

Pectobacterium carotovorum subsp. *carotovorum* – the causal agent of broccoli soft rot in Serbia

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ABSTRACT

Soft rot symptoms were observed on broccoli plants in several commercial fields in the western part of Serbia. Six strains of bacteria were isolated from diseased tissues and identified as *Pectobacterium carotovorum* subsp. *carotovorum* using conventional bacteriological and molecular methods. All strains were non-fluorescent, gram-negative, facultative anaerobes, oxidase-negative and catalase-positive, causing soft rot on potato and carrot slices and did not induce hypersensitive reaction on tobacco leaves. They grew in 5% NaCl and at 37°C, did not produce acid from α -methyl glucoside, sorbitol and maltose, nor reducing substances from sucrose, but utilized lactose and trehalose, and did not produce indole or lecithinase. The investigated strains showed characteristic growth on Logan's medium and did not produce blue pigmented indigoidine on GYCA medium nor "fried egg" colonies on PDA. The identity of strains was confirmed by ITS-PCR and ITS-RFLP analyses and by sequence analysis of the 16S rRNA gene. In a pathogenicity assay, all strains caused tissue discoloration and soft rot development on inoculated broccoli head tissue fragments.

Keywords: Broccoli; *Pectobacterium carotovorum* subsp. *carotovorum*; Soft rot; 16S rRNA gene; ITS-PCR-RFLP

INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica* Plenck) is one of the most important herbaceous biennial crops of the *Brassicaceae* family that originates in the Mediterranean. High nutritive value and many health benefits make broccoli one of the most valuable vegetable crops worldwide. Broccoli has been grown in Europe for centuries. In Serbia, it is grown in smaller fields,

compared to other vegetables, but its production and consumption have increased significantly in recent years. However, the increase in broccoli production, both in fields and greenhouses, has contributed to frequent occurrence of various diseases of this crop.

Bacterial diseases frequently affect broccoli production, especially when environmental conditions are favorable for disease development. Soft rot caused by the pectolytic bacteria *Pectobacterium carotovorum* subsp. *carotovorum*

and *Pectobacterium carotovorum* subsp. *atrosepticum*, and by *Pseudomonas* spp. (*Pseudomonas marginalis* and *Pseudomonas fluorescens*) is a widespread and economically important disease of broccoli and other *Brassicaceae* (Canaday et al., 1991; Koike et al., 2007). Similarly, black rot of broccoli, caused by *Xanthomonas campestris* pv. *campestris*, occurs frequently and causes V-shaped lesions on leaf margins and blackened veins associated with systemic movement of the pathogen in plant (Williams, 1980).

During 2012, soft rot symptoms on broccoli plants were observed in Šabac and Bogatić localities, where the vegetable is grown intensively. After the first harvest, water-soaked areas were observed on broccoli stem tissue, followed by maceration, which progressed into soft rot decay of entire plants. Disease incidence was approximately 30%. The type of symptoms observed on broccoli tissue indicated a possible infection with phytopathogenic bacteria. As symptoms of soft rot can be caused by bacteria belonging to two genera, *Pectobacterium* and *Pseudomonas*, our research focused on studying the etiology of the disease and identifying the causal agent of broccoli soft rot in Serbia.

MATERIAL AND METHODS

Isolation of bacteria

Samples of broccoli plants showing symptoms of soft rot were collected after the first harvest of broccoli heads in September and October of 2012. Isolation of the pathogen was carried out using fragments cut out with sterile scalpel from border area between apparently healthy tissue and diseased dark brown tissue. The samples were homogenized in a few drops of sterile distilled water using pestle and mortar. After a few minutes, a loopful of resulting homogenate was streaked on nutrient agar medium (NA, Torlak, Belgrade) and incubated for 2-3 days at 27°C.

Pathogenicity test

Pathogenicity of the isolated strains was tested by inoculation of broccoli head tissue fragments. Inoculum was prepared from a 24 h old culture of tested strains grown on NA and suspended in sterile distilled water. Concentration was adjusted to approx. 10^8 CFU/ml ($OD_{600} = 0.3$). Three florets per strain were inoculated by pricking petals with a syringe and hypodermic needle and depositing a droplet of bacterial suspension at the point of inoculation. Sterile distilled water was used as a negative control, while *P. carotovorum* subsp. *carotovorum*, strain KFB 85, was used as a positive control. The inoculated

florets were placed in a sealed plastic container and incubated under high humidity conditions at 28°C. Tissue discoloration and development of soft rot around inoculation points were monitored over the next 48-72 h.

