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Scientific paper

Ralstonia solanacearum – a New Threat to Potato Production in Serbia

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SUMMARY

A survey of ware potatoes (a total of 1127 samples) from localities in Serbia during two consecutive years resulted in detection and identification of *R. solanacearum* in 17 tuber samples. The monitoring detected the causal agent of bacterial wilt and brown rot of potato in three districts of Vojvodina province. In 2011, the infection by *R. solanacearum* was confirmed in 7 samples of ware potato tubers (varieties – Saturna, Pirol, Hermes, Panda) in West Bačka and South Bačka Districts. In 2012, the infection by *R. solanacearum* was confirmed in 10 potato tuber samples (Lady Claire, Desiree, Panda, Red Fantasy and Vineta varieties) from two districts: South Bačka and Central Banat. Bacterial strains obtained from positive samples were identified as *R. solanacearum* biovar 2 using PCR/RFLP analysis, pathogenicity test on tomato transplants, and nutritional, enzymatic and biovar determination tests. To our best knowledge, these are the only findings of *R. solanacearum* infection in ware potatoes in Serbia. *R. solanacearum* was not detected in tomato or any other host plant tested in this study. Furthermore, the bacterium was not found in any of the water samples tested, including those originating from areas in which the bacterium was found in ware potato samples.

Keywords: Bacterial wilt; Brown rot; Potato; Identification; PCR

INTRODUCTION

Ralstonia solanacearum (Smith) Yabuuchi et al. (1995) is the causal agent of bacterial wilt, infecting over 450 plant species from 44 families, including many crops that are important economically. This complex bacterial species has been subdivided into five host-specific races and five biovars based on their biochemical properties (Hayward, 1991). Due to its wide range of hosts, the pathogen is present worldwide, mainly in warmer and more humid regions. Race 3 (equivalent to biovar 2) is adapted to temperate climates because of its lower temperature optimum compared to the other races. It has a narrow host range and exhibits high virulence on potato, tomato and other solanaceous crops. Moreover, recent outbreaks of this race in Europe have been also described on *Pelargonium* spp. (Hudelson et al., 2002; Janse et al., 2004). Being a highly heterogeneous and complex pathogen, *R. solanacearum* is one of the major constraints in the production of many important vegetables, among which potato is a major concern for

the European region. Moreover, race 3 appears to have been responsible for outbreaks of brown rot of potato in several European countries (Belgium, France, Germany, Italy, The Netherlands, Portugal, Spain, Turkey, UK) in the past two decades (Grousset et al., 1998; Hayward et al., 1998; Stefani et al., 2005). The devastating nature of this soil-borne pathogen and a lack of effective control measures are the main reasons for its quarantine status both in the EPPO region and in Serbia (EU, 1998; Pravilnik, 2009, 2010). It is listed as an EPPO A2 quarantine organism and the bacterium is under eradication wherever it occurs in the EU or other EPPO countries. The occurrence of different races and strains of the pathogen with varying virulence under different environmental conditions presents a serious threat to European and Mediterranean potato and tomato production. In addition, to prevent the introduction of *R. solanacearum* to new territories and to limit its spread, the European Union Council issued Directive 98/57 for mandatory control (EU, 1998) aiming to eradicate the organism. Latent infections in seed potato tubers have resulted in the spreading of this organism both locally and internationally, and effective control of brown rot is dependent on reliability of pathogen detection at the latent stage (Ciampi et al., 1980). Therefore, all EPPO members have undertaken the obligation to conduct monitoring of potato and tomato crops, including ware potato tubers, tomatoes and other host plants and water to enable early detection of the pathogen (Weller et al., 2000).

