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CHARACTERIZATION OF *PSEUDOMONAS SAVASTANOI* PV. *GLYCINEA* ISOLATES FROM VOJVODINA

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Abstract

Pseudomonas savastanoi pv. *glycinea*, causal agent of soybean bacterial blight in Vojvodina province, was isolated during 2005 from infected soybean leaves of several cultivars. Pathogenicity of the isolates obtained was proved by inoculation of soybean plants at cotyledon stage (Balkan cultivar) and by hypersensitive reaction (HR) on tobacco leaves. The bacterium was identified serologically (agglutination test) and with molecular technique (PCR). The strains which caused bacterial blight of soybeans in Vojvodina were proved to be *P. savastanoi* pv. *glycinea*. Their cultural, biochemical and physiological characteristics are given.

Key words: *Pseudomonas savastanoi* pv. *glycinea*, soybean, bacterial blight, identification

Introduction

Bacterial blight of soybean, caused by *Pseudomonas savastanoi* pv. *glycinea*, is a common and very widespread disease of soybean both in Serbia and throughout the world. In seasons with rainy springs, the bacterial blight occurs regularly in the province of Vojvodina, often with great severity (Balaž et al. 1995). Under favourable conditions the disease may cause great damage, especially in early-maturing cultivars (Vidić and Balaž 1997).

Infected seed and plant debris are the main source of inoculum.

The symptoms can be easily seen on leaves of diseased plants, in the form of small, water-soaked spots, with bacterial exudates appearing on the underside of the leaves. A chlorotic halo appears around the spots as a result of coronatine production.

The aim of the work was to characterize the population of 10 *P. savastanoi* pv. *glycinea* isolates from leaves of soybean with bacterial blight symptoms in Vojvodina.

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Materials and methods

Isolation procedure

Leaves with characteristic bacterial blight symptoms were collected from 23 soybean fields located in nine major soybean-producing areas in Vojvodina region. Leaves were placed in paper bags and transported to the laboratory.

Isolations of the pathogen was carried out from diseased soybean leaves, using standard procedure of smearing macerated tissues across the nutritive surface (Schaad 1980, Lelliott and Stead 1987, Arsenijević 1997). Morphological, biochemical and physiological characteristics of investigated isolates were performed with conventional methods given by Lelliott et al. (1966) and Schaad et al. (2001).

Screening bacteria pathogenicity

Hypersensitivity reaction on tobacco leaves (HR)

Pathogenicity of the obtained isolates was tested on tobacco plants (*Nicotiana tabacum*) with the method of Klement (1963). Bacterial water suspension of each isolate (10^6 cfu/ml) was infiltrated into mesophyll of a fully expanded tobacco leaf. HR was observed after 24 h.

Pathogenicity to soybean seedlings

Inoculum of each isolate was prepared from 48-hour-old bacterial cultures grown on Nutrient Sucrose Agar (NSA) medium. The bacteria were suspended in sterile water and inoculum concentration was adjusted to approximately 10^8 cfu/ml with McFarlands scale. Five plants were inoculated with each isolate by spraying with aqueous bacterial suspension.

Inoculated plants were covered with polyethylene bags and incubated for 48 h in a moisture chamber (95% humidity). Afterwards plants were kept under laboratory conditions and daily observed for the occurrence of bacterial symptoms for 15 days.

During the experiments plants were watered regularly and wetted with hand sprayer. Check plants were sprayed with distilled water.

Morphological and cultural properties

Growth characteristics of the cultures in question were determined on the basis of colour appearance, size and shape of colonies developed on nutritive Meat Peptone Agar (MPA) and NSA media (Arsenijević 1997, Schaad et al. 2001).

King's medium B (King et al. 1954) was also used for green fluorescent pigment production. Fluorescence was observed under UV light.

