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Sources of resistance in *Musa* to *Xanthomonas campestris* pv. *musacearum*, the causal agent of banana xanthomonas wilt

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It is claimed that, with the exception of *Musa balbisiana*, all banana varieties are susceptible to bacterial wilt caused by *Xanthomonas campestris* pv. *musacearum* (Xcm). Despite being resistant to Xcm infection, *M. balbisiana* is not preferred for breeding because it belongs to the BB genome subgroup, while most edible bananas are of the A genome. To identify potential sources of resistance to Xcm, 72 banana accessions representing the *Musa* genetic diversity were evaluated in an outdoor confined potted trial. The midribs of the youngest leaf of 3-month-old banana plants were inoculated with 10^8 CFU mL^{-1} of Xcm isolate USY13P, and symptom development assessed weekly for 4 months. Results confirmed that *M. balbisiana* genotypes are indeed resistant to Xcm. Varieties within the *Musa acuminata* subsp. *zebrina* (AA) set were further identified as potentially useful sources of Xcm resistance. These findings reveal the potential to develop banana and plantain varieties with tolerance to Xcm.

Keywords: banana, genotype, molecular breeding, resistance, xanthomonas wilt

Introduction

Bananas are an important staple and income-generating crop for many farmers in tropical and subtropical climates (Padam *et al.*, 2014). Bananas belong to the genus *Musa*, which together with *Ensete* and *Musella*, belong to the family Musaceae. Previously, the genus *Musa* was divided into the section *Ingentimusa* with chromosome number $2n = 14$; *Callimusa* and *Australimusa* with chromosome number $2n = 20$ (*Musa beccarii*, which is part of *Callimusa* section, has 18 chromosomes); and *Eumusa* and *Rhodochlamys* with chromosome number $2n = 22$ (Christelová *et al.*, 2011a). Information on the ploidy level of the germplasm is essential in breeding programmes because it influences fertility (Suman *et al.*, 2012).

Research has shown that banana is highly vulnerable to diseases due to the genetic uniformity (Tripathi *et al.*, 2008). Banana bacterial wilt, also known as xanthomonas wilt, caused by *Xanthomonas campestris* pv. *musacearum* (Xcm), is regarded as the most devastating disease of banana in East and Central Africa (Nakato *et al.*, 2018). Xcm transmission is by insect vectors, contaminated garden tools and infected planting material (Nakato *et al.*, 2018). Insect infection through the inflorescence occurs for cultivars that shed their bracts; however, all cultivars are susceptible to infection by tools that are used for deleafing and desuckering or weeding of the annual crops that are mixed in banana plantations (Nakato *et al.*, 2018). In addition to reducing yield, xanthomonas wilt can kill banana plants (Nakato *et al.*, 2018).

Xcm is a rod-shaped, aerobic, Gram-negative bacterium that is motile by a single flagellum (Bradbury, 1986; Smith *et al.*, 2008). Biochemical characteristics, including urease production, hydrolysis of aesculin, production of hydrogen sulphide from peptone, catalase production and utilization of sorbitol, dulcitol and salicin revealed that Xcm belongs to *X. campestris* (Bradbury, 1986). Further characterization using fatty acid methyl ester (FAMEs) analysis, *gyrB* gene sequence and rep-PCR revealed that Xcm was closely related to *Xanthomonas vasicola* pv. *vasculorum* (Xvv) and *Xanthomonas vasicola* pv. *holcicola* (Xvh) (Aritua *et al.*, 2008) and renaming of Xcm to *Xanthomonas vasicola* pv. *musacearum* was proposed. Due to lack of adequate pathogenicity studies on *X. vasicola* species and the annulment of the

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proposed naming of *X. vasicola* pathovars, and despite suggestions gained from comparative genomics (Wasukira *et al.*, 2012, 2014), renaming of Xcm has not been done (Aritua *et al.*, 2008; Karamura *et al.*, 2015). However, results from whole genome sequencing clearly show that *Xanthomonas* causing wilt of banana is not close to *X. campestris* (Studholme *et al.*, 2010).

Initial population genetic studies separated Xcm into two sublineages (Wasukira *et al.*, 2012). However, in a recent study, using polymorphic multilocus number of tandem repeat analysis (MLVA) markers on a larger Xcm collection, 12 clusters were identified (Nakato, 2018). Although most of the clusters were consistent with the sublineage classification suggested by Wasukira *et al.* (2012), an unexpected diversity was observed in the clusters that were not assigned to any sublineage (Nakato, 2018). Further analyses are underway to determine if the isolates within the different clusters differ in virulence.

Xcm can survive in plant residues for up to 3 months (Ssekiwoko *et al.*, 2006). Cultural practices such as single diseased stem removal (Nakato *et al.*, 2018), breaking off the male bud with a forked stick and sterilization of garden tools with sodium hypochlorite (Ssekiwoko *et al.*, 2006; Nakato *et al.*, 2018) have been recommended to control xanthomonas wilt. Although effective, the adoption of these practices has been low and variable, probably because of the high demand of labour and resource requirements from smallholder farmers (Nakato *et al.*, 2018). Resistant or tolerant accessions may be the most promising option. However, previous reports revealed that all banana accessions except *Musa balbisiana* were susceptible to Xcm (Ssekiwoko *et al.*, 2006; Tripathi *et al.*, 2008; Kebede & Gemmeda, 2017). Despite being resistant to Xcm, *M. balbisiana* is not preferred for breeding because it belongs to the BB genome subgroup, while most edible bananas are of the A genome. Therefore, genetic engineering has been proposed as the only method for developing Xcm resistant varieties.

Genetically modified bananas with resistance to Xcm have been developed using non-banana genes and field tested in Uganda (Namukwaya *et al.*, 2012). Most countries do not have biosafety regulations for the adoption and use of genetically modified plants, which hinders the adoption and use of transformed banana accessions. Therefore, there is a need to find sources of resistance to Xcm within the available banana germplasm.

