Uganda Journal of Agricultural Sciences, 2012, 13 (2): 1-19 ISSN 1026-0919

Molecular diagnostic tools targeting different taxonomic levels of Xanthomonads aid in disease management

J. Adriko[1,3], E.R. Mbega[1,2], R.B. Mabagala[2], C.N. Mortensen[1], E.G. Wulff[1], W.K. Tushemereirwe[3], J. Kubiriba[3] and O.S. Lund[1]

[1]Danish Seed Health Centre for Developing Countries, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Hoejbakkegaard Alle 30 DK-2630, Taastrup, Denmark
[2]African Seed Health Centre, Department of Crop Science and Production, Sokoine University of Agriculture, P. O. Box 3005, Morogoro, Tanzania
[3]National Agricultural Research Laboratories, Kawanda, P. O. Box 7065, Kampala, Uganda

Author for correspondence: adrikoj@yahoo.com

Abstract

Effective plant disease management requires quick, accurate and specific diagnostic techniques, which in turn help in disease surveillance, regulation of material movement and ensure good quality planting material. When investigating emerging diseases with no existing specific diagnostic protocols, it can be useful to apply tools detecting all members of a genus as one. On the other hand, the banana xanthomonas wilt devastating East and Central Africa had no specific detection tool available over ten years after its first report. In this article, we present molecular diagnostic tools developed for genus, species and pathovar specific detection of *Xanthomonas campestris* pv. *musacearum* (Xcm). The tools included; i) primers developed based on the internal transcribed spacer region (ITS) of the ribosomal DNA (X-ITS) and a xanthan biosynthetic gene (*gumD*) (X-gumD) for genus level *Xanthomonas* detection; ii) primers based on the hypothetical protein NZ_ACHT01000085 (NZ085) for specific detection of the species *X. vasicola* and the general secretion protein D NZ_ACHT01000280 (GspDm) for specific detection of Xcm. The X-ITS and X-gumD primers specifically amplified DNA from xanthomonads giving 254 and 402 bp fragments, respectively without amplifying DNA of non-xanthomonads. PCR primers NZ085 specifically amplified a 349 bp fragment from DNA of Xcm, *X. vasicola* pv. *holcicola* (Xvh) and *X. axonopodis* pv. *vasculorum* (Xav) proposed to belong to the species *X. vasicola*. The GspDm primers amplified a 265bp DNA fragment of Xcm isolates tested with no DNA amplification of other plant associated-bacteria, including the two closely related Xvh and Xav. This provides a promising disease detection approach for both unknown and suspected pathogens.

Key words: Banana xanthomonas wilt, molecular diagnosis, PCR, multiplex PCR, *Xanthomonas*, *Xanthomonas campestris* pv. *musacearum*

Introduction

Xanthomonads are gram-negative, mostly yellow bacteria that are predominantly plant–associated and not encountered in other environments (Hayward, 1993). They cause disease symptoms that include but not limited to wilt, necrosis, gummosis and vascular or parenchy-matous symptoms on leaves, fruits or stems of nearly 400 plant hosts; including rice, banana, citrus, bean, tomato, pepper,

sugarcane, manioc (cassava), cotton and wheat (Leyns *et al*., 1984; Bradbury, 1986; Hayward, 1993; Schaad *et al*., 2001). Banana xanthomonas wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) that was first reported in Ethiopia on enset (*Ensete ventricosum*) (Yirgou and Bradbury, 1968), is a major threat to banana production in East and Central Africa (Tripathi *et al*., 2009). It affects all banana cultivars grown in the region causing total crop yield loss where the disease is established (Eden-Green, 2004).

Accurate identification of the pathogen is the basic requirement for effective disease management (Narayanasamy, 2011). Identification and classification of plant pathogenic bacteria is historically based on phenotypic characteristics such as symptoms caused, presence of specific antigens for serology analysis (ELISA), biochemical characteristics, substrate utilization profiles (BIOLOG), fatty acid composition (FAME) and multilocus enzyme profiles (MLEE) (Bochner 1989; Griffin *et al*., 1991; Verniere *et al*., 1993; Louws *et al*., 1999). Symptomatology has a limitation in that similar symptoms can be caused by different pathogens, or be easily confused for those caused by other biotic and abiotic factors. Reproducibility of phenotypic analyses is uncertain and the phenotypic features may not reflect the true identity of organisms (Louws *et al*., 1999). Isolation of pathogen in pure culture is often hampered by the presence of saprophytic or non-target bacteria which can overgrow the slow-growing pathogen (Narayanasamy, 2011) even on semi-selective media. Pathogenicity tests are also useful but however in a disease complexity involving two or more pathogens; it is difficult to identify the primary pathogen and the secondary invaders of the affected plant tissues (Narayanasamy, 2011). Biochemical tests are labor intensive and time consuming while serological methods have high development costs and at times less sensitive. Molecular based tools provide a fast, accurate and specific alternative for pathogen detection (Alvarez *et al*., 2004). Several PCR protocols are published for specific detection of individual *Xanthomonas* species (Palacio-Bielsa *et al*., 2009). A few PCR protocols amplifying a high number of *Xanthomonas* species have also been published. These protocols were based on 16S rDNA (Maes, 1993) and the *hrp* gene cluster of *Xanthomonas campestris* pv. *vesicatoria* (Leite *et al*., 1994), respectively. In our studies, these two protocols when tested against a wide range of xanthomonads and non-xanthomonads did not provide a fully adequate genus-specific identification of strains. Recently, a semi-selective medium for xanthomonads, Xan-D, was described (Lee *et al*., 2009) taking advantage of the gene *estA*, involved in Tween 80 hydrolysis and being conserved across the genus. This tool combined the use of the semi-selective medium and a PCR that specifically detects *estA* from xanthomonads (Lee *et al*., 2009). A number of PCR-based methods have been developed to detect Xcm (Lewis Ivey *et al*., 2010; Adikini *et al*., 2011), but these methods also did not show adequate specificity of Xcm detection in evaluations conducted with a collection of xanthomonads including genetically closely related reference strains.

We thus intended to develop fast molecular-based specific PCRs for detection of members of the genus *Xanthomonas* and Xcm at the genus, species and pathovar levels independent

of medium isolation. In addition, we intended to include internal controls for PCR amplification allowing quality control of general PCR amplification when applied to either pure bacterial DNA or to crude DNA extracted from plant tissue. The latter was obtained by multiplexing PCR with primers targeting bacterial 16S rDNA.

