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ETIOLOGICAL STUDY OF BLOSSOM BLAST OF PEAR IN SOUTHEAST SERBIA (REGION OF LESKOVAC)

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Abstract. The paper studied and biochemical characteristics of pathogenic strains of bacteria isolated from necrotic pear flower buds in the region of Leskovac. Strains were gram negative, fluoresce on King's medium B (KB), does not produce oxidase, produce levan and HR caused by tobacco. On the basis of pathogenicity tests, biochemical and physiological properties and molecular methods (PCR) revealed that these symptoms cause the Krušce Psudomnoas syringae, more widespread and economically štetninji pear pathogen not only in Leskovac, but throughout Serbia.

Key words: Pear, Pseudomnas syringae, blossom blast, Leskovac

Introduction

Peach is the territory of the Jablanica grown on 700 acres, with the present tendeniciju further increase the surface area. Dominated by the variety William and then Santa Maria, Butira, Vettel, Boskova bottle. Successful production of pears in the territory of Jablanica region affected by the presence of plant pathogenic organisms, including of course the top priority *Erwinia amylovora*, fire blight prouzokovač pome fruit trees, and phytopathogenic fungus *Venturia pirina*..

In recent years, however, is a phenomenon observed extensive necrosis of flower buds of pear. Symptoms observed after flowering, especially after the rainy and cold weather. Nekrotirane flowers, and the surrounding leaves get black, but the disease does not spread to several years in industry, but remains located in the zone of flowering branches on which to form cancer-wound. Since the symptoms exhibited different from those caused by *E. amylovora* aim of this study was to determine the causal agent of disease and study its characteristics.

These symptoms caused by the phytopathogenic bacteria *Pseudomonas syringae* and the goal was to determine whether the bacteria cause necrosis of pear flowers (blossom blast) and study its pathogenic and bacteriological characteristics.

Materials and methods

Samples with typical symptoms of pear trees were collected in Leskovac, in the period 2008-2010, year, which plantations were performed modern and intensive cultural practices in order to achieve high quality production.

Isolation of pathogens was done using the standard smear on mesopeptonsku medium enriched with sharozom (NAS) and King's medium B (KB) [KING et al., 1954], which are widely used for isolation of Pseudomonas bacteria from diseased fruit. Separated individual colonies, which produce levan and fluoresce on King's medium B were inoculated on the surface mesopeptonsku enriched with 2% glycerol for storage in a collection [ARSENIJEVIC, 1997; GAVRILOVIC, 2006].

Pathogenicity of the studied isolates was tested by infiltration of bacterial suspensions (107 cfu/ml) in the mesophyll leaf tissue of tobacco (cv Samsun) in order to test to cause hypersensitivity reactions (HR) and inoculation of immature pear fruits, cherries and lemon, bean pods and leaves of lilac [ARSENIJEVIC 1997; CLEMENT, 1990; GAVRILOVIC, 2006].

Of bacteriological characteristics were studied to identify the most important P. syringae: distinguishing Gram create fluorescentog pigment on King's medium B, glucose metabolism; stvarnje Levan, oxidase activity, pectinase, arginine dehidrolaze (spades), hydrolysis of gelatin and esculin, the creation of tyrosinase and metabolism of tartrate (GATT).

BOX PCR

DNA isolation

Total genomic DNA was isolated from bacteria using a modified procedure described by Ausubel, 1992. Bacterial cultures were grown in the U.S. medium supplemented 48 h at 25 ° C. Bacterial cells were washed with sterile water and centrifuged at 4,000 × g, 10 min at 4 ° C. Pellet was resuspended twice with 0.85% NaCl and once with 0.1 M $NaPO_4$ buffer (pH 6.8). Cells were then treririane with 10% sodium dodecyl sulfate (SDS) and mixed with 20 mg proteinase K at 37 °C, 1 h. Then, NaCl was added to the final koncentrcije of 5 M and DNA was purified using the solution of 10% ammonium hexadecyltrimethyl bromide (CTAB) in 1 M NaCl at 65 ° C, 10 min. The whole procedure was followed with phenol-chloroform and chloroform extraction. DNA was then purified using izopropanolske precipitation. Purified DNA was dissolved in Tris-EDTA (TE, 10 mM Tris, 1 mM EDTA, pH 8.0) and its volume was measured at 260 nm spectrofotometrijski.

