IDENTIFICATION AND GENETIC CHARACTERISATION OF *XANTHOMONAS CAMPESTRIS* pv. *CAMPESTRIS* AS AN OILSEED RAPE PATHOGEN IN SERBIA

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SUMMARY

During 2010, black rot symptoms were observed in oilseed rape plants grown in a commercial plot in Serbia. Ten bacterial isolates obtained from diseased plants, and identified as *Xanthomonas campestris* pv. *campestris* (*Xcc*) based on pathogenicity, physiological and biochemical tests, PTA-ELISA and 16S rDNA sequences analysis, were investigated in detail. Strains were characterized by comparing them by rep-PCR fingerprints using ERIC and $(GTG)_5$ primers. The 16S rDNA sequences of strains TUr1 and TUr6 were deposited in GenBank under accession Nos. KF057196 and KF057197, respectively. Phylogenetic analysis of the 16S regions showed high similarity level for oilseed rape representative strains and *Xcc* strains of different origin isolated from kale, cabbage and broccoli.

Key words: black rot, oilseed rape, ERIC, $(GTG)_5$, 16S rDNA.

INTRODUCTION

The plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*) causes black rot in a large number of crucifers worldwide (Alvarez, 2000). Typical disease symptoms include V-shaped yellowing of leaf margins as an early symptom following hydathode infection. Advanced systemic infections can cause darkened leaf veins and stem vascular tissue, extensive leaf yellowing, leaf wilting and necrosis (Popović *et al*., 2013; Williams, 1980). The disease is favored by warm, humid conditions and can spread rapidly through rain dispersal and irrigation water. In warm and wet conditions black rot losses may exceed 50%, due to the rapid spread of the disease and extensive killing of host tissues (RPD 924, 1999). Because of its agricultural and industrial importance, the molecular genetics of *Xcc* have attracted particular attention for over two decades. The whole-genome sequences of three different *Xcc* strains have been recently published, ATCC 33913 (synonym 528 T), B100 and 8004 (da Silva *et al*., 2002; Vorhölter *et al*., 2008; Qian *et al*., 2005). The genome consists of a single chromosome *ca.* 5.0 Mb in size, has no plasmids, a GC content of approximately 65% and an average predicted number of coding DNA sequences (CDS) of 4308. The diversity of *Xantomonas* spp. from different geographical origins has recently been studied using rep-PCR (Jensen *et al*., 2010; Lema *et al*., 2012; Zhai *et al*., 2010), pulsed-field gel electrophoresis (PFGE) (Quezado-Duval *et al*., 2004), or a combination of rep-PCR and other methods, such as Biolog and FAME analysis (Massomo *et al*., 2003), AFLP and PFGE (Valverde *et al*., 2007). A primer set designed from randomly selected fragments of DNA obtained after rep-PCR analysis specific to *Xcc* was also utilized for detection purposes (Fargier and Manceau, 2007).

In Serbia, *Xcc* strains isolated from several crucifers (cabbage, kale, broccoli) have previously been identified and described (Popović *et al*., 2013). This study reports the presence of *Xcc* on oilseed rape (*Brassica napus*), which is recorded for the first time as a host in Serbia. Pathogenicity, physiological, biochemical and serological tests of *Xcc* strains and their 16S rDNA sequence analysis were used for pathogen identification. A comparative analysis of 14 *Xcc* isolates from oilseed rape and other hosts and 6 other *Xanthomonas* species was done by rep-PCR fingerprinting.

MATERIALS AND METHODS

Sample collection. In September 2010, leaves of oilseed rape with v-shaped necrotic lesions surrounded by yellow halos on the leaf margins were collected. Symptoms were observed on the local cv. Slavica (IFVC, Novi Sad) grown in a 3 ha field located in the Bačka region (Vojvodina, Serbia). 'Slavica' was planted earlier than winter oilseed rape cultivars that are usually grown in the same area. Average disease incidence on 3-month-old plants was 45%.

Bacterial isolation. Diseased leaves were rinsed with sterile distilled water (SDW) and dried at room temperature before isolation. Leaf sections *ca.* 3 mm in diameter

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taken from the margin of necrotic leaf tissue were macerated in SDW, and the extract was streaked onto nutrient agar (NA) and yeast extract-dextrose-calcium carbonate (YDC) agar. Plates were incubated at 28°C for 3 days. Ten representative isolates that showed yellow colonies with typical morphology, and which were Gram-negative, catalase positive, and oxidase-negative were selected for further study.

