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Full Length Research Paper

Identification and characterization of strains of *Xanthomonas campestris pv. Vesicatoria* from Tanzania by biolog system and sensitivity to antibiotics

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Thirty five strains of *Xanthomonas campestris* pv. vesicatoria collected from different tomato-producing areas in three Regions (Morogoro, Arusha and Iringa), representing three different ecological conditions in Tanzania were characterized using their carbon source utilization profiles by the GN Microplate system, and their sensitivity to antibiotics. Although most of the strains could not be identified by the Biolog system as *X vesicatoria pv. vesicatoria*, the strains were found to differ in their pattern of carbon source utilization, and were clustered into three major groups. A similar pattern was observed in antibiotic sensitivity of the strains. The results indicated the presence of variations within the Tanzanian populations *of X. campestris pv. vesicatoria* on the basis of carbon source utilization patterns and their sensitivity to antibiotics. These findings also indicate the existence of *X. campestris* pv*. vesicatoria* strains in Tanzania that are different from those included in the Biolog database.

Key words: Bacterial spot, tomato, Tanzania, Biolog, antibiotics.

INTRODUCTION

The bacterial spot disease of tomato (Lycopersicon esculentum Mill.) is caused by *X.c. pv. vesicatoria* (Doidge) Dye. The disease is considered to be a major constraint to tomato production all over the world (Zitter, 1985; Watterson, 1986; Scott et al., 1989; Yu et al., 1995; Venette et al., 1996, Blancard, 1997). It attacks every part of the tomato plant. Symptoms of bacterial spot appear on leaves, flowers, petioles, stems and roots. Infection on leaves causes defoliation, resulting in reduced marketable fruit weight for both staked and unstaked tomatoes (Dougherty, 1978; Pohro-nezny and Volin, 1983), and increased exposure of fruits to sun scald. But the main economic effect of the disease is the reduction in fruit weight and quality. Bacterial spots on the fruits have been shown to account for up to 52% weight loss in infected fruits (Jones et al., 1986). Resistance to bacterial spot in the host plant has been reported to be the most effective means of control (Yu et al., 1995, Blancard, 1997). Other methods of managing the disease include field sanitation and seed treatment with sodium hypochlorite (CABI, 2005).

It is not clear when bacterial spot of tomato was first detected in Tanzania, but field surveys conducted in the country in 1997 and 1998 (Black et al., 2001) showed that the disease was widespread in tomato and pepper fields in all the vegetable-growing regions of the country. The incidence of the disease was found to vary widely between years and fields, and ranged between <5 – 90%. Other surveys conducted in 1998-2000 in Arusha, northern Tanzania (Kaaya et al., 2003) confirmed the widespread occurrence of bacterial spot disease in the region.

Various investigations have shown that there is wide degree of variation within *X.c. pv. vesicatoria* (Stall et al., 1994; Vauterin et al., 1995). The presence of sub-specific variation in the pathogen has proved to be a major obstacle to the effective use of resistant tomato varieties in the management of bacterial spot disease. Therefore, one of the key pre-requisites for breeding of tomato varieties with resistance to the disease in any given environment is determination of variation within the existing populations of the pathogen. The present study, therefore, aimed at characterizing the strains of *X.c.* pv.

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Table 1. A list of antibiotics used to screen the sensitivity of *Xanthomonas campestris pv. vesicatoria* strains collected from Tanzania.

Antibiotic	Solvent	Concentration
Gentamycin	Water	50 µg/ml
Chloramphenicol	Absolute alcohol	20 µg/ml
Polymixin B sulphate	Water	50 µg/ml
Streptomycin sulphate	water	50 µg/ml
Lincomycin hydrochloride	water	10 µg/ml
Vancomycin	water	50 µg/ml
Phosphomycin	water	50 µg/ml
Bacitracin	water	50 µg/ml
Ampicillin	water	50 µg/ml
Cephazoline	water	10 µg/ml

vesicatoria from Tanzania by Biolog and sensitivity to antibiotics.

MATERIALS AND METHODS

Collection of diseased tomato samples

Diseased tomato samples showing typical bacterial spot symptoms were collected randomly from 30 locations in the tomato-growing areas of Tanzania. The survey covered 10 locations in Arusha, two in Morogoro, and 19 in Iringa Regions. From each field, tomato fruits showing typical bacterial spot symptoms were collected, placed in paper bags and brought to the laboratory for the isolation of the target pathogen. From the samples collected, thirty five strains of were isolated.