Materials and methods

Bacterial isolates

For genus level detection, 45 xanthomonads of 25 different species covering the strains reported in the phylogenetic studies of the genus *Xanthomonas* (Gurtler and Stanisich, 1996; Parkinson *et al*., 2007; 2009; Young *et al*., 2008) were used in the analyses together with fifteen non-*Xanthomonas* bacterial strains. Two members of the *Xanthomonadaceae* family most closely related to *Xanthomonas*, namely *Stenotrophomonas maltophila* and *Xylella fastidiosa*, were included in the tests. Five bacterial isolates recovered from tomato seed samples collected from Tanzania were included in the study. These isolates were previously identified based on results from pathogenicity testing, Biolog identification and 16S rRNA sequencing (Mbega, 2011). Isolates 73 and 167 identified as *X. gardnerii* and *X. arboricola* pv. *poinsettiicola*, respectively were used as *Xanthomonas* positive controls while isolates 27, 36 and 38 identified as *Stenotrophomonas* spp. (Mbega, 2011) served as negative controls of the studies. To test tools for detection of Xcm, twelve Xcm strains were tested together with 51 other *Xanthomonas* and eight non-*Xanthomonas* reference strains. In these tests, xanthomonads genetically closely related to Xcm, as per earlier studies namely; Xvh and Xav (Aritua *et al*., 2008), were tested to establish specificity of developed Xcm molecular diagnostic tool.

The majority of bacterial strains were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), England, while the other sources included the American Type Culture Collection (ATCC), USA and Plant Research International (IPO), Netherlands. The *X. translucens* pv. *undulosa* B498 isolate was provided by Dr. Norman W. Schaad (personal communication), and *X. axonopodis* pv. *phaseoli* No. 17, from the Danish Seed Health Centre (DSHC), Denmark. Five of the Xcm strains were acquired from the National Banana Research Programme (NBRP, NARO) in Uganda.

PCR reactions

For *Xanthomonas* genus level detection, the X1623 (designed from 16-23 *Xanthomonas* ITS region) primer mix comprised of X1623-F3j (GGCGGG GACTTCGAGTCCCTAA), X1623-F3k (GGCGGGGACTTCGAGTTCCTAA), X1623-F3c (CGGGGACCTCGA GTCC CTA), X1623-F3d (GCGGGGA CTTA GAGTCCCTA), and X1623-R2 (CTGCA GGATACTGCCGAAGCA), constituting 12.5, 2.1, 8.3, 2.1, and 25 pmol, respectively. The X-ITS primers were run singly and multiplexed with 5 pmol each of 16S rDNA primers P16SF1 (5´GCCAGCAGCCGCGGTAATAC3´) and P16SR2 (5´GCGCTCGTTGCGG GACTTA3´). The 25µl PCR reaction consisted of 5X GoTaq PCR buffer (Promega); 2 mM $MgCl_2$ (Promega); 200 µM of each deoxynucleotide triphosphate (Promega); and 1.25 U of GoTaq DNA polymerase (Promega). The amplification process involved an initial denaturation of

3 min at 95 °C, followed by 28 cycles of denaturation of 94 °C for 15 s, annealing at 67 °C for 10s, elongation at 72 °C for 10 s. The final extension was 72 °C for 3 min. The X-gumD PCR had 25 pmol each of primers X-gumD-Fw7 (5´GGCCGCGA GTTCTACATGTTCAA3´) and X-gumD-R7 (5´CACGATGATGCGGATATCCA GCCACAA3´) which were run singly and in multiplex with 3.75 pmol each of P16S primers P16S Fw3 (CGTGGGGAG CGAACAGGATTA) and P16S Rv3 (CTTGACGGGCGGTGTG TACAA). The 25 µl PCR reaction consisted of 5X GoTaq PCR buffer (Promega); 1.5 mM $MgCl_2$ (Promega); 200 µM of each deoxynucleotide triphosphate (Promega); and 1.25 U of GoTaq DNA polymerase (Promega). The amplification process involved an initial denaturation of 3 min at 95°C, followed by 30 cycles of denaturation of 95°C for 20s, annealing at 66°C for 15s, elongation at 72°C for 15 s. The final extension was 72°C for 3 min.

For specific Xcm detection, primers GspDm-F2 (GCGGTTACAACACCG TTCAAT) and GspDm-R3 (AGGT GGAGTTGATCGGAATG) designed from Xcm genomic sequence contig NZ_ACHT01000280 encoding general secretion protein D (ZP_06489699) were used while primers NZ085-F3 (CGTGCCATGTATGCGCTGAT) and NZ085-R3 (GAGCGGCATAGTGCGA CAGA) designed from the Xcm genomic sequence contig NZ_ACHT01000085 encoding a hypothetical protein (ZP_06488508) were used for species-specific detection of *X. vasicola*. These primer sets were also run with 16S prokaryotic ribosomal DNA primers P16S-F3 (CGTGGGGAGCGAACAGGATTA) and P16S-R3 (CTTGACGGGCGGTGTG TACAA) (Adriko *et al*., 2012). The 25 µl PCR reaction consisted of 1.5mM of $MgCl_2$ (Promega), 5X GoTaq flexi PCR buffer (Promega), 200 µM of each deoxynucleotide triphosphate (Promega), 25 pmol of each primer, 1.25 U of GoTaq DNA polymerase (Promega) and 50ng of bacterial DNA template. 6.25 pmol of each P16S primer was used in multiplex PCR reaction mixture. The amplification process was performed in Eppendorf mastercycler gradient PCR machine (Eppendorf) and involved an initial denaturation of 3 min at 95°C, followed by 32 cycles of denaturation of 95°C for 20 s, annealing at 64°C for 15s, elongation at 72°C for 13 s. The final extension was 72°C for 3 min.

The amplified PCR products were separated by horizontal gel electrophoresis in 1.5% agarose gels in 0.5x TBE (Tris-borate EDTA) buffer at 50V/cm for 45 minutes. The pre-stained gel with ethidium bromide (0.5 µg/ml) was then visualised under UV transilluminator and photographed with Fujifilm instant black and white Professional film.

Results

X-ITS primers were robust and efficient in specific detection of xanthomonads in pure cultures

When the RS 21 and RS 22 primers (Leite *et al*., 1994) were tested in PCR reactions, they were not robust enough in *Xanthomonas* detection, while the 16S rDNA primers (Maes, 1993) gave the delineated 480 bp product in all the xanthomonads but also in *Ralstonia solanacearum* (NCPPB 2315), *Stenotrophomonas maltophilia* (NCPPB 1974, ATCC13637) and *Xylella fastidiosa* (ATCC 700964) (Figure 1, Table 1). The X-ITS primers amplified all the tested *Xanthomonas* strains giving a product with the expected size of 254 bp,

