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Full Length Research Paper

Xanthomonas campestris pv. *campestris* race 1 is the main causal agent of black rot of Brassicas in Southern Mozambique

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Severe outbreaks of bacterial black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) were observed in *Brassica* production fields of Southern Mozambique. The causal agent of the disease in the Mahotas and Chòkwé districts was identified and characterised. In total, 83 *Xanthomonas*-like strains were isolated from seed samples and leaves of cabbage and tronchuda cole with typical symptoms of the disease. Forty-six out of the 83 strains were found to be putative *Xcc* in at least one of the tests used: Classical biochemical assays, enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, Biolog identification system, polymerase chain reaction (PCR) with specific primers and pathogenicity tests. The ELISA tests were positive for 43 strains. Biolog identified 43 strains as *Xanthomonas*, but only 32 as *Xcc*. PCR tests with primers targeting a fragment of the *hrpF* gene were positive for all 46 strains tested. Three strains were not pathogenic or weakly pathogenic and all other strains caused typical black rot symptoms in brassicas. Race type differentiation tests revealed the *Xcc* strains from Mozambique as members of race 1. The prevalence of this pathogenic race of the *Xcc* pathogen in Mozambique should be considered when black rot resistant cultivars are evaluated or introduced into the production regions of this country.

Key words: Cole crops, cabbage, tronchuda, landraces, seeds, black rot, enzyme-linked immunosorbent assay (ELISA), Biolog, polymerase chain reaction (PCR), race-type.

INTRODUCTION

In many African countries, including Mozambique, cabbage (*Brassica oleracea* L. var. *capitata* L.) and other leafy brassicas rank among the most important vegetables. One serious threat to sustainable production of *brassicas* is black rot disease caused by *Xanthomonas campestris* pv. *Campestris* (Pammel) Dowson (*Xcc*) The typical leaf symptom of black rot is V-shaped lesions on the leaf margin with black veins (Alvarez, 2000). Other observed symptoms are characterised by necrosis and sudden collapse of large areas of mesophyll in advance of blackening of veins (Alvarez et al., 1994; Massomo et al., 2003). Cabbage heads affected by *Xcc* loose market value and may rot in the field or soon after harvest (Massomo et al., 2003). The black rot pathogen is seed-borne and can survive in plant debris and crucifer weeds (Williams, 1980; Alvarez, 2000). Control of black rot has proven to be difficult due to its seed-borne nature, to the

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low efficiency of chemical control, and the lack of resistant cultivars (Williams, 1980; Onsando, 1992; Alvarez, 2000; Massomo et al., 2003).

The genus *Xanthomonas* has a wide host range, and based on host specificity, 123 pathovars of *Xanthomonas campestris* were recognised (Dye et al., 1980). However, DNA-DNA hybridization studies proposed that *Xanthomonas campestris* consists of *Xcc* and not more than five additional pathovars (*aberrans, armoraciae, barbareae, incanae* and *raphani*), all causing diseases in crucifers (Vauterin et al., 1995). The validity of these pathovars has been questioned and strains from these six pathovars were shown to produce only three distinct disease phenotypes: black rot due to *Xcc*, leaf spot disease of crucifers and solanaceous hosts caused by *X. campestris* pv. *raphani*, and bacterial blight of ornamental crucifers typically caused by *X. campestris* pv. *incanae* (Fargier and Manceau, 2007). Several races of *Xcc* were also described based on interactions with differential varieties. Kamoun et al. (1992) reported five different races of *Xcc* based on the reaction with certain cultivars of turnip (*B. rapa*) and mustard (*B. juncea*). Vicente et al. (2001) described six races and Fargier and Manceau (2007) added three new races within *Xcc*. Races 1 and 4 were reported as the most common and aggressive races in *B. oleracea* crops (Vicente et al., 2001; Lema et al., 2012; Mulema et al., 2012).

Xcc is known to be composed by genetically, serologically and pathogenically diverse groups of strains (Alvarez et al., 1994). Methods for detection of *Xcc* from seeds currently recommended by the International Seed Testing Association (ISTA) are based on plating seed extracts onto semi-selective agar substrates and confirmation of identity of the isolated bacteria by pathogenicity tests (ISTA, 2007). However, differentiation of *Xcc* strains from closely related pathovars that are pathogenic on other *brassicas* is not possible based solely on morphological and biochemical characters, and it is often difficult by pathogenicity tests (Franken, 1992). The Biolog identification system, based on carbon source utilization tests, was used to characterise *Xcc* to the species level by Mguni et al. (1999) and Massomo et al. (2003). Monoclonal and polyclonal antibodies were used in routine identification and in the characterisation of *Xcc* strains by Franken (1992), Berg et al. (2005) and Chidamba and Benzuidehnout (2012).

