Pestic. Phytomed. (Belgrade), 22 (2007) 113-119
Pestic. fitomed. (Beograd), 22 (2007) 113-119

UDC: 632.95:679.25.634.711
Scientific Paper * Naučni rad

First Report of Tumorigenic *Agrobacterium radiobacter* on Raspberry in Serbia

Svetlana Milijašević[1], Veljko Gavrilović[2], Svetlana Živković[2], Nenad Trkulja[2] and Joanna Pulawska[3]

[1] Institute of Pesticides and Environmental Protection, Belgrade, Serbia
[2]Institute of Plant Protection and Environment, Belgrade, Serbia
[3]Research Institute of Pomology and Floriculture, Skierniewice, Poland

SUMMARY

During the spring of 2003, gall symptoms on the roots and crowns of young raspberry plants cv. Vilamette were observed near Valjevo. Phytopathogenic bacteria were isolated from diseased plant samples. Based on the pathogenic, morphological, differential biochemical and physiological characteristics, the isolated strains were identified as tumorigenic *Agrobacterium radiobacter* (biovar 1 *Agrobacterium*). In order to confirm the identity of isolated strains by polymerase chain reaction (PCR) primers complementary to *tms2* gene located on the Ti plasmid were used. In the first PCR protocol using a tms2F1 + tms2R2 primer pair, 617 bp products specific for tumorigenic *Agrobacterium* strains were amplified. The second PCR protocol, using a tms2F1 + tms2B primer pair, amplified the expected 458 bp products. On the basis of multiplex PCR with primers complementary to chromosomal gene coding for 23S rRNA, the isolated strains were classified as biovar 1 *Agrobacterium* (*A. radiobacter).* This is the first report of tumorigenic *A. radiobacter* on raspberry in Serbia.

Keywords: Tumorigenic *Agrobacterium;* Crown gall; Raspberry; Identification; PCR

INTRODUCTION

A tendency to expand raspberry production in Serbia has resulted in extensive planting of new orchards, especially during the past decade. Import of nursery stocks without previous plant health inspection and planting of uncertified material have enabled introduction of many raspberry pathogens, including quarantine and economically important organisms. Besides the fungus *Phytophthora fragariae* var. *rubi*, the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* van Hall was reported as a raspberry pathogen in Serbia a few years ago (Gavrilović et al., 2003, 2004).

Tumorigenic *Agrobacterium,* the causal agent of crown gall, and *Agrobacterium rubi,* which causes cane gall of raspberry, are bacterial diseases that seriously threaten raspberry production in all main production areas of this crop. Crown gall is the more widespread of the two diseases because it affects not only raspberries but as many as some 800 different plant species belonging to 90 plant families, including a number of major

fruit trees, grapevines and ornamentals (DeCleen and DeLey, 1976; Canfield and Moore, 1991).

During the spring of 2003, gall symptoms on the roots and crowns of young raspberry plants cv. Vilamette were observed in a nursery near Valjevo. Since tumorigenic *Agrobacterium* had not been recorded on raspberry plants in Serbia before, the aim of this study was to identify the pathogen using conventional methods (isolation, pathogenicity and bacteriological determinative tests) and to confirm the identity of isolated strains using polymerase chain reaction (PCR).

MATERIAL AND METHODS

Pathogen isolation

The bacteria were isolated from collected samples of diseased raspberry plants cv. Vilamette using young, cream-coloured fresh galls. Gall surface was washed under running water, surface sterilized for 10 minutes in 1% sodium hypochlorite solution and rinsed with sterile water prior to isolation. The small pieces of tumor tissue were macerated, soaked for four hours in sterile distilled water to allow difussion of bacteria into the liquid and bacterial suspension was streaked on nutrient agar plates (NA) (Schaad et al., 2001). Plates were incubated at 25°C and examined after 2-3 days. Presumptive colonies were purified by streaking onto PDA + $CaCO_3$ and nutrient agar. Single cell colonies were transferred on to NA slants and stored at 4°C. Three strains were chosen for further studies.

The strains were compared with the reference strain of tumour-inducing *A. tumefaciens* KFB 096 (obtained by courtesy of A. Obradović, originated from Hungary, collection of S. Sule).