Potato is grown on about 100,000 ha in Serbia, mainly on private farms, with an average yield of about 10-15 t/ha (Rekanović et al., 2012). There are several potato pathogens affecting the production of this important crop. Unlike fungal diseases that could be avoided by fungicide treatment, bacterial pathogens are much more difficult to control. In Serbia, *R. solanacearum* is on the A1 list of quarantine pests (Pravilnik, 2010). It was recorded for the first time at a single site near Gornji Milanovac, Moravica District (www.minpolj.gov.rs) in 2010. Although eradication measures were applied, a survey was conducted to estimate the current status of this pathogen in Serbia and monitor latent infections of *R. solanacearum* in ware potato tubers, tomatoes, solanaceous weed plants and water samples according to the Program of Measures for Plant Health Protection 2011-2012 issued by the Serbian Ministry of Agriculture, Forestry and Water Management. The results of that survey are presented in this paper.

MATERIAL AND METHODS

The survey was conducted in all districts of Northern Serbia (Vojvodina Province), and in Eastern Serbia (Braničevo, Bor and Zaječar Districts) and Central Serbia (Podunavlje and Pomoravlje Districts). Potato tuber samples were collected from those districts through the assistance of the Agricultural Extension Service. The samples were taken either from fields during harvest or from warehouses after harvest. Additionally, the Inspection Service collected warehouse samples suspected of infection with *R. solanacearum*. The number of tested tuber samples was 703 in 2011 and 424 in 2012, each sample consisting of 200 tubers. A total of 86 tomato and weed host plant samples from the same districts were also tested from July to September of both years. Water samples were collected from watercourses and surface water (rivers, canals, wells, lakes) used for irrigation of potato fields from July to September. During 2011, 45 water samples were tested, while in 2012 the number of water samples was doubled (89) due to an increased number of samples taken from the crop fields in which the bacterium had been found in potato tubers the previous year.

Detection of *R. solanacearum* in asymptomatic potato tubers

Potato tuber testing was conducted on extracts from vascular tissue, using selective isolation and PCR analysis as primary screening tests (EU, 1998; Pravilnik, 2009).

Selective isolation

Selective isolation was carried out on validated semiselective medium - SMSA modified by Elphinstone et al. (1996), using the dilution plating technique. Fifty microliters of concentrated sample extract was used per plate, each dilution in three replicates. Plates were incubated at 28°C and examined after 4-7 days. Presumptive colonies were purified by subculturing on Sucrose Peptone Agar (SPA) (Lelliott and Stead, 1987). Single cell colonies were transferred on to King's B medium (KBM) slants and stored at 4°C for further studies. The isolated strains were compared with the standard reference strain of *R. solanacearum* biovar 2 (race 3) NCPPB 4156 (equivalent strain designations = CFBP 3857, PD 2762).

DNA extraction, polymerase chain reaction test

Total DNA was extracted from tuber vascular tissue (200 tubers per sample) and purified using DNeasy Plant Kit (Qiagen, Germany) according to the manufacturer's

instructions. PCR reactions were performed in an Eppendorf Master Cycler. The validated PCR protocol of Seal et al. (1993) with oligonucleotide primers OLY-1/Y-2 was used. The reactions contained: 1 X PCR Master mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM each dNTPs), 1 µl of each primer (20 µM) and 2 µl of template DNA in 25 µl volume. Reaction mix without template DNA was used as negative control and the reference strain of *R. solanacearum* NCPPB 4156 was used as positive control. Amplification products were visualized in 1% agarose gel stained with ethidium bromide and observed in UV transilluminator.

Identification of *R. solanacearum* strains

To confirm the identity of strains, presumptive colonies isolated on SMSA medium were identified by PCR/RFLP analysis, pathogenicity test on tomato transplants, nutritional and enzymatic tests and biovar determination.

PCR/RFLP analysis

PCR tests were conducted for each strain and the reference strain according to PCR protocols described by Seal et al. (1993) and Pastrik and Maiss (2000) with the oligonucleotide primer pair Ps-1/Ps-2. To prepare template DNA, cultures were grown on nutrient agar (NA) for 24 hours. For each strain, a single colony was suspended in 100 µl of sterile distilled water in a microvial. Closed vials were heated at 100°C for four minutes. Microvials with heated bacterial suspensions were transferred into ice and, after cooling, pulse-centrifuged. PCR reactions were performed in the Eppendorf Master Cycler. For both PCR protocols, PCR reaction content was as explained above, with the exception of template DNA volume 1µl in 25 µl reaction. Amplified products were visualized as decribed above.