Molecular detection methods based on DNA-DNA hybridisation studies (Vauterin et al., 1995), rRNA gene analysis (Simões et al., 2007), repetitive sequence-based PCR (rep-PCR) (Louws et al., 1994; Massomo et al., 2003; Valverde et al., 2007; Jensen et al., 2010; Chidamba and Benzuidehnout, 2012) and amplified fragment length polymorphism (AFLP) (Valverde et al., 2007) have been increasingly applied for species and pathovar differentiation. Genetic variation of strains of the black rot pathogen affecting brassicas in Tanzania has been reported (Massomo et al., 2003). PCR tests have been reported in the detection and identification of the black rot pathogen from *brassicas* (Berg et al., 2005; Fargier and Manceau, 2007; Zacchardelli et al., 2007; Mathis et al., 2009).

Black rot of *brassicas* has been reported in Mozambique, an important producer and consumer of *Brassica* crops (Segeren et al., 1994). The disease remains a serious problem in major brassica production areas of the country. In 2007, an outbreak of the disease was reported causing severe damage on seed-beds, and field plants under sprinkler and furrow-irrigation of Boane, Mahotas and Chòkwé districts of Southern Mozambique. A high disease incidence (up to 70%) was observed affecting popularly grown brassicas (Bila, 2008). At the time, the source of inoculum and health status of seed and seedlings were not known. However, there were strong indications that the outbreaks could be related to seed-borne infections of the *Xcc* pathogen.

The main objectives of this study were (i) to isolate and verify the identity of strains causing black rot like symptoms of brassica in Southern districts of Mozambique using different methods including classical biochemical assays, enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, Biolog identification system, polymerase chain reaction (PCR) with specific primers and pathogenicity tests; and (ii) to characterise the identified strains by race typing on differential *Brassica* spp. genotypes.

MATERIALS AND METHODS

Seed samples

One seed sample of *Brassica oleracea* L. var. *capitata* L. (cultivar 'Copenhagen Market'), DSHC accession number 48.623, and two of Portuguese tronchuda coles "Couve Tronchuda" (*B. oleracea* L. var. *costata* DC.; Dias, 1995), DSHC accession numbers 48.622 (imported) and 48.624 (locally produced in Mahotas district), were collected in 2007 and tested in 2008 for the presence of *Xcc* by liquid assays.

Collection of diseased leaf samples

Leaf samples from seed-bed and field plants of cabbage and Portuguese tronchuda cole landraces showing black rot symptoms in the fields, were collected from farms in areas of Chókwé and Mahotas districts, Southern Mozambique in August-September, 2007 (Bila, 2008). Seventy eight farms were visited and the incidence of black rot disease in *Brassica* landraces and cultivars was evaluated. Leaf samples were collected from 40 farms from plants showing black rot symptoms. Samples were placed in paper bags and taken to the laboratory for the isolation of the pathogen. The isolations were conducted from the cabbage cultivars 'Copenhagen Market', 'Glory F_1', 'Glory of Enkhuizen' and 'Starke', and from Portuguese tronchuda cole landraces grown locally for several years.

Table 1. Reference strains used in this study.

Strain	Strains (race-type)[a]	Geographical origin	Host of origin	Reference strains used in tests
Acidovorax avenae subsp*. avenae*	NCPPB 3356	Nepal	*Oryza sativa*	ELISA
Pseudomonas syringae pv. *maculicola*	NCPPB 2039	New Zealand	*Brassica oleracea* var. *botrytis*	ELISA, Biolog, PCR
Xanthomonas campestris pv. *armoraciae*	NCPPB 1930	New Zealand	*Armoracia lapathifolia*	PCR
Xanthomonas campestris pv. *campestris*	SM35, 36, 37, 38, 55, 56, 74, 75 (race 1)	Tanzania	*Brassica oleracea* var. *capitata*	Biolog, race-typing
	NCPPB 3207 (race 1)	Zimbabwe	*Brassica oleracea* var. *capitata*	Isolation colony morphology, biochemical tests, ELISA, PCR, Biolog, pathogencity, race-typing
	HRI 3811 (race 1)	USA	*Brassica oleracea* var. *capitata*	Race-typing
	NCPPB 2031 (race 1)	South Africa	*Brassica oleracea* var. *capitata*	Race-typing
	NCPPB528 (race 3)	UK	*Brassica oleracea* var. *gemnifera*	Race-typing
	HRI 1279A (race 4)	UK	*Brassica oleracea* var. *capitata*	Race-typing
	HRI 3880 (race 5)	Australia	*Brassica oleracea* var. *capitata*	Race-typing
	B147[b] (race 5)	Hawaii, USA	NG	Isolation colony morphology, biochemical tests, ELISA, PCR, Pathogenicity, race-typing
	HRI 6181 (race 6)	Portugal	*Brassica rapa*	Race-typing
Xanthomonas campestris pv. *raphani*	NCPPB 1946	USA	*Raphanus sativus*	Pathogenicity

