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Identification and characterisation of *Xanthomonas campestris* pv. *campestris* strains from Tanzania by pathogenicity tests, Biolog, rep-PCR and fatty acid methyl ester analysis

Said M.S. Massomo[1,2], Hanne Nielsen[2], Robert B. Mabagala[3], Keld Mansfeld-Giese[4], John Hockenhull[1] and Carmen N. Mortensen[2,*]
[1]*Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark;* [2]*Danish Government Institute of Seed Pathology for Developing Countries, Thorvaldsensvej 57, DK-1871 Frederiksberg C., Denmark;* [3]*Sokoine University of Agriculture, P.O. Box 3005, Morogoro, Tanzania;* [4]*Danish Institute of Agricultural Sciences, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark;* [*]*Author for correspondence (Phone: +45 35 28 37 27; Fax: +45 35 28 37 01; E-mail: cnm@kvl.dk)*

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Abstract

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is a major disease constraint to cabbage production by smallholder farmers in Africa. Variability exists within the pathogen, and yet differentiation of Xcc strains from other closely-related xanthomonads attacking crucifers is often difficult. The Biolog system, fatty acid methyl ester analysis using microbial identification system (MIS), rep-PCR and pathogenicity tests were used to identify and characterise Xcc strains from Tanzania. Great diversity was observed among Xcc strains in their Biolog and rep-PCR profiles. Specific rep-PCR genomic fingerprints were linked to some geographical areas in the country. Most of the Xcc strains were clustered in two groups based on their fatty acid profiles and symptom expression in cabbage although some deviant strains were found. Each of the methods allowed a degree of identification from species, pathovar to the strain level. Biolog and MIS identified all Xcc strains at least to the genus level. Additionally, Biolog identified 47% of Xcc strains to the pathovar and 43% to strain level, whereas MIS identified 43% of the strains to pathovar level. In the absence of a database, the utility of rep-PCR for routine diagnosis of strains was limited, although the procedure was good for delineation of Xcc to the strain level. These findings indicate the existence of Xcc strains in Tanzania that are distinct from those included in Biolog and MIS databases. The limitations noticed warrant continued improvement of databases and inclusion of pathogenicity testing, using universally susceptible cultivars, as an integral part of strain identification.

Introduction

Xanthomonas campestris pv. *campestris* (Xcc), the cause of black rot of crucifers, is a seed-borne bacterium which occurs worldwide (Williams, 1980; Alvarez, 2000). Black rot has become the major disease constraint to smallholder cabbage (*Brassica oleracea* var. *capitata*) growers in Africa where substantial crop losses are experienced especially during the warm and wet seasons (Day et al., 1992; Onsando, 1992; Mguni, 1996; Massomo, 2002). The seed-borne phase of this bacterium is an important means of survival and long distance dissemination. High disease incidence may arise when seed lots with as low as 0.05% infection are used (Schaad et al., 1980). Isolation of Xcc from seeds has been improved by using semi-selective agar media (Schaad, 1989; Franken, 1992). However, differentiation of Xcc strains from closely related pathovars of *X. campestris* (Xc) attacking other brassicas is not possible on the basis of morphological and biochemical

characteristics and is often difficult by pathogenicity testing (Franken, 1992). The relative homogeneity of Xc pathovars associated with brassica has also been demonstrated by DNA–DNA hybridisation studies (Vauterin et al., 1995), fatty acid methyl ester (FAME) analysis (Yang et al., 1993) and SDS–PAGE protein patterns (Minsavage and Schaad, 1983; Thaveechai and Schaad, 1986). Other methods used to type bacteria include phage typing, sero-typing, plasmid profiling and rRNA sequencing (Alvarez et al., 1994; de Bruijn et al., 1996). More recently, genomic fingerprinting by PCR amplification, with primers specific to the highly conserved, repetitive elements such as the 35–40 bp repetitive extragenic palindromic (REP) sequence, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) and the 154 bp BOX element, was used successfully to characterise a large number of bacteria and differentiate closely related strains of bacteria (Louws et al., 1994; Versalovic et al., 1994; 1995; de Bruijn et al., 1996). Fatty acid profiling, based on analysis of complex, stable cellular FAME profiles, offers a valuable, rapid and accurate method for identification of many bacteria at the species level and often at the pathovar level (Sasser, 1990; Stead et al., 1992; Weller et al., 2000). Mguni et al. (1999) characterised Xcc to the strain level by the carbon source utilisation pattern in the Biolog identification system. Accurate and rapid methods for differentiation of strains within Xcc from closely related brassica pathovars of Xc are necessary in diagnosis, epidemiological studies and control of black rot.

The present studies were conducted to evaluate the suitability and utility of the Biolog system, FAME analysis using the microbial identification system (MIS), rep-PCR and pathogenicity tests for identification and characterisation of Xcc strains from Tanzania, to determine the extent and distribution of Xcc variation in Tanzania, and to explore the relationship between genomic fingerprints, FAME profiles, carbon source utilisation patterns and pathogenicity of Xcc strains.

Materials and methods

Collection of leaf samples

Ninety farmers' fields with diseased vegetable brassica crops (cabbage, *Brassica oleracea* var. *capitata*; cauliflower, *B.o.* var. *botrytis*; collard/kale, *B.o.* var. *acephala* and Chinese cabbage, *B. rapa* var. *pekinensis*) were inspected in the major brassica growing areas of Tanzania between April and August 1998 (Table 1). Villages lying between altitudes of 800 and 1800 m were selected to represent the different agroecological zones in the districts of Arumeru-Arusha and Lushoto-Tanga. In the Morogoro region, sampling was limited to Mgeta in the Uluguru mountains (900–1200 m). From each field, infected leaves were collected, placed in paper bags and transported to the laboratory for further processing. Leaves were dried at room temperature (25–27 °C) between sheets of absorbent paper in a wooden press before the isolation of causal bacteria associated with black rot symptoms.

Bacterial isolation

From each sample, leaf tissue segments of ca. 2 × 3–4 mm were excised from lesion margins. The leaf segments were teased in drops of 0.85% sterile saline solution and left to stand for 5 min in a lamina airflow chamber. Loopfuls of the saline were streaked on pre-chilled plates of nutrient starch cycloheximide agar (NSCA) (Schaad and Kendrick, 1975). Plates were inspected for the presence of pale yellowish convex mucoid starch hydrolysing bacterial colonies after two days of incubation at 28 °C. The colonies were purified on yeast dextrose carbonate (YDC) agar and stored at −80 °C on porcelain beads in Protect® tubes (Protect System, Bury, UK).