Pathogenicity tests

Pathogenicity of the strains isolated from tumors was tested on sterilised and aseptically cut carrot disks by inoculation with bacterial suspension. Inoculum (containing 10^7 cfu/ml) was prepared by culturing bacteria in liquid medium 523 (10 g sucrose, 8 g casein - acid hydrolysate, 4 g yeast extract, 2 g K_2HPO_4, 0.3 g $MgSO_4$ x 7 H_2O and 1 L H_2O) (Schaad et al., 2001).

Carrot disks treated in the same way with distilled water served as negative control and disks inoculated with the reference KFB 096 were used as positive control. Inoculated carrot slices were placed on moistened sterile filter paper in Petri dishes and kept at room temperature for three weeks.

Pathogenicity of *Agrobacterium* strains was also tested on young raspberry plants inoculated with bacterial suspension (10^8 cfu/ml) prepared from each strain (Schaad et al., 2001). Plants inoculated with water served as negative control, while plants inoculated with the reference strain served as positive control. The presence of galls was checked after four weeks. To fulfill Koch's postulates, bacteria were reisolated from galls.

Biochemical characteristics

Characterization of the pathogen was carried out by determining the following set of biochemical characteristics: Gram reaction, metabolism of glucose, catalase activity, Kovac's oxidase test, acid production from erythritol, 3-ketolactose production, citrate and L-tyrosine utilization, growth and pigmentation in ferric ammonium citrate broth and growth in 2% sodium chloride (Lelliott and Stead, 1987; Schaad et al., 2001).

Polymerase chain reaction (PCR)

To confirm the identity of strains isolated from raspberry plants, a PCR test was conducted from each isolate and from a culture of the reference strain according to the PCR protocol of Pulawska and Sobiczewski (2005). To prepare template DNA, cultures were grown on NA for 24 hours. For each strain, a single colony was suspended in 100 µl of sterile distilled water in a microvial. PCR reactions were performed in Eppendorf thermocycler. Primers were synthesized by Fermentas (Lithuania).

PCR was conducted using primers specific for detection of tumorigenic agrobacteria (complementary to the *tms*2 gene) with the following sequence:

- tms2B:
  5` GGA GCA CTG CCG GGT GCC TCG GGA 3`
- tms2F1:
  5` TTT CAG CTG CTA GGG CCA CAT CAG 3`
- tms2R2:
  5` TCG CCA TGG AAA CGC CGG AGT AGG 3`.

In the first step, PCR was conducted using the tms2F1 + tms2R2 primer pair (Pulawska and Sobiczewski, 2005) where the expected PCR products are 617 base pairs (bp). In the second PCR using the primers tms2F1 and tms2B (Sachadyn and Kur, 1997; Pulawska and Sobiczewski, 2005) the expected products are 458 bp.

DNA amplification was performed in a total volume of 25 µl. All 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 1 µl of template DNA. Sterile deionized water was used as negative control, and the reference strain of *A. tumefaciens* was used as positive control.

Amplification conditions were: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1.5 min, and a final extension step at 72°C for 10 min.

To determine the biovar/species of isolated agrobacteria, a multiplex PCR based on the 23S rRNA gene was performed according to Puławska et al. (2006). Five primers designed by Pulawska et al. (2006) with the following sequence:

- UF :
  5` GTAAGAAGCGAACGCAGGGAACT 3`
- B1R:
  5` GACAATGACTGTTCTACGCGTAA 3`
- B2R:
  5` TCCGATACCTCCAGGGCCCCTCACA 3`
- AvR:
  5` AACTAACTCAATCGCGCTATTAAC 3`
- ArR:
  5` AAAACAGCCACTACGACTGTCTT 5`

were used for multiplex PCR.

DNA amplification was performed in a total volume of 15 µl in Thermal Cycler Trio-Thermoblock (Biometra, Germany). All reactions were performed in PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl, 0.1% Triton X-100) with 1.5 mM $MgCl_2$, 200 µl of each dNTPs, 1µl of each primer and 0.5 U of termostable DNA polymerase (Promega, Madison, USA). The amplification conditions were: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 67°C for 1 min, extension at 72°C for 1.5 min and a final extension step for 10 min.

PCR products were analysed in 1.5% agarose gel run at 100 V for 30 min. in TBE buffer, stained in ethidium bromide and observed in UV transilluminator.