[a]NCPPB, National Collection of Plant Pathogenic Bacteria; HRI, School of Life Sciences (ex-Warwick HRI), The University of Warwick, Wellesbourne, UK; SM, from Massomo et al. (2003); Strain B147 provided by A. Alvarez, Hawaii, USA; NG: not given.

Reference strains

Information on the reference strains used in the different tests is presented in Table 1. The strains were grown on yeast dextrose calcium carbonate (YDC) agar plates to ensure purity and incubated at 28°C for 2 days. Strains were stored at -80°C on porcelain beads of Protect Bacterial Preservers (Protect System, Bury, UK) until use for the tests.

Isolation of strains

Isolation of potential *Xcc* strains from commercial and locally produced seed samples was performed according to the standardised liquid plating assay (ISTA, 2007). Plant material was rinsed in tap water and then dried at room temperature between sheets of absorbent paper. Bacteria were isolated from *Brassica* leaves showing black rot symptoms by excising sections from the lesions margins, which were placed on a glass slide with a drop of sterile saline solution (0.85% NaCl) and observed for the presence of bacterial ooze. Loopfuls of bacterial suspension were streaked onto mCS20ABN and FS agar (ISTA, 2007). Reference *Xcc* strains NCPPB 3207 and B147 served as positive controls. A sterility check using saline solution was included as a negative control. Plates were incubated at 28°C for 3 to 4 days and were observed for the presence of putative *Xcc* colonies.

Suspected bacterial strains isolated from seed and leaf sections that showed to be positive for starch hydrolysis on mCS20ABN and FS agar plates were streaked onto YDC agar and incubated at 28°C for 2 days. Single yellow pigmented colonies were transferred to another YDC agar plate to secure purity. Strains were stored at -80°C on porcelain beads of Protect Bacterial Preservers (Protect System, Bury, UK) until further identification.

Characterization and identification of the isolated bacterial strains

Preliminary characterization of the strains

Putative *Xcc* strains isolated from seed samples and leaf sections (Table 2) were subjected to Gram reaction, Oxidase reaction, oxidative/fermentative utilisation of glucose, arginine dihydrolase and nitrate reduction reaction tests

Table 2. Identification and characterisation of 46 isolates of *Xanthomonas campestris* pv. *campestris* and related bacteria isolated from *Brassica oleracea* seeds and leaf samples obtained in the Chókwé and Mahotas districts of Mozambique.

Strain Number	Origin *Brassica* crop	ELISA[a]	Biolog[b] (Similarity value)	PCR[c]	Pathogenicity[d]	Race[e]
Isolated from commercial seeds obtained in Chókwé:						
22A	Portuguese tronchuda	+	*Xcr* (0.54)	+	+	1
22B	Portuguese tronchuda	+	*Xcc* (0.91)	+	+	1
22C	Portuguese tronchuda	+	*Xcc* (0.81)	+	+	1
22D	Portuguese tronchuda	+	*Xcc* (0.82)	+	+	1
22E	Portuguese tronchuda	+	*Xcc* (0.78)	+	+	1
22F	Portuguese tronchuda	+	*Xcc* (0.68)	+	+	1
22G	Portuguese tronchuda	ND	*Xcc* (0.71)	+	+	1
23A	Cabbage	+	*Xcc* (0.82)	+	+	1
23B	Cabbage	+	No ID	+	+	1
23C	Cabbage	+	*Xanthomonas*	+	+	1
23D	Cabbage	+	*Xanthomonas*	+	+	1
23E	Cabbage	+	*Xcb* (0.81)	+	+	1
23F	Cabbage	+	*Xanthomonas*	+	+	1
23G	Cabbage	+	*Xcp* (0.82)	+	+	1
Isolated from farm saved seed in Mahotas:						
24E	Portuguese tronchuda	-	*Xcc* (0.68)	+	+	1
Isolated from a leaf sample from Chókwè:						
9	Cabbage	+	*Xanthomonas*	+	+	1
Isolated from leaf samples from Mahotas:						
43	Cabbage	+	*Xcc* (0.74)	nd	+	1
50	Cabbage	-	*Xcc* (0.76)	+	-	-
51	Cabbage	+	Sp (0.73)	+	+	1
52	Cabbage	+	*Xcp* (0.80)	+	+	1
53	Cabbage	+	*Xcc* (0.75)	+	+	1
54	Cabbage	+	*Xcc* (0.76)	+	+	1
55	Cabbage	+	No ID	+	+	-
56	Cabbage	+	*Xcc* (0.83)	+	+	1
57	Cabbage	+	*Xcc* (0.84)	+	+	1
58	Cabbage	+	*Xcr* (0.73)	+	+	1
59	Cabbage	+	*Xcc* (0.83)	+	+	1
60	Cabbage	+	*Xcc* (0.67)	+	+	1
61	Cabbage	+	*Xcc* (0.61)	+	+	1
62	Cabbage	+	*Xcc* (0.76)	+	+	-

