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RESEARCH ARTICLE

Study of *Xanthomonas campestris* pv. campestris isolates originating from soil and *Brassica spp*. seeds

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ABSTRACT

Xanthomonas campestris pv. *campestris* (Xcc) is a harmful pathogen of the *Brassicaceae* agricultural plants in Serbian agro-ecological conditions. The bacterium survives in a soil, plant residues, seeds, wild, and weedy plants. The aim of this study was to characterize Xcc isolates that persist in field soil and seeds where they represent a risk to cause disease of *Brassicaceae* plants. Collection of soil samples was carried out in the period 2015 - 2017 in four locations regions Vojvodina with cabbage cultivation in monoculture or with crop rotations. The cabbage seed samples were collected in the same period in Futog and Despotovo locations (agricultural farms) and in local farmer markets. All isolations were done on SX and YDC agar plates with the addition of cycloheximide with 250 mg/l. Based on the pathogenicity tests, morphological and biochemical characteristics, 28 identified Xcc isolates were selected, 12 from soil and 16 from seeds. Molecular confirmation of the Xcc isolates was done using multiplex PCR (m-PCR) with Xcc-specific primer sets DLH 120/125 and Zup 2309/2310. In all selected isolates and in the reference Xcc strain NCPPB1144 the expected 370 and 619 bp PCR fragments were amplified. Characterization of isolates by rep-PCR fingerprinting using BOX A1R and $(GTG)_5$ primers resulted in informative patterns. Cluster analysis grouped the strain Xcc NCPPB 1144 together with all Xcc isolates, while other tested Xanthomonads were clustered separately. Based on the similarity level of 90%, we obtained three out of 12 as representative isolates from soil and four out of 16 representative seeds isolates. The characterization of Xcc isolates from soil and seeds will enable their comparison with those pathogenic isolated from leaves of *Brassicaceae* plants.

Key words: Black rot pathogen; *Xanthomonas* spp.; multiplex-PCR; rep-PCR

INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc) is an economically important widespread pathogen of many cultivated *Brassicaceae* plants present across the World (Vicente and Holub, 2013; Laala et al., 2021) and particularly in Serbia (Balaž, 2005; Popović et al., 2013; 2014; Vlajić et al., 2017; Popović et al., 2019; Jelušić et al., 2020). It infects many species of cruciferous crops, including such agriculturally important crops as *Brassica oleracea* plants (cabbage, broccoli, cauliflower, and kohlrabi), *B. rapa* (turnips, oilseeds, Oriental plants), *B. napus* (canola, Sweed), radish, and ornamentals (Jensen et al., 2010; Rathaur et. al., 2015), as well as the wild and weedy brassicas (Ignatov et al., 2007). Damages are primarily due to reduction of yield, storage ability, and the market quality.

The major source of inoculum is infected seeds (Vicente and Holub, 2013), and bacteria in soil (Lopez et al., 1999; Gazdik et al., 2021), infected weeds of *Brassicaceae* family and infected plant debris. Reduction of inoculum by removal of plant residues after harvest, as well as crop rotation for a minimum 3 - 4 years are important strategy for the pathogen control. Xcc can persist in the soil on infected plant debris for at least 24 months (Köhl et al., 2011). Classical identification methods and advanced molecular techniques, especially those based on polymerase chain reaction (PCR), are very useful in diagnosis of this bacterial disease (Singh et al., 2015). Repetitive DNA polymerase chain reaction-based fingerprinting (rep-PCR) is highly reproducible and discriminative method extensively used to assess the genetic diversity of Xcc strains (Singh et al., 2011; Mulema et al., 2012). Massomo et al. (2003) found

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that 76 Xcc strains clustered according to the geographical area of isolation. Results of rep-PCR fingerprinting allowed the separation of Xcc from closely related pathovar *X. campestris* pv. *raphani* strains, and sometimes showed a tendency for strains of the same race among Xcc and *X. campestris* pv. *raphani* to cluster together (Vicente et al., 2006). Jelušić et al. (2020) confirm the presence of diversity among Xcc isolates originating from Serbian winter oilseed rape using three rep-PCR primers.

The aim of this study was the identification, characterization and pathogenicity evaluation for Xcc isolates obtained from soil with and without presence of hosts. The same objective study was for isolate of Xcc obtained from cabbage, cauliflower and rapeseed seeds (certified commercial and saved seed), which represent a high risk for the disease appearance in cultivated *Brassicaceae* plants.

MATERIALS AND METHODS

Bacterial isolation from soil samples

Collection of soil samples was carried out in the period 2015 - 2017 from 5 locations: Kovilj, Despotovo, Rimski šančevi, Futog, Gospodjinci (Vojvodina, Serbia).

Total of 21 fields were assessed, of which 19 fields was in crop rotation (corn, soybean, wheat, cabbage, and cauliflower), while 2 fields were in monoculture (cabbage was grown during 3-4 period). The size of fields was between 0.5 to 1 ha.