Bacterial strains

One hundred and twenty-four strains were included in this study (Table 1). Of these, strains SM 10–SM 122 were isolated in 1998. Eight Xcc strains (SM 123–SM 131) from Tanzania were provided by Zakia Abubakar, Plant Protection Division, Zanzibar, Tanzania. Five strains of Xcc (SM 132–SM 136) were obtained from Cames Mguni, Plant Protection Research Specialist Services, Zimbabwe. Five other strains of Xcc used in the Biolog test were obtained from the following culture collections: strains Xcc 14, Xcc 95 and Xcc 143, from the Asian Vegetable Research Development Centre, Taiwan, strain 1803 from United States Department of Agriculture, USA and strain IPO 1011 from Plant Research Institute BV (formerly Research Institute of Plant Protection), Wageningen, The Netherlands. Finally, *X.c.* pv. *campestris* strains NCPPB 2031, NCPPB 3207, *X.c.* pv. *armoraciae* (Xca) NCPPB 1930,

Table 1. The source and characteristics of bacterial strains tested by viscosity, pathogenicity, Biolog, fatty acid analysis and rep-PCR procedures

Strain (SM) no.	Viscosity test[1]	HR[1]	Symptom type[2]	FAME		Biolog identification[4] (similarity values)		rep-PCR groups[5]		Source of bacterial strains	
				Group	Identification[3]			BOX	REP	Crop[6]	Field location/supplier
10	+	+	1a	II	Xca (0.786)	Xanth.	XccB (0.273)	1	2	1	*Arumeru, E. Kioga 1*
11	+	+	1a	II	Xca (0.747)	Xanth.	XccB (0.276)	1	nd	1	*Arumeru, E. Kioga 1*
12	+	+	1a	I	Xcr (0.694)	nd[7]	nd	5	4	1	*Arumeru, E. Kioga 2*
13	+	+	1b	I	Xav (0.633)	XccA (0.595)	na	5	3	1	*Arumeru, E. Kioga 2*
14	+	+	2b	II	Xcc (0.837)	nd	nd	1	2	1	*Arumeru, E. Kioga 2*
15	+	+	1b	II	Xca (0.760)	nd	nd	1	nd	1	*Arumeru, E. Kioga 3*
16	+	+	1b	II	Xcc (0.803)	Xanth.	XccB (0.429)	1	2	1	*Arumeru, E. Kioga 3*
17	+	+	1b	I	Xav (0.628)	Xanth.	Xcr (0.303)	5	4	1	*Arumeru, Kikuletwa 4*
18	+	+	1b	I	Xca (0.798)	Xanth.	XccB (0.364)	5	2	1	*Arumeru, Olturoto 5*
19	+	+	1b	I	Xcr (0.813)	XccA (0.664)	na[8]	1	nd	1	*Arumeru, Olturoto 6*
20	+	+	1b	I	Xca (0.846)	XccB (0.668)	na	1	1	4	*Arumeru, Makiba 7*
21	+	+	1b	II	Xcc (0.851)	nd	nd	1	1	4	*Arumeru, Makiba 7*
25	+	+	1b	II	Xcc (0.912)	XccB (0.574)	na	1	1	4	*Arumeru, Makiba 8*
26	+	+	1b	II	Xcc (0.913)	Xanth.	XccB (0.440)	1	nd	4	*Arumeru, Makiba 8*
27	+	+	1b	II	Xcc (0.827)	Xanth.	XccB (0.386)	1	1	4	*Arumeru, Makiba 8*
28	+	+	1a	II	Xcc (0.869)	XccA (0.569)	na	1	1	4	*Arumeru, Makiba 8*
29	+	+	1b	II	Xcc (0.766)	nd	nd	1	nd	4	*Arumeru, Makiba 9*
30	+	+	1b	III	Xcc (0.861)	XccB (0.510)	na	1	1	4	*Arumeru, Makiba 10*
31	+	+	1b	III	Xcc (0.852)	XccA (0.511)	na	1	nd	4	*Arumeru, Makiba 12*
32	+	+	1b	II	Xcc (0.882)	nd	nd	1	nd	1	*Arumeru, O. Sapuk 13*
33	+	+	1b	II	Xcc (0.837)	nd	nd	1	2	1	*Arumeru, O. Sapuk 13*
34	+	+	1b	II	Xcc (0.853)	nd	nd	1	2	1	*Arumeru, O. Sapuk 13*
35	+	+	1b	nd	Xcr (0.689)	XccB (0.597)	na	6	nd	5	*Arumeru, Makiba 15*
36	+	+	1b	II	Xcc (0.838)	Xanth.	Xcr (0.398)	1	1	1	*Arumeru, Ngiresi 16*
37	+	+	1b	II	Xcc (0.880)	XccB (0.713)	na	1	1	1	*Arumeru, Ngiresi 16*
38	+	+	1b	I	Xca (0.851)	XccB (0.606)	na	7	3	1	*Arumeru, O. Sapuk 18*
39	+	+	1b	I	Xca (0.841)	XccB (0.514)	na	7	3	1	*Arumeru, O. Sapuk 19*
40	+	+	1b	II	Xcc (0.790)	nd	nd	nd	2	2	*Arumeru, O. Sapuk 20*
41	+	+	1a	II	Xcc (0.832)	Xanth.	XccB (0.318)	nd	nd	2	*Arumeru, O. Sapuk 20*
42	+	+	1b	II	Xcc (0.717)	Xanth.	XccB (0.368)	nd	2	4	*Arumeru, Tengeru 43*
43	+	+	1b	II	Xcc (0.818)	Xc.d (0.808)		1	nd	2	*Arumeru, O. Sapuk 21*
44	+	+	1a	I	Xcr (0.668)	Xanth.	Xvt (0.445)	6	2	2	*Arumeru, O. Sapuk 22*
45	+	+	1a	I	Xcr (0.742)	nd	nd	nd	nd	2	*Arumeru, O. Sapuk 22*
46	+	+	1a	II	Xcc (0.824)	XccB (0.507)	na	1	nd	1	*Arumeru, Ngiresi 17*
47	+	+	1a	I	Xca (0.844)	Xanth.	XccB (0.449)	nd	nd	1	*Arumeru, O.Sambu 23*
49	+	+	1b	II	Xcc (0.756)	XccB (0.535)	na	nd	1	1	*Arumeru, O.Wass 25*
50	+	+	1b	II	Xcc (0.838)	nd	nd	1	nd	1	*Arumeru, Oleigeruno 25*
51	+	+	1b	V	Xca (0.799)	XccB (0.878)	na	1	1	1	*Arumeru, Oleigeruno 27*
52	+	+	1b	II	Xcr (0.640)	Xanth.	XccB (0.328)	1	nd	1	*Arumeru, Olgilai 28*

Table 1. (*Continued*)