Bacterial isolation

Tissue segments of about 2 mm^2 were excised from advancing lesion margins on symptomatic tomato fruits. The tissue segments were teased in a few drops of sterile distilled water (SDW), and allowed to stand for 10-15 min in a lamina airflow chamber. A loopful of the resulting suspension was streaked onto plates of nutrient agar (NA) and incubated for 72 h at 25-28°C and observed for colony growth.

Presumptive colonies of the pathogen (pale to strongly yellow, mucoid colonies) were purified by sub-culturing single colonies, following procedures described by Bradbury (1970). One colony of the purified presumptive pathogen from each sample was selected and retained on NA slants for further tests.

Identification of bacterial strains

Presumptive *X. c.* pv. *vesicatoria* colonies and reference strains of the pathogen were identified using physiological and biochemical characteristics, including Gram reaction, nitrate reduction, Kovac's oxidase reaction, starch hydrolysis, oxidative metabolism of glucose and the hypersensitive reaction on tobacco. In addition, the bacteria were subjected to pathogenicity tests on tomato cv. Tanya (a tomato variety with susceptibility to bacterial spot).

Carbon source utilization

Preparation of bacterial inoculum, incubation and analysis: A typical colony was selected from each *X. c* pv. *vesicatoria* strain on nutrient glucose agar (NGA) plate and streaked on Tripticase Soy agar (TSA). After about 48 h, the bacteria were harvested from the TSA plates and suspended in 25 ml sterile 0.8 % saline solution in centrifuge tubes. Harvesting of the bacteria was done with a moistened cotton swab. The swabs were rolled gently across the surface of the medium to avoid any removal of the agar medium along with the bacteria. The harvested bacteria were washed by centrifuging at 6000 x g for 18 min to remove extracellular polysaccharides, cell debris and other exogenous substances which might be catabolised by the bacteria to produce false positives (Adams and Martin, 1964; Black and Sweetmore, 1994). The resulting pellets were re-suspended in about 20 ml of sterile saline. The suspension was adjusted to an optical density (OD) of 0.3 at 620 nm (corresponding to about 108 cfu ml-1). The GN Microtiter plates were pre-warmed at 28 ± 1℃ for about 10 min, before they were inoculated with the *X. c. pv. vesicatoria* suspend-sion at 150 µl per well, using a multi-channel pipette. The inoculated plates were then incubated at 28 ± 1℃. Utilization of the carbon source substrates was evaluated visually at 24 and 48 h. Reduction of the tetrazolium dye to violet formazan was recorded as positive for utilization of the compounds. Based on the differences in intensity of the purple colour, the reactions were categorised into four classes as strongly positive (class 3), moderately positive (class 2), borderline (class 1) and negative (0).

Data were analysed using the programme for bacterial identi-fication (Biolog's Microlog TM1, Release 3.50, Biolog Inc., Hayward, CA). A similarity index of ≥ 0.5 was considered a correct identi-fication for the *X. c. pv. vesicatoria* strains. Characterisation of the strains for their diversity and clustering was done by subjecting the data to hierarchical analysis using SAS version 9.1 statistical soft-ware (SAS Institute Inc, Cary, NC, USA). The resulting dendogram is presented as Figure. 1.

Sensitivities to antibiotics: Ten antibiotics were screened for their ability to inhibit the growth of *X. c. pv. vesicatoria* in vitro. Thirty *X. c. pv. vesicatoria* strains were used in the experiment. The antibiotics used were gentamycin sulphate, chloramphenicol, polymixin B sulphate, streptomycin sulphate, lincomycin hydrochlo-ride, vanco-mycin, phosphomycin, bacitracin, ampicillin and cepha-zolin. These were prepared as shown in Table 1.