Pathogenicity. Pathogenicity tests with 10 selected isolates grown for 48 h on YDC at 28°C, were carried out on cv. Slavica plants using three methods: (i) spraying a bacterial suspension (10^8 CFU ml^{-1} in sterile water) onto the leaf surfaces of 4-week-old oilseed rape plants; (ii) stabbing the major veins of each of the first two true oilseed rape leaves with the tip of a sterile toothpick that had been dipped into a colony of the appropriate strain, and (iii) immersing oilseed rape cotyledons into a bacterial suspension (10^8 CFU ml^{-1} in sterile water). SDW was used as negative control treatment. A reference strain, *Xcc* NCPPB 1144 (National Collection of Plant Pathogenic Bacteria, UK) isolated from *Brassica oleracea* (cabbage), was used as a positive control. Tests plants (two plants for each method of inoculation and each bacterial strain or control treatment) were covered with plastic bags and maintained in a greenhouse at 25±1°C and 80% relative humidity. Re-isolations were done onto YDC by streaking margins of necrotic tissue macerated in SDW.

Phenotypic characterization. All isolates were characterized by the methods of Dye (1962) and Lelliot and Stead (1987). For all tests, cultures were grown on YDC at 28°C for 48 h. Oxidative/fermentative (OF) medium supplemented with glucose was used to determine the type of metabolism for each strain. Strains were Gram-stained and tested for: Kovacs' oxidase reaction; nitrate reduction; catalase, levan, hydrogen sulfide and indole production; starch, gelatine and aesculin hydrolysis; Tween 80 lypolysis; growth at 35°C; tolerance to 0.10 and 0.02% triphenyl-tetrazolium chloride (TTC) and acid production from d-arabinose, arginine, dulcitol, galactose, d-glucose, maltose, mannose, sorbitol, sucrose and xylose.

Plate Trapped Antigen (PTA) ELISA. Strains suspected to be *Xcc* were tested using a commercial ELISA kit (ADGEN Phytodiagnostics, UK) following the manufacturer's instructions. Bacterial suspensions (3.10^8 CFU ml^{-1}) were prepared in sterile water using pure cultures grown on the YDC for 48 h at 27°C.

Genetic characterization. Total bacterial DNA was extracted as follows: single colonies grown on YDC plates were transferred to 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), resuspended and heated at 95°C for 10 min, immediately cooled on ice for 3 min, centrifuged at 12,000 rpm for 5 min, and the supernatants were kept at –20°C until needed. The strains used for genotyping were: *Xcc* originating from oilseed rape (TUr1- TUr10), cabbage (TKu7), broccoli (TBr11) and kale (TKe5), *Xcc* reference strain NCPPB 1144 (*Brassica oleracea var. capitata*, UK), *X. arboricola* pv. *pruni* NCPPB 3156 (*Prunus persica*, Italy), *X. hortorum* pv. *pelargonii* NCPPB 3330 (*Pelargonium zonale*, UK), *X. fragariae* NCPPB 2473 *Fragaria vesca*, Italy, *X. vesicatoria* NCPPB 1431 (*Lycopersicon esculentum*, Hungary), *X. axonopodis* pv. *phaseoli* GSPB 1241 (*Phaseolus vulgaris*) (Goettinger Sammlung Phytopathogener Bakterien, Germany) and *X. axonopodis* pv. *phaseoli* var. *fuscans* CFBP 6165 (*Phaseolus vulgaris*, Canada) (La Collection Française de Bactéries Phytopathogènes, France).

rep-PCR. Amplification reactions were conducted with primer set ERIC1R/ERIC2 for ERIC and $(GTG)_5$ primer for BOX PCR. PCR was carried out in a 50 µl reaction vol. using GreenTaq Dream master mix (Thermo Scientific, Lithuania) with 1 µl of template DNA and 0.1 µmol of each primer in an Eppendorf Master Cycler with the temperature profile recommended by de Bruijn (1992). The cycling programs started with an initial denaturation at 95°C for 7 min followed by 35 cycles of a three-step PCR program: 1 min denaturation at 94°C, 1 min at 52°C (ERIC) or 53°C (BOX) for primer annealing and 8 min at 65°C for primer extension, followed by a final extension at 65°C for 16 min. Amplification reactions were repeated at least twice for each bacterial strain. PCR products (10 µl) were separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5× Tris-borate-EDTA buffer for 3 h at 5V/cm. Gels were stained with ethidium bromide (0.5 µg ml^{-1}), and DNA migration patterns were analyzed visually. Fragment size was determined by comparison with the DNA molecular weight markers GeneRuler DNA Ladder mix SM0331 (Thermo Scientific, Lithuania).

