Pestic. Phytomed. (Belgrade), 30(3), 2015, 179–185
DOI: 10.2298/PIF1503179R

UDC 632.35:634.10(497.16)
Original scientific paper

Monitoring of *Erwinia amylovora* in Montenegro

Dragana Radunović[1*], Veljko Gavrilović[2], Katarina Gašić[2] and Marija Krstić[3]

[1]Biotechnical Faculty, Mihaila Lalica 1, 81000 Podgorica, Montenegro
[2]Institute for Plant Protection and Environment, Teodora Drajzera 9, 11040 Belgrade, Serbia
[3]Ministry of Agriculture and Rural Development, Rimski trg bb, 81000 Podgorica, Montenegro
*(*draganaradunovic@t-com.me)*

Received: August 14, 2015
Accepted: October 26, 2015

SUMMARY

Recent studies of *Erwinia amylovora* in Montenegro, conducted from 2012 to 2014, indicated that the bacterium was widespread in the northern, continental part of the country, where the most important fruit-growing regions are situated. The presence of the bacterium was confirmed on quince, pear, apple, medlar and hawthorn. Pathogenic, cultural and biochemical characteristics of *E. amylovora* strains sampled from pome fruit species and indigenous flora in Montenegro had been studied previously.

In the present study, serological tests were used for identification of *E. amylovora* strains originating from pome fruit trees and indigenous plants. Monitoring of *E. amylovora* and collection of samples with symptoms of bacterial fire blight from different hosts and locations were performed in Montenegro from 2012 to 2014. Isolation of the bacterium on nutrient medium produced a large number of isolates, whose pathogenicity was confirmed on immature pear fruits. Twenty-seven strains of the bacterium, originating from three pome fruit species (quince, pear and apple) and one indigenous species (hawthorn) were selected for serological analyses. Two applied serological methods, ELISA and IF test, enabled rapid detection of the bacterium and simultaneous examination of a large number of samples over a short period of time. Serological analyses showed high homogeneity in antigenic structure of the studied *E. amylovora* strains sampled from quince, pear, apple and hawthorn from nine locations in Montenegro.

Keywords: Fire blight; Pome fruits; Hawthorn; Montenegro

INTRODUCTION

E. amylovora (Burrill) Winslow is the causal agent of fire blight, one of the most destructive diseases of fruit and ornamental plants worldwide, and one of the most harmful bacterial diseases of cultivated plants (van der Zwet & Beer, 1999). In addition to reducing yield and quality of fruits, fire blight also shortens the life span of fruit trees, often causing their complete decay (van der Zwet & Keil, 1979; Panić & Arsenijević, 1996).

Because of its harmfulness, *E. amylovora* has been placed on the A2 EPPO quarantine list and its presence has been confirmed in 51 countries (OEPP/EPPO, 2013). Economic importance of fire blight is likely to increase in the future, primarily because the problem of *E. amylovora* control has not yet been adequately solved. The importance of integrated control management, including the selection of resistant genotypes, mechanical and chemical measures, is emphasized in literature, as well as the use of biological agents (van der Zwet & Beer, 1999; Steiner, 2000; Psallidas & Tsiantos, 2000; Turechek, 2004).

The bacterium was observed for the first time in Montenegro in 1996, and it was on several pear trees around Bijelo Polje (Arsenijević & Gavrilović, 2007). It was experimentally confirmed in 2003 on apple samples from the vicinity of Nikšić (Obradović et al., 2003). Recent studies included *E. amylovora* monitoring in the northern and central parts of Montenegro (Balaž et al., 2012; Radunović & Gavrilović, 2013), and examination of pathogenic, cultural and biochemical characteristics of *E. amylovora* strains originating from pome fruit species and indigenous plants (Radunović et al., 2013). The bacterium was confirmed on quince (*Cydonia oblonga),* pear (*Pyrus communis*), apple (*Malus domestica*), medlar (*Mespilus germanica*) and hawthorn (*Crategus* sp.). The most severe infection was observed on quince, while the disease occurred sporadically on pear and apple (Balaž et al., 2012).

