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Characterization of *Xanthomonas campestris* pv. *campestris* in Algeria

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Summary. *Xanthomonas campestris* pv. *campestris* (*Xcc*) causes the black rot of cruciferous plants. This seed-borne bacterium is considered as the most destructive disease to cruciferous crops. Although sources of contamination are various, seeds are the main source of transmission. Typical symptoms of black rot were first observed in 2011 on cabbage and cauliflower fields in the main production areas of Algeria. Leaf samples displaying typical symptoms were collected during 2011 to 2014, and 170 strains were isolated from 45 commercial fields. *Xcc* isolates were very homogeneous in morphological, physiological and biochemical characteristics similar to reference strains, and gave positive pathogenicity and molecular test results (multiplex PCR with specific primers). This is the first record of *Xcc* in Algeria. Genetic diversity within the isolates was assessed in comparison with strains isolated elsewhere. A multilocus sequence analysis based on two housekeeping genes (*gyrB* and *rpoD*) was carried out on 77 strains representative isolates. The isolates grouped into 20 haplotypes defined with 68 polymorphic sites. The phylogenetic tree obtained showed that *Xcc* is in two groups, and all Algerian strains clustered in group 1 in three subgroups. No relationships were detected between haplotypes and the origins of the seed lots, the varieties of host cabbage, the years of isolation and agroclimatic regions.

Keywords. *Brassicaceae,* seed-borne bacterium, genetic diversity, multilocus sequence analysis.

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INTRODUCTION

Black rot caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*), is the most widespread disease of cruciferous crops. It was first reported in the United States of America in 1895 by Pammel (Swing and Civerolo, 1993), and

is considered one of the most destructive bacterial diseases of vegetables (Williams, 1980). Under optimal temperature and humidity conditions, the disease can cause losses up to 100% on sensitive brassica varieties (Mgonja and Swai, 2000; Janse and Wenneker, 2002; Massomo *et al*., 2004). Several *Brassica* species can be infected (cabbage, cauliflower, kale, broccoli, turnip, radish and mustard). Cabbage and cauliflower are the most susceptible (Kocks and Zadoks, 1996). Many weeds can also be infected by the bacterium, and these ensure the conservation of the pathogen throughout the year as inoculum sources (Schaad and White, 1974; Rat and Chauveau, 1985). *Xcc* is a seed-borne pathogen, this being the primary inoculum source (Cook *et al*., 1952; Schaad *et al*., 1980; Schultz and Gabrielson, 1986), but *Xcc* can survive for months in leaves and other plant debris in the soil.

Black rot management is mainly based on prophylactic measures, which include eradication of inoculum reservoirs especially crop debris and cruciferous weeds, and certified pathogen-free seed lots (Janse and Wenneker, 2002). One contaminated seed among 10 000 healthy seeds can initiate a black rot outbreak after planting (Cook *et al*., 1952; Schaad *et al*., 1980; Laala *et al*., 2015).

Xcc has not been previously reported in Algeria. A survey conducted by Emmanouilidis dates back to 1976; but reported the absence of black rot in the Algerian territory. Nevertheless, similar symptoms of black rot were observed in 2011 (Figure 1) in several cabbage and cauliflower producing areas of Algeria. The symptoms observed were yellow V-shaped, chlorotic lesions on host leaf margins and blackening of vascular tissue (Janse and Wennekek, 2002; Tsygankova *et al*., 2004).

Figure 1. Symptoms of black rot in a cauliflower crop (Zéralda, Wilaya of Algeries, Algeria), with yellow V- shaped lesions at margin of a leaf (blue arrow).

The purpose of this study was to collect symptomatic samples, and to identify and characterize pathogen strains isolated in Algeria and to assess the diversity of their populations.One hundred and seventy isolates were identified as *Xcc* and characterized with a range of biochemical, biological and molecular tests. Seventy-seven isolates were selected to study their genetic diversity by Multilocus Sequence Analysis (MLSA), based on two housekeeping genes, the *gyrB* and *ropD* which code, respectively, for a DNA gyrase B and the sigma factor 70.

MATERIALS AND METHODS

Collection of samples

During surveys conducted from 2011 to 2014, three hundred and fifteen samples of Brassica leaves with typical symptoms of black rot were collected. The sampling was carried out in 76 fields, from 35 cabbage crops (*Brassica oleracea* subsp. *capitata)* and 41 of cauliflower crops (*Brassica oleracea* subsp. *botrytis*), located in the Wilaya of Algiers, Boumerdes, Tipaza, Ain Defla, Skikda, Mostaganem and Tizi Ouzou regions of Algeria (Figure 2). During sampling, climatic conditions were 20 to 30°C, and 80% humidity on average.

The leaf samples were labelled, stored in plastic bags and transported to the Phytosanitary laboratory at the Centre National de Contrôle et de Certification des semences et plants (CNCC), El Harach, Alger, and were stored at 4°C until use (1 to 2 d). The samples and their main characteristics are listed in Table 1.