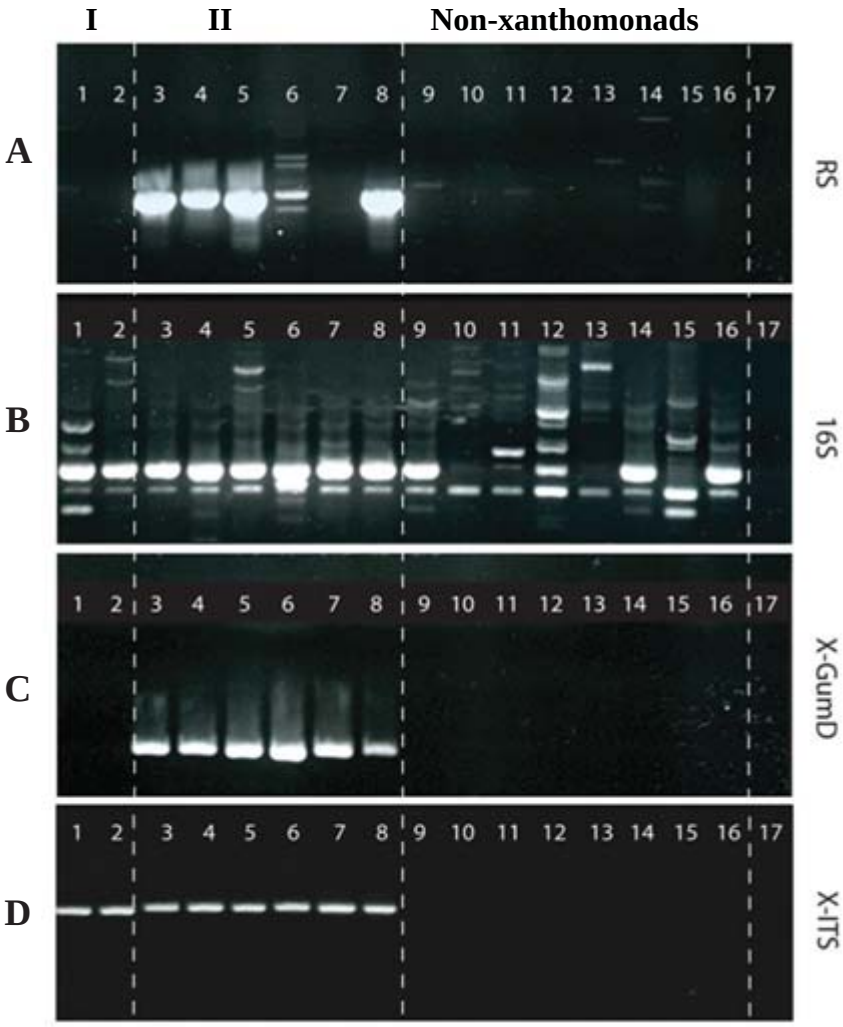

Figure 1. DNA amplification from cultures of xanthomonads Groups I and II and non-xanthomonads using RS (A), 16S rRNA (B), X-gumD (C), and X-ITS (D) primers. Samples are *Xanthomonas albilineans* NCPPB 1830 (1), *X. hyacinthi* NCPPB 205 (2), *X. codiaei* NCPPB 3443 (3), *X. cassava* NCPPB 101 (4), *X. oryzae* pv. *oryzae* NCPPB 3002 (5), *X. vesicatoria* NCPPB 422 (6), tomato isolate 73 (7), tomato isolate 167 (8), *Stenotrophomonas maltophilia* ATCC 13637 (9), *Burkholderia glumae* NCPPB 2391 (10), *Pseudomonas syringae* pv. *tomato* NCPPB 269 (11), *Xylella fastidiosa* ATCC 700964 (12), *Acidovorax avenae* subsp. *avenae* NCPPB 1011 (13), tomato isolate 27 (14), tomato isolate 36 (15), tomato isolate 38 (16) and water (17).

while none of the non-xanthomonads gave the corresponding band of expected size in either case (Figure 1, Table 1). The results suggested that these primers were quite specific and suitable for screening of bacterial cultures and in their differentiation as xanthomonads and non-xanthomonads.

The X-gumD primers categorized xanthomonads into two groups

PCR assays using the X-gumD primers on DNA of the various bacterial strains appeared to be efficient in differentiating *Xanthomonas* strains of the two established phylogenetic groups (Hauben *et al*., 1997; Young *et al*., 2008). The

Table 1. Bacterial isolates used in experiment and comparison of *Xanthomonas* detection specificity and robustness of X-gumD and X-ITS primers with previously developed primers using single target and multiplex PCR assays

Bacterial species/strains	Host	*Xanthomonas* Group	Primers				Multiplex PCR primers	
			RS21, RS22	16S rRNA, X.16SrDNA	X-gumD	X-ITS	X-gumD / P16S	X-ITS / P16S
Xanthomonas albilineans NCPPB 1830	Sugarcane	I	–	+	–	+	-/+	+/+
X. hyacinthi NCPPB 205	Hyacinth	I	–	+	–	+	-/+	+/+
X. theicola NCPPB 4353	Tea	I	–	+	–	+	-/+	+/+
X. translucens pv. *translucens* NCPPB 2389	Barley	I	–	+	–	+	-/+	+/+
X. t. pv. *undulosa* B498	Wheat	I	–	+	–	+	-/+	+/+
X. sacchari NCPPB 4341	Sugarcane	I	–	+	vw+	+	vw+/+	+/+
X. arboricola pv. *celebensis* NCPPB 1832	Banana	II	+	+	+	+	+/+	+/+
X. axonopodis NCPPB 457	Axonopus	II	+	+	+	+	+/+	+/+
X. a. pv. *aurantifolii* NCPPB 4377	Citrus	II	+	+	+	+	+/+	+/+
X. a. pv. *cyamopsidis* NCPPB 637	GuarGum	II	+	+	+	+	+/+	+/+
X. a. pv. *glycines* NCPPB 1124	Soy bean	II	–	+	+	+	+/+	+/+
X. a. pv. *manihotis* NCPPB 2965	Cassava	II	+	+	+	+	+/+	+/+
X. a. pv. *phaseoli* No 17	Beans	II	+	+	+	+	+/+	+/+
X. a. pv. *vasculorum* NCPPB 206	Maize	II	+	+	+	+	+/+	+/+
X. a. pv. *vignicola* NCPPB 555	Cowpea	II	+	+	+	+	+/+	+/+
X. a. pv. *vignicola* NCPPB 638	Cowpea	II	+	+	+	+	+/+	+/+
X. bromi NCPPB 4343	Rescue brome grass	II	+	+	+	+	+/+	+/+
X. campestris var. *aberrans* NCPPB 2986	Cabbage	II	+	+	+	+	+/+	+/+
X. c. var. *armoraciae* NCPPB 1930	Horseradish	II	+	+	+	+	+/+	+/+
X. c. pv. *barbareae* NCPPB 983	Garden yellow rocket	II	+	+	+	+	+/+	+/+
X. c. pv. *campestris* NCPPB 528	Cabbage	II	+	+	+	+	+/+	+/+

Table 1. Contd.