A previous study to identify banana accessions resistant to Xcm was conducted on 43 accessions: mainly the East African Highland banana (EAHB, AAA) and a few diploid, triploid and tetraploid varieties (Ssekiwoko *et al.*, 2006; Tripathi *et al.*, 2008). Of these, only the wild banana *M. balbisiana* was resistant to Xcm. In this study, the number of banana accessions was expanded to include the entire genetic diversity found in *Musa*, including diploid AA accessions that were not screened in previous studies (Tripathi *et al.*, 2008). Such germplasm constitutes a valuable genetic resource for banana breeding programmes aimed at producing resistant varieties. This study was designed to provide answers to the following questions: (i) is there resistance to Xcm within the banana germplasm? (ii) Can this resistance be used in a breeding programme? (iii) Is the resistance coming from a specific set of germplasm, so that targeted screening can be conducted to expand the sources of resistance? To answer these questions, a set of 72 banana accessions comprising representatives of the entire *Musa* diversity were screened under controlled conditions for response to Xcm following artificial inoculation.

Materials and methods

Plant materials

This study was conducted at the International Institute of Tropical Agricultural (IITA) Sendusu station (0°31′30″N; 32°36′54″E) and at the National Agricultural Research Laboratories, Kawanda, Uganda (0°24′25″N; 32°32′07″E). A total of 72 banana accessions from the IITA germplasm collection with different ploidy levels were screened in an outdoor confined pot trial for response to Xcm in the period October 2015 to January 2016 and March 2016 to June 2016 (Table 1). For each accession, 12 plants were raised from disease-free corms. Corms from healthy suckers were pared and treated with dursban (chlorpyrifos, belonging to phosphorothioate group of organophosphorus pesticides) for 20 min to eliminate nematodes and weevils prior to planting. The pots were filled to 3/4 full with a mixture of sterilized top forest soil and sand (3:1) and arranged in a completely randomized design. Plants were watered every other day, three days a week.

Xcm isolation, maintenance and inoculum preparation

Xcm was isolated from the pseudostem of a Kayinja (ABB subgroup) banana plant with symptoms, harvested from Kifu National Forest Reserve in Mukono District, Uganda in December 2014. Sections (3 g) of inner parts of the pseudostems were aseptically macerated in 3 mL sterile distilled water using a sterile mortar and pestle. One millilitre of the resulting suspension was serial diluted in sterile distilled water. Twenty microlitres from the 10^{-2} dilution was spread on a plate of semiselective yeast peptone glucose agar (YPGA) growth medium (Mwangi *et al.*, 2007), the plate sealed with Parafilm and incubated at 24 ± 1 °C for 72 h. Single colonies with a yellow, convex, mucoid morphology typical of Xcm were harvested and further purified by streaking onto fresh YPGA plates. Xcm colonies were confirmed using Xcm-specific primers (Adriko *et al.*, 2012) and further characterized as sublineage II by PCR (Wasukira *et al.*, 2012), before storage at −80 °C under the code USY13P, at NARO, Kawanda, Uganda. To prepare inoculum, Xcm culture USY13P was revived and multiplied in YPG broth and incubated at 28 °C for 48 h. The inoculum was adjusted with sterile distilled water to 10^8 colony-forming units per mL (approximately 0.5 OD_{600}) using a spectrophotometer (Thermo Fisher Scientific) before inoculation.

Screening procedure and disease assessment

Nine of the 12 plants for each accession were inoculated with Xcm 3 months post-planting. A 100 µL solution of freshly

Table 1 Banana accessions from the IITA germplasm collection evaluated for response to *Xanthomonas campestris* pv. *musacearum* (Xcm) inoculation and grouped into disease reaction types[a] based on disease index[b]. The accessions were further genetically characterized using SSR markers and grouped into clusters[c] based on morphological descriptors.

							1st evaluation		2nd evaluation	
Accession	Cultivar name	Genotype	Expected ploidy	Observed ploidy	Set	Cluster[c]	Disease index[b]	Reaction type[a]	Disease index	Reaction type
MMC 192	*Musa balbisiana*	BB	2×	2×	*M. balbisiana* cluster	VII	18 a	R	8 a	R
ITC1179	Monyet	AA	2×		*M. acuminata* subsp. *zebrina* cluster	X	117 a	T	265 ab	T
ITC1177	*M. acuminata* subsp. *zebrina*	AA	2×	2×	*M. acuminata* subsp. *zebrina* cluster	X	139 a	T	333 bc	MS
ITC0116	Saba	ABB	3×	3×	ABB Bluggoe/Monthan cluster	XII	144 a	T	207 ab	T
ITC1178	Buitenzorg[d]	AA	2×		AA cv. ISEA 2 cluster	IX	161 a	T	146 ab	T
ITC1120	Tani	BB	2×		*M. balbisiana* cluster	VII	163 a	T	146 ab	T
ITC0019	IC2	AAAA	4×	4×	Related to AA cv. African cluster	IX	176 a	T		
ITC0396	Pelipita	ABB	3×	3×	*M. balbisiana* cluster	VII	201 a	T	159 ab	T
ITC1224	Kikundi	AAA	3×		AAA/Lujugira/Mutika	X	201 a	T		
ITC0246	Cameroun	BB	2×	2×	*M. balbisiana* cluster	VII	228 a	T	212 ab	T
Not known	*M. acuminata* subsp. *zebrina*	AA	2×		*M. acuminata* subsp. *zebrina* cluster	X	233 a	T		
ITC0243	Pisang Rajah	AAB	3×		AAB/Pome	IX	238 a	T		
ITC0728	Maia Oa	AA	2×	2×	*M. acuminata* subsp. *zebrina* cluster	X	255 a	T		
ITC0837	Yalim	AA	2×		AA cv. *banksii* s.l. cluster	XI	289 a	MS		
ITC1345	Pisang Kra	AA	2×		*M. acuminata* subsp. *malaccensis* cluster	III	295 a	MS		
ITC0084	Mbwazirume	AAA	3×		AAA/Lujugira/Mutika	X	312 ab	MS		
ITC1468	Kahuti	AA	2×	2×	AA cv. African	IX	328 ab	MS		
ITC1454	Makyugu 1	AA	2×		AA cv. African	IX	328 ab	MS		
ITC1462	Suu	AAA	3×	3×	AAA/Lujugira/Mutika	X	333 ab	MS		
ITC0654	Petite Naine	AAA	3×	3×	AAA/Cavendish	IX	334 ab	MS		
ITC1139	*M. acuminata* subsp. *zebrina*	AA	2×		*M. acuminata* subsp. *zebrina* cluster	X	343 ab	MS		
ITC1467	Kisanga Machi	AA	2×		AA cv. ISEA 2 cluster	IX	345 ab	MS		
ITC1459	Mlema	AAA	3×	3×	AAA/Lujugira/Mutika	X	362 ab	MS		
ITC0058	Cacambou	ABB	3×		ABB Bluggoe/Monthan cluster	XII	366 ab	MS		
ITC0966	Zebrina (G.F.)	AA	2×	2×	Does not cluster with other subsp. *zebrina*		368 ab	MS		
ITC0364	Silver Bluggoe	ABB	3×	3×	ABB Bluggoe/Monthan cluster	XII	407 ab	MS		
ITC1457	Haa Haa	AAA	3×		AAA/Lujugira/Mutika	X	410 ab	MS		
ITC1594	Mshare	AA	2×	2×	AA cv. African	IX	415 ab	MS		
ITC0078	Who-gu	AAA	3×		AA cv. IndonTriNG	IX	430 ab	MS		
ITC0944	Wambo[de]	AA	2×	3×	AAB plantain + plantain-like cluster	XIII	433 ab	MS		
ITC1319	FHIA-18	AAAB	4×	4×	AAB/Pome	IX	438 ab	MS		
ITC1458	Ilayi Red	AAA	3×		AAA/Lujugira/Mutika	X	439 ab	MS		
ITC0393	Truncata	AA	2×		*M. acuminata* subsp. *burmannicoides*/*burmannica*/*siamea*/*truncata*	I	460 ab	MS		
ITC1461	Ntebwa	AAA	3×	3×	AAA/Lujugira/Mutika	X	462 ab	MS		
ITC0574	Robusta	AAA	3×	3×	AAA/Cavendish	IX	467 ab	MS		
ITC1452	Huti (Shumba Nyeelu)	AA	2×	2×	AA cv. African	IX	469 ab	MS		
ITC1451	Kitarasa	AAA	3×	3×	AAA/Lujugira/Mutika	X	471 ab	MS		
ITC0768	Lacatan	AAA	3×		AAA/Cavendish	IX	473 ab	MS		