By the method of random sampling, up to 10 cm deep a larger number of subsamples were taken and 1 representative sample (weighed 0.5 kg) was made. Soil aliquots (10 g) were suspended in sterile distilled water (SDW; 100 ml) and placed on an electromagnetic stirrer for 10 min. The small aliquots of the standard soil dilution series (10^{-1}, 10^{-2}, 10^{-3}) were streaked on the surface of plates with SX agar (Schaad and White, 1974), and YDC medium (Schaad, 1980) with addition of cycloheximide ($C_{15}H_{23}NO_4$ ≥ 96%; ROTH®) (250 mg/l) and then incubated at temperature 28 ± 1°C.

The isolation was done in three replications. Typical colonies for Xcc were collected from both mediums, isolates from the same soil sample were compared by rep-PCR and selected (total 15), then were grown in nutrient broth and stored in 30% glycerol (v/v) at −80 °C.

Bacterial isolation from seed samples

The seed samples (total 34) were collected at 2015 - 2017. Samples of saved seeds cabbage originated from two localities (Futog and Despotovo), and certified commercial seeds (cabbage, cauliflower and rapeseed) were obtained at the local agricultural markets. The weight of the samples was 0.025 kg. Extraction of bacteria from the seed samples was carried out according to the International Seed Testing Association (ISTA, 2014) and Chitarra et al. (2002) methods. Typical colonies (total 19) for the genus *Xanthomonas* were selected and grown in nutrient broth and stored in 30% glycerol (v/v) at−80 °C.

Pathogenicity

Pathogenicity of all isolates collected from soils and seed samples was tested using two artificial inoculation methods: (i) infiltration of the bacterial suspension in the central nerve of cabbage leaf and (ii) spraying a leaves of cabbage, cauliflower, kale, broccoli and oil seed rape.

(i) Bacterial suspension in SDW with 10^7 CFU mL^{-1} was prepared from 48h old culture grown on YDC and infiltrated into cabbage leaf mesophyll with syringe and hypodermal needle (Obradović and Arsenijević, 1999; Radunović and Balaž, 2012). The inoculated and control plants were incubated in a moist chamber for 7 days, and kept further in climatic chamber at 25°C with 50% relative humidity under 16 h light. Bacteria were re-isolated on YDC medium from the inoculated leaves to confirm the causing agent for the developed symptoms.

(ii) Five plant species were included in this pathogenicity test: cabbage variety - Futoški, cauliflower variety - Snežna grudva, kale variety - Gvozdena glava, broccoli hybrid Naxos F1 and oilseed rape variety - Slavica. Seeds first were disinfected in a mixture of Funomil 0.07% (thiophanate-methyl 700g kg^{-1}) and the antibiotic streptomycin sulfate 0.02% for 5 min, washed in distilled water and dried on filter paper. After drying, seeds were sown in containers filled with a mixture of sterile substrate and sand in a ratio of 3:1. The plants were grown for five weeks at 25°C with a day night light regime 16/8. Artificial inoculation was performed in the phenophase of four leaves (BBCH 14), which is according to Fargier and Manceau (2007) the most suitable for Xcc inoculation. Spraying was performed using a hand sprayer, with a bacterial suspension (≤10^7 CFU mL^{-1}) prepared from a 48 h bacterial culture grown on YDC medium. Inoculated plants were kept in a chamber, under the same conditions of temperature (25°C) and light with high relative humidity (approximately 50%). Symptoms were monitored on daily basis, and results were recorded on the fifth, seventh, and twelfth day after inoculation.

The both inoculation experiments were performed in three replications, using the Xcc strain NCPPB1144 (National Collection of Plant Pathogenic Bacteria, UK) as a positive control, while leaves infiltrated or sprayed only with SDW were served as negative control.

Biochemical characteristics

For the all tested isolates the following biochemical reactions were examined: Gram reaction, catalase and oxidase activity, oxidative - fermentative metabolism of glucose (O/F test), hydrolysis of starch, gelatine, aesculin and Tween-80, tolerance to 0.1% and 0.02% TTC (triphenyl tetrazolium chloride), reduction of nitrates, production of indole and hydrogen sulphide and growth at 35°C (Schaad et al., 2001).

Molecular identification

For DNA isolation, selected colonies of bacteria grown for 48 h on YDC were transferred and homogenized in 1.5 ml Eppendorf tubes containing 1 ml of sterile saline solution (0.9% NaCl) and centrifuged for 5 min at 8000 rpm, the supernatant was removed, and 500 µl of 0.5 N NaOH was added. Incubation was performed at 65ºC for 10 min, followed by vortexing at 1000 rpm. Per 5 µl of the suspension was transferred into new tubes where 495 µl of nuclease free water was added. The isolated DNA solution was stored at -20ºC.

