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Clavibacter michiganensis subsp. *michiganensis*, Bacterial Canker of Tomato: 1. Conventional and Molecular Identification

Svetlana Milijašević[1], Biljana Todorović[1] and Jelica Balaž[2]

[1] *Institute of Pesticides and Environmental Protection, Belgrade*
[2]*Plant Protection Departmant, Faculty of Agriculture, Novi Sad*

SUMMARY

In May 2006, tomato leaves with watersoaked areas between leaf veins were detected in glasshouses in Padinska Skela. The early-stage symptoms were followed by rapid desiccation to white and pale brown necrosis and subsequently by wilting of entire tomato plants. In June 2006, symptoms of bacterial canker and wilt were also recorded in many greenhouses in the Jablanica County in southern Serbia.

Samples of diseased tomato plants were collected from several tomato-growing regions in Serbia in order to identify the causal agent using conventional identification methods (isolation, pathogenicity and bacteriological determinative tests). Another objective of this study was to confirm the identity of isolated bacterial strains by polymerase chain reaction (PCR method).

According to the morphological characteristics observed on NA and NBY media, biochemical characteristics, hypersensitive response in four-o'clock plant leaves and pathogenicity test on tomato seedlings, the investigated strains were identified as *C. michiganensis* subsp**.** *michiganensis*. To confirm the identity of isolated strains two PCR protocols were used. Amplification of expected length DNA fragments, 614 bp and 270 bp, respectively, confirmed that the investigated strains belonged to *C. michiganensis* subsp**.** *michiganensis*.

Keywords: Bacterial canker; Bacterial wilt; Tomato; Identification; PCR; *Clavibacter michiganensis* subsp. *michiganensis*

INTRODUCTION

Bacterial canker of tomato (*Lycopersicon esculentum*), caused by *Clavibacter michiganensis* subsp. *michiganensis*, is a serious concern for growers in all major tomato-producing areas. The organism causes bacterial wilt and canker, which is considered to be the most important bacterial disease of tomato as it can drastically reduce yield and quality, thus causing substantial economic losses both in greenhouses and in open-field production (Chang et al., 1992; Gitaitis, 1991; Hadas et al., 2005). Besides, it usually occurs erratical-

ly so that even years of absence or limited occurrence can be followed by an epidemic (EPPO/CABI, 2005). Although listed as a quarantine organism under the European Community Plant Health Directive 77/93/EEC (Anonymous, 1995) it is quite widely distributed in EPPO member-states (EPPO/CABI, 1998). In Serbia, *C. michiganensis* subsp. *michiganensis* is on the A2 list of pests regulated as quarantine, and it occurs only in some areas with restricted distribution (Anonymous, 1999, 2006).

Generally, *C. michiganensis* subsp. *michiganensis* causes systemic infection of tomato. Symptoms caused by this bacterium depend on the place of production (glasshouse or field), plant age at the time of infection, cultural practices, cultivars, etc. Bacterial canker is a vascular and parenchymatal disease with a wide range of symptoms which include necrosis of the leaf margin, stunting, wilting, vascular discoloration and eventual plant death. The pathogen also induces leaf-spots as a result of local infection, as well as small, raised, superficial necrotic spots on the fruit surrounded by white halos, referred to as "bird's eye lesions" (Gitaitis, 1991; EPPO/CABI, 2005).

However, bacterial canker of tomato has not been recorded in Serbia since it was first described by Šutić (1957). In May 2006, tomato leaves with watersoaked areas between leaf veins were observed in a glasshouse crop in Padinska Skela. These early-stage symptoms were followed by rapid desiccation to white and pale brown necrosis and subsequently by wilting of entire tomato plants. In June 2006, symptoms of bacterial canker and wilt were also recorded in various greenhouses in the Jablanica County in southern Serbia.

Since wilt symptoms caused by *C. michiganensis* subsp. *michiganensis* may be confused with other systemic diseases caused by phytopathogenic fungi *Fusarium* spp. and *Verticillium* spp. and disorders caused by other bacteria: *Pseudomonas corrugata* (tomato pith necrosis), *Ralstonia solanacearum* and *Pectobacterium carotovorum* (wilting), samples of diseased tomato plants were collected from several tomato-growing regions in Serbia in order to identify the causal agent by conventional identification methods (isolation, pathogenicity and bacteriological determinative tests).