Bacterial isolation and purification

One hundred and seventy isolates were obtained from symptomatic samples, using the protocol of Schaad

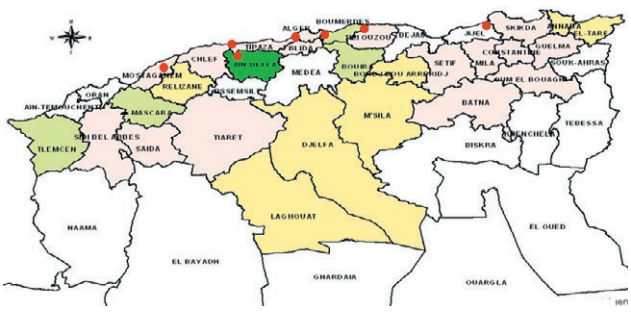

Figure 2. Surveyed and sampled *Brassica*-growing areas (red circles) in Algeria (from 2011-2014).

Table 1. *Xanthomonas campestris* pv. *campestris* isolate designations, their hosts, source regions, and haplotypes formed with concatenated partial sequences, for 77 isolates ontained in Algeria.

Isolate	Host	Host variety	Region of isolation	Field No.	Year of isolation	Conc. haplotype
XccDZ111	*Brassica oleracea* spp. *capitata*	Smilla	Staoueli ITCMI	green house 1	05/10/2011	H1
XccDZ112	*Brassica oleracea* spp. *botrytis*	Yebouze	Staoueli ITCMI	green house 2	05/10/2011	H1
XccDZ112.6	*Brassica oleracea* spp. *botrytis*	Arizona	Rouiba	2	10/10/2011	H1
XccDZ11272	*Brassica oleracea* spp. *botrytis*	Arizona	Rouiba	2	10/10/2011	H1
XccDZ113.1	*Brassica oleracea* spp. *capitata*	Solo	Rouiba	3	10/10/2011	H1
XccDZ113.5	*Brassica oleracea* spp. *capitata*	Solo	Rouiba	3	10/10/2011	H1
XccDZ114.9	*Brassica oleracea* spp. *capitata*	Bitch	Rouiba	4	10/10/2011	H1
XccDZ114.10	*Brassica oleracea* spp. *capitata*	Bitch	Rouiba	4	10/10/2011	H2
XccDZ115.1	*Brassica oleracea* spp. *botrytis*	Arizona	Tipaza	5	27/10/2011	H1
XccDZ116.1	*Brassica oleracea* spp. *botrytis*	Arizona	Tipaza	6	27/10/2011	H3
XccDZ116.2	*Brassica oleracea* spp. *botrytis*	Arizona	Tipaza	6	27/10/2011	H3
XccDZ1230.2	*Brassica oleracea* spp. *capitata*	Chebli	SidiFredj	30	07/01/2012	H4
XccDZ1230.3	*Brassica oleracea* spp. *capitata*	Chebli	SidiFredj	30	07/01/2012	H4
XccDZ1230.4	*Brassica oleracea* spp. *capitata*	Chebli	SidiFredj	30	07/01/2012	H4
XccDZ1230.5	*Brassica oleracea* spp. *capitata*	Chebli	SidiFredj	30	07/01/2012	H1
XccDZ1241.3	*Brassica oleracea* spp. *capitata*	Smilla	Staoueli	41	06/04/2012	H1
XccDZ1242.3	*Brassica oleracea* spp. *capitata*	Chebli	SidiFredj	42	06/04/2012	H5
XccDZ1243.1	*Brassica oleracea* spp. *capitata*	Chebli	Staoueli	43	06/04/2012	H6
XccDZ1243.2	*Brassica oleracea* spp. *capitata*	Chebli	Staoueli	43	06/04/2012	H1
XccDZ1243.3	*Brassica oleracea* spp. *capitata*	Chebli	Staoueli	43	06/04/2012	H1
XccDZ1244.1	*Brassica oleracea* spp. *capitata*	Spacestar	Staoueli	44	06/04/2012	H1
XccDZ1244.2	*Brassica oleracea* spp. *capitata*	Spacestar	Staoueli	44	06/04/2012	H1
XccDZ1244.3	*Brassica oleracea* spp. *capitata*	Spacestar	Staoueli	44	06/04/2012	H7
XccDZ1246.1	*Brassica oleracea* spp. *capitata*	Yerbouze	Staoueli	46	06/04/2012	H1
XccDZ1246.2	*Brassica oleracea* spp. *capitata*	Yerbouze	Staoueli	46	06/04/2012	H1
XccDZ1358	*Brassica oleracea* spp. *capitata*	Standard	Boumerdès	58	21/02/2013	H1
XccDZ1360	*Brassica oleracea* spp. *capitata*	Canapatchi	K. El Khechna	60	18/03/2013	H1
XccDZ1360.1	*Brassica oleracea* spp. *capitata*	Canapatchi	K. El Khechna	60	18/03/2013	H1
XccDZ1375.2	*Brassica oleracea* spp. *capitata*	Yerbouze	Aïn Taya	75	23/06/2013	H1
XccDZ1375.3	*Brassica oleracea* spp. *capitata*	Yerbouze	Aïn Taya	75	23/06/2013	H8
XccDZ1375.6	*Brassica oleracea* spp. *capitata*	Yerbouze	Aïn Taya	75	23/06/2013	H1
XccDZ1376.9	*Brassica oleracea* spp. *capitata*	Yerbouze	Aïn Taya	76	23/06/3013	H9
XccDZ1382.2	*Brassica oleracea* spp. *capitata*	Yerbouze	Ouled Moussa	82	23/06/2013	H1
XccDZ1384.3	*Brassica oleracea* spp. *capitata*	Yerbouze	Ouled Moussa	84	23/06/2013	H1
XccDZ.1388.2	*Brassica oleracea* spp. *capitata*	Arizona	Mostaganem	88	25/03/2013	H20
XccDZ1388.4	*Brassica oleracea* spp. *capitata*	Arizona	Mostaganem	88	23/03/2013	H1
XccDZ1490.43	*Brassica oleracea* spp. *botrytis*	Atlas	Zéralda	90	17/01/2014	H1
XccDZ1490.54	*Brassica oleracea* spp. *botrytis*	Atlas	Zéralda	90	17/01/2014	H1
XccDZ1493.12	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H10
XccDZ1493.31	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H1
XccDZ1493.32	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H9
XccDZ1493.34	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H1
XccDZ1493.51	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H1
XccDZ1493.54	*Brassica oleracea* spp. *botrytis*	Spacestar	Zéralda	93	17/01/2014	H1
XccDZ14100.6	*Brassica oleracea* spp. *capitata*	Sakata	Aïn Taya	100	06/05/2014	H1
XccDZ14101.20	*Brassica oleracea* spp. *capitata*	Raissa	Aïn Taya	101	06/05/2014	H1
XccDZ14101.21	*Brassica oleracea* spp. *capitata*	Raissa	Aïn Taya	101	06/05/2014	H1