*Multiplex-PCR***.** DNA of the collected 28 Xcc isolates was amplified in m-PCR with specific primer sets DLH 120/125 (Berg et al*.,* 2005), and Zup 2309/2310 (Rijlaarsdam et al., 2004) (Table 1). PCR amplification was carried out in 20 µl reaction volume using GreenTaq Dream Master Mix (Thermo Scientific, Lithuania) with 1 µl of template DNA and 0.2 µmol of primers Zup 2309/Zup2310 and 0.6 µmol of primers DLH120/DLH125.

The program comprised of 3 min initial denaturation at 95°C followed by 6 cycles of 40s at 95°C, 40s at 63°C with a touchdown of 1°C per cycle, and 40s at 72°C. PCR was continued for 29 cycles of 40s at 95°C, 40s at 58°C, and 40s at 72°C. A final extension was performed for 5 min at 72°C.

In all m-PCR reactions, the Xcc strain NCPPB1144 was used as a positive control. PCR products (7 µl) were separated by electrophoresis (BlueMarine™ 200) in 2% (w/v) agarose gels in 0.5X Tris-borate-EDTA buffer for 1 h at 90V and stained with ethidium bromide (0.5 µg ml^{-1}). For fragment size estimation DNA molecular weight markers SERVA FastLoad 100bp DNA Ladder (ServaElectrophoresis GmbH, Heidelberg, Germany) were used.

Molecular characterization by rep-PCR.

Characterization of Xcc isolates by rep-PCR analysis was done using $(GTG)_5$ (GTG GTG GTG GTG GTG) and BOX A1R (CTA CGG CAA GGC GAC GCT GAC G) primers (Versalovic et al., 1994).

rep-PCR was carried out in a 25 µl reaction volume using GreenTaq Dream MasterMix (Thermo Scientific, Lithuania) with 1 µl of template DNA and 100 pmol of each primer in an Eppendorf MasterCycler Personal thermocycler (Germany). The amplification conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles (denaturation at 94°C for 1 min, annealing at 53°C for 1 min, primer extension at 65°C for 8 min) and final extension at 65°C for 16 min. PCR amplification was repeated at least twice for each Xcc isolate.

rep-PCR products (8 µl) were separated by electrophoresis (MAXIGEL Eco 2, APELEX) in 1.5% (w/v) agarose gels in 0.5X Tris-borate-EDTA buffer for 3 h at 5V/cm and stained with ethidium bromide (0.5 µg ml^{-1}). For fragment size estimation DNA molecular weight markers MassRuler Express DNA Ladder Mix Reverse SM1293 (SM1293) and GeneRuler DNA Ladder Mix SM0331 (SM0331) (Thermo Scientific, Lithuania) were used.

The reproducible bands ranging from 200 bp to 3 kb in the rep-PCR fingerprinting patterns were counted and transformed to 0/1 matrix to calculate simple matching coefficient (SSM) and compare of Xcc isolates and reference strains of several *Xanthomonas* species (Table 2). Cluster analysis based on UPGMA (unweighted pair group arithmetic average - linkage algorithm) was performed using STATISTICA 8 software.

Table 1: Primer pairs used in this study

Primer	Sequence (5´-3´)	Length of product	Reference
DLH 120	CCG TAG CAC TTA GTG CAA TG	619 bp	Berg et al. (2005)
DLH 125	GCA TTT CCA TCG GTC ACG ATT G		
Zup 2309	AAA TCA GGG GGA TGC GGT GG	370 bp	Rijlaarsdam et al. (2004)
Zup 2310	TCC GGC CAG GGT CGA TAC AGT G		

Table 2: Reference strains

Code	Strains	Host	Origin
NCPPB 1144	Xcc	cabbage	UK
NCPPB 4321	*X. perforans*	pepper	UK
NCBI KX512833	*X. euvesicatoria*	pepper	USA
KFB 29	*X. vesicatoria*	pepper	Serbia (curator prof. dr A. Obradović)
KBNS 204	*X. axonopodis* pv. *phaseoli*	bean	Serbia (curator prof. dr J. Balaž)

RESULTS AND DISCUSSION

Bacterial isolation from soil and seed samples

Presence of Xcc bacteria was confirmed in the collected seed (cabbage, cauliflower, oil rape) and soil samples during 2015 - 2017. In a three-year period, 15 isolates from 21 soil samples and 19 isolates from 34 seed samples were collected. The presence of Xcc was not recorded in 6 soil samples and 15 seed samples. For the genus *Xanthomonas* one typical colony per sample was selected for subculture.

Typical Xcc colonies were formed after 4 days of incubation on SX medium (mucoid and glistening, 3 - 4 mm in diameter, surrounded by clearly visible zone of starch hydrolis) and on YDC (yellow, glistening and mucoid 3 mm in diameter). The presence of Xcc colonies of 8 soil samples was obtained on YDC medium, while Xcc of 15 samples on SX medium was confirmed. Comparing the selected colonies from the same soil sample (total of 8) by rep-PCR, the obtained patterns showed similarities grater than 96%, suggesting the same isolate grown on different medium.