Table 2. Contd.

64	Cabbage	+	*Xcc* (0.59)	+	+	1
66	Cabbage	+	*Xcc* (0.52)	+	+	1
67	Cabbage	+	*Xcc* (0.65)	+	+	1
68	Cabbage	+	*Xcc* (0.74)	+	+	1
69	Cabbage	+	*Xcr* (0.68)	+	+	1
70	Cabbage	+	*Xcc* (0.68)	+	+	1
72	Cabbage	+	*Xcc* (0.70)	+	+	1
73	Portuguese tronchuda	+	*Xcc* (0.71)	+	+	1
74	Cabbage	+	*Xcr* (0.66)	+	+	1
75	Cabbage	+	*Xcc* (0.74)	+	+	1
76	Cabbage	+	*Xcc* (0.62)	+	+	1
77	Cabbage	+	*Xcc* (0.83)	+	+	1
78	Cabbage	+	*Xcc* (0.82)	+	+	1
79	Cabbage	+	*Xcc* (0.69)	+	+	1
83	Cabbage	-	*Xcc* (0.82)	+	+	1
84	Cabbage	+	*Xcc* (0.69)	+	+	1

[a]Symbols: + and – indicate species was detected or not detected, respectively; nd, not done; [b]Abreviations of names of strains are as follows: *Xcc*, *X. campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani; Xcp*, *X. campestris* pv. *poinsettiicola*; *Xcb*, *X. campestris* pv. *begoniae;* Sp, *Sphingomonas parapaucimobilis*; No ID, No identification whenever similarity value was bellow 0.5; [c]According to Berg et al. (2005). [d]Pathogenicity tests conducted in Savoy cabbage plants. 'Wirosa $F_{1'}$; results recorded 10 and 14 days after inoculation: +, black rot V-shaped lesions; -, no symptoms observed. [e] Determined following inoculations on differential cultivars ('Just Right' turnip, 'Cob60' rape, 'Seven Top' turnip, 'FBLM2' mustard, 'Miracle F_1' cawliflower and 'SxD1' cole).

(Lelliot and Stead, 1987). Reference Xcc strains NCPPB 3207 and B147 were included as positive controls.

Enzyme-linked immunosorbent assay (ELISA)

All the putative *Xcc* strains isolated from seed and leaf samples were tested by indirect ELISA with monoclonal antibodies (Agdia Inc., 303380 Country Road 6, Elkhart, Indiana 46514 USA) for identification of *X. campestris* pv. *campestris* following the instructions of the manufacturer. *Acidovorax avenae* subsp. *avenae* NCPPB 3353 a non-related plant pathogen to cabbage and *Pseudomonas syringae* pv. *maculicola* NCPPB 2039, a leaf spot pathogen of cabbage, served as negative controls for the ELISA tests. The reference *Xcc* strains NCPPB 3207 and B147 were used as positive controls. Wells in which color developed were an indication of positive results, while wells in which there was no significant color development indicated negative results.

Biolog identification

Bacterial strains were tested for ability to metabolise carbon sources using Biolog GN Microtiter Plates (Microlog 2, version 3.5, Biolog Inc., Hayward, CA, USA) following instructions of the manufacturer. The *Xcc* strains NCPPB 3207 and *Pseudomonas syringae* pv. *maculicola* NCPPB 2039 served as the positive and negative controls, respectively. *Xcc* strains isolated from Tanzania (SM35, SM36, SM38, SM55 and SM56 and SM75) by Massomo et al. (2003) were also included in the Biolog tests as positive controls.