The obtained Ps-1/Ps-2 PCR amplicons were subjected to RFLP analysis with *Taq*I restriction enzyme (Fermentas, Lithuania) according to the manufacturer's instruction. RFLP products were separated and visualized in 1% agarose gel or 8% polyacrylamide gel, stained and visualized as described above.

Pathogenicity test

Pathogenicity of the isolated strains was tested using a syringe inoculation test on tomato seedlings (EU, 1998; Pravilnik 2009). An inoculum of approximately 10^6 colony forming units (CFU) was prepared from 24-48 h old cultures of the isolated strains and the reference strain NCPPB 4156 in sterile distilled water. Tomato seedlings, cv. Saint Pierre, were grown in the sterile plant growing substrate "B medium course" (Floragard, Germany). Four seedlings at the third true leaf stage were inoculated per strain, including the reference one, by injection into the stem at the cotyledons. The plants injected with sterile water served as negative control. Treated plants were covered with plastic bags for 48 hours, and incubated at 26°C and >70% relative humidity for two weeks and observed daily for wilting, chlorosis, stunting and epinasty. The bacterium was reisolated by dilution plating on SMSA from wilting plants by removing a 1 cm stem section from 2 cm above the inoculation point. Suspected colonies were subcultured and identified as described above (EU, 1998; Pravilnik, 2009).

Nutritional and enzymatic tests

The following set of nutritional and enzymatic tests was conducted: fluorescent pigment production, Gram reaction, metabolism of glucose, catalase activity, Kovac's oxidase test, growth at 40°C, growth in 1% and 2% NaCl, arginine dihydrolase activity, levan formation, aesculin hydrolysis, starch hydrolysis and gelatin hydrolysis (Lellott and Stead, 1987; Schaad et al., 2001).

Biovar determination

In order to determine the biovar of the isolated and confirmed *R. solanacearum* strains, we tested their ability to utilize/oxidaze the following disaccharides and alcohols: maltose, lactose, D(+) cellobiose, mannitol, sorbitol and dulcitol (Hayward, 1964; Hayward et al., 1990).

Detection of *R. solanacearum* in tomato and weed samples

Samples of tomato plants were prepared by cutting 1 cm sections just above the main stem, and macerating them in extraction buffer (50 mM phosphate buffer, pH 7.0) (EU, 1998) using extraction bags. For other hosts, 1 cm fragments from the base of each stem just above the soil level, or 1-2 cm sections from underwater stems in the case of *Solanum dulcamara,* were used and prepared in the same way. A total of 86 samples were tested using selective isolation as described above for potato extracts (EU, 1998; Pravilnik, 2009).

Detection of *R. solanacearum* in water samples

During 2011, 45 water samples, from watercourses and surface water used for irrigation of potato production fields, were tested. In the 2012 survey, 89 water samples were tested, of which 30 samples originated from South Bačka (27) and Central Banat Districts: (3). Prior to isolation, water samples were concentrated by centrifuging 30 ml sub-samples at 10,000 g for 10 minutes at 4°C, discarding the supernatant and resuspending the pellet in 1 ml pellet buffer (50 mM phosphate buffer, pH 7.0) (EU, 1998). Concentration was not conducted for water samples from potato processing and sewage effluents. Samples were tested using selective isolation as described above (EU, 1998; Pravilnik, 2009).

RESULTS

In 2011, *R. solanacearum* was detected and identified in seven samples of potato tuber extracts (out of 703 tested) from one producer and confirmed in seven more samples additionally sampled from the same potato lots by the Inspection Service, all in Bačka region (Table 1). In 2012, *R. solanacearum* was found in ten potato samples (out of 424 tested) from three producers and also confirmed after repeated sampling by the Inspection Service from the same lots originating from two districts: South Bačka and Central Banat. Data on the origin of the investigated *R. solanacearum* strains are given in Table 1.