Ready-made paper discs of about 6 mm diameter used in the experiments were purchased from the open market. Bacterial strains evaluated for sensitivity to antibiotics were prepared by suspending a loopful of 48 h – old bacterial culture in 20 ml of sterile distilled water (SDW). The resulting bacterial suspension was then adjusted to an optical density of 0.06 at 620 nm (which corres-ponded to ca. 108 cfu ml-1). One micro litre of the bacterial sus-pension was spread on NGA surfaces in Petri dishes using a Drigal-

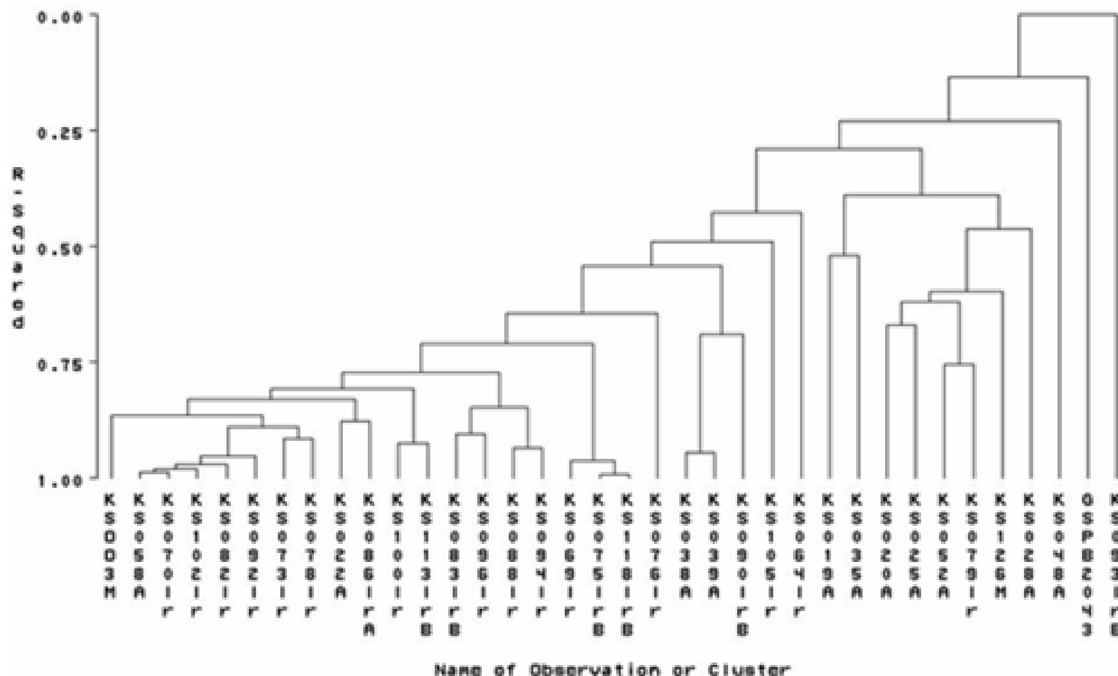

Figure 1. Grouping of *Xanthomonas campestris* pv. vesicatoria strains from Arusha, Iringa and Morogoro, Tanzania based on metabolism of 95 carbon sources by the Biolog database.

ski spatula. Then, paper discs were soaked in the antibiotic solution, laid on the agar surface and labelled accordingly. Inoculated plates were incubated at 28°C for 48 h and observed daily for growth inhibition zones around the paper discs. Radial inhibition zones around each disc, corresponding to the level of antibiotic activity for each treatment, were measured using Verneer Callipers. Data obtained were subjected to hierarchical analysis using SAS version 9.1 statistical software (SAS Institute Inc, Cary, NC, USA). A dendogram showing relationship between the *X.c. pv. vesicatoria* strains on the basis of sensitivity to antibiotics was prepared.

RESULTS

Bacterial strains

Thirty five strains of *X. c. pv. vesicatoria* were collected from Arusha, Iringa and Morogoro regions of Tanzania. A summary of the *X. c. pv. vesicatoria* strains collected and their reaction to various characterization tests is presented in Table 4. All strains tested were pathogenic on tomato cv. Tanya, causing dark brown lesions on leaves.

Carbon source utilization

The 35 strains of *X. c. pv. vesicatoria* differed widely in utilization of carbon sources in the MicroPlates. Twenty three carbon sources (D-fructose, α-D-glucose, D-manose, methyl pyruvate, monomethyl succinate, Cis-aconitic acid, D-gluconic acid, D,L-lactic acid, bomo succinic acid, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine hydroxy L-proline, L-ornithine, Urocanic acid, Inosine, glycerol and glucose-6-phosphate) were utilized by all the *X. c. pv. vesicatoria* strains tested, and only one substrate (I-erythritol) was not utilised by any strain. Differences in the carbon source utilization patterns of the *X. c. pv. vesicatoria* strains tended to be influenced by geographical origin (Figure 1). Although the differences in this pattern were not significant in most cases, statistically significant differences ($P \leq 0.05$) were observed in the way strains from Morogoro, Arusha and Iringa utilized 10 of the carbon sources (Table 2). Whereas none of the *X. c. pv. vesicatoria* strains from Morogoro utilised γ-hydroxybutyric acid, 54.5 and 40.9% of the strains from Arusha and Iringa, respectively, utilised the carbon source (Table 3). A similar trend was observed in the way the strains utilised α-ketobutyric acid. Statistically significant differ-ences ($P \leq 0.05$) were also observed in the way the strains utilised Cebacic acid, Hydroxy L-proline, L-leucine, L-phenyl alanine, L-threonine and 2-amino ethanol (Table 2).

