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Full Length Research Paper

Xanthomonads and other yellow-pigmented *Xanthomonas*-like bacteria associated with tomato seeds in Tanzania

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Tomato (*Solanum lycopersicum* L.) seeds habour unique bacterial community that can be pathogenic or beneficial to their host. *Xanthomonas* causing bacterial leaf spot (BLSX) on tomato and other yellow-pigmented xanthomonads-like bacteria (XLB) that closely resemble BLSX were obtained from tomato seeds collected from Northern, Central and Southern highland regions of Tanzania. A total of 73 strains were isolated from 52 seed samples of 15 tomato cultivars. Results obtained with Biolog and sequence analysis of the 16S rRNA gene showed that samples originating from Central Tanzania harbored the most diverse populations of XLB and BLSX as compared to Northern and Southern Tanzania. The predominant bacterial genera in tomato seeds were *Stenotrophomonas*, *Sphingomonas*, *Chryseobacterium*, *Xanthomonas*, *Pantoea* and *Flavobacterium*. All strains identified by Biolog as *Xanthomonas* with exception of *Xanthomonas campestris* pv. *malvacearum*, were pathogenic on tomato and pepper plants. Strains identified by Biolog as *Sphingomonas sanguinis* and *Sphingomonas terrae* also incited black rot symptoms on pepper leaves. However, bacterial strains belonging to the genus *Stenotrophomonas, Chryseobacterium*, *Pantoea* and *Flavobacterium* were not pathogenic on tomato and pepper. Phylogenetic analysis showed that strains of the genus *Xanthomonas* are more closely related to *Stenotrophomonas* and *Pantoea* compared to the other bacterial genera found in tomato seeds.

Key words: *Xanthomonas*, yellow-pigmented bacteria, seed, tomato, phylogeny.

INTRODUCTION

Members of the genus *Xanthomonas* are Gram-negative, aerobic, yellow pigment-producing bacteria that belong to the class of γ-proteobacteria, many of which are plant pathogens (Hayward, 1993). Bacterial leaf spot (BLS) caused by *Xanthomonas* spp. is one of the most serious seed-borne diseases of tomato (*Solanum lycopersicum* L.) in Tanzania (Black et al., 2001; Kaaya et al., 2003, Shenge, 2007). Four species of the genus *Xanthomonas*, namely *Xanthomonas euvesicatoria* (Xeu), *Xanthomonas vesicatoria* (Xv), *Xanthomonas perforans* (Xp) and *Xanthomonas gardneri* (Xg), are associated with BLS of tomato (Jones et al., 2004, 2006). In addition to these species, *Xanthomonas campestris* pv. *raphani* and *Xanthomonas arboricola* have been reported as BLS pathogens of tomato (Westcott and Horst, 2001; Punina et al., 2009) and hot pepper plants, respectively (Myung et al., 2010).

Infested tomato seed is often reported as the primary source of the pathogen (McGuire and Jones, 1989). Therefore, a number of methods for detection of BLS pathogens in tomato seeds have been developed, including liquid assays with semi-selective media (McGuire et al., 1986, McGuire and Jones, 1989;

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Table 1. Tomato seed samples collected from three growing zones in Tanzania used in the current study.

Variety/cultivar	DSHC accession number	Origin of seed sample		
		Northern zone	Central zone	Southern highland zone
Cal-J	48638, 48667, 48668, 48674, 48678	+	+	+
Tanya	48639, 48647, 48649, 48651, 48656, 48662, 48664, 48667, 48669, 8728, 48729, 48730, 48834, 48836, 48837, 48838, 48862	+	+	+
Money maker	48644, 48653	+	+	+
Romeco	48646	-	-	+
Tengeru 97	28654, 48659, 48671	+	+	+
Vikima DK*	48676	-	+	-
Onyx	48670, 48672, 48679	-	+	-
Mpwipwi-local	48658	-	-	+
Mshumaa	48648, 48657, 48660,	+	+	+
RomaVFN	48655, 48673, 48680	+	+	+
Kituruma-local	48642	+	-	-
Israel	48652	-	-	+
Dumdum-local	48640, 48641,48645, 48650, 48663, 48666, 48675, 48681, 48835	+	+	+
Rio Grande	48677	-	+	-
VF 311	48643	-	+	-

DSHC=Danish Seed Health Centre; -=absent; +=commonly found; * named by farmers as 'Vikima DK' (name of the supplier company).

