CHARACTERISTICS OF *ERWINIA AMYLOVORA* STRAINS ISOLATED FROM ORNAMENTAL SHRUBS

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Abstract
This paper presents the characteristics of *Erwinia amylovora* strains isolated from *Cotoneaster horizontalis* and *Pyracantha coccinea* on the territory of Nis District in Serbia, as well as biochemical and physiological characteristics of these strains. Identification of *Erwinia amylovora* strains was made according to internationally recognized methods, such as: Biolog test, immunofluorescence, ELISA test and agglutination test, and biochemical and physiological characteristics of the isolates of *Erwinia amylovora* were determined by levan production, oxidase and catalase activity, hydrolysis of gelatin and esculin, VP - test, MR - test, and glucose metabolism. Characteristics of the isolated strains fully coincide with the control strains which were isolated from pome fruits.

Key words: *Erwinia amylovora*, fire blight, *Cotoneaster horizontalis*, *Pyracantha coccinea*.

Introduction

Erwinia amylovora is the causative agent of fire blight of pome fruit trees and other rosaceous plants. The most important bacteria hosts in Serbia are: pear, apple, medlar and quince, and other plants of the species *Sorbus* (Vojinović 2006, Gavrilović et al. 2008) *Cotoneaster* (Balaz et al. 2004), *Crategus* (Vojinović et al. 2009), *Pyracantha*, *Chaenomeless* and *Photinia*. This bacterium is on the international list of quarantine organisms (34 Bulettin OEPP / EPPO, 2008). It is also on the A2 list of quarantine harmful organisms in Serbia, and it seriously affects the production of pome fruits in each country where it is registered (Gavrilović et al., 2009). A number of ornamental shrubs have also been reported as important hosts of the bacterium *Erwinia amylovora* (Sherald 2007). Since ornamental plants from the species *Pyracantha* and *Cotonaster* are grown in many parks in city centers, the examination of characteristics of *Erwinia amylovora* strains isolated from these plants is very important. *Cotoneaster horizontalis* and *Pyracantha coccinea* have an important place in the flora of parks. Identification test done in a plant pathology laboratory indicates the existence of this bacterium in ornamental shrubs (Bastas et al. 2012).

Material and methods

Identification of *Erwinia amylovora* was made according to internationally recognized methods, such as: Biolog test, immunofluorescence, ELISA test and agglutination test.

Biolog TEST

The Biolog GP2 MicroPlate is designed for identification and characterization of a very wide range of aerobic gram-positive bacteria (Vojinović 2010). The Biolog GP2 MicroPlate performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a “metabolic fingerprint”. These fingerprint reaction patterns provide a vast amount of information conveniently contained in a single Biolog MicroPlate. The metabolic fingerprint patterns are compared and identified using the MicroLog™ database software (Brennan et al. 2002). The procedure is fast and simple, involving only 5 steps, and requiring only 2 to 3 minutes hands-on time per sample (Brennan et al. 2002).

1) A pure culture of a bacterium was grown on a Biolog Universal Growth agar plate (Biolog catalog No 70101 for a 500 g jar of dehydrated powder.) Bacillus spp. was grown on a Biolog Universal Growth agar plate with 25% Maltose.

2) The bacteria was swabbed from the surface of the agar plate, and suspended to a specified density in GN/GP Inoculating Fluid (Biolog catalog No 72101).
3) 150 µl of bacterial suspension was pipetted into each well of the GP2 MicroPlate (Biolog catalog No 1014).
4) The MicroPlate was incubated at 30°C or 35 °C (depending upon the nature of the organism) for 4-24 hours.
5) The MicroPlates was read either visually or with the Biolog MicroStation or OmniLog System and compared to the GP Database (Biolog catalog No 22404A) and a result is displayed.