RESULTS

Disease symptoms

Crown gall is characterized by tumor-like swellings or galls on the roots and crowns of diseased plants near the soil surface. In the early stages of symptom development, galls are spherical, tumor-like, white in color and having rough and spongy consistency. With age they become brown, woody knots (Figure 1). The plant may show water stress and nutrient deficiency symptoms since the movement of water and nutrients through the plant is disrupted.

Figure 1. Crown gall symptoms on raspberry – natural infection
Slika 1. Simptomi bakterioznog raka na malini – prirodna infekcija

Pathogenicity tests

All tested strains caused small, green tumors on carrot disks three weeks after inoculation (Figure 2A). No changes were observed on carrot slices inoculated with water (Figure 2B), while the positive control strain *A. tumefaciens* KFB 096 induced tiny, green tumors three weeks after inoculation (Figure 2C).

On the basis of tests carried out on raspberry plants all strains were characterized as tumor-inducing. The investigated strains, including the reference one, induced galls on young raspberry plants four weeks after inoculation (Figure 2D), while no symptoms were observed on plants that served as negative control.

Biochemical characteristics

Convex, circular with an entire edge, cream-coulored, glistening colonies were selected for further study. The results of biochemical tests of bacterial strains were as follows: Gram negative; oxidative metabolism of glucose; catalase positive; oxidase pos-

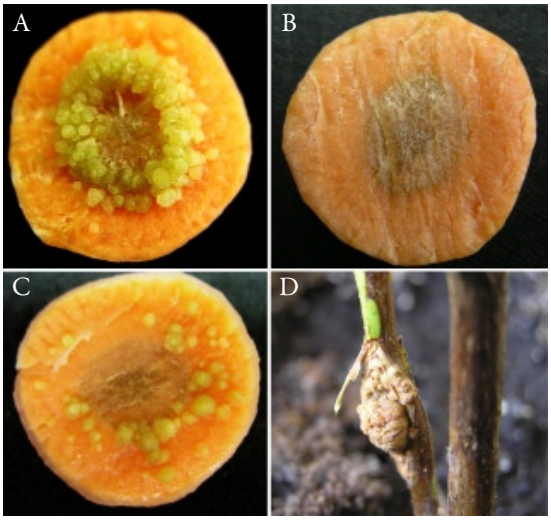

Figure 2: A = Small, green tumors on carrot disks three weeks after inoculation with strain M29; **B** = Carrot slices inoculated with water; **C** = Small, green tumors on carrot disks inoculated with positive control strain *A. tumefaciens* KFB 096; **D** = Galls on young raspberry plants – artificial inoculation
Slika 2: A = sitni, zeleni tumorčići na diskovima mrkve tri nedelje nakon inokulacije sojem M29; **B** = kriške mrkve inokulisane vodom; **C** = sitni, zeleni tumorčići na diskovima mrkve inokulisanim kontrolnim sojem *A. tumefaciens* KFB 096; **D** = tumori na mladim biljkama maline – veštačka inokulacija

itive, no acid from erythrotol, production of 3-ketolactose, utilization of citrate, growth in ferric ammonium citrate broth and in 2% NaCl (Table 1). Based on biochemical characteristics, and pathogenicity test on carrot disks the investigated strains were identified as biovar 1 *Agrobacterium (A. radiobacter).*

Polymerase chain reaction (PCR)

Two PCRs were conducted in order to confirm the identity of isolated strains. In the first step, using tms2F1 + tms2R2 primers, 617 bp PCR products specific for tumorigenic *Agrobacterium* strains were detected (Figure 3). In the second trial, using the tms2F1 + tms2B primer pair, PCR products of expected size (458 bp) characteristic for this bacterium were amplified (Figure 4). Therefore, we confirmed that the strains isolated from tumors in raspberry plants were tumorigenic *Agrobacterium* strains.