(continued)

Table 1 (continued)

Accession	Cultivar name	Genotype	Expected ploidy	Observed ploidy	Set	Cluster[c]	1st evaluation		2nd evaluation	
							Disease index[b]	Reaction type[a]	Disease index	Reaction type
ITC1464	Ntindii I	AAA	3×	3×	AAA/Lujugira/Mutika	X	477 ab	MS		
ITC1544	Mlelembo	AA	2×	2×	AA cv. African	IX	478 ab	MS		
ITC1349	Pisang Serun 400	AA	2×	2×	*M. acuminata* subsp. *malaccensis* cluster	III	481 ab	MS	270 ab	T
MMC 016	Tereza	AAA	3×	3×	AAA/Lujugira/Mutika	X	484 ab	MS		
ITC1466	Nshonowa	AA	2×	2×	AA cv. African	IX	490 ab	MS		
ITC0609	Pahang	AA	2×	2×	Closest accession ITC0610	I	495 ab	MS		
ITC1465	Ibwi	AAA	3×		AA cv. African	IX	496 ab	MS		
ITC0947	Duningi	AAB	3×	3×	AA cv. IndonTriPh	X	500 abc	MS		
ITC1456	Huti RB	AA	2×		AA cv. African	IX	511 abc	HS		
ITC0868	Porapora	AA	2×		AA cv. *banksii* s.l. cluster	XI	512 abc	HS		
ITC0814	Bagul	AA	2×		AA cv. *banksii* s.l. cluster	XI	518 abc	HS		
MMC 167	Sukari Ndiizi	AAB	3×	3×	AAB Silk cluster	VIII	520 abc	HS		
ITC0526	Kluai Namwa Khom	ABB	3×		ABB Pisang Awak cluster	VIII	528 abc	HS		
ITC1305	Paji	AA	2×		AAA/Lujugira/Mutika	X	528 abc	HS		
ITC0897	*M. acuminata* subsp. *banksii*	AA	2×		AA subsp. *banksii* s.l. cluster	XI	538 abc	HS		
MMC 020	Kibuzi	AAA	3×	3×	AAA/Lujugira/Mutika	X	548 abc	HS		
ITC0595	Pagatau	AAA	3×		AA cv. IndonTriNG	IX	549 abc	HS		
ITC0840	Kuspaka	AA	2×		AA cv. *banksii* s.l. cluster	XI	554 abc	HS		
ITC0164	Rugondo	AAA	3×		AAA/Lujugira/Mutika	X	556 abc	HS		
ITC1143	Gia Hui	ABB	3×		*M. acuminata* subsp. *burmannicoides/ burmannica/siamea/ truncata*	I	560 abc	HS		
ITC0087	Kayinja	ABB	3×	3×	ABB Pisang Awak cluster	VIII	566 abc	HS		
ITC1243	Kokopo	AA	2×			IX	573 abc	HS		
ITC1348	Pisang Serun 404	AA	2×	2×	*M. acuminata* subsp. *malaccensis* cluster	III	578 abc	HS	571 d	HS
ITC0310	Morong Princesa	AA	2×		AA cv. ISEA 2 cluster	IX	589 abc	HS		
ITC0074	*M. acuminata* subsp. *malaccensis*	AA	2×		*M. acuminata* subsp. *malaccensis* cluster	III	595 bc	HS		
ITC0946	Merik	AAA	3×	3×	AA cv. IndonTriPh	X	596 bc	HS		
ITC1318	SH-3436-9	AAAA	4×	4×	Related to AA cv. African cluster	IX	600 bc	HS		
ITC0629	Selangor 2[e]	AA	2×	4×	Related to AA cv. African cluster	IX	606 bc	HS		
ITC0259	Galeo	AA	2×		AA cv African	IX	626 cd	HS		
ITC0312	Pisang Jari Buaya	AA	2×		AA cv. Pisang Jari Buaya	I	677 cd	HS		
TARS 18062	Pitu	AA	2×		AA cv. African	IX	678 cd	HS		
ITC1000	Gunih	AA	2×				678 cd	HS		
ITC0610	Tuu Gia	AA	2×			I	687 cd	HS	306 ab	MS
ITC0250	*M. acuminata* subsp. *malaccensis*	AA	2×		*M. acuminata* subsp. *malaccensis* cluster	III	745 cd	HS	371 bc	MS
LSD							278***		171***	

Means in the columns followed by the same letter are not significantly different ($P \leq 0.05$) by LSD.