The results of this study have shown that identification of the *X. c. pv. vesicatoria* strains from Tanzania, and the reference strains from GSPB (Göttingen, Germany) and Serbia, using the Biolog Identification System was very poor, as none of the strains was identified correctly. The names given with similarity values below 0.500 refer to closest-matched identity given by the Biolog system. Out of the 34 *X. c. pv. vesicatoria* strains from Tanzania, 15

Table 2. Carbon source utilization patterns of *X. c.* pv. *vesicatoria* strains from Arusha, Iringa and Morogoro regions of Tanzania.

Region	No. of strains	α-Cyclodextrin	D-Galactonic acid lactone	γ-Hydroxybutyric acid	α-Ketobutyric acid	Cebacic acid	Hydroxy L-proline	L-leucine	L-phenyl alanine	L-threonine	2-amino ethanol
Morogoro	2	50.00*[a]	100.00[a]	0[b]	0[b]	50.00[a]	50.00[a]	0.00[b]	0[b]	100.00[a]	50.00[a]
Arusha	11	18.18[b]	27.27[b]	54.54[a]	9.09[ab]	72.72[a]	36.36[a]	36.36[a]	54.54[a]	72.72[ab]	18.18[b]
Iringa	22	18.18[b]	63.63[ab]	40.90[a]	13.63[a]	9.09[b]	18.18[b]	18.18[b]	18.18[ab]	50.00[b]	4.54[b]
Lsd		14.981	52.072	33.908	12.397	25.129	33.267	33.267	43.479	42.413	28.835
CV (%)		22.96	36.07	47.02	72.210	25.38	34.84	59.83	79.12	25.20	52.47

Means followed by the same letter within a column are not statistically different (P≤ 0.05) by DMRT.
*Percentage of strains that utilized the following carbon sources.

Table 3. Nearest-matched identification of *X. campestris pv. vesicatoria* strains from Tanzania by the Biolog Microlog database.

Strain	Identification	SIM
KSOO3M	*P. s. pv. aptata*	0.205
KS019A	*P. fluorescens Biotype G*	0.59
KS020A	*P. fluorescens Biotype A*	0.124
KS022A	*Vibrio metschnikovii*	0.334
KS025A	*P. flourescens Biotype A*	0.278
KS028A	*Vibrio metschnikovii*	0.271
KS035A	*P. flourescens Biotype A*	0.315
KS038A	*P. cissicola (Xanthomonas-like*	0
KS039A	*Vibrio metschnikovii*	0.22
KS048A	*Brevundimonas vesicularis*	0.465
KS052A	*P. flourescens Biotype A*	0.319
KS058A	*Vibrio metschnikovii*	0.304
KS064Ir	*Vibrio metschnikovii*	0.233
KS069Ir	*P. flourescens Biotype A*	0.111
KS070Ir	*Vibrio metschnikovii*	0.385
KS073Ir	*Vibrio metschnikovii*	0.199
KS075IrB	*Vibrio metschnikovii*	0.288
KS076Ir	*Vibrio metschnikovii*	0.082
KS078Ir	*Vibrio metschnikovii*	0.324
KS079Ir	*P.flourescens Biotype A*	0.162
KS082Ir	*Vibrio metschnikovii*	0.243
KS083IrB	*Vibrio metschnikovii*	0.266
KS086IrA	*P.flourescens Biotype A*	0.303
KS088Ir	*Brevundimonas vesicularis*	0.111
KS090IrB	*P. s. pv. aptata*	0.332
KS092Ir	*P. s. pv. Aptata*	0.332
KS093IrB	*P. viridilivida*	0.324
KS094Ir	*Vibrio metschnikovii*	0.3
KS096Ir	*P. s. pv. Pisi*	0.128
KS100Ir	*Vibrio metschnikovii*	0.298
KS102Ir	*P. s. pv. aptata*	0.296
KS105Ir	*P.flourescens Biotype A*	0.245
KS113IrB	*Vibrio metschnikovii*	0.211
KS118IrB	*Vibrio metschnikovii*	0.242
KS126M	*Acinobacter calcoaceticus genospecies*	0.111
GSPB 2043	*P. fluorescens*	0.344
XG101	*P.cissicola (Xanthomonas-like*	0.397
93-1	*X. c. pv. Poinsetticola*	0.48
XV-56	*X. c. pv. Poinsetticola*	0.753
75-3	*P.cissicloa*	0.426
98-118	*Sphingomonas terrae*	0.414