Sijam et al., 1992), along with biochemical tests and or the Biolog identification system (Jones et al., 1993; Shenge et al., 2007), nucleic acid analysis (Leite et al., 1995; Kuflu and Cuppels, 1997; Obradodivic et al., 2004; Mbega et al., 2010) and pathogenicity tests (Lelliot and Stead, 1987; ISF, 2007). Seed-associated *Xanthomonas*-like bacteria (XLB) that closely resemble the BLS-causing xanthomonads (BLSX) are also commonly found in tomato seeds, but currently very limited information on these bacterial communities is available (Gitaitis et al., 1987). These microorganisms grow fast on agar media and appear to interfere with the isolation and ability of recovery of the BLSX from tomato seeds (ISF, 2007). At present, most of the existing data on bacterial microflora of tomato seed is restricted to seed-borne pathogens (Black et al., 2001; Kaaya et al., 2003; Shenge et al., 2007) and does not include information on example other bacterial species, their distribution and genetic relationships. Tomatoes grown from locally-produced seeds have been reported to harbour unique populations of xanthomonads (Gitaitis et al., 1987; Punina et al., 2009). Knowledge on these microbial populations can be useful to improve the isolation of pathogenic bacteria from tomato seed in seed health assays and to reveal their pathogenic or pro-biotic potential in tomato and related hosts.

This study aimed at 1) assessing genus and/ or species composition of yellow-pigmented, Xanthomonads and *Xanthomonas*-like bacteria associated with tomato seed in three different tomato-growing zones in Tanzania; 2) evaluating the pathogenicity of the different bacterial strains on tomato and sweet pepper, and 3) examining their genetic relationship based on phylogenetic analysis of 16S rRNA gene sequences.

MATERIALS AND METHODS

Seed samples

Farmer-saved seeds of tomato were collected from three main tomato-growing zones in Tanzania namely the Northern, Central and Southern highland zones, in July 2008. A total of 52 tomato seed samples of 15 different commonly-grown cultivars were randomly collected from 45 different locations (Table 1). The survey covered one farm in Kilimanjaro, five in Iringa, seven in Tanga, seven in Mbeya, nine in Rukwa and sixteen farms in Morogoro regions.

Isolation of bacteria from seed

The hand halving method was used to obtain working seed samples for seed health assays using a standardized procedure described by International Seed Testing Association (ISTA, 2005). For isolation of bacteria, sub-samples of 1000 to 10000 seeds were incubated in phosphate buffer saline (PBS)-Tween buffer (0.05 M of PO_4, pH 7.2; 8.5 g of NaCl; 20 ml of Tween 20; 1000 ml of distilled water) for 14 to 20 h at 4 to 10°C (ISF, 2007). Aliquots of 100 µL from the undiluted, 1:10 and 1:100 dilutions in duplicate were plated onto: (1) mTMB medium (10 g of bacto peptone, 0.1 g of H_3BO_3, 10 g of KBr, 0.25 g of $CaCl_2$ anhydrous, 15 g of bacto agar, 10 ml of Tween 80, 65 mg of cephalexin, 12 mg of 5-fluorouracil, 0.2 g of tobramycin sulphate, 100 mg of cycloheximide and 1000 ml of

distilled water) (McGuire et al., 1986) and (2) CKTM medium (Sijam et al., 1992) (2 g of soya peptone, 2 g of tryptone, 1 g of glucose, 1 g of L-glutamine, 6 g of L-histidine, 0.8 g of $(NH4)_2$ HPO_4, 1 g of KH_2PO_4, 0.4 g of $MgSO_4.7H_2O$, 0.25 g of $CaCl_2$ anhydrous, 15 g of Bacto agar, 10 ml of Tween 80, 65 mg of cephalexin, 12 mg of 5-fluorouracil, 0.4 g of tobramycin sulphate, 100 mg of bacitracin, 10 g of neomycin sulphate, 100 mg of cycloheximide and 1000 ml of distilled water).

Inoculated plates were incubated at 28°C for 3 to 7 days and were observed for the presence of yellow-pigmented colonies daily. Reference strains of BLSX were used as positive controls and included strains obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), UK. namely, *X. euvesicatoria* (NCPPB 2968), *X. vesicatoria* (NCPPB 422), *X. vesicatoria* (NCPPB 476), *X. perforans* (NCPPB 4321), *X. gardneri* (NCPPB 881) and strains isolated from tomato (XVT 12 and XVT 28) and pepper (XVP 129 and XVP 196) received as *X. campestris* pv. *vesicatoria* (courtesy of Dr Jaw-Fen Wang from the World Vegetable Centre in Taiwan). Two yellow-pigmented bacterial pathogens of tomato served as negative controls in the evaluations, namely strain NCPPB 2445 (*Pseudomonas corrugata*) and IPO 542 (*Clavibacter michiganensis* subsp. *michiganensis* - obtained from the Culture Collection of Plant Research International in Wageningen, Netherlands).

Identification of bacterial strains

Preliminary identification tests

Yellow-pigmented *Xanthomonas* and *Xanthomonas-like* colonies isolated from tomato seed samples onto mTMB and CKTM media were transferred to nutrient agar (NA), incubated at 28°C for 24 h and stored at 80°C on porcelain beads of the Protect Bacterial Preservers (Protect System, Bury, UK) until use. Preliminary identification of the strains was conducted by Gram-reaction (potassium hydroxide (KOH) solubility test), oxidase reaction, starch hydrolysis test and oxidative-fermentative (O/F) metabolism of glucose (Lelliot and Stead, 1987).