[a]Disease rating scale based on wilt incidence to evaluate relative resistance of banana accessions to Xcm infection. Resistant (R), no plants wilted; tolerant (T), <30% plants wilted; moderately susceptible (MS), >30% and <50% plants wilted; and highly susceptible (HS), >50% plants wilted.

[b]Disease index based on percentage of plant area that is diseased during a given period of time.

[c]Accessions grouped together based on the morphological traits-based classification.

[d]Accessions that grouped into sets different from the expected.

[e]Accessions with ploidy level different from the expected.

diluted Xcm inoculum was injected into the midrib of the youngest leaf with a syringe fitted with 28-gauge needle (Ssekiwoko *et al.*, 2006). The remaining three plants served as controls and were inoculated with sterile distilled water. The midrib inoculation imitates tool infection that is common during deleafing and detrashing, which are farmer practices conducted routinely to maintain the banana field.

The plants were observed weekly for four months and banana xanthomonas wilt (BXW) symptom expression and severity recorded. Parameters recorded included time to symptom expression, BXW symptom characteristics such as leaf wilting, and whole plant death. Disease severity was scored using a scale of 0–3, adopted from Winstead & Kelman (1952), and modified as follows: 0, no disease symptoms; 1, necrosis of inoculated leaf; 2, wilting of uninoculated leaves; and 3, death of entire plant. The severity score was used to compute the disease index using Equation 1:

$$\text{Disease index(DI)} = (((1 * A) + (2 * B) + (3 * C))/\text{No. of plants}) * 100 \quad (1)$$

where A = number of plants with inoculated leaf showing symptoms, B = number of plants with uninoculated leaves showing symptoms, and C = number of wilted plants.

The time interval between inoculation and appearance of disease symptoms, and complete wilting (days post-inoculation, DPI) was computed by counting the number of days from inoculation to symptom development. Classification of plants into resistant and susceptible categories was based on a scale developed by Tripathi *et al.* (2008) and modified as follows: resistant (R), no plants wilted; tolerant (T), <30% plants wilted; moderately susceptible (MS), >30% and <50% plants wilted; and highly susceptible (HS), >50% plants wilted.

The inoculation was repeated on a subset comprising 12 accessions that had a resistant or tolerant response from the first screening and a few that were highly susceptible. Similarly, these plants were inoculated with Xcm at 3 months post-planting and data collected as above.

Area under disease progress curve (AUDPC)

The AUDPC is a useful quantitative summary of disease intensity over time and for comparative analyses among genotypes, varieties or treatments. The average data of each score at weekly intervals was used to compute the AUDPC according to the formula reported by Forbes *et al.* (1993). The AUDPC was calculated using Equation 2:

$$\text{AUDPC} = \sum_{i=1}^{N_i-1} \left(\frac{Y_i + Y_{i+1}}{2}\right)(t_{i+1} - t_i) \quad (2)$$

where t = time in weeks of each reading, Y = percentage of affected plants at each reading, N = number of readings, and i = reading.

Genetic characterization of the banana accessions

Two methods were used to confirm identity of accessions used: (i) estimation of ploidy level, and (ii) genotyping using simple sequence repeat (SSR) markers. Ploidy level of each accession was estimated by flow cytometry as described by Doležel *et al.* (2007). A small piece of young banana cigar leaf midrib was chopped into a glass Petri dish containing 500 μL of ice cold Otto I buffer. The homogenate was filtered through a 50 μm nylon filter into a sample tube and incubated for 1–5 min on ice. Otto II buffer (1 mL) containing the fluorescent dye 4-6-diamidino phenyl indole (DAPI) was added before ploidy measurement using Sysmex CyFlow flow cytometer equipped with UV excitation and detectors for DAPI fluorescence (Christelová *et al.*, 2017). Molecular characterization using SSR markers targeting 19 loci was conducted as described by Christelová *et al.* (2011b, 2017). The SSR markers are motifs of 1–6 bp repeats tandemly arranged in the genomes of eukaryotic and prokaryotic organisms (Christelová *et al.*, 2011b). The SSR loci were amplified using specific primers (Hippolyte *et al.*, 2010) that were adjusted by 5′-M13 tails to enable the use of a universal fluorescently labelled primer according to Schuelke (2000). Four different fluorophores were used for the primer labelling (6-carboxyfluorescein (6-FAM), VIC, NED and PET; Applied Biosystems), allowing for subsequent multiplexing of the reactions.

Data analysis

Principal component analysis

Principal component analysis (PCA) was performed to evaluate the relative contribution of disease incubation, incidence and severity to the observed variability among the different accessions, and to identify the variables that contributed most to the data structure. The analysis was performed using the correlation matrix function in GenStat v. 17 (VSN International Ltd 2014).

Disease assessment

To assess variation among accessions for time (DPI) to symptom expression, DPI to complete wilting, disease index and AUDPC, a one-way analysis of variance (ANOVA, no blocking) was performed using GenStat v. 17. Means were separated using least significant difference at 95% confidence level.

Mean values from ANOVA results were used to perform cluster analysis with FactoMineR package in R that permits multivariate exploratory data analysis (Husson *et al.*, 2008). Based on Euclidean distances, hierarchical clustering using the ward.D2 method in R was used to group banana accessions into relatively homogenous units or disease reaction types using disease incubation, incidence and severity data (time to symptom expression, time to complete wilting and AUDPC).

