Research Papers

Characterization of \textit{Xanthomonas campestris} pv. \textit{campestris} in Algeria

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Summary. \textit{Xanthomonas campestris} pv. \textit{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 Algeria 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 report 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 (\textit{gyrB} and \textit{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. \textit{Brassicaceae}, seed-borne bacterium, genetic diversity, multilocus sequence analysis.

INTRODUCTION

Black rot caused by \textit{Xanthomonas campestris} pv. \textit{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).

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

| Isolate   | Host variety | Host | 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. capitata | 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              |
| XccDZ1246.3| Brassica oleracea spp. capitata | Yerbouze | Staoueli | 46 | 06/04/2012 | H1              |
| XccDZ12358| Brassica oleracea spp. capitata | Standard | Boumerdès | 58 | 21/02/2013 | H1              |
| XccDZ12360| Brassica oleracea spp. capitata | Canapatchi | K. El Khechna | 60 | 18/03/2013 | H1              |
| XccDZ12360.1| Brassica oleracea spp. capitata | Canapatchi | K. El Khechna | 60 | 18/03/2013 | H1              |
| XccDZ12375.2| Brassica oleracea spp. capitata | Yerbouze | Ain Taya | 75 | 23/06/2013 | H1              |
| XccDZ12375.3| Brassica oleracea spp. capitata | Yerbouze | Ain Taya | 75 | 23/06/2013 | H8              |
| XccDZ12376.6| Brassica oleracea spp. capitata | Yerbouze | Ain Taya | 75 | 23/06/2013 | H1              |
| XccDZ12376.9| Brassica oleracea spp. capitata | Yerbouze | Ain Taya | 76 | 23/06/2013 | H9              |
| XccDZ12382.2| Brassica oleracea spp. capitata | Yerbouze | Ouled Moussa | 82 | 23/06/2013 | H1              |
| XccDZ12384.3| Brassica oleracea spp. capitata | Yerbouze | Ouled Moussa | 84 | 23/06/2013 | H1              |
| XccDZ1388.2| 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 | H1              |
| 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 | Ain Taya | 100 | 06/05/2014 | H1              |
| XccDZ14101.20| Brassica oleracea spp. capitata | Raissa | Ain Taya | 101 | 06/05/2014 | H1              |
| XccDZ14101.21| Brassica oleracea spp. capitata | Raissa | Ain Taya | 101 | 06/05/2014 | H1              |

(Continued)
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 cephalaxine 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 sub-culturing of a single colony on YPGA and on GYCA (yeast extract 5 g L^{-1}, glucose 10 g L^{-1}, CaCO₃ 40 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 (http://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 1. (Continued).

| Isolate | Host          | Host variety               | Region of isolation | Field No. | Year of isolation | Conc. haplotype |
|---------|---------------|----------------------------|---------------------|-----------|-------------------|-----------------|
| XccDZ14101.22 | Brassica oleracea spp. capitata | Raissa | Ain Taya | 101 | 06/05/2014 | H7 |
| XccDZ14101.27 | Brassica oleracea spp. capitata | Raissa | Ain Taya | 101 | 06/05/2014 | H1 |
| XccDZ14102.28 | Brassica oleracea spp. botrytis | Arizona | Ain Taya | 102 | 06/05/2014 | H1 |
| XccDZ14102.29 | Brassica oleracea spp. botrytis | Arizona | Ain Taya | 102 | 06/05/2014 | H1 |
| XccDZ14/102.30 | Brassica oleracea spp. botrytis | Arizona | Ain Taya | 102 | 06/05/2014 | H1 |
| XccDZ14/102.34 | Brassica oleracea spp. botrytis | Arizona | Ain Taya | 102 | 06/05/2014 | H7 |
| XccDZ14/102.38 | Brassica oleracea spp. botrytis | Arizona | Ain 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 | H1 |
| XccDZ14/104.61b | Brassica oleracea spp. botrytis | Arizona | Tizi Ouzou | 104 | 25/06/2014 | H12 |
| 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 | Ain Dafla | 106 | 16/11/2014 | H17 |
| XccDZ14/106/127 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H16 |
| XccDZ14/106/129 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H16 |
| XccDZ14/106/130 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H18 |
| XccDZ14/106/131 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H1 |
| XccDZ14/106/132 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H1 |
| XccDZ14/106/134 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H1 |
| XccDZ14/106/135 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H19 |
| XccDZ14/106/136 | Brassica oleracea spp. botrytis | Arizona | Ain Dafla | 106 | 16/11/2014 | H1 |
| XccDZ14/106/137 | Brassica oleracea spp. botrytis | Arizona | Ain 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.
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 MgCl2, 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 YPGAvc 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 gyrB (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 MgCl2, 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 gels in 1× TBE buffer, stained with ethidium bromide, and visualized using UV transilluminator on a Gel Doc 2000 (Bio-Rad).