Characteristic Xcc colonies of 34 isolates - 15 from soil samples and 19 from seed were selected for further study.

Pathogenicity

For the obtained isolates, pathogenicity was confirmed by artificial inoculation of cabbage leaves and spraying a leaves of cabbage, cauliflower, kale, broccoli and oil seed rape.

(i) The method of infiltration bacterial suspension in the central nerve. In three days after inoculation (d.a.i), 28 from 34 isolates and Xcc strain NCPPB1144 caused discoloration and tissue collapse at the inoculation site on cabbage leaves. In 5 d.a.i., the necrosis spread from the inoculation site to petiole and blackening of leaf veins occurred, while on the seventh day the leaf tissue was completely dead. Similar pathogenicity testing results are reported by Radunović and Balaž (2012) using Xcc isolates derived from cabbage and collard. No pathological changes were observed on the plants inoculated with water until the end of the experiment. Based on pathogenic, morphological, biochemical - physiological and molecular characteristics, the reisolated bacteria (in total 28 reisolates) were identified as Xcc, fulfiling the Koch postulate.

(ii) Spraying method. The first symptoms on the leaves of inoculated plants (cabbage, cauliflower, kale, broccoli and oil seed rape) using the spraying method was observed on the fifth day after inoculation, in the form of tiny watery spots distributed along the edge of the leaf. On 7 d a i the spot began to spread of the interior, since 12 d a i, typical symptoms in the shape of "V" were formed on the edge of the leaves, and the appearance of a yellow halo was noticed between healthy and diseased tissue. The same symptoms development was reported by Fargier and Manceau (2007). Most of the tested isolates and Xcc strain NCPPB1144 caused the symptoms on the all tested host plants (cabbage, cauliflower, kale, broccoli and oil seed rape). Isolates from soil Z6, Z10 and Z13 did not cause symptoms on the leaves of the tested plants. Probably, the mentioned isolates reduced the activity of the T2SS system and the secretion of enzymes required for the degradation of the cell wall over time in the soil, which was reported earlier by Vicente and Holub (2013) and LENG et al. (2019). Same authors stated that when mechanical tissue damage occurs, the bacteria penetrate unhindered and cause diseases symptoms. No pathological changes were observed on the plants inoculated with water until the end of the experiment.

Based on results achieved in both inoculation experiments further testing was conducted with 28 isolates, 12 from soil and 16 from seeds (Table 3).

Biochemical characteristics

The all selected Xcc isolates were Gram-negative, catalase-positive, oxidase-negative and showed oxidative - fermentative metabolism of glucose. All isolates hydrolyzed starch, gelatine, aesculin and Tween 80. Nitrate reduction was negative, and medium with 0.1% and 0.02% TTC inhibited the growth of the tested isolates. Isolates produced indole and hydrogen sulphide and grew at 35°C. The same reaction was obtained for the type Xcc strain NCPPB1144.

All the isolates obtained from soil and seed samples had the same biochemical characteristics as reported for isolates from diseased cabbage, kale, broccoli (Obradović et al., 2000; Popović et al., 2013) and collard plants (Radunović and Balaž, 2012).

Molecular identification

For all tested isolates and the strain Xcc NCPPB 1144, fragments of 370 and 619 bp were amplified in m-PCR, confirming that they belong to Xcc (Figs. 1 and 2). Grimault

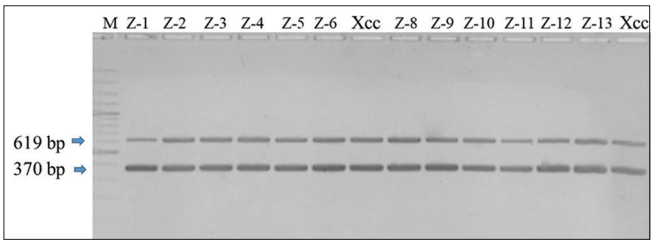

Fig 1: Identification of Xcc isolates from soil by m-PCR using primers DLH 120/125 (619 bp) and Zup 2309/2310 (370 bp). M – ladder (SERVA FastLoad 100bp DNA Ladder, ServaElectrophoresis GmbH, Heidelberg, Germany); Z1 – Z13 isolates, R – reference strain NCPPB 1144 Xcc