Enrichment DASI-ELISA
The only commercial kit for Enrichment DASI-ELISA has been validated in the ring tests. It is based on the monoclonal antibodies and technique described by Gorris et al. 2005. As positive controls, use aliquots of a sample extract that previously gave a negative result on testing were used, mixed with 108 cells of *Erwinia amylovora* per mL. As negative controls, include a sample extract that has previously given a negative result for E. amylovora and a suspension of a non-*Erwinia amylovora* strain in PBS were used. The necessary amount of the enriched extracts and controls was boil in a water bath (or in a thermoblock) at 100° for 10 min before ELISA, making sure that the tubes are not opened. The remaining enriched samples for isolation and /or PCR were keep. The boiled samples was process to ELISA (once at room temperature) on the same day or they were freeze at −20 °C for subsequent analysis. This heat treatment is necessary for optimum sensitivity and specificity using the monoclonal antibodies obtained by Gorris et al. (1996a). The appropriate dilution of rabbit anti-E. amylovora polyclonal immunoglobulins in carbonate buffer, pH 9.6. was prepared 200 µL to each well of a Nunc Polysorp (or equivalent) ELISA plate was added. Incubate at 37 °C for 4 h or at 4 °C for 16 h.was perfomed. The wels were washed three times with washing buffer. 200 µL per well of the plant macerates previously enriched in the two media and boiled was added. Two wells per sample enriched on each medium, and two of the positive and negative controls were used. The negative controls of the extraction buffer and of the enrichment media were used (previously prepared as additional negative controls of the enrichment). Incubation for 16 h at 4 °C was the step. The wells were next washed three times with washing buffer (as above). The appropriate dilution of specific *Erwinia amylovora* monoclonal antibodies was prepared in PBS plus 0.5% bovine serum albumin (BSA) and added 200 µL to each well. Incubate was at 37 °C for 2 h. The wells were washed three times with PBS-Tween. The appropriate dilution of goat antimouse immunoglobulins conjugated with alkaline phosphatase in PBS was prepared. 200 µL to each well was added. Incubate was at 37 °C for 2 h. The wells were washwed three times as above. 1 mg mL−1168 Erwinia amylovora (Bulletin OEPP/EPPO, 2008) alkaline phosphatase substrate (p-nitrophenylphosphate) in substrate buffer was prepared. 200 µL to each well was added. Incubation was at room temperature and reading was at 405 nm after 30, 45, and 60 min. The ELISA test was negative if the average optical density (OD) reading from duplicate sample wells was < 2x OD of that in the negative sample extract control wells (provided the OD for the positive controls were above 1.0 after 60 min incubation and were greater than twice the OD obtained for negative sample extracts). The ELISA test was positive if the average OD readings from duplicate sample wells was > 2x OD in the negative sample extract wells provided that 2x average OD readings in all negative control wells were lower those in the positive control wells. Negative ELISA readings in positive control wells indicate that the test has not been performed correctly and/or the reagents were not well prepared. Positive ELISA readings in negative control wells indicate cross-contaminations or non-specific antibody binding has occurred. In either case, the test should be repeated or a second test based on a different biological principle should be performed.

Immunofluorescence (IF)
For this test it is necessary to use a validated source of antibodies to *Erwinia amylovora* (Ivanović et al. 2012). Three commercial antibodies have been validated in the ring test. It is recommended that

