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Does *Xanthomonas campestris* pv. *musacearum* Colonize Banana Cord Root Tissue?

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Abstract

Xanthomonas wilt of banana and enset (XW) caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) is a devastating bacterial disease. Infection of pre-flowering stage plants result in leaf yellowing/wilting and eventual plant death. Floral infections result in wilting of male bud bracts, followed by decaying of the rachis, premature fruit ripening and bunch rotting, and eventual death of the plant. The movement of Xcm in infected plants is systemic. However, the presence of Xcm in cord roots of banana plants has not yet been investigated. Cord roots of symptomatic pre-flowering stage and inflorescence-infected plants of an East African highland banana cultivar mixture (AAA-EA) and 'Pisang Awak' (ABB) were examined for Xcm presence, in naturally infested farmers' fields and after artificial inoculation. Pre-flowering stage plants were inoculated by cutting the three oldest leaves with a contaminated knife, while a Xcm suspension was smeared on male flower/bract scars of flowering plants. In addition, pre-flowering stage plants were inoculated by cutting the cord roots with a contaminated knife and drenching the surrounding soil with Xcm suspension. Plants were monitored during 12 months for symptom development. Overall, the presence of Xcm in the cord roots was significantly lower compared to corms. A higher incidence of cord root infection was observed in the artificially inoculated plants compared to plants assessed in farmers' fields. The incidence of cord root infections in inflorescence-infected plants increased with progressing disease development for both varieties. The results suggest that cord roots could contribute to garden tool transmission, for example during weeding. Therefore, hand weeding and herbicide use are advised when diseased mats are present in a field. Long incubation periods and latent infections were noted after artificial cord root inoculations. The development and deployment of diagnostic kits sensitive to latent infections for routine surveillance is therefore recommended for effective XW management.

INTRODUCTION

Xanthomonas wilt of banana and enset (XW) caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) was first reported in Ethiopia in 1968 (Yirgou and Bradbury, 1968 and 1974). XW is currently present in Uganda (Tushemereirwe et al., 2003), the Democratic Republic of Congo (Ndungo et al., 2004), Rwanda (Muhinyuza and Gaidashova, 2006), Tanzania (Mgenzi et al., 2006), Kenya (Anon, 2006; Mbaka et al., 2009) and Burundi (Carter et al., 2010). Similar to other banana bacterial wilts (Bugtok and Moko blood disease), XW is

primarily spread by insects, mainly bees and flies, from an oozing inflorescence (male bud or rachis) to a healthy one (Tinzaara et al., 2007) and by garden tools (Yirgou and Bradbury, 1974; Addis et al., 2010). The disease affects plants in both the pre-flowering and flowering stage. Infected plants in the pre-flowering stage show leaf wilting/yellowing symptoms and eventual death of the plant; such infections mainly occur through contaminated garden tools. The first visible symptom of a floral infection in banana is wilting of male bud bracts, followed by drying of the rachis, premature fruit ripening and drying and, eventually, wilting and death of the entire plant (Ssekiwoko et al*.,* 2006). Internal pseudostem cross-sections show yellow bacterial ooze, while cross sections of the fruits show rusty brown stains (Thwaites et al., 2000; Tushemereirwe et al., 2003; Biruma et al., 2007).

Welde-Michael et al. (2008) showed that Xcm isolates could not survive in the soil for more than 9 days. By the 9th day, the bacterial population is reduced to levels that cannot initiate infection. Bacteria require either wounds or natural openings such as hydathodes and stomata to enter a plant (Manners, 1993). In pot trials that established the effect of nematode-inflicted cord root cortex damage on soil-borne transmission of Xcm, incidences of 50% in 'Pisang Awak' (ABB) and 33% in East African highland banana (AAA-EA) were recorded (Meki et al., 2010). In contrast, 0% infection was observed in 'Pisang Awak' and 17% in AAA-EA plants for the treatment without nematodes. It was concluded that the presence of nematodes led to damage of roots and the resulting wounds on the root system enhanced entry of Xcm. Addis et al. (2010) also reported a low Xcm incidence level when cord roots of 'Pisang Awak' banana plants were cut with a contaminated tool compared to cutting the above ground parts. Together, these studies suggest that banana cord roots may be an important entry route for Xcm infection. Thus the ability of Xcm to enter cord roots of mature banana plants especially AAA-EA needs to be elucidated.

In a previous study conducted by Ssekiwoko et al. (2006), 56% of the true stems (from soil level upwards) were free of Xcm for banana plants with shrivelling bract symptoms. However, plants with more advanced symptoms (decaying rachis, premature ripening of fruits and whole bunch rotting/drying) had bacteria at the base of the plant, and in parts of the corm of both the mother plant and the attached suckers. The presence of Xcm in cord roots was not investigated. The objectives of the current study were therefore to determine the presence of Xcm in the cord roots of symptomatic florally infected and symptomatic pre-flowering stage AAA-EA and 'Pisang Awak' banana plants and the ability of Xcm to enter plants through wounds on the cord roots.