Strain (SM) no.	Viscosity test[1]	HR[1]	Symptom type[2]	FAME		Biolog identification[4] (similarity values)		rep-PCR groups[5]		Source of bacterial strains	
				Group	Identification[3]			BOX	REP	Crop[6]	Field location/supplier
53	+	+	1b	II	Xcc (0.811)	XccB (0.517)	na	1	nd	1	*Arumeru, Olgilai 29*
54	nd	nd	3	nd	nd	nd	nd	nd	nd	1	*Arumeru, Olgilai 30*
55	nd	nd	2b	I	Xcc (0.887)	XccB (0.565)	na	1	2	1	*Arumeru, Olgilai 31*
56	+	+	2b	II	Xcc (0.847)	XccA (0.657)	na	1	1	2	*Arumeru, Olgilai 32*
57	+	+	2b	IV	Xcr (0.628)	XccA (0.569)	na	3	4	3	*Arumeru, Olgilai 33*
58	+	+	2b	II	Xcc (0.746)	Xcc	XccB (0.456)	3	4	5	*Arumeru, Olgilai 34*
59	+	+	1a	I	Xca (0.888)	Xcc	XccB (0.335)	1	1	1	*Arumeru, Olgilai 35*
60	+	+	1b	II	Xcc (0.834)	nd	nd	nd	1	1	*Arumeru, O.Sambu 24*
62	+	nd	1b	II	Xcc (0.732)	nd	nd	1	1	1	*Arumeru, Patanumbe 36*
63	+	+	1a	II	Xcc (0.852)	XccA (0.504)	na	1	1	4	*Arumeru, Patanumbe 37*
64	+	+	1b	I	Xca (0.858)	nd	nd	1	nd	4	*Arumeru, Patanumbe 37*
65	nd	nd	1b	I	Xcr (0.854)	nd	nd	1	nd	4	*Arumeru, Patanumbe 39*
66	+	+	1a	I	Xcr (0.878)	XccA (0.669)	na	1	1	4	*Arumeru, Patanumbe 40*
67	+	+	1a	I	Xca (0.903)	Xanth.	XccA (0.287)	1	nd	4	*Arumeru, Patanumbe 40*
69	nd	nd	1a	I	Xca (0.712)	nd	nd	1	nd	4	*Arumeru, Patanumbe 40*
70	+	+	1a	III	Xcc (0.803)	nd	nd	1	nd	4	*Arumeru, Patanumbe 41*
71	+	+	1b	II	Xcr (0.866)	XccB (0.504)	na	7	nd	5	*Arumeru, Patanumbe 42*
72	+	+	1a	I	Xcr (0.855)	Xanth.	XccB (0.457)	1	nd	5	*Arumeru, Tengeru 44*
73	+	+	1b	II	Xcc (0.847)	Xanth.	XccB (0.467)	1	nd	4	*Arumeru, Valesca 45*
74	+	+	1b	nd	nd	nd	nd	1	nd	1	*Lushoto, Dochi 46*
75	+	+	1b	II	Xcc (0.826)	Xanth.	XccA (0.226)	2	nd	1	*Lushoto, Dochi 47*
76	+	+	1b	II	Xcc (0.773)	XccA (0.669)	na	2	nd	1	*Lushoto, Luandai 48*
77	+	+	1b	II	Xcc (0.801)	XccA (0.666)	na	2	nd	1	*Lushoto, Luandai 49*
78	+	+	1a	I	Xca (0.856)	Xanth.	XccA (0.249)	1	nd	1	*Lushoto, Gegestal 50*
79	+	+	1b	II	Xcc (0.838)	XccB (0.559)	na	1	nd	1	*Lushoto, Mabawani 51*
80	+	+	1a	II	Xcc (0.740)	XccB (0.529)	na	2	nd	1	*Lushoto, Magamba 52*
81	+	+	1a	II	Xcr (0.800)	Xanth.	—	1	2	1	*Lushoto, Magamba 53*
82	+	+	1b	II	Xca (0.783)	Xanth.	Xcr (0.487)	1	nd	1	*Lushoto, Magamba 54*
83	+	+	1b	I	Xca (0.744)	XccA (0.581)	na	2	nd	1	*Lushoto, Magila 55*
84	+	+	1a	II	Xcc (0.812)	Xanth.	Xcr (0.409)	1	nd	1	*Lushoto, Magila 56*
85	+	+	1b	II	Xcc (0.717)	XccB (0.538)	na	2	nd	1	*Lushoto, Ngulu 57*
86	+	+	1a	I	Xca (0.773)	Xanth.	XccB (0.495)	1	2	1	*Lushoto, Ngulu 58*
87	+	+	1a	II	Xcr (0.787)	Xanth.	Xc.d (0.485)	1	2	1	*Lushoto, Ngulu 58*
88	+	+	1b	I	Xca (0.633)	Xanth.	XccA (0.360)	1	2	1	*Lushoto, Shashui 59*
89	+	+	1b	I	Xcr (0.748)	nd	nd	1	2	1	*Lushoto, Shashui 60*
90	+	+	2a	I	Xca (0.861)	Xanth.	Xcr (0.349)	1	2	1	*Lushoto, Shashui 60*
91	+	+	1a	I	Xcc (0.893)	XccA (0.587)	na	1	nd	1	*Lushoto, Shashui 61*

92	+	+	1b	II	Xca (0.818)	XccA (0.575)	na	2	nd	1	*Lushoto, Soni 62*
93	+	+	1b	I	Xcc (0.899)	XccA (0.566)	na	1	nd	1	*Lushoto, Soni 63*
94	+	+	1a	I	Xcc (0.634)	Xanth.	XccA (0.318)	2	2	1	*Lushoto, Soni 63*
95	+	+	1a	nd	Xca (0.775)	Xcr (0.786)	na	1	2	1	*Lushoto, Soni 64*
96	+	+	1a	I	Xcr (0.685)	Xanth.	na	1	nd	1	*Lushoto, Ubiri 65*
97	+	+	1a	I	Xca (0.840)	XccB (0.563)	na	1	2	1	*Lushoto, Ubiri 65*
98	+	+	2a	II	Xcc (0.845)	Xanth.	Xcr (0.268)	nd	2	1	*Lushoto, Ubiri 66*
99	+	−	4	nd	nd	Xanth.	Xcr (0.318)	7	nd	1	*Arumeru, O.Wass 67*
102	+	+	1b	nd	nd	XccB (0.715)	na	2	2	4	*Arumeru, Tengeru 71*
103	+	+	1a	I	Xca (0.718)	Xc.d (0.833)	na	1	1	1	*Arumeru, Tengeru 72*
104	+	+	1b	I	Xcr (0.906)	Xanth.	XccB (0.368)	1	1	2	*Arumeru, Tengeru 73*
105	+	+	1b	I	Xca (0.879)	XccA (0.597)	na	1	1	1	*Arumeru, Tengeru 74*
106	+	+	1a	I	Xca (0.879)	Xanth.	Xc.p (0.474)	1	2	1	*Arumeru, Tengeru 75*
107	+	+	1a	nd	Xca (0.797)	XccB (0.715)	na	1	2	1	*Arumeru, Tengeru 75*
108	+	+	1b	I	Xcr (0.767)	XccA (0.694)	na	1	2	1	*Arumeru, Tengeru 76*
109	+	−	3	II	Xcr (0.752)	Xcc	XccB (0.333)	7	nd	1	*Arumeru, Tengeru 77*
110	+	+	1b	I	Xcc (0.851)	Xanth.	XccA (0.446)	1	1	1	*Arumeru, Olturoto 78*
111	+	+	1a	I	Xca (0.726)	Xanth.	Xc.d (0.297)	1	2	1	*Arumeru, Olturoto 79*
113	+	+	1a	nd	Xca (0.778)	Xcr (0.750)	na	1	1	1	*Arumeru, Tengeru 76*
114	+	+	1a	nd	Xca (0.770)	Xanth.	Xcr (0.250)	2	5	1	*Lushoto, Magamba 52*
115	+	+	1a	I	Xca (0.774)	Xcr (0.557)	na	1	2	1	*Lushoto, Ngulu 58*
116	+	+	1a	I	Xca (0.766)	Xc.vi (0.534)	na	1	2	1	*Lushoto, Magila 56*
117	+	+	1a	nd	nd	Xanth.	Xcr (0.403)	1	2	1	*Morogoro, Mgeta 81*
118	+	+	1a	nd	nd	XccA (0.694)	na	1	2	1	*Morogoro, Mgeta 82*
119	+	+	1a	nd	nd	Xanth.	Xc.fici (0.489)	1	2	1	*Morogoro, Mgeta 83*
120	+	+	1a	nd	nd	Xanth.	XccA (0.446)	1	2	1	*Morogoro, Mgeta 84*
121	+	+	1a	nd	nd	Xanth.	XccA (0.461)	1	2	1	*Morogoro, Mgeta 85*
122	+	+	1a	nd	nd	Xanth.	Xc.beg (0.212)	1	2	1	*Morogoro, Mgeta 86*
123	+	+	1a	II	Xcr (0.774)	nd	nd	6	nd	na	*Arumeru 1 (Z.Abubakar)*
124	+	+	1a	II	Xcc (0.794)	nd	nd	3	nd	na	*Iringa, 2-1 (Z.Abubakar)*
125	−	−	3	nd	nd	nd	nd	6	5	na	*Iringa, 2.2 (Z.Abubakar)*
126	+	+	1a	I	Xca (0.679)	nd	nd	6	nd	na	*Kilimanjaro,3 (Z.Abubakar)*
127	+	+	2a	I	nd	nd	nd	6	5	na	*Mbeya, 4 (Z.Abubakar)*
129	−	−	3	nd	nd	nd	nd	nd	nd	na	*Arumeru, 6 (Z.Abubakar)*
130	+	+	1a	I	Xcr (0.849)	nd	nd	1	1	na	*Arumeru, 7 (Z.Abubakar)*
131	+	+	1b	I	Xcr (0.833)	nd	nd	1	1	na	*Iringa, 8 (Z.Abubakar)*
132	+	+	nd	I	Xca (0.782)	Xanth.	na	1	2	na	*Zimbabwe, 8 (C.Mguni)*
133	+	+	nd	nd	Xcc (0.388)	Xcc	Xcc B (0.304)	7	5	na	*Zimbabwe, 10 (C.Mguni)*
134	+	+	nd	I	Xca (0.690)	XccA (0.707)	na	1	2	na	*Zimbabwe, 95 (C.Mguni)*
135	+	+	nd	I	Xca (0.844)	Xanth.	XccA (0.452)	1	2	na	*Zimbabwe, 97 (C.Mguni)*
136	+	+	nd	IV	Xcr (0.701)	XccB (0.770)	na	4	4	na	*Zimbabwe, 107 (C.Mguni)*
141	+	−	4	IV	Xcr (0.453)	nd		nd	nd		*Xca NCPPB 1930*

