 1 for 'Pisang Awak', and at all disease symptom stages for 'Matooke'.

Additional trials, using artificial inoculation of inflorescences, are needed in order to relate the speed of bacterial movement in different plant parts to the rate of appearance of visible disease symptoms.

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Tables

Table 1. Percentage of plants with *Xanthomonas* wilt (Xcm) in different organs of mother plants and attached suckers of two banana cultivars, 'Pisang Awak' (PA) and 'Matooke' (MA), at four stages of disease development[1, 2].

Disease development stage	True stem length free of Xcm (%)		Plants with Xcm (%)									
			Mother plant						Attached sucker			
			True stem		Leaf sheaths		Corm		Corm		Leaf sheaths	
	PA	MA	PA	MA	PA	MA	PA	MA	PA	MA	PA	MA
1. Wilting male bud	62a*	11a**	100a*	100a*	46a*	33c**	0c**	33b*	0c*	0b*	0b*	0b*
2. Decaying rachis	23b*	5a**	100a*	100a*	48a*	50b*	27b*	33b*	0c*	0b*	0b*	0b*
3. Premature fruit ripening	14b*	0a**	100a*	100a*	50a**	83a*	29b**	67a*	14b*	0b**	0b*	0b*
4. Bunch rotting	0b*	0a*	100a*	100a*	52a*	33c**	53a**	67a*	28a**	33a*	9a**	33a*

[1]Means in a column followed by the same letter are not significantly different according to Tukey's HSD test (P<0.05).

[2]Means in a row followed by the same number of stars for a given plant part are not significantly different according to Tukey's HSD test (P<0.05).

Table 2. Presence of plants with *Xanthomonas* wilt (Xcm) in the different parts of the corm of two banana cultivars, 'Pisang Awak' and 'Matooke', at four stages of disease development[1, 2].

Disease development stage	Plants with Xcm (%)					
	'Matooke'			'Pisang Awak'		
	Cortex	Layer of mangin	Inner cylinder	Cortex	Layer of mangin	Inner cylinder
1. Wilting male bud	0d***	33c*	17c**	0c*	0b*	0b*
2. Decaying rachis	17c***	50b*	33b**	8b***	33a*	21a**
3. Premature fruit ripening	50a**	67a*	50a**	0c**	33a*	0b**
4. Bunch rotting	33b**	67a*	33b**	30a*	36a*	27a*

[1]Means in a column followed by the same letter are not significantly different according to Tukey's HSD test (P<0.05).

[2]Means in a row followed by the same number of stars for a given plant part are not significantly different according to Tukey's HSD test (P<0.05).

Table 3. Percentage of plants with *Xanthomonas* wilt (Xcm) in leaf sheaths 1 (youngest) to 5 (oldest) of two banana cultivars, 'Pisang Awak' and 'Matooke', at four stages of disease development[1, 2].

Disease development stage	Plants with Xcm (%)									
	'Matooke'					'Pisang Awak'				
	1	2	3	4	5	1	2	3	4	5
1. Wilting male bud	20b*	20c*	20c*	0c**	0b**	8c*	9c*	8b*	0c***	5c**
2. Decaying rachis	0c**	25c*	25b*	25b*	0b**	8c****	14b***	29c*	23b**	7c****
3. Premature fruit ripening	50a**	67a*	33a***	67a*	33a***	18b***	36a**	44a*	27a***	25a***
4. Bunch rotting	0c**	33b*	0d**	0c**	0b**	50a*	38a**	35b**	21b***	21b***

[1]Means in a column followed by the same letter are not significantly different according to Tukey's HSD test ($P<0.05$).

[2]Means in a row followed by the same number of stars for a given plant part are not significantly different according to Tukey's HSD test ($P<0.05$).

Table 4. Insertion point height on the true stem, expressed as percentage of true stem height from plant base, for leaf sheaths inserted above corm level for two, ready to harvest, banana cultivars, 'Matooke' and 'Pisang Awak' (n=5)[1].

Leaf number	Matooke	Pisang Awak
Flag leaf	90a	81a
1	75b	65b
2	56c	46c
3	36d	29d
4	20e	18e
5	10f	11f
6	5g	7g
7	3gh	5h
8	1h	3hi
9	1h	1i

[1]Means in a column followed by the same letter are not significantly different according to Tukey's HSD test ($P<0.05$).