British Biotechnology Journal
3(4): 556-574, 2013

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Improved Sample Preparation for PCR-Based Assays in the Detection of Xanthomonads Causing Bacterial Leaf Spot of Tomato

E. R. Mbega[1,2,4*], J. Adriko[1,3], C. N. Mortensen[1], E. G. Wulff[1] , O. S. Lund[1] and R. B. Mabagala[2]

[1]Danish Seed Health Centre for Developing Countries, Department of Agriculture and Ecology, Faculty of Life Science, University of Copenhagen, Denmark.
[2]African Seed Health Centre, Department of Crop Science and Production, Faculty of Agriculture, Sokoine University of Agriculture, P.O.BOX 3005, Morogoro, Tanzania.
[3]National Agricultural Research Laboratories, Kawanda, Uganda.
[4]Agricultural Research Institute Ilonga, P.O.BOX 33, Kilosa, Morogoro, Tanzania.

Authors' contributions

This work was carried out in collaboration between all authors. This work was part of PhD study of authors ERM, JA designed Xan 7 primers and author CNM was the main project and main supervisor of the PhD studies. Authors RBM, OSL and EGW were co-supervisors of the PhD studies. All authors read and approved the final manuscript.

Research Article

Received 17th March 2013
Accepted 21st May 2013
Published 27th August 2013

ABSTRACT

Aims: To develop a sampling procedure for PCR-based screening of bacterial leaf spot (BLS)-causing xanthomonads without DNA extraction from infected tomato plants.
Place and Duration of Study: University of Copenhagen, Denmark and Sokoine University of Agriculture, Morogoro, Tanzania between July 2008 and November 2010.
Methodology: Flinders Technology Associates (FTA®) plant cards and Chromatography paper or Whatman® paper strips (WPS) were spotted with bacterial suspensions from 24-h-old cultures from reference strains of BLS-causing xanthomonads, or sap obtained by grinding or hand maceration of plant tissue, were used as templates in PCR reactions or isolation of live bacterial cells on Nutrient agar (NA) media. Samples were tested by PCR with Xan 7 genus/-specific *Xanthomonas* primers or in multiplex with 26S rRNA primers. Isolation of bacteria was done by streaking aliquots of 75 µl of a suspension from a disc

**Corresponding author: Email: mbegaernest@yahoo.co.uk;*

(2-mm-punch by Harris Micro Punch®) in triplicate, removed from each of the FTA plant card and WPS onto NA media.
Results: The FTA plant card spotted with pure cultures of reference strains of xanthomonads and sap from grinding or direct maceration of plant tissue resulted in more clear PCR bands (402 bp) and (594 bp of rRNA gene in multiplex) than the WPS samples. Sensitivity of detection by the FTA paper-based PCR was ≈ 5.0 x 10^2, while that of the WPS was > 1.0 x 10^3 CFU/ml. The WPS (but not the FTA) was proved to be useful for saving living bacteria cells for up to one week of storage at ambient temperatures.
Conclusion: Both FTA plant card and WPS can be used for PCR detection of BLS-causing xanthomonads in tomato. However, the FTA plant card is recommended as it produced clearer PCR products than WPS. WPS is recommended for experiments requiring isolation of live bacterial cells on NA media.

Keywords: FTA; Whatman; diagnosis; bacteria; tomato.

1. INTRODUCTION

Bacterial diseases including bacterial spot or bacterial leaf spot (BLS) have been estimated to cause 30-45% losses of tomato production in Tanzania [1]. BLS has been considered to be the most serious and a major constraint in tomato production, with an incidence ranging from < 5% to 90% in Tanzania [1,2,3]. Jones et al. [4] reported *Xanthomonas euvesicatoria* (Xeu), *X. vesicatoria* (Xv) *X. perforans* (Xp) and *X. gardneri* (Xg) as the major causal agents of BLS. Other organisms reported to be pathogenic on tomato include *X. campestris* pv. *raphani* [5] and *Xanthomonas arboricola* [6,7]. Diseases caused by pathogenic bacteria still occur at unacceptably high frequencies in both industrialised nations and developing countries. Recent BLS outbreaks have been reported in Tanzania [7]. Large number of species and races of the BLS-associated xanthomonads and symptom similarities with those caused by other bacterial and fungal plant pathogens have made the diagnosis of the disease complicated. Different protocols such as serological tests [8,9,10], semi-selective media [11,12,13], biochemical tests [14], nucleic acid analysis [7,15,16,17,18], and pathogenicity tests [19,20] have been reported to detect the BLS-causing xanthomonads in tomato. However, most of these procedures are complicated, costly and often rely on isolation of pure bacterium culture, DNA extraction and testing methodology. Therefore, development of low cost and simple tools for effective sampling, handling, storage and testing was needed for better detection and diagnosis of the BLS pathogens from diseased plants.