The strains were also tested for hypersensitivity on tobacco cv. White Barley (Klement et al. 1990). The suspension of bacteria (approx. 10^8 CFU/ml) was injected with a syringe and hypodermic needle into tobacco leaves. *Pseudomonas syringae* pv. *syringae*, strain KFB 0103, was used as a positive, while sterile distilled water was used as a negative control. Necrosis of the infiltrated area after 24 h was considered a positive reaction.

Physiological and biochemical characteristics

Strains were analyzed using standard bacteriological and differential physiological and biochemical tests. The following bacterial characteristics were studied: Gram reaction; fluorescence on King's medium B (KB); oxidase, catalase and lecithinase activity; oxidative/fermentative metabolism (O/F test); metabolism of α -methyl glycoside, sorbitol, maltose, lactose and trehalose; production of indole and reducing substances from sucrose; growth at 37°C and in 5% NaCl; growth and appearance of colonies on Logan's medium, Potato-Dextrose Agar (PDA) and Glucose-Yeast Extract-CaCO₃ Agar (GYCA) (Arsenijević, 1997; Cother & Sivasithamparam, 1983; Lelliott & Stead, 1987; Obradović, 1999; de Boer & Kelman, 2001).

Pectolitic activity of the strains was checked by inoculating potato tuber and carrot slices. Plant material was washed, then sprayed with 95% ethanol and allowed to dry at room temperature. Potato tuber and carrot slices were cut by sterile scalpel and inoculated by placing a 24 h old culture of each strain in the centre of each slice, using bacteriological loop. Inoculated plant material was incubated at room temperature for 24-48 h to allow soft rot symptoms to develop.

Molecular identification

In order to detect and differentiate *Pectobacterium* species, PCR assay using universal primers (L1/G1) developed for the 16S-23S intergenic transcribed spacer (ITS) region and restriction fragment length polymorphism (RFLP) analyses (Toth et al., 2001) was done. DNA templates were prepared by heating bacterial suspensions (approx. 10^8 CFU/ml) at 95°C for 10 min. The PCR reaction was performed in a total volume of 50 μ l reaction mixture containing 1 \times PCR Master Mix (Fermentas, Thermo Fisher Scientific, Waltham, MA), 0.4 μ M of each primer and 2 μ l of template DNA. Lysates were incubated on ice for 5 min and centrifuged for 5 min at 8000 rpm. The temperature profile was as follows: initial

denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min; and final extension at 72°C for 2 min. PCR reaction was conducted in a Thermo Cycler 2720 (Applied Biosystem, USA). PCR products were separated by 2% agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained in ethidium bromide (1 µg/ml) and visualized under UV light by a digital imaging camera (Vilber Lourmat, France).

PCR products were further digested with restriction endonucleases *RsaI* and *HhaI* (*CfoI*) (Fermentas, Thermo Fisher Scientific, Waltham, MA), as recommended by the manufacturer. The digests were resolved by electrophoresis in 2.2% agarose gel at 55 V for 2 h, followed by staining and visualization as previously described.

16S rDNA sequence analysis

PCR was performed using the primers fD1 and rP2, which have been designed for most eubacterial 16S ribosomal DNA and amplify a fragment of about 1500 bp in the 16S rRNA gene (Weisburg et al., 1991). The reaction mixture (50 µl) consisted of: 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂), 20 µM of each primer, 0.2 mM deoxynucleoside triphosphate (each), 2.5 U Taq DNA polymerase and 2 µl of template DNA prepared as described previously. PCR amplification reactions were performed in a Thermo Cycler 2720 (Applied Biosystem, USA) using the following conditions: initial denaturation at 94°C for 4 min; 35 cycles consisting of 94°C for 1 min, 58°C for 1 min and 72°C for 3 min, and final extension cycle at 72°C for 15 min before cooling at 4°C. Amplified PCR products were resolved by 1.5% agarose gel electrophoresis in TAE buffer, stained and visualized as previously described.