MATERIALS AND METHODS

The presence of Xcm in cord roots and corms was assessed in naturally infected plants in farmers' fields in Luwero district, a XW hotspot and in artificial inoculation experiments in an isolated location in Kifu forest, Mukono district, central Uganda. Two varieties, 'Pisang Awak' and East African highland banana (cultivar mixture), were used in this study. For the farmer fields in Luwero district, observations were made on farms and symptomatic plants identified and sampled for laboratory isolation of Xcm. For the Kifu artificial inoculation experiments, twenty plants per cultivar in the pre-flowering stage (9 months old) were inoculated by cutting the three oldest leaves at the leaf petioles using a knife contaminated by dipping in Xcm suspension between each cut. Conversely, 40 flowering plants per cultivar were inoculated by smearing a bacterial suspension on the

male flower and male bract scars with a soft brush. The inoculum was prepared from aseptically excised transverse sections of plant parts (flower stalks and pseudostems) obtained from symptomatic plants and macerated in sterile deionised distilled water in order to prepare the Xcm suspension. Twenty µL of the suspension were then transferred onto a semi-selective growth medium of cellobiose cephalexin agar (CCA) (Mwebaze et al., 2006) and incubated at 24^{o}C for 72 hours. Colonies with a yellow, convex, mucoid morphology typical of Xcm colonies were harvested, suspended in sterile distilled water and adjusted to a concentration of $1x10^{8}$ cfus/ml (~0.5OD) by dilution.

In both farmers' fields and Kifu trials, plants in the pre-flowering stage were sampled after observing the characteristic leaf yellowing and wilting symptoms in one to three leaves. For florally-infected plants, purposive sampling was done at the four XW symptom stages (wilting male-bud, decaying rachis, premature fruit ripening and rotting bunch). In farmers' fields, 14 (9 'Pisang Awak' and 5 AAA-EA) plants in the pre-flowering stage and 36 (17 'Pisang Awak' and 19 AAA-EA) plants with floral infection symptoms were sampled. In the Kifu trials, 13 plants in the pre-flowering stage (6 'Pisang Awak' and 7 AAA-EA) and 42 (21 'Pisang Awak' and 21 AAA-EA) florally inoculated plants were sampled. From each sampled plant, cross sections of six randomly selected cord roots and the corm were aseptically removed for laboratory analysis using a knife or machete sterilised in household bleach (5.3% NaOCl).

Laboratory work was conducted at the National Agricultural Research Laboratories, Kawanda, Uganda. In the laboratory, cord root samples were cleaned with tap water; surface sterilized by dipping in household bleach (5.3% NaOCl) for 3 min and rinsed with sterile water. Transverse sections (1 mm thick) were then cut midway along each root sample, chopped, crushed and suspended in distilled sterile water to obtain a bacterial suspension. The suspension was serially diluted (suspension: water 1: 9) five times and 10 µl of each dilution plated on a semi-selective medium of CCA. The plates were then incubated at 24^{o}C for 72 hours and observed for Xcm growth, characterized by predominantly shiny, dome-shaped, circular, smooth, mucoid and yellow colonies. For corm tissues, surface sterilization was not necessary, as the samples had no soil contamination. The outer layer of a cross-section of the corm tissue was peeled off and a cross-section of the remaining tissue, including the cortex, layer of margin and inner cylinder was scrapped off, chopped, crushed and suspended in distilled water. Other procedures were similar to those for root samples. The number of plants which were positive for Xcm in the corm and root tissues was then expressed as a percentage of the total number of sampled plants.

To determine the ability of Xcm to enter through wounds on cord roots, ten 9-month old banana plants of each cultivar were inoculated at Kifu forest by cutting 6 to 10 cord roots using a contaminated knife. The knife was dipped in a Xcm suspension (see below) between each cutting treatment. The surrounding soil was subsequently drenched with a suspension (200 ml per plant) of Xcm. The bacterial suspension was prepared by mixing Xcm colonies (grown on CCA media) with distilled sterile water and diluting the suspension to a concentration of $1x10^{8}$ cfus/ml (~0.5OD). These plants were then observed for a period of 12 months for development of XW symptoms. At 12 months, latent infection in asymptomatic plants was assessed by culturing suspensions of leaf petiole tissues from the youngest leaves on semi-selective CCA media and through PCR amplification of Xcm DNA fragments (650bp) using specific primers (Adikini et al., 2011). Data collected included percentage XW

incidence and percentage of asymptomatic plants with latent infection. Means were separated using least significance difference (lsd) at 5% and analysis of variance (ANOVA) was performed using the GenStat 11th Edition (VSN International Ltd, 2008).