(44.11%) were identified as Vibrio metschnikovii with similarity indices of 0.082-0.324, eight strains (23.52%) were identified as *Pseudomonas fluorescens* Biotype A with similarity indices of 0.111-0.319, four (11.76%) were identified as *Pseudomonas aptata* with similarity indices of 0.205-0.332, two (5.88%) were identified as *Brevundimonas vesicularis* with similarity indices of 0.111 and 0.465, while one each was identified as Pseudomonas cissicola (SIM = 0.218), *Pseudomonas pisi* (SIM = 0.128), Acinobacter calcoaceticus genospecies 1 (SIM=0.111)

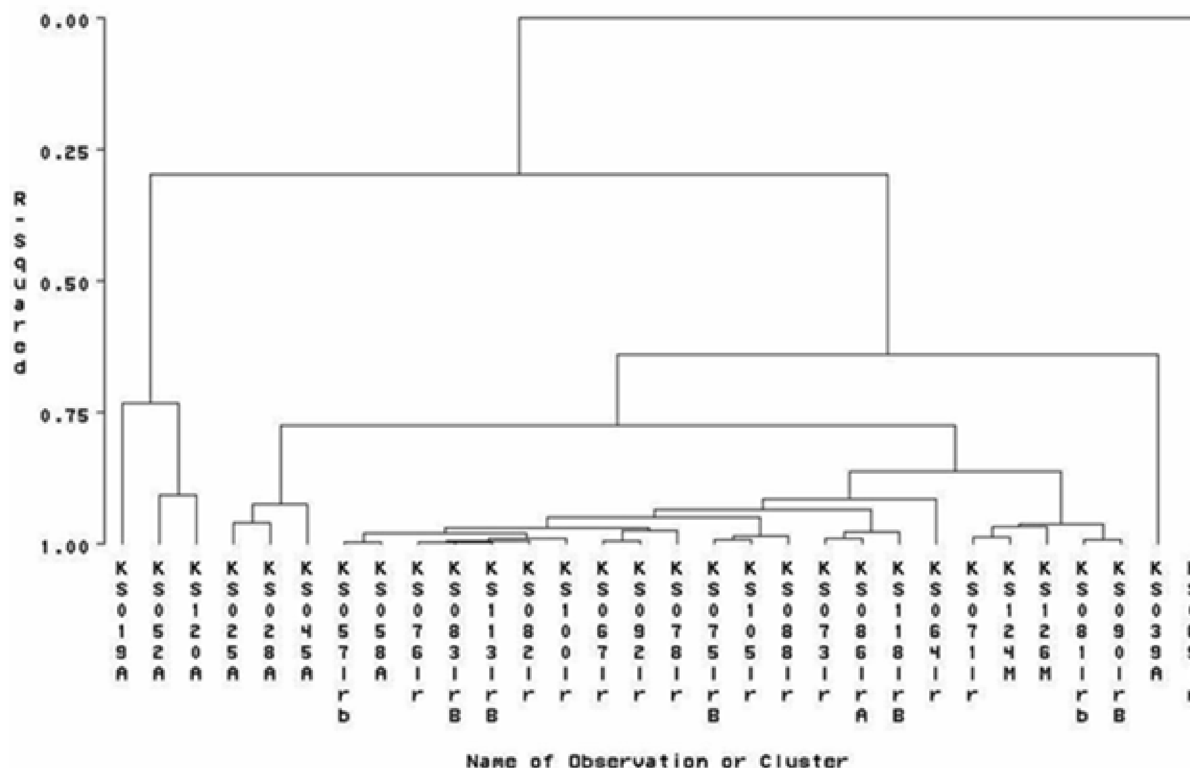

Figure 2. Dendrogram showing grouping of strains of *Xanthomonas campestris* pv. vesicatoria from Arusha, Iringa and Morogoro, Tanzania based on sensitivity to various antibiotics.