Table 3: Isolates with soil and seed origin

Origin	Isolate	Date of isolation	Host	Locality
Soil	Z-1	September 2th 2015	cabbage (cv*. Futoški)	Kovilj
	Z-2	September 17th 2015	cabbage (cv. Futoški)	
	Z-3	September 17th 2015	cabbage (cv. Futoški)	R. šančevi
	Z-4	September 17th 2015	cabbage (monoculture) (cv. Futoški)	Futog
	Z-5	October 5th 2015	cabbage (monoculture) (cv. Futoški)	
	Z-6	April 12th 2016	wheat (cv. NS40s)	
	Z-8	September 5th 2016	cabbage (cv. Futoški)	Despotovo
	Z-9	September 5th 2016	cauliflower (sv. unknown)	
	Z-10	September 5th 2016	cabbage (monoculture) (sv. Futoški)	
	Z-11	September 29th 2016	cabbage (cv. Futoški)	R. šančevi
	Z-12	September 20th 2017	cabbage (cv. Futoški)	Futog
	Z-13	October 2nd 2017	cabbage (cv. Futoški)	Gospođinci
Seed	S-1	June 8th 2015	cabbage (cv. Futoški)	Futog
	S-2	June 8th 2015	cabbage (cv. Futoški)	
	S-3	June 8th 2015	cabbage (cv. Futoški)	
	S-4	June 8th 2015	cabbage (cv. Futoški)	
	S-5	June 8th 2015	cabbage (cv. Futoški)	
	S-6	June 24th 2015	cabbage (cv. Futoški)	Despotovo
	S-7	June 29th 2015	cabbage (cv. Futoški)	Novi Sad
	S-8	July 14th 2015	cabbage (cv. Futoški)	
	S-9	April 05th 2016	cabbage (cv. Orion)	
	S-10	April 05th 2016	cauliflower (cv. Snežna grudva)	
	S-11	June 12th 2016	cabbage (cv. Srpski melez)	Futog
	S-12	June 12th 2016	cabbage (cv. Futoški)	
	S-13	June 12th 2016	cabbage (cv. Orion)	
	S-14	August 02nd 2016	oil rape (cv. Slavica)	Novi Sad
	S-15	August 02nd 2016	oil rape (cv. Slavica)	
	S-16	May 11th 2017	cabbage (cv. Futoški)	Despotovo

*cv - cultivar

et al. (2012) recommended to use primer combination DLH 120/125 and ZUP 2309/2310 for identification of Xcc on Brassica seeds. Peňázová et al. (2015) used the same primers during the evaluation of different methods for Xcc extraction and detection from cabbage leaves, and stated that primers DLH120/125 were reproducible to determine *X. campestris* isolates whereas primers ZUP2309/2310 were specific for the pathovar *campestris*.

Molecular characterization of Xcc isolates by rep-PCR

Patterns obtained by rep-PCR amplification using two BOX primers (GTG and BOX A1R) for Xcc isolated from soil are shown in Fig. 3 and 4, respectively. The results of joint analysis of the GTG and BOX A1R patterns for each isolates are shown on the dendrogram (Fig. 5). Rep-PCR analysis of 12 Xcc isolates showed their high similarity to the reference strain Xcc NCPPB 1144. This Xcc cluster had 46% difference from the second cluster that contained *X. perforans* NCPPB 4321 and *X. euvesicatoria* NCBI KX512833, while *X. vesicatoria* KFB 29 and *X. axonopodis* v. *phaseoli* KBNS 204 formed a third cluster.

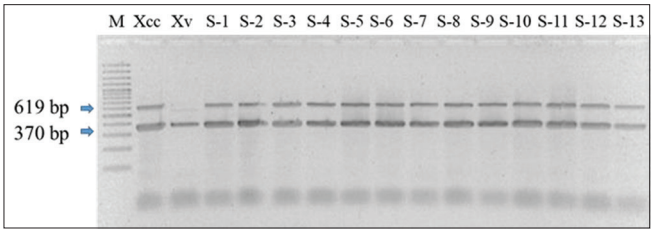

Fig 2: Identification of Xcc isolates from seeds by m-PCR using primers DLH 120/125 (619 bp) and Zup 2309/2310 (370 bp). M – ladder (SERVA FastLoad 100bp DNA Ladder, ServaElectrophoresis GmbH, Heidelberg, Germany); R – reference strain NCPPB 1144 Xcc; Xv - *X. vesicatoria* KFB 29; S1 – S13 isolates

Among isolates Z-1, Z-5, Z-6 and Z-13 maximal homology was observed, such as Z-2 with Z-3 and Z-11 with Z-12, while mutual similarities were 93%. Those soil isolates originated from Kovilj (2), Rimski šančevi and Gospodjinci and three from Futog locations formed one branch with the strain Xcc NCPPB 1144. Other soil isolates clustered in separate branch of this subcluster with the 79% similarity. Three isolates from soils under different host plants, cabbage and cauliflower, in Despotovo clustered together with 90% similarity and in the same branch as Z-4 (under cabbage) from Futog.

Xcc isolates originated from seeds were also subjected to PCR amplification using two rep-PCR primers and resulting patterns are shown in Figs. 6 and 7. Cluster analysis of two fingerprinting data of 16 Xcc isolates is shown on the dendrogram (Fig. 8).