Bacterial species/strains	Host	*Xanthomonas* Group	Primers				Multiplex PCR primers	
			RS21, RS22	16S rRNA, X.16SrDNA	X-gumD	X-ITS	X-gumD / P16S	X-ITS / P16S
X. c. pv. *carotae* NCPPB 3440	Carrot	II	–	+	+	+	+/+	+/+
X. codiaei NCPPB 3443	Freijo cordia-wood	II	+	+	+	+	+/+	+/+
X. c. pv. *incanae* NCPPB 937	Stock plant	II	+	+	+	+	+/+	+/+
X. c. pv. *musacearum* NCPPB 4387	Banana	II	+	+	+	+	+/+	+/+
X. c. pv. *raphani* NCPPB 1946	Radish	II	+	+	+	+	+/+	+/+
X. cassavae NCPPB 101	Cassava	II	+	+	+	+	+/+	+/+
X. citri subsp. *citri* NCPPB 410	Orange	II	+	+	+	+	+/+	+/+
X. citri subsp. *malvacearum* NCPPB 210	Cotton	II	+	+	+	+	+/+	+/+
X. cucurbitae NCPPB 2597	Squash	II	–	+	+	+	+/+	+/+
X. euvesicatoria NCPPB 2968	Pepper	II	+	+	+	+	+/+	+/+
X. fragariae NCPPB 2949	Strawberry	II	+	+	w+	+	w+/+	+/+
X. fuscans subsp. *fuscans* IPO 482	Bean	II	+	+	+	+	+/+	+/+
X. gardneri NCPPB 881	Tomato	II	+	+	+	+	+/+	+/+
X. hortorum pv. *pelargonii* NCPPB 305	Geranium	II	+	+	+	+	+/+	+/+
X. melonis NCPPB 3434	Melon	II	–	+	+	+	+/+	+/+
X. oryzae pv. *oryzae* NCPPB 3002	Rice	II	+	+	+	+	+/+	+/+
X. o. pv. *oryzicola* NCPPB 1151	Rice	II	+	+	+	+	+/+	+/+
X. perforans NCPPB 4321	Tomato	II	+	+	+	+	+/+	+/+
X. pisi NCPPB 762	Pea	II	–	+	+	+	+/+	+/+
X. pruni NCPPB 416	Plum	II	w+*	+	+	+	+/+	+/+
X. pruni NCPPB 3155	Plum	II	w+*	+	+	+	+/+	+/+
X. sesame NCPPB 631	Sesame	II	+	+	+	+	+/+	+/+

Table 1. Contd.

Bacterial species/strains	Host	*Xanthomonas* Group	Primers				Multiplex PCR primers	
			RS21, RS22	16S rRNA, X.16SrDNA	X-gumD	X-ITS	X-gumD / P16S	X-ITS / P16S
X. vasicola pv. *holcicola* NCPPB 2417	Sorghum	II	+	+	+	+	+/+	+/+
X. vesicatoria NCPPB 422	Tomato	II	w+*	+	+	+	+/+	+/+
Tomato isolate 73 (*X. gardneri*)	Tomato	II	–	+	+	+	+/+	+/+
Tomato isolate 167 (*X. arboricola* pv. *poinsettiicola*)	Tomato	II	+	+	+	+	+/+	+/+
Acidovorax avenae subsp. *avenae* NCPPB 1011	Maize	NX	–	–	–	–	–/+	–/+
A. a. subsp. *citrulli* ATCC 29625	Melon	NX	–	–	–	–	–/+	–/+
Burkholderia glumae 2391		NX	–	–	–	–	–/+	–/+
Clavibacter michiganensis subsp. *michiganensis* IPO 542	Tomato	NX	–	–	–	–	–/+	–/+
Dickeya dadantii NCPPB 3090	Rice	NX	–	–	–	–	–/+	–/+
Pantoea agglomerans NCPPB 2971	Wisteria	NX	–	–	–	–	–/+	–/+
Pectobacterium carotovorum subsp. *carotovorum* NCPPB 1280	Potato	NX	–	–	–	–	–/+	–/+
Pseudomonas corrugata NCPPB 2445	Potato	NX	–	–	–	–	–/+	–/+
P. savastanoi pv. *phaseolicola* NCPPB 1321	Bean	NX	–	–	–	–	–/+	–/+
P. syringae subsp. *syringae* NCPPB 1417	Rice	NX	–	–	–	–	–/+	–/+
P. s. subsp. *tomato* NCPPB 269	Tomato	NX	–	–	–	–	–/+	-/+
Ralstonia solanacearum NCPPB 2315	Banana	NX	–	+	–	–	–/+	-/+
Stenotrophomonas maltophilia ATCC13637	Various	NX	–	+	–	–	–/+	-/+
S. maltophilia NCPPB 1974	Various	NX	–	+	–	–	–/+	–/+

Table 1. Contd.

Bacterial species/strains	Host	*Xanthomonas* Group	Primers			Multiplex PCR primers	
			RS21, RS22	16S rRNA, X.16SrDNA	X-gumD	X-ITS	X-gumD / P16S	X-ITS / P16S
Xyllela fastidiosa ATCC 700964	Grape	NX	–	+*	–	–	–/+	–/+
Tomato isolate 27 (*Stenotrophomonas* sp.)	Tomato	NX	–	+	–	–	–/+	–/+
Tomato isolate 36 (*Stenotrophomonas* sp.)	Tomato	NX	–	–	–	–	–/+	–/+
Tomato isolate 38 (*Stenotrophomonas* sp.)	Tomato	NX	–	+	–	–	–/+	–/+

Symbols: I are group I *Xanthomonas*, II group II *Xanthomonas* and NX the Non-*Xanthomonas* bacteria; + and – indicate species was detected or not detected, respectively; w+ and vw+ are weak and very weak reactions, respectively; +* indicates expected band size plus bands of other sizes, –* is amplification of fragments other than expected target size; +/+ is positive *Xanthomonas* target and 16S rDNA amplification, and –/+ is no *Xanthomonas* target amplification but positive 16S rDNA amplification.

primers allowed efficient DNA amplification in group II *Xanthomonas* strains (represented by *X. campestris*) giving a 402bp product, with only DNA of *X. fragariae* (NCPPB 2949) being weakly amplified. The test results also showed no amplification in strains of the group I xanthomonads (represented by *X. albilineans*); however, a very weak band of DNA amplification was observed with *X. sacchari* NCPPB 4341 which is also a member of group I. The amplification of DNA from this strain was fainter when compared to the bands observed with *X. fragariae* NCPPB 2949 DNA. The DNA from the non-xanthomonads was not amplified (Figure 1, Table 1).

The 16S prokaryotic rDNA primers provided internal control in multiplex PCR with X-gumD and X-ITS primers in the screening of pure bacterial cultures

When P16S primers were used as internal controls in multiplex PCR with the developed PCR primers X-ITS, double bands of more or less equal intensity, corresponding to the amplification of X-ITS (254bp) and the 16S ribosomal DNA (596bp), were observed in DNA from xanthomonads resulting from amplification of both the prokaryote and *Xanthomonas* targets, while only the P16S rDNA sequence was amplified in the genomic DNA of the non-xanthomonads (Figure 2). In multiplex PCR assay of the X-gumD with P16S primers; double bands were obtained with the members of group II and *X. sacchari* (NCPPB 4341), corresponding to the amplification of the *gumD* gene (402bp) and the 16S ribosomal DNA (641bp). In the rest of the group I xanthomonads and non-xanthomonads, only the band corresponding to the 16S ribosomal DNA gene was observed.

Multiplex PCR showing double bands of similar intensity of X-gumD and P16S amplifications generally distinguished *Xanthomonas* strains of group II from the members of group I (Figure 2, Table 1).