Our monitoring of weather conditions that facilitate the appearance of fire blight symptoms in different climate regions of Montenegro confirmed their close interconnection. Quince, pear and apple plantings with high infection level, and individual highly infected trees of these fruit species in northeastern (location Bijelo Polje) and western (location Nikšić) parts of the country, were the hotbeds from which the bacteria spread to new areas and new host plants (Radunović et al., 2013).

The objective of this investigation was to conduct a nationwide monitoring of *E. amylovora*, and serological identification and examination of antigenic characteristics of *E. amylovora* strains originating from pome fruit trees and indigenous plants from different locations in Montenegro.

MATERIAL AND METHODS

Monitoring, sampling and isolation of the bacterium and pathogenicity tests

The monitoring of *E. amylovora* was conducted from 2012 to 2014. Health status in smaller and larger pome fruit plantations was checked, as well as free-standing trees in farmyards in the northern, central and southern parts of the country. Fire blight symptoms were monitored on susceptible fruit and indigenous species expected to host the bacterium. During the health check, samples with symptoms were collected, and later analyzed in the laboratory.

The bacterium was isolated from diseased shoots, fruits and flowers on meat peptone agar (MPA), nutrient sucrose agar (NSA) and King's B media, using standard bacteriological procedures (Lelliott & Stead, 1987; Arsenijević, 1997; Schaad et al., 2001). The bacterial culture was kept on slanted meat peptone medium with 2% glycerol (NAG), at 4°C in a fridge. All tests were carried out using 24 h old bacterial cultures grown on NSA (Arsenijević, 1997; Schaad et al., 2001).

Hypersensitivity reaction of tobacco was tested using plants of the variety Burley. Bacterial suspension in sterile distilled water (approx. 10^8 CFU/ml) was infiltrated by medical needle into inter-neural leaf tissue. Necrosis of the infiltrated area as a positive result was observed 24 h after inoculation.

Pathogenicity of the strains was tested by inoculation of immature pear fruits (Villiams variety) using a bacterial suspension of approximately 10^8 CFU/ml. Three fruits per strain were inoculated using syringes and medical needles. The inoculated fruits were placed in a sealed plastic container and incubated under high humidity conditions at 25°C. Fruit necrosis with bacterial exudate, as a positive reaction, was recorded 3-5 days after inoculation. Fruits inoculated with sterile distilled water were used as the negative control, while the reference strain NCPPB 595 was the positive control.

Serological analyses

Serological characteristics of the investigated bacterial strains were examined using two serological methods: ELISA and IF test. Twenty-seven *E. amylovora* strains originating from three pome fruit species (quince, pear and apple) and one species of indigenous flora (hawthorn) were sampled from nine locations in Montenegro and selected for the analyses (Table1).

In all tests, the characteristics of the investigated strains were compared to those of the reference *E. amylovora* strain NCPPB 595 obtained from the National Collection of Plant Pathogenic Bacteria, Great Britain.

ELISA test

The applied method was an indirect enzyme-linked immunosorbent assay - Plate Trapped Antigen PTA (ELISA) (Paulin, 2000; Schaad et al., 2001; Janse, 2006), in which a microtiter plate and polyclonal antiserum specific for *E. amylovora* detection (Identikit PTA General Y, ADGEN Phytodiagnostics, Neogen Europe, Ltd., Scotland, UK) were used. Testing was carried out according to the manufacturer's instructions and PTA procedure (OEPP/EPPO, 2013). The tested samples and controls (positive and negative), as well as the reference strain NCPPB 595, were pipetted into the wells of microtiter plate in two replicates each. Through several steps of incubation and rinsing, antibodies and enzyme-linked (conjugated) antibodies were added, and the formed antigen-antibody complex was detected by enzyme reaction.