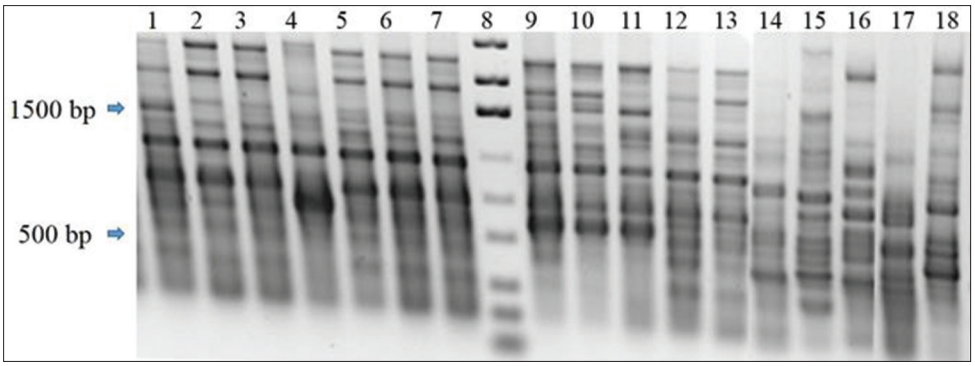

Fig 3: Rep-PCR of Xcc isolated from soil and some related strains obtained by (GTG)5 primer. Lines 1-7: Z1- Z6 and Z8; line 8: SM1293 (MassRuler Express DNA Ladder Mix Reverse SM1293, Thermo Scientific, Lithuania); lin. 9-13: Z9-Z13; lin. 14-18: *X. perforans* NCPPB 4321, *X. vesicatoria* KFB 29, *X. euvesicatoria* NCBI KX512833, *X. a. pv. phaseoli* KBNS 204, Xcc NCPPB 1144.

All isolates from seeds clustered with Xcc NCPPB 1144 in separate clusters, while reference strains *X. perforans* NCPPB 4321, *X. vesicatoria* KFB 29, *X. euvesicatoria* NCBI KX512833 and *X. axonopodis* pv. *phaseoli* KBNS 204 clustered in the second cluster differing about 65% from the first cluster. The first cluster contained 3 subclusters mutually 30% different; reference strain Xcc NCPPB 1144 was the only member of one of them. Five Xcc isolates

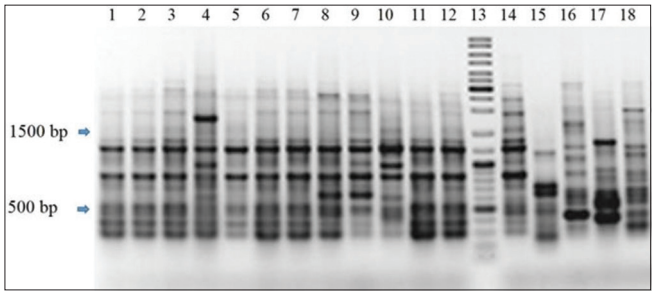

Fig 4: Rep-PCR of Xcc isolated from soil and some related strains obtained by BOX A1R primer. Lines 1-12: Z1-Z6 and Z8-Z13; line 13: SM0331 (GeneRuler DNA Ladder Mix SM0331, Thermo Scientific, Lithuania); lin. 14-18: Xcc NCPPB 1144, *X. a. pv. phaseoli* KBNS 204, *X. perforans* NCPPB 4321; *X. vesicatoria* KFB 29; *X. euvesicatoria* NCBI KX512833.

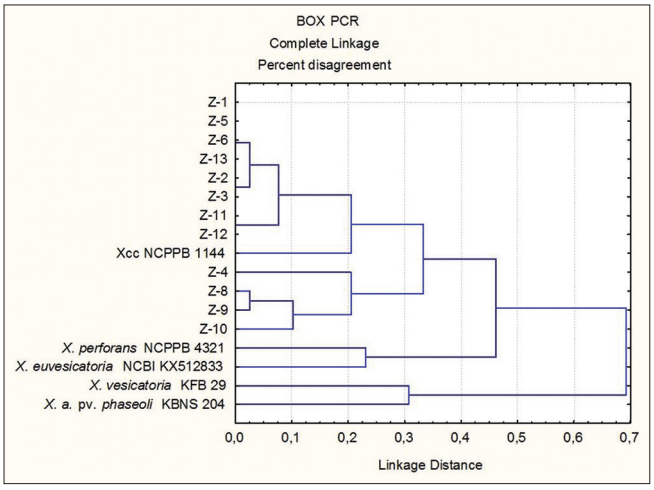

Fig 5: Dendrogram of genetic similarity of 12 Xcc isolates from soil and some related strains based on (GTG)5 and BOX A1R fingerprinting data.

from seeds formed one subcluster and showed up to 8% differences. Maximum similarity was shown in cabbage seed isolates from Futog collected in two years with cauliflower seed isolate from Novi Sad. The third subcluster divided into two branches (19% different) which involved 3 and 8 isolates, showing similarities from 81% to 100%. Three cabbage seed isolates from the same year in Futog showed similarity above 92% and 84% similarity with other cabbage seed isolates - two from Futog isolated in two successive years and three from Despotovo and Novi Sad in the same year. Maximum similarity was shown in oil rape seed isolate from Novi Sad (2016) and cabbage seed isolate from Despotovo (2017), which were in the same subcluster with another oil rape seed isolate from Novi Sad.