Biolog identification

Identification of the bacterial strains isolated was done based on their ability to metabolize carbon sources using the Biolog Identification System (MicroLog™ 2, version 4.2, Biolog Inc., Hayward, CA, USA). A single colony of each strain cultured on NA was inoculated onto Biolog Universal Growth medium and incubated for 24 h at 28°C. Colonies were harvested with a sterile moistened cotton swab, suspended in sterile inoculating fluid and adjusted at 590 nm to match Biolog GN turbidity standards. Aliquots of 150 µL of bacterial suspensions were loaded into each well of the Biolog GN2 microtiter plates. Bacterial suspensions made from 24-h-old cultures from reference (positive and negative) strains previously described under isolation of bacteria from the seed were included. Readings were conducted using the Biolog microplate reader (ICN Flow Titertek® Multiscan Plus, Version 2.03, Lab-Systems, Finland) after 24 h of incubation at 28°C, and results were analysed with the MicroLog software version 4.2 (Biolog Inc., Hayward, CA, USA). A strain was considered to be identified if the similarity index obtained was of at least 0.5 after 24 h of incubation. Identification to the next match was not attempted.

Pathogenicity tests

In order to assess the pathogenic potential of the isolated bacterial strains, pathogenicity tests were conducted on tomato and sweet pepper (*Capsicum annuum* L.) cultivars that are known to be susceptible to BLSX. Therefore, seed lots of the tomato cultivar Tanya and Moneymaker and the sweet pepper cultivar Early Calwonder, found to be free of BLS pathogen(s) by liquid assays (ISF, 2007), were used in the pathogenicity tests. Prior to sowing, the seeds were surface-disinfected in 70% ethanol for 1 min, followed by immersion in 1% of sodium hypochlorite for 3 min, rinsed three times consecutively in sterile distilled water and left to dry under the flow cabinet for 1 to 2 h. Seeds were then sown in pots of 8 cm diameter containing peat soil (Pindstrup substrate No.2, Pindstrup Mosebrug A/S, Denmark) and sand (3:1), respectively and kept in growth chamber at 25 to 30°C under high relative humidity (> 85%). The abaxial surfaces of four 14-day-old tomato and 21-day-old sweet pepper seedlings per strain were spray-inoculated to runoff with an inoculum suspension of 10^8 CFU/ml (OD_{600} = 0.01) prepared from 24 h-old bacterial cultures grown on NA at 28°C. The inoculated seedlings were subsequently covered with polyethylene bags and kept in the growth chamber for 14 days. Seedlings sprayed with sterile saline water (containing 0.85% of NaCl) alone were used as a negative control and seedlings sprayed with suspensions prepared from the BLSX reference strain cultures served as positive controls.

The plants were examined for symptoms 14 days (Shenge et al., 2007) after inoculation, and scored as negative when no obvious symptoms were observed. Water-soaked lesions which developed into dark brown spots on the leaves were scored as positive for BLS disease and isolations of the pathogen were made from symptomatic leaves (ISF, 2007). Additionally, a small tissue section from the margins of the lesion was cut off using a sterile surgical blade and comminuted in a drop of sterile saline solution (0.85% NaCl) on a sterile microscope slide, and observed for bacterial ooze under the compound microscope at ×10, ×40 and ×100 magnifications. Loopfuls from the suspensions were then spread onto NA plates and incubated at 28°C for 72 h. Identification of the re-isolated bacteria was conducted using Biolog Identification System .

Bacterial identification and phylogenetic analysis using 16S rRNA gene sequences

Bacterial DNA was extracted from 24 to 48-h-old pure cultures growing on NA with the DNeasy purification kit for Gram-negative bacteria (DNeasy Blood and Tissue Kit, Qiagen) as described by the manufacturer's instructions. In addition to the 73 isolated bacterial strains, DNA of *X. euvesicatoria* (NCPPB 2968), *X. vesicatoria* (NCPPB 422 and 476), *X. perforans* (NCPPB 4321), *X. gardneri* (NCPPB 881), and strains from the World Vegetable Centre, Taiwan, Culture Collection XVT 12, XVT 28, XVP 129 and XVP 196 submitted as *X. campestris* pv *vesicatoria* (Tables 2 and 3) were extracted and used as reference strains. The primer pairs (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1522R: 5′-AAGGAGGTGATCCARCC GCA-3′) developed by Johnson (1994) that amplified the 16S rRNA gene region of bacteria were used in the polymerase chain reaction (PCR) amplification assays. PCR reactions conducted in Eppendorf PCR tubes comprised of 25 µL containing 1.5 µL of 1.5 mM $MgCl_2$, 5 µL of Flexi buffer (5x), 4 µL of 1.25 mM dNTP, 0.25 µL of 10 pmol of each primer, 12.75 µL of sterile distilled water, 0.25 µL of 1.25 units of *Taq* polymerase (Promega™, Madison, Wisconsin, USA) and 1 µL of DNA (10-100 ng). PCR reactions were performed in a master cycler gradient machine (Eppendorf 22331 Hamburg, Germany). Reactions were run for 30 cycles, each consisting of 20 s at 95°C, 15 s at 64°C, and 15 s at 72°C, with initial denaturation of 3 min at 95°C and final extension of 3 min at 72°C . A 10 µL aliquot of each amplified PCR product was separated on 1% agarose gel stained with 12 µL of ethidium bromide (0.5 µg/ml) in 0.5X TBE (Tris-borate EDTA) at 50 V/cm for 45 min.

