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Biochemical and molecular tools reveal two diverse *Xanthomonas* groups in bananas

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ABSTRACT

Xanthomonas campestris pv. *musacearum* (Xcm) causing the banana *Xanthomonas* wilt (BXW) disease has been the main xanthomonad associated with bananas in East and Central Africa based on phenotypic and biochemical characteristics. However, biochemical methods cannot effectively distinguish between pathogenic and non-pathogenic xanthomonads. In this study, gram-negative and yellow-pigmented mucoid bacteria were isolated from BXW symptomatic and symptomless bananas collected from different parts of Uganda. Biolog, Xcm-specific (GspDm), *Xanthomonas vasicola* species-specific (NZ085) and *Xanthomonas* genus-specific (X1623) primers in PCR, and sequencing of ITS region were used to identify and characterize the isolates. Biolog tests revealed several isolates as xanthomonads. The GspDm and NZ085 primers accurately identified three isolates from diseased bananas as Xcm and these were pathogenic when re-inoculated into bananas. DNA from more isolates than those amplified by GspDm and NZ085 primers were amplified by the X1623 primers implying they are xanthomonads, these were however non-pathogenic on bananas. In the 16–23 ITS sequence based phylogeny, the pathogenic bacteria clustered together with the Xcm reference strain, while the non-pathogenic xanthomonads isolated from both BXW symptomatic and symptomless bananas clustered with group I xanthomonads. The findings reveal dynamic *Xanthomonas* populations in bananas, which can easily be misrepresented by only using phenotyping and biochemical tests. A combination of tools provides the most accurate identity and characterization of these plant associated bacteria. The interactions between the pathogenic and non-pathogenic xanthomonads in bananas may pave way to understanding effect of microbial interactions on BXW disease development and offer clues to biocontrol of Xcm.

1. Introduction

Banana *Xanthomonas* wilt (Bxw) disease caused by *Xanthomonas campestris* pv. *musacearum* (Yirgou and Bradbury, 1968) Dye (Xcm) has been reported to be the most serious disease of bananas in Eastern and Central Africa (Tripathi et al., 2009) affecting all major banana cultivars grown (Tushemereirwe et al., 2003). Proper diagnosis is vital for the adoption of management options. The diagnosis of the causal agent *X. campestris* pv. *musacearum* (Yirgou and Bradbury, 1968) Dye has been based on biochemical tests including use of semi-selective agar media, pathogenicity tests and molecular based assays (Mwangi et al., 2007; Tripathi et al., 2007; Ssekiwoko et al., 2006; Aritua et al., 2007a,b, 2008, 2009). Variations in morphological characteristics of bacteria are generally insufficient to distinguish between bacteria species and also phenotypic features do not necessarily reflect genetic relatedness of bacterial isolates (Narayanasamy, 2011; Louws et al., 1999). On the other hand, pathogenicity tests in use may not distinguish between the primary pathogen and the secondary invaders of the affected plant tissues in a disease complex involving two or more pathogens (Narayanasamy, 2011). A DNA-based approach to study relationships between species in the genus *Xanthomonas* was based on a multilocus sequence analysis (MLSA) where four gene sequences; the chaperone protein dnaK (*dnaK*), tonB-dependent receptor (*fyuA*), DNA gyrase subunit B (*gyrB*) and RNA polymerase sigma factor (*rpoD*) were used to identify the allelic mismatches at the loci of different xanthomonads (Young et al., 2008). These studies found two groups in the genus *Xanthomonas* group I and group

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II suggesting that the genus *Xanthomonas* could be represented by two separate genera. *X. campestris* pv. *musacearum* isolates have also previously been characterized using *gyrase* B gene sequencing, DNA fingerprinting and fatty acid methyl esters (FAME) analysis and these studies revealed the homogeneity of Xcm isolates (Aritua et al., 2007a,b, 2008, 2009). The studies also showed Xcm has close genetic relationship with *Xanthomonas vasicola* pv. *holcicola* (Xvh) and *X. axonopodis* pv. *vasculorum* (Xav) resulting in a proposal to rename the pathogen as *X. vasicola* pv. *musacearum* (Aritua et al., 2008). The ITS sequences are markers commonly used in phylogenetic studies of bacteria because of their high variability as compared to 16S rDNA sequences (Leblond-Bourget et al., 1996). Plant-associated microbes including bacteria play different roles in plant health, some causing disease whereas others prevent diseases. Additionally, some bacteria that do not cause disease rather promote host growth (Leach et al., 2002).

The objective of the study was thus to characterize Gram negative yellow pigmented bacteria isolated from bananas in Uganda by the carbon source utilization in the Biolog identification system, use of PCR primers targeting *X. campestris* pv. *musacearum* at the pathovar, species and genus levels and to establish their identity and phylogenetic relationships using ITS sequence analysis.

2. Materials and methods

2.1. Sample collection

Healthy and symptomless banana plant samples of the four main common use types, namely; plantain, East African highland or cooking banana (matooke), beer and dessert-type bananas were collected from districts in Eastern, Western and Central regions of Uganda in 2009–2010 (Fig. 1). An average of eight samples were collected per district, four being symptomatic plants and four asymptomatic. In Ntungamo, only four symptomless plant samples were collected as we did not come across any plants showing BXW symptoms. Samples for bacteria isolation comprised of pseudostems 4 cm long with diameter of 5 cm cut with machetes sterilized using 1% sodium hypochlorite (NaOCl) and sterile scalpel blades. The cut surfaces of the pseudostems were wiped with tissue paper and each sample placed in a separate paper envelope to avoid cross contamination. Samples were transported to the laboratory for bacterial isolation.