The PCR products of two strains were purified and directly sequenced in both directions (Macrogen Europe) using the same primers as for PCR amplification. The obtained sequences were edited and assembled using PREGAP 4 and GAP 4 of the Staden software package (Staden et al., 1998) and then compared with available sequences deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) using nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) for nucleotide (blastn) alignments.

RESULTS

Isolation of bacteria and pathogenicity tests

Bacteria were isolated from soft rotted broccoli tissue on nutrient agar, showing shiny, greyish white, round, 2 mm in diameter colonies after 48 h of incubation.

Although a large number of isolates were collected from two localities (Šabac and Bogatić), six strains were selected for further investigation based on their pectolytic activity.

Symptoms of tissue discoloration and soft rot of broccoli florets developed within 48 to 72 h after inoculation (Figure 1). No symptoms developed on control florets. Soft rot appeared on the inoculated carrot and potato tuber slices 24 h after inoculation, indicating a strong pectolytic activity of the studied strains. The strains did not induce hypersensitive reaction on tobacco leaves.



Figure 1. Pathogenicity assay. Tissue discoloration and soft rot of broccoli floret inoculated with one of the studied strains (above), and no symptoms developed on negative control (below) 48 h after inoculation.

Physiological and biochemical characteristics

All investigated strains were non-fluorescent, gram-negative, facultative anaerobes, oxidase and lecithinase negative, and catalase positive. They grew at 37°C and in yeast salts broth medium containing 5% NaCl, did not produce acid from α -methyl glucoside, sorbitol and maltose, nor reducing substances from sucrose, but utilized lactose and trehalose, and did not produce indole (Table 1). The investigated strains formed light red, 1.5-mm-diameter colonies on Logan's medium, and did not produce blue pigmented indigoidine on GYCA medium nor "fried egg" colonies on PDA. These characteristics were typical for the bacterium *P. carotovorum* subsp. *carotovorum*.

Molecular identification

PCR amplification of the 16S-23S rRNA intergenic transcribed spacer region (ITS) using L1 and G1 primers resulted in two bands of 535 and 580 bp separated by electrophoresis in 2% agarose gel (data not shown). An analysis of ITS-PCR products confirmed that the strains isolated in this study belong to *Pectobacterium* spp. ITS-PCR products were digested using *RsaI* and *HhaI* restriction enzymes, and profiles characteristic for *P. carotovorum* subsp. *carotovorum* were obtained (Figure 2).

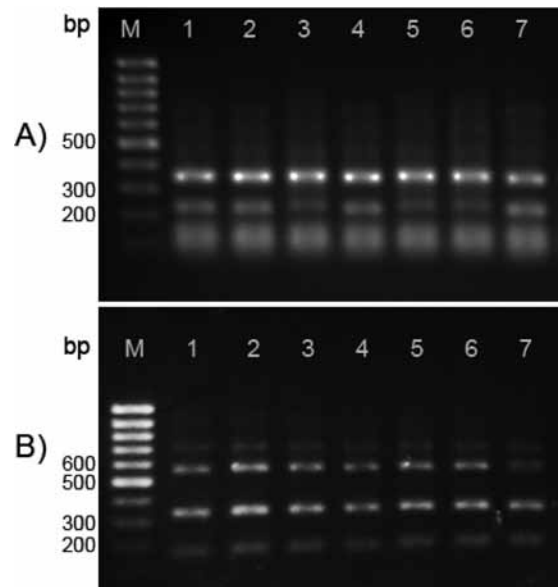


Figure 2. ITS-PCR-RFLP profiles of the studied strains acquired using *HhaI* (A) and *RsaI* (B) restriction enzymes. Lane M, marker (MassRuler Low Range DNA Ladder, Fermentas, Thermo Fisher Scientific, Waltham, MA); Lanes 1-6 bacterial strains isolated in this study; Lane 7 - KFB 85 (*P. c. ssp. carotovorum* control strain).

Table 1. Biochemical and physiological characteristics of the studied strains compared to other *Pectobacterium* species of the soft rot group and *Dickeya* spp.