the titre and dilution of use are determined for each new batch of antibodies (Ivanović et al. 2012). The IF test should be performed on freshly prepared sample extracts. If the extracts stored at −80 °C under glycerol are used, remove glycerol it by adding 1 mL of PBS, centrifuge for 15 min at 7000 g, discard supernatant and resuspend in PBS (Jerffreger 2012). For each set of tests, prepare a positive control slide using a suspension of 106 cells mL^{-1} of a known pure culture of *Erwinia amylovora* (Jerffreger 2012). For large-scale survey work, it is recommended to include blind positive control slides (Ivanović et al. 2012). Use PBS and an aliquot of a sample extract, which was negative by several techniques, as negative controls. Use undiluted macerates and 1 : 10 and 1 : 100 dilutions in PBS to spot windows of the IF slides. Prepare one slide for each sample and its dilutions. Allow to air-dry and fix by flaming or by absolute or 95° ethanol according to the characteristics of the antibodies used. Store slides at −20 °C until required. Use the monoclonal or polyclonal antibodies at the appropriate dilutions in PBS. Spot 25 –30 µL per well. Incubate slides in a moist chamber for 30 min at room temperature. Use of two dilutions of the antibodies is advised when working with polyclonal antibodies, to detect cross reactions with other bacteria. Shake droplets off the slide and rinse slides carefully with PBS. Wash 10 min with the same buffer. Carefully remove excess moisture. Dilute the appropriate FITC conjugates in PBS: antimouse for the monoclonal antibodies (GAM-FITC) and antirabbit (GAR-FITC) or antigoat. Cover the windows of all slides with the corresponding diluted conjugate and incubate in a moist chamber for 30 min at room temperature. Repeat the washing step. Pipette 5 –10 µL 0.1 m phosphate-buffered glycerol mountant with antifading (0.5% phenylenediamine or other) on each window and apply a cover slip. View slides under oil immersion at 500 –1000× magnification by scanning windows across 2 diameters at right angles and around the perimeters. Calculate the number of cells per mL = l of the sample, according to EU (1998). The test is negative if green fluorescing cells with morphology typical of *Erwinia amylovora* are observed in positive controls but not in sample windows. The test is positive if green fluorescing cells with typical morphology are observed in positive control and sample windows, but not in negative control windows. As a population of 103 cells per mL is considered the limit of reliable detection by the IF test, for samples with > 103 cells per mL, the IF test is considered positive. For samples with < 103 cells per ml, the result of the IF test may be considered doubtful. In such a case, further testing or re-sampling should be performed. Samples with large numbers of incomplete or weakly fluorescing cells compared to the positive control need further testing, with different dilutions of antibody or pellet or a second source of antibodies.

Agglutination test

Suspected *Erwinia amylovora* colonies levan-positive, non fluorescent in King's B medium, can be tested for slide agglutination mixing them in a drop of PBS with a drop of *Erwinia amylovora* specific antiserum (not diluted or only at 5 or 10 fold dilution) on a slide (Gavrilović et al. 2007).

Biochemical and physiological characteristics of the isolates of Erwinia amylovora were determined using the following parameters:

Production of levan on a medium supplemented with sucrose (NAS)

Levan is a complex polysaccharide macromolecule which is produced by sucrose metabolism. Bacteria that produce levan on media supplemented with sucrose form very prominent and large colonies, which are shiny, smooth and slimy (levan type) (Gavrilović et al. 2008). A mesopeptone medium supplemented with 5% sucrose was used for the production of levan (Vojinović et al., 2008).

Gelatin hydrolysis

The nutrient medium of peptone, yeast extract and gelatin was dispensed (5 ml) into test tubes (16 x 160 mm) and sterilized in an autoclave at 121^{o}C for 15 minutes. Three test tubes with the medium were inoculated with each isolate. The results were read at 3, 7, 14 and 21 day. Before reading, the test tubes were placed in a refrigerator at 4^{o}C for 30 minutes. If there is no hardening of the medium after this period, the bacteria break down gelatin.

Esculin hydrolysis
The corresponding medium was angled after sterilization and after solidification it was inoculated with the bacteria from 24-hour-old culture. The results were read after 4 days of growth at 27°C. A positive test resulted in a color change of the medium to dark brown.

Glucose metabolism (O / F)
After sterilization and cooling to 50 ° C the filter-sterilized glucose solution was added to Hugh-Leifson medium, to make a final concentration of 1%. Four test tubes were inoculated with each analyzed isolate. In each group half of the test tubes were sealed with sterile paraffin oil to create anaerobic conditions. The results were read after three days of growth in a thermostat at 27°C. A positive test resulted in a color change of the medium to yellow, under aerobic and anaerobic conditions.

Catalase production
The concentration of 20% H_2O_2 solution was used as a test reagent to detect the presence of catalase. A small amount of a well-isolated, 24-hour-old colony was taken with a bacteriological loop and emulsified in a drop of H_2O_2 on a microscopic plate. A positive test resulted in the appearance of gas bubbles and formation of free oxygen.

Oxidase activity
Kovacs method was used (Vojinović et al. 2009). The strips of filter paper were soaked in 1% solution of tetra-methyl-*p*-phenylenediamine-dihydrochloride. A well-isolated colony from the angled medium was taken with a glass rod and streaked on the filter paper strips. Microorganisms are oxidase positive when the color changes to dark purple within 10 to 15 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 30 to 40 seconds.