Detection of *R. solanacearum* in asymptomatic potato tubers

Selective isolation

Typical milky white, flat, irregular, fluidal colonies with pink to dark red centre appeared after 3-4 days of growth on semiselective SMSA medium in 17 positive samples (Figure 1). Presumptive colonies were subcultured on SPA and used for identification tests. Two strains per sample were chosen for further tests.

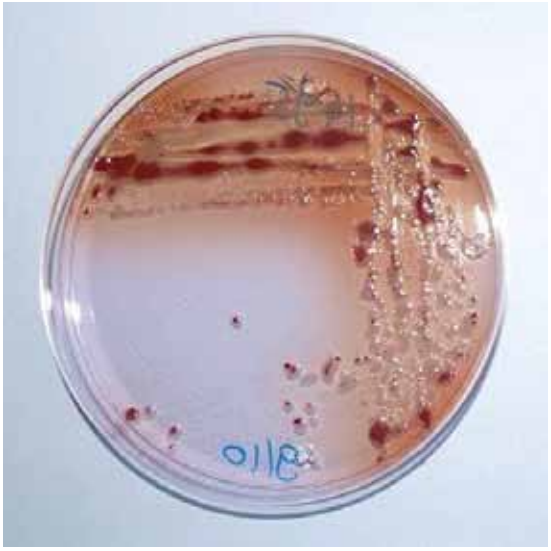

Figure 1. Colony morphology on semiselective SMSA medium after 3-4 days growth.

Table 1. Origin of investigated *R. solanacearum* strains

District	Cultivar	Number of positive samples	Strain designation
West Bačka*	Hermes	2	Rs 32/11, Rs 49/11
West Bačka*	Pirol	1	Rs 31/11,
South Bačka*	Hermes	1	Rs 10/11
South Bačka*	Saturna	1	Rs13/11
South Bačka*	Panda	2	Rs 14/11, Rs 15/11
Central Banat**	Red Fantasy	6	Rs 94/12, Rs 95/12 Rs 96/12, Rs 97/12, Rs 98/12, Rs 99/12
Central Banat**	Vineta	1	Rs 103/12
South Bačka**	Lady Claire	1	Rs 69/12
South Bačka**	Desiree	1	Rs 57/12
South Bačka**	Panda	1	Rs 77/12

*Sites inspected in 2011
**Sites inspected in 2012

PCR

According to the PCR protocol of Seal et al. (1993) with OLY1/Y2 primer pair, PCR products of expected size (288 bp) were amplified in 17 samples after DNA extraction (data not shown).

Identification and biovar determination

PCR/RFLP analysis

The strains were identified based on amplification of a 288 bp product with the specific primers OLY1/Y2 (Seal et al., 1993) (Figure 2) and 553 bp product with specific primers Ps-1/Ps-2 (Pastrik and Maiss, 2000) (Figure 3).

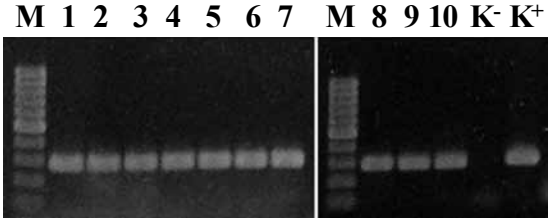

Figure 2. Agarose gel 1% showing products of selected *R. solanacearum* strains amplified with OLI1/Y2 primer pair. Samples:1-Rs 94/12, 2-Rs 95/12, 3-Rs 96/12, 4-Rs 97/12, 5-Rs 98/12, 6-Rs 99/12,7-103/12, 8-69/12, 9-57/12, 10-77/12, K^--negative control, K^+-positive control (strain NCPPB 4156). M-100 bp DNA marker (Fermentas, Lithuania); fragment sizes in base pairs from top to bottom: 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100.