Comparing the patterns of isolates on the basis of rep-PCR using BOX A1R and $(GTG)_5$ primers, we obtained three representatives from soil on the similarity level of 90%: Z-1, Z-4 and Z-8, and four representative seeds isolates S-1, S-2, S-5 and S-14.

A worldwide comparison of Xcc population using rep-PCR confirmed that the technique was valuable for confirming pathogen diversity. Characterization by rep-PCR fingerprinting and the genetic diversity estimation of Xcc strains from field - cultivated crucifer plants has been carried out in Israel and other geographic locations. Using this method, 22 isolates divided into 3 different genotypes, showing high diversity into this pathovar (Valverde et al., 2007). Italian populations of 141 Xcc isolates from leaves was distributed in three main clusters using BOX-PCR, and no obvious relationships were observed between the strains comprised in each cluster and their geographic origin or host plant (Zaccardelli et al., 2008). Xcc population, originated from cabbage fields in five major cabbage-growing districts in Nepal, has been collected from 39 cabbage plants, 4 cauliflower plants, and 1 leaf mustard plant and characterized by the combined rep-PCR. Obtained profiles clearly separated the Nepalese Xcc strains from other *Xanthomonas* spp. and pathovars

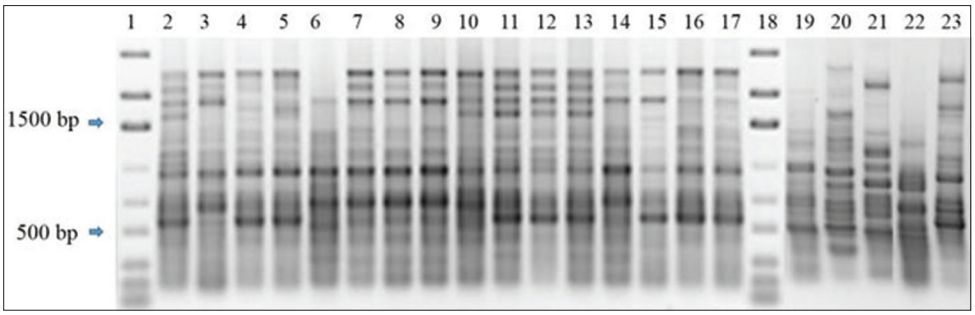

Fig 6: Rep-PCR of Xcc isolated from seeds and some related strains obtained by (GTG)5 primer. Line 1: SM1293 (MassRuler Express DNA Ladder Mix Reverse SM1293, Thermo Scientific, Lithuania); lin. 2-17: S1-S16; line 18: SM1293; lin. 19-23: X. *perforans* NCPPB 4321, X. *vesicatoria* KFB 29, *X. euvesicatoria* NCBI KX512833, X. *a. pv. phaseoli* KBNS 204, Xcc NCPPB 1144.

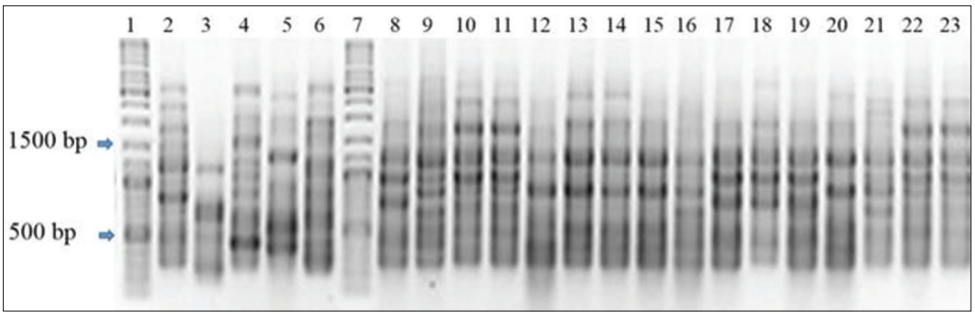

Fig 7: Rep-PCR of Xcc isolated from seeds and some related strains obtained by BOX A1R primer. Line 1: SM0331 (GeneRuler DNA Ladder Mix SM0331, Thermo Scientific, Lithuania); lin. 2-6: Xcc NCPPB 1144, *X. a. pv. phaseoli* KBNS 204, *X. perforans* NCPPB 4321, *X. vesicatoria* KFB 29, *X. euvesicatoria* NCBI KX512833; line 7: SM0331; lin. 8-23: S1-S16.