Polymerase chain reaction (PCR)

Extraction of bacterial DNA from 45 out of 46 putative *Xcc* strains from Mozambique was conducted from 24 to 48 h pure culture grown on Nutrient agar (NA) with the DNeasy purification kit for Gram-negative bacteria (DNeasy Blood and Tissue Kit, Qiagen) following instructions of the supplier. PCR tests were conducted with primers pair, DLH120 (5'-CCGTAGCACTTAGT-GCAATG-3') and DLH125 (5'-GCATTTCCATCGGT-CACGAT TG-3') designed to amplify a 619 bp fragment of the 3' end of *hrpF* gene from *X. campestris* (Berg et al., 2005) with minor modifications. Reference *Xcc* strains NCPPB 3207, B147, and *X. campestris* pv. *armoraciae* NCPPB 1930 were used as positive controls while *Pseudomonas syringae* pv. *maculicola* NCPPB 2039 and buffer alone served as negative control for the PCR tests. Additionally, *Xcc* strains

isolated from Tanzania (SM35, SM36, SM38, SM74 and SM75) by Massomo et al. (2003) were tested in the PCR assays as positive controls.

Pathogenicity tests

The pathogenicity assays were conducted by inoculating the two upper leaves of 3 to 4 leaf stage *Brassica* seedlings of the savoy cabbage cv. Wirosa F1 (*B. oleracea* L. var. sabauda L.) with known susceptibility to all *Xcc* strains (Vicente et al., 2001; ISTA, 2007) which were grown in 800 cm^3 pots in sterile soil (Pindstrup substrate number 2, Pindstrup Mosebrug A/S, Denmark). A small amount of bacterial growth directly from a 24 h YDC culture was scraped with a sterile insect pin. Six major veins of the two youngest leaves were stab inoculated near the leaf edges with the insect pin loaded with bacterial growth. Four plants were inoculated per isolate. Reference *Xcc* strains NCPPB 3207 and B147 were included as the positive control for the pathogenicity tests, while stabbing with a clean insect pin served as the negative control. Furthermore, *Xcc* strains isolated from Tanzania (SM35, SM36, SM38, SM55, SM56, SM74 and SM75) by Massomo et al. (2003) were also included in the pathogenicity tests as positive controls. Inoculated plants were grown in a growth chamber at 20 to 25°C. Plants were recorded for typical black rot symptoms in form of progressive V-shaped, yellow necrotic lesions with blackened veins after 5 to 14 days.

Race determination

For the race determination, 46 bacterial strains isolated from seeds and leaves from Mozambique as well as eight *Xcc* strains (SM 35, 36, 38, 55, 56, 75 and 74) isolated from Tanzania (Massomo et al., 2003) (Table 2) were tested. While the reference *Xcc* strains NCPPB 3207, NCPPB 2030, HRI3811 (race 1), NCPPB 528 (race 3), HRI 1279A (race 4), HRI 3880 and B147 (race 5), and HRI6181 (race 6) served as positive controls, sterile water was used as the negative control in the race determination tests.

The improved set of differential *Brassica* spp. reported by Jensen et al. (2010), consisting of the susceptible control Savoy cabbage 'Wirosa F_1' and the differentials 'Just Right' turnip (*B. rapa* L.), 'Cob 60' rape (*B. napus* L.), 'Seven Top' turnip (*B. rapa* L.), 'PIC1' mustard (*B. carinata* L.), 'FBLM2' mustard (*B. juncea* (L.) Czernj. and Coss), 'Miracle F_1' cawliflower (*B. oleracea* L. var. *botrytis* L.) and 'SxD1' cole (*B. oleracea* L.) were used in the race determination tests.