Duplication of DNA bar

Reproduction of the DNA bar is done in 25 mL reaction mixture that consisted of 67 mM Tris-HCl (pH 8.8), 25 mM $MgCl_2$, 125 micron dNTP, 2 U Taq DNA polymerase (Fermentas, Lithuania) and 100 pmol of primer BOXA1R. Sterile distilled water was used as negative control. BOX A primer sequence (BOXAlR [5'-Ctac GGCAAGGCGACGCTGACG-3']) [LUPSKI, 1992]. PCR program for the duplication is performed on the device model Mastercycler personal (Eppendorf, Hamburg, Germany.) According to the following procedure [de BRUIJN, 1992]: an initial cycle of 7 min at 95 ° C, followed by 30 cycles of denaturation at 94 ° C for 1 min , hybridization of primers 1 min at 52 ° C and polymerization of 8 min at 65 ° C and then a final cycle of polymerization of 16 min at 65 ° C. The amplified DNA fragments were separated by the process of electrophoresis in 1.5% agarose gel and 0.5 x TAE buffer at a voltage 80 V. The fragments were stained by dipping the gel in a solution of ethidium bromide (100 mg / 100 ml) for 20 min and observed under UV light for transillumination

As control strains in this study were used CFBP 1582 (*P. syringae pv syringae*), CFBP 2119 (tobacco leaves morsptunorum) and CFBP 1430 (*Erwinia amylovora*), all originating from the National Collection of Plant Pathogenic Bacteria, Angers, France.

RESULTS

Isolation of bacteria and pathogenicity test

Bacteria on the surface mesopeptonskoj enriched with 5% sucrose (NAS) formed pale gray colonies levan type, diameter 2 to 2.5 mm, and at King's medium B produces intense fluorescent dye.

Strains cause HR in tobacco and necrosis of inoculated immature pear fruits (cultivar Viljami, Santa Maria), cherries (the variety Burlat, out and sambarst) and lemon (variety unknown) manifested in the form of black necrotic spots ulegnutih diameter of 3-5 mm. Symptoms appear on the fruits of cherry, but after 24 hours of inoculation, and the fruits of pear and lemon after 2-3 days. Necrosis of leaf lilac starts from the leaf handles, immersed in a suspension of bacteria and very fast operation veins pervading the whole list, which becomes black. On inoculated bean pods around the sites of infiltration of a suspension of bacteria are observed characteristic brown spots with conspicuous orange halo.

They are identical in pathogenicity tests behave control strain CFBP 1582 *(P.s.pv.syringae*), while the control strain CFBP 2119 (*P.s.pv. morsprunorum*), cause HR in tobacco and necrosis of sweet cherry, while the inoculated bean pods caused bledomrke spots around Places of infiltration, which is seen as a negative result. On inoculated pear fruit, lemon leaves and lilac, this strain does not cause necrotic lesions *(Table 1)*. Based on the results of tests it was concluded that the pathogenicity of the isolates from pear characterized pathogenicity characteristic of *Ps pv. syringae.*

Control strain CFBP 1430 (*E. amylovora)* on pear fruit inoculated and shake cause necrosis accompanied by abundant production of bacterial exudate. This strain are inoculated lemon fruit, bean pods and leaves of lilac, since these plants are not hosts of E. amylovora.

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Bacteriological characteristics

The studied isolates were gramnegative, fluoresce on King's medium B and glucose metabolized exclusively under aerobic conditions (oxidative); produce levan but a negative reaction in tests oxidase activity arginindehidrolaze and pectinase; hydrolyzed gelatin and esculin but negative reaction in tests to create tyrosinase and tartrate metabolism. *(Table 2)*

Control strain CFBP 1582 (*P.s.pv.syringae*) when these tests behave identically načim the strains isolated from pear, whereas the control strain CFBP 2119 (*P.s. pv morsprunorum)* does not decompose gelatin and esculin, but it is metabolized by tyrosinase and tartarate *(Table 2)*.

Based on the results of differential biochemical tests for *P. S. pv. syringae* and *P.s. pv. morsprunorum* (GATT) strains isolated from necrosis of the affected branches and flowers are classified in pear pv. syringae.