Table 1. *E. amylovora* strains examined by ELISA and IF tests

Number	Strain	Host plant	Location
1.	Du-1	quince	Berane
2.	Du-2	quince	Berane
3.	Du-3	quince	Andrijevica
4.	Du-4	quince	Andrijevica
5.	Du-5	quince	Nikšić
6.	Du-6	quince	Nikšić
7.	Du-7	quince	Bijelo Polje
8.	Du-8	quince	Pljevlja
9.	Du-9	quince	Plav
10.	Du-10	quince	Petnjica
11.	Du-11	quince	Cetinje
12.	Kr-1	pear	Bijelo Polje
13.	Kr-2	pear	Bijelo Polje
14.	Kr-3	pear	Nikšić
15.	Kr-4	pear	Nikšić
16.	Kr-5	pear	Berane
17.	Kr-6	pear	Berane
18.	Kr-7	pear	Bar
19.	Ja-1	apple	Bijelo Polje
20.	Ja-2	apple	Berane
21.	Ja-3	apple	Berane
22.	Ja-4	apple	Plav
23.	Ja-5	apple	Petnjica
24.	Gl-1	hawthorn	Bijelo Polje
25.	Gl-2	hawthorn	Bijelo Polje
26.	Gl-3	hawthorn	Bijelo Polje
27.	Gl-4	hawthorn	Bijelo Polje
28.	NCPPB 595	pear	Great Britain

The results were obtained visually, based on colour change in the plate and by measuring the absorbance with a spectrophotometer at the wavelength of 405 nm (ELISA automatic plate reader - Universal Microplate Reader EL x 800 BioTek Instruments, USA). The results were read after 30 and 60 minutes. If the mean absorbance value was two or more times higher than the mean absorbance value of the negative control, it was considered a positive reaction, as well as the colour change to yellow.

Immunofluorescence (IF) test

An immunofluorescence (IF) test was carried out according to ADGEN Phytodiagnostic instructions (Neogen Europe, Ltd., Scotland, UK), using specific antibodies for the detection of *E. amylovora* and following the IF procedure (OEPP/EPPO, 2013). The applied method was indirect immunofluorescence (IIF), where the binding of specific antibodies to bacterial cells as antigens was visualized by fluorescent markers (Paulin, 2000; Schaad et al., 2001; Janse, 2006).

The bacterial suspension with a concentration of 10^7 CFU/ml was prepared from an overnight culture of isolates in sterile distilled water. The prepared bacterial samples, positive and negative control, and the reference strain were placed into antigen fields of the microscopic plates. After incubation at room temperature and rinsing, specific antibodies were added into each field. Incubation and rinsing were repeated, and then secondary, non-specific antibodies with attached fluorescein-isothiocyanate (FITC) were added. Readings on the fluorescence microscope Olympus BX51 (100x immersion lens) were used for observing the presence of fluorescent bacterial cells with corresponding morphology.

RESULTS AND DISCUSSION

The results of the conducted monitoring showed that *E. amylovora* was widespread in Montenegro, especially in its northern and western fruit-growing regions. Fire blight symptoms were found on quince, pear, apple, medlar and hawthorn. The highest levels of infection and damage were observed on quince. The flowering period of that fruit species coincides with the rainy period of the year and optimum temperatures for flower infection, which makes quince the most vulnerable host. Lower infection levels on apple and pear are associated with lower temperatures than optimum for the infection during the flowering stage and intensive growth of their shoots.

It is important that fire blight symptoms were also observed on hawthorn plants found on uncultivated land around orchards and by roadsides. Hawthorn infection is a potential a source of inoculum, important for spreading of the bacteria to pome fruit species (Radunović et al., 2013).

Diseased plant parts with symptoms of fire blight were collected from quince, pear, apple and hawthorn trees on nine locations in Montenegro. A large number of isolates was obtained by isolation of the bacterium from diseased shoots, fruits and flowers. Typical convex and mucous colonies (levan type) were formed on NSA medium, which is an important characteristic of *E. amylovora*. Colonies formed on King B medium were white, shiny and without fluorescence, which is also typical for that bacterium.

In the pathogenicity test on immature pear fruits, all studied strains caused necrosis with bacterial exudates three days after inoculation. Complete decay of inoculated fruits was observed after 5-6 days of inoculation.

The symptoms did not develop on fruits inoculated with water (Figure 1). All strains also caused hypersensitivity reaction of tobacco plants after 18-24 h in the form of inter-neural tissue necrosis.