Flinders Technology Associates (FTA®) plant card is a Whatman paper that has been treated with a patented chemical formulation that lyses cells, captures and immobilizes nucleic acids in the paper matrix. In addition to having denaturing and chelating properties, a free radical trap, nucleic acid damage is prevented (www.whatman.com). The chemicals are non-organic and safe to use [21]. Successful application of FTA in sampling, recovery and molecular characterisation studies has been reported [21,22,23,24,25,32,33,38]. In this study, we developed a sample preparation protocol collected on FTA plant cards or Whatman chromatography paper (Whatman International Ltd, Maidstone, England) that is also referred to as Whatman paper strip (WPS) and used as template in PCR. The bacterial DNA templates prepared from artificially-inoculated and naturally-infected field tomato plants were compared with those from the pure bacterial cultures in PCR. The results showed

successful application of the FTA plant cards in sample preparation for the PCR detection of BLS-causing xanthomonads of tomato in field surveys.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Four bacterial strains namely *Xanthomonas euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321 and *X. gardneri* NCPPB 881 (NCPPB= National Collection of Plant Pathogenic Bacteria, UK), representing the four main groups of BLS-causing xanthomonads of tomato [4], were used is this study. *Pseudomonas corrugata* NCPPB 2445 was included and used as a negative control in some experiments. All bacterial strains used in this study were obtained from NCPPB, Sand Hutton, York, United Kingdom. Strains were grown on NA medium (meat extract 3 g, Bacto Peptone 5 g, Bacto Agar 20 g, distilled water 1000 ml) for 24-48 h at 28ºC. The strains of *Xanthomonas* were used for the inoculation of tomato seeds used in the preliminary evaluation of the sampling methods.

2.2 Seed Inoculation with *Xanthomonas* Strains

One-thousand seeds of tomato cultivar Tanya were surface-disinfected in 70% ethanol for 1 min, followed by dipping in 1% sodium hypochlorite for 3 min and rinsed three times in sterile distilled water. After disinfection, the seeds were transferred to Petri dishes containing sterile filter papers and allowed to air-dry for 6 h in a laminar flow chamber. The surface-disinfected seeds (500 seeds per bacterial strain) were inoculated with bacterial suspensions of 10^8 CFU/ml ($OD_{600} = 0.01$) prepared from 24-48 h-old cultures of *X. vesicatoria* NCPPB 422, *X. gardneri* NCPPB 881, *X. euvesicatoria* NCPPB 2968 and *X. perforans* NCPPB 4321 strains; OD600 values were measured using a Nanodrop (ND 100 Spectrophotometer, NanoDrop Technologies, Inc). The inoculation was done by vacuum infiltration of tomato seeds for 30 min with 5 ml of the bacterial suspension. After infiltration, the inoculated seeds were allowed to air-dry under the laminar air flow cabinet for 6 h and stored at 4-10ºC until use.

2.3 Growing of Plants

Eight inoculated seeds per bacterial strain were sown in pots of 8 cm diameter containing sand and peat soil (Pindstrup substrate No.2, Pindstrup Mosebrug A/S, Denmark) (1:3), respectively. The pots were covered with polyethylene bags and kept in the growth chamber at 28ºC under high relative humidity (>85%). Seeds treated with sterile saline water (0.85% of NaCl) were used as negative control. After seven days, the polyethylene bags were removed from the pots, and the seedlings were allowed to grow for up to 28 days after sowing.

2.4 Sampling of DNA using FTA Plant Card and WPS

Two types of membranes, Flinders Technology Associates (FTA®) plant card (Cat No. WB120065) and Chromatography paper 3MM Chr (Cat No. 3030614) (Whatman International Ltd, Maidstone, England) that we referred as Whatman paper strip (WPS) were tested in this study. Two methods of extraction were used to prepare sample from infected plants. In the first method, one gram of leaf tissue from 28 day-old diseased tomato plants was crushed in 3 ml of sterile distilled water using sterile mortars and pestles. Plant extracts

of 75 µl were applied onto FTA plant cards and WPS. In the second sampling method two-three diseased leaf samples were macerated with the help of fingers in a plastic bag and the plant sap was pressed through a small hole made with a sterile needle onto FTA plant cards and WPS. In both methods, the paper membranes were allowed to air-dry for 1h under laminar air cabinet and then stored at room temperature (25 ± 2ºC) until use.

2.5 Recovery of Bacteria from FTA Plant Card and WPS

Three discs were removed from the dried spotted areas from each of the FTA plant card and WPS using a 2-mm-punch (Harris Micro Punch®). The individual discs were each placed into PCR tubes with 200 µl of sterile distilled water for 15 min and thoroughly mixed by pipetting. Aliquots of 75 µl were streaked onto NA plates using a sterile triangular glass rod. Samples spotted by sterile distilled water served as a negative control. Bacterial suspensions from 24-h-old cultures from the reference strains of the four *Xanthomonas* species were used as positive controls. A suspension prepared from *P. corrugata* NCPPB 2445 was used as a negative control.The plates were incubated at 28ºC for 96 h. The bacterial colonies were counted, and four putative yellow colonies with morphology similar to the reference strains were transferred to the NA plates. The identity of the isolates was initially conducted by Gram reaction [14]. Gram-positive isolates were discarded. Further identification was conducted using the Biolog identification system, pathogenicity tests and PCR with the Xan7 primers.

2.6 Biolog Identification

Gram-negative bacteria recovered from the membranes were identified using Biolog Identification System (MicroLog™ 2, version 4.2, Biolog Inc., Hayward, CA, USA). Single colonies of each isolate cultured on NA plates were inoculated onto Biolog Universal Growth medium plates and incubated for 24 h at 28 ºC. Colonies were harvested with a wet sterile 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 microplates. Readings of inoculated Biolog GN plates 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 28ºC. An isolate was considered to be identified if the similarity index obtained was of at least 0.5 after 24 - 48 h. The closest next match in identification was not attempted.