Table 1. (*Continued*)

Strain (SM) no.	Viscosity test[1]	HR[1]	Symptom type[2]	FAME		Biolog identification[4] (similarity values)		rep-PCR groups[5]		Source of bacterial strains	
				Group	Identification[3]			BOX	REP	Crop[6]	Field location/supplier
142	+	nd	3	IV	Xap (0.497)	XccB (0.594)	na	na	nd		*Xcr NCPPB 1946*
143	+	+	1a	III	Xcc (0.707)	nd		1	1		*Xcc NCPPB 2031 (South Africa)*
144	+	+	3	IV	Xcr (0.577)	Xcab (0.629)	na	nd	nd		*Xcab NCPPB 2986 (Australia)*
145	+	+	1b	III	Xcc (0.787)	nd		1	2		*Xcc NCPPB 3207 (Zimbabwe)*
146	+	+	2a	IV	Xcr (0.670)	XccB (0.672)	na	4	nd		*Xcc B147 (Alvarez, A., Hawaii)*
147	+	nd	4	IV	Xcr (0.548)	nd		nd	nd		*Xci NCPPB 937 (USA)*

[1]Viscosity test/Hypersensitivity reaction: + is positive and − is negative.
[2]Symptom type: 1a – V-shaped lesions, with extensive chlorosis followed by wilting, 1b – V-shaped lesions, without extensive chlorosis followed by wilting, 2a – Necrotic spots, surrounded by a yellow halo, followed by severe blight and wilting, 2b – Necrotic spots, followed by severe blight and wilting, 3 – Other symptoms atypical of black rot and 4 – Non-pathogenic, no symptoms.
[3]Identification abbreviations are as follows: Xanth – *Xanthomonas*, Xcc – *Xanthomonas campestris* pv. *campestris*, Xca – *X.c.* pv. *armoraciae*, Xcr – *X.c.* pv. *raphani*, Xcv – *X. axonopodis* pv. *vesicatoria*, Xc.vi – *X.c.* pv. *vitians*, Xcab – *X.c.* pv. *abberans*, Xc.d – *X.c.* pv. *dieffenbachiae*, Xc. beg – *X.c.* pv. *begoniae*.
[4]The names in the adjacent column refer to closest matched identity given by Biolog system whenever similarity value was below 0.5.
[5]Groupings by banding pattern of rep-PCR products (see Figure 3a and b).
[6]Crop: 1 = Cabbage, *Brassica oleracea* var. *capitata*, 2 = Cauliflower, *B.o.* var. *botrytis*, 3 = Chinese cabbage, *Brassica chinensis*, 4 = Collard, *B.o.* var. *acephala* and 5 = Kale, *B.o.* var. *acephala*.
[7]nd = not done.
[8]na = not available/not applicable.

X.c. pv. *raphani* (Xcr) NCPPB 1946, *X.c.* pv. *aberrans* (Xcab), NCPPB 2986 and *X.c.* pv. *incanae* NCPPB 937, obtained from National Collection of Plant Pathogenic Bacteria, UK and Xcc strain B147, obtained from A. Alvarez, University of Hawaii, were used as reference strains.

Identification of strains

Presumptive Xcc isolates and reference strains were tested for Xcc-determinative characteristics, including viscosity of bacterial suspension (Pierce et al., 1990), indirect ELISA using Xcc-specific monoclonal antibodies (Agdia, Inc. 1998), Gram reaction, nitrate reduction, Kovacs' oxidase reaction, starch hydrolysis, oxidative metabolism of glucose and hypersensitive reaction in pepper plants (Lelliot and Stead, 1987). Pathogenicity was confirmed by inoculating susceptible cabbage cv. Copenhagen Market by the cotyledon pricking method (Anonymous, 1985) and foliar spraying of 4–6-week-old plants with bacterial inoculum, grown on YDC agar for 48 h at 28 °C, harvested and adjusted to 10^8 cfu/ml in 0.85% saline solution. Plants were incubated in polyethylene humid chambers for 24 h in growth rooms maintained at 28 °C with 14/10 h light regime. Thereafter, they were removed from the humid chambers and kept under the same light and temperature conditions. Types of symptoms induced by spray inoculation were recorded 14–21 days after inoculation (DAI). The following three tests were chosen for further characterisation of the strains.

a. Fatty acid methyl ester analysis. Fatty acids were methylated, extracted and analysed using standard and recommended procedures for gas chromatographic FAME analysis (Sasser, 1990). Analysis was performed with a Hewlett Packard gas chromatograph HP5890 CG using the aerobe method TSBA40 and the library TSBA Version 4.1, followed by analysis of data by the MIS software 6.0, from MIDI Inc. (Newark, Del., USA).

b. Biolog metabolic fingerprinting. Strains were tested for ability to metabolise carbon sources using Biolog GN microtiter plates (Microlog 2, Version 3.5, Biolog Inc., Hayward, CA). A single colony of each strain cultured on Difco nutrient agar (NA) was inoculated onto TSA plates and incubated for 24 h at 28 °C. Bacterial colonies were harvested with a sterile moistened cotton swab, suspended in sterile saline solution and adjusted at 590 nm to match the Biolog GN MicroPlate system's turbidity standards. Aliquots of 150 µl of bacterial suspensions were loaded into each well of the microplates. Visual reading of the carbon source utilisation patterns in each MicroPlate was done after incubation for 24 h at 28 °C. The metabolic fingerprints of each strain were entered into a computer and compared with the Biolog database. Furthermore, the fingerprints of the strains were changed into binary codes and analysed by the MIX program (mixed algorithm Version 3.572c), included in the PHYLIP package. A phenogram was generated using the DRAWGRAM program (Felsenstein, 1993).