All tested *Agrobacterium* isolates amplified only 184 bp long PCR product in multiplex PCR with five

Table 1. Biochemical and physiological characteristics of the investigated *Agrobacterium* strains
Tabela 1. Biohemijsko-fiziološke odlike proučavanih sojeva *Agrobacterium*

Strains Sojevi	Investigated strains Proučavani sojevi	Control strain Kontrolni soj (KFB 096)
Origin Poreklo	Serbia Srbija	Hungary Mađarska
Tests: Testovi:		
Gram stain Bojenje po Gramu	-	-
Glucose (O/F[1]) metabolism O/F[1] metabolizam glukoze	O	O
Oxidase activity Aktivnost oksidaze	+	+
Catalase production Stvaranje katalaze	+	+
Acid from erythritol Stvaranje kiseline iz eritritola	-	-
3-ketolactose production Stvaranje 3-ketolaktoze	+	+
Citrate utilization Korišćenje citrata	-	-
L-tyrosine utilization Korišćenje L-tirozina	-	-
Ferric ammonium citrate broth test Porast u feriamonijum citratnom rastvoru	+	+
Growth in 2% NaCl Tolerantnost prema 2% NaCl	+	+

+ = Positive reaction; - = Negative reaction
O = Oxidative metabolism of glucose
1 = Oxidative-fermentative metabolism of glucose
+ = Pozitivna reakcija, - = Negativna reakcija
O = Oksidativni metabolizam glukoze
1 = Oksidativno-fermentativni metabolizam glukoze

primers complementary to 23S rRNA gene (Figure 5). This product is characteristic only of biovar 1 strains (Pulawska et al., 2006).

DISCUSSION

Although *Agrobacterium* as the causal agent of crown gall has been present in Serbia for a long time, rasp-

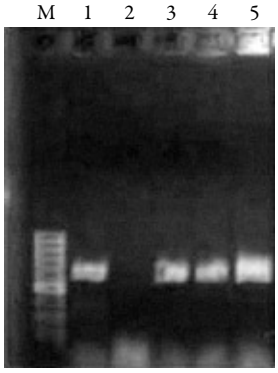

Figure 3. Amplification of 617 bp PCR products with DNA of following *Agrobacterium* strains: **1** = Positive control strain KFB 096; **2** = Negative control; **3** = M29; **4** = M32; **5** = M33; **M** = Molecular weight marker 100 bp ladder
Slika 3. Amplifikacija PCR produkata veličine 617 bp sledećih sojeva: **1** = pozitivna kontrola soj KFB 096; **2** = negativna kontrola; **3** = M29; **4** = M32; **5** = M33; **M** = molekularni marker (100 bp)

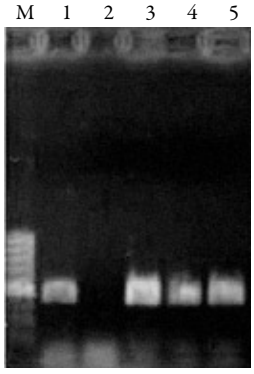

Figure 4. Amplification of 458 bp PCR products with DNA of following *Agrobacterium* strains: **1** = Positive control strain KFB 096; **2** = Negative control; **3** = M29; **4** = M32; **5** = M33; **M** = Molecular weight marker 100 bp ladder
Slika 4. Amplifikacija PCR produkata veličine 458 bp sledećih sojeva: **1** = pozitivna kontrola soj KFB 096; **2** = negativna kontrola; **3** = M29; **4** = M32; **5** = M33; **M** = molekularni marker (100 bp)

berry has become its new host. The bacteria that induce crown gall are responsible for great losses, first of all in nursery production of fruit trees, roses and grapevines worldwide (Kennedy and Alcorn, 1980). Economic losses caused by tumorigenic agrobacteria are not only related to damages in diseased plants but also to the prohibition of commercialization of plants with tumors, which makes the damage even more serious. Besides the use of healthy planting material, it is very important from a practical point of view to determine whether soils in the fields assigned to nurseries are free of tumor-inducing bacteria (Pulawska and Sobiczewski, 2005). These facts indicate the importance of application of rapid, sensitive and specific methods in diagnosis of tumorigenic agrobacteria.