2.7 Pathogenicity Tests

Pathogenicity tests of the yellow-pigmented bacteria recovered from the WPS were conducted with four 14-day-old tomato seedlings cv. Tanya *per* isolate. Tomato seedlings were spray-inoculated to runoff (on their abaxial leaf surface) with bacterial suspensions of 10^8 CFU/ml (OD_{600} = 0.01) prepared from 24-h-old bacterial cultures. Inoculated seedlings were then covered with polyethylene and kept in the growth chamber bags for seven days. The seedlings were left in the growth chamber for up to 14 days after inoculation. Seedlings sprayed with sterile saline water (0.85% of NaCl) were used as negative control, and seedlings sprayed with suspensions prepared from each of the four reference xanthomonads served as positive controls. The plants were examined for symptoms 14 days after inoculation, and scored as negative when no obvious symptoms were observed [26]. Water-soaked lesions, which developed into dark brown spots on the leaves, were scored as positive for BLS symptoms.

2.8 Preparation of Bacterial DNA from Pure Cultures on FTA Plant Cards and WPS

To avoid DNA extracts of the bacteria isolated from WPS, bacterial colonies from NA plates were spotted onto an FTA plant card and WPS by suspending a loopful of 24-h-old bacterial growth in 500 µl of sterile distilled water in an Eppendorf tube and vortexing for 30 s. The bacterial suspension of 50 µl was applied as a single spot on the paper, and the paper was air-dried for 1 h at room temperature. The spotted FTA plant cards and WPS were stored at room temperature until use. Using a Harris Micro Punch instrument, discs 2-mm in diameter were taken from spots made on the FTA plant card and WPS. To avoid contamination, the punch was cleaned three times in soapy water and rinsed thrice with sterile distilled water, followed by brief drying on the flame prior and after making each punch. Processed FTA plant card punches were either assayed immediately or stored at 4ºC.

2.9 PCR Tests

The sample discs were each placed in separate Eppendorf tubes. The discs from the WPS were used directly, whereas the FTA plant card discs were washed and air-dried for 1 h at room temperature following the manufacturer's instructions (www.whatman.com). Detection of the BLS-causing xanthomonads was done by PCR using genus *Xanthomonas*-specific primers Xan7(X-gumD-fw 5`GGCCGCGAGTTCTACATGTTCAA and X-gumD-rv 5`CACGATGATGCGGATATCCAGCCACAA) developed by Adriko [28]. The PCR amplification was conducted by placing the discs in 25-µl PCR master mix containing 12.75 µl of sterile distilled water, 1.5 µl of 1.5 mM $MgCl_2$, 5 µl of buffer 5x, 4 µl of 1.25 mM dNTP, 0.25 µl of 40 µM of each primer; and 0.25 µl of 1.25 units of *Taq* polymerase (Promega™, Madison, Wisconsin, USA. In a multiplex PCR, 0.25 µl of 40 µM host mitochondrial (m26S F4/R3) primers (m26S F4 5'ACCAGGGGGTAGCGACTGTTTATT and m26S R3 5'CCCCAGGATGTGATGAGTCGACAT) [28] were included in a master mix as internal control. Reactions were run in an Eppendorf mastercycler gradient PCR machine (Eppendorf 22331, Hamburg, Germany) for 40 cycles, each consisting of 30 s at 95°C, 15 s at 66°C, and 20 s at 72°C, with initial denaturation of 3 min at 95°C and final extension of 3min at 72°C. The amplified PCR products of 9-µl were run on a 1.5 % agarose gel pre-stained with 12 µl of 0.5 µg/ml ethidium bromide at 50 V in 0.5x TBE (Tris-borate EDTA) buffer for 40 min and visualised on a UV transilluminator. Photographs were taken using Fujifilm instant black and white Professional Polaroid film.

2.10 Sensitivity of the PCR Assay with FTA and WPS Spotted with Plant Sap

Plant sap from artificially-inoculated tomato plants cv. Tanya was serially-diluted in healthy plant sap in a ratio of 1:5, 1:25, 1:125, 1:625 and 1:1325 (diseased plant: healthy plant). Diluted saps of 75 µl were spotted onto the FTA plant cards and WPS. The paper membranes were allowed to air-dry as previously described, stored at room temperature and used in the PCR reactions. To estimate the number of cells printed on the FTA plant card and WPS, samples of 75 µl in duplicates from each dilution were also plated on NA and incubated at 28ºC for 96 h. Sap prepared from healthy plants was used as a negative control. Suspensions from 24-h-old bacterial cells of the four reference strains grown on nutrient agar plates were prepared in sterile saline solution (0.85% NaCl) to obtain colony counts of 10^8 CFU/ml (OD_{600} = 0.01) and were used as positive controls for the test. Total bacterial counts on the inoculated NA plates were conducted 96 h after incubation. Putative yellow-pigmented colonies with a similar morphology as that of the reference strains were

tested by PCR using DNA templates spotted on the FTA plant card and WPS. The PCR detection limits with the plant tissue prints was compared to the results of the serially-diluted samples on the plant FTA cards and WPS, which were related to total numbers of the bacterial cell counts. FTA plant card and WPS printed with sterile saline solution served as a negative control for the reactions.

2.11 Field Surveys, Collection and Testing of Samples on FTA Plant Cards

Field surveys were conducted in the main tomato-growing areas in Tanzania during March-April 2010 using the DNA samples collected on the FTA plant cards only. A total of 117 farms were visited. The survey covered 27 farms in Iringa, 22 in Mbeya, 18 in Tanga, 15 in Morogoro, 15 in Kilimanjaro, 13 in Dodoma, 5 in Arusha regions and 2 in Mara regions. Within each tomato farm, the incidence of BLS was assessed in five 2 x 2 m^2 quadrants, one at each corner, and at the centre of the field [3]. An average of 10 plants was assessed along a diagonal line within each quadrant, and the number of plants showing typical symptoms of BLS was recorded. The BLS incidence in each field was calculated in percentage by dividing the number of infected plants over the total number (10 plants x 5 quadrants) of plants sampled in the surveyed fields.