RESULTS AND DISCUSSION

Xcm bacteria were more often observed in corm tissue than in root tissue, in both 'Pisang Awak' and AAA-EA plants, and this was significant ($P<0.05$) in symptomatic pre-flowering plants (Table 1) and highly significant ($P<0.01$) in florally-infected plants (Tables 2 and 3). In pre-flowering stage symptomatic plants in farmers' fields, Xcm was only isolated in cord roots of 11% of 'Pisang Awak' plants, though all corms of both varieties contained the bacteria (Table 1). The artificially tool-inoculated pre-flowering stage plants had a significantly higher number of plants with Xcm in their cord roots. A cord root incidence of 57% and 33% was observed for AAA-EA and 'Pisang Awak', respectively, while the corm incidence was respectively 100% and 66.7% (Table 1).

In symptomatic florally-infected plants in farmers' fields (Table 2), Xcm was only isolated from cord roots in 14.3% of AAA-EA plants at the premature fruit ripening stage, despite a high corm incidence from the decaying rachis stage onwards (between 82.5% and 100%) in both varieties. In contrast, more plants had Xcm in their cord roots in artificially inoculated flowering plants (Table 3), with significant differences ($P<0.05$) observed between the different infection stages. Cord root colonization increased with progress in disease development for both varieties. Incidence in AAA-EA increased from 20% at the decaying rachis stage to 83.3% at the bunch rotting stage; and in 'Pisang Awak' plants from 16.7% at premature fruit ripening stage to 60% at bunch rotting stage. These results suggest that banana cord roots are eventually colonized by Xcm bacteria when an infection occurs through above-ground plant parts. However, root infection is slow when compared to disease incidence observed in corm tissue, suggesting a possible difficulty in colonization of root tissues by Xcm.

Ssekiwoko et al. (2006) reported that when Xcm invades the corm, the bacteria first colonize the layer of Mangin (a cambium ring in the corm), followed by the inner cylinder and finally the outer cylinder. He also reported more plants to have bacteria in the layer of Mangin compared to the corm's central cylinder or the cortex layer because Xcm mainly infects the vascular system. The inner cylinder and the cortex are largely a mass of starchy parenchyma, while the layer of Mangin is a mass of vascular bundles (Stover and Simmonds, 1987). As such, Xcm does not seem to spread easily in the inner cylinder and the cortex (Ssekiwoko et al., 2006). Based on this information and on the fact that cord roots are formed from the layer of Mangin and subsequently grow through the cortex tissue into the soil (Stover and Simmonds, 1987), one would expect cord roots, just like corm tissues (i.e. cortex and central cylinder), to be rapidly colonised by Xcm, which is not confirmed by our results. Banana cord roots (5-8 mm thick) are white and fleshy at first, if healthy, and later in life become somewhat corky (Stover and Simmonds, 1987). This corky nature of root tissues could be responsible for the delayed and low colonization rate of root tissues in symptomatic plants.

Only 30% of 'Pisang Awak' and AAA-EA plants showed visible disease symptoms 12 months after inoculation through cord root cutting (Table 4). However, for an additional 29% of asymptomatic plants of both varieties, bacteria could either be isolated on CCA

media and or detected through PCR amplification of Xcm-specific fragments. Moreover, long incubation periods after plant inoculation with Xcm through cord roots were recorded, from 128 to 357 days in AAA-EA and from 186 to 369 days in 'Pisang Awak' plants. These findings confirm that the transmission efficiency through cord roots is low, in contrast to above-ground tissues. Results are in agreement with Addis et al. (2010) who reported a 25% Xcm infection level when banana cord roots were cut with a contaminated tool. Ashagari (1985) also reported a low transmission efficiency of Xcm (30%) when enset cord roots were cut with a contaminated knife compared to a 100% transmission rate when enset leaf petioles were cut with a contaminated tool.

CONCLUSION AND RECOMMENDATIONS

This study showed that Xcm does colonize banana cord root tissue. However, the colonization of cord roots is slower compared to banana corm tissue. The results confirm the low Xcm transmission efficiency in cord roots. Thus XW tool infections occur mainly through above-ground plant parts. Nevertheless, the study suggests that cord roots could contribute to garden tool transmission, for example during weeding. Therefore, hand weeding and herbicide use is advised when diseased mats are present in a field. Cord root infected plants also exhibited long disease incubation periods, with latent infections in some plants. This complicates the management of XW and partially explains resurgence of the disease where it was previously eradicated. Development and deployment of diagnostic kits sensitive to latent infections for routine surveillance is thus recommended for the effective management of the disease.