The NZ085 primers specifically detect Xcm, Xvh and Xav

Amplification tests on genomic DNA from 63 strains of *Xanthomonas* representing 23 species and 16 pathovars, and eight non-xanthomonads, was performed using NZ085-F3 and NZ085-R3 primers in PCR and the primers successfully amplified a 349-bp fragment from genomic DNA of Xcm as predicted. However, specific amplification of DNA was also observed for genomic DNA of the closely related strains Xvh and Xav, resulting in DNA fragments of identical size (Figure 3, Table 2). In contrast, no amplification of DNA was observed for the other *Xanthomonas* and non-*Xanthomonas* strains tested using the NZ085 primers. The amplification of DNA of Xcm, Xvh and Xav support the indication of a close genetic relationship between these bacteria, as suggested by Aritua *et al*. (2007ab; 2008).

GspDm primers are specific for detection of Xcm

PCR tests with GspDm primers on reference strains of bacteria resulted in amplification of DNA specifically from Xcm and not from other xanthomonads, including the closely related Xvh and Xav strains. This demonstrates a high specificity of the GspDm PCR and its potential for use as a diagnostic tool of Xcm (Figure 3, Table 2).

Use of internal controls with NZ085 and GspDm PCRs for quality checking of negative results in Xcm diagnosis

Multiplex PCRs were carried out on 31 bacterial strains using either NZ085 or GspDm primers alongside 16S primers in order to provide an internal control for detecting genomic bacterial DNA. The NZ085 primers positively amplified DNA

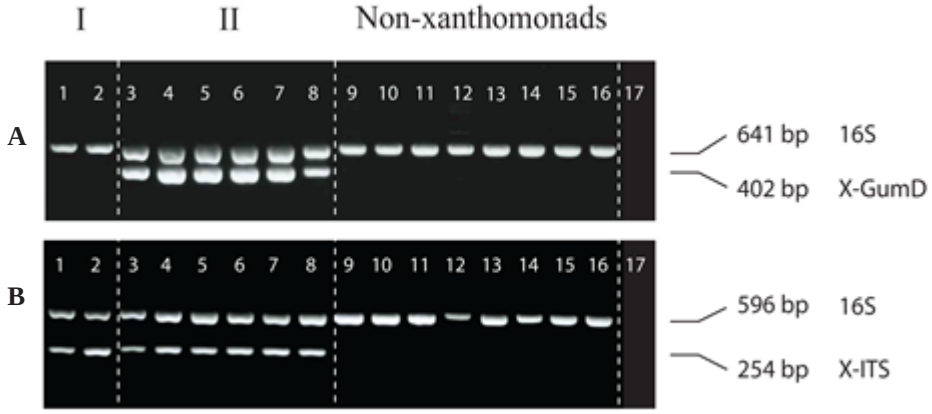

Figure 2. Multiplex PCR amplification of DNA from pure cultures of xanthomonads (groups I and II) and non-xanthomonads by X-gumD (A) and X-ITS (B) primers. Samples are *Xanthomonas albilineans* NCPPB 1830 (1), *X. hyacinthi* NCPPB 205 (2), *X. codiaei* NCPPB 3443 (3), *X. cassava* NCPPB 101 (4), *X. oryzae* pv. *oryzae* NCPPB 3002 (5), *X. vesicatoria* NCPPB 422 (6), tomato isolates 73 (7), and tomato isolate 167 (8) identified as *Xanthomonas*, *Stenotrophomonas maltophilia* ATCC 13637 (9), *Burkholderia glumae* NCPPB 2391 (10), *Pseudomonas syringae* pv. *tomato* NCPPB 269 (11), *Xylella fastidiosa* ATCC 700964 (12), *Acidovorax avenae* subsp. *avenae* NCPPB 1010 (13), Tomato isolates 27 (14), 36 (15), and 38 (16) identified as *Stenotrophomonas*, and water (17).