From plants assessed in each farm, 3-4 leaf/fruit samples from suspect BLS infected plants were randomly picked and packed in transparent plastic bags. A part of each sample from the collected plant material was macerated by hand while placed in a plastic bag, and extracts were pressed through a small hole made with a sterile needle. Samples were directly spotted on FTA plant cards. The papers were allowed to air-dry under field conditions for 15 min. The remaining sample and FTA plant cards were transported to the African Seed Health Centre laboratory based at Sokoine University of Agriculture, Morogoro, Tanzania, for the isolation of the BLS-causing xanthomonads.

2.12 Isolation of Xanthomonads from Tomato Field Plants with BLS Symptoms

Isolation of the BLS-causing xanthomonads from tomato samples was conducted in a concurrent study aimed to characterise and document the presence of xanthomonads associated with BLS in Tanzania (data not shown). A small piece of 1 x 1 mm^2 was removed from margin of a leaf and placed on a drop of sterile distilled water on a sterilized glass slide and was covered by a cover slip for observation of bacterial ooze under a compound microscope. Samples found positive for bacterial ooze were used for isolation of bacteria. To isolate the bacteria, a small piece from the margin of the lesion was removed and placed onto a sterile glass slide with a drop of sterile distilled water; bacteria was allowed to rest for 60 s, and a loopful of the suspension was streaked in duplicates onto NA medium plates. The inoculated plates were incubated at 28 ºC for 96 h. Four suspect pale-to-yellow pigmented *Xanthomonas*-like colonies *per* sample were isolated and transferred onto the NA plates for further testing. Pure cultures of putative *Xanthomonas* isolated from the tomato samples were tested for Gram-reaction using 3% Potassium hydroxide (KOH) solubility test [14]. The pure bacterial cultures of the Gram-negative bacterial isolates were then printed on FTA plant cards and WPS and were tested by PCR.

3. RESULTS

3.1 Recovery of Bacteria from FTA Plant Card and WPS

Xanthomonads, other yellow-pigmented and cream Gram-negative bacteria were only recovered from the WPS samples (Table 1). The yellow-pigmented isolates were identified by Biolog system as members of the genera *Xanthomonas*, *Sphingomonas* and *Pantoea*, while cream isolates were identified as *Pseudomonas* (Table 1). Results of Biolog identification of isolates recovered from the inoculated WPS membrane as xanthomonads were similar to the results obtained for the reference *Xanthomonas* strains. The strains were identified as *X. c* pv. *dieffenbachiae, X. c. pv begoniae* B, *X. c.* pv. *dieffenbachiae* and *Xanthomonas* sp, respectively (Table 1). Isolation of the yellow-pigmented bacteria and xanthomonads from WPS was possible one week after storage at room temperature (data not shown). Further attempts were not made for the recovery of the bacteria under prolonged storage conditions. No xanthomonads were recovered from the FTA plant cards printed with artificially inoculated tomato plants with the four reference strains of the bacterial leaf spot pathogens of tomato. However, only a few white-cream pigmented bacterial colonies were recorded which were restricted to the point of inoculation on the NA plates. These bacteria were Gram positive in the KOH-solubility tests and therefore were not tested by PCR (Table 1).

The bacterial isolates identified by Biolog system as xanthomonads appeared to be pathogenic on tomato plants and induced similar leaf spot symptoms as those observed in plants inoculated with the four *Xanthomonas* reference strains (Table 1). The yellow-pigmented bacterial isolates identified by Biolog as *Sphingomonas* and *Pantoea* species did not induce disease symptoms on the inoculated tomato plants (Table 1). Disease symptoms were not observed on seedlings sprayed with sterile saline water (containing 0.85% of NaCl) control.

3.2 PCR Tests

PCR products of 402 bp were obtained from the bacterial DNA templates spotted on both FTA plant cards and WPS from pure cultures of bacterial suspensions of the four *Xanthomonas* reference strains using the genus-specific primers Xan 7 (Table 1). Irrespective of whether the sample was obtained by grinding or by direct maceration of plant tissue in plastic bags prior to application on FTA membranes, the PCR amplified the target bacterial DNA (Fig. 1). Suspensions added to the FTA plant card from tissue maceration or from pure cultures resulted in more clear PCR bands than those from the WPS samples. PCR obtained by grinding the BLS-infected plant tissue samples prior to printing on the FTA plant cards produced two bands in a multiplex PCR reaction, indicating DNA amplifications of both *Xanthomonas* spp. (402 bp) and host plant mitochondrial rRNA (594 bp) (Fig. 2 A-i). For PCR with BLS infected samples collected onto WPS, only one amplicon of 402 bp corresponding to the bacterial DNA was observed (Fig 2 B-i). The results also showed that host primers set (m26S F4/R3) failed to amplify host plant DNA on FTA plant cards and WPS samples (Fig 2 A-ii and B-ii) printed directly with sap from hand maceration conducted in plastic bags. However, bacterial DNA of the leaf spot-causing pathogens was amplified irrespective of the paper membrane used. Even though the use of host primers as internal controls to PCR reactions did not seem to work with the samples processed by hand maceration in plastic bags, the technique was selected for field sample collection as it was

easy to apply under field conditions, where tools for grinding and sterile distilled water are difficult to obtain and/or use.