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Tables

Table 1. Incidence of *Xanthomonas campestris* pv. *musacearum* (Xcm) in corms and cord roots of symptomatic pre-flowering stage AAA-EA and 'Pisang Awak' (ABB) banana plants (i.e. with 2 to 3 yellowing/wilting leaves characteristic of Xanthomonas wilt) in farmers' fields and on station trials in Kifu forest.

Trial type	% of plants with Xcm in the corm or cord roots					
	AAA-EA			Pisang Awak		
	n	Corm	Cord root	n	Corm	Cord root
Farmers' fields	5	100.0*	0.0**	9	100.0*	11.1 **
On-station trial	7	100.0*	57.1*	6	66.7*	33.3*
CV%	45.9					
Lsd (5%)	60.5^{S}					

Means in rows with the same number of star(s) for a given cultivar (i.e. AAA-EA or Pisang Awak') are not significantly different.

No significant difference ($P<0.05$) in incidences observed between means for a given plant part across cultivars.

^{S} Significant differences in Xcm incidence between the corm and cord root tissues for the different varieties at $P<0.05$.

Table 2. Incidence of *Xanthomonas campestris* pv. *musacearum* (Xcm) in the corms and cord roots of AAA-EA and 'Pisang Awak' (ABB) plants growing in farmers' fields in Luwero district, central Uganda at various stages of disease development after an inflorescence infection.

Symptom stage	% of plants with Xcm in the corm or cord roots					
	AAA-EA			Pisang Awak		
	n	Corm	Cord root	n	Corm	Cord root
Wilting male bud	5	0a*	0a*	4	0a*	0a*
Decaying rachis	3	100b**	0a*	2	100b**	0a*
Premature fruit ripening	7	100b**	14.3b*	7	100b**	0a*
Bunch and plant rotting	4	82.5c**	0a*	4	100b**	0a*
CV%	16.2					
Lsd - plant parts (5%)	7.13^{HS}					
Lsd - infection stages (5%)	10.09^{HS}					

Means in rows with the same number of stars are not significantly different at $P<0.05$.
Means in the same column with the same letter are not significantly different at $P<0.05$.
[HS] Highly significant differences exist in Xcm incidence between the corm and root cord tissues and between infection stages in both varieties at $P<0.01$.

Table 3. Incidence of *Xanthomonas campestris* pv. *musacearum* (Xcm) in the corm and cord roots of AAA-EA and 'Pisang Awak' (ABB) plants at various stages of disease development following artificial inoculation of the inflorescence in a trial at Kifu forest, Mukono district, central Uganda.

Symptom stage	% of plants with Xcm in the corm or cord roots					
	AAA-EA			Pisang Awak		
	n	Corm	Cord root	n	Corm	Cord root
Wilting male bud	5	20.0a*	0.0b**	6	0.0b**	0.0b**
Decaying rachis	5	20.0a*	20.0a*	4	50.0b**	0.0c***
Premature fruit ripening	5	80.0a*	20.0b**	6	83.3a*	16.7b**
Bunch and plant rotting	6	100.0a*	83.3b**	5	100.0a*	60.0c***
CV%			30.2			
Lsd - plant parts (5%)			6.16[HS]			
Lsd - infection stages (5%)			8.71[HS]			

Means in rows with the same number of stars are not significantly different at $P<0.05$.
Means in the same column with the same letter are not significantly different at $P<0.05$.
[HS] Highly significant differences exist in Xcm incidence between the corm and root cord tissues and between infection stages in both varieties at $P<0.01$.

Table 4. Xanthomonas wilt incidence (based on visible symptoms), incubation period and latent infection levels (based on CCA media and PCR amplification of *Xanthomonas campestris* pv. *musacearum* (Xcm) DNA fragments) in 9-month-old pre-flowering stage AAA-EA and 'Pisang Awak' (ABB) plants. Plants were assessed 12 months after inoculation with Xcm by cutting cord roots of plants with a contaminated knife and subsequent drenching of the surrounding soils with a suspension of the bacteria.

	[1]Incidence (%) based on visible symptoms (n=10)	[2]Incubation period (days)	Plants with latent infection (%)	
			Based on [3]CCA media (n=10)	Based on [4]PCR (n=10)
AAA-EA	30	128 - 357	29.6	0.0
Pisang Awak	30	186 - 369	14.3	29.6

[1] Visible symptoms are wilted and yellowing leaves.
[2] Incubation period based on the time from inoculation to first visible symptoms.
[3] CCA - cellubiose cephalexin agar.
[4] PCR - polymerase chain reaction.