Cabbage plants were raised from seed sown in peat soil as earlier described, in a greenhouse with a day/night temperature of 20/18°C, venting at 23/18°C (day/night), with supplementary lightning to give 16 h days. Bacterial strains were grown on nutrient agar medium at 28°C for 48 h. Bacterial culture growth was scraped from the plates and suspended in 10 ml of sterile distilled water to produce a turbid suspension (10^8 CFU/ml) with OD_{600} = 0.01 determined by using a Nanodrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc.). Leaves of four-week-old plants were inoculated according to Vicente et al. (2001) by clipping secondary veins near the leaf margins using a mouse tooth forceps with teeth wrapped in absorbent cotton wool dipped into the bacterial suspension. Leaves inoculated with cotton wool dipped in sterile water served as negative control. Ten points of inoculations were made per leaf, and the three youngest leaves on each plant were inoculated. After inoculation the plants were kept at 27/25°C (day/night) and maintained under humid conditions covered with plastic and' ventilating every second day. Two to three plants were inoculated per strain, and the inoculations were repeated if the first results indicated that the strain was weakly or non-pathogenic. Black rot disease symptoms were assayed on a scale of 0 to 3 based on the relatively size of the largest lesion on the leaf according to Vicente et al. (2001). The presence or absence of symptoms was recorded two and three weeks after inoculation.

RESULTS

Bacterial isolation

Eighty-three *Xanthomonas*-like strains were isolated from seed and leaf samples using two semi-selective media, mCS20ABN and FS agar (ISTA, 2007). Forty-six out of the 83 strains were selected for further identification based on colony morphology on YDC medium and ability to hydrolyse starch. Out of the 46 strains, fifteen were isolated from three seed samples of the cabbage 'Copenhagen Market' (DSHC accession number 48.623), and Portuguese tronchuda coles (DSHC accession number 48.622 and 42624) using the liquid plating assay. Thirty-one strains isolated from leaves of the cabbage 'Glory F_1', 'Glory of Enkhuizen', 'Copenhagen Market' and 'Starke' from fields of Mahotas and Chòkwé were selected for further identification and characterisation. Thirty of these strains were obtained from plants growing in fields located in Mahotas while only one putative *Xcc* strain was obtained from Chókwè.

Characterization and identification of the bacterial strains

Preliminary characterization of the strains

All the 46 strains, 15 originating from seed (Table 2) and 31 from leaf samples (Table 2), were considered to be putative *Xcc*, as they were found to be Gram negative, negative or weak positive in the oxidase test, nitrate negative or weak positive, with oxidative metabolism of glucose and showed to hydrolyse starch on mCSABN20 and FS agar. These 46 strains were subjected to further identification by ELISA, Biolog, PCR and pathogenicity tests.

ELISA

Forty-three out of 46 strains isolated from both seed and leaf samples were positive in ELISA tests (Table 2). The strain 24E isolated from kale seed and strains 50 and 83 isolated from cabbage leaves were negative in the ELISA tests (Table 2). Positive test results were observed with the reference *Xcc* strains (NCPPB 3207 and B147) that were used as positive controls. Cross reactions were observed with the negative controls, *Acidovorax avenae* subsp. *avenae* NCPPB 3356 and *Pseudomonas syringae*

pv. *maculicola* NCPPB 2039.

Biolog identification

The Biolog system identified 39 out of the 46 tested strains as pathovars of *Xanthomonas campestris*, with 32 of these strains identified as *X. campestris* pv. *campestris* (*Xcc*), four as *X*. *campestris* pv. *raphani* (*Xcr*), two as *X*. *campestris* pv. *poinsenttiicola* (*Xcp*) (valid name: *X*. *axonopodis* pv. *poinsenttiicola*) and one as *X*. *campestris* pv. *begoniae* (*Xcb*) (valid name: *X*. *axonopodis* pv. *begoniae*), with similarity values between 0.52 and 0.91. In addition, while four of the tested strains were only identified at genus level as *Xanthomonas*, one of the strains was identified as *Sphingomonas parapaucimobilis* (strain 51); two strains (strain 23B isolated from cabbage seed, and strain 55 from cabbage leaf) were not identified by Biolog (Table 2). The reference *Xcc* strain NCPPB 3207 was identified as *Xcc* (similarity value 0.59), while *Pseudomonas syringae* pv. *maculicola* NCPPB 2039 strain was identified as *Pseudomonas syringae* pv. *pisi* (similarity value 0.75) (data not shown).

Polymerase chain reaction (PCR)

PCR tests amplified a 619 bp fragment corresponding to part of the *hrpF* gene from the total DNA of all the 46 putative *Xcc* strains isolated from seed and leaf samples of *Brassica* (Table 2), including the positive control *Xcc* reference strains (B147 and NCPPB 3207), *Xcr* NCPPB 1930 and *Xcc* strains isolated from Tanzania (SM35, SM36, SM38, SM74 and SM75). Weak reactions were observed with one of the negative controls, namely *Pseudomonas syringae* pv. *maculicola* NCPPB 2039 (data not shown).