Genetic characterization and ploidy confirmation

The PCR-amplified fragments from the 19 SSR loci were scored for presence or absence and the genetic diversity among individual accessions was evaluated using Nei's genetic distance coefficient (Nei, 1973). Subsequently, the genetic distance matrix was used for hierarchical clustering using unweighted pair group method with arithmetic mean (UPGMA; Michener & Sokal, 1957). A dendrogram was constructed based on the results of UPGMA analysis and visualized in FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The dissimilarity index threshold of 0.25 was used to assign accessions into groups.

Results

Symptom expression

Thirty-eight accessions had localized necrosis (tissue necrosis around the point of inoculation) and this was observed starting 7 DPI to 77 DPI (Fig. 1). Thirty-four

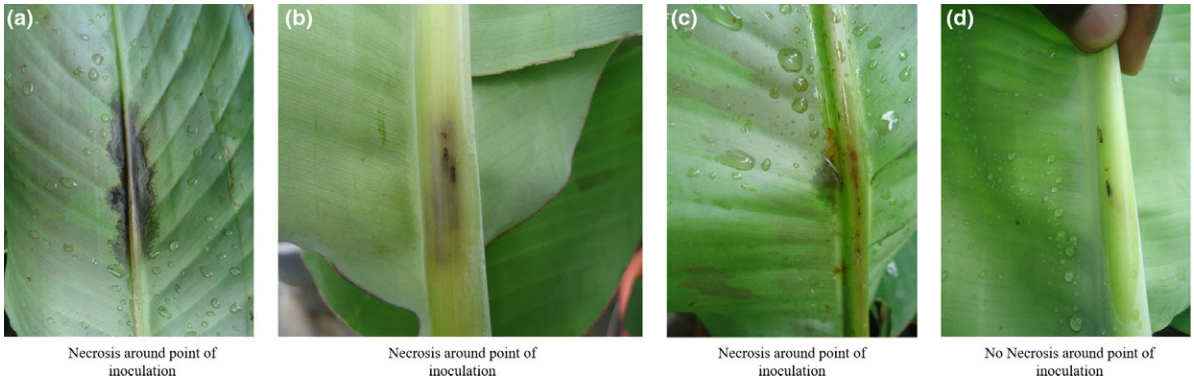

Figure 1 Banana leaves showing the point of inoculation for *Xanthomonas campestris* pv. *musacearum* inoculum at 14 days post-inoculation for representative accessions. Leaves a–c indicate the different levels of necrosis around the point of inoculation observed in treated plants for 53 % of the accessions. Leaf d is indicative of accessions that did not show any necrosis around the point of inoculation.

of the accessions did not show localized necrosis around the point of inoculation (Fig. 1). However, this localized necrosis was not related to the final disease resistance scoring. The inoculated leaf in *M. balbisiana* did not develop necrosis at the point of inoculation and no disease symptoms were observed on the inoculated leaf and beyond (Fig. 1d).

Banana accession response to Xcm inoculation

Principal component analysis revealed that DPI to complete wilting was the most reliable factor for differentiating response of banana accessions to Xcm infection (Table 2). DPI to complete wilting accounted for 66 % of the variability among banana accessions and disease index (DI) accounted for 30 %. Therefore, DPI to complete wilting and DI were used in subsequent analyses. Significant differences ($P < 0.05$) were observed in reaction of accessions to Xcm infection (Table 1). The lowest DI value was reported for *M. balbisiana* while the highest was reported for *M. acuminata* subsp. *malaccensis* (Table 1). Fifty-nine accessions were susceptible (MS and HS) to Xcm infection, 12 had a tolerant reaction, and one was resistant (Table 1). All accessions screened, except *M. balbisiana*, had some or all plants that wilted completely (Table 1). None of the accessions was immune. Accessions with a susceptible reaction clustered separately from those with a tolerant reaction, with *M. balbisiana* as an out-group (Fig. 2). Figure 3 shows symptom development in representative accessions per disease reaction type.

Table 2 Principal component scores from correlation matrix of disease progression variables in banana accessions inoculated with *Xanthomonas campestris* pv *musacearum* to evaluate relative contribution of disease parameters to the overall disease assessment

	Principal component scores	
Variable	PC1	PC2
Disease index	0.473	**0.773**
DPI to complete wilting	**0.552**	−0.634
DPI to symptom expression	−0.687	0.022
Percentage variation	65.8	29.9
Cumulative percentage variation	65.8	95.7

Rescreening results did not change much from that observed in the initial screening (Table 1). Significant differences ($P < 0.05$) were observed in accessions' response to Xcm (Table 1). As previously observed in the initial analysis, *M. balbisiana* presented the lowest DI while Pisang Serun 404 displayed the highest DI (Table 1). Of the 12 accessions, seven accessions had a tolerant response, four accessions were susceptible and only one was resistant (Table 1). Using the disease reaction types, some accessions had the same response as observed during the initial screening, while others had a different response (Table 1). For example, *M. balbisiana* was resistant in both analyses; similarly, Buitenzorg, Saba, Tani, Pelipita, Cameroun and Monyet were tolerant and Pisang Serun 404 was highly susceptible in both analyses (Table 1). However, Pisang Serun 400, Tuu Gia, *M. acuminata* subsp. *zebrina* and *M. acuminata* subsp. *malaccensis* differently (Table 1). Tuu Gia and *M. acuminata* subsp. *malaccensis* changed from highly susceptible to moderately susceptible, Pisang Serun 400 changed from moderately susceptible to tolerant, and *M. acuminata* subsp. *zebrina* from tolerant to moderately susceptible.

Genetic variation among the accession

Ploidy analysis revealed that 32 of the 34 accessions for which ploidy level was determined had the same values for both the expected and observed ploidy levels (Table 1). However, accessions Wambo and Selangor, both with an expected ploidy level of 2× were confirmed to be 3× and 4×, respectively (Table 1).

