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 10⁸ 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 *Ingentimum* 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 *Emusa* 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...
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 accesses 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 promising option 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 Agricultural (IITA) Sendusu station (0°31’30”N, 32°36’54”E)80°31″0″C under the code USY13P, at NARO, Kawanda, 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 105 colony-forming units per mL (approximately 0.5 OD600) 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 based on disease index. The accessions were further genetically characterized using SSR markers and grouped into clusters based on morphological descriptors.

| Accession | Cultivar name | Genotype | Expected ploidy | Observed ploidy | Set | Cluster | 1st evaluation | 2nd evaluation |
|------------|---------------|-----------|-----------------|-----------------|-----|---------|----------------|---------------|
| MMC 192    | *Musa balbisiana* | BB | 2x | 2x | *M. balbisiana* cluster | VII | 18 a R | 8 a R |
| ITC1177    | Monyet | AA | 2x | 2x | *M. acuminata* subsp. *zebrina* cluster | X | 117 a T | 265 ab T |
| ITC1177    | *M. acuminata* subsp. *zebrina* | AA | 2x | 2x | *M. acuminata* subsp. *zebrina* cluster | X | 139 a T | 333 bc MS |
| ITC0116    | Saba | ABB | 3x | 3x | ABB Bluggoe/Monthan cluster | XII | 144 a T | 207 ab T |
| ITC1178    | Buitenzorg | AA | 2x | 2x | AA cv. ISEA 2 cluster | IX | 161 a T | 146 ab T |
| ITC1120    | Tani | BB | 2x | 2x | *M. balbisiana* cluster | VII | 163 a T | 146 ab T |
| ITC0019    | IC | AAAA | 4x | 4x | Related to AA cv. | IX | 176 a T | |
| ITC0396    | Peliapita | ABB | 3x | 3x | *M. balbisiana* cluster | VII | 201 a T | 159 ab T |
| ITC1224    | Kikundi | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 201 a T | |
| ITC0246    | Cameroun | BB | 2x | 2x | *M. balbisiana* cluster | VII | 228 a T | 212 ab T |
| ITC0243    | Pisang Rajah | AAB | 3x | 3x | AAB/Pome | IX | 238 a T | |
| ITC0728    | Maia Oa | AA | 2x | 2x | *M. acuminata* subsp. *zebrina* cluster | X | 255 a T | |
| ITC0837    | Yalim | AA | 2x | 2x | AA cv. *banksii* s.l. cluster | XI | 289 a MS | |
| ITC1345    | Pisang Kra | AA | 2x | 2x | *M. acuminata* subsp. *malaccensis* cluster | III | 295 a MS | |
| ITC0084    | Mbwazirume | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 312 ab MS | |
| ITC1466    | Kahuti | AA | 2x | 2x | AA cv. African | IX | 328 ab MS | |
| ITC1454    | Maikyugwa 1 | AA | 2x | 2x | AA cv. *F. cymosa* | IX | 328 ab MS | |
| ITC1462    | Suu | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 333 ab MS | |
| ITC0654    | Petite Naine | AAA | 3x | 3x | AAA/Cavendish | IX | 334 ab MS | |
| ITC1139    | *M. acuminata* subsp. *zebrina* | AA | 2x | 2x | *M. acuminata* subsp. *zebrina* cluster | X | 343 ab MS | |
| ITC1467    | Kisaiga Machi | AA | 2x | 2x | AA cv. ISEA 2 cluster | IX | 345 ab MS | |
| ITC1459    | Mlema | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 362 ab MS | |
| ITC0058    | Cacambou | ABB | 3x | 3x | ABB Bluggoe/Monthan cluster | XII | 366 ab MS | |
| ITC0966    | Zebrina (G.F.) | AA | 2x | 2x | Does not cluster with other subsp. *zebrina* | | 368 ab MS | |
| ITC0364    | Silver Bluggoe | ABB | 3x | 3x | ABB Bluggoe/Monthan cluster | XII | 407 ab MS | |
| ITC1457    | Haa Haa | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 410 ab MS | |
| ITC1594    | Mshare | AAA | 3x | 3x | AA cv. African | IX | 415 ab MS | |
| ITC0078    | Who-gu | AAA | 3x | 3x | AA cv. IndonTriNG | IX | 430 ab MS | |
| ITC0944    | Warnbo | AA | 2x | 2x | AAB plantain + plantain-like cluster | XIII | 433 ab MS | |
| ITC1319    | FHIA-18 | AAAB | 4x | 4x | AAB/Pome | IX | 438 ab MS | |
| ITC1458    | Ilai Red | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 439 ab MS | |
| ITC0393    | Truncata | AA | 2x | 2x | *M. acuminata* subsp. *bumannicoides/bumanni* | I | 460 ab MS | |
| ITC1461    | Ntebwa | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 462 ab MS | |
| ITC0574    | Robusta | AAA | 3x | 3x | AAA/Cavendish | IX | 467 ab MS | |
| ITC1452    | Huti (Shumbi Nyeelu) | AA | 2x | 2x | AA cv. African | IX | 469 ab MS | |
| ITC1451    | Kitarasa | AAA | 3x | 3x | AAA/Lujugira/Mutika | X | 471 ab MS | |
| ITC0768    | Lacatan | AAA | 3x | 3x | AAA/Cavendish | IX | 473 ab MS | |
Table 1 (continued)