Table 2. Identification and characterization of yellow-pigmented bacterial strains isolated from tomato seed samples from different growing regions of Tanzania using Biolog and pathogenicity test.

Bacterial genus identified by Biolog (total number of strains within the same genus in percentage)*	Bacterial identification by Biolog (number of strains in percentage)	Origin of seed sample			Pathogenicity test		
		Northern zone	Central zone	Southern highland	Tn[1)]	Mk[2)]	EC[3)]
Chryseobacterium (19.2)	*Chryseobacterium* sp. (4.1)	-	+	+	-	-	-
	C. gleum/indologenes (15.1)	-	+	+	-	-	-
Flavobacterium (1.4)	*Flavobacterium tirrenicum* (1.4)	-	+	-	-	-	-
Pantoea (2.7)	*Pantoea* sp. (2.7)	-	+	-	-	-	-
Sphingomonas (23.3)	*Sphingomonas* sp. (5.5)	+	+	-	-	-	-
	S. parapaucimobilis (4.1)	-	+	+	-	-	-
	S. sanguinis (12.3)	+	+	+	-	-	v
	S. terrae (1.4)	-	+	-	-	-	v
Stenotrophomonas (31.5)	*Stenotrophomonas* sp. (4.1)	-	+	-	-	-	-
	S. maltophilia (27.4)	+	+	+	-	-	-
Xanthomonas (8.2)	*Xanthomonas* sp. (2.7)	-	+	-	+	+	+
	X. campestris pv.*begoniae* A (1.4)	-	-	+	+	+	+
	X. campestris pv. *malvacearum* (1.4)	+	-	-	-	-	HR
	X. campestris pv. *poisenttiicola* (2.7)	-	+	+	+	+	+
NA	No identification (15.1)	-	+	+	-	-	-

*Percentage of a total of 73 strains. A strain was considered to be identified if the similarity index value obtained was ≥ 0.5 after 24 h. [1)] Tn = Tomato cv. Tanya; [2)] MK = tomato cv. Moneymaker; [3)] EC = sweet pepper cv. Early Calwonder; - = absent/no response; + = present/positive response; HR = hypersensitive response; v= variable black rotting symptoms; NA = not applicable; ND = not done.

A 1200 bp band present in each strain was then visualized on a UV transilluminator. Purification of the final PCR product was conducted using Fermentas DNA Extraction Kit (product number KO513, www. Fermentas.com). The purified PCR products were quantified using a Nanodrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc) and sent to Eurofins MWG Operon (www. eurofinsdna.com) or Starseq (www.starseq.com) for sequencing. All obtained sequences were blast searched in the NCBI database (http://www.ncbi.nlm.nih.gov/) and compared with sequences in the database. The identification of the bacterial strains was based on the closest similar sequence deposited at NCBI.

To investigate the phylogenetic relationship of the bacterial strains, 73 sequences of the 16S rRNA gene obtained from the isolated strains from tomato and nine sequences obtained from BLSX reference strains were aligned by ClustalX (Thompson et al., 1997) and edited in the Bio-Edit program. Phylogenetic analysis was conducted using the MEGA4 (Tamura et al., 2007) software and the Maximum Parsimony method (Eck and Dayhoff, 1966) was used as the criterion. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 (Felsenstein, 1985; Nei and Kumar, 2000) in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included were 1st, 2nd, 3rd and non- coding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion options).

Table 3. Identification of yellow-pigmented bacterial strains and reference strains from tomato seed samples from different growing regions of Tanzania using 16S rRNA gene sequences.

Code	Genus	16S rRNA identification (%)*	Origin of seed sample		
			Northern zone	Central zone	Southern highland
18, 19, 20, 21, 24, 30, 32, 34, 40, 42, 87, 111, 115, 131 and 157	*Chryseobacterium*	20.5	-	+	+
110	*Flavobacterium*	1.4	-	+	-
164 and 169	*Pantoea*	2.7	-	+	-
7, 8, 9,10, 23, 31, 114, 116,118,123, 138, 139, 142, 143,146, 153, 155, 156, 159, 160, 161, 168 and 171	*Sphingomonas*	31.5	+	+	+
1, 2, 4, 5, 6, 11, 25, 26, 27, 28, 33, 36, 38, 41, 43, 88, 90, 124, 126, 144, 147, 149, 150, 151 and 152	*Stenotrophomonas*	34.2	+	+	+
39, 55, 73, 93, 100, 167 and 172	*Xanthomonas*	9.6	+	+	+
Reference strains 12 NCPPB 422[a], 13 NCPPB 476[b], 15 NCPPB 4321[c], 16 NCPPB 881[d], 17 NCPPB 2968[e], XVT 12, XVT 28, XVP 129, XVP 196	*Xanthomonas*	ND	ND	ND	ND

* Percentage of a total of 73 strains. NCPPB, National Collection of Plant Pathogenic Bacteria, UK, [a] = *Xanthomonas* reference strains: *X. vesicatoria* NCPPB 422, [b] = *X. vesicatoria* NCPPB 476 strain received as *X. campestris* pv. *vesicatoria*, [c] = *X. perforans* NCPPB 4321, [d] = *X. gardneri* NCPPB 881 and [e] = *X. euvesicatoria* NCPPB 2968; XVT 12, XVT28, XVP 129 and XVP 196 = The World Vegetable Center culture collection strains, Taiwan, received as *X. campestris* pv. *vesicatoria* isolated from tomato (XVT) and pepper (XVP). ND = Not done.