Genomic fingerprint comparisons among bacterial strains were performed by measuring the reproducible bands ranging from 200 bp to 3 kb band sizes. The presence (1) or absence (0) of each band was converted into binary data and cluster analyses were performed using the STATISTICA 7 program.

PCR amplification of the bacterial 16S rRNA gene. The 16S rRNA gene was amplified using universal bacterial primers fDl and rD1 (Weisburg *et al*., 1991). PCR amplification was carried out in a 50 µl reaction vol. using GreenTaq Dream master mix (Thermo Scientific, Lithuania) with 1 µl of template DNA and 0.1 µmol of each primer. DNA amplification was performed with the following temperature profile: an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 58°C), and extension (2 min at 72°C); and a final extension at 72°C for 5 min. Amplified DNA was examined by electrophoresis for 2 h at 5 V cm^{-1} in a 1.2% (w/v) agarose gel with the GeneRuler DNA Ladder mix SM0331 (Thermo Scientific, Lithuania).

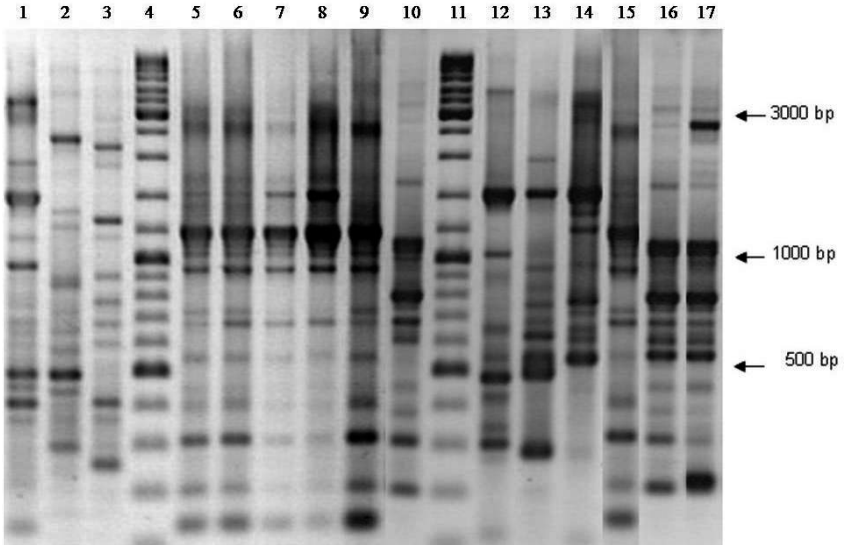

Fig. 1. ERIC-PCR of 10 indigenous *Xcc* strains isolated from *Brassica napus* and some related strains. Lanes 1-3, *X. vesicatoria* (NCPPB 1431), *X. a.* pv. *phaseoli* (GSPB 1241), *X. a.* pv. *phaseoli* var. *fuscans* (CFBP 6165); lanes 4 and 11: marker (GeneRuler DNA Ladder mix SM0331, Thermo Scientific, Lithuania); lanes 5-9, oilseed rape strains TUr1, TUr5, TUr6, TUr8, TUr10; lane 10, *Xcc* (NCPPB 1144); lane 12, *X. a.* pv. *pruni* (NCPPB 3156); lane 13, *X. h.* pv. *pelargonii* (NCPPB 3330); lane 14, *X. fragariae* (NCPPB 2473); lane 15, TKU7 strain from cabbage; lane 16, TBR11 strain from broccoli; lane 17, TKE5 strain from kale.

Phylogenetic anaylsis. The 16S rDNA of strains TUr1 and TUr6 were sequenced (IMGGI SeqService Belgrade) and deposited under accession Nos. KF057196 and KF057197, respectively, and compared with published sequences of the 16S rRNA gene from GenBank, using the algorithm BLAST (Altschul *et al*., 1997).