### 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      | 63°C                  | CCGTAGACCTAGTGCAATGGCA TTTCCATCGTACGATT | 619         | Berg et al., 2005 |
|             | DLH125^b      |                       |                    |                           |            |
| rrs (Universal Primers) | 1052–F^a     | 63°C                  | GCATGGTTTGCAGCTCGTTA CCGCTACCTTGTTGAC   | 441         | Eden et al., 1991 |
|             | Bac–R ^b      |                       |                    |                           |            |
| atpD        | P-X-ATPD-F^a  | 62°C                  | GGGCAAGATCGTTCCAGAT GCTTCTGTCAGGTTGAT  | 868         | Fargier et al., 2011 |
|             | P-X-ATPD-R^a  |                       |                    |                           |            |
| dnaK        | P-X-DNAR-F^a  | 62°C                  | GGTTATGACCTGCGACCAC ACCTTCGGCATACGGGTCT | 1,034       | Fargier et al., 2011 |
|             | P-X-DNAR-R^b  |                       |                    |                           |            |
| gyrB        | emigyrB1F^a   | 63°C                  | TGGCGCGCAAGATCGTCACCAC GCCTGGTCTCGATGAAAGTC | 904         | Fargier et al., 2011 |
|             | emigyrB4R^b   |                       |                    |                           |            |
| rpoD        | emirpo11F^a   | 63°C                  | ATGGCCAAGCAGCTCGTGC AACCTGTAAACCGCGGTTAAT | 1,313       | Fargier et al., 2011 |
|             | emirpo13R^b   |                       |                    |                           |            |

^a Forward primers; ^b reverse primers.
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 campestris 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
Xanthomonas campestris in Algeria

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 out-group, 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

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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              |          |          |          |          |
|-----------------|------------------|--------------------|----------|----------|----------|----------|
| XccDZ114.10     | ATCC 33913/ Xcc-C-168 | gyrB 734/735* (99%) | rpoD 574/577 (99%) | atpD 661/667(99%) | dnaK 778/779 (99%) |
| Xcc pv. vesicatoria 85-10 | 639/736 (87%) | 539/578 (93%) | 630/666 (95%) | 736/779 (94%) |
| XccDZ113.5      | ATCC 33913/ Xcc-C-168 | gyrB 734/735 (99%) | rpoD 577/577(100%) | atpD 663/667 (99%) | dnaK 777/779 (99%) |
| Xcc pv. vesicatoria 85-10 | 639/736 (87%) | 639/736 (87%) | 542/578 (94%) | 735/779 (94%) |
| XccDZ1388.2     | ATCC 33913/ Xcc-C-168 | gyrB 734/735 (99%) | rpoD 576/577(99%) | atpD 663/667 (99%) | dnaK 776/779 (99%) |
| Xcc pv. vesicatoria 85-10 | 639/736 (87%) | 541/578 (94%) | 630/666(95%) | 734/779 (94%) |

* 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 %                 |
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 ICMP 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 et al., 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. Yerrouze). 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, varieties, 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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