Methyl red and VP test (acetoin production)
This test is used to detect acetoin that produce some bacteria from the family *Enterobacteriaceae*. The test was performed by adding a few drops of 5% alpha-naphthol solution and a few drops of 40% potassium hydroxide solution to the laboratory-prepared Voges-Proskauer medium (Torlak, Belgrade), and the results were read after the second and the fifth day. If the color turns red within 2 hours, the test is positive. The methyl red test was used as an indicator of the final pH value of the end-products derived from the breakdown of glucose. Methyl red turns red when the pH of the medium is 4.2 or less.

Breeding characteristics of Erwinia amylovora isolates were determined using the following parameters:

Growth at 34°C and 36°C and in a medium containing 5% and 7% NaCl
Growth at 34°C and 36°C was monitored on the medium of yeast extract and mineral salts. The medium was dispensed as 5 ml portions into 16x160 mm test tubes and sterilized by autoclaving at 115°C, the usual treatment being 20 minutes at a pressure of 1.2 atmospheres. Three test tubes with the medium were inoculated with each isolate. After inoculation, test tubes with the medium were incubated in a water bath that maintained the proper temperature for 14 days. If the medium becomes turbid, the bacteria will grow at the mentioned temperature. In order to study the growth of *Erwinia amylovora* in the presence of 5% and 7% NaCl, test tubes with the inoculated medium were maintained in a thermostat at 27° C for 14 days. A positive test resulted in turbidity of the culture medium. Non-inoculated media were used as controls.

Results and discussion

A total of 10 *Erwinia amylovora* strains were analyzed in this study. They were isolated from two different host plants in various geographic regions. Biolog test is a very reliable and simple method for the identification of *Erwinia amylovora* (Vojinović 2010). This test results showed that all strains analyzed were affiliated with the bacterium *Erwinia amylovora*, and similarity index ranged from 0,65 to 0,93. Different types of carbon dispensed into the microwells, in reaction with the bacterial suspension produce a color change in microwells to purple due to redox reactions. All analyzed strains metabolize 17 to 23 carbon compounds: n-acetyl-d-glucosamine, d-fructose, d-galactose, gentiobiose, α-d-glucose, m-inositol, d-mannitol, d-mannose, β-methyl-d-glucoside, d-psicose, d-sorbitol, sucrose, d-trehalose, succinic acid monomethyl ester, d-gluconic acid, succinic acid, bromosuccinic acid, l-aspartic acid, l-glutamic-acid, inosine, glycerol, α-d-glucose-1-phosphate, d-glucose-6-phosphate. The strains isolated from *Cotoneaster horizontalis* and *Pyracantha coccinea* react positively with the corresponding serum in ELISA test, they are positive in immunofluorescence tests and they cause agglutination reaction (Table 1).

Table 1. Methods for the identification of Erwinia amylovora

Strain	Elisa test	IF test	Agglutination test	Biolog test similarity index
Pc-1	+	+	+	0,72
Pc -2	+	+	+	0,93
Pc -3	+	+	+	0,91
Pc -4	+	+	+	0,67
Pc -5	+	+	+	0,87
Ch-1	+	+	+	0,65
Ch 2	+	+	+	0,75
Ch -3	+	+	+	0,72
Ch -4	+	+	+	0,73
Ch -5	+	+	+	0,65
Ap-55 control	+	+	+	0,91

These strains produce levan, they are catalase positive, they hydrolyze gelatin and produce acetoin. They showed a negative reaction for oxidase production, esculin hydrolysis and methyl red test. All analyzed strains metabolize glucose under aerobic and anaerobic conditions (O / F test), as shown in Table 2.

Table 2. Biochemical and physiological characteristics of the strains of Erwinia amylovora

Strain	Levan production	Gelatin hidrolysis	Esculin hidrolysis	Glucose metabolism		Catalase activity	Oxidase activity	VP test	Methyl red test
				O	F				
Pc-1	+	+	-	+	+	+	-	+	-
Pc -2	+	+	-	+	+	+	-	+	-
Pc -3	+	+	-	+	+	+	-	+	-
Pc -4	+	+	-	+	+	+	-	+	-
Pc -5	+	+	-	+	+	+	-	+	-
Ch-1	+	+	-	+	+	+	-	+	-
Ch 2	+	+	-	+	+	+	-	+	-
Ch -3	+	+	-	+	+	+	-	+	-
Ch -4	+	+	-	+	+	+	-	+	-
Ch -5	+	+	-	+	+	+	-	+	-
Ap-55 control	+	+	-	+	+	+	-	+	-

Strains used in this study produce acids from the following carbohydrates: fructose, glucose, ribose, sucrose, sorbitol, mannitol and glycerol. They showed a negative result for dulcitol and inositol (Table 3 and 4).