A phylogentic analysis of the 16S rRNA genes was performed using the Maximum Likelihood method (Tamura and Nei, 1993). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (above the branches). The analysis involved 11 nucleotide (nt) sequences. All positions containing gaps and missing data were eliminated. There were a total of 1495 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

PCR testing for *Xanthomonas gardneri*. To further confirm the identity of our isolates, a PCR assay was done using sets of primers according to Koenraadt *et al.* (2009). The reference strain included for comparison was *X. gardneri* NCPPB 881 (*Lycopersicon esculentum*, former Yugoslavia). To prepare template DNA of each strain, including the reference strain, bacterial cells were grown for 24 h on NA at 27°C, suspended in sterile distilled water to achieve an optical density of 0.3 at 600_{nm} (OD_{600} ~ 10^8 CFU ml^{-1}), then diluted to 10^6 CFU ml^{-1} and centrifuged. The PCR reaction was performed in an Eppendorf Master thermocycler using primers XgF/R (Metabion International, Germany). DNA fragments were amplified in a reaction mixture volume of 25 µl, containing: 1× PCR Master Mix (Fermentas, Lithuania), 0.2 µM of each primer and 2 µl of template DNA. *Pseudomonas syringae* pv. *glycinea* strain NCPPB 3318 (pathovar reference strain, *Glycine max*, Italy) and reaction mix without addition of DNA as blank were used as negative controls. The amplification conditions were denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing at 55°C for 1 min and extension at 7°C for 1 min. A final DNA extension step was at 72°C for 5 min. The PCR product of each strain was separated by electrophoresis in a 1.5% (w/v) agarose gel with addition of ethidium bromide (final concentration of 0.5 µg ml^{-1}) run at 100 V for 40 min in 1× TBE buffer. A low range DNA ladder (50-1500 bp; SM1103, Fermentas, Lithuania) was used for comparison with the expected size of the amplified fragments. Gels were observed in a UV transilluminator and photographed.

RESULTS

In this study, 10 representatives *Xcc* isolates (TUr1-TUr10) obtained from V-shaped lesions from oilseed rape were characterized. After 48 h incubation, typical colonies were small (up to 2 mm), yellow on NA or yellow, translucent, circular, and raised on YDC medium (up to 3-5 mm) (TUr1-TUr10).

In pathogenicity tests these isolates and the reference strain caused typical yellow lesions that turned into necrotic spots about seven days post inoculation (dpi). The spots coalesced within 21 dpi to form necrotic areas. Plants inoculated with SDW remained symptomless. Re-isolated