RESULTS

Isolation and preliminary identification of bacterial strains

A total of 73 *Xanthomonas* and *Xanthomonas*-like colonies were isolated on CKTM and mTMB agar. Two to seven days after incubation, the colonies were yellow to pale yellow, 0.5-2 mm in diameter, circular and entire with smooth margins. Most of the isolated bacteria that hydrolyzed starch were surrounded by clear and milky zones with crystal precipitates due to Tween lipolysis on the CKTM and mTMB media (data not shown). The bacteria were all Gram-negative and two out of 73(2.7%) strains were facultative anaerobes, while 97.3% were aerobes with oxidative metabolism of glucose in O/F tests. All colonies of the isolated bacteria grew faster (2-3 days) on mTMB and NA agar media than on CKTM agar (4-7 days) (data not shown).

Biolog identification

The Biolog identification results are presented in Table 2. The genera of the isolated bacteria in tomato seeds were *Stenotrophomonas* (31.5%), *Sphingomonas* (23.3%), *Chryseobacterium* (19.2%), *Xanthomonas* (8.2%), *Pantoea* (2.7%) and *Flavobacterium* (1.4%). *Stenotrophomonas maltophilia* was the most frequently isolated bacterial species with 27.4% of the strains (20 out of 73 strains), followed by *Chryseobacterium gleum / indologenes* with 15.1% and *Sphingomonas sanguinis* with 12.3%. Less frequently isolated strains were bacterial species identified as *Sphingomonas parapaucimobilis* (4.1%), *X. campestris* pv. *poisenttiicola* (2.7%), *Flavobacterium tirrenicum* (1.3%), *Sphingomonas terrae* (1.4%), *X. campesrris* pv. *begoniae* (1.4%) and *X. campesrris* pv. *malvacearum* (1.4%) (Table 2). Some strains were only possible to identify at the genus level namely, *Chryseobacterium* sp. (4.1%), *Pantoea* sp. (2.7%), *Sphingomonas* sp. (5.5%), *Stenotrophomonas* sp. (4.1%) and *Xanthomonas* sp. (2.7%) (Table 2). Moreover, 21 out of 73 bacterial strains (corresponding to 15.1%) were not identified by the Biolog Identification System (Table 2).

The Biolog test results also show that bacterial strains found in seed samples of the Northern zone of Tanzania were identified as *Sphingomonas* sp., *S. sanguinis*, *S. maltophilia* and *X. campestris* pv. *malvacearum*. With exception of *X. campestris* pv. *begoniae* and *X. campestris* pv. *malvacearum*, all the bacterial genera and species were found in the seed samples originating from the Central zone (Table 2). In the Southern highland zone, *Chryseobacterium* sp., *Chryseobacterium*

gleum/indologenes, *S. parapaucimobilis*, *S. sanguinis*, *S. maltophilia*, *X*. *campestris* pv. *begoniae*, and *X*. *campestris* pv. *poisenttiicola* were found colonizing tomato seeds (Table 2). A total of 15.1% of the isolated bacterial strains originated from the Central and the Southern highland zones were not identified by Biolog (Table 2). The system also failed to correctly identify at the species level the four main BLSX reference strains (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*) (data not shown). However, Biolog seemed to be very useful to identify these and other reference strains to their correct genus level (data not shown).

Pathogenicity tests

Pathogenicity tests of the bacterial strains isolated from tomato seeds indicated that bacteria from the genera *Chryseobacterium, Flavobacterium, Stenotrophomonas* and *Pantoea* did not induce any symptoms on tomato and pepper (Table 2). The strains identified by Biolog as *Xanthomonas* sp., *X. campestris* pv. *begoniae* A and *X. campestris* pv. *poisenttiicola* were pathogenic on both tomato cv. Tanya and Moneymaker and Early Calwonder pepper plants. The symptoms produced by these strains were similar to those produced by the positive reference control strains and consisted of water-soaked lesions, which became circular to irregularly shaped, dark brown spots on the leaves. A bacterial strain identified by Biolog as *X. campestris* pv. *malvacearum* was non-pathogenic on tomato plants. However, it induced a hypersensitive reaction in sweet pepper plants (Table 2). The results also indicate that two species of the genus *Sphingomonas*, namely *S. sanguinis* and *S. terrae*, produced variable black rot symptoms (or brown vein discoloration) on the margins of the inoculated sweet pepper plants, being pathogenic on this host (Table 2). These two *Sphingomonas* species were non-pathogenic on tomato plants. Water-soaked and / or black rot symptoms were not observed in plants sprayed with sterile saline water (negative) control.