Biochemical and physiological properties

The bacterial cultures were tested for: Gram reaction, oxidase and catalase production, presence of arginine dihydrolase, potato soft rot, O/F test, levan produc-

tion, acid production from carbohydrates, gelatine liquefication, aesculin and starch hydrolysis and reduction of nitrate (Lelliott et al. 1966, Arsenijević 1997, Schaad et al. 2001).

Gram reaction

Gram reaction was tested by mixing bacteria with a drop in 3% solution of KOH (Suslow et al. 1982).

Enzymatic activity

Presence of oxidase. Kovacs' method was used. A colony of 24–48 h grown on nutrient agar slope was applied with platinum loop and the production of a distinct purple colour in 10 s was recorded as a positive result.

Presence of catalase. A loopful of bacterial a 24–48 h growth from nutrient agar slope was smeared on a slide and covered by a drop of 20% hydrogen peroxide (Schaad et al. 2001).

Presence of arginine dihydrolase. Thornley's medium was used to detect formation of alkali from arginine compounds (medium would turn red in four days due to NH_3) under anaerobic conditions. A fresh culture was stabbed into a tube with medium, then covered with sterile parafine oil and incubated at 27°C (Thornley 1960).

Potato soft rot (Lelliott et al. 1966). Tranverse slices, 7–8 mm thick, of washed, alcohol flamed, peeled potato tubers were placed in Petri dishes and the surface of the slice flooded immediately with sterile distilled water until the water in the dish reached about halfway up the slice. Slices were inoculated heavily with growth from a nutrient agar slope into a nick made in the centre of each slice. As positive result was recorded if most of the slice had rotted.

Utilization of carbon compounds

Oxidative/Fermentative test (O/F). The basal medium used was that of Ayers, Rupp & Johnson (1919) with peptone 0.2%, NaCl 0.5%, K_2HPO_4 0.03%, Oxoid agar (No. 3) 0.35%, bromthymol blue 0.003% in distilled water, pH 7.1. A 10% (w/v) aqueous solution of sucrose was sterilized by filtration, or by momentary autoclaving, and added aseptically to the hot, sterile, basal medium to give a final concentration of 1% (w/v). The production of yellow colour which indicated acid production, in the unsealed tube, was scored as a positive reaction.

Production of levan. Plates of nutrient agar containing 5% sucrose were streaked and large, white, domed, mucoid colonies were assumed to indicate to production of levan.

Acid production from carbohydrates. The yellow colouring of synthetic base containing 1% of sugar (xylose, glucose, mannose, galactose, fructose, maltose, lactose, sucrose, starch, raffinose, inositol, dulcitol and glycerol) after 15 days of bacteria incubation indicated a positive result.

Degradation of macromolecules

Presence of gelatinase. Gelatine liquefaction was tested on nutrient gelatine containing: Bacto yeast extract 3 g, Oxoid peptone 5 g, gelatine 120 g, distilled wa-

ter up to 1000 ml. Tubes were placed at 4°C for 30 min before the results were recorded. Liquefaction was only scored as positive when the medium flowed, a very viscid type of liquefaction which flowed slowly was ignored.

Aesculin hydrolysis. Bacteria were incubated for three days at 25°C according to Lelliott and Stead (1987). The brown colouring of medium indicated positive reaction.

Soluble starch hydrolysis. Bacteria were grown on Nutrient Agar (NA) medium containing 0.2% soluble starch (w/v). The absence of clear zone around the colonies after 10 days was an indication of absence of hydrolysis.

Utilization of nitrogen compounds

Reduction of nitrate. Test of nitrate reduction involve growing the test bacteria in a nitrate medium and than testing for the presence of nitrite using specific reagent. The medium contained: peptone 10 g, K_2HPO_4 5 g, yeast extract 1 g, KNO_3 1 g, agar 2 g, distilled water up to 1000 ml. If nitrate to nitrite reduction has taken place, the surface turned red after upon addition of Follet and Ratcliff's reagent (positive test result).