c. Genomic fingerprinting. Two rep-PCR protocols, namely BOX-PCR and REP-PCR, were used. A total of 108 Xc strains were subjected to BOX-PCR genomic fingerprinting using BOX AIR primer (5′-CTACggCAAggCgACgCTgACg-3′). Seventy-one representative Xcc strains were furthermore subjected to REP-PCR, using REP 1R (5′-IIIICgICgICATCIggC-3′) and REP 2I (5′-ICgICTTATCIggCCTAC-3′) primer set (Versalovic et al., 1991; 1994). The PCR procedure used was as described by Versalovic et al. (1994) with minor modifications. Bacterial suspensions from 48 h cultures, grown on NA at 28 °C, were adjusted to 0.1 optical density at 600 nm. Aliquots of 25 µl from bacterial suspensions were mixed in a 50 µl PCR-reaction mixture. The mixture contained: 1.5 pmol/µl of the respective primer(s), 200 or 250 mM of each of the four deoxynucleotide triphosphates in BOX- or REP-PCR, respectively, and 2 or 3 units of *taq* DNA polymerase (Promega, Madison, WI) in storage buffer A, 1X reaction buffer (Magnesium free, 500 mM KCl, 100 mM Tris–HCl, pH 9.0 at 25 °C, 1% Triton X 100), 3 mM $MgCl_2$ and 4% or 5% dimethyl sulfoxide (v/v). The PCR reactions were performed with a Perkin-Elmer 2400 DNA thermal cycler (Perkin Elmer Corp.). Amplification conditions were: an initial denaturation at 95 °C for 7 min, followed by 33 cycles of denaturation at 94 °C for 1 min, annealing at 53 or 44 °C for 1 min with BOX or REP primers, respectively, extension at 65 °C for 8 min and a final extension at 65 °C for 15 min followed by cooling at 4 °C. Ten microlitre aliquots of amplified PCR products were separated by electrophoresis in a 1% agarose gel (7.5 × 10 cm) in 1X Tris-acetate buffer for 1 h and stained with ethidium bromide. Control lanes with water, Xc and/or Xcc reference strains were included in each PCR run. Gels were visualised on a UV transilluminator (TFX-20M, Vilber Lourmat, France), and

photographed using Polaroid type 55 films (Polaroid (UK) Ltd, Hertfordshire, England). The banding patterns were scored visually according to their presence or absence at specific positions for each strain.

Results

Identification of bacterial strains

One hundred and thirteen putative Xc strains were isolated from the brassica leaf samples based on colony characteristics on NSCA medium and Gram reaction. Of these, strains SM 10–SM 122 were identified as xanthomonads based on oxidative utilisation of glucose, nitrate test, starch hydrolysis, viscosity test, ELISA and hypersensitive reactions on pepper (Table 1). Viscosity tests of bacterial suspensions effectively distinguished all xanthomonads from other yellow pigmented strains (Table 1). The monoclonal antibodies used reacted positively with all Xcc strains and cross-reacted with reference strains of Xcr and Xcab, but not with Xca.

Pathogenicity tests

Of 127 strains tested from leaf samples and other sources, 111 strains were pathogenic to cabbage (Table 1). Pathogenic strains induced symptoms which were arbitrarily categorised into five groups, ranging from characteristic to non-characteristic black rot symptoms (Table 1). Ninety-nine strains in symptom group 1a and 1b initially caused small greyish spots around the stomata 5 DAI, followed by characteristic necrotic V-shaped lesions on the margins of leaves with chlorosis 14–21 DAI. Subsequently, vein blackening and wilting of leaves occurred. Forty-five strains in group 1a (Table 1) caused extensive chlorosis and sudden collapse of vein-delimited mesophyll tissue, reminiscent of blight. These symptoms usually occurred before vein blackening and wilting of plants. Three strains in group 2a, together with Xcc reference strain B 147, initially caused yellow haloes around stomata that later turned into severe necrotic spots. These spots coalesced within 14–21 DAI and formed extensive tan necrotic blotches in the leaf lamina and distorted leaf margins. However, although strains in this group had restricted vascular infection, plants were eventually killed by leaf loss. Five strains in group 2b caused symptoms similar to those of group 2a, except for the absence of yellow haloes around the stomata. Four non-Xc strains in group 3 caused localised leaf necrosis and/or drying of inoculated leaves. One xanthomonad, strain SM 99, was non-pathogenic to cabbage. The reference strain of Xcab (NCPPB 2986) consistently induced black necrotic blotches around hydathodes and in the leaf, while Xcr (NCPPB 1946) caused localised necrotic spots around stomata of inoculated leaves.

Fatty acid methyl ester analysis

All Xc test strains subjected to FAME analysis were identified as xanthomonads, 45 strains out of the 104 were identified as Xcc, and the other strains were identified to the pathovar level as Xca (35 strains), Xcr (22 strains) and *X. axonopodis* pv. *vesicatoria* (2 strains) (Table 1). The relatedness of strains based on FAME profiles is shown in Figure 1. Four main clusters of strains were differentiated. Almost all Xcc test strains (96 out of 100 tested) were placed in clusters I and II (Euclidian distances ranging from 0 to 5.0). The third cluster contained five Xcc strains including the reference strains Xcc NCPPB 2031 and

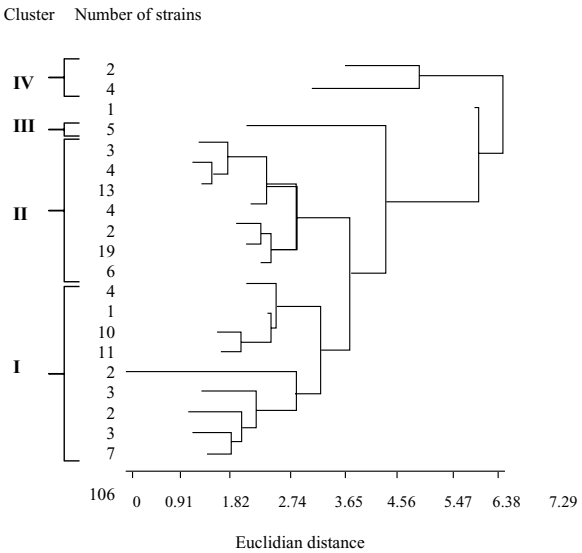

Figure 1. Dendogram showing the relationships among strains of *X. campestris* based on fatty acid methyl ester analysis. Clusters I–III contained Xcc test strains and two reference strains of Xcc NCPPB 2031 and NCPBB 3207. Cluster IV consisted of Xca NCPPB 1930, Xcab NCPPB 2986, Xcr NCPPB 1946, Xcc B 147 and a test strain SM 57.

Xcc NCPPB 3207. The fourth cluster consisted of Xcc strain B 147, a test strain SM 57 and other non-Xcc strains such as Xcr, Xca and Xcab.