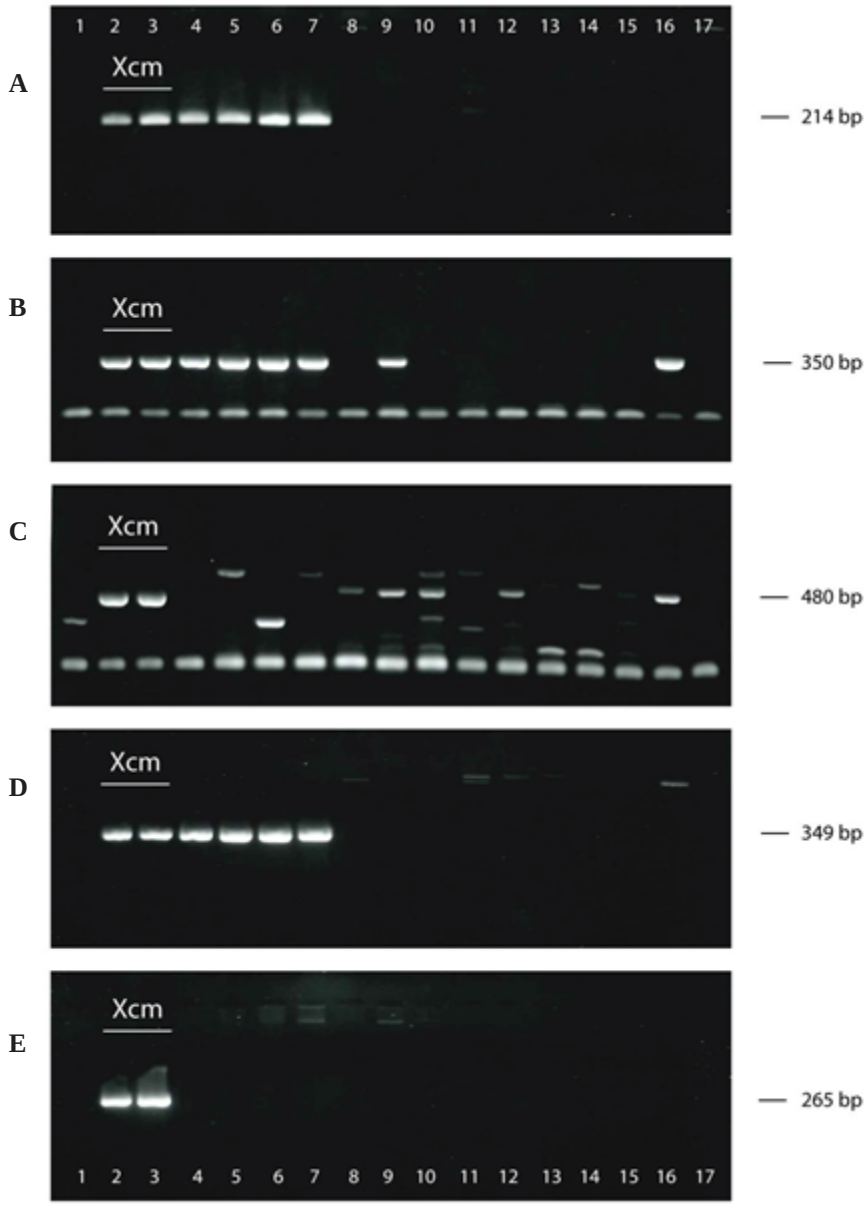

Figure 3. Results with tests on 17 xanthomonads using a PCR based on BXW-1 and BXW-3 primers (Lewis Ivey at al 2010) (A), Xcm primers 35 and 44 (Adikini et al 2011) (B and C), NZ085 primers (D), and the GspDm primers (E) revealing the detection coverage of BXW-1 and BXW-3, Xcm 35 and 44, and NZ085 primers as well as the specificity of the GspDm primers to diagnose Xcm strains. The lanes are *Xanthomonas melonis* NCPPB 3434 (1), *X. campestris.* pv. *musacearum* NCPPB 2005 (2) and NCPPB 4387 (3), *X. vasicola* pv. *holcicola* NCPPB 2417 (4) and NCPPB 1060 (5), *X. axonopodis* pv. *vasculorum* NCPPB 206 (6) and NCPPB 889 (7), *X. oryzae* pv. *oryzae* NCPPB 3002 (8), *X. axonopodis* NCPPB 457 (9), *X. a.* pv. *malvacearum* NCPPB 210 (10), *X. fuscans* subsp. *fuscans* IPO 482 (11), *X. a.* pv. *phaseoli* DSHC No. 17 (12), *X. perforans* NCPPB 4321 (13), *X. euvesicatoria* NCPPB 2968 (14), *X. a.* pv. *citri* NCPPB 410 (15), *X. a.* pv. *manihotis* NCPPB 2965 (16) and *X. c.* pv. *campestris* NCPPB 528 (17).

Table 2. Bacterial reference strain and field samples tested using NZ085 and GspDm Primers for Xcm detection

Bacterial strain name	Hosts	Source	Collection number	Amplification by NZ085	Amplification by GspDm
Xanthomonas campestris pv. *musacearum*	Banana	National Banana Research Program, NARO- Uganda (NBRP)	MKN 00108	+	+
	Banana	NBRP	MKN 00208	+	+
	Banana	NBRP	MKN 00308	+	+
	Banana	NBRP	WKG00508	+	+
	Banana	NBRP	MBR 00608	+	+
	Ensete	National Collection of Plant Pathogenic Bacteria (NCPPB)	NCPPB 2005	+	+
	Banana	NCPPB	NCPPB 4387	+	+
	Banana	NCPPB	NCPPB 4386	+	+
	Banana	NCPPB	NCPPB 4392	+	+
	Banana	NCPPB	NCPPB 4389	+	+
	Banana	NCPPB	NCPPB 4378	+	+
	Banana	NCPPB	NCPPB 2251	+	+
X. vasicola pv. *holcicola*	Sorghum	NCPPB	NCPPB 2417	+	–
	Sorghum	NCPPB	NCPPB 1060	+	–
X. axonopodis pv. *vasculorum*	Maize,	NCPPB	NCPPB 206,	+	–
	Sugarcane		NCPPB 889	+	–
X. albilineans	Sugarcane	NCPPB	NCPPB 1830	–	–
X. arboricola pv. *celebensis*	Banana	NCPPB	NCPPB 1832	–	–
X. axonopodis	Axonopus	NCPPB	NCPPB 457	–	–
X. a. pv. *aurantifolii*	Citrus-lemon	NCPPB	NCPPB 4377	–	–

Table 2. Contd.

Bacterial strain name	Hosts	Source	Collection number	Amplification by NZ085	Amplification by GspDm
X. a. pv. *citri*	Citrus	NCPPB	NCPPB 410	–	–
	Citrus	NCPPB	NCPPB 3234	–	–
	Citrus	NCPPB	NCPPB 3655	–	–
X. a. pv. *glycines*	Soybean	NCPPB	NCPPB 1124	–	–
X. a. pv. *malvacearum*	Cotton	NCPPB	NCPPB 210	–	–
	Cotton	NCPPB	NCPPB 633	–	–
X. a. pv. *manihotis*	Cassava	NCPPB	NCPPB 2965	–	–
X. a. pv. *phaseoli*	Bean	Danish Seed Health Centre (DSHC)	DSHC No. 17	–	–
X. a. pv. *vignicola*	Cowpea	NCPPB	NCPPB 638	–	–
	Cowpea	NCPPB	NCPPB 555	–	–
X. bromi	Rescue Brome grass	NCPPB	NCPPB 4405	–	–
		NCPPB	NCPPB 4343	–	–
X. campestris pv. *aberrans*	Cabbage	NCPPB	NCPPB 2986	–	–
X. c. pv. *campestris*	Cabbage	NCPPB	NCPPB 528	–	–
	Cabbage	NCPPB	NCPPB 2031	–	–
	Cabbage	NCPPB	NCPPB 3207	–	–
X. c. pv. *sesami*	Sesame	NCPPB	NCPPB 631	–	–
X. carotae	Carrot	NCPPB	NCPPB 1422	–	–
X. cassavae	Cassava	NCPPB	NCPPB 101	–	–
X. cucurbitae	Cucumber	NCPPB	NCPPB 2597	–	–
X. euvesicatoria	Pepper	NCPPB	NCPPB 2968	–	–
X. fragariae	Strawberry	NCPPB	NCPPB 2949	–	–

Table 2. Contd.

Bacterial strain name	Hosts	Source	Collection number	Amplification by NZ085	Amplification by GspDm
X. fuscans subsp. *fuscans*	Bean	NCPPB	NCPPB 1056	–	–
	Bean	NCPPB	NCPPB 1402	–	–
	Bean	Research Institute for Plant Protection (IPO) – Netherlands	IPO 482	–	–
X. gardneri	Tomato	NCPPB	NCPPB 881	–	–
X. hyacinthi	Hyacinth	NCPPB	NCPPB 205	–	–
X. melonis	Melon	NCPPB	NCPPB 3434	–	–
X. oryzae pv. *oryzae*	Rice	NCPPB	NCPPB 3002	–	–
	Rice	NCPPB	NCPPB 1150	–	–
	Rice	NCPPB	NCPPB 1153	–	–
	Rice	NCPPB	NCPPB 1154	–	–
	Rice	NCPPB	NCPPB 1936	–	–
	Rice	NCPPB	NCPPB 3363	–	–
X. oryzae pv. *oryzicola*	Rice	NCPPB	NCPPB 1632	–	–
	Rice	NCPPB	NCPPB 1151	–	–
	Rice	NCPPB	NCPPB 2921	–	–
X. perforans	Tomato	NCPPB	NCPPB 4321	–	–
X. pisi	Peas	NCPPB	NCPPB 762	–	–
X. pruni	Plum	NCPPB	NCPPB 3155	–	–
X. sacchari	Sugarcane	NCPPB	NCPPB 4341	–	–
X. theicola	Tea	NCPPB	NCPPB 4353	–	–
X. vesicatoria	Tomato	NCPPB	NCPPB 422	–	–
Acidovorax avenae	Rice	NCPPB	NCPPB 1010	–	–
Burkholderia glumae	Rice	NCPPB	NCPPB 2391	–	–
Pantoea agglomerans	Grass species	NCPPB	NCPPB 2971	–	–

Table 2. Contd.