| Accession | Cultivar name | Genotype | Expected ploidy | Observed ploidy | Set | Cluster | 1st evaluation | 2nd evaluation |
|-----------|---------------|----------|-----------------|-----------------|-----|---------|---------------|---------------|
| ITC1464   | Ntindi I      | AAA      | 3x              | 3x              | AAA/Lujugira/Mutika | X     | 477 ab | MS            |
| ITC1544   | Mielembro     | AA       | 2x              | 2x              | AA cv. African     | IX    | 478 ab | MS            |
| ITC1349   | Pisang Serum 400 | AA    | 2x              | 2x              | M. acuminata subsp. malaccensis cluster | III | 481 ab | MS | 270 ab | T |
| MMC 016   | Tereza        | AAA      | 3x              | 3x              | AAA/Lujugira/Mutika | X     | 484 ab | MS            |
| ITC1466   | Nshonowa      | AA       | 2x              | 2x              | AA cv. African     | IX    | 490 ab | MS            |
| ITC0609   | Pahang        | AA       | 2x              | 2x              | Closest accession  | I     | 495 ab | MS            |
| ITC1465   | Ibwi          | AAA      | 3x              |                  | AA cv. African     | IX    | 496 ab | MS            |
| ITC0947   | Duntingi      | AAB      | 3x              | 3x              | AA cv. IndonTriPh  | X     | 500 ab | MS            |
| ITC1456   | Huti RB       | AA       | 2x              |                  | AA cv. African     | IX    | 511 ab | HS            |
| ITC0868   | Porapora      | AA       | 2x              |                  | AA cv. banksii s.l. cluster | XI | 512 ab | HS            |
| ITC0814   | Bagui         | AA       | 2x              |                  | AA cv. banksii s.l. cluster | XI | 518 ab | HS            |
| MMC 167   | Sukari Ndizi  | AAB      | 3x              | 3x              | AAB Silk cluster   | VII   | 520 ab | HS            |
| ITC0526   | Kluaí Namwa   | ABB      | 3x              |                  | ABB Pisang Awak cluster | VII | 528 ab | HS            |
| ITC1305   | Paji          | AA       | 2x              |                  | AA/Lujugira/Mutika | X     | 528 ab | HS            |
| ITC0897   | M. acuminata subsp. banksii | AA | 2x | AA subsp. banksii s.l. cluster | XI | 538 ab | HS |
| MMC 020   | Kibuzi        | AAA      | 3x              | 3x              | AAA/Lujugira/Mutika | X     | 548 ab | HS            |
| ITC0595   | Pagatau       | AAA      | 3x              |                  | AA cv. IndonTriNG | IX    | 549 ab | HS            |
| ITC0840   | Kusapaka      | AA       | 2x              |                  | AA cv. banksii s.l. cluster | XI | 554 ab | HS            |
| ITC0164   | Rugondo       | AAA      | 3x              |                  | AAA/Lujugira/Mutika | X     | 556 ab | HS            |
| ITC0946   | Merik         | AAA      | 3x              |                  | M. acuminata subsp. burmannicoides | I | 560 ab | HS |
| ITC0310   | Morong Princesa | ABB | 3x | 3x | ABB Pisang Awak cluster | VIII | 566 ab | HS |
| ITC0007   | M. acuminata subsp. malaccensis cluster | AA | 2x | 2x | M. acuminata subsp. malaccensis cluster | III | 578 ab | HS |
| ITC0087   | Kayinja       | ABB      | 3x              | 3x              | ABB Pisang Awak cluster | VIII | 571 d | HS |
| ITC1243   | Kokopo        | AA       | 2x              |                  | AA cv. ISEA 2 cluster | IX | 589 ab | HS |
| ITC1348   | Pisang Serum 404 | AA | 2x | 2x | M. acuminata subsp. malaccensis cluster | III | 595 bc | HS |
| ITC0310   | Morong Princesa | ABB | 3x | 3x | ABB Pisang Awak cluster | VIII | 566 ab | HS |
| ITC0087   | M. acuminata subsp. malaccensis | AA | 2x | 2x | M. acuminata subsp. malaccensis cluster | III | 578 ab | HS |
| ITC0946   | Merik         | AAA      | 3x              | 3x              | AA cv. IndonTriPh  | X     | 596 bc | HS            |
| ITC1318   | SH-3436-9     | AAAA     | 4x              | 4x              | Related to AA cv. African cluster | IX | 600 bc | HS |
| ITC0629   | Selangor 2s  | AA       | 2x              | 4x              | Related to AA cv. African cluster | IX | 606 bc | HS |
| ITC0259   | Galeo         | AA       | 2x              |                  | AA cv African      | IX    | 626 cd | HS            |
| ITC0312   | Pisang Jari Buaya | AA | 2x | 2x | AA cv. Pisang Jari Buaya | I | 677 cd | HS |
| TARS 1806 | Pitu          | AA       | 2x              |                  | AA cv. African     | IX    | 678 cd | HS            |
| ITC1000   | Gunih         | AA       | 2x              |                  | AA cv. African     | IX    | 678 cd | HS            |
| ITC0610   | Tuu Gia       | AA       | 2x              |                  | Related to AA cv. African cluster | I | 687 cd | MS |
| ITC0250   | M. acuminata subsp. malaccensis | AA | 2x | 2x | M. acuminata subsp. malaccensis cluster | III | 745 cd | MS |