Metabolic fingerprinting

The Biolog system identified the strains as Biolog type XccA or XccB whenever similarity values exceed 0.5. The Xcc strains with similarity values below 0.5 were either identified as Xcc or simply as *Xanthomonas* (Xanth). All 89 strains tested were identified at least to the *Xanthomonas* genus level. Of these, 41 strains were identified only to the genus level (*Xanthomonas*) with the closest matched identities as shown in Table 1, and four strains were identified to the pathovar level (Xcc). Eighteen strains were identified as XccA and additional 20 as XccB with similarity values of up to 0.878. Three strains, with similarity values between 0.557 and 0.786, were named as Xcr, and the rest were identified as *X. dieffenbachiae* (2 strains) and *X. vitians* (1 strain). The relatedness of the Xcc strains is presented in Figure 2, Xcc strains which belonged to the two Biolog types XccA and XccB were generally grouped in clusters A and B, respectively. Most of the Xcc strains identified as Xcr were included in cluster A. The Biolog database species profile for Xcab was closely related to cluster B, whereas reference strain Xcr was placed between the two clusters. The XccA and XccB Biolog types were found to be widespread in Tanzania.

The following 32 substrates were utilised by more than 95% of the Xcc strains: α-D-glucose, α-keto glutaric acid, α-keto butyric acid, acetic acid, bromo succinic acid, cellobiose, dextrin, D-fructose, D-galactose, D-mannose, D-melibiose, D-psicose, D-saccharic acid, D-trehalose, gentiobiose, glycerol, glycogen, L-fucose, L-glutamic acid, L-proline, L-serine, L-theronine, malonic acid, maltose, methyl pyruvate, mono methyl succinate, *N*-acetyl-D-glucosamine, propionic acid, succinic acid, sucrose, Tween 40 and Tween 80. The following 29 substrates were not utilised by any Xcc strains; α-cyclodextrin, α-D-lactose, γ-hydroxy butyric acid, γ-amino butyric acid, 2,3-butanediol, 2-amino ethanol, adonitol, D,L-carnitine, D-arabitol, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-mannitol, D-serine, D-sorbitol, L-arabinose, L-phenylalanine, L-pyroglutamic acid, L-rhamnose, *meso*-inositol, *N*-acetyl-D-galactosamine, phenyl ethylamine, *p*-hydroxy phenyl acetic acid, putrescine, quinic acid, sebacic acid, thymidine and xylitol. In addition, eight substrates were not utilised by more than 95% of the strains: α-ketovaleric acid, β-methyl D-glucoside, D-glucuronic acid, glucoronamide, hydoxy L-proline, itaconic acid, L-histidine and *i*-erythritol. The remaining 26 substrates were utilised in a variable manner by Xc strains; α-hydroxybutyric acid, β-hydroxybutyric acid, alaninamide, cis-aconitic acid, citric acid, D,L-α-glycerol phosphate, D,L-lactic acid, D-alanine, D-raffinose, formic acid, glucose-1-phosphate, glucose-6-phosphate, glycly-L-aspartic acid, glycly-L-glutamic acid, inosine, lactulose, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-leucine, L-ornithine, succinamic acid, turanose, uridine and urocanic acid.

Genomic fingerprinting

The two rep-PCR protocols used gave reproducible genomic PCR profiles consisting of approximately 400–3000 bp. With BOX-PCR, Xcc strains generated more than seven fingerprint patterns that were distinct from those of closely-related Xc pathovars (Table 1 and Figure 3a). The majority of Xcc strains (71 out of 108) showed fingerprint pattern named BOX-PCR 1, hereafter abbreviated as B1. The rest of the strains were assembled in groups B2 (10 strains), B3 (3 strains), B5 (4 strains) and B6 (6 strains). Almost all strains in group B2 originated from Lushoto district, Tanzania. Fingerprint pattern B4 was only found in a strain from Zimbabwe. Group B7 included seven bacterial strains with assorted fingerprints dissimilar to any of the patterns of Xc strains in the other groups. Strains of Xcc having BOX-fingerprint pattern B1 were separated with REP-PCR into two groups, namely R1 and R2 (Figure 3b). Most (59 out of 71) of the Xcc strains analysed by REP-PCR displayed a genomic fingerprint pattern highly similar to that observed with groups R1 and R2, the latter being the most widespread type in Tanzania (Table 1 and Figure 3b). Strains in group R1 were never isolated from the samples collected from Mgeta and Lushoto, Tanzania, whereas strains in group R3 were only obtained from samples collected in a localised area in Arumeru, Arusha, Tanzania. Group R4 was closely matched only with five strains. In addition, four other Xcc strains, assembled in group R5, showed various genomic fingerprint patterns that were distinct from fingerprints of the other Xcc groups and the Xc reference strains used in this study.