(Continued)

Table 1. (Continued).

Isolate	Host	Host variety	Region of isolation	Field No.	Year of isolation	Conc. haplotype
XccDZ14101.22	*Brassica oleracea* spp. *capitata*	Raissa	Aïn Taya	101	06/05/2014	H7
XccDZ14101.27	*Brassica oleracea* spp. *capitata*	Raissa	Aïn Taya	101	06/05/2014	H1
XccDZ14102.28	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Taya	102	06/05/2014	H1
XccDZ14102.29	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Taya	102	06/05/2014	H1
XccDZ14/102.30	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Taya	102	06/05/2014	H1
XccDZ14/102.34	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Taya	102	06/05/2014	H7
XccDZ14/102.38	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Taya	102	06/05/2014	H1
XccDZ14/104.60	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.61a	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H11
XccDZ14/104.61b	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H12
XccDZ14/104.61c	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H13
XccDZ14/104.64	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.66a	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H14
XccDZ14/104.66b	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.67	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.68	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.69a	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.69b	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H1
XccDZ14/104.69c	*Brassica oleracea* spp. *botrytis*	Arizona	Tizi Ouzou	104	25/06/2014	H15
XccDZ14/105.75	*Brassica oleracea* spp. *botrytis*	Solo	Zéralda/Azurplage	105	12/09/2014	H16
XccDZ14/106/126	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H17
XccDZ14/106/127	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H16
XccDZ14/106/129	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H16
XccDZ14/106/130	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H18
XccDZ14/106/131	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H1
XccDZ14/106/132	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H1
XccDZ14/106/134	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H1
XccDZ14/106/135	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H19
XccDZ14/106/136	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H1
XccDZ14/106/137	*Brassica oleracea* spp. *botrytis*	Arizona	Aïn Dafla	106	16/11/2014	H1
CFBP 1121*	*Brassica oleracea* cv. *bullata gemmifera.*	///	France	///	1967	
CFBP 5241 * (ATCC 33915)	*Brassica oleracea* var. *gemmifera*	///	United Kingdom	///	1957	

*Type strain.

et al. (2001). Each sample was rinsed with tap water and then dried. Small sections of affected tissue were taken and crushed using a sterile scalpel and soaked in a small volume (approx. 2 mL) of sterile distilled water for 15-20 min to allow diffusion of bacterial cells.

Aliquots of 50 µL of tenfold dilutions (10^{-1} to 10^{-8}) of the suspension were plated onto YPGACvc medium (yeast extract 7 g L^{-1}, peptone 7 g L^{-1}, glucose 7 g L^{-1}, agar 15 g L^{-1}, pH 7), supplemented with cephalexine 25 mg L^{-1}, vancomycin 0.5 mg L^{-1} and cycloheximide 100 mg L^{-1}). The plates were then incubated for 24 h at 28°C and observed daily for bacterial growth. *Xcc*-like colonies (colour and shape) were purified by repeated subculturing of a single colony on YPGA and on GYCA (yeast extract 5 g L^{-1}, glucose 10 g L^{-1}, $CaCO_3$ 40 g L^{-1} agar 15 g L^{-1}, pH 7) medium (Lelliot and Stead, 1987).

Two reference strains of *Xanthomonas campestris* pv. *campestris* (CFBP 1121 and 5241) were used for this study, obtained from the Collection Française de Bactéries Associées aux Plantes (CFBP), Angers, France (https://www6.inrae.fr/cirm_eng/CFBP-Plant-Associated-Bacteria).

All purified isolates were preserved at -20°C and -80°C in microtubes, containing 1 mL of YP medium (yeast extract 7 g L^{-1}, peptone 7 g L^{-1}) supplemented with 20% glycerol.