3.3 PCR Sensitivity Tests on FTA Plant Card and WPS

Detection limits of PCR were tested using prints on the FTA plant card and WPS with a series of 5-fold dilutions of plant sap from infected tomato plant (Table 2). The detection limits were 1:625 from the FTA plant card and 1:25 from WPS, respectively. Dilution of 1:625 was corresponding to bacterial colony counts on NA of as low as 2.65×10^2, 4.30×10^2, 4.56×10^2 and 4.59×10^2 CFU/ml for *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*, respectively, and dilution of 1:25 was corresponding to $> 1 \times 10^3$ CFU/ml of bacteria on NA (Table 2). The PCR products were amplified from FTA and WPS samples printed with suspension of pure bacterial cultures of the reference strains irrespective of paper membranes used. The results demonstrated the suitability of application of FTA plant cards in the collection of bacterial DNA samples on field surveys. Therefore, FTA plant cards were selected for application in tomato field surveys in Tanzania.

3.4 Surveys and PCR Tests with FTA membranes

Surveys conducted in the tomato-growing regions indicated that the leaf spot disease caused by xanthomonads was widespread in Tanzania (Table 3). The incidence of bacterial spot during this survey ranged from 2% to > 80% (Table 3). PCR tests showed 22 out of 117 (18.8%) field samples collected on the FTA plant cards tested positive for *Xanthomonas* (Fig. 3). The number of samples positive for BLS-causing xanthomonads per location covered in the current study is shown in Table 3. No attempts were made to re-isolate other pathogens causing leaf spot symptoms from tomato samples.

3.5 PCR Tests with FTA Membranes Templates from Bacterial Cultures

A total of 136 yellow-pigmented Gram-negative bacteria were isolated from infected field samples collected during field surveys conducted in 2008-2010 in Tanzania. Thirty (EMBE 1-30) out of 136 were identified as xanthomonads. The results also showed that both FTA plant card and WPS spotted with xanthomonads suspensions equally produced clear signals in PCR tests (Fig. 4).

Table 1. Recovery and identification of bacterial strains from FTA plant card and WPS prints made from tomato plants inoculated with BLS-causing xanthomonads

Paper membrane /treatment	Bacterial strain	Biolog identification (Sim values)[1]	Total No. CFU in 75 µl on NA[2]	Pathogenicity test (tomato cv. Tanya)	PCR test (Xan 7)
WPS					
	Xanthomonas euvesicatoria NCPPB 2869	*X. campestris* pv. *dieffenbachiae* (0.650)	409	+	+
		Sphingomonas yanoikuyae	11	-	-
	X. vesicatoria NCPPB 422	*X. c* pv. *begoniae* B (0.650)	59	+	+
		Sphingomonas sp.	7	-	-
		Pseudomonas fuscovaginae (0.500)	2	-	-
	X. perforans NCPPB 4321	*X.c* pv. *dieffenbachiae* (0.550)	90	+	+
		Sphingomonas sp.	8	-	-
	X. gardneri NCPPB 881	Genus *Xanthomonas*	85	+	+
		Sphingomonas sp.	5	-	-
FTA plant card[3)]					
	X. euvesicatoria NCPPB 2869	ND	3	NA	+
	X. vesicatoria NCPPB 422	ND	2	NA	+
	X. perforans NCPPB 4321	ND	1	NA	+
	X. gardneri NCPPB 881	ND	2	NA	+
Reference strains [4)]					
	X. euvesicatoria NCPPB 2968	*X.c* pv. *dieffenbachiae* (0.800)	ND	+	+
	X. vesicatoria NCPPB	*X. c* pv. *begoniae* B	ND	+	+

422	(0.520)			
X. perforans NCPPB 4321	*X.c* pv. *dieffenbachiae* (0.650)	ND	+	+
X. gardneri NCPPB 881	*X. c* pv. *begoniae* B (0.530)	ND	+	+
Pseudomonas corrugata NCPPB 2445	*P. corrugata* (0.600)	ND	ND	-
Sterile distilled water	Na	0	-	-

[1]Biolog identification of Gram-negative bacteria only. [2]Each value is an average of counts from three NA plates of three independent discs from prints made from plant sap (1 disc/200 µl of sterile distilled); aliquots of 75 µl were streaked onto NA plates in triplicates. [3]Gram-positive, white-pigmented colonies on FTA discs were discarded. [4]Reference strains from pure cultures suspension plated on NA. WPS = Whatman paper strip; ND = Not done and NA= not applicable. NCPPB = National Collection of Plant Pathogenic Bacteria, UK.