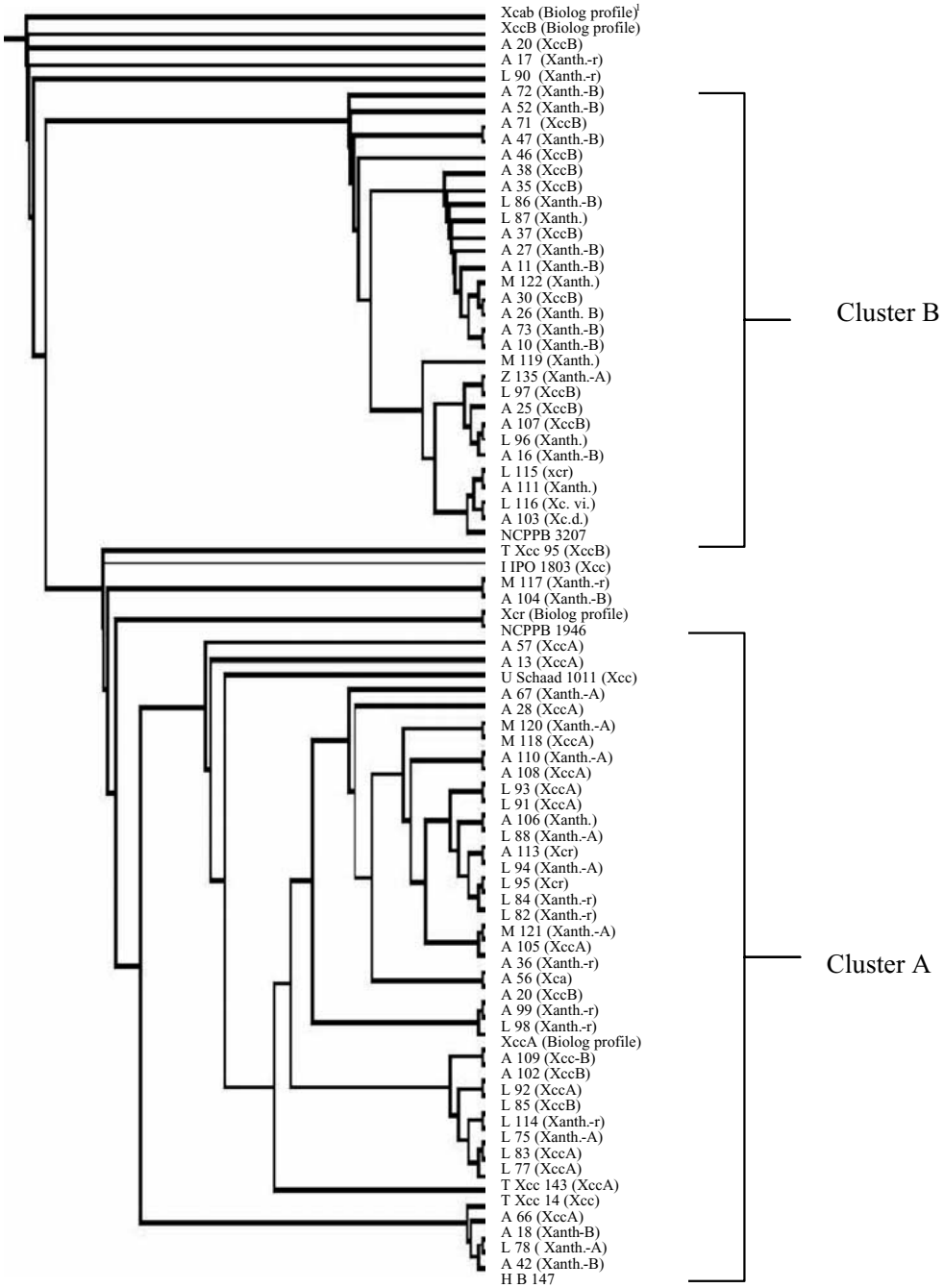

Figure 2. A Phenogram showing the relationships among the strains of *X. campestris* based on differential utilisation of the 95 carbon substrates available in the Biolog GN MicroPlate. [1]First letter refers to the location in Tanzania (or elsewhere) where the strain was obtained: L – Lushoto, A – Arusha, M – Mgeta, U – USA, T – Taiwan, Z – Zimbabwe, H – Hawaii and I – The Netherlands. Numbers refer to the strain numbers as in Table 1. The names in brackets refer to the name and/or closest matched identity given by Biolog GN database, i.e Xcc-A – *Xanthomonas campestris* pv. *campestris*, closest match XccA. The Xc.vi. and Xc.d. refer to *X.c.* pv. *vitians* and *X.c.* pv. *dieffenbachiae*, respectively. Species profiles in the database i.e. Xcab, XccA, XccB, and Xcr are metabolic fingerprints of species.

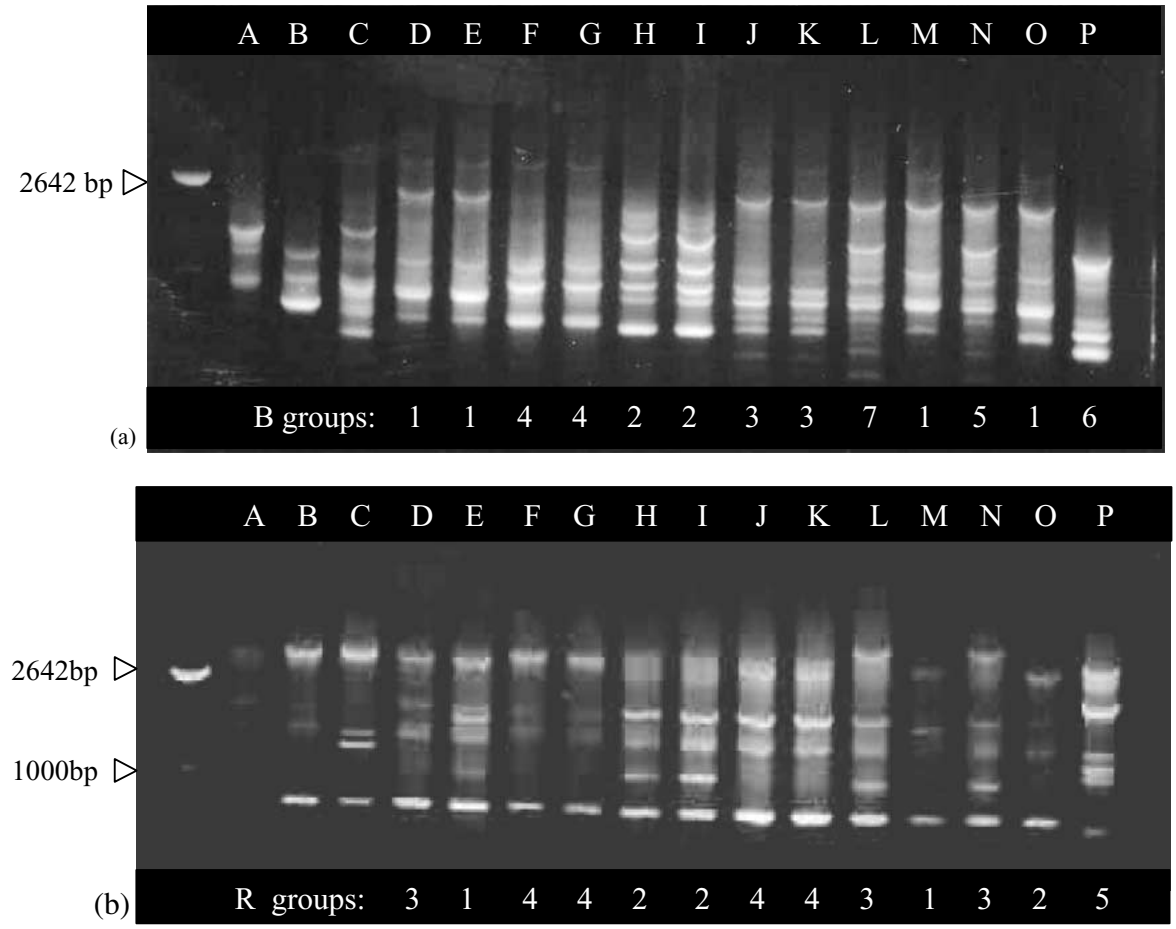

Figure 3. (a) Genomic fingerprint pattern of selected *X. campestris* strains showing the BOX-PCR groupings (B groups). (b) Genomic fingerprint pattern of selected *X. campestris* strains showing the REP-PCR groupings (R groups). Lane: A – *X. campestris* pv. *armoraciae* NCPPB 1930; B – *X.c.* pv. *abberans*; C – *X.c.* pv. *raphani*; D – *X.c.* pv. *campestris* NCPPB 3207; E – *X.c.* pv. *campestris* NCPPB 2031; F – *X.c.* pv. *campestris* B 147; G – SM 136; H – SM 94; I – SM 77; J – SM 58; K – SM 57; L – SM 38; M – SM 25; N – SM 13; O – SM 10; P – SM 125.

Discussion

Symptoms caused by Xcc in cabbage depend upon several factors such as cultivar, plant age (Schaad and Alvarez, 1993), light and temperature (Williams et al., 1972), humidity, strain of the pathogen and even the method used for inoculation (Franken, 1992). However, in the true leaves, blackened vascular tissues and V-shaped chlorotic to necrotic lesions along the leaf margins are characteristic symptoms of black rot (Cook et al., 1952; Williams, 1980; Onsando, 1992; Alvarez, 2000). Most of the Xcc strains in this study caused systemic black rot symptoms in cabbage. We observed 'blight' symptoms from some Xcc strains, characterised by necrosis and sudden collapse of large areas of mesophyll in advance of blackening of veins. Similar symptoms have been described by Alvarez et al. (1994). Strains SM 57 and the Xcc reference strain B 147 caused tan necrotic blotches, mesophyll collapse and severe blight-like symptoms. A similar reaction was observed for two of the strains used by Alvarez et al. (1994), who suggested that such strains were aggressive variants of Xcc. However, field evaluation of cabbage varieties for resistance against Xcc, revealed that these strains caused less severe black rot compared to the typical strains of Xcc when plants were inoculated through the roots or foliage (Wulff, 2000; Massomo, 2002). Based on the atypical rep-PCR

fingerprints, FAME profile and symptom expression in cabbage observed in this study, the reference strain B 147 does not appear to be a true Xcc but rather a member of the leaf spotting Xc group. Aberrant symptoms of black rot were also observed in studies conducted in Thailand (Thaveechai and Schaad, 1986) and Oklahoma (Zhao et al., 2000). In separate studies (unpublished), we have also observed that some strains that cause blight conforming to the description of Alvarez et al. (1994) do not always induce such symptoms in plants inoculated at the cotyledon stage. In the present study, the term 'blight' was used for symptoms characterised by a sudden mesophyll collapse and formation of tan necrotic blotches.