Table 2. Primers used for PCR amplification and sequencing of Algerian isolates of *Xanthomonas campestris* pv. *campestris*.

Target gene	Primers names	Annealing temperature	Sequences (5'-3')	Expected fragment size (bp)	References
hrpF	DLH120[a] DLH125[b]	63°C	CCGTAGCACTTAGTGCAATGGCA TTTCCATCGGTCACGATTG	619	Berg *et al.,* 2005
rrs (Universal Primers)	1052–F[a] Bac–R [b]	63°C	GCATGGTTGTCGTCAGCTCGTTA CGGCTACCTTGTTACGACT	441	Eden *et al.,*1991
atpD	P-X-ATPD-F[a] P-X-ATPD-R[b]	62°C	GGGCAAGATCGTTCAGAT GCTCTTGGTCGAGGTGAT	868	Fargier *et al.,* 2011
dnaK	P-X-DNAK-F[a] P-X-DNAK-R[b]	62°C	GGTATTGACCTCGGCACCAC ACCTTCGGCATACGGGTCT	1,034	Fargier *et al.,* 2011
gyrB	emigyrB1F[a] emigyrB4R[b]	63°C	TGCGCGGCAAGATCCTCAAC GCGTTGTCCTCGATGAAGTC	904	Fargier *et al.,* 2011
rpoD	emirpo11F[a] emirpo13R[b]	63°C	ATGGCCAACGAACGTCCTGC AACTTGTAACCGCGACGGTATTCG	1,313	Fargier *et al.,* 2011

[a] Forward primers; [b] reverse primers.

Bacterial characterization

Isolates were tested for cytochrome oxidase activity (Klement *et al.,* 1990), esculine hydrolysis (Lelliot *et al.,* 1987), oxidative and fermentative metabolism of glucose as described previously (Hugh and Leifson, 1953). The hypersensitive host response was monitored in tobacco leaf tissues (*Nicotiana tabacum* Xanthi) after infiltration of 10^8 cfu mL^{-1} bacterial suspensions from 24 h cultures.

Molecular characterization was performed on DNA extracts obtained by boiling bacterial suspensions of 10^6 cfu mL^{-1} for 10 min and subsequently keeping these on ice for 15 min. The suspensions were then centrifuged at 10 000 rpm for 5 min and stored at -20°C. Amplifications were performed by multiplex PCR according to (Laala *et al*., 2015). Each reaction was performed in a final volume of 20 µL containing: 1× Green GoTaq Flexi Buffer (Promega), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.5 µM of each specific primer DLH 120/LH125 (Berg *et al*., 2005), 0.05 µM of each universal bacterial primer (used to validate the PCR reaction) 1052-F/Bac-R (Eden *et al*., 1991), 0.05 U of GoTaq Flexi DNA polymerase and 3 µL of template DNA. The primer sequences used in this study are presented in Table 2.

PCR reactions were conducted with a C1000 thermocycler (Bio-Rad). The programme consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of 40 s at 95°C, 40 s at 63°C (touchdown to 58°C over the first six cycles) and 40 s at 72°C. The amplification products were separated on 1.5 % agarose gels in 1× TBE buffer, stained with ethidium bromide, and visualized using UV transilluminator on a Gel Doc 2000 (Bio-Rad).

Pathogenicity tests

Presumptive isolates were tested for their pathogenicity on cauliflower seedlings (cv. Arizona) after seed contamination, according to Laala *et al.* (2015). Symptoms were observed 7 to 14 d after sowing inoculated seeds. A positive control (*Xcc* strain CFBP 5241) and a negative control (sterile distillate water) were included in the assays. Koch's postulates were confirmed by symptomatic leaves onto YPGACvc medium and by PCR characterization of re-isolated bacterial colonies.

Housekeeping gene amplification and sequencing

Sequences from three isolates (Xcc DZ114.10, Xcc DZ113.5 and Xcc DZ1388.2) were selected from the main varieties cultivated in Algeria to be compared with those available in the database with BLASTN (Altschul *et al*., 1990). Fragments of four housekeeping genes were sequenced for each isolate, including gyr*B* (DNA gyrase subunit B), *rpoD* (RNA polymerase sigma-70 factor), *atpD* (ATP synthase beta chain), and DnaK (heat shock protein 70), according to (Fargier *et al.,* 2011) (Table 2). The amplifications were each carried out in a thermocycler (iCycler; Bio-Rad), in a final volume of 20 µL containing 1× green GoTaq Hot Start buffer, 1.5 µL of 25 mM $MgCl_2$, 0.2 mM of dNTP, 0.5 µM of each primer and 1U of GoTaq Hot Start DNA polymerase (Promega) and 3 µL of target DNA. The amplification program consisted of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at Tm (Table 2) and 1.30 min at 72°C, and finished with 72°C for 5 min. The PCR products were analysed on 1.5% agarose

gel in 1× TBE buffer. The two strands of the amplicons obtained from the three isolates were sequenced by the GATC Biotech laboratory, Germany. The sequence electropherograms were analysed using the software 4 peaks 1.7.2 (https://nucleobytes.com/4peaks/index.html). The DNA sequences were aligned using the Multalin software (Corpet, 1988: http://multalin.toulouse.inra.fr/multalin/).