2.2. Bacterial isolation and reference strains

The pseudostems were surface sterilized with 70% ethanol for a minute followed by 1% NaOCl for 3 min. The material was then rinsed three times in sterile distilled water and blotted to dry. Samples of approximately 1 g were crushed in 1 ml of sterile distilled water in a Petri dish. The suspension was then diluted tenfold and 100 μl of the diluted suspension was spread on semi-selective YPGA (Yeast extract-5 g l^{-1}, Peptone-5 g l^{-1}, Glucose-4 g l^{-1}, Agar-12 g l^{-1}) medium containing antibiotics cephalexin (40 mg l^{-1}), 5-fluorouracil (10 mg l^{-1}) and cycloheximide (120 mg l^{-1}) (Mwangi et al., 2007). The inoculated plates were incubated at 28 °C for 48–72 h and mucoid yellow-pigmented bacteria with morphological characteristics similar to the reference strain of *X. campestris* pv. *musacearum* were purified on nutrient agar (NA) medium. Pure bacteria cultures were obtained after successive transfer of single colonies on new NA plates after every two to three days.

2.3. Biolog identification

The purified bacterium was first identified using the Biolog GEN III MicroPlate™ (Biolog Inc., Hayward, CA, USA). Single colonies from pure bacterial cultures grown on NA plates were transferred onto Biolog universal growth (BUG™) agar (Biolog Inc., Hayward, CA) and incubated at 28 °C for 24 h. Colonies were picked using a sterile moistened Biolog cotton swab, suspended in sterile inoculating fluid, IF-A (Biolog Inc., Hayward, CA) and concentration adjusted to match Biolog GEN III turbidity standards. Aliquots of 100 μl of bacterial suspensions were loaded into each well of the Microplates. Each bacterial isolate was inoculated on a separate Biolog GEN III MicroPlate™. The reference strain *X. campestris* pv. *musacearum* NCPPB 2005 was used as the positive control. Readings of inoculated Biolog GEN III microplates were conducted using the Biolog MicroPlate reader (ICN Flow Titertek® Multiscan Plus, Version 2.03, Lab- Systems, Finland) after 24–48 h of incubation at 28 °C. An isolate was considered to be identified if the similarity index obtained was at least 0.5 after 24–48 h. The next match identification was not attempted. Bacterial isolates shown to be Gram positive by the Biolog test were discarded while the Gram negative ones were preserved at −80 °C in Protect Bacterial Preservers (Technical Service Consultants Limited) until use.

Table 1
Reference bacterial strains used in the study.

Bacterial species/strains	*Xanthomonas* Group	Host plant
Xanthomonas albilineans NCPPB 1830	Group I	Sugarcane
X. hyacinthi NCPPB 205	Group I	Hyacinth
X. theicola NCPPB 4353	Group I	Tea
X. translucens pv. translucens NCPPB 2389	Group I	Barley
X. translucens pv. *undulosa* B498[a]	Group I	Wheat
X. sacchari NCPPB 4341	Group I	Sugarcane
X. axonopodis NCPPB 457	Group II	Axonopus
X. axonopodis pv. *manihotis* NCPPB 2965	Group II	Cassava
X. axonopodis pv. *phaseoli* DSHC No 17[b]	Group II	Beans
X. axonopodis pv. *vasculorum* NCPPB 206	Group II	Sugarcane
X. axonopodis pv. *vignicola* NCPPB 638	Group II	Cowpea
X. campestris pv. *cordiaei* NCPPB 3443	Group II	Jenny wood
X. campestris pv. *musacearum* NCPPB 2005	Group II	Banana
X. cassavae NCPPB 101	Group II	Cassava
X. citri subsp. *citri* NCPPB 410	Group II	Citrus
X. citri subsp. malvacearum NCPPB 210	Group II	Cotton
X. euvesicatoria NCPPB 2968	Group II	Tomato
X. fuscans subsp. *fuscans* IPO 482[c]	Group II	Beans
X. oryzae pv. *oryzae* NCPPB 3002	Group II	Rice
X. perforans NCPPB 4321	Group II	Tomato
X. pruni NCPPB 3155	Group II	Plum
X. vasicola pv. holcicola NCPPB 2417	Group II	Sorghum
X. vesicatoria NCPPB 422	Group II	Tomato
Stenotrophomonas maltophilia ATCC13637[d,*]	NA	NG
Xyllela fastidiosa ATCC 700964[d,*]	NA	Grape

NA = non applicable; NG = not given.
[a] Norman W. Schaad (personal communication).
[b] Danish Seed Health Center (DSHC).
[c] Research Institute for Plant Protection (IPO), Netherlands.
[d] American Type Culture Collection (ATCC).
[*] Purified genomic DNA of *Stenotrophomonas maltophilia* and *Xyllela fastidiosa* used.

2.4. Extraction of bacterial DNA

Genomic DNA was extracted from twenty two bacterial isolates comprising those identified in preliminary Biolog GEN III identification as xanthomonads (12 isolates), *Stenotrophomonas maltophilia* (1), *Sphingomonas capsulata* (1), *Sphingomonas parapaucimobilis* (1), *Vogesella indigofera* (1), *Pseudomonas fulva* (1), *Flavobacterium* (1), plus 4 of 19 isolates for which Biolog identification gave no ID (Tables 2 and 3). DNA was also extracted from 23 reference strains of *Xanthomonas* (Table 1). Several samples were used for PCR analysis including those not identified as *Xanthomonas* to ensure accurate identification as these isolates phenotypically appeared like xanthomonads. Biolog is reported to have accuracy levels of as low as 54% (Noble, 1995) necessitating complementary confirmatory tests. The bacteria preserved at −80 °C in Protect Bacterial Pre-