BOX - PCR

Possibility of using BOX primers to identify *P. syringae* originating from the pear is not considered.Our hypothesis was that the repetitive DNA sequences in bacteria originating from *P.syringae* pear to be identical in all isolates regardless of origin. These repetitive sequences will be shown in the form of DNA fragments that vary in size.

Multiplying the total DNA isolated from bacteria *P. syringae* originating with pear using BOX primer sequences are obtained DNA fragment size of 100 bp to 4000 bp, which together form, image, characteristic for each isolate. The fingerprints were compared with fingerprints obtained from isolates from apples, cherries, raspberries and plums, as well as control isolates CFBP 1582 (*P.s.pv. syringae*) and CFBP 2119 (*P. s .pv morsprunorum*). Prints of reference and the tested isolates are shown in Figure 4 BOX repetitive DNA sequences obtained from isolates of *P. syringae* from pear originated from different localities have yielded identical fingerprints on the gel. Fingerprints showed a clear difference from the control isolates and in comparison to isolates originating from other fruit *(Figure 4)*.

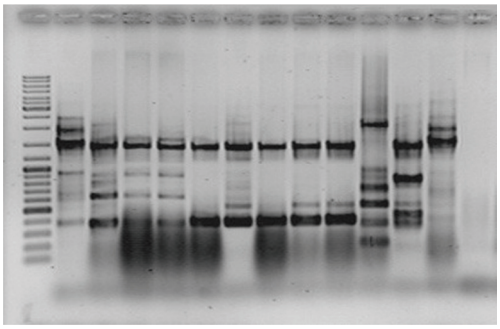

Figure 1. Agarose gel electrophoresis of BOX-PCR fingerprint patterns obtained from *Pseudomonas syringae.* Lane 1 show the DNA molecular size marker (GeneRuler^{TM} DNA Ladder Mix), CFBP-2119 (lanes 2), CFBP-1582 (lanes 3), *P. syringae* isolates from sour cherry (lane 4-5), *P. syringae* isolates from pear (lane 6-10), *P. syringae* isolate from raspberry (lane 11), *P. syringae* isolate from apple (lane 12); *P. syringae* isolate from plum (lane 13) and negative control (lane 14).

Discussion

Pseudomonas syringae parasitizes numerous types of stone and pome fruit trees worldwide and is one of the economically important pathogens of plants [ARSENIJEVIC, 1997; SCORTICHINI et al., 2003, NATALINI et al., 2006; KENELLY et al., 2007]. Pear is definitely among the more susceptible hosts of this bacterium, and the damage that may occur because of infection with the bacteria depend on environmental factors, susceptibility of the species, age, etc. pear tree.

Spots and Cervantes (1994) reported outbreak of extinction pear trees caused by the bacteria, after an extremely low lows during the winter months. The appearance of reddish flowers and leaves of pear high intenzitata noticed in South Africa has also resulted in great damage [MANSVELT and HATINGH, 1986].

Studying *P. syringae* as parasites of pear [NATALINI et al., 2006] also suggest a widespread distribution of these bacteria as pathogens of pear and significant losses in some years may cause.

The bacterium is a pathogen of pear in Serbia for the first time described in the early seventies, causing dry branches, downy shoots, and necrosis of leaves and flowers

[ARSENIJEVIC, 1970]. Intensification of production and the newly planted pear in the previous decade, were found massive infection of pear trees by the bacteria and observed significantly damages result. Particularly on young trees aged 3-6 years, grafted on quince as a vegetative medium [GAVRILOVIC et al., 2003, 2008].

Observed two types of symptoms: necrosis and drying of perennial trees and pear trees, followed by education of the cancer and tan flowers [GAVRILOVIC et al., 2003; GAVRILOVIC, 2006]. In South Serbia (Leskovac), however have been seen so far only symptoms blight blossom. However, during his visit to pear orchards have noticed symptoms of necrosis of the fruits, leaves and shoots of pear, as, on the basis of literature information is often symptomatic [MANSVELT and HATINGH, 1986].

Symptoms of the disease are visually very similar to those caused by *Erwinia amylovora*, the agent of bacterial blight of pome fruit trees, but there are some very important differences and specificities. Clusters caused by arson *P. syringae* is limited to the inflorescence and floral handles, bacteria does not spread further through the arms but remains localized in the region of floral branches on which the form of early cancer. Another important difference is the absence of bacterial exudate, which in the case of infection with *E. aymlovora* abundantly appears on the diseased fruit bodies. Furthermore, *P. syringae* in diseased branches cause necrosis followed by peeling of the epidermis, and *E. amylovora* not.