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

*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.

*bDisease index based on percentage of plant area that is diseased during a given period of time.

*cAccessions grouped together based on the morphological traits-based classification.

*dAccessions that grouped into sets different from the expected.

*eAccessions 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 (Sekiwoko et al., 2006). The remaining three plants served as controls and were inoculated with sterile distilled water. The midrib inoculation imitates soil infection that is common during feeding 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:

\[
\text{Disease index (DI)} = \frac{([1 \times A] + [2 \times B] + [3 \times C])}{\text{No. of plants}} \times 100
\]

(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 reading, 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:

\[
\text{DI} = \frac{([1 \times A] + [2 \times B] + [3 \times C])}{\text{No. of plants}} \times 100
\]

(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.

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:

\[
\text{AUDPC} = \sum_{i=1}^{N} \left( \frac{Y_i + Y_{i+1}}{2} \right) \left( t_{i+1} - t_i \right)
\]

(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 Dolezel 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 (Christelova et al., 2017). Molecular characterization using SSR markers targeting 19 loci was conducted as described by Christelova et al. (2011b, 2017). The SSR markers are motifs of 1–6 bp repeats tandemly arranged in the genomes of eukaryotic and prokaryotic organisms (Christelova 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
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 the 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.

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* differed (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.

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**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.

| Variable                     | Principal component scores | Percentage variation | Cumulative percentage variation |
|------------------------------|----------------------------|----------------------|--------------------------------|
|                               | 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 |                       |                               |
Figure 2 Dendrogram 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.
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 Christelov *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 (Christelov *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.
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 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 dendrogram and how they clustered with the core subsets identified by Christelová *et al.* (2017). The different blocks of accessions are differentiated by colour.
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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