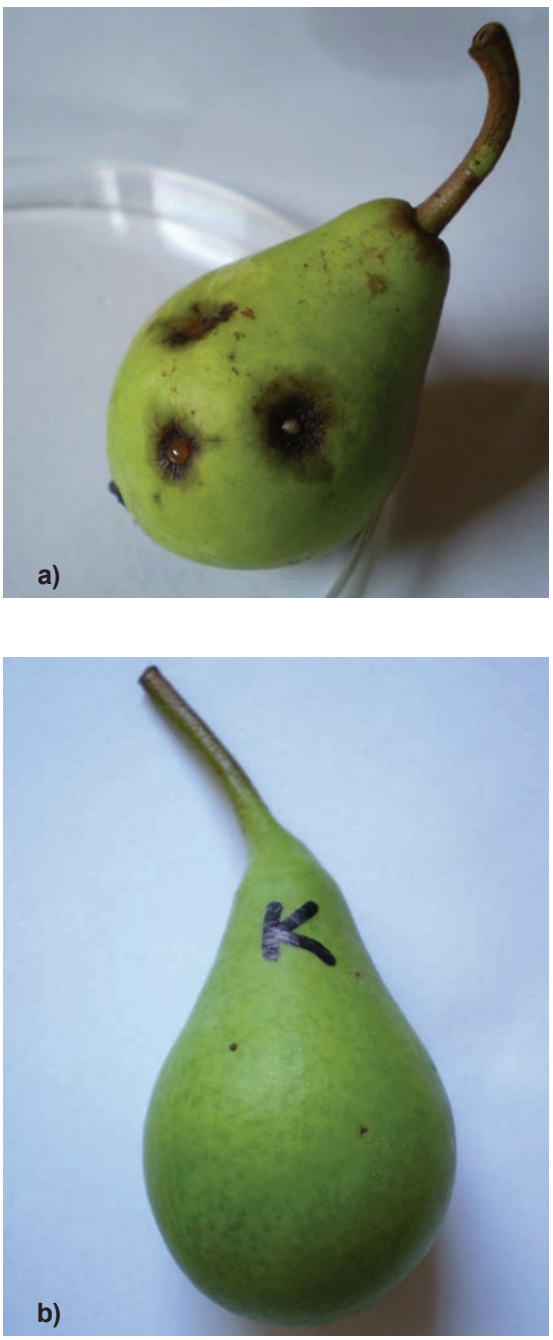

Figure 1. Pathogenicity test on immature pear fruits.
a) Necrosis with drops of bacterial exudate developed 3 days after inoculation
b) Negative control – no sympthoms

Twenty-seven *E. amylovora* strains, originating from quince, pear, apple and hawthorn, were selected for further serological analysis. The PTA ELISA serological method applied on microtiter plates revealed that all investigated strains reacted with specific antibodies and had the same serological characteristics as *E. amylovora* (Figures 2 and 3). The applied method of indirect immunofluorescence (IIF) with labelled secondary antibodies revealed positive reaction of all investigated strains with specific antibodies. Positive reaction was observed in all investigated strains, as well as the reference *E. amylovora* strain NCPPB 595 (Figures 4 and 5). Based on their serological characteristics examined in the ELISA and IF tests, all investigated strains were identified as belonging to the species *E. amylovora*.

Intensive studies of *E. amylovora* in Montenegro, carried out over the period from 2012 to 2014, confirmed the presence of this bacterium in all fruit growing regions of the country (Balaž et al., 2012; Radunović & Gavrilović, 2013). Considering the fact that pome fruit growing areas in Montenegro expand every year (Statistički godišnjak Crne Gore, 2013), the risk of future infection of a large number of trees by this bacterium is high, which could reduce the number of quince, pear and apple trees. Rapid identification of this bacterium, using modern serological and molecular methods, is therefore very important because it reduces the time needed for its detection and enables prompt implementation of adequate control measures (McLaughlin et al., 1989; Gavrilović, 2009).