Multilocus Sequences Analysis (MLSA) and phylogenetic analysis

The purposes of this approach were to study the genetic diversity of *Xcc* isolated in Algeria in comparison with strains from different countries, based on the diversity of the housekeeping genes, and to identify potential inoculum sources.

Ninety-six isolates were selected for a phylogenetic study from the 170 *Xcc* isolates identified by their microbiological, biochemical and molecular traits. Two housekeeping genes (*gyrB* gene and *rpoD*) were chosen for this study. The *gyrB* gene is a good marker of specificity between species, while the *rpoD* gene is a good marker of discrimination within a species (Fischer-Le Saux, personal communication). The amplification conditions were the same as those defined above. The quality and yield of each amplicon were verified by loading 10 µL of the reaction product onto 1.5% agarose gel in 1× TBE buffer, and also by Nano-Drop ND-2000 (ThermoFisher Scientific). The 96 amplicons were sequenced at the GATC Biotech laboratory, Germany.

DNA sequences were analysed using the software 4 peaks 1.7.2. and were aligned using the Bioedit program (Hall, 1999). Only sequences having average lengths of 500 bp were used for analyses. The sequence data of the two genes were concatenated according to the alphabetical order of the gene, the *gyrB* gene followed by the *rpoD* gene, using the GENEIOUS software Version 4.8.5 (BIOMATTERS).

Phylogenetic analyses were carried out on individual genes as well as on the concatenated sequences using the DNA Sequence polymorphism (DnaSP) software version 5.10 (Rozas *et al.,* 2010). This analysis is based on the Tajima's method (Tajima, 1996), allowing a multiple alignment by determining pairwise nucleotide diversity. The differentiation between the haplotypes is based on the presence of a single nucleotide different per sequence (Wicker *et al.,* 2012; Tancos *et al*., 2015).

The phylogenetic tree was constructed using Mega software version 6.06 (Tamura *et al*., 2013) based on the Maximum likelihood (ML) method (Tamura and Nei, 1993). This software allows quick analysis of a large number of sequences, and evolutionary phylogenetic reconstruction based on statistical bootstrap analysis at 1000 replications. The tree was constructed with the DNA sequences of each gene and with the concatenated sequences. Sequences of *Xcc* strains (Xcc-C168, Xcc-C278, ICMP 6541, strain 0407, strain 0470, ICMP 13, CFBP 5241, 70 genome, ICMP 4013, ATCC 33913, 21080, Xcc-8004), published on the NCBI database were used for comparisons with sequences from the Algerian strains, and the sequence from *X. campestris* pv. *vesicatoria* (*Xcv*) 85-10 (Thieme *et al.,* 2005) was used to root the tree.

RESULTS

Characterization of Xanthomonas campestis *pv.* campestris *isolates*

Out of the 315 symptomatic samples collected between 2011 and 2014, 170 isolates showed typical morphological colonies on the YPGACvc medium. After 24 h incubation, the bacterial colonies were round, yellow, mucoid, convex and shiny.

All these isolates were Gram negative and correspond to the biochemical characteristics as previously described (Schaad, 1980; Lelliott and Stead, 1987). They were glucose oxidative (Hugh and Leifson, 1953). The activity of levansucrase and catalase were positive, and the isolates induced HRs on tobacco leaves. They did not display cytochrome oxidase (strike oxidase strips: Fluka Analytical) and did not reduce nitrates.

The multiplex PCR designed for *Xcc* identification (Laala *et al*., 2015) generated amplicons with expected sizes (619 and 441 pb) for the 170 isolates tested.

Pathogenicity assays

All the plants inoculated with bacterial suspensions (1 10^7 cfu mL^{-1}) of the Algerian *Xcc* isolates and the reference strain (CFBP 5242) showed symptoms of rotting within 7-14 d after sowing. No symptoms were observed in plants inoculated with sterile water. Re-isolations were performed on YPGACvc from germinated seedlings. Resulting bacterial colonies were yellowish, circular, and mucoid, and were identified as *Xcc* by multiplex PCR (Laala *et al*., 2015), confirming the Koch's postulates.

Amplification of housekeeping genes

Sequence amplifications from the three isolates of *Xcc* (Xcc DZ114.10, Xcc DZ113.5 and Xcc DZ1388.2) using four housekeeping genes, gave four bands of

Table 3. Comparison of housekeeping gene DNA sequences of Algerian strains of *Xanthomonas campestris* pv. *campestris* with that of other strains of *Xanthomonas campestris* (*X.c.*).

Algerian strains	Reference strains	Genes			
		*gyr*B	*rpo*D	*atp*D	*dna*k
XccDZ114.10	ATCC 33913/ Xcc-C-168	734/735[a] (99%)[b]	574/577 (99%)	661/667(99%)	778/779 (99%)
	X.c. pv. *vesicatoria* 85-10	639/736 (87%)	539/578 (93%)	630/666 (95%)	736/779 (94%)
XccDZ113.5	ATCC 33913/ Xcc-C-168	734/735 (99%)	577/577(100%)	663/667 (99%)	777/779 (99%)
	X.c. pv. *vesicatoria* 85-10	639/736 (87%)	639/736 (87%)	542/578 (94%)	735/779 (94%)
XccDZ1388.2	ATCC 33913/ Xcc-C-168	734/735 (99%)	576/577(99%)	663/667 (99%)	776/779 (99%)
	X.c. pv. *vesicatoria* 85-10	639/736 (87%)	541/578 (94%)[b]	630/666(95%)	734/779 (94%)

[a] Ratio of identical nucleotides; [b] identity level

Table 4. Genetic diversity of *Xanthomonas campestris* pv. *campestris* isolates assessed by MLSA.