Pathogenicity tests

With exception of the strain 50, all the 46 tested strains were pathogenic to savoy cabbage 'Wirosa' (Table 2). Characteristic black rot symptoms were induced by 45 of the tested strains and by the *Xcc* reference strains (NCPPB 3207 and B147).

Race determination

The reactions obtained following inoculation of *Brassica* differentials revealed 43 out of 46 putative *Xcc* strains from Mozambique and eight *Xcc* strains from Tanzania as members of race 1 (Table 2). These isolates induced black rot symptoms on the *Brassica* cultivars: Savoy cabbage 'Wirosa F_{1}', 'Just Right' turnip, 'Seven Top' turnip, 'Cob 60' rape, 'Miracle' cawliflower and 'SxD1' cole, but did not induce symptoms on 'PIC1' and 'FBLM2' mustards. The same result was obtained with the reference *Xcc* strains of race 1 (HRI 3811, NCPPB 3207 and NCPPB 2031) and *Xcc* strains isolated from Tanzania (SM35, SM36, SM38, SM74 and SM75). No apparent symptoms were observed in any of the *Brassica* cultivars inoculated with isolates 50, 55 and 62 from Mozambique. Phenotype interactions of reference *Xcc* strains of race 3 (NCPPB 528), race 4 (HRI 1279A), race 5 (HRI 3380 and B147) and race 6 (HRI 6181), in the differential *Brassica* cultivars, followed the pattern described by Vicente et al. (2001). Plants inoculated with water (negative control) did not show any symptom of black rot.

DISCUSSION

The present study confirms the presence of the black rot pathogen in the Southern region of Mozambique. *X. campetris* pv. *campestris* (*Xcc)* was detected in seed and leaf samples from the brassica producing areas of Mahotas and Chòkwé. Fourty-six out of 83 *Xanthomonas* like strains isolated from seed and leaf samples, were found to be *Xcc* in at least one of the applied tests. Although a limited number of seed samples were tested in this investigation, the current results still reveal the presence of the black rot pathogen in seeds from Mozambique. This result confirms that seeds are an important source of inoculum of *Xcc* and supports the need for improvement of the seed health status of *Brassica* in the Southern part of the country. From field growing plants, thirty-one strains were isolated indicating the presence of the pathogen in the farmers' fields. However, most of the strains isolated from leaves were found in the district of Mahotas as compared to Chókwè. This very limited number of strains could be explained by the poor storage conditions available for leaf samples collected in Chókwè. The antibodies for detection of *Xcc* used in the ELISA tests reacted positively with 43 strains from Mozambique. The tests failed to identify some of the strains, for example, strains 24E and 83 which were later identified as race 1, an indication that these strains might be different *Xcc* genotypes not recognised by the antibodies used, resulting thus in false negatives. In addition, problems with cross reactions were also observed with the negative reference controls (*Acidovorax avenae* subsp. *avenae* NCPPB 3356 and *Pseudomonas syringae* pv. *maculicola* NCPPB 2039). Cross reactions of *Xcc* antibodies with other *X. campestris* pathovars and non-xanthomonads have been reported (Franken, 1992; Berg et al., 2005). As indicated by other authors like Franken (1992), the final identification of the strains cannot rely on ELISA tests alone, but it should be complemented with pathogenicity tests.

Based on the Biolog nutritional profile, 14 out of 15 isolates from seeds and 29 out of 31 isolates from leaves

were identified as *Xanthomonas*. Of these isolates, 8 isolates from seeds and 24 isolates from leaves were identified as *Xcc*. The Biolog system misidentified some isolates as *X*. *campestris* pv. *raphani* (*Xcr*), *X*. *campestris* pv. *poinsenttiicola* (*Xcp*) (valid name: *X*. *axonopodis* pv. *poinsenttiicola*), *X*. *campestris* pv. *begoniae* (*Xcb*) (valid name: *X*. *axonopodis* pv. *begoniae*) and other *Xanthomonas* spp.