Bacterial identification and phylogenetic analysis using 16S rRNA gene sequences

Phylogenetic analysis of the 16S rRNA gene sequences of the bacterial strains isolated from tomato seeds indicate that the main genera were *Stenotrophomonas* (34.2%), *Sphingomonas* (31.5%), *Chryseobacterium* (20.5%), *Xanthomonas* (9.6%), *Pantoea* (2.7%) and *Flavobacterium* (1.4%) (Table 3). The results obtained with the phylogenetic analysis of the 16S rRNA gene sequences show that the strains were grouped in six different clusters, namely *Stenotrophomonas*, *Xanthomonas*, *Pantoea*, *Sphingomonas*, *Chryseobacterium* and *Flavobacterium* (Figure 1).

Cluster I comprised *Stenotrophomonas* spp. strains, which were found to be closely related to *Xanthomonas* sp. that are pathogenic to tomato (Figure 1). This cluster was supported by a bootstrap similarity of 89%. The bacterial strain previously identified by Biolog as *X. campestris* pv. *malvacearum* (strain 124) was placed in cluster I by the 16S rRNA gene sequences (Figure 1 and Table 3). Cluster II contained *Xanthomonas* spp. most of which had 52% bootstrap similarity values (Figure 1). The reference strains included in this study for each type of strains of the four species of BLSX:*X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422 and 476, *X. perforans* NCPPB 4321 and *X. gardneri* NCPPB 881; four reference *Xanthomonas* spp; XVP 196, XVP 129, XVT 28, XVT 12 received as *X. campestris* pv. *vesicatoria* from the World Vegetable Centre culture collection and six pathogenic *Xanthomonads* (Nos. 39, 55, 73, 100, 167 and 172), isolated from the tomato seed samples with Danish Seed Health Centre (DSHC) accession numbers. 48.675, 48.670, 48.681, 48.639, 48.730 and 48.838, respectively, from Tanzania clustered together in this group (Figure 1). The blast search analysis of the 16S rRNA sequences of these pathogenic *Xanthomonads* isolated from tomato and identified by Biolog as *X. campestris* pv. *poisenttiicola* (strains 55 and 172) and *X. campestris* pv. *begoniae* A (strain 73) indicate that the sequences matched most closely to *X. gardneri* partial 16S rRNA gene type strain DSM 19127T with GenBank accession FR749911.1, while the strain identified by Biolog as *Xanthomonas* sp., namely strain 167 matched most closely to both *X*. *arboricola* pv. *poisenttiicola* strain LMG 8676 16S rRNA genome sequence data with GenBank accessions GU144260.1 and *X. gardneri* partial 16S rRNA gene type strain DSM 19127T with GenBank accession FR749911.1 (Table 4). Strains numbered 39, 93 and 100 matched most closely to a *Xanthomonadaceae* bacterium NML93-0792, *X. campestris* pv. *vesicatoria* complete genome and *Xanthomonas* sp. 3C_3 16S rRNA gene with GenBank accession EU313793.1, AM039952.1 and AY689031.1, respectively (Table 4). Cluster III consisted of *Pantoea* spp. These species were closely related to *Xanthomonas* and were supported by a bootstrap value of 53% similarity (Figure 1). Clusters IV, V and VI consisted of *Sphingomonas*, *Chryseobacterium* and *Flavobacterium* species, respectively (Figure 1). These clusters were not closely related to the genus *Xanthomonas*.

DISCUSSION

The present study show that tomato seed from three different tomato-growing zones in Tanzania harbored a diverse community of yellow-pigmented BLSX and XLB strains. In total, 73 bacterial strains were isolated from 52 tomato seeds from 15 different cultivars and varieties. Results obtained with Biolog show that samples that