A dendogram generated using SSR marker data separated the 72 banana accessions into 22 clusters at 0.25 Nei's dissimilarity index with relatively significant bootstrap support (>35%) (Table 1; Fig. 4). The susceptible accessions were present in 20 out of the known 22 sets

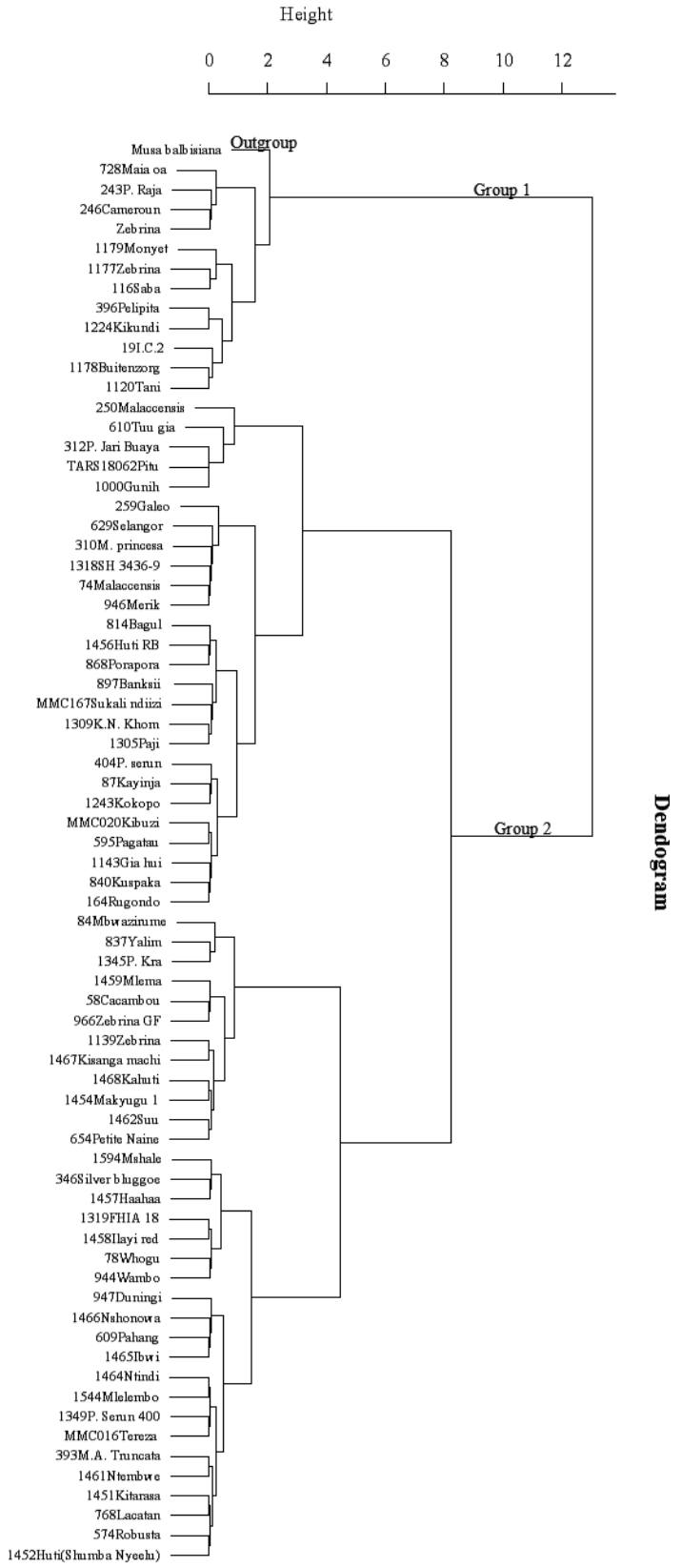

Figure 2 Dendogram of hierarchical cluster analysis using WARD. D2 method for banana accessions based on Euclidean distances for time to symptom development, time to complete wilting and disease index.

Figure 3 Symptom development for representative accessions for different reaction types at 35 days post-inoculation. The varieties are *Musa balbisiana* (resistant), Pelipita (tolerant), Ilayi Red (moderately susceptible) and Pitu (highly susceptible).

but the resistant and tolerant accessions clustered in the sets of *M. balbisiana* and *M. acuminata* subsp. *zebrina* only (Table 1). Three accessions, i.e. Buitenzorg, Ibwi and Wambo, grouped into different sets from the expected. For example, although Buitenzorg should group within the *M. acuminata* subsp. *zebrina* set, it grouped as AA cv. ISEA 2; similarly Ibwi, which is within the Lujugira/Mutika set, grouped into the AA cv. African set, and Wambo, which is AA cv. African, grouped into the plantain set. This could be a case of mislabelling or misclassification.

The accessions were further grouped into clusters as presented by Christelová *et al.* (2017) (Table 1). The putative tolerant accessions clustered into VII, IX, X, XI and XII, while the susceptible accessions clustered into I, III, VIII, IX, X, XI, XII and XIII (Table 1; Fig. 5). Clusters IX, X, XI and XII contained accessions with both susceptible and tolerant reactions while cluster VII only contained tolerant accessions, and clusters I, III and VIII only contained susceptible accessions (Table 1; Fig. 5). These results confirm resistance in cluster VII with genotypes with BB and ABB background, and suggest cluster X with only genotypes with pure A genomic configurations needs to be targeted for screening. The cluster X comprised the following genotypes: AA, AAA and AAB (Christelová *et al.*, 2017).

Discussion

Breeding for host plant resistance to pathogens is an important aspect for sustainable crop production. It is commonly believed and supported with data from this study that *M. balbisiana* with the BB genome configuration is the only source of BXW resistance (Ssekiwoko *et al.*, 2006; Tripathi *et al.*, 2008), thus providing limited options for banana breeding for BXW resistance. The observed tolerance to BXW in some genotypes with AAB and ABB background is, therefore, presumed to have originated from the B genome.

Necrosis around the point of inoculation indicates an intense reaction of the plant to infection, stimulated by specific elicitors of the pathogen, which evoke a hypersensitive-like reaction (HR-like). However, this lethal necrosis did not prevent further spread of the pathogen and thus is not a resistance attribute to Xcm. The inoculated leaf in *M. balbisiana* developed such symptoms, but the bacteria were not able to spread and colonize other parts of the plant, implying that *M. balbisiana* may have some degree of vertical resistance for possible exploration in breeding programmes.