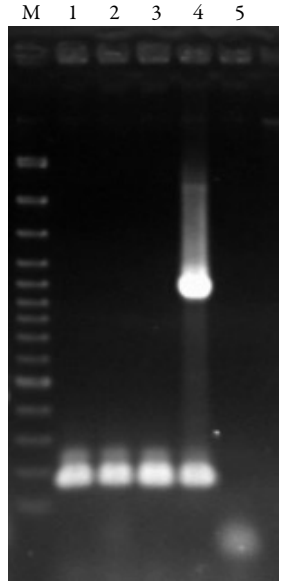

Figure 5. Electrophoresis gel showing PCR products obtained in multiplex PCR with DNA of following *Agrobacterium* strains: **1** = M29; **2** = M32; **3** = M33; **4** = Positive control – DNA of strains C58 (biovar 1 – product 184 bp) and 123 (biovar 2 – product 1066 bp); **5** = Negative control; **M** = Molecular weight marker 100 bp ladder
Slika 5. Agarozni gel sa PCR produktima dobijenim u multiplex PCR reakciji sa DNK sledećih sojeva; **1** = M29; **2** = M32; **3** = M33; **4** = pozitivna kontrola – DNK sojeva C58 (biovar 1 – produkt 184 bp) i 123 (biovar 2 – produkt 1066 bp); **5** = negativna kontrola; **M** - molekularni marker (100 bp)

However, sometimes it is very difficult to detect agrobacteria, due to a small number of bacterial cells present in tumors (Lopez, 1991; Schaad et al., 2001), which makes it hard to prove infections caused by *Agrobacterium* species. Disease diagnosis using conventional methods is based on the isolation of agrobacteria on non-selective or selective media (Schroth et al., 1965; New and Kerr, 1971; Roy and Sasser, 1983), followed by pathogenicity tests on herbaceous plants (tomato, tobacco, sunflower, etc.), carrot disks or their original hosts and identification on the basis of biochemical and physiological characteristics (Lelliott and Stead, 1987; Schaad et al., 2001). However, the main disadvantages of these methods are that they are time-consuming, and very often not

sensitive enough. PCR-based techniques have enabled the development of sensitive methods appropriate for large-scale routine analyses. Several PCR protocols for detecting the crown gall causal agent from bacterial cultures, plant material and soil have been designed so far (Nesme et al., 1990; Dong et al., 1992; Haas et al., 1995; Sawada et al., 1995; Pulawska and Sobiczewski, 2005; Pulawska et al., 2006). Most of these molecular methods are based on amplification of the Ti plasmid. However, using the primers designed by Nesme et al. (1990) it was not possible to detect all strains of agrobacteria. These primers allowed only the detection of the nopaline type of pTi, but not other types (octopine, etc). Moreover, other primers designed for the detection of bacteria harbouring Ti or Ri plasmids have also proved to be selective in detection of tumorigenic agrobacteria (Sawada et al., 1995).

Our investigation included PCR amplifications conducted using different sets of primers in order to identify strains isolated from raspberry plants. Primers complementary to the *tms*2 gene have proved to be highly specific and allowed the detection of tumorigenic agrobacteria regardless of their biovar/species affiliation and the type of Ti plasmid (Pulawska and Sobiczewski, 2005). On the other hand, primers complementary to the chromosomal gene coding for the larger subunit of ribosomal RNA – 23S rRNA, allowed us to classify the tested strains as biovar 1 agrobacteria (*A. radiobacter*). These results are in agreement with results obtained on the basis of biochemical features (Table 1). This is the first report of tumorigenic *Agrobacterium radiobacter* (biovar 1) on raspberry in Serbia.

REFERENCES

Canfield, M. L. and Moore, L. W.: Isolation and characterization of opine-utilizing strains of *Agrobacterium tumefaciens* and fluorescent strains of *Pseudomonas* spp. from rootstocks of *Malus*. Phytopathology, 81: 440-443, 1991.

DeCleen, M. and DeLey, J.: The host range of crown gall. Botan. Rev., 42: 389-466, 1976.

Dong, L.-C., Sun, C.-W., Thies, K.L., Luthe, D.S. and Graves, C.H.: Use of polymerase chain reaction to detect pathogenic strains of *Agrobacterium*. Phytopathology, 82: 434-439, 1992.

Gavrilović, V., Milijašević, S. i Arsenijević, M.: Dalja proučavanja bakterije *Pseudomonas syringae* pv. *syringae* parazita maline u nas. Zbornik rezimea I simpozijuma o malini Srbije i Crne Gore, Čačak, 2003, str.104-105.

Gavrilović, V., Milijašević, S. i Arsenijević, M.: *Pseudomonas syringae* parazit maline u Srbiji. Jugoslovensko voćarstvo, 38: 183-190, 2004.