and Pseudomonas viridilivida (SIM=0.324). The reference strains (GSPB 2043, XG101, 93-1, XV-56, 75-3 and 98-118)* were identified as *Pseudomonas fluorescens* (SIM 0.344), Pseudomonas cissicola (SIM = 0.397), *Xanthomonas campestris* pv. *poinsetticola* (SIM =0.480), *Xanthomonas campestris* pv. *poinsetticola* (SIM = 0.753), *Pseudomonas cissicola* (SIM= 0.426) and *Sphingomonas terrae* (SIM =0.414), respectively.

The dendogram of *X. c. pv. vesicatoria* strains based on the Biolog Identification System is shown as Figure 1. According to the Biolog dendrogram, strains of *X. c.* pv. *vesicatoria* from Tanzania formed three distinct clusters, which seemed to confirm a strong relationship to geographical origin. The first and largest cluster consisted of strains from Iringa. This was a homogeneous grouping that was punctuated in three places by strains from Arusha. The second cluster consisted of strains from Arusha, while the third cluster consisted of a mixture of strains from Morogoro and Arusha.

Sensitivity of *X.c. pv. vesicatoria strains* to antibiotics

The results of the sensitivity of *X. c. pv. vesicatoria* strains to the antibiotics are presented in Table 3. The results indicated that all the strains from the three regions in Tanzania were sensitive to gentamycin, streptomycin and ampicillin. Sensitivity to the other antibiotics ranged between 16.66 to 96.66%. Sensitivity to the antibiotics (especially lincomycin, vancomycin and bacitracin) appeared to be influenced by geographical origin of the strains. The strains from Arusha showed resistance to lincomycin and vancomycin, while those from Iringa were sensitive to these antibiotics. All the strains from Arusha and Morogoro were sensitive to bacitracin, whereas 47.61% of strains from Iringa showed resistance to the antibiotic. Slight differences in the way the strains responded to cephazoline were also observed. Whereas all the strains from Arusha and the strains from Morogoro were resistant to cephazoline, only 42.85% of strains from Iringa showed resistance to the antibiotic (Table 3). A dendrograms showing relatedness of the *X.c. pv. vesicatoria* strains on the basis of their sensitivity to antibiotics showed major grouping based on geographical origin of the strains (Figure 2).

DISCUSSION

Thirty five strains of *X. c. pv. vesicatoria* collected from Arusha, Iringa and Morogoro were identified and characterised by Biolog and sensitivity to antibiotics. Based on the results in the current study, it was observed that the

Table 4. Sensitivity *of Xanthomonas campestris* pv. *vesicatoria* strains from Arusha, Iringa and Morogoro regions in Tanzanian to some antibiotics.