Tripathi *et al.* (2008) and Ssekiwoko *et al.* (2006, 2015) documented the ability of *M. balbisiana* to resist Xcm infection. For example, Ssekiwoko *et al.* (2015) explored the mechanisms of resistance in *M. balbisiana* and concluded that neither quorum sensing nor the HR played a part in *M. balbisiana* reaction to Xcm infection. However, the authors noted that *PR3* genes played a role in delaying symptom expression. Pathogens are only able to cause disease symptoms in a plant after reaching a population threshold that permits symptom expression. Ssekiwoko *et al.* (2015) further noted that Xcm disables the plant's defence system (explaining why most banana accessions succumb to Xcm infection), but *M. balbisiana* was able to re-express its defence genes after 72 h.

Thirteen other sources of resistance apart from varieties with the BB genotype were found tolerant. Their genomic configurations and ploidy levels were AA, AAA, AAB, ABB and AAAA. Tolerance in some genotypes with AA shows that tolerance is derived from some A genome accessions, while the AAB and ABB resistance could be from the B genome. This opens new possibilities in banana breeding as several important banana subgroups such as the EAHB belong to the AAA subgroup. Moreover, this is useful for AAB and ABB breeding as well, as the B genome contains the endogenous banana streak virus (eBSV), which is activated when plants are stressed, although some efforts are being made to generate eBSV-free B genotypes by breeding (Noumbissié *et al.*, 2016). Interestingly, accessions with the A genome with tolerance to Xcm were also identified.

Based on the molecular characterization of the banana accessions, it was observed that genotypes belonging to the *M. balbisiana* set were resistant and those belonging to the *M. acuminata* subsp. *zebrina*, and Yalim

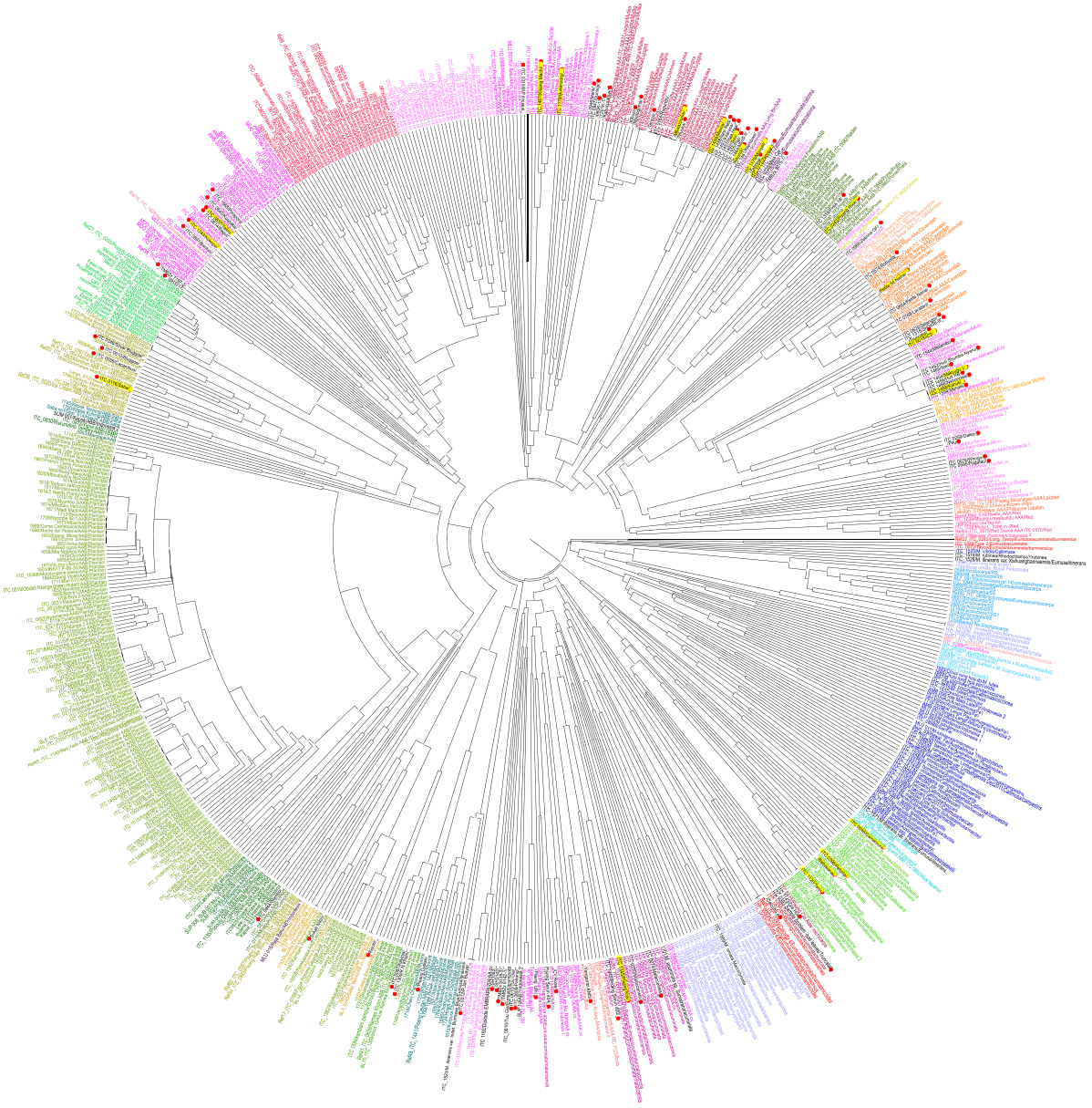

Figure 4 Diversity tree of known genetic pool of banana accessions. The figure shows the position of screened accessions (with a red dot and highlighted in yellow) on the dendogram and how they clustered with the core subsets identified by Christelová *et al.* (2017). The different blocks of accessions are differentiated by colour.

belonging to the subspecies *banksii*, were tolerant. The *M. balbisiana* set is known to harbour traits for biotic stress tolerance (Robinson & Sauco, 2010). This is important as the subspecies *zebrina* and *banksii* contributed to the formation of the triploid AAA/Lujugira/Mutika set of the EAHB (Christelová *et al.*, 2017). It was also observed that the accession Kikundi of the EAHB was tolerant, while the other 12 accessions tested were susceptible. IC2 (AAAA), Saba (ABB), Pelipita (ABB) and Pisang Raja (AAB) were also tolerant. The AA cv. African set contains cultivars found in East Africa and islands of the Indian Ocean and is the proposed progenitor of the subgroup AAA/Cavendish and AAA Gros Michel (Christelová *et al.*, 2017). The AAB/ Bluggoe-Monthan set is still under investigation. According to Christelová *et al.* (2017), there is limited intraspecific diversity within *M. balbisiana* unlike in *M. acuminata*. Several authors agree that on average, the constitution of the A genome of *M. acuminata* and clones with AA genome is approximately 12 % larger than the B genome of *M. balbisiana*, with small intraspecific variation in nuclear DNA found in a number of wild *M. acuminata* diploid and parthenocarpic bananas and large variation exhibited among triploid cultivars (Kamaté *et al.*, 2001).