Identification of *E. amylovora* had been conducted by conventional methods, which included the examination of pathogenic, cultural and biochemical characteristics of its strains originating from pome fruit and indigenous species from different locations in Montenegro (Radunović et al., 2013).

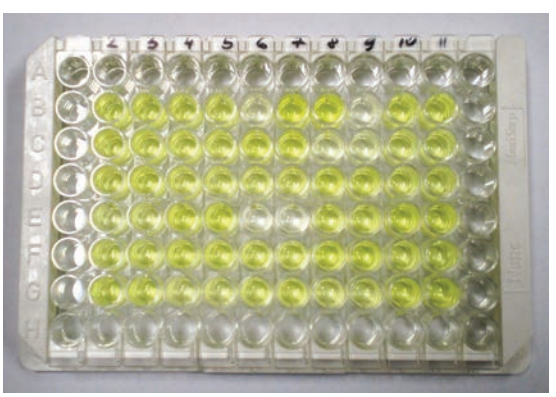

Figure 2. ELISA test. Visual detection of the colour change to yellow in microtiter plate wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.865 1	0.949 1	0.994 7	0.977 7	0.627 13	1.156 595	1.229 595	0.508 13	1.227 23	1.319 23	
C		0.815 2	0.953 2	1.045 8	1.023 8	1.205 14	1.396 14	0.614 18	1.601 18	1.040 24	1.025 24	
D		0.759 3	0.852 3	0.876 9	0.896 9	0.931 15	1.395 15	1.335 19	1.412 19	1.115 25	1.033 25	
E		0.697 4	0.790 4	1.040 10	1.023 10	0.412 K⁻	0.420 K⁻	1.148 20	1.163 20	1.644 K⁺	1.504 K⁺	
F		0.854 5	0.929 5	1.056 11	1.164 11	1.163 16	1.156 16	1.487 21	1.441 21	0.995 26	0.845 26	
G		0.843 6	0.829 6	0.638 12	0.652 12	1.120 17	1.147 17	0.727 22	0.652 22	1.298 27	1.168 27	
H												

Figure 3. ELISA test. Arrangement of investigated strains in microtiter plate and absorbance values recorded by spectrophotometer (after 60 minutes). (K– negative control; K+ positive control; 595 – reference strain)

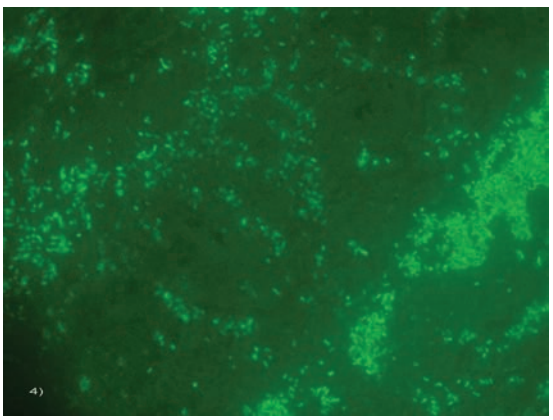

Figure 4. IF test. Yellow-green fluorescence of bacterial cells, observed under fluorescence microscope (Du-3 strain).

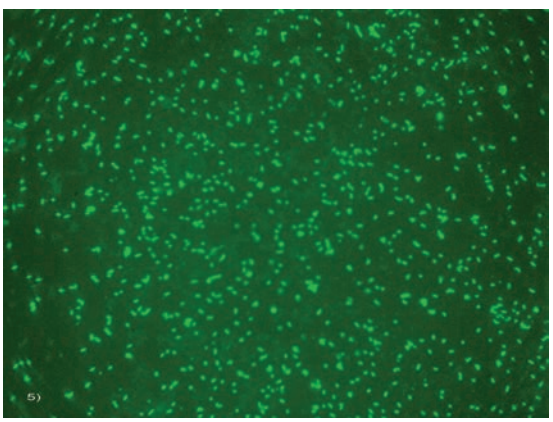

Figure 5. IF test. Yellow-green fluorescence of bacterial cells, observed under fluorescence microscope (reference strain NCPPB 595).