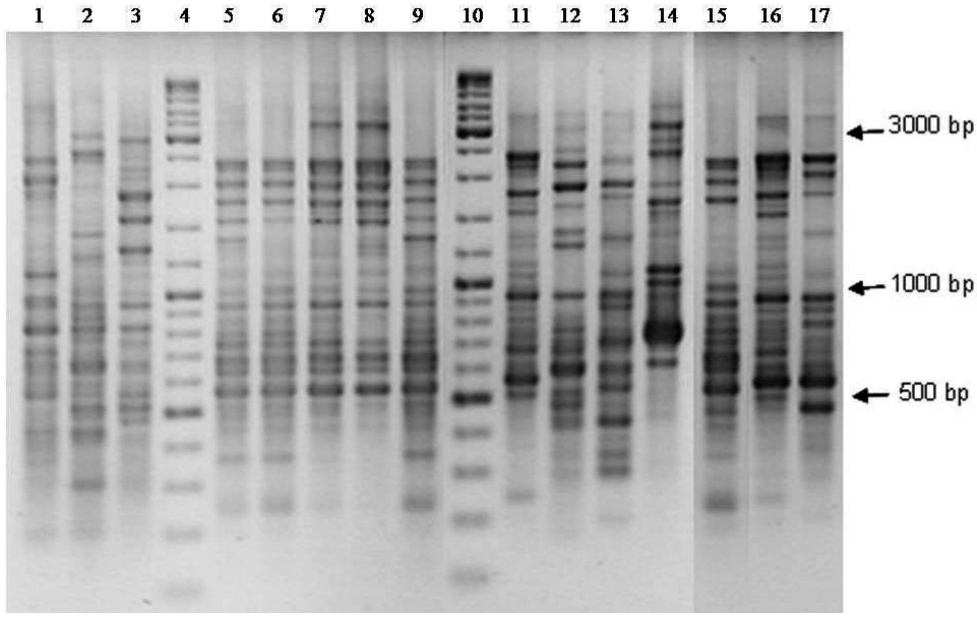

Fig. 2. BOX -PCR of 10 *Xcc* strains from *Brassica napus* and some related strains obtained by $(GTG)_5$ primer. Lanes 1-3, *X. vesicatoria* (NCPPB 1431), *X. a.* pv. *phaseoli* (GSPB 1241), *X. a.* pv. *phaseoli* var. *fuscans* (CFBP 6165); lanes 4 and 10, marker (GeneRuler DNA Ladder mix SM0331,Thermo Scientific, Lithuania); lanes 5-9, oilseed rape strains TUr1, TUr5, TUr6, TUr8, TUr10; lane 11, *Xcc* (NCPPB 1144); lane 12, *X. a.* pv. *pruni* (NCPPB 3156); lane 13, *X. h.* pv. *pelargonii* (NCPPB 3330); lane 14, *X. fragariae* (NCPPB 2473); lane 15, TKU7 strain from cabbage; lane 16, TBR11 strain from broccoli; lane 17, TKE5 strain from kale.

strains showed the same colony morphology as described above. Bacterial isolates grew at 35°C, produced levan from sucrose, hydrogen sulfide, and indole; did not reduce nitrate; hydrolyzed Tween 80, starch, gelatin, and aesculin; did not show tolerance to 0.10 and 0.02% triphenyltetrazolium chloride; and produced acid from d-arabinose, arginine, dulcitol, galactose, d-glucose, maltose, mannose, sorbitol, sucrose and xylose. All isolates and the reference strain tested by PTA-ELISA reacted with *Xcc*-specific polyclonal antibodies.

rep-PCR. ERIC PCRs, conducted using the primer set ERIC1R/ERIC2, yielded 35 bands varying from 130 to 3000 bp (Fig. 1). All *Xcc* TUr strains showed a similar pattern, with several diagnostic and few polymorphic bands. Two patterns could be differentiated at the similarity level of 98% according to cluster analysis (data not shown). The $(GTG)_5$ primer, used for BOX analysis instead of BOX A1R, yielded a total of 24 bands varying from 110 to 3000 bp (Fig. 2). Reference strain *Xcc* NCPPB 1144 belonged to the same cluster as the TUr strains. Using $(GTG)_5$, we compared TUr strains and selected a representative strain for each pattern: TUr1 (representing patterns for TUr2, TUr3, TUr4, TUr 9), TUr5, TUr6 (TUr7), TUr8, TUr10.

Cluster analysis of fingerprinting data obtained by ERIC and $(GTG)_5$ is shown in Fig. 3. The highest similarity (98%) was observed between TUr1- TUr5 and TUr6-TUr8 strains. All TUr strains were similar to the reference TKU7 strain from cabbage and formed one group. Together with the TBR11 strain from broccoli, the TKE5 strain from kale and the reference strain *Xcc* NCPPB 1144 (about 63% homology), the TUr group formed a *Xcc* subcluster that differed by 51% from the *X. axonopodis* pv. *phaseoli* (GSPB 1241) subcluster. The second cluster (differing by more than 57%) contains the other type strains tested: *X. arboricola* pv. *pruni* NCPPB 3156, *X. hortorum* pv. *pelargonii* NCPPB 3330, *X. fragariae* NCPPB 2473, *X. vesicatoria* NCPPB 1431 and *X. axonopodis* pv. *phaseoli* var. *fuscans* CFBP 6165.