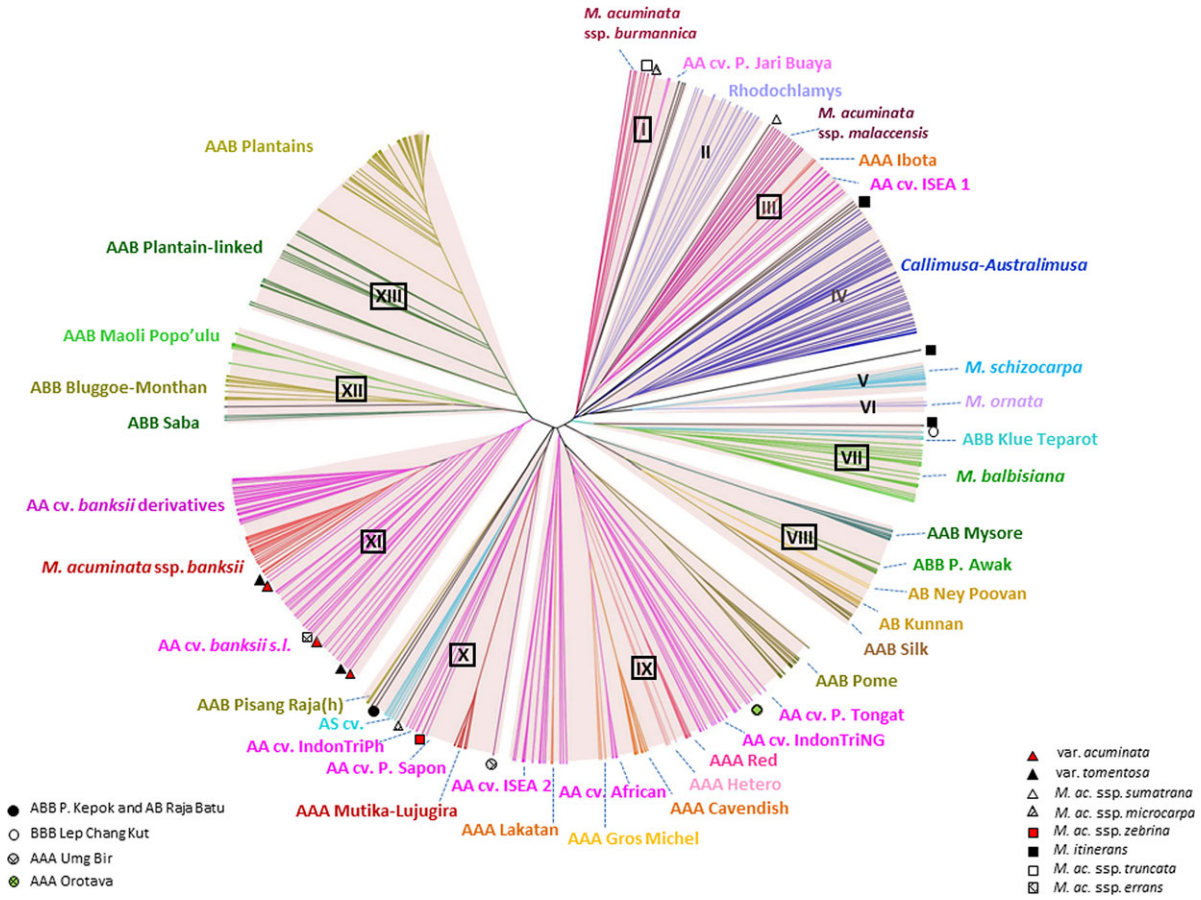

Figure 5 UPGMA dendogram showing the clusters (with a bold square around them), based on morphological descriptors, into which the screened accessions were categorized. The 72 accessions grouped into clusters I, III, VII, VIII, IX, XI, XII and XIII as indicated by Christelová *et al.* (2017). The individual sets of the clustered accession are indicated in Table 2 (the diagram was adopted and modified from Christelová *et al.*, 2017).

Although intraspecific genetic diversity is an important attribute deemed critical for additive and dominance effects (Tilman *et al.*, 2001), there is limited understanding of the impact on population performance (Moore *et al.*, 2014). However, it could be assumed that it influences the apparent variation within the A genome to phytopathogens.

The present study has shed light on avenues to explore the genetic diversity in *Musa* Xcm resistance and tolerance. It has confirmed resistance of some genotypes with the B genome to Xcm, but most importantly highlights that the A genome also contains resistance genes in two subspecies of *M. acuminata* which can be used in breeding of bananas and plantains. The A genome is preferred for breeding because most edible bananas are of the A genome. Buitenzorg and Monyet, both belonging to different sets and clusters, were tolerant, hence useful in future breeding programmes as sources of resistance to Xcm.

Currently, a mapping population comprising 180 lines, resulting from the crossing of Monyet (AA) (tolerant) and Kokopo (AA) (highly susceptible) is available to further explore the genetics of Xcm resistance. This population will be evaluated for response to Xcm and used to identify quantitative trait loci associated with Xcm resistance. In addition, it would be worthwhile to study the variability in tolerant genotypes, through measurements of pathogen load, proliferation and/or analyses of gene expression profiles to explore mechanisms of tolerance. A selection of representative accessions from each cluster should be screened using Xcm isolates representing the two Xcm sublineages observed through genetic analysis using single nucleotide polymorphism (SNP) and MLVA markers.

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