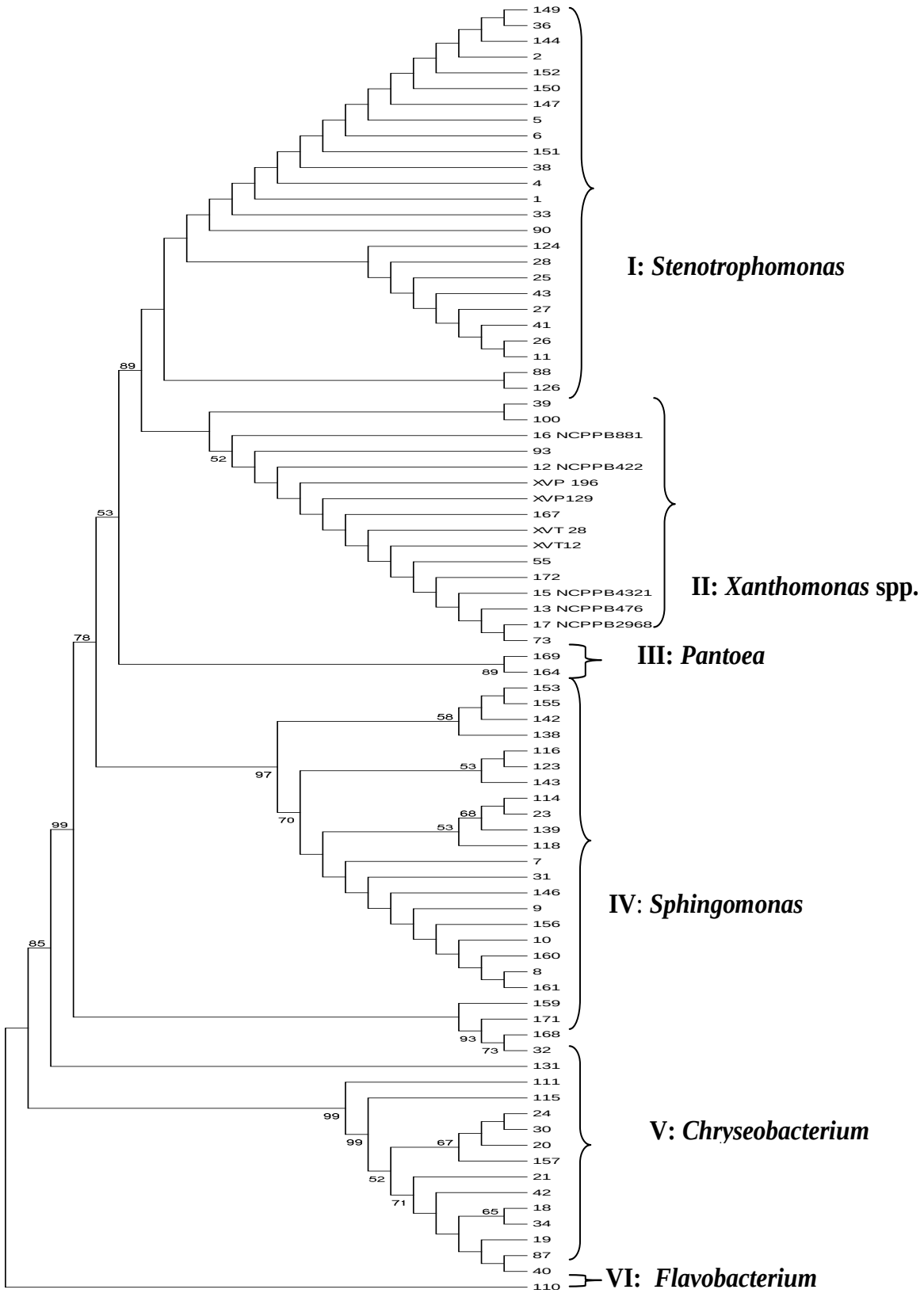

Figure 1. Phylogenetic analysis of 73 bacterial strains from tomato seeds from Tanzania and reference strains of *Xanthomonads* isolated from tomato and pepper using Close-Neighbor-Interchange algorithm method for 16S rRNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 4. *Xanthomonas* species isolated from tomato seed identified by Biolog and 16S rRNA sequence analysis.

Strain	Biolog identification		16S rRNA gene analysis			
	Organism	SIM	Organism	GenBank accession	Query coverage (%)	Max ID (%)
39	*Xanthomonas* sp.	NA	*Xanthomonadaceae* bacterium	EU313793.1	99	99
55	*Xanthomonas campestris* pv. *poisenttiicola*	0.53	*X. gardneri* type strain 19127 T	FR749911.1	100	100
73	*X. campestris* pv. *begoniae* A	0.78	*X. gardneri* type strain 19127 T	FR749911.1	99	97
93	No ID	NA	*X. campestris* pv.*vesicatoria* complete genome	AM039952.1	96	95
100	No ID	NA	*Xanthomonas* sp. 3C_3	AY689031.1	98	99
167	*Xanthomonas* sp.	NA	*X. arboricola* pv. *poinsetttiicola* str.LMG 8676	GU1444260.1	87	96
172	*Xanthomonas campestris* pv. *poisenttiicola*	0.74	*X. gardneri* type str.19127 T	FR749911.1	87	96
			X. gardneri type strain 19127 T	FR749911.1	100	99

NA= Not applicable; Max ID = maximum identity; SIM = similarity index; No ID = no identity.

originated from Central Tanzania harbored the most diverse population of XLB and BLSX, as compared to Northern and Southern Tanzania. The predominant bacterial genera isolated from tomato seeds were identified by Biolog as *Stenotrophomonas*, *Sphingomonas*, *Chryseobacterium*, *Xanthomonas*, *Pantoea* and *Flavobacterium*. Even though the Biolog identification system was a useful and robust tool in the identification of yellow-pigmented bacteria associated with tomato seed, it failed to correctly identify *Xanthomonads* and other XLB to the species or pathovar levels (data not shown). Such results indicate the limitations of the Biolog database in its application, pointing to the need for expansion of the *Xanthomonads* database in the system. The present results are in agreement with those reported by other researchers on the limitations of the Biolog system in the identification of plant pathogenic *Xanthomonads* in various crop-pathogen systems (Jones et al., 1993; Massomo et al., 2003; Shenge et al., 2007). Those limitations are associated with differences between metabolic profiles of the strains in the current study and those of *Xanthomonads* used in the Biolog GN database (Jones et al., 1993; Massomo et al., 2003; Shenge et al., 2007). Despite the limitations of the Biolog identification system, it is still a useful and relatively simple and robust tool for the identification of plant pathogenic bacteria when combined with other identification methods such as pathogenicity tests and PCR.