PCR amplification of the partial 16S rRNA gene. The partial 16S rDNA sequence of two representative strains from oil seed rape (TUr1 and TUr10) was amplified using the fD1/rD1 primer set and determined by the IMGGI SeqService facility in Belgrade. The 1510 bp and 1503 bp 16S rDNA sequences of TUr1 and TUr6, deposited under accession Nos. KF057196 and KF057197, respectively, were compared to those from GenBank. The greatest similarity (99% homology) was found with *Xcc* strains ATCC 33913 and B100 and with *Xanthomonas gardneri* type strain DSM 19127T and *X. gardneri* CNPH496. A dendrogram resulting from the phylogenetic analysis of TUr1 and TUr6 strains based on the 16S rRNA gene is shown in Fig. 4. The analysis involved the almost complete sequences of 16S rRNA gene (1495 bp) of nine *X. campestris* and two *X. gardneri* strains from the NCBI database by multiple sequence alignments and the phylogenetic trees was constructed using the NJ (Neighbor-Joining) method. TUr sequences were compared with Serbian strains from kale (TKE5), broccoli (TBR11) and cabbage (TKU7). The highest similarity level (99.98%) TUr strains showed to strain TKE5.

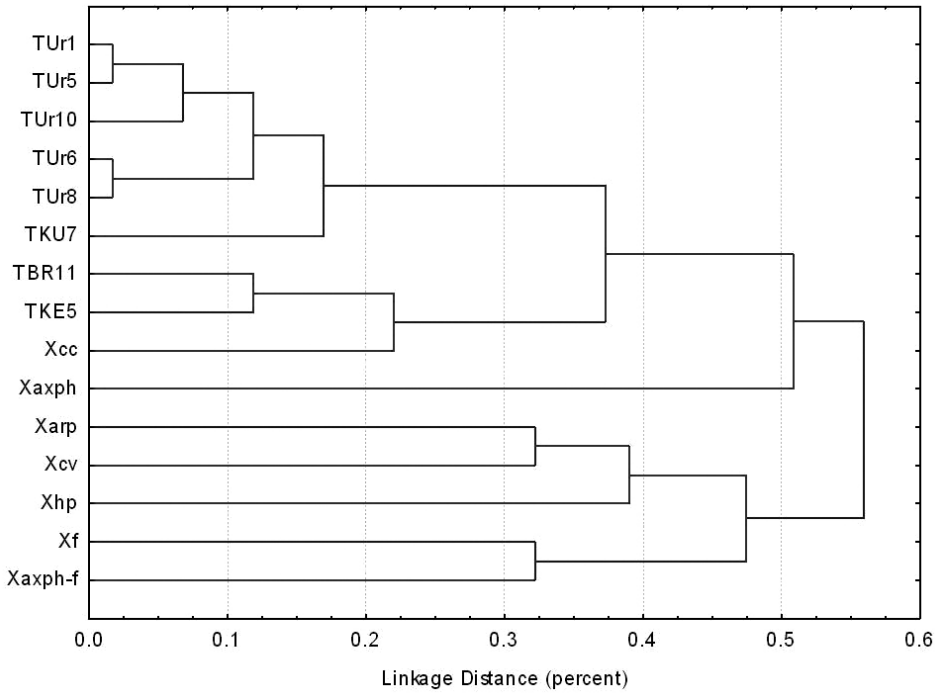

Fig. 3. Dendrogram of genetic similarity of 10 *Xcc* strains from *Brassica napus* and some related strains based on ERIC and BOX-$(GTG)_5$ fingerprinting data. TUr1, TUr5, TUr6, TUr8, TUr10 oilseed rape strains; Xcv, *X. vesicatoria* (NCPPB 1431); Xarp, *X. a.* pv. *pruni* (NCPPB 3156); Xhp, *X. h.* pv. *pelargonii* (NCPPB 3330); Xf, *X. fragariae* (NCPPB 2473); Xaxph, *X. a.* pv. *phaseoli* (GSPB 1241); Xaxph-f, *X. a.* pv. *phaseoli* var. *fuscans* (CFBP 6165); *Xcc*, *X. c.* pv. *campestris* (NCPPB 1144); TKU7, *Xcc* strain from cabbage; TBR11, *Xcc* strain from broccoli; TKE5, *Xcc* strain from kale.

PCR testing for *Xanthomonas gardneri*. To clearly discriminate tested *B. napus* isolates, tentatively identified as *Xcc* from *X. gardneri*, the PCR specific primers for detection of this bacterium were applied to all tested strains and reference strain NCPPB 881. No amplification of 154 bp bands specific for *X. gardneri* was obtained for TUr strains, which confirmed that all of them belong to *Xcc* (Fig. 5).