In recent years, there has been a substantial interest in developing efficient and sensitive assays for detection and identification of *C. michiganensis* subsp. *michiganensis* (Gitaitis et al., 1991; Hadas et al., 2005). In addition to conventional detection methods, PCR-based procedures have been developed (Dreier et al., 1995; Santos et al., 1997; Pastrik and Rainey, 1999). Drier et al. (1995) designed specific primers for detection of *C. michiganensis* subsp. *michiganensis* derived from the plasmid-borne pathogenicity gene *pat*-1. A few years later, Pastrik and Rainey (1999) designed oligonucleotide primers derived from the 16S-23S rRNA intergenic spacer region, also specific for the detection of this bacterium. Therefore, the other objective of this study was to confirm the identity of the isolated bacterial strains by PCR method, after they were previously identified conventionally.

MATERIAL AND METHODS

Isolation of bacteria and growth conditions

Eleven bacterial strains were isolated from the diseased tomato plant parts collected from two different regions in Serbia (Table 1). Wilting plants were cut at the basis of the stem and broken petioles and dark-yellow to brown discoloration of the vascular tissues on the cut surfaces were looked for. Using a scalpel disinfected by dipping in ethanol and flaming, small cuttings of vascular tissue were made and immediately immersed in sterile distilled water and allowed to soak for 10 minutes for diffusion of bacteria (Schaad et al., 2001; EPPO/CABI, 2005). The bacterial exudate was streaked on Nutrient Agar (NA), and Nutrient Broth Yeast extract agar (NBY) and plates were incubated at 26°C for 4-7 days. Isolations from leaf spots were made by using young spots previously surface-disinfected by wiping with a tissue paper drenched in 70% ethanol. Spots were removed with disinfected scalpel blade, transferred to a small volume of sterile distilled water and soaked for 15-20 minutes for difussion of bacteria (EPPO/CABI, 2005). The leaf tissue was macerated and bacterial suspension was streaked on NA and NBY media. Plates were incubated at 26°C and examined after four days. Typical light yellow, round, semifluidal, 2-3 mm colonies which developed after 3-4 days were transfered to NBY medium. Presumptive colonies were purified by subculturing on Nutrient Glucose Agar (NGA) and Yeast Peptone Glucose Agar (YPGA). Single cell colonies were transferred to King's B medium (KBM) slants and stored at 4°C for further studies. The strains were compared with the reference strain of *C. michiganensis* ssp. *michiganensis* CFBP 4999 (equivalent strain designation = NCPPB 2979) obtained from Collection Française des Bactéries Phytopathogènes (CFBP).

Table 1. Strains from tomato leaves and stems
Tabela 1. Sojevi iz listova i stabljika paradajza

Strain Soj	Locality Lokalitet	Plant organ Biljni organ
P-2	Padinska Skela	leaf (list)
P-4	Padinska Skela	leaf (list)
P-5	Padinska Skela	leaf (list)
P-7	Padinska Skela	leaf (list)
P-8	Padinska Skela	leaf (list)
P-10	Padinska Skela	leaf (list)
P-12	Padinska Skela	leaf (list)
P-68	Lebane	stem (stablo)
P-69	Lebane	stem (stablo)
P-71	Leskovac	stem (stablo)
P-72	Leskovac	stem (stablo)

Pathogenicity test

Pathogenicity of the isolated strains was tested using a tomato seedling test (Lelliott and Stead, 1987; EPPO/CABI, 2005). Bacterial suspension was prepared from the isolated strains and the reference strain (CFBP 4999) by suspending a single colony in 100 µl of sterile distilled water. Tomato seedlings cv. Saint Pierre were grown in sterile plant growing substrate ”B medium course” (Floragard, Germany) at 26°C and >70% relative humidity. Seedlings were inoculated at the second true leaf stage for each strain, including the reference one, by injection into the stem at the cotyledons and kept under plastic bags for 48 hours. The plants injected with water served as negative control. As of the fifth day, plants were observed for wilting and stem canker for another twenty days. The bacterium was isolated from wilting plants by removing a 1cm stem section from 2 cm above the inoculation point. Suspect colonies were subcultured and identified. Strains were also subjected to hypersensitive reaction using four-o'clock plants (*Mirabilis jalapa*) (Gitaitis, 1990; Carlton et al., 1998).