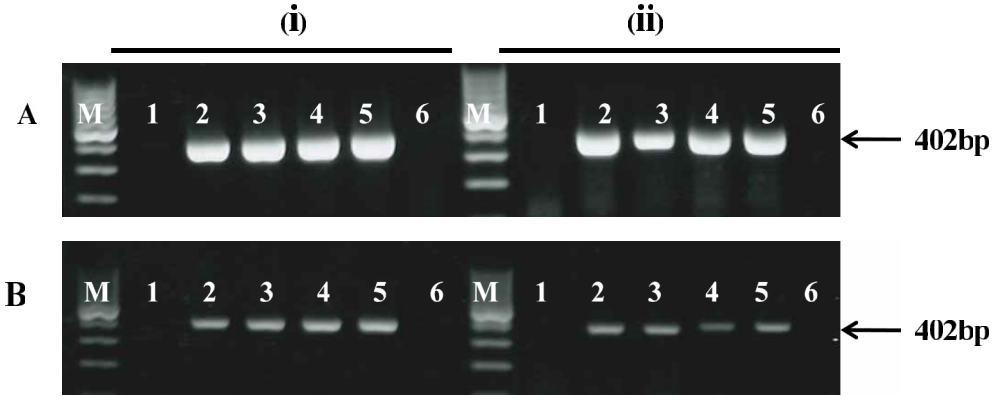

Fig. 1. PCR detection of bacterial genomic DNA of xanthomonads from infected tomato plants on FTA plant cards (A) and Whatman paper strips (B) using *Xanthomonas* genus-specific (Xan7) primers. (i) Leaf prints prepared by grinding of infected tomato tissue; (ii) leaf samples macerated by hand in plastic bag. Molecular weight marker 1000 bp (lane M), water control (lane 1); Lanes 2-6, leaf prints originated from tomato plants infected with *X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321 and *X. gardneri* NCPPB 881, respectively, and healthy plant control (Lane 6). NCPPB = National Collection of Plant Pathogenic Bacteria, UK

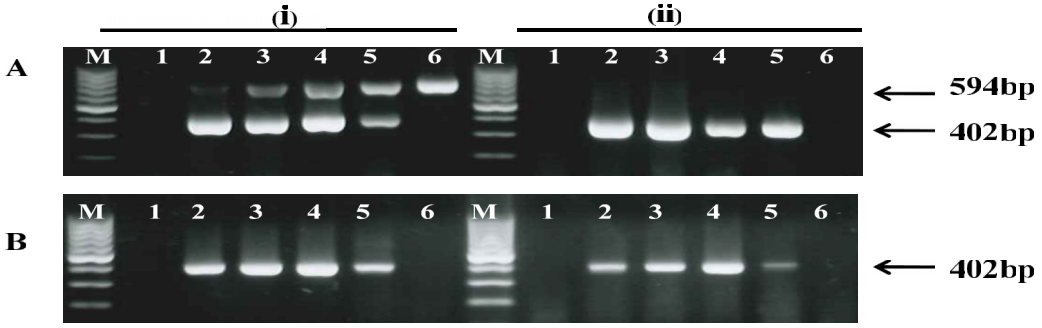

Fig. 2. Multiplex PCR detection of xanthomonads from infected tomato plant tissue with Xan 7 primers (402 bp) and plant M26S primers (594 bp) on FTA plant cards (A) and Whatman paper strips (B). Leaf prints from macerated plant extract by grinding (i) and macerated plant extract in plastic bags (ii). Molecular weight maker, 1000 bp (Lane M), water control (Lane 1); Lanes 2-5, leaf prints originated from tomato plants infected with *X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321, *X. gardneri* NCPPB 881, respectively, and healthy plant control (Lane 6). NCPPB = National Collection of Plant Pathogenic Bacteria, UK

Table 2. Detection limits of *Xanthomonas* PCR-based assays with FTA and WPS membranes used as DNA templates from tomato plant extract

Bacterial strain	Dilution ratio (v/v)	CFU/ml[1]	FTA-PCR[2]	WPS-PCR[2]
Xanthomonas euvesicatoria NCPPB 2968	undiluted	>1000	+	+
	1: 5	>1000	+	+
	1:25	>1000	+	W
	1:125	897	W	-
	1:625	265	W	-
	1: 3125	58	-	-
X. vesicatoria NCPPB 422	undiluted	>1000	+	+
	1:5	>1000	+	W
	1:25	996	+	W
	1:125	876	W	-
	1: 625	430	W	-
	1:3125	130	-	-
X. perforans NCPPB 4321	undiluted	>1000	+	+
	1:5	>1000	+	+
	1:25	>1000	+	W
	1:125	456	W	-
	1: 625	120	-	-
	1:3125	65	-	-
X. gardneri NCPPB 441	undiluted	>1000	+	+
	1:5	>1000	+	W
	1:25	>1000	+	W
	1:125	753	+	-
	1: 625	459	W	-
	1:3125	78	-	-
Healthy plant	Undiluted	0	-	-

[1] Only yellow-pigmented colonies with morphology similar to pure culture of reference strains were counted at different dilutions (v/v) comprising of sap of infected plant material / sap of healthy tomato tissue. Each value is an average of counts from two NA plates. NCPPB = National Collection of Plant Pathogenic Bacteria, UK. [2] PCR tests conducted using genus-specific Xanthomonas PCR Xan 7 primers developed by Adriko, (2011); PCR products generated from serially diluted cells of bacterial leaf spot reference strains: += positive amplification; W = weak band; -= negative amplifications; 75 µl was added to each filter for dilutions 1:5, 1:25, 1:125, 1:625 and 1:1325

Table 3. Evaluation of FTA plant cards in tissue prints from tomato plants showing bacterial leaf spot symptoms conducted in Tanzania in 2010

Locality	Region	No. of fields	Disease incidence range (%)[1]						No. of positive samples[2]
			0	1-20	21-40	41-60	61-80	>80	
Tukuyu	Mbeya	4	1	0	0	1	1	1	0/4
Uyole	Mbeya	18	0	0	2	8	5	3	1/18
Ilula/Kilolo	Iringa	27	0	4	6	7	8	2	4/27
Hai	Kilimanjaro	15	0	2	7	2	4	0	2/15
Tengeru	Arusha	5	0	0	0	2	3	0	3/5
Lushoto	Tanga	18	0	8	6	1	3	0	5/18
Mgeta	Morogoro	10	0	2	3	5	0	0	2/10
Doma	Morogoro	5	0	0	0	3	2	0	2/5
Msalato	Dodoma	6	1	0	1	2	2	0	1/6
Makutopora	Dodoma	7	0	4	3	0	0	0	1/7
Musoma	Mara	2	0	0	0	2	0	0	1/2
Total		117	2	20	28	33	28	6	22/117

[1]Disease incidence range based on leaf/fruit spot symptoms on plants in the growing area of Tanzania.
[2]Leaf samples were spotted on FTA plant cards and used as templates in PCR tests conducted using genus-specific Xanthomonas PCR Xan7 primers developed by Adriko, (2011).