DISCUSSION

The present study shows that *Xcc* can cause bacterial black rot disease on *Brassica napus* var. *napus* (oilseed rape) under field conditions in Serbia. To our knowledge this is the first time that *Xcc* is reported from oilseed rape as a new host in Serbia. Symptoms were observed on the domestic winter cv. Slavica, planted earlier than other winter oilseed rape cultivars usually grown in this area. This fact can be epidemiologically important because it shows that certain winter cultivars of oilseed rape are more susceptible to *Xcc* when planted early.

The YDC medium proved to be very useful for the successful isolation of *Xcc* from this host. Yellow, translucent, circular, and raised bacterial colonies were routinely isolated from all symptomatic plants, the yellow color originating from the water-insoluble yellow pigments (xanthomonadins) (Schaad, 1988).

The 10 isolates studied were Gram-negative and showed typical *Xcc* features, such as catalase test-positive, negative for oxidase, oxidative utilization of glucose, growth at 35°C, positive for levan production from sucrose, hydrogen sulfide and indole production; negative for nitrate reduction; positive for Tween 80, starch, gelatin, and aesculin hydrolysis; no tolerance to 0.10 and 0.02% triphenyltetrazolium chloride; utilisation of d-arabinose, arginine, dulcitol, galactose, d-glucose, maltose, mannose, sorbitol, sucrose and xylose (Adhikari and Basnyat, 1999; Alvarez *et al*., 1994; Popovic *et al*., 2013; Schaad and Alvarez, 1993; Swings *et al*., 1993). The results of biochemical and physiological characteristics of tested strains suggested all strains to be very homogeneous. Following isolation and identification, tests were carried out on young oilseed rape plants to determine the pathogenicity and virulence of the strains. All of them caused typical disease symptoms on artificially inoculated oilseed seedlings and young plants in growth chamber assays as reported by Cook *et al*. (1952) and Williams *et al*. (1972). The same bacterium was reisolated from artificially inoculated plants, fulfilling Koch's postulates and bringing to the conclusion that the pathogen responsible for black rot disease of oilseed rape in Serbia is *Xcc*.

A high level of polymorphism among *X. axonopodis* pv. *manihotis* strains originating from different edaphoclimatic zones was observed in Colombia on the basis of REP and ERIC PCR (Restrepo *et al*., 2000). Genetic diversity of

Fig. 4. Phylogenetic analysis using almost complete 16S rRNA gene sequences (1495 bp) of *X. campestris* strains TUr1 and TUr6 isolated from oilseed rape. The phylogenetic trees were inferred from multiple sequence alignments using similarity matrix method implemented in MEGA 5 and phylogenetic trees were constructed using the Neighbor-Joining method. Bar: 5 substitutions per 1000 nucleotides.

X. arboricola pv. *juglandis* strains from different geographical areas was clearly shown by rep-PCR (Scortichini *et al*., 2001). Biolog, rep-PCR and fatty acid methyl ester analysis was used by Massomo *et al*. (2003) for assessing the correlation of geographical origin with different genotypes of *Xcc* from Tanzania. Valverde *et al*. (2007) used rep-PCR, AFLP and PFGE to analyze the genetic diversity among *Xcc* strains from field-cultivated crucifer plants showing typical black rot symptoms in Israel and other geographic locations, representing the six known races of this pathogen. The combined rep-PCR patterns and obtained clusters were in agreement with AFLP and PFGE cluster analyses. Characterization of *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans* isolated from common bean (Mahuku *et al*., 2006), of Italian populations of *Xcc* (Zaccardelli *et al*., 2008), and the evaluation of genetic diversity of highly virulent strains of *X. campestris* pv. *malvacearum* (Zhai *et al*., 2010) were done by rep-PCR fingerprinting. The same method was also used to assess the genetic diversity of pathogenic *Xcc* strains from Nepal, isolated from cabbage, cauliflower, and leaf mustard (*Brassica juncea*) with typical lesions. Recently, however, a rep-PCR analysis of *Xcc* strains from northwestern Spain showed no clear relationship to race, crop or geographical origin (Lema *et al*., 2012). Our results are consistent with the results by Jensen *et al*. (2010) who used combined rep-PCR analysis to prove that *Xcc* strains clustered separately from other *Xanthomonas* spp. and pathovars from other hosts, as it was the case in our study.