Pathogen identification using serological methods

Agglutination test (express agglutination test). For serological identification express agglutination test with commercial antibodies of *Pseudomonas savastanoi* pv. *glycinea* (Express Kit, NEOGEN Europe Ltd., Scotland, UK) was used. A mix of 20 μl of test reagent (antiserum) with a small quantity of tested bacterial colonies, was placed on testing card with applicator sticks. Within 60 s positive reaction was clearly indicated as granular agglutination. In the case of negative reaction the drop of test reagent remaind transparent (no agglutination). The results obtained were compared with positive and negative controls of Express Kit.

Pathogen molecular identification by PCR

Identification of the bacterium *Pseudomonas savastanoi* pv. *glycinea* by polymerase chain reaction (PCR) is based on the multiplication of DNA fragments controlling synthesis of phytotoxin coronatine (Bereswill et al. 1994). Besides *P. savastanoi* pv. *glycinea*, there are also several other patovars of the pathogen that are capable of producing coronatine. These are *tomato*, *atropurpurea*, *maculicola* and *morsprunorum* (Cuppels et al. 1990, Wiebe and Campbell 1993).

Isolates were tested for their identity with *P. savastanoi* pv. *glycinea* with a biomolecular method of PCR according to Bereswill et al. (1994) and Schaad et al. (2001). A ready-made PCR mixture (Eppendorf Master Mix-Fermentas) for 100 reactions and a set of primers amplifying diagnostic PCR products 650 base pairs (bp) in size were used. The primer sequences were as follows:

Primer 1: 5`-GGC GCT CCC TCG CACTT-3`
Primer 2: 5`-GGT ATT GGC GGG GGTGC-3`

A coronatine-producing isolate of *P. savastanoi* pv. *glycinea* NCPPB 3318 served as control. An isolate of *Xanthomonas campestris* pv. *vesicatoria* served as a negative control and a blank (chemicals only). Whole cells were used as the DNA sample with no prior DNA isolation and a bacterial water suspension was used at 10^6 cfu/ml.

24 μl of the PCR mixture and 1 μl of bacterial suspension were added into each Eppendorf tube. The suspension and the buffer were mixed together in a homogenizer and transferred into the preheated block of the PCR apparatus.

The PCR conditions were set on the gradient PCR apparatus. The block of the machine was heated to 95°C and the eppendorfs with the samples were placed into it. The program was started after closing the lid of the machine.

DNA fragments obtained by the chain reaction were detected on a 2% agarose gel with 9 μl of ethidium bromide. 10 μl of the PCR product were applied onto each gel plate. Electrophoretic separation of the components was performed at room temperature for 60 min (constant voltage of 100 V and maximum current power of 5 V/cm). After that, the gel was viewed on a transilluminator and photographed. PCR tests in which specific amplification products 650 bp in size were detected were considered positive, provided the same product was not identified in samples used as negative control or a blank.