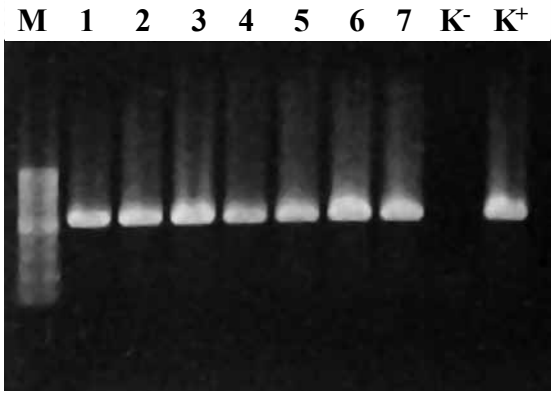

Figure 3. Agarose gel 1% showing products of selected *R. solanacearum* strains amplified with PS1/PS2 primer pair. Samples:1-Rs 32/11, 2-Rs 49/11, 3-Rs 31/11, 4-Rs 10/11, 5-Rs 13/11, 6-Rs 14/11, 7-Rs 15/11, K^--negative control, K^+-positive control (strain NCPPB 4156). M-100 bp DNA marker (Fermentas, Lithuania); fragment sizes in base pairs from top to bottom: 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100.

Restriction analysis of the Ps-1/Ps-2 PCR amplicons using *Taq*I restriction enzyme yielded profiles (two fragments around 450 bp and 100 bp) visually identical with all strains from Serbia and the reference strain NCPPB 4156 (Figure 4, 5).

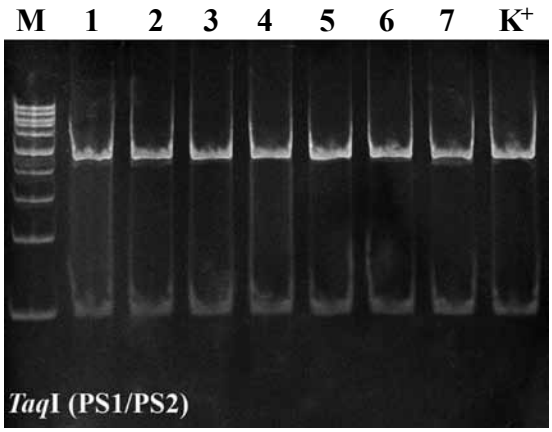

Figure 4. Polyacrylamide gel 8% showing the *Taq*I restriction fragment length polymorphism patterns on PS1/PS2 amplified fragments of selected *R. solanacearum* strains: 1-Rs 32/11, 2-Rs 49/11, 3-Rs 31/11, 4-Rs 10/11, 5-Rs 13/11, 6-Rs 14/11, 7-Rs 15/11, K^+-positive control (strain NCPPB 4156). M-100 bp DNA marker (Fermentas, Lithuania); fragment sizes in base pairs from top to bottom: 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100.

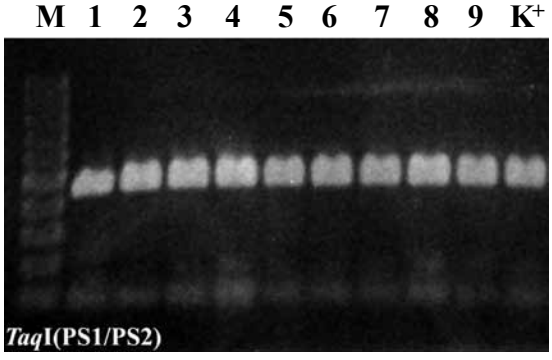

Figure 5. Agarose gel 1% showing the *Taq*I restriction fragment length polymorphism patterns on PS1/PS2 amplified fragments of selected *R. solanacearum* strains:1-Rs 94/12, 2-Rs 95/12, 3-Rs 96/12, 4-Rs 97/12, 5-Rs 98/12, 6-Rs 99/12, 7-103/12, 8-69/12, 9-57/12, K^+-positive control (strain NCPPB 4156). M-100 bp DNA marker (Fermentas, Lithuania); fragment sizes in base pairs from top to bottom: 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100.

Pathogenicity test

Tomato plants inoculated with the investigated and reference strains (Figure 6) showed wilting 4-7 days after inoculation. Plants injected with sterile distilled water remained healthy. Bacteria were isolated from the diseased plants and identified.

Nutritional and enzymatic identification tests

The isolated strains conformed closely to the set of nutritional and enzymatic features that are characteristic of *R. solanacearum*. The results of these tests are shown in Table 2.