Gene	Fragment size (pb)	No. of sequences selected	No. of haplotypes	No. of polymorphic sites	% of polymorphic sites
gyrB	737	94	6	9	1.22%
rpoD	575	83	20	61	10.6 %
Concatened Sequences	1,312	77	20	68	5.18 %

the expected sizes of 904 bp for the *gyrB* gene, 1313 bp for the *rpoD* gene, 868 bp for the *atpD* gene and 1034 bp for the *dnaK* gene (Fargier *et al.,* 2011). The DNA sequences of the four genes from the three Algerian isolates showed high percentage similarities of 98 to 100%, in comparison with *Xcc* sequences, and less than 96% similarity with other *Xanthomonas* species available in the NCBI database (Table 3). The partial nucleotide sequences of the strains *Xcc* DZ114.10, *Xcc* DZ113.5 and *Xcc* DZ1388.2 were deposited in the NCBI database under the following accession numbers: the *atpD* gene (KU556302 to KU556304), the *DnaK* gene (KU556305 to KU556307), the *gyrB* gene (KU556308 to KU556310) and *rpoD* (KU556311 to KU556312).

The strains were deposited in the CFBP, under the numbers CFBP 8405, CFBP 8406, CFBP 8407.

Phylogenetic analysis of housekeeping genes

Of the 170 of *Xcc* isolates, 96 representatives of all the sampled regions were selected for examination of genetic diversity. Two loci, the *gyrB* and the *rpoD* genes were used. DNA fragments of the two genes were sequenced. Ninety-four sequences of 735 bp for the *gyrB* gene, 83 sequences of 577 bp for the *rpoD* gene and 77 sequences concatenated (1312 bp) were used.

The phylogenetic analysis carried out by the DnaSP (Rozas *et al*., 2010) revealed the presence of polymorphism among the strains. The number of polymorphic nucleotide sites varied from nine to 68 (Table 4).

The *rpoD* locus displayed greatest discriminating power, which corroborates the MLSA studies published by Fargier *et al*. (2011), Lange *et al*. (2016) and Popović *et al*. (2019). Twenty haplotypes were obtained for this gene with a variable site level reaching 10.6%, compared with 1.22% for *gyrB* (Table 4). The concatenation of the two loci grouped the 77 strains into 20 haplotypes with 68 polymorphic sites (Table 4).

Fifty strains were of haplotype H1 and these were isolated from different areas throughout the sampling period (Table 1). Fourteen haplotypes out of 20 (H2 to H20) were each of a single isolate. The phylogenetic tree constructed with the partial sequences of the *gyrB* gene showed strong homology between the Algerian strains and the 33 *Xcc* reference sequences. The *Xcv* was an outgroup, as expected (data not showed). The *rpoD* gene was more polymorphic. The *Xcc* strains clustered in two groups, among which the Algerian strains clustered in group I associated with 13 reference strains isolated from many international locations. No strains isolated in Algeria clustered in group II (Figure 3). The *Xcv* was associated to *Xcc* group II with a high bootstrap value. This could be explained by the presence of a recombination event between *Xcc* and *Xcv* within the *rpoD* gene.

The phylogenetic tree showed that the Algerian isolates were in three subgroups within group I (Figure 3). The first subgroup (Ia) contained the majority of