High homogeneity in the antigenic structure of *E. amylovora* strains, originating from quince, pear, apple and hawthorn, was revealed in this study by serological techniques. Serological methods enabled rapid bacterial diagnosing and simultaneous examination of a large number of samples within a short period of time, as well as low-concentration bacterial detection in plant material.

The investigated *E. amylovora* strains, sampled from different hosts and locations, showed a high uniformity in pathogenic, biochemical and serological characteristics, which has also been reported by other authors (Arsenijević et al., 1994; Obradović et al., 2003; Gavrilović et al., 2008; Radunović et al., 2013). The reference strain NCPPB 595 showed identical characteristics, so that we inferred that the *E. amylovora* population in Montenegro is homogeneous regarding these characteristics.

ACKNOWLEDGEMENT

The study was funded by the Ministry of Science and Ministry of Agriculture and Rural Development of Montenegro as part of the 2012-2015 National Research Project „Serological and Molecular Characterization of *Erwinia amylovora* Strains (Burrill; Winslow *et. al.)* Obtained from Different Plant Species in Montenegro".

REFERENCES

Arsenijević, M. (1997). *Bakterioze biljaka* (*3.* izd). Novi Sad: S Print, Univerzitet u Novom Sadu, Poljoprivredni fakultet.

Arsenijević, M., Đurišić, S., & Mitrev, S. (1994). Serološko identifikovanje izolata bakterije *Erwinia amylovora* parazita jabučastih voćaka. *Zaštita bilja, 45*(4), 273-278.

Arsenijević, M., & Gavrilović, V. (2007). *Praktični priručnik o bakterioznoj plamenjači voćaka i ukrasnih biljaka*. Beograd: Institut za zaštitu bilja i životnu sredinu.

Balaž, J., Radunović, D., & Krstić, M. (2012). Status of *Erwinia amylovora* in Montenegro. In *Proceedings of International Symposium: Current Trends in Plant Protection* (pp 373-378). Belgrade, Serbia: Institute for Plant Protection and Environment.

Gavrilović, V. (2009). *Erwinia amylovora* - parazit jabučastih voćaka u Srbiji. In *Zbornik radova, Inovacije u voćarstvu, II savetovanje* (pp 107-117). Beograd: Poljoprivredni fakultet.

Gavrilović, V., Milijašević, S., Todorović, B., Živković, S., & Trkulja, N. (2008). *Erwinia amylovora* - prouzrokovač nekroze korenovog vrata stabla jabuke. *Pesticidi i fitomedicina, 23*(1), 17-23.

Janse, J.D. (2006). *Phytobacteriology - Principles and practice*. Boston, MA: CABI Publishing.

Lelliott, R.A., & Stead, D.E. (1987). Methods for the diagnosis of bacterial disease of plants. In *British Society for Plant Pathology* (p 200). Oxford: Blackwell Scientific Publications.

Mclaughlin, R.J., Chen, T.A., & Wells, J.M. (1989). Monoclonal antibodies against *Erwinia amylovora*: Characterization and evaluation of a mixture for detection by enzyme-linked immunosorbent assay. *Phytopathology, 79*(5), 610-613.

Obradović, A., Vučinić, Z., & Gavrilović, V. (2003). Epifitotična pojava bakteriozne plamenjače dunje u Srbiji i Crnoj Gori. In *Zbornik rezimea VI savetovanje o zaštiti bilja* (Zlatibor) (p 84). Beograd: Društvo za zaštitu bilja Srbije.

OEPP/EPPO (2013). Diagnosis of *Erwinia amylovora*. Protocol for the diagnosis of quarantine organism. SMT Project SMT-4-CT98-2252. *Bulletin* OEPP/EPPO Bulletin, *43*, 21-45.

Panić, M., & Arsenijević, M. (1996). *Bakteriozna plamenjača voćaka i ukrasnih biljaka - Erwinia amylovora*. Beograd: Zajednica za voće i povrće. Novi Sad: Poljoprivredni fakultet.

Paulin, J.P. (2000). *Erwinia amylovora*: General characteristics, biochemistry and serology. In J.L. Vanneste (Ed.), *Fire blight: The disease and its causative agent, Erwinia amylovora*. (pp 87-117). Hamilton, New Zealand: CABI Publishing.