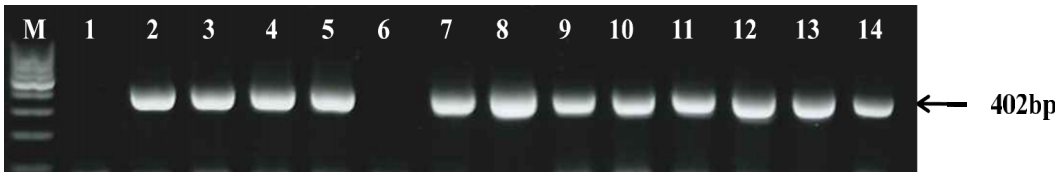

Fig. 3. PCR detection of bacterial genomic DNA of BLS-causing xanthomonads from field tomato plants on FTA plant card using the *Xanthomonas* genus-specific (Xan7) primers. Lanes, M = Molecular weight maker, 1000 bp, Lane 1, water control; Lanes 2-6, leaf prints originated from tomato plants infected with *X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321, *X. gardneri* NCPPB 881 and healthy plant control, respectively; Lanes 7-8, 9-10, 11-12 and 13-14 are leaf prints originated from field tomato plants growing at Mvomero in Morogoro, Msalato in Dodoma, Ilula in Iringa and Lushoto in Tanga region, respectively. NCPPB = National Collection of Plant Pathogenic Bacteria, UK.

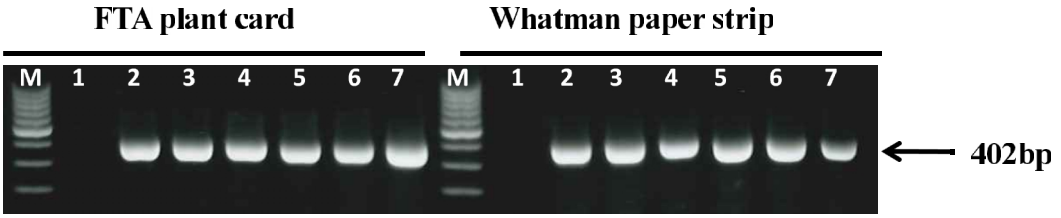

Fig. 4. PCR detection of bacterial genomic DNA of xanthomonads from pure culture suspensions spotted on FTA plant cards and Whatman paper strip using the *Xanthomonas* genus-specific (Xan7) primers. Molecular weight marker 1000 bp (lane M), water control (lane 1); Lanes 2-5, pure culture from *X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321 and *X. gardneri* NCPPB 881, respectively; Lanes 6 and 7, pure cultures from bacteria isolated from infected field tomato plants, EMBE 10 and EMBE 29, respectively. NCPPB = National Collection for Plant Pathogenic Bacteria, UK. EMBE = bacterial strains isolated by Ernest Mbega from infected field tomato plants in Tanzania

4. DISCUSSION

This study demonstrated the application of FTA plant cards and WPS in sampling, handling, storage and detection of xanthomonads associated with BLS in Tanzania. The technologies were evaluated with tomato plants raised from seeds that were artificially inoculated with the four main BLS-causing strains [4] and with tomato field plants from the Northern, Central and Southern Highlands in Tanzania.

Of the two paper membranes used in the study, the WPS appeared to retain live bacterial cells when isolation was done one week after storage at room temperature (data not shown). The Biolog system used to identify members of the genera *Xanthomonas*, *Sphinogomonas*, *Pseudomonas* and *Pantoea* did not appear to be reliable. The Biolog system was not able to identify correctly the four reference strains used as positive controls to the species level, i.e. *X. euvesicatoria* NCPPB 2968, *X. vesicatoria* NCPPB 422, *X. perforans* NCPPB 4321 and *X. gardneri* NCPPB 881 were misidentified as *X. c* pv. *dieffenbachiae, X. c.* pv. *begoniae* B, *X.c* pv. *dieffenbachiae* and *Xanthomonas* sp, respectively. Such observation has been associated with differences between metabolic profiles of the strains used in this study and those of xanthomonads used in the Biolog GN database and as reported by other studies [26,29,30]. Despite of the limitations of the Biolog identification system, it was still a useful and relatively simple and robust tool for the identification of plant pathogenic bacteria when combined with other identification methods [7]

The pathogenicity of all recovered xanthomonads to induce BLS symptoms similar to those observed in plants inoculated with the reference strains of the BLS pathogens implied that the isolated xanthomonads from the WPS were indeed those previously inoculated on the plants. Such observations demonstrated the ability of the WPS to store live bacterial cells that could be used for isolation of the bacterial pathogen for later identification.

The isolation of *Sphingomonas*, *Pseudomonas* and *Pantoea* spp. from the WPS was not a surprise as it is already known that these bacterial can be found in leaf surfaces of tomato both in field and screen house conditions [7,31]. Gram-negative bacterial cells were not recovered from FTA plant cards even if the FTA plant cards were immediately used after

sampling. In general such observations indicated the suitability of FTA in handling quarantine organisms as only DNA, not the live bacterial cells, were stored in the membranes. Several studies have demonstrated the successful application of FTA technology in many biological systems [21,22,23,24,32,33]. Therefore, FTA plant card can be a reliable tool for collection, handling, storage and retrieval of xanthomonads DNA for PCR assays as bacterial DNA was retained on the FTA plant card.