Characterization of the pathogen

Biochemical characteristics

Pathogen characterization was carried out by determining the following set of phenotypic properties: Gram reaction, metabolism of glucose, catalase activity, Kovac's oxidase test, levan formation, aesculin hydrolysis, starch hydrolysis, casein hydrolysis, H_2S production from peptone, acid production from mannose and mannitol and the use of sodium acetate as carbon source (Lellott and Stead, 1987; Schaad et al., 2001).

Polymerase chain reaction tests

To confirm the identity of the isolated strains, PCR test was conducted from each isolate and from a culture of a reference strain according to PCR protocols of Drier et al. (1995) and Pastrik and Rainey (1999). To prepare template DNA, cultures were grown on NBY medium 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 95°C for 15 minutes. Microvials with heated bacterial suspensions were transferred into ice and, after cooling, pulse-centrifuged. PCR reactions were performed in Eppendorf Master Cycler. Primers were synthesized by Fermentas (Lithuania).

For the PCR protocol designed by Drier et al. (1995), oligonucleotide primers with the following sequence were used:

CMM-5: 5' GCG AAT AAG CCC ATA TCA A 3'
and
CMM-6: 5' CGT CAG GAG GTC GCT AAT A 3',

producing a 614 bp amplification product. PCR conditions were: 1 cycle of 2 min. at 96°C; 30 cycles of 60 s at 96°C; 90 s at 55°C; 60 s at 72°C; 1 cycle of 10 min. at 72°C.

According to the PCR protocol of Pastrik and Rainey (1999), oligonucleotide primers with the following sequence were used:

PSA-4: 5' TCA TTG GTC AAT TCT GTC TCC C 3'
and
PSA-R: 5' TAC TGA GAT GTT TCA CTT CCC C 3',

producing a 270 bp amplification product. PCR conditions were: 1 cycle of 2.5 min. at 94°C; 30 cycles of 30 s at 94°C; 20 s at 63°C; 45 s at 72°C; 1 cycle of 10 min. at 72°C.

For both PCR protocols, 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 2.5 µl of template DNA from bacterial colonies previously prepared in 25 µl volume. Sterile deionized water was used as negative control, and a reference strain of *C. michiganensis* subsp. *michiganensis* (CFBP 4999) was used as positive control. Amplification products were anylized in 2% agarose gel run at 100 V for 40 min. in TBE buffer and stained with ethidium bromide and observed in UV transiluminator.

RESULTS

Disease symptoms

Bacterial canker is a vascular and parenchymatal disease with a wide range of symptoms. Superficial symptoms on the upper surfaces of leaves are roughly circular and slightly raised white spots, about 1 mm in diameter. In the early stages of symptom development, the spots are difficult to distinguish. In a young crop they are normally first observed on mature leaves that are at least 1 m above the soil surface when plants are about 1.5-2 m high. The spots may expand, merging with others, and become surrounded with necrotic tissue. Spots may also occur on stems, petioles, peduncles and calyxes. These spots are 1-3 mm in diameter, raised, creamy white at first. These areas often turn light to dark brown later on. Superficial symptoms can be found on fruit of any stage but are usually first seen on green fruit. Small, white spots appear over any part of surface. The spots develop 3-6 mm in diameter, have dark brown/black centers, become raised and surrounded by distinct white halo, and are usually termed „bird's eye lesions". The first symptoms of systemic infection can be observed as small areas of interveinal tissue which turn dull green and have watersoaked appearance (Figure 1) before becoming desiccated and light brown (Figure 2). Lesions usually increase in size and are eventually accompanied by irreversible wilting, which results in the leaf death. Fruits of systemically infected plants may fail to develop and fall or ripen unevenly. They can appear marbled with longitudinal chlorotic streaks and internal bleaching of vascular tissues. Plants with external symptoms of advanced systemic infection almost always show one