Biolog was used in the identification of *X. campestris* in *Brassica* spp. in studies conducted in Tanzania (Massomo et al., 2003) and Zimbabwe (Mguni, 1999). As indicated by Zhao et al. (2000) and Massomo et al. (2003), Biolog identification tends to be reliable only to species level. In the present study, the system was able to identify 32 strains out of 46 (70%) as *Xcc*; four strains were identified as the closely related pathovar *Xcr*. The system was indeed more reliable at the genus level with 43 out of 46 strains (93%) being identified as *Xanthomo-nas*. Two strains could not be identified; strain 55 was weakly pathogenic or non-pathogenic whilst the strain 23B produced typical black rot symptoms in pathogenicity tests. Strain 51 was misidentified as *Sphingomonas parapaucimobilis* in the Biolog test, as the strain proved to give a positive result in the pathogenicity tests and identified as race 1. These limitations revealed the need for upgrading the Biolog identification system to include new strains with metabolic fingerprints different from those already found in the Biolog GN database as suggested by Massomo et al. (2003). Black et al. (2000) have also discussed the shortcomings of commercially available databases in the identification of subtropical and tropical strains of bacteria.

The PCR tests with the primers DLH120 and DLH125 that target the *hrpF* gene (Berg et al., 2005) showed to be able to detect all the 46 isolated strains as well as the reference strains of *Xcc* and other *X*. *campestris* pathovars (that is, *armoraciae*) affecting *brassicas*. This was expected as the primers developed by Berg et al. (2005) were reported to be specific to the *X. campestris* pathovars (that is, *campestris*, *armoraciae*, *raphani* and *incanae*) (Berg et al., 2005). However, the PCR tests showed also a weakly positive reaction with the negative reference strain *Pseudomonas syringae* pv. *maculicola*, indicating the possibility of detection of false positives. The use of other primers in singlex or in multiplex PCR reported for the diagnosis of the *Xcc* pathogen (Fargier and Manceau, 2007; Zacchardelli et al., 2007; Mathis et al. 2009) could be included in future identification studies.

Results obtained from the pathogenicity tests showed that with the exception of the strain 50 (negative in ELISA, but positive in the Biolog and PCR tests), all the strains from seeds and leaves from Mozambique were pathogenic to Savoy cabbage 'Wirosa F_1' producing black rot symptoms and should therefore be considered *X. campestris* pv. *campestris*. This included some strains not identified as *Xcc* by Biolog and ELISA. The pathogenicity tests showed that only pathovar campestris was present as none of the strains produced other symptoms. Although the Biolog system indicated that some strains might be *X. campestris* pv. *raphani*, we did not observe leaf spot and lesions in the brassicas tested typical of pathovar *raphani* (Vicente et al., 2006).

The present study revealed the presence of race 1 of *Xcc* in *Brassica* seed and crops in the two surveyed areas of the Southern part of Mozambique. Different studies have reported the predominance of race 1 of *Xcc* in *Brassica* crops cultivated in Africa and elsewhere (Lema et al., 2012). Race 1 appeared to be the most widespread *Xcc* race worldwide, with race 4 as the second most common race (Vicente et al., 2001; Mulema et al., 2012). Mulema et al. (2012) reported race 1 in Tanzania, but not in Uganda; this was explained by the small sampling population. A limited number of *Xcc* strains isolated by Massomo et al. (2003) representing the rep-PCR groups of the pathogen in Tanzania were found to belong to race 1 in the present study. Race 4 was suggested to be the most important *Xcc* race in Kenya and Uganda (Mulema et al., 2012) and South Africa (Chidamba and Bezuidenhout, 2012).

In the present study, most of the strains from Mozambique were isolated from *B. oleracea* varieties. Vicente et al. (2001) also reported that there could be a relationship between race and host of origin, as isolates belonging to race 1, 2, 3 and 4 were all isolated from *B. oleracea* crops or weeds growing in association with *B*. *oleracea* crops. Future surveys carried out in different growing seasons, ecological zones and other *brassicas* in Mozambique are to be conducted in order to document the prevalent types of *Xcc* strains in the country.

Each of the methods used in this study allowed a degree of identification from species, pathovar to the strain level. However, there were some inconsistencies in the results with the different methods used in the identification of *Xcc*. While 45 strains were positive in pathogenicity tests, only 43 were positive in race differentiation tests. Strain 50 was negative both in pathogenicity and race differentiation tests; while strains 55 and 62 although positive in preliminary pathogenicity tests inducing typical V-shape lesions could not be race differentiated. Since these strains did not induce black rot symptoms in any of the other tested *Brassica* varieties in the race typing tests either, this could be an indication that these strains either lost their pathogenicity or that the strains could be other variants of *X. campestris*.

This investigation confirms the presence of the black rot pathogen *X. campestris* pv. *campestris* race 1 in the cole fields in Southern Mozambique. The presence of race 1 of the pathogen should be considered when resistant cultivars are bred and introduced into regions in Mozambique as a part of integrated disease management strategies to achieve the control of this important disease of brassicas.

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