Results

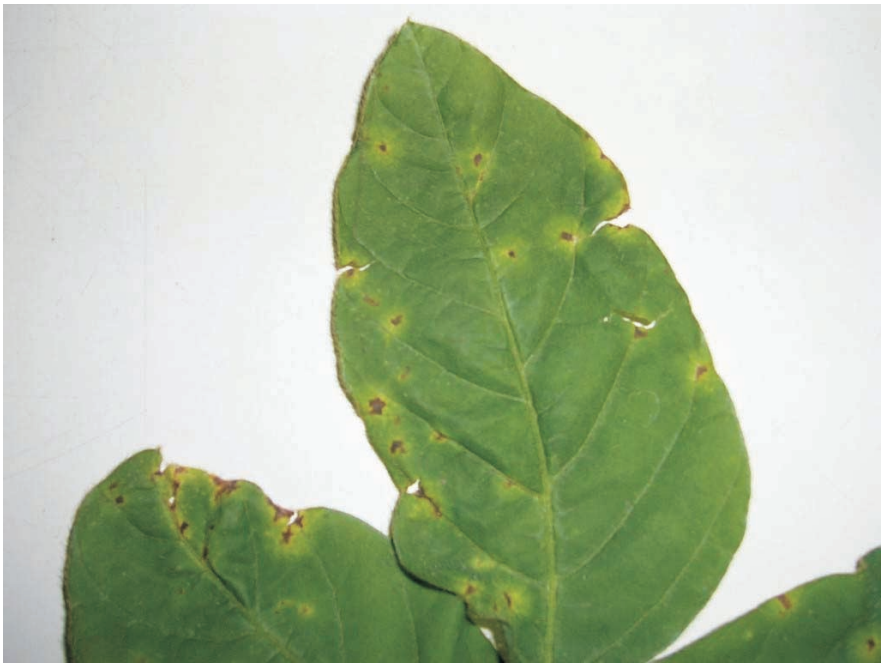
Disease symptoms

Pseudomonas savastanoi pv. *glycinea* attacks all of the above-ground plant organs of soybean, but the most typical symptoms appear on leaves and pods. Symptoms of bacterial blight of soybean can be observed on plants in the field during spring time in June, especially after a rainy period. They are easily visible on leaves in the form of small, water-soaked green spots, while on the underside of the leaves bacterial exudates can be seen. At lower temperatures, a chlorotic halo appears around the spots as the phytotoxin coronatine is produced (Phot. 1).

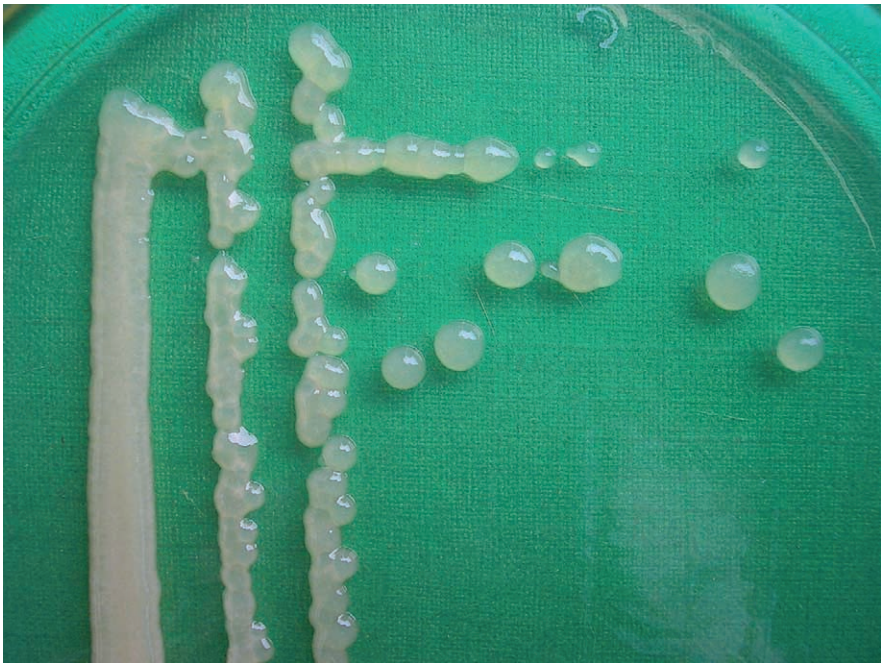
Pathogenicity

All the 10 isolates tested (Psg1-Psg10) caused necrosis of tobacco leaf tissue between veins after 24 h from its infiltration, which indicates typical HR reaction. The rapid death of plant cells lead to necrosis of the inoculated tissue with no appearance of usual watersoaked spots.

All isolates also produced typical symptoms of soybean bacterial blight on soybean seedlings, 15 days after inoculation, causing greasy and individual spots. The first symptoms appeared three days after inoculation caused by Psg5 isolate. Later tissue reaction was characteristic of Psg3 isolate inoculated (five days after inoculation) pointing out to its lower pathogenicity. No differences in pathogenicity were found between other isolates.