Figure 6. Wilting of tomato transplants 4 days after inoculation with *R. solanacearum* strains and negative control (right bottom corner)

Table 2. Nutritional and enzymatic tests

Tests	Serbian strains (17)	NCPPB 4156
FP	-	-
O/F test	O+/F-	O+/F-
Oxidase	+	+
Catalase	+	+
Growth at 40°C	-	-
Growth in 1% NaCl	+	+
Growth in 2% NaCl	-	-
Arginine dihydrolase activity	-	-
Gelatin hydrolysis	-	-
Starch hydrolysis	-	-
Aesculin hydrolysis	-	-
Levan production	-	-
Gram reaction	-	-

Legend: FP – fluorescent pigment production; O/F test – oxidative metabolism of glucose;
+ – positive result, - – negative result

Biovar determination

All strains utilized maltose, lactose, and D (+) cellobiose, but not mannitol, sorbitol and dulcitol. According to these results, the investigated strains were classified as biovar 2 of *R. solanacearum* (Table 3).

Table 3. Biovar determination tests

Tests Utilisation of	**Serbian strains (17)**	**NCPPB 4156**
Maltose	+	+
Lactose	+	+
D (+) Cellobiose	+	+
Mannitol	-	-
Sorbitol	-	-
Dulcitol	-	-

Detection of *R. solanacearum* in tomato and weed samples

A total of 47 tomato and 39 other host plants were tested in 2011 and 2012, respectively, and there were no positive records of *R. solanacearum* presence.

Detection of *R. solanacearum* in water samples

R. solanacearum was not detected in any of the tested water samples regardless of origin.

DISCUSSION

Our survey of ware potatoes (a total of 1127 samples) from localities in Serbia during two consecutive years resulted in detection and identification of *R. solanacearum* in 17 tuber samples. For confirming infection with the bacterium, sampling and detection procedures were repeated for positive samples. The results of monitoring showed that all positive samples originated from Vojvodina province (West Bačka, South Bačka and Central Banat Districts). To our best knowledge, these are the only findings of *R. solanacearum* infection in ware potatoes in Serbia. In 2011, *R. solanacearum* was confirmed in 7 samples of ware potato tubers (Saturna, Pirol, Hermes and Panda varieties) in West Bačka and South Bačka Districts. In 2012, *R. solanacearum* was confirmed in 10 potato tuber samples (Lady Claire, Desiree, Panda, Red Fantasy and Vineta varieties) originating from two districts: South Bačka and Central Banat.

The bacterium was isolated from symptomless potato tubers on SMSA medium where it produced irregular-shaped, white colonies with pink to red centers, as described for *R. solanacearum* (EU, 1998). In contrast to a report by Trigalet et al. (1998) that some French strains from different hosts had been less mucoid and showed variation in colony morphology, all Serbian strains were mucoid, indicating abundant exopolysaccharide production. In addition, no variation in colony morphology of different strains was observed on SMSA and SPA media. We also observed that all Serbian strains changed the color of SMSA medium into dark brown after 2-3 days of incubation.

Pathogenicity tests revealed that the isolated bacteria induced wilt symptoms on inoculated tomato transplants 4-7 days after inoculation, followed by rapid plant death. A study of aggressiveness of French strains of *R. solanacearum* had shown that there was a great variability in pathogenicity among them, but no correlation could be found between aggressiveness and host or place of origin (Trigalet et al., 1998). Serbian strains showed pathogenicity in tomato inoculation tests. However, all strains tested in this study derived from potato tubers (none from other host plants) and we did not study pathogenicity in other hosts but tomato, nor other inoculation techniques were used that would allow any decisive conclusion on their aggressiveness.