strain	Gentamycin sulphate	Chloram-phenicol	Polymixin B sulphate	Streptomycin sulphate	Lincomycin hydrochloride	Vancomycin	Phospho mycin	Bacitracin	Ampicillin	Cephazolin	Water control
KS019A	8.18	1.84	0.8	3.29	0	11.99	40.31	0	13.75	11.23	0
KS025A	6.62	2.27	1.72	3.25	0	0	18.96	0	13.86	4.55	0
KS028A	8.53	1.22	2.62	2.57	0.83	0.44	15.98	0	13.33	2.82	0
KS039A	9.37	0.63	0	2.96	0.61	0	16.52	0	11.04	16.38	0
KS045A	6.14	0	2.57	5.35	0	0	14	0	10.36	2.13	0
KS052A	8.17	2.31	0	5.03	0	0.81	32.94	0	15.63	5.63	0
KS057Irb	8.39	1.47	0	5.09	0.75	0.59	19.21	0.67	2.1	1.26	0
KS058A	8.3	0	0.52	4.55	0	0.76	19.57	1.09	1.36	0.43	0
KS064Ir	9.32	0	0	6.06	0.74	0.95	15.87	0.52	3.43	0	0
KS067Ir	7.79	0	0	5.43	0	0	20.82	0	3.38	1.79	0
KS069Ir	7.24	0	0	4.43	0	20.45	0	4.71	1.25	0	0
KS071Ir	6.26	2.03	0	5.58	0	0	23.76	0	9.6	0	0
KS073Ir	7.12	4.03	0	6.72	0	0	20.75	3.43	3.38	2.42	0
KS075Ir	6.1	3.03	0	6.53	0	0	23.75	0	3.38	0	0
KS076Ir	6.52	0.81	0	5.61	0	0	18.69	0	3.1	0	0
KS078Ir	7.53	1.23	0	4.75	0	0	22.06	2.25	4.64	0	0
KS081Irb	7.17	2.69	0	6.35	0	0	23.42	0	7.02	2.11	0
KS082Ir	6.12	2.12	0	5.55	0	0	20.76	0.72	3.53	0	0
KS083IrB	6.34	0	0.8	6.38	0	0	20.26	0	3.46	0	0
KS086IrA	8.25	3.41	0	5.81	0	0	22.42	2.08	4.26	2.55	0
KS090IrB	8.7	0.99	0.53	5.7	0	0	24.06	0	2.72	0	0
KS092Ir	6.03	4.5	0	5.56	0	0	21.58	0	7.94	3.03	0
KS100Ir	5.8	0	0	4.23	0	0	20.28	0	4.48	2.07	0
KS105Ir	6.27	0.62	2.51	5.8	0	0	22.37	0	1.57	0	0
KS113IrB	6.67	1.56	0.87	6.6	0	0	19.77	0	4.85	0	0
KS118IrB	6.39	0.54	1.29	5.46	0	0	21.11	0.68	3.27	0	0
KS120A	7.2	1.74	0.58	6.96	0	0	34.22	4.88	5.02	2.73	0
KS124M	10.74	0.89	3.68	4.32	0	0	24.81	0	14.99	10.69	0
KS126M	6.33	1.31	0.95	4.73	0	0	21.54	0	9.52	2.98	0
KS128M	6.78	1.13	1.26	4.12	0.92	0	21.54	0	8.2	1.85	0

Biolog Identification system was not very reliable and accurate in identifying and characterizing *X. c. pv. vesicatoria*. Several authors have reported the limited reliability of the Biolog system in the identification of plant pathogenic bacteria (Jainkittivong et al., 1989; Jones et al., 1993; Black and Sweetmore, 1994; Khatri-Chhetri, 1999; Black et al., 2000; Massomo et al., 2003). In the current study, none of the *X. c. pv. vesicatoria* strains from Tanzanian were correctly identified, even at the genus level. Similarly, out of six reference strains (one from the GSPB collection, and five from Obradovic, A (from Serbia)) only two were identified to the genus level. The poor identification of the strains is likely indicative of the existence of *X. c. pv. vesicatoria* strains with metabolic profiles that differ significantly from those of the strains used in the Biolog GN database. There is, therefore a need for further widening of the *X. c. pv. vesicatoria* data-base in the Biolog identification system to include strains from various geographical regions that represent ecological systems.

It has already been shown with other species of phytopathogenic bacteria that variations in the utilization of organic substrates by the same pathovar do often occur (Schaad, 1988). These differences could be due to different testing methods applied, differences between strains due to environmental and nutritional influences, and the state of the MicroLog plates. The suitability of the Biolog system and other commercially available databases of metabolic profiles for the identification of Subtropical and tropical strains of bacteria, including the bacterial spot pathogen has been criticised by other users (Black et al., 2000).

Although the identification of the *X .c. pv. vesicatoria* strains by the Biolog system was poor, it proved very useful in characterising the strains. A dendrogram of the carbon source utilization profiles of the strains showed that the strains generally clustered according to geographical origin, although the strains from Morogoro tended to have more similar metabolic profiles to those from Arusha than to those from Iringa (Figure 1).

In general, the response of the *X. c. pv. vesicatoria* strains to antibiotics and their grouping into three main clusters (Figure 2) were similar to those of the carbon source utilization patterns (Figure 1). Even though several antibiotics have been approved for use in agriculture, only streptomycin and tetracycline have been used extensively for plant disease control. Among the *X. c. pv. vesicatoria* strains evaluated for antibiotic sensitivity, none of them was resistant to streptomycin (Table 3).

Although on a global level, the use of antibiotics on plants to control diseases is low relative to the total use, application of antibiotics in the agro-ecosystem presents unique circum-stances that could impact the build up of resistance-to-antibiotics genes in the environment. Currently, there is no known record of antibiotic application for plant disease control on a commercial level in Tanzania, therefore the ecological risk does not exist or can be considered as minimal.

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