Test	Studied strains	<i>P. c. ssp. carotovorum</i> KFB 85	<i>P. c. ssp. atrosepticum</i> (Lit.)	<i>Dickeya</i> spp. (Lit.)
Gram reaction	-	-	-	-
Fluorescence on King's medium B	-	-	-	-
Oxidase activity	-	-	-	-
Catalase activity	+	+	+	+
Lecithinase activity	-	-	-	+
Glucose metabolism	OF	OF	OF	OF
Acid production from:				
α -methyl glucoside	-	-	+	-
lactose	+	+	+	+
maltose	-	-	+	-
sorbitol	-	-	-	-
trehalose	+	+	+	-
Growth at 37°C	+	+	-	+
Growth in 5% NaCl	+	+	+	-
Indol production	-	-	-	+
Reducing substances from sucrose	-	-	+	+
Hypersensitivity on tobacco	-	+/-	+	+
Pathogenicity assay				
Broccoli	+	+		
Potato	+	+		
Carrot	+	+		

+ positive reaction; - negative reaction; +/- weak reaction; OF - oxidative-fermentative metabolism of glucose; Lit. - literature data (Arsenijević, 1997; de Boer & Kelman, 2001).

Two strains, KBI 1 and KBI 2, were further characterized by partial sequencing of their 16S rRNA gene. The sequences (approx. 1350-bp) were aligned and analyzed using the Staden software package. The sequences of KBI 1 (GenBank acc. no. KC527051) and KBI 2 (GenBank acc. no. KC527052) showed 100% identity with *P. carotovorum* subsp. *carotovorum* sequences previously deposited in GenBank (Nabhan et al., 2012).

DISCUSSION

Broccoli production and consumption have increased significantly in recent years, which has also contributed to frequent occurrence of various diseases of that crop. Bacterial soft rot is a destructive disease of broccoli causing significant crop losses (Canaday et al., 1991). The disease affects both floret and stem tissue. It appears first as a water-soaked lesion followed by maceration and soft rotting of tissue (Ludy et al., 1997). Campbell et al. (1995) reported that soft rot of broccoli, caused by two genera of bacteria, *Pectobacterium* and *Pseudomonas*, had led to crop losses of between 30% and 100%, costing the UK industry £9.5 million annually (as cited in Darling et al. 2000).

P. c. ssp. carotovorum is one of the major soft rotting causal agents of vegetable and ornamental plants worldwide (Bradbury, 1986; Arsenijević, 1997). In Serbia, this bacterium has already been confirmed as a pathogen of potato, pepper, carrot, celery, parsley, eggplant, lettuce, cabbage, cauliflower and fennel (Arsenijević et al., 1996; Obradović, 1996; Arsenijević et al., 1997; Jovanović & Arsenijević, 1998; Jovanović, 1998; Obradović, 1999; Gavrilović et al., 2001; Gavrilović et al., 2009). Ivanović et al. (2009) reported *P. c. ssp. carotovorum* as an economically important pathogen of calla lily, causing significant losses in production of this ornamental plant. Based on earlier research in Serbia, cultivated *Brassica* spp. have proved to be very susceptible to that bacterium, confirming it as the causal agent of soft rot of seed cabbage plants (Arsenijević & Obradović, 1996). Warm and wet conditions favour the infection, although disease may develop over a wide range of temperatures. The bacterium enters plant tissue primarily through wounds, often created by insect feeding or during harvest. Severe damage should be expected after hailstorms since the bacteria can easily infect plants through wounds caused by hailstones (Arsenijević, 1997). Insects and irrigation water are also effective modes of bacterial spreading (Bhat et al., 2010).