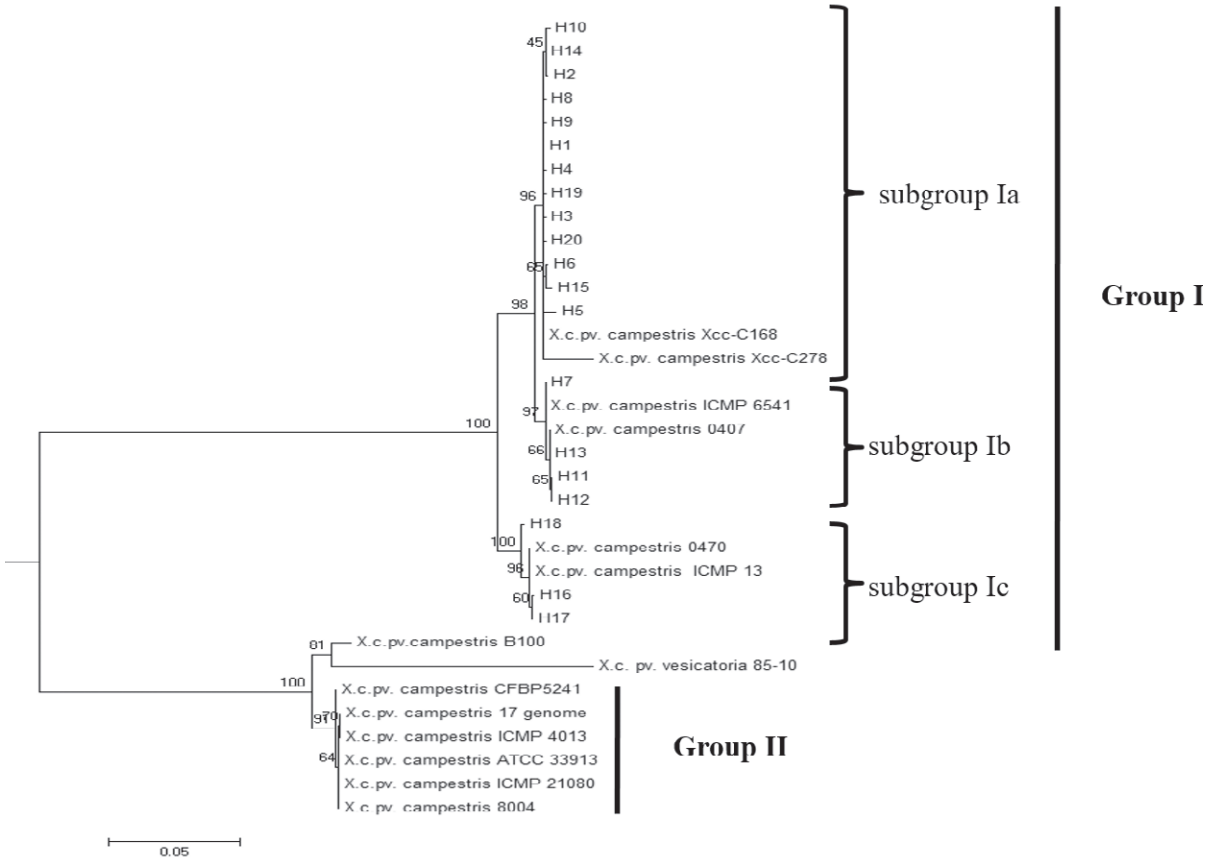

Figure 3. Maximum likelihood tree constructed with concatenated partial sequences of two housekeeping gene (*gyrB* and *rpoD*) of 20 Algerian haplotypes and 13 *Xanthomonas campestris* pv. *campestris* international strains, and rooted with *Xanthomonas campestris* pv. *vesicatoria*. Bootstrap percentages obtained for 1000 replicates are indicated at each node.

Algerian isolates with 13 haplotypes. It also included two strains isolated in India (*Xcc*-C168 and *Xcc*-C278) (Rathaur *et al.,* 2015; Singh *et al.,* 2016). Subgroup Ib contained four Algerian haplotypes and ICMP 6541 isolated in New Zealand, and 0407 isolated in New York State, United States of America. The subgroup Ic included three Algerian haplotypes, and *Xcc* 0470 from New York State and ICMP 13 from the United Kingdom. Furthermore, the strains 0407 and 0470 isolated in New York State were separated in two distinct subgroups, which is in accordance with the study of Lange *et al*. (2016). Results obtained by DnaSP software showed that the 20 haplotypes identified in Algeria displayed homology with strains isolated from many international regions. The Algerian isolates grouped in the haplotype H1 with strong similarity to Xcc-168 strain isolated in India (Rathaur *et al.,* 2015), and haplotype H7 was very similar to strain ICPM 6541 isolated in New Zealand (Young *et al*., 2008) (Figure 3). However, none of the strains isolated in Algeria fitted into the clusters of group 2, which suggests that Algerian population Algeria did not cover all of the recognized diversity of *Xcc*.

DISCUSSION

This study reports for the first time the presence of *Xcc* in Algeria, after the investigation conducted in cabbage and cauliflower fields from 2011 to 2014. A total of 170 *Xcc* isolates was obtained from 45 fields. These isolates were identified as *Xcc* by morphological, physiological and molecular tests. The analysis of four housekeeping genes (*gyrB*, the *rpoD*, the *atpD* and the *dnaK*) performed on partial DNA sequences of three representative Algerian strains showed 98 to 100% similarity with homologous *Xcc* sequences available in the GenBank database (Table 3). Results obtained from this survey indicate the importance of black rot in Algeria, and how widespread the disease is in the main Brassica growing areas of the country. However, the sampling

method used did not allow assessment of how the disease spreads in this country, because each region was not monitored each year.

The MLSA approach was conducted for the genetic studies. This is a powerful tool for study of phylogenetic relationships at the inter- and intraspecies levels, by analyzing sequences of two or more housekeeping genes from a large number of bacterial isolates (Hanage *et al*., 2006; Fargier *et al*., 2011). Genes encoding housekeeping proteins are universally distributed among bacteria, present in single copies and dispersed in the genomes (Hanage *et al*., 2006; Youseif *et al*., 2014). In the present study, the MLSA results in general revealed the existence of genetic diversity within Algerian *Xcc* isolates. A total of 20 haplotypes were obtained displaying 68 polymorphic nucleotide sites (Table 4). However, no correlation was found between haplotypes with species and host variety, year of isolation or geographic origin of host plants. This strongly indicates that inoculum came from seeds that would have been imported and distributed in the country. This also supports the conclusions of previous studies (Zaccardelli *et al*., 2008; Fargier *et al*., 2011; Rathaur *et al.,* 2015; Lange *et al*., 2016; Bella, 2019). Fargier *et al*. (2011) described two groups of *Xcc* from MLSA using genomic sequences of seven housekeeping genes. The present study confirmed the occurrence of two genomic groups within *Xcc*, and showed that all the strains isolated in Algeria belonged to the group *Xcc* I of Fargier *et al*. (2011). Therefore, the MLSA haplotypes of Algerian isolates were not unique, as no distinct clustering was present when Algerian *Xcc* haplotypes were compared with the internationally collected haplotypes.