Yet despite these differences for the reliable detection of disease causing mechanisms, the isolation of bacteria and to study its effects are. Especially since similar symptoms to Krušce are also caused by pathogenic fungi and phytoplasma [STAROVIĆ et al., 2007], and joint infection by bacteria P. pear syringae and E. amylovora [PANIC and ARSENIJEVIC, 1996, GAVRILOVIC, 1998]

Isolation *P.syringae* possible during the spring and summer, and most successful immediately after onset of disease, since then its activity is greatest. For these purposes have proved to be suitable substrates mesopeptonska with sucrose (NAS) and King's medium B, and the characteristics of colonies of bacteria (making levana and fluorescence) of these media have an important diagnostic character [ARSENIJEVIC, 1997; KIERNICK-BROWN and SANDS, 2001; Gavrilovic , 2006].

The test applied for the pathogenicity test are very reliable, easily achievable and are widely used for this purpose [BURKOWITZ and RUDOLPH, 1994; SCORTICHINI et al., 2003; NATALINI et al., 2006; GAVRILOVIC, 2006]. Preference should we still give those tests in which a reliable result obtained after 1 - 3 days after inoculation and these are the results of our testing pathogenicity of nesazrelim fruits of cherry, pear and lemon green beans and green beans [GAVRILOVIC, 2006]. If the isolation of P. syringae performed during the autumn and winter months for the pathogenicity test can be used pear seedlings and cotyledons of peach leaves, which are available throughout the year [ENDERT and RITCHIE, 1984; GAVRILOVIC, 2006].

In terms of biochemical characteristics of isolates studied with pears exhibit exceptional uniformity and show typical features of Pseudomnas syringae pv. syringae. Although isolates of P. syringae are characterized by high biochemical activity, for reliable identification of greatest importance, the results of tests of the GATT, which have the appearance of PCR methods were of great importance for the differentiation of PV. syringae and pv. morsptunorum, as the two most common pathogenic variety of bacteria [LATORRE and JONES, 1979; BURKOWITZ and RUDOLPH, 1994; ARSENIJEVIC, 1997]. The lack of this group of tests is a relatively long period to obtain valid results. Also been isolated from strains that are characterized by intermediate properties in terms of biochemical characteristics of this group. The diversity of the population of P. syringae in these tests was found in those isolated from certain species of stone fruit trees. With respect to the results of these tests with the pear isolates studied demonstrated a marked uniformity of hydrolyzed gelatin and esculin, does not create and is not metabolized tyrosinase tartarate [SOBICZEVSKI, 1984, BALAZS et al., 1988; GAVRILOVIC 2006].

Application of the method lanačane reaction (PCR) using BOX primers proved to be very dependable for the detection of P. syringae and other bacterial species. Using hand ERIC and REP primers can be estimated potential differences among strains, which is of great importance for the study of plant

pathogenic bacteria. Determining differences among strains is important for studying the epidemiology of bacteria, the elaboration of measures for its control but is also significant for the phytosanitary services and reliable detection of pathogens, especially quarantine [SCORTICHINI, 2005; GASIC et al., 2009].

Using these methods were differences in the strains of *P. syringae* originating from different hosts, and confirmed the existence of diversity among isolates from different localities geogeafskih.

Litle et al. (1998) using the ERIC PCR method have found that strains of *P. s. pv. syringae* originating from stone fruit orchards in California, significantly different from the strains of bacteria isolated from other hosts and within populations represent a separate group (cluster). Natalini et al. (2006) point out that using REP and BOX PCR primers A1 utvrđne three different groups of *P. syringae pv. syringae* from pear originated from Greece, Italy, Spain and England.

Using BOX primers showed significant differences among strains of *P. syringae* originating from different types of fruit in Serbia. Isolates from pears showed a marked uniformity among themselves, but are partially different from the control strain *P. s. pv.syringae* originating from France. This suggests that the further study of these bacteria as pathogens of pear in the U.S. must use the ERIC and REP primers and regions of our study 16SrRNK strains as precisely as they position themselves in relation to isolates from pear originated from other sites [IVANOVIC et al., 2009].

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