Phot. 1. Symptoms on soybean leaf (photo by M. Ignjatov)



Phot. 2. Bacterium colonies on nutrient agar (photo by M. Ignjatov)

Reisolations of Psg-like bacteria from the inoculated soybean plants were positive in all cases.

Phenotypic characteristics of Psg-like isolates

Three to four days after the isolation, large, shiny, white, and bulging colonies of bacteria were formed on the nutrient medium containing sucrose (NSA) (Phot. 2).

Table 1

Phenotypic characteristics of Psg-like isolates

Biochemical test	Reference strain NCPPB 3318	Investigated strains Psg1-Psg10	Schaad 1980, Schaad et al. 2001
Gram reaction – 3% KOH test	+	+	+
LOPAT test			
levan	+	+	+
oxidase	–	–	–
potato soft rot	–	–	–
arginine dihydrolase	–	–	–
HR on tobacco	+	+	+
Acid is produced from:			
xylose	+	+	+
glucose	+	+	+
mannose	+	+	+
galactose	+	+	+
fructose	+	+	+
maltose	–	–	–
lactose	–	–	–
sucrose	+	+	+
dextrine	–	–	–
raffinose	+	+	+
inositol	+	+	+
dulcitol	–	–	–
Hydrolysis of:			
gelatin	–	–	–
aesculin	–	–	–
starch	–	–	–
Reduction of nitrate	–	–	–
O/F test	O	O	O
Catalase	+	+	+

10 of the isolates were chosen for further study (Psg1-Psg10). Morphological, biochemical and physiological features of studied isolates and reference strain NCPPB 3318 were similar. All isolates were aerobic and oxidase and arginine dihydrolase negative. The 10 isolates formed catalase, hydrolized aesculin and starch, did not liquiefy gelatine or reduce nitrate. All isolates produced acid from: glucose, galactose, xylose, arabinose, mannose, saccharose, rafinose and inositol (Table 1).

The results of LOPAT tests demonstrated that the investigated isolates belonged to Ia group of fluorescent plant pathogenic *Pseudomonas*.

Agglutination test (express agglutination test)

The agglutination test gave positive reaction for all the isolates tested, so we can conclude that they all represented *Pseudomonas savastanoi* pv. *glycinea* (Fig. 1).