Haplotype H1 contained approx. 50 strains isolated from several *Brassica* varieties and from 21 fields over all collection periods. It also contained two strains collected from a nursery seedling, strain Xcc DZ11.1 isolated from a cauliflower (cv. Smilla) leaf and strain Xcc DZ11.2 isolated from a cabbage (cv. Yerbouze). It cannot be confirmed that these two isolates derived from a seed lot infected with the same inoculum, because haplotype H1 is widely distributed in Algeria and internationally. Strains Xcc DZ114.9 and Xcc DZ114.10 were collected from one plant but were of different haplotypes, respectively, haplotype H1 and H2. This indicates that these isolates were from different everal sources, either a seed lot contaminated by more than one haplotype, from two different seed lots sowed in the field, or from infected seed and with different environmental origins (Schaad and White,1974; Alvarez and Cho, 1978; Schaad and Dianese 1981; Chun and Alvarez; 1983; Alvarez and Lou 1985).

The phylogenetic tree obtained showed that Algerian *Xcc* isolates were of three subgroups with a high bootstrap values. The majority of *Xcc* isolates clustered in subgroup Ia. This included 13 haplotypes, and the isolates were collected from different conditions over the period 2011-2014, as well as Xcc-C168 isolated from Uttar Pradesh (India) and Xcc-C278 isolated from Delhi (India) (Rathaur *et al.,* 2015, Singh *et al.,* 2016). The subgroup Ib contained haplotypes H7, H11, H12 and H13, as well as ICMP 6541 from New Zealand and 0407 (Fresco) from New York State (Lange *et al*., 2016). Subgroup Ic contained three haplotypes (H16, H17 and H18) were included in subgroup Ic, along with strain 470 (Kaiten) isolated in New York State (Lange *et al.,* 2016) and ICMP 13 (CFBP 2350) isolated in 1957 in the United Kingdom. This suggests that the *Xcc* population isolated in the present study covered the full diversity of group *Xcc* I described by Fargier *et al*. (2011). Previous studies have been conducted to trace the origins of *Xcc* isolates using molecular characterization techniques based on PCR or housekeeping MLSA (Ignatov *et al*., 2007; Zaccardelli *et al*., 2008; Jensen *et al*., 2010; Mulema *et al*., 2011; Lema *et al*., 2012; Rathaur *et al*., 2015; Lange *et al*., 2016; Bella *et al*., 2019), but these methods were not suitable for tracing particular inoculum sources in the Algerian situation described here. This was probably due to the international trading of Brassica seed lots, since the pathogen is a seed-borne bacterium (Cook *et al*., 1952; Weller and Saettler, 1980; Blackeman, 1991). Fields may therefore contain strains from several origins conserved in plant debris (Schaad and Dianese, 1981), or strains may be associated with cruciferous weeds. The preliminary results presented here revealed the existence of diversity within Algerian isolates. However the MLSA approach, using two housekeeping genes, and the type of sampling, were not discriminating enough to trace relationships between genetic diversity within Algerian strains and seed lot origins, varities, years or regions.

New approaches are currently under development, such as multilocus variable number tandem repeat analysis (MLVA) (Vogler *et al.,* 2006; Bui Thi Ngoc *et al.,* 2009). MLVA is widely used to study genetic diversity and deduce patterns of the spread of bacterial pathogens (Achtman, 2008; Cunty *et al*., 2015), and this methodology would allow increased understanding of the structure of Algerian *Xcc* populations. This pathogen is occurring in the main market gardens in the Algiers, Mostaghenem, Ain Defla regions, and in Tipaza in western Algeria and Tizi Ouzou and Boumerdes in the east of the country. It would be useful to extend the survey area in western and eastern parts of Algeria where cabbages are grown. Although first symptoms of black rot were reported in Algeria only at the beginning of this study (2011), the level of diversity of recorded haplo-

types in Algeria indicates that *Xcc* was introduced in the country before that year.

The present study provides a basis to assess the relative roles of host weeds and infected plant debris versus that of contaminated seed lots in the epidemiology of the black rot of cruciferous plants in Algeria. Control of seed-borne pathogens is important for appropriate disease management, and to prevent their introduction and spread. To this aim, research on control and certification of vegetable seeds is currently being promulgated by the Ministry of Agriculture of the Republic of Algeria. This should allow improved management of seed-borne pathogens and improve agricultural production. This is likely to include the use of certified pathogen-free propagative materials (seeds and seedlings) coupled with appropriate agronomic practices such as destruction of crop debris and wild hosts, and seedbed rotation.

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COMPLIANCE WITH ETHICAL STANDARDS

Studies in this article do not involve human participants nor animals.

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