Psallidas, P.G., & Tsiantos, J. (2000). Chemical control of fire blight. In J.L. Vanneste (Ed.), *Fire blight: The disease and its causative agent, Erwinia amylovora* (pp 199-234). Hamilton, New Zealand: CABI Publishing.

Radunović, D., & Gavrilović, V. (2013). Monitoring *Erwinia amylovora* u Crnoj Gori. In *Zbornik rezimea radova XII Savetovanje o zaštiti bilja* (Zlatibor) (pp 172-173). Beograd: Društvo za zaštitu bilja Srbije.

Radunović, D., Gavrilović, V., & Krstić, M. (2013). Bacterial characteristics of *Erwinia amylovora* strains originating from pome fruits and ornamental plants in Montenegro. Zaštita bilja/*Plant Protection (Belgrade), 64*(1), 7-13.

Schaad, N.W., Jones, J.B., & Chun, W. (2001). *Laboratory guide for identification of plant pathogenic bacteria,(3rd ed.)*. St. Paul, MN: APS Press.

Statistički godišnjak Crne Gore. (2013). Podgorica: Zavod za statistiku Crne Gore.

Steiner, P.W. (2000). Integrated orchard and nurery management for the control of fire blight. In J. Vanneste (Ed.), *Fire blight: The disease and its causative agent, Erwinia amylovora* (pp 339-359). Hamilton, New Zealand: CABI Publishing.

Turechek, W.W. (2004). Apple diseases and their management. In S.A.M.H. Naqvi (ed.), *Diseases of fruits and vegetables, diagnosis and management, Vol. 1*.(pp 1-108) The Netherlands: Kluwer Academic Publishers.

van der Zwet, T., & Beer, S.V. (1999). Fire blight: Its nature, prevention and control. A practical guide to integrated diseases management. *Agricultural Bulletin, 631, 97,*

van der Zwet, T., & Keil, H.L. (1979). Fire blight: A bacterial disease of Rosaceous plants. In *Fire blight – A bacterial disease of Rosaceous plants* (p 200). Washington, DC: U.S. Department of Agriculture.

Monitoring *Erwinia amylovora* u Crnoj Gori

REZIME

Novija proučavanja *Erwinia amylovora* u Crnoj Gori, sprovedena u periodu od 2012 do 2014. godine, pokazuju da je ova bakterija široko rasprostranjena u severnom, kontinentalnom delu zemlje, gde se nalaze i najznačajniji voćarski regioni. Prisustvo bakterije potvrđeno je na dunji, krušci, jabuci, mušmuli i glogu. U prethodnim istraživanjima proučene su patogene, odgajivačke i biohemijske odlike sojeva *E. amylovora* poreklom sa jabučastih voćnih vrsta i biljaka spontane flore u Crnoj Gori.

U ovom radu primenjeni su serološki testovi u identifikaciji sojeva *E. amylovora* poreklom sa jabučastih voćaka i biljaka spontane flore. Monitoring *E. amylovora* i sakupljanje uzoraka sa simptomima bakteriozne plamenjače izvršeno je u periodu od 2012 do 2014. godine, sa različitih domaćina i lokaliteta u Crnoj Gori. Izolacijom bakterije na hranljive podloge dobijen

je veći broj izolata, čija je patogenost potvrđena na zelenim plodovima kruške. Za serološke analize odabrano je 27 sojeva ove bakterije, poreklom sa tri jabučaste voćne vrste (dunja, kruška i jabuka) i jedne vrste iz spontane flore (glog). Primenjene su dve serološke metode: ELISA i IF test, koje su omogućile brzu detekciju bakterije i istovremeno ispitivanje velikog broja uzoraka za kratko vreme. Serološkim analizama utvrđena je visoka homogenost u antigenoj strukturi proučavanih sojeva *E. amylovora* poreklom sa dunje, kruške, jabuke i gloga, iz 9 lokaliteta u Crnoj Gori.

Ključne reči: Bakteriozna plamenjača; Jabučasto voće; Glog; Crna Gora