Fig. 1. First symptoms of systemic infection - watersoaked spots
Sl. 1. Početni simptomi sistemične infekcije

Fig. 2. Systemic symptoms - brown necrotic areas on lamina
Sl. 2. Simptomi sistemične infekcije – mrke nekrotične površine na listu

Fig. 3. Stem cross-sections - vascular tissue varies in colour from yellow to brown
Sl. 3. Poprečni presek stabljike – boja vaskularnog tkiva varira od žute do mrke

Fig. 4. Spliting of petioles
Sl. 4. Rascep peteljke ploda

or more internal symptoms. In stem cross-sections, the vascular tissue varies in colour from yellow to brown (Figure 3). Occasionally, cavities can develop in the pith. Brown streaking and spliting of stems and petioles can also occur (Figure 4).

Bacterial strains and their phenotypic characteristics

A survey of greenhouses with tomato production in Serbia resulted in the isolation of numerous bacterial strains. Eleven strains were selected for further study.

On NA medium plates, the bacterial colonies were small, 2-3 mm in diameter developed within 3-4 days, light yellow, round and semifluidal (Figure 5). Colonies become deeper yellow and glistening with longer incubation. The bacterial colonies on the NBY were smooth, yellow, mucoid, circular, convex with entire margins (Figure 6).

Tomato plants inoculated with the investigated strains (Figure 7) and reference strain (Figure 8) showed canker symptoms after 15 days and leaves

Fig. 5. Bacterial colonies on the NA medium after 4 days
Sl. 5. Bakterijske kolonije na MPA podlozi nakon 4 dana

Fig. 6. Bacterial colonies on the NBY medium after 4 days
Sl. 6. Bakterijske kolonije na NBY podlozi nakon 4 dana

Fig. 7. Pathogenicity test - stem canker – strain P-4
Sl. 7. Test patogenosti – rak stabljike – soj P-4

Fig. 8. Pathogenicity test - stem canker – reference strain (CFBP 4999)
Sl. 8. Test patogenosti - rak stabljike – kontrolni soj (CFBP 4999)

turned yellow. Plants injected with sterile distilled water remained healthy. Bacteria were isolated from the diseased plants and identified.

Necrosis was observed on four-o'clock plant (*Mirabilis jalapa*) leaves within 24 hours of infiltration with bacterial cells.

The results of physiological and biochemical tests of the bacterium were as follows: Gram positive; oxidative metabolism of glucose; catalase positive; oxidase negative; levan negative; aesculin hydrolysis positive; starch hydrolysis positive; casein hydrolysis negative; H_2S produced from peptone; acid produced aerobically from mannose but not from mannitol; and sodium acetate used as a carbon source (Table 2).

According to the morphological characteristics on NA and NBY media, biochemical characteristics, hypersensitive response in four-o'clock plant leaves and pathogenicity test on tomato seedlings, the investigated strains were identified as *C. michiganensis* subsp. *michiganensis*.

Polymerase chain reaction tests

To confirm the identity of isolated strains, two PCR protocols were used. According to the PCR protocol designed by Drier et al., PCR products of expected size (614 bp) were amplified from all investigated strains, previously identified by conventional methods as *C. michiganensis* subsp. *michiganensis*, as well as from the reference strain, while water control was negative (Figure 9).

Table 2. Biochemical characterization of the pathogen
Tabela 2. Biohemijska karakterizacija patogena

Strains Sojevi	Investigated strains (11) Proučavani sojevi	Reference strain (4999) Kontrolni soj (4999)
Origin Poreklo	Serbia Srbija	France Francuska
Gram stain Bojenje po Gramu	+	+
Glucose (O/F) metabolism O/F[1] metabolizam glukoze	O	O
Levan production Stvaranje levana	–	–
Oxidase activity Aktivnost oksidaze	–	–
Four-o'clock plant hypersensitive reaction Hipersenzitivna reakcija noćurka	+	+
Starch hydrolysis Razlaganje skroba	–	–
Aesculin hydrolysis Hidroliza eskulina	+	+
Casein hydrolysis test Hidroliza kazeina	–	–
Catalase production Stvaranje katalaze	+	+
H_2S production test Stvaranje H_2S	+	+
Carbohydrate utilisation: sodium acetate Korišćenje ugljenika iz: natrijum acetata	+	+
Acid produce from: Stvaranje kiseline iz:		
· mannitol/manitola	–	–
· mannose/manoze	+	+