The bacterial strains isolated from diseased broccoli tissue were identified as *P. c. subsp. carotovorum* based on their biochemical and physiological characteristics and confirmed by species and subspecies specific PCR and RFLP analyses of the 16S–23S ITS region, and 16S rRNA sequence analysis. Although *P. c. subsp. carotovorum* can be easily isolated on different nutrient media, nutrient agar has proved very suitable because characteristic colonies appear after 24–48 h of incubation. Other authors have also reported advantages of using this medium for *P. c. ssp. carotovorum* isolation (Arsenijević, 1997; Jovanović, 1998; Obradović, 1999). Using different biochemical tests, *P. c. ssp. carotovorum* can be differentiated from other *Pectobacterium* spp. (Arsenijević, 1997; de Boer & Kelman, 2001; Ivanović et al., 2009). Unlike *P. c. ssp. atrosepticum* or *Dickeya* spp. (syn. *Pectobacterium chrysanthemi*), the investigated strains grew in 5% NaCl and at 37°C, did not produce acid from α -methyl glucoside, sorbitol and maltose, nor reducing substances from sucrose, but utilized lactose and trehalose, and did not produce indole or lecithinase. In addition, the isolated strains showed characteristic growth on Logan's medium, and did not produce blue pigmented indigoidine on GYCA medium or "fried egg" colonies on PDA. The bacterium *Pseudomonas marginalis* has also been reported as the causal agent of soft rot of broccoli (Wimalajeewa et al., 1987). The bacterium has a wide host range too, but unlike the *P. c. ssp. carotovorum*, it produces levan and oxidase, fluorescent pigment on KB medium and utilizes glucose only in aerobic conditions (oxidatively) (Bradbury, 1986; Arsenijević, 1997).

All strains in our study showed pectolytic activity on potato tuber and carrot slices but failed to induce hypersensitive reaction of tobacco. Previously, *P. c. ssp. carotovorum* strains had been shown to vary in their ability to cause HR in tobacco and the presence of type III secretion system (Yap et al., 2004; Kim et al., 2009; Baghaee-Ravari et al., 2011; Nabhan et al., 2012).

In addition to studying biochemical and physiological characteristics of the isolated strains, their identity was also confirmed by PCR and RFLP analyses of the ITS region, as well as an analysis of 16S rRNA sequences. ITS-PCR followed by ITS-RFLP were reliable methods for classifying the strains of *Pectobacterium* spp. The obtained RFLP profiles of the studied strains were in compliance with results previously reported (Toth et al., 2001; Golkhandan et al., 2013).

As the production of broccoli is currently increasing in Serbia, more attention should be paid to the presence of *P. c. ssp. carotovorum*, which could cause significant

production losses. As the bacterium can successfully survive in plant debris and soil, infected field residues make an important source of inoculum facilitating bacterial dispersal. Therefore, one of the measures for suppressing and controlling the bacterium could be crop rotation with soft rot-resistant plants, primarily legumes. Chemical control has limited efficiency, and priority should be given to cultural practices.

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Pectobacterium carotovorum subsp. *carotovorum* prouzročivač vlažne truleži brokolija u Srbiji

REZIME

Simptomi vlažne truleži brokolija uočeni su u nekoliko komercijalnih zasada u zapadnoj Srbiji. Sojevi bakterija izolovani su iz obolelog biljnog tkiva i identifikovani kao *Pectobacterium carotovorum* subsp. *carotovorum* korišćenjem konvencionalnih bakterioloških i novijih molekularnih metoda. Svi proučavani sojevi bili su nefluorescentni, Gram-negativni, fakultativno-anaerobni, oksidaza i lecitinaza negativni, katalaza pozitivni, prouzrokujući vlažnu trulež cvetnih drški brokolija, kriški krompira i kriški mrkve, ali nisu indukovali hipersenzitivnu reakciju duvana. Svi sojevi razvijaju se pri 37°C i u prisustvu 5% NaCl, ne proizvode kiselinu iz α -metil glukozida, sorbitola i maloze, niti redukujuće supstance iz saharoze, ali razlažu laktozu i trehalozu, i ne stvaraju indol. Proučavani sojevi ispoljili su karakterističan razvoj na Loganovoj diferencijalnoj podlozi, i nisu proizvodili plavi pigment na GYCA podlozi niti "fried egg" kolonije na PDA podlozi. Identifikacija sojeva potvrđena je korišćenjem ITS-PCR i ITS-RFLP, kao i analizom sekvenci 16S rRNA gena. Prilikom izvođenja testa patogenosti, svi sojevi prouzrokovali su obezbojavanje tkiva i vlažnu trulež cvetnih drški brokolija.

Ključne reči: Brokoli; *Pectobacterium carotovorum* subsp. *carotovorum*; vlažna trulež; 16S rRNA gen; ITS-PCR-RFLP