In the pathogenicity tests, all strains identified as members of the genus *Xanthomonas* (except the strain identified by Biolog as *X*. *campestris* pv. *malvacearum*) were highly pathogenic in tomato plants and sweet pepper, inducing BLS symptoms. Indeed, the strain identified as *X*. *campestris* pv. *malvacearum* by Biolog was identified by 16S rRNA gene sequence analysis as *Stenotrophomonas* sp., indicating a divergence in the results obtained by the two methods. However, the results obtained with the pathogenicity test could indicate that the strain should be identified as *Stenotrophomonas* due to the lack of pathogenicity in tomato and pepper. With the exception of *S. sanguinis* and *S. terrae*, all other XLB strains were non-pathogenic to tomato and sweet pepper plants. Up to now, few *Sphingomonas* strains have been reported as plant pathogens. For example, strains of *Sphingomonas suberifaciens* (Syn. *Rhizomonas suberifaciens*) were found to be the causal agent of corky root of lettuce (Van Bruggen et al., 1993). *S. suberifaciens* was for a long time the only confirmed plant pathogen in the four *Sphingomonas*-related genera (Takeuchi et al., 2001). In 2002, a strain of *Sphingomonas* sp. was reported as a novel pathogen of yellow Spanish melon fruits, causing brown spot (Buonaurio et al., 2002). The present study shows the potentiality of *S. sanguinis* and *S. terrae* as pathogens of sweet pepper.

The identification of the strains using 16S rRNA gene sequences appeared to correlate with the Biolog results, as the main bacterial genera identified with this method were *Stenotrophomonas*, *Xanthomonas*, *Pantoea*, *Sphingomonas*, *Chryseobacterium* and *Flavobacterium*. Using 16S rRNA gene sequences, the highest bacterial diversity was found in the Central zone of Tanzania, confirming the previous results obtained with Biolog. Phylogenetic analysis of the obtained gene sequences

showed that the bacterial strains obtained from tomato seeds were grouped into six different groups comprising the six different identified genera (Figure 1). Of the six identified groups, *Stenotrophomonas* spp. and *Pantoea* spp. were the ones most closely related to *Xanthomonas* group (Figure 1). Similar results were found by Nesme et al. (1995) and Moore et al. (1997), who both indicated that the genera *Xanthomonas* and *Stenotrophomonas* are closely related.

The analysis of the 16S rRNA gene sequences may facilitate studies of many tropical bacterial strains, thus contributing to the identification to genus level of the XLB and BLSX organisms. Despite the relatively low number of *Xanthomonads* isolated from tomato seed during the present study, a large variation was found among the strains of which 4 out of 7 matched most closely to the *X. gardneri* type strain DSM 19127T with GenBank accession FR749911.1, implying that the possible identity of these *Xanthomonads* isolated from tomato seed from Tanzania could be *X. gardneri* (Table 4). However, one out of six strains (strain 167, Table 4) matched most closely to both *X. arboricola* pv. *poisenttiicola* strain LMG 8676 and *X. gardneri* strain DSM 19127T, implying that the other possible identity of these *Xanthomonads* strains could be *X. arboricola* (Table 4). However, more work is needed to characterize these *Xanthomonads* in order to correctly prove their identity because the 16S rRNA gene analysis currently in use for characterization of prokaryotes is more suitable for identification of genera of bacteria and less adapted with regard to species identification (Janda and Abbott, 2007), as observed also in the current study.

The predominance of XLB strains in seeds indicated that tomato seed is a good habitat for them. As most of the strains were not pathogenic on tomato, one could speculate if these bacteria could provide some benefits to tomato in form of for example natural protection against pathogens or other pro-biotic properties. Some authors have indicated that *Stenotrophomonas maltophilia* has a biocontrol role against the soil-borne phytopathogenic fungus *Pythium ultimum in vitro* (Column et al., 1997), *Rhizoctonia solani* of tall fescue (*Festuca arundinacea* Schreb.) (Giesler and Yuen, 1998) and *Ralstonia solanacearum* race 3 biovar 2 the causal agent of potato brown rot (Messiha et al., 2007). Some studies reported that *Pantoea* spp. have a strong *in vitro* antifungal activity against fungal pathogens of tomato, such as *Botrytis cinerea*, *Fulvia fulva* and *Alternaria solani* (Junichiro et al., 2007). The role of these microbes interacting with tomato and the potential role of bacterial organisms isolated from tomato during this study could be explored in biological control or other pro-biotic programmes.

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