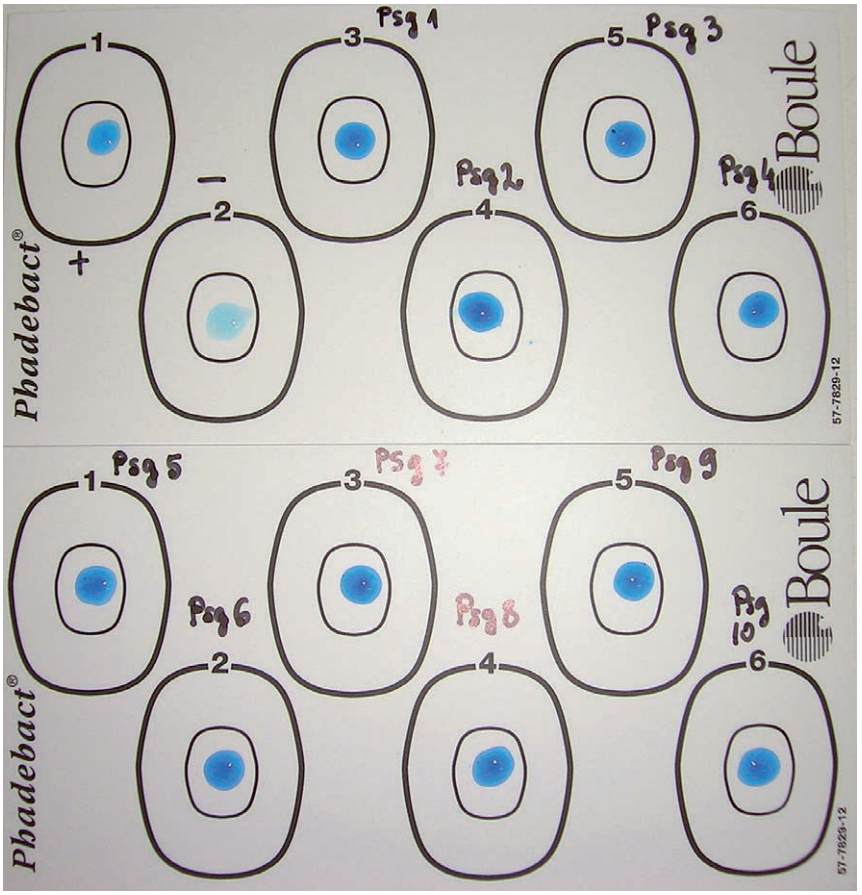
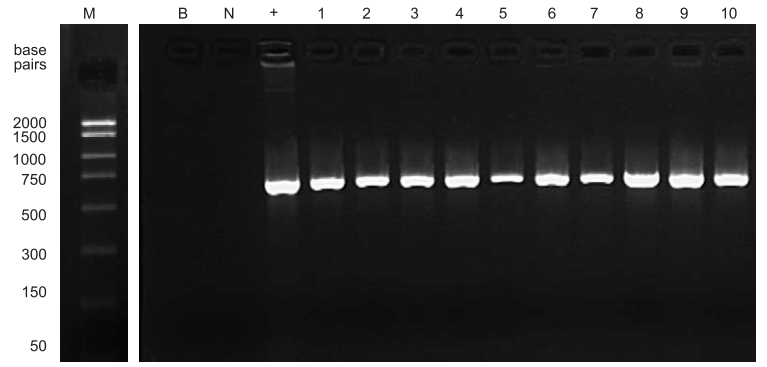


Fig. 1. Positive results clearly indicated as granular agglutination (1+, Psg1-Psg10); no agglutination in the negative control (2–)

Molecular identification method using PCR

In samples of all the isolates a nucleic acid fragment 650 base pairs in size and specific to *Pseudomonas savastanoi* pv. *glycinea* strains producing coronatine was detected (Phot. 3).



Phot. 3. 1.5% agarose gel and products of amplification of specific DNA fragment of *cfl* gene 650 bp: M – PCR marker, 50–2000 bp, B – blank, N – negative control, + – positive control (*Pseudomonas savastanoi* pv. *glycinea* NCPPB 3318), 1–10 – samples (Psg1-Psg10, respectively) (photo by M. Ignjatov)

Discussion

All the isolates studied, originating from diseased soybean plants, belonged to *Pseudomonas savastanoi* pv. *glycinea*. They were very homogeneous in morphological, physiological and biochemical characteristics and did not differ from the reference strain NCPPB 3318 obtained from National Collection of Plant Pathogenic Bacteria, United Kingdom.

Pathogenicity test on tobacco showed that all the tested isolates caused hypersensitive reaction after 24 h, as the consequence of incompatible relationship between pathogen and host. Pathogenicity test on host plants showed that all the isolates caused characteristic greasy and individual spots on cotyledons. The optimum temperature for typical symptoms development, spots with yellowish-green halo resulting from coronatine synthesis is 18°C (Budde and Ullrich 2000), which is important for incubation of plants after artificial inoculation. Moriwaki et al. (1996) studied 25 *P. savastanoi* pv. *glycinea* isolates and found that in seven of them formation of spots was accompanied by the appearance of a chlorotic halo.