A study of phenotypic characteristics universal for *R. solanacearum* strains showed that all isolated strains in this study had the same characteristics in nutritional and enzymatic tests as the reference strain NCPPB 4156 and therefore they were identified as *R. solanacearum*. PCR analysis of the Serbian strains from potato samples with two primer pairs specific for *R. solanacearum* confirmed the presence of the bacterium (EU, 1998). RFLP analysis of the Ps-1/Ps-2 amplicons yielded profiles identical to the reference strain of *R. solanacearum* and they were in agreement with those described for *R. solanacearum* confirming identification of the bacterium (EU, 1998; Pravilnik, 2009). The confirmed strains were classified as biovar 2 *R. solanacearum* on the basis of their ability to utilize or oxidase three hexose alcohols and three disaccharides (Table 3).

R. solanacearum was not detected in tomato or any of the other host plants tested in this study. Furthermore, the bacterium was not found in any of 134 water samples tested, including those originating from areas in which the bacterium was found in ware potato samples. It is noteworthy that outbreaks of the bacterium in several European countries (UK, The Netherlands, Belgium, France, Spain) during the past two decades have been mostly associated with contamination

of surface water used for irrigation, or the use of irrigation water from sources in which infected *S. dulcamara* was found to be growing (Janse, 1996; Grousset et al., 1998; Elphinstone et al., 1998; Caruso et al., 2000). Furthermore, *Urtica dioica* has also been reported to be a reservoir of the bacterial wilt pathogen (Wenneker et al., 1999). It has been hypothesized that this bacterium can overwinter on the roots of *S. dulcamara* rather than persist freely in water (Elphinstone et al., 1998; Janse et al., 1998). The results reported by Caruso et al. (2000) showed that populations of *R. solanacearum* biovar 2 in a Spanish river had not been highest in the proximity of the potato field in which an outbreak of brown rot had been first detected, and indicated that the bacterium was found more frequently in *S. dulcamara* plants close to sites with higher levels of the pathogen.

Our results showed that even though a higher number of water samples (27) was collected in South Bačka District after findings of *R. solanacearum* infection in tubers in that district over the previous year and 14 water samples were additionally taken from within the area of bacterial infection, the results of laboratory testing were also negative. We could assume that one of the reasons for negative results of water samples is the fact that the semiaquatic weed *S. dulcamara* as the main bacterium reservoir is not that common in our waterways.

Although all contaminated potato tubers were destroyed and eradication measures ordered and conducted by the authorities of the Ministry of Agriculture, Forestry and Water Management on all infected sites in order to prevent the spread and to eradicate potato brown rot, monitoring of the bacterium presence will be continued.

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Ralstonia solanacearum – nova pretnja proizvodnji krompira u Srbiji

REZIME

Pregledom merkantilnog krompira (ukupno 1127 uzoraka) iz lokaliteta u Srbiji tokom dve uzastopne godine, *R. solanacearum* je detektovana i identifikovana u 17 uzoraka krtola krompira. Rezultati su pokazali da je prouzrokovač mrke truleži krtola i bakteriozne uvelosti krompira registrovan samo u 3 okruga u Vojvodini. Tokom 2011. godine, *R. solanacearum* je potvrđena u 7 uzoraka krtola merkantilnog krompira (sorte: Saturna, Pirol, Hermes, Panda) u Zapadnobačkom i Južnobačkom okrugu. Tokom 2012. godine, *R. solanacearum* je potvrđena u 10 uzoraka krtola merkantilnog krompira (sorte: Lady Claire, Desiree, Panda, Red fantasy, Vineta) lociranih u dva okruga: Južnobački i Srednjebanatski. Sojevi izolovane bakterije identifikovani su kao *R. solanacearum* biovar 2 na osnovu PCR/RFLP analize, testa patogenosti na rasadu paradajza, biohemijskih odlika bakterije i testova za određivanje biovara. Prema našim saznanjima, ovo su jedini nalazi *R. solanacearum* u merkantilnom krompiru u Srbiji. Istraživanje je pokazalo i da *R. solanacearum* nije detektovana u biljkama paradajza kao ni u drugim testiranim biljkama domaćinima ove bakterije. Takođe, bakterija nije detektovana ni u jednom uzorku vode, uključujući i one poreklom iz područja gde je bakterija nađena u uzorcima krtola krompira.

Ključne reči: Bakteriozno uvenuće; mrka trulež; krompir; identifikacija; PCR