This study also demonstrated that both FTA plant card and WPS can be used for the detection of xanthomonads causing bacterial leaf spot in tomato. However, amplification of the target DNA from FTA plant cards was stronger than from WPS. Such observations might be due to the quality of paper used. FTA plant cards are treated with patented chemical formulations (www.whatman.com). These chemicals are reported to contain strong buffers, free radical traps and protein denaturants that lyses cell membranes on contact, and physically entrap DNA, and stabilize and protect DNA from nuclease, oxidation, UV damage and from degradation [21,34,35]. Unlike FTA plant cards, WPS samples are neither treated, nor washed, giving it high possibilities for PCR inhibition by free radicals or samples being degraded through denaturation.

The study has also demonstrated the importance of sample preparation and its impact on the retrieval of plant DNA on FTA plant card and WPS. Pounding the leaf material directly onto the FTA paper by applying moderate pounding/pressure with a blunt object such as a pestle [36,37] has been recommended but is usually difficult to apply under field conditions. In our study, we tested two other sample preparation methods. The first method involved grinding the BLS-infected plant tissue samples prior to printing them on the FTA plant cards and WPS. The detection of both bacterial and host DNA in a multiplex PCR reaction with FTA plant card (but not WPS) implied the FTA preserved DNA better. Inclusion of internal amplification control appeared to be suitable for evaluation of false negative results. However, implementation of this method under field condition is difficult. The second method which involved macerating the samples by hand in plastic bags prior to printing on FTA plant card was tested and selected for use in field survey even though only bacterial DNA was amplified. This technique allowed the detection of the target xanthomonads by PCR tests. This is simple and fast, and it facilitated the collection and handling of many samples while minimising the risks of contamination. The failing of host PCR primers in amplifying the plant DNA from macerating the sample by hand while in a plastic bag prior to application to FTA plant cards and WPS could be due lack of stability of the rRNAs or their genes in storage condition, or existence of plant inhibitors such as polyphenolic compounds and polysaccharides [37].

The PCR detection limits was as low as 1:625 for the FTA plant card compared to 1:25 of the WPS, indicating the FTA plant card is more efficient in DNA preservation. Therefore, the results further proved suitability of the FTA plant cards for collection of DNA from field samples.

The FTA plant cards were proved to be useful in sample preparation by evaluation of field samples. The high incidence (> 80%) of leaf spot symptoms observed during the current survey could probably be explained by the presence of other disease-causing organisms which induce similar symptoms in tomato plants. The similarities of symptoms caused by other microorganisms such as pseudomonads on tomato leaves have been reported [1,2]. Such results indicate the significance of using PCR in the screening of tomato samples suspected of being infected with the BLS-causing xanthomonads. Use of FTA plant cards

along with PCR tests can provide quick and reliable means for detection of the BLS-causing xanthomonads, thus avoiding confusion with other leaf spot-causing organisms of tomato.

In the present study, we have also demonstrated that both FTA plant cards and WPS can be used for sample preparation for PCR in quick screening of pure cultures for identification of the BLS-causing organisms to the genus level. We have shown that PCR tests produced clear and similar signals from the FTA plant cards and WPS spotted with suspensions of xanthomonads. The use of FTA plant cards or WPS with pure bacterial culture provide a fast means of screening bacterial cultures without extraction of DNA. This technology can be useful for storage of DNA from bacterial isolates infecting tomato plant samples. In our study, PCR amplification of the xanthomonad DNA fragment was possible from FTA plant card samples spotted with suspensions of pure cultures or plant sap that had been stored under room temperature for about three years (data not shown). Detection of the BLS-causing xanthomonads from samples printed on WPS was very inconsistent, and DNA amplification was rarely possible after one year of storage at room temperature (data not shown).

5. CONCLUSION

In conclusion, WPS appeared to have the ability to retain live bacterial cells up to one week of storage at room temperature. Since both paper membranes did not require an additional DNA extraction step prior to PCR tests, a fast screening of bacterial cultures by PCR was achieved. However, the FTA plant card is recommended for collection of bacterial DNA templates as they produced a more clear PCR products than WPS. The FTA plant cards can be used for quick screening of BLS-causing organisms of tomato both in infected field plant samples and pure cultures of BLS xanthomonads. The WPS could also be used along with FTA plant cards when re-isolation of the bacterial agents is required. Collection and handling of the tomato samples with FTA plant cards that could be stored at ambient temperature is easy and, therefore, it can be used by extension workers, plant pathologists and plant quarantine agents. Future studies may be needed to improve the application of FTA plant card in multiplex PCR for simultaneous detection of BLS xanthomonads. The successful detection of the BLS organisms could be explained in part by the robust nature of the PCR tests which need limited target templates for amplification. The successful amplification of the bacterial genomic DNA targets directly from washed membrane discs gives possibilities for further exploration of application of the FTA technology in sampling of bacterial DNA for PCR tests in the detection of other plant pathogens of vegetable crops in Tanzania and other countries.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

We thank the Danish Development Assistance (DANIDA) through ENRECA project No. 731 for providing funds for this research and Hanne Nielsen for technical assistance.

COMPETING INTERESTS

Authors declare that no competing interests exist.

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