All the isolates produced acid from xylose, glucose, mannose, galactose, fructose, sucrose, raffinose, inositol and dulcitol, as it was earlier described by other authors (Schaad 1980, Fahy and Persley 1983, Lelliott and Stead 1987). All the isolates metabolized glucose oxidatively, they were catalase positive, did not hydrolyse gelatine, aesculin or starch and did not reduce nitrate.

On the basis of LOPAT tests results it was concluded that tested isolates had characteristics of Ia fluorescence group of phytopathogenic bacteria belonging to *Pseudomonas*. The results are in agreement with the results reported by Lelliott et al. (1966) and Arsenijević (1997).

Antigenes of tested bacterial isolates were linked to antibodies of *P. savastanoi* pv. *glycinea*, forming large molecular aggregates visible as granular sediments. Positive reaction was clearly indicated as granular agglutination with all the isolates. Identification of phytopathogenic bacteria by conventional methods requires a lot of materials and labour and is time-consuming. In an agglutination test, the antibody is coated on the surface of an inert carrier particle, and a positive antigen-antibody reaction results in clumping/agglutination of the carrier particles which can be visualized by the naked eye or under a microscope. Agglutination tests are more sensitive than other precipitation tests and can be carried out with lower concentrations of reagents than that necessary for precipitation tests (Koenig et al. 1979, Walkey et al. 1992, Hughes and Ollennu 1993). Although the precipitation and agglutination tests lack the sensitivity of other serological assays, they are excellent methods for detecting viruses and bacteria that occur in a reasonable concentration in plants.

Bender et al. (1989) found in their studies that the gene responsible for coronatine synthesis was located on a specific bacterial plasmid. Later, Bereswill et al. (1994) developed a molecular method for bacteria identification based on the chain multiplication of DNA fragments responsible for the synthesis of the phytotoxin coronatine. Some more recent studies indicate that there are *P. savastanoi* pv. *glycinea* strains that have no gene for the production of coronatine, which creates a possibility of obtaining false negative results with this method (Zaccardelli et al. 2003). Although the latest studies suggest the existence of so-called Cor^- strains with no gene for coronatine production, such strains were not identified in our study. The biomolecular method of polymerase chain reaction was used for the first time here to determine isolates obtained from commercial soybean cultivars in Vojvodina.

On the basis of pathogenic, morphological, biochemical, physiological, serological and molecular characterization, we concluded that the studied isolates belonged to *Pseudomonas savastanoi* pv. *glycinea*.

The development of molecular biology techniques and introduction of molecular methods for rapid pathogen identification is particularly important in the case determining quarantine and economically important plant pathogens, transmitted by seed, since their presence cannot be reliably determined otherwise in a short time. The method has the advantage of being quick and reliable and thus able to meet the demands and requirements of modern trends in agriculture.

Streszczenie

CHARAKTERYSTYKA IZOLATÓW *PSEUDOMONAS SAVASTANOI* PV. *GLYCINEA* Z WOJWODINY

W 2005 roku wyizolowano z zakażonych liści kilku odmian soi patogen powodujący bakteryjną plamistość soi, groźną i często występującą w Wojwodinie chorobę. Patogeniczność uzyskanych izolatów oraz izolatu wzorcowego (National Collection of Plant Pathogenic Bacteria, United Kingdom – NCPPB 3318) wykazano w doświadczeniach infekcyjnych na roślinach soi w stadium liścieni ('Balkan') oraz na podstawie reakcji nadwrażliwości (HR) na liściach tytoniu. Badane izolaty scharakteryzowano metodami biochemiczno-fizjologicznymi. Identyfikację bakterii potwierdzono metodą serologiczną (test aglutynacji) z przeciwciałami *Pseudomonas savastanoi* pv. *glycinea* NEOGEN Europe Ltd., Scotland, UK, oraz metodą molekularną (PCR) – na podstawie wytwarzania fitotoksyny koronatyny.

Wszystkie badane izolaty, powodujące bakteryjną plamistość soi w Wojwodinie, należały do *P. savastanoi* pv. *glycinea*.

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