+ = 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

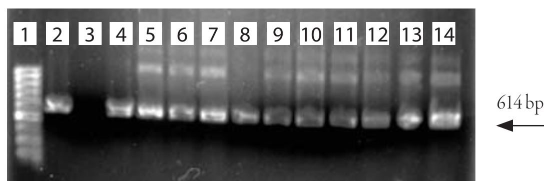

Fig. 9. Amplification of 614 bp DNA fragment
Sl. 9. Amplifikacija DNK fragmenta veličine 614 bp

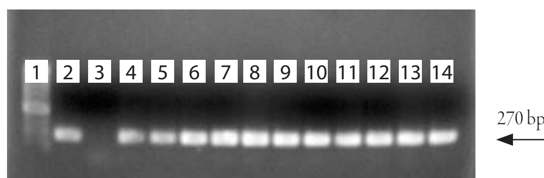

Fig. 10. Amplification of 270 bp DNA fragment
Sl. 10. Amplifikacija DNK fragmenta veličine 270 bp

Legend:

1 = Ledder (100bp)
2 = Strain CFBP 4999 (positive control)
3 = Negative (water) control
4-14 = Tested strains

Legenda:

1 = Marker (100 bp)
2 = Soj CFBP 4999 (pozitivna kontrola)
3 = Negativna (vodena) kontrola
4-14 = Testirani sojevi

Using the PCR protocol of Pastrik and Rainey (1999), amplicons of the expected size (270 bp) were detected in all investigated strains, as well as in the reference strain, while water control was negative (Figure 10).

Amplification of DNA fragments of 614 bp according to the Drier et al. (1995) protocol and DNA fragments of 270 bp according to the Pastrik and Rainey (1999) protocol confirmed that the investigated strains belonged to *C. michiganensis* subsp. *michiganensis*.

DISCUSSION

Favourable climatic conditions for disease development and limited possibilities of controling bacterial diseases are the most common reasons for further spread of important plant pathogenic bacteria in Serbia. Although bacterial canker of tomato was originally described in late 1950s (Šutić, 1957) the disease had not posed a serious threat for a long time. However, widespread production of tomatoes in small greenhouses and poor phytosanitary measures obviously contributed to high disease incidence over the recent period, especially because the pathogen is highly contagious under protected cultivation. Direct spread from a few primary infections occurs during culture manipulation (EPPO/CABI, 2005) and once the disease has appeared it is very difficult to keep it under control. In May 2006, wilt symptoms were recorded in glasshouses in Padinska Skela, and later on, in June 2006, in several greenhouses owned by small growers in the Jablanica County. Wilt and canker symptoms had been previously observed by some tomato growers in 2005 too, but no data has been published on the etiology of this disease. Since similar symptoms could be caused by some other pathogenic organisms, early and accurate pathogen identification is a very important step in disease control. The results of this study showed that *C. michiganensis* subsp. *michiganensis* was associated with canker and wilt symptoms in tomato crops in greenhouses. The results of conventional identification methods were confirmed with PCR-based methods because of their reliable, fast and sensitive nature. Seedborne nature of this pathogen necessitates the use of these methods to make sure that the protective measures are undertaken in time.

Tomato production in the same greenhouse year after year, planting of unsertified seeds, use of the same substrates and pots for transplant production, and generally low hygiene in protected crop production make this problem even more serious. Growers should therefore be informed by plant protection experts about this arising problem so that further spread could be prevented, considering the quarantine status of this bacterium in Serbia.

REFERENCES

Anonymous. Commission Directive 95/4/EC Amendment of 21 February 1995 to the European Community Plant Health Directive (77/93/EEC). Off. J. Europ. Commu., L44: 56-60, 1995.