(Jensen et al., 2010). Seventy-five Xcc isolates recovered from infected fields in north-western Spain have been subjected to rep-PCR for differentiation among the nine existing Xcc races and the combined fingerprints clustered into four groups, without clear relationship to race, crop or geographical origin (Lema et al., 2012). On the basis of the fingerprint patterns generated by rep-PCR and RAPD, 40 Xcc strains isolated from cabbage, radish, cauliflower, turnip and kohlrabi grown on different geographic regions of northern Iran, were differentiated into seven clusters at 76% similarity level, but geographical origin of

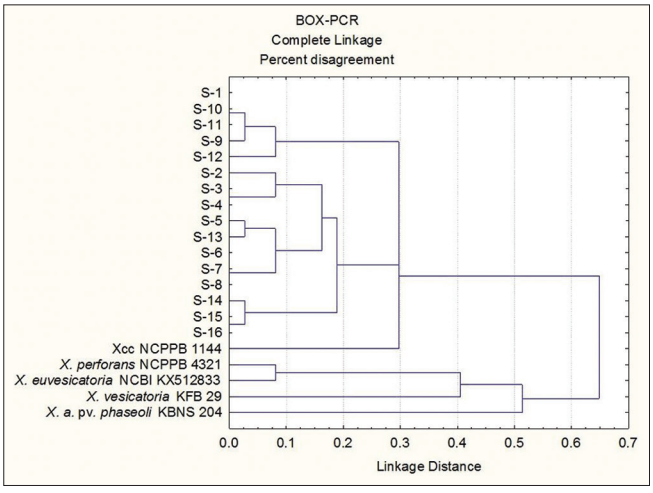

Fig 8: Dendrogram of genetic similarity of 16 Xcc isolates from seeds and some related strains based on (GTG)5 and BOX A1R fingerprinting data.

strains does not correlate with the RAPD and rep-PCR clusters (Rouhrazi and Khodakaramian, 2014). Genetic characterization of 75 Xcc strains from 12 agro-climatic regions of India was carried out using combined rep-PCR to determine the distribution pattern of races and diversity of the population. Some isolates obtained from the same source produced similar profiles, bur other strains showed different profiles despite belonging to the same race, as well as some isolates had similar profiles despite belonging to different races (Rathaur et al., 2015). The high diversity of 95 isolates *X. campestris* spp. infecting crucifers in New York state, and assessed by multiple methods, including rep-PCR using the BOX-A1R primer, was confirmed by 15 unique fingerprint patterns generated from isolates collected in the 5 years. No correlation between BOX patterns and their spatial distribution as found, and very little fingerprint overlap from year-to-year except for one farm where the same fingerprint was isolated over a 5 years (Lange et al., 2016). Popović et al., 2019 estimated the diversity of 147 Xcc isolates sampled from broccoli, cabbage, cauliflower, collard greens, kale, kohlrabi and the winter oilseed rape, collected from different regions in Serbia. The higher level of genetic diversity was found in winter oilseed rape isolates compared to isolates from the other hosts, where ERIC and REP-PCR showed better distinguishing potential than BOX A1R. In our investigation, Xcc isolates collected from different soils and from seeds clustered together with Xcc

NCPPB 1144, and separate to the reference strains from different pathovars - *X. a.* pv. *phaseoli* KBNS 204, *X. perforans* NCPPB 4321, *X. vesicatoria* KFB 29 and *X. euvesicatoria* NCBI KX512833. These results are consistent with earlier investigation of Xcc isolated from leaves of cabbage, kale and broccoli from Serbia (Popović et al., 2013) and results of Jensen et al. (2010) who also obtained *Xcc* strains in a separate cluster from other *Xanthomonas* spp. and pathovars from other hosts.

CONCLUSIONS

For isolation of Xcc from seed has been used YDC agar with the addition of cycloheximide, and for isolation of Xcc from soil, more samples were successfully isolated on SX agar than YDC. Based on pathogenicity and biochemical tests, as well as molecular identification by m-PCR (primers pairs DLH 120/125 and Zup 2309/2310) isolates were identified as Xcc.

Characterization of isolates by rep-PCR using BOX A1R and $(GTG)_5$ primers resulted in informative patterns and enabled comparison of isolates. On the pattern similarity level of 90%, three representative isolates from soil (Z-1, Z-4 and Z-8) and four representative seed isolates (S-1, S-2, S-5 and S-14) was chosen.

To the best of our knowledge, this is the first rep-PCR characterization and comparison of Xcc isolates from soil and seeds of cabbage, cauliflower and oil rape from different locations and years of isolation in Vojvodina.

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Authors' contributions

All the listed authors had participated in the manuscript. Vlajić, S., Iličić, R., Kozoderović, G., Ignjatov, M., Jošić, D.: performed analysis and wrote manuscript. Maširević, S., Gvozdanović - Varga, J. and Jošić, D.: research supervision. All authors revised the document critically and approved the final manuscript for submission to the Journal.

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