Reproducible genomic rep-PCR fingerprints were produced for all tested oilseed rape *Xcc* strains. Most bands, differing in intensity, were present in all TUr strains. Five BOX PCR $(GTG)_5$ patterns showed several polymorphic bands. Dendrograms obtained with each individual set of primers showed the separation of TUr strains into two ERIC groups at 98% similarity level and three $(GTG)_5$ groups at a similarity level of 92%.

Although the most frequent occurring pattern was that of the TUr1 strain, the tested population of 10 strains is too small for drawing ultimate conclusions. Further investigation will involve a larger number of *Xcc* strains from oilseed rape. Several studies have shown differences in the ability of rep-PCR primers to reveal diversity in *Xanthomonas* spp. Trindade *et al*. (2005) compared several field strains of *X. campestris* pv. *viticola* providing information on their diversity and origin. Although combined analysis of the patterns obtained with primers REP, ERIC and BOX showed a high degree of similarity among those Brazilian strains, ERIC patterns revealed more polymorphic bands among the strains than the other two sets of primers. ERIC primers revealed a higher level of genetic diversity than REP and BOX primers also in a study on *X. axonopodis* pv. *phaseoli* and its var. *fuscans* (Lopez *et al*., 2006) and *Xcc* races (Jensen *et al*., 2010).

BOX analyses in previous investigations were mostly performed using the BOX A1R primer. Our investigation showed the primer $(GTG)_5$, a BOX type, to be more discriminative than BOX A1R (data not shown) and ERIC for

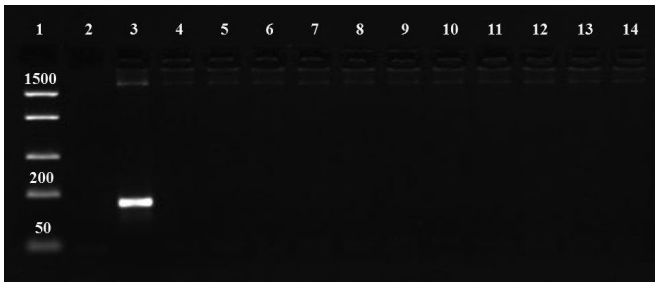

Fig. 5. PCR using *X. gardneri*-specific primers XgF/R showing that amplifications from *B. napus* strains were all negative. The only positive band is from the *X. gardeneri* type strain NCPPB 3318. Lane 1, Marker [Low Range DNA Ladder SM1103 (50-1500 bp), Fermentas, Lithuania]; lane 2, SDW; lane 3, *X. gardneri* NCPPB 881 reference strain (154 bp amplicon); lane 4, *Pseudomonas syringae* pv. *glycinea* NCPPB 3318; lanes 5-14. oilseed rape strains TUr1-TUr10.

Xcc strains from oilseed rape. The genomic patterns of *Xcc* TUr strains were shown to be very homogeneous and quite distinctive from other *Xanthomonas* strains isolated from the same area. TUr strains showed the highest similarity (83%) with *Xcc* TKU7 strain isolated from cabbage and the lowest (62.5%) with a group of strain comprising *Xcc* TBR11 from broccoli, *Xcc* TKE5 from kale (Popović *et al*., 2013) and the *Xcc* type strain (NCPPB 1144) from cabbage.

In this study, 16S rDNA sequences of two representative strains showed a high similarity level to sequences of *X. gardneri* type strain DSM 19127T, *X. gardneri* CNPH496 (99%) and *Xcc* strains ATCC 33913 and B100 (99%) from the NCBI database. MEGA5 phylogenetic analysis of these regions revealed high levels of similarity among all Serbian *Xcc* strains that grouped in the two closest branches (~98%). The highest similarity (99.98%) was observed for the representative strain TUr6 from oilseed rape and strain TKE5 from kale. In a *X. gardneri*-specific PCR none of tested TUr strains yielded band specific to *X. gardneri.* All these molecular test results allow us to conclude that all tested strains from oilseed rape belong to *Xcc*.

Based on pathogenicity tests that completed Koch's postulates, sequence analysis, as well as the results of physiological, biochemical and serological tests, it seems plausible to conclude that the bacterial strains from oilseed rape investigated in this study are identifiable as *Xcc*. It is the first time that this pathogen is reported on oilseed rape in Serbia.

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