Anonymous. Pravilnik o zdravstvenom pregledu useva i objekata za proizvodnju semena, rasada i sadnog materijala i zdravstvenom pregledu semena, rasada i sadnog materijala. Sl. list SRJ, 66, 1999.

Anonymous: Pravilnik o izmenama i dopunama Pravilnika o zdravstvenom pregledu useva i objekata za proizvodnju semena, rasada i sadnog materijala i zdravstvenom pregledu semena, rasada i sadnog materijala. Sl. glasnik RS, 39, 2006.

Carlton, W.M., Braun, E.J. and Gleason, M.L.: Ingress of *C. michiganensis* subsp. *michiganensis* into tomato leaves through hydatodes. Phytopathology, 88: 525-529, 1998.

Chang, R.J., Ries, S.M. and Pataky, J.K.: Reduction in yield of processing tomatoes and incidence of bacterial canker. Plant Dis., 76: 805-809, 1992.

Dreier, J., Bermpohl, A. and Eichenlaub, R.: Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology, 85: 462-468, 1995.

EPPO/CABI: Map 253. **In**: Distribution Maps of Quarantine Pests for Europe. CAB International, Wallingford, UK, 1998.

EPPO/CABI: *Clavibacter michiganensis* subsp. *michiganensis*. EPPO Bull., 35: 275-283, 2005.

Gitaitis, R.D.: Induction of a hyprsensitive like reaction in four-o'clock by *C. michiganensis* subsp. *michiganensis*. Plant Dis., 74: 58-60, 1990.

Hadas, R., Kritzman, G., Klietman, F., Gefen, T. and Manulis, S.: Comparison of extraction procedures and determination of the detection treshhold for *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds. Plant pathol., 54: 643-649, 2005.

Klement, Z., Rudolf, 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.

Pastrik, K.H. and Rainey, F.A.: Identification and differentiation of *C. michiganensis* subspecies by Polymerase Chain Reaction-based techniques. J. Phytopathol., 147: 687-693, 1999.

Santos, M.S., Cruz, L., Norskov, P. and Rasmussen, O.F.: A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain. Seed Sci. Technol., 25: 581-584, 1997.

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

Šutić. D.: Bakterioze crvenog patlidžana. Institut za zaštitu bilja, Beograd. Posebna izdanja, doktorska disertacija, 1957, 1-67.

Clavibacter michiganensis subsp. *michiganensis*, bakteriozni rak paradajza: 1. Konvencionalna i molekularna identifikacija

REZIME

Tokom maja 2006. godine zapažene su vlažne pege na lišću paradajza gajenom u staklenicima u Padinskoj Skeli. Ovi početni simptomi praćeni su brzim sušenjem lisnog tkiva, pojavom bele i svetlomrke nekroze i uvenućem celih biljaka. U junu 2006. godine, simptomi bakterioznog raka i uvenuća paradajza takođe su zapaženi i u mnogim plastenicima u Jablaničkom okrugu.

Uzorci obolelih biljaka paradajza sakupljeni su sa nekoliko lokaliteta u Srbiji, u cilju identifikacije prouzrokovača bolesti korišćenjem konvencionalnih metoda za identifikaciju (izolacija, patogenost i bakteriološki testovi). Drugi cilj istraživanja bio je da potvrdimo identitet izolovanih sojeva bakterija primenom lančane reakcije polimeraze (PCR metod).

Prema morfološkim karakteristikama na mesopeptonskoj (MPA) i NBY podlozi, biohemijsko-fiziološkim odlikama, hipersenzitivnoj reakciji na noćurku (*Mirabilis jalapa*) i patogenim odlikama na rasadu paradajza, izolovani sojevi su identifikovani kao *C. michiganensis* subsp. *michiganensis*. Identifikacija izolovanih sojeva bakterija potvrđena je korišćenjem dva PCR protokola. Amplifikovani su DNA fragmenti očekivane dužine, 614 bp i 270 bp, čime je potvrđeno da testirani sojevi pripadaju bakteriji *C. michiganensis* subsp. *michiganensis*.

Ključne reči: Bakteriozni rak; bakteriozno uvenuće; paradajz; identifikacija; PCR; *Clavibacter michiganensis* subsp. *michiganensis*