Haas, J.H., Moore, L.W., Ream, W. and Manulis, S.: Universal PCR primers for detection of phytopathogenic *Agrobacterium* strains. Appl. Environ. Microbiol., 61: 2879-2884, 1995.

Kennedy, B.W. and Alcorn, S.M.: Estimates of U. S. crop losses to prokaryote plant pathogens. Plant Dis., 64: 674-676, 1980.

Klement, Z., Rudolph, K. and Sands, D.: Methods in phytobacteriology. Akademiai Kiado, Budapest, Hungary, 1990.

Lelliott, R.A. and Stead, D.E.: Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications, Oxford, UK, 1987.

Nesme, X., Leclerc, M.C. and Bardin, R.: PCR detection of an original endosymbiont: the Ti plasmid of *Agrobacterium tumefaciens*. In: Endocytobiology (Nardon, P., Gianinazzi-Pearson, V., Greinier, A.M., Margulis, L. and Smith, D., eds.). Institut National de la Recherche Agronomique, Paris, France, 1990, pp. 47-50.

New, P.B. and Kerr, A.: A selective medium for *Agrobacterium radiobacter* biotype 2. J. Appl. Bacteriol., 34: 233-236, 1971.

Pulawska, J. and Sobiczewski, P.: Development of a semi-nested PCR based method for sensitive detection of tumorogenic *Agrobacterium* in soil. J. Appl. Microbiol., 98: 710-721, 2005.

Pulawska, J., Willems, A. and Sobiczewski, P.: Rapid and specific identification of four *Agrobacterium* species and biovars using multiplex PCR. System. Appl. Microbiol., 29: 470-479, 2006.

Roy, M.A. and Sasser, M.: A medium selective for *Agrobacterium tumefaciens* biotype 3. Phytopathology, 73: 810, 1983.

Sachadyn, P. and Kur, J.: A new PCR system for *Agrobacterium tumefaciens* detection based on amplification of T-DNA fragment. Acta Microbiol. Polon., 46: 145-156, 1997.

Sawada, H., Ieki, H. and Matsuda, I.: PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. App. Environ. Microbiol., 61: 828-831, 1995.

Schaad, N., Jones, J.B. and Chun, W.: Laboratory guide for identification of plant pathogenic bacteria. APS Press, St. Paul, Minnesota, USA, 2001.

Schroth, M.N., Thompson, J.P. and Hidebrand, D.C.: Isolation of *Agrobacterium tumefaciens – A. radiobacter* group from the soil. Phytopathology, 55: 645-647, 1965.

Prvi nalaz tumorogenih sojeva *Agrobacterium radiobacter* na malini u Srbiji

REZIME

Tokom proleća 2003. godine, zapaženi su tumori na korenu i korenovom vratu mladih biljaka maline sorte Vilamette u okolini Valjeva. Iz obolelih uzoraka izolovane su fitopatogene bakterije. Proučavanjem patogenih, morfoloških i diferencijalnih biohemijsko-fizioloških karakteristika izolovanih sojeva, utvrđeno je da proučavani sojevi pripadaju bakteriji *Agrobacterium radiobacter* (biovar 1). U cilju potvrde identiteta izolovanih sojeva lančanom reakcijom polimeraze (PCR), korišćeni su prajmeri komplementarni *tms*2 genu lociranom na Ti plazmidu. U prvoj PCR reakciji izvedenoj korišćenjem tms2F1 + tms2R2 para prajmera, detektovani su fragmenti nukleinske kiseline veličine 617 baznih parova (bp), specifični za tumorogene sojeve *A. tumefaciens*. U drugoj PCR reakciji korišćenjem para prajmera tms2F1 i tms2B su amplifikovani PCR produkti očekivane veličine 458 bp, karakteristični za ovu bakteriju. Na osnovu multiplex PCR reakcije korišćenjem prajmera komplementarnih na hromozomalni gen koji kodira 23S rRNA sojevi izolovani iz tumora na korenu maline svrstani su u biovar 1 *Agrobacterium* (*A. radiobacter*). Ovo je prvi nalaz ove bakterije na malini u Srbiji.

Ključne reči: Tumorogeni sojevi; *Agrobacterium;* bakteriozni rak korena; malina; identifikacija; PCR