Bacterial strain name	Hosts	Source	Collection number	Amplification by NZ085	Amplification by GspDm
Ralstonia solanacearum	Banana	NCPPB	NCPPB 2315	–	–
Pectobacterium carotovorum subsp. *carotovorum*	Potato	NCPPB	NCPPB 1280	–	–
Pseudomonas corrugata	Tomato	NCPPB	NCPPB 2445	–	–
Xyllela fastidiosa[a]	Grape	American Type Culture Collection	ATCC 700964	–	–
Stenotrophomonas maltophilia	Various plants	NCPPB	NCPPB 1974	–	–

[a]*Xyllela fastidiosa* purified genomic DNA
+ is positive amplification, – is no amplification

from the Xcm, Xvh and Xav isolates giving two bands, one corresponding to the NZ_ACHT01000085 fragment (349 bp) and the other corresponding to the 16S ribosomal DNA fragment (641 bp). Results with the GspDm primers showed double bands for Xcm isolates corresponding to the 16S ribosomal DNA fragment (641 bp) and the *gspD* gene fragment (265 bp). For all other reference strains tested (13 Xanthomonas and seven non-Xanthomonas) only one band corresponding to the 16S ribosomal fragment (641 bp) was observed (Figure 4).

Discussion

Bacterial diseases, including those caused by members of the genus *Xanthomonas*, are always being reported in new places, as revealed by reports of British Society for Plant Pathology (http://www.ndrs.org.uk/) and the American Phyto pathological Society (http://apsjournals.apsnet.org/loi/pdis) among others, and often are reported as emerging diseases in the tropics. This situation thus calls for detection tools that can readily be used in diagnosis and support an effective management of such diseases. Early and accurate detection and identification of new cases of *Xanthomonas* diseases as provided by molecular based methods (Louws *et al*., 1999; López *et al*., 2003; Narayanasamy, 2011) will aid to speed up their management. From the study, we showed that PCR tests with primers designed from the EPS (xanthan) synthesis pathway gene *gumD* (X-gumD) and 16S – 23S ITS region (X-ITS) are effective and robust in the detection of the members of the genus *Xanthomonas*. These gene targets were very specific and quite effective in the detection of a larger

number of xanthomonads than those previously published PCR-based *Xanthomonas* diagnostic tools without giving false positive results. The *gumD* based PCR proved to be a useful tool in grouping xanthomonads into the two published groups (Hauben *et al*., 1997; Young *et al*., 2008) as the PCR did not detect or give very weak amplification with xanthomonads from group I.

Strategic management and control of the banana *Xanthomonas* wilt is likely to benefit from specific and reliable diagnostic laboratory tools to detect the Xcm pathogen. In order to provide a simple PCR method with complete specificity with regard to Xcm detection, two independent target sequences of genomic Xcm DNA were tested based on assumed Xcm specificity. The first target primers NZ085 turned out to give a similar level of PCR specificity as reported for the PCR method described above (Lewis Ivey *et al*., 2010) detecting Xcm as well as Xav and Xvh strains. The second set of primers targeting the *gspD* gene of *X. c.* pv. *musacearum* provided a more specific Xcm detection in pure culture and banana plant samples. Twelve pathogenic reference *X. c.* pv. *musacearum* strains isolated from various African countries were correctly identified using these primer pairs, while no other bacteria was detected.

The use of internal controls in the form of prokaryotic gene target in combination with pathogen specific detection is highly valuable for a diagnostic tool for the following two reasons: 1) Specificity allows unambiguous identification of the pathogen; 2) Efficient multiplexing with ribosomal primers allows the immediate quality assessment of negative results regarding the Xcm amplicon (if the general prokaryotic or plant ribosomal amplicon is observed in the absence of a pathogen specific band, the PCR result may be attributed to the absence of the pathogen and not to a general inhibition of the PCR reaction). Multiplex PCR with internal controls in the diagnosis of *Xanthomonas* species have previously been reported (Glick *et al.*, 2002; Berg *et al*., 2005; Robène-Soustrade *et al*., 2010).

In conclusion, this study indicated that the primer sets developed were highly specific for either xanthomonads or Xcm detection, thus the PCR assays can be considered as reliable and useful methods in the detection and diagnosis of xanthomonads and Xcm, respectively. The findings imply that the developed PCR-based *Xanthomonas* diagnostic tools when used together offer robust and specific detection of *Xanthomonas* and could be effective in the diagnosis of *Xanthomonas* from infected plants. Additionally, the X-gumD primers tested in parallel to the X-ITS primers effectively discriminated the tested *Xanthomonas* strains to belong to groups I or II. Also, based on the observations and arguments described above we hereby propose the GspDm PCR as a tool for Bxw diagnosis. The proposed PCR has the potential to assist Bxw disease diagnosis and disease monitoring without confusing it with the Moko and blood disease incited by *Ralstonia solanacearum* which cause similar symptoms on all banana genotypes (Eden-Green and Seal 1993; Thwaites *et al*., 2000) and the plant quarantine organism *X. arboricola* pv. *celebensis* known to cause banana leaf stripe symptoms reported from Indonesia.

Acknowledgement

This work was made possible through funding from DANIDA under the

ENRECA project LIFE - 731. The contribution of Hanne, B. Nielsen of Danish Seed Health Centre is acknowledged and highly appreciated.

References

Adikini, S., Tripathi, L., Beeda, F., Tusiime G., Magembe, E.M. and Kim, D.J. 2011. Development of a specific molecular tool for detecting *Xanthomonas campestris* pv. *musacearum*. *Plant Pathology* 60:443-452.

Adriko, J., Aritua, V., Mortensen, C.N., Tushemereirwe, W.K., Kubiriba,J. and Lund, O.S. 2012. Multiplex PCR for specific and robust detection of *Xanthomonas campestris* pv. *musacearum* in pure culture and infected plant material. *Plant Pathology* 61:489-497.

Alvarez, M.A. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annu. Rev. Phytopathol.* 42:339-66.

Aritua, V., Nanyonjo, A., Kumakech, F. and Tushemereirwe, W.2007a. Rep-PCR reveals a high genetic homogeneity among Ugandan isolates of *Xanthomonas campestris* pv. *musacearum*. *Afr. J. Biotechnol.* 6: 179-83.

Aritua, V., Parkinson, N., Thwaites, R., Heeney, J.V, Jones, D.J., Tushemereirwe, W., Crozier, J., Reeder, R., Stead, D.E. and Smith, J. 2008. Characterization of the *Xanthomonas* sp. causing wilt of enset and banana and its proposed reclassification as a strain of *X. vasicola*. *Plant Pathology* 57:170-177.