Table 3. Breaking down of monosaccharides and oligosaccharides from the analyzed strains of *Erwinia amylovora*

Strain	Fructose	Glucose	Ribose	Sucrose
Pc-1	+	+	+	+
Pc -2	+	+	+	+
Pc -3	+	+	+	+
Pc -4	+	+	+	+
Pc -5	+	+	+	+
Ch-1	+	+	+	+
Ch 2	+	+	+	+
Ch -3	+	+	+	+
Ch -4	+	+	+	+
Ch -5	+	+	+	+
Ap-55 control	+	+	+	+

Table 4. Metabolism of polyhydric alcohols and polysaccharides

Strain	Sorbitol	Manitol	Inositol	Glycerol	Dulcitol
Pc-1	+	+	-	+	-
Pc -2	+	+	-	+	-
Pc -3	+	+	-	+	-
Pc -4	+	+	-	+	-
Pc -5	+	+	-	+	-
Ch-1	+	+	-	+	-
Ch 2	+	+	-	+	-
Ch -3	+	+	-	+	-
Ch -4	+	+	-	+	-
Ch -5	+	+	-	+	-
Ap-55 control	+	+	-	+	-

All *Erwinia amylovora* isolates inoculated in a liquid medium grow at 34^{o}C, but not at 36^{o}C. They grow in a liquid medium with 5% NaCl, but not in a medium with 7% NaCl. A positive test resulted in turbidity of the culture medium in test tubes (Table 5).

Table 5. Growth at a 34 ^{o}C and 36 ^{o}C and in a medium containing 5% and7% NaCl

Strain	Growth at a temperature of		Containing NaCl	
	34^{o}C	36^{o}C	5%	7%
Pc-1	+	-	+	-
Pc -2	+	-	+	-
Pc -3	+	-	+	-
Pc -4	+	-	+	-
Pc -5	+	-	+	-
Ch-1	+	-	+	-
Ch 2	+	-	+	-
Ch -3	+	-	+	-
Ch -4	+	-	+	-
Ch -5	+	-	+	-
Ap-55 control	+	-	+	-

Characteristics of *Erwinia amylovora* strains isolated from *Cotoneaster horizontalis* and *Pyracantha coccinea* on the territory of Nis District fully coincide with the control strains and data from the literature (Brennan et al., 2002, Schaad et al., 2002, Beer, 2005, Triplett et al., 2006, Postinikova et al., 2008, Smits et al., 2010, Vojinović 2010, Balaž et.al 2012, Bastas 2012, Bastas et al. 2012, Ivanović et al.2012.).

Conclusions

Erwinia amylovora, the causative agent of fire blight, has been experimentally detected up to now in almost all major areas of the Republic of Serbia where pome fruits and ornamental shrubs are cultivated. In Nis district, it is detected on apple, pear, quince, medlar, rowan, *Cotoneaster horizontalis* and *Pyracantha coccinea*. Characteristics of the isolated strains fully coincide with the control strains and with the strains already detected. Identification of *Erwinia amylovora* strains was made according to internationally recognized methods, such as: Biolog test, immunofluorescence, ELISA test and agglutination test. Biochemical and physiological characteristics of the isolates of *Erwinia amylovora* were determined by levan production, oxidase and catalase activity, hydrolysis of gelatin and esculin, VP - test, MR - test, and glucose metabolism. Breeding characteristics were determined with the growth of *Erwinia amylovora* at 34^{o}C and 36^{o}C and in a medium containing 5% and 7% NaCl. Characteristics of *Erwinia amylovora* strains isolated from *Cotoneaster horizontalis* and *Pyracantha coccinea* on the territory of Nis District fully coincide with the control strains and data from the literature. Any possible differences could be determined by the use of new methods which are not available at present.

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