When the MIS was used for FAME analysis, all the Xcc were identified correctly to the species level. However, the ability of this system to identify Xcc strains to pathovar level was limited. The limitation of MIS in identification of xanthomonads has previously been reported (Yang et al., 1993; Weller et al., 2000). This may be attributable to inadequacies in the library entries in terms of number and/or diversity of strains in the respective databases. Results from this study corroborate the findings of Yang et al. (1993), who demonstrated that Xcc strains and other Xc strains isolated from brassica crops were closely related in their FAMEs. Most of the Xcc strains from Tanzania were grouped together tightly, in clusters I and II, with considerable heterogeneity in FAME profiles within the two clusters, these strains differed slightly in their FAMEs from the Xcc reference strains used. Since bacterial cell membrane, hence fatty acid composition respond to changes in environment (Weller et al., 2000), the observed heterogeneity of FAME profiles might be a reflection of the agroecological differences of the locations where leaf samples were collected. Similar observations were made in an earlier FAME analysis of Xcc strains from Tanzania (N. Kaaya, 1997 unpublished report). Analyses based on FAME composition also revealed the existence of a few Xcc strains that were more closely related to the reference strains of Xcr, Xca and Xcab than to Xcc (Figure 2).

The Biolog GN MicroPlate system correctly identified all Xcc strains to species level, but it was less accurate for pathovar and strain differentiation. It was consistently observed that some Xcc strains were identified only to the genus or species level, while other strains were invariably identified to the strain level as Biolog XccA and XccB types (Table 1). In addition, other Xcc strains were wrongly identified as Xcr. This anomaly was also observed by Mguni et al. (1999), who considered 'Xcc' as a Biolog type. Jones et al. (1993) reported 70% correct identification of Xcc with an earlier Version 3.0 of the Biolog GN system. Such variation or inconsistency may be an indication of the existence of Xcc strains with metabolic fingerprints different from those used in the Biolog GN database. Indeed, the suitability of commercially available databases of metabolic profiles for the identification of subtropical and tropical strains of bacteria was recently criticised by Black et al. (2000). The latest version of Biolog 4.1 does not designate Xcc strains to the strain level, which may be a reflection of the difficulties observed in the earlier versions. However, the Biolog GN MicroPlate system is a useful diagnostic procedure as it is quick and easy to use for routine identification of bacterial strains. It seems likely that the efficiency of the Biolog GN MicroPlate system in the identification of Xcc strains can be improved by expanding the database. In this study, Xcc strains identified as Biolog type XccA, including B 147 and SM 57, were generally associated with blight symptoms whereas Biolog type XccB strains were associated with the characteristic V-shaped lesions, blackening of veins and wilting, but without extensive chlorosis and sudden collapse of the mesophyll tissue. Mguni (1996) made similar observations. The relative predominance of Biolog XccA strains in Lushoto and Morogoro areas as opposed to the more widespread occurrence of Biolog XccB strains in Tanzania may be an indication of ecological adaptation.

Using the rep-PCR technique, variations were observed among strains of Xcc from brassica fields in Tanzania, and specific fingerprint patterns were linked to geographical areas. The predominance of rep-PCR groups R1 and R2 suggests limited dissemination or adaptability of other Xcc strains, such as rep-PCR group R3, in Tanzania. Linkage of rep-PCR profiles to geographical origin has recently been reported in *X. arboricola* pv. *arboricola* isolates from Persian walnut (*Juglans regia*) (Scortichini et al., 2001). Louws et al. (1994) considered that selection for a specialised niche could affect the distribution of repetitive sequences, leading to fingerprints unique to specific pathovars or strains. Sharples and Llyod (1990) noted that evolution of bacterial genomes is often linked with repeated DNA elements. Therefore, the relative restricted nature of strains of rep-PCR genomic fingerprint group B2 isolated from cabbage in Lushoto district, may perhaps be due to the evolution of strains adapted in this locality.

Alvarez et al. (1994) described the correlation between groups formed by RFLP and those formed by serology and pathogenicity tests and inferred that Xca and Xcr were synonymous in nature, a claim made earlier by Black and Machmud (1983). Tamura et al. (1994) reported that the host range of Xca was restricted to horse radish while Sahin and Miller (1997) reported the occurrence of a pathotype of Xca that attacked kale and radish (*Raphanus sativus*). In contrast, Vicente et al. (1998) could not confirm the existence of Xca and doubted the distinction between Xcc and other brassica pathovars. While such differences may be an indication of the complex nature of Xc pathovars attacking brassica, some of the problems associated with the differentiation of the pathovars of Xc are specifically related to their taxonomy and nomenclature. We observed close relatedness between the FAME profiles of Xcr and Xca, but their rep-PCR patterns were different. Remarkable similarity in rep-PCR fingerprints, FAME profiles and symptom expression in cabbage was observed between strains SM 57, Xcr strain NCPPB 1946 and Xcc strain B 147, which all caused symptoms atypical of black rot in cabbage (Table 1). However, there were no apparent correlations in rep-PCR, fatty acid and symptom expression among the rest of Xcc strains which caused characteristic black rot symptoms. Thus, even in the case of strains from Lushoto district where unique rep-PCR and Biolog fingerprinting were predominant, no correlation was found between the groupings made by the two methods. Strains of Xcc that produced characteristic black rot symptoms were previously found to be highly similar in their membrane protein profiles (Minsavage and Schaad, 1983).

The data obtained from this study confirm previous findings describing the heterogeneity within Xcc strains. Genetic diversity within other pathovars of xanthomonads affecting rice (Leach et al., 1992), cassava (Verdier et al., 1994) and tomato/pepper (Louws et al., 1995) has been described. Chen et al. (1994) provided evidence that the pathovar status in Xc may, in some cases, be determined by a very few genes independent of phylogenetic background. Each of the methods tested allowed a certain extent of identification of strains from species, pathovar to the strain level. The Biolog and MIS methods were generally less certain for pathovar and strain differentiation. We found the rep-PCR technique to be extremely reliable, reproducible, rapid and highly discriminatory in the study of diversity of Xcc. This further confirm the utility of rep-PCR for differentiation of closely-related strains of bacteria and the potential usefulness for studying bacterial evolution in specific ecological areas.While the unique fingerprint profiles of Xcc generated by rep-PCR could be a useful tool in diagnosis and in differentiation of strains, without a database the utility of rep-PCR for routine identification of Xcc strains is limited. These limitations warrant continued improvements and creation (in case of rep-PCR) of such databases as well as inclusion of pathogenicity testing, using universally susceptible cultivars, as an integral part of strain identification.

The present results indicate the existence of Xcc strains in Tanzania that are distinct from those used to create the Biolog and MIS databases. With the suggested existence of pathogenic variants (races) in Xcc (Ignatov et al., 1998; Vicente et al., 2001; Taylor et al., 2002), knowledge about the genetic diversity of Xcc in the locality will be essential whenever disease management strategies are based on host plant resistance.

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