Aritua, V., Parkinson, N., Thwaites, R., Tushemereirwe, W. and Smith, J. 2007b.Molecular epidemiology of *Xanthomonas campestris* pv. *musacearum*, the causal agent of Xanthomonas wilt of banana and enset. In: Programme and Abstracts of ISHS D ProMusa Symposium: Recent Advances in Banana Crop Protection for Sustainable Production and Improved Livelihoods, Greenway Woods Resort, White River, South Africa, September 10–14, 2007. 32.

Berg, T., Tesoriero, L. and Hailstones, D.L 2005. PCR-based detection of *Xanthomonas campestris* pathovars in *Brassica* seed. *Plant Pathology* 54: 416-427.

Bochner, B.R. 1989. Sleuthing out bacterial identities. *Nature (London)* 399:157-158.

Bradbury, J.F.1986. *Xanthomonas* Dowson 1939. pp. 198–260. In: J. F. Bradbury (Ed.). Guide to Plant Pathogenic Bacteria, CAB International Mycological Institute, Slough.

Eden-Green, S. 2004. How can the banana *Xanthomonas* wilt be halted? *InfoMusa* 13:38-41.

Eden-Green, S. and Seal, S. 1993. Bacterial diseases of banana and plantain in Southeast Asia. In: Ganry, J. (Ed.). Breeding Banana and Plantain for Resistance to Diseases and Pests. Montpellier, France: CIRAD, INIBAP. pp. 115-21.

Glick, D.L., Coffey, C.M. and Sulzinski, M.A. 2002. Simultaneous PCR detection of the two major bacterial pathogens of Geranium. *J. Phytopathol.* 150:54-59.

Griffin, D.E., Dowler, W.M., Hartung, J. S. and Bonde, M.R. 1991. Differences

in substrate utilization among isolates of *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pv. *oryzicola* from several countries. *Phytopathology* 81:1222.

Gurtler, V. and Stanisich, V.A. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiol.* 142:3-16.

Hauben, L., Vauterin, L., Swings, J. and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.* 47:328-335.

Hayward, A.C.1993. The hosts of *Xanthomonas*. pp. 1-199. In: J.G. Swings and E.L. Civerolo (Eds.), *Xanthomonas*, Chapman and Hall, London, United Kingdom.

Lee, Y-A., Sung, A-N., Liu, T-F. and Lee, Y-S. 2009. Combination of chromogenic differential medium and *esta*-specific PCR for isolation and detection of phytopathogenic *Xanthomonas* spp. *Appl. Environ. Microbiol.* 75:6831-6838.

Leite, P.R. Jr., Minsavage, V.G., Bonas, U. and Stall, E.R. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the Hrp genes of *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Environ. Microbiol.* 60:1068-1077.

Lewis Ivey, L.M., Tusiime, G. and Miller, A.S. 2010. A polymerase chain reaction assay for the detection of *Xanthomonas campestris* pv. *musacearum* in banana. *Plant Disease* 94:109-114.

Leyns, F., DeCleene, M., Swings, J.G. and Deley, J. 1984. The host range of the genus *Xanthomonas*. *Bot. Rev.* 50: 308-356.

López, M.M., Bertolini, E., Olmos, A., Caruso, P., Gorris, T. M., Llop, P., Penyalver, R. and Cambra, M. 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *Int. Microbiol.* 6:233-243.

Louws, F.J., Rademaker, J.L.W. and de Bruijn, F.J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Ann. Rev. Phytopathol.* 37:81-125.

Louws, F.J., Rademaker, J.L.W. and de bruijn, F.J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: Diversity, Detection, and Disease diagnosis. *Ann. Rev. Phytopathol.* 37:1-125.

Maes, M. 1993. Fast classification of plant-associated bacteria in the *Xanthomonas* genus. *FEMS Microbiol. Lett.* 113:161-166.

Mbega, R.E. 2011. Detection, characterization and control of *Xanthomonas* spp. causal agents of bacterial leaf spot of tomato in Tanzania. PhD Thesis, University of Copenhagen, Denmark.

Narayanasamy, P. 2011. Diagnosis of Bacterial Diseases of Plants. pp. 233-246. In: P. Narayanasamy (Ed.). Microbial Plant Pathogens-Detection and Disease Diagnosis: Bacterial and Phytoplasmal Pathogens, Vol. 2. Springer, Dordrecht, Heidelberg, London, New York.

Palacio-Bielsa, A., Cambra, M.A. and López, M.M. 2009. PCR detection and identification of plant-pathogenic bacteria: Updated review of protocols (1989-2007). *J. Plant Pathol.* 91:249-297.

Parkinson, N., Cowie, C., Heeney, J. and Stead, D. 2009. Phylogenetic structure

of *Xanthomonas* determined by comparison of gyrB sequences. *Int. J. Syst. Evol. Microbiol.* 59:264-74.

Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J. and Stead, D. 2007. Phylogenetic analysis of *Xanthomonas* species by comparison of partial *gyrase* B gene sequences. *Int. J. Syst. Evol. Microbiol.* 57: 2881-2887.

Robène-Soustrade, I., Legrand, D., Gagnevin, L., Chiroleu, F., Laurent, A. and Pruvost, O. 2010. Multiplex nested PCR for detection of *Xanthomonas axonopodis* pv. *allii* from onion seeds. *Appl. Environ. Microbiol.* 76:2697-2703.

Schaad, W.N., Jones, J.B. and Lacy, H.G. 2001. *Xanthomonas*. pp. 175-200. In: N.W. Schaad, J.B. Jones, and W. Chun (Ed.), Laboratory Guide for Identification of Plant Pathogenic Bacteria. APS Press, St Paul, Minnesota, USA.

Simões, H.N.T., Gonçalves, R.E., Rosato, B.Y. and Mehta A. 2007. Differentiation of *Xanthomonas* species by PCR-RFLP of *rpf*B and *atp*D genes. *FEMS Microbiol. Lett*. 271:33-39.

Thwaites, R., Eden-Green, S.J. and Black, R. 2000. Diseases caused by bacteria. pp. 213-239. In: Diseases of Banana, Abaca and Enset. D.R. Jones (Ed.). CABI Publishing, Wallingford.

Tripathi, L., Mwangi, M., Abele, S., Aritua V., Tushemereirwe, K.W. and Bandyopadhyay, R. 2009. Xanthomonas wilt, a threat to banana production in East and Central Africa. *Plant Disease* 93:440-51.

Verniere, C., Pruvost, O., Civerolo, E.L., Gambin, O., Jaquemoud-Collet, J.P. and Luisetti, J. 1993. Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Appl. Environ. Microbiol.* 59:243-249.

Yirgou, D. and Bradbury, J.F. 1968. Bacterial wilt of enset (*Ensete ventricosum*) incited by *Xanthomonas musacearum* sp. *Phytopathology* 58:111-112.

Young, J.M., Park, C.D., Shearman, H.M. and Fargier, E. 2008. A multilocus sequence analysis of the genus *Xanthomonas*. *Syst. Appl. Microbiol.* 31:366-377.