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A NEW BACTERIAL DISEASE ON BLUBERRY (*VACCINIUM CORYMBOSUM*) CAUSED BY *PSEUDOMONAS* SPP.

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Abstract: In 2011, leaf spot disease was observed on the blueberry (*Vaccinium corymbosum*) cv. Nelson growing on a commercial field located in Central Poland. The disease symptoms could be seen as russet brown, irregular spots. The diameter of the spots was 0.3–0.5 cm, and the spots often coalesced. From these leaf spots, a fluorescent bacterium was repeatedly isolated in almost pure culture. Polymerase chain reaction (PCR) using primers Ps-for and Ps-rev, specific for *Pseudomonas* spp. confirmed that they belong to this genus. Based on LOPAT tests [levan production from sucrose (L), presence of oxidase (O), pectolytic activity on potato (P), the presence of arginine dihydrolase (A), hypersensitivity reaction on tobacco (T)], 6 isolates were classified to the LOPAT group Ib – group of *Pseudomonas syringae* subsp. *savastanoi* and *Pseudomonas delphini*, and one isolate to group Ia – *P. syringae*. All isolates caused a hypersensitivity reaction on tobacco plants, and symptoms similar to those under natural conditions, when young leaves of blueberry cv. Nelson were inoculated. Sequence analysis of 16S rRNA and *rpo*B genes showed the highest similarity of 6 studied strains to the species *P. avellanae*. Further taxonomic study is necessary to enable definitive classification of these isolates. It is the first time that a bacterial disease caused by the *Pseudomonas* spp. was observed in Poland.

Key words: pathogencity test, *Pseudomonas* spp., *rpo*B, *Vaccinium corymbosum*, 16S rRNA

INTRODUCTION

The cultivation of blueberry (*Vaccinium corymbosum*) is becoming increasingly important in Poland. This is due to the attractiveness of the fruit, the high demand of the Polish market and the demand from abroad, and therefore the profitability of the crop. The acreage of blueberry in Poland has increased every year, and at present is estimated to be over 3,000 hectares. For many years blueberry plants have been considered resistant or tolerant to diseases. Intensive chemical protection was not required for blueberry in Poland. However, nowadays, both the disease and pests are becoming an increasing problem in the cultivation of this plant species. Fungal diseases like fusicoccum canker (*Godronia cassandrae*), gray mold (*Botrytis cinerea*), brown rot (*Monilinia vaccinii-corymbosi*), and anthracnose (*Colletotrichum acutatum*) are considered to be the most dangerous diseases of blueberry in Poland. *V. corymbosum* is very rarely infected by bacterial pathogens which can affect the yield. The only bacterial pathogen described in Poland is tumorogenic *Agrobacterium* spp. causing crown gall (Puławska 2011). In the United States and other countries, the bacterial canker caused by *Pseudomonas syringae* was observed (http://blueberries.msu.edu/uploads/files/Bacterial canker.pdf; Vaughan 1956; Gierrero and Lobos 1989; Dicarlo and Punja 2012). Also, as reported in New Jersey, USA, the causal agent of bacterial leaf spot *Pseudomonas andropogonis* can affect growth of blueberry cuttings, contributing to poor establishment of plants during the first year of growth (Kobayashi *et al*. 1995). Moreover, in Georgia, USA, the bacterial leaf scorch caused by the new emerging pathogen *Xylella fastidiosa* resulted in abnormally developed shoots with a reduced number of flower buds (Chang *et al*. 2009).

Monitoring conducted in 2011 on a 5 ha of high bush blueberry field, showed the presence of spots on leaves of the blueberry cv. Nelson. The symptoms observed were brown regular and irregular elongated spots with a diameter of approximately 0.3–0.5 cm, often along the midrib, and sometimes merging with each other. Two types of spots were observed: brown spots with black necrotic tissue in the centres, and brown rust spots without black necrosis in the middle of the tissue (Fig. 1).

The aim of this study was to isolate, characterise, and identify the causal agent of blueberry leaf spot.

MATERIALS AND METHODS

Isolation of bacteria

From the samples of diseased leaf tissue of blueberry cv. Nelson, the pieces of tissue from between the border of the healthy and diseased tissue, were surface sterilized with 70% ethanol and macerated in sterile distilled water. The resulting mixture was transferred to the microbiological culture media: YNA (0.5% yeast extract, nutrient agar 2.3%, Difco) and King's B (King *et al*. 1954), and incubated at 27°C for 3 days.

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Phenotypic characteristics

For all bacterial isolates obtained, the Gram reaction with 3% KOH (Suslov *et al*. 1982), LOPAT tests [levan production from sucrose (L), presence of oxidase (O), pectolytic activity on potato (P), the presence of arginine dihydrolase (A), hypersensitivity reaction on tobacco (T)], and catalase production (Lelliott and Stead 1987; Schaad 2001) were determined. The reference strain *P. s.* pv. *syringae* LMG1247^T was included in all tests.

Preliminary identification of isolates by polymerase chain reaction (PCR)

Genomic DNA of bacteria was isolated according to the method of Aljanabi and Martinez (1997) with modification described by Kałużna *et al*. (2012) and then it was tested in PCR reaction with primers Ps-for and Ps-rev (Widmer *et al*. 1998) specific to bacteria belonging to the *Pseudomonas* genus, according to the reaction conditions as originally described. The reference strain *P. s.* pv. *syringae* LMG 1247^T was included for comparison.

Pathogenicity test

The pathogenicity of isolates was tested on leaves of 2-year-old blueberry plants cv. Nelson. Young leaves were inoculated with a 10^7 cells/ml suspension of a 2-day-old culture from King's B by: 1) infiltration of the leaf using a hypodermic syringe and 2) by cutting the tips of the leaves with scissors previously dipped in the bacterial suspension. The infiltrated leaves and cut tips were subsequently incubated under high humidity (plants were covered with plastic bags) at room temperature in a greenhouse. One plant inoculated with sterile distilled water was used as the negative control. The formation and development of disease symptoms were observed up to 8 weeks after inoculation. Re-isolations were made from infected tissue. Re-isolates were confirmed to be the same *Pseudomonas* species by colony morphology, phenotypic characters, and PCR.

Identification of isolates based on sequence analysis of 16S rRNA and *rpo*B genes

All *Pseudomonas* isolates were identified by sequence analysis of 16S rRNA and *rpo*B genes. The primers fD1 and 800r (Weisburg *et al*. 1991; Drancourt *et al*. 1997) were used for amplification of 16SrRNA. Primers LAPS and LAPS27 (Ait Tayeb *et al*. 2005) were used for *rpo*B with PCR conditions described in the above noted papers. The sequences obtained were assembled using the SeqMan Lasergene package (DNASTAR, Inc., Madison, WI) and compared with sequences deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Dendrograms including most closely related species and pathovars were constructed using the method of Maximum Likelihood with the MEGA5 program (Tamura *et al*. 2011).

PCR with primers specific for *P. avellanae* and *P. s.* pv. *actinidiae*

Based on the results obtained from the analysis of the 16S rRNA and *rpo*B gene sequences, the specific primers for *P. avellanae* (*Pa*) (Scortichini and Marchesi 2001) and *P. s.* pv. *actinidiae* (*Psa*) (Rees-George *et al*. 2010) were chosen for further identification using PCR.

RESULTS AND DISCUSSION

Seven fluorescent bacterial isolates were obtained from those blueberry leaves exhibiting disease symptoms. The isolates' fluorescence and colony morphology on King B media indicated that they belonged to the genus *Pseudomonas*. However, after a few days, it was observed that 6 of them produced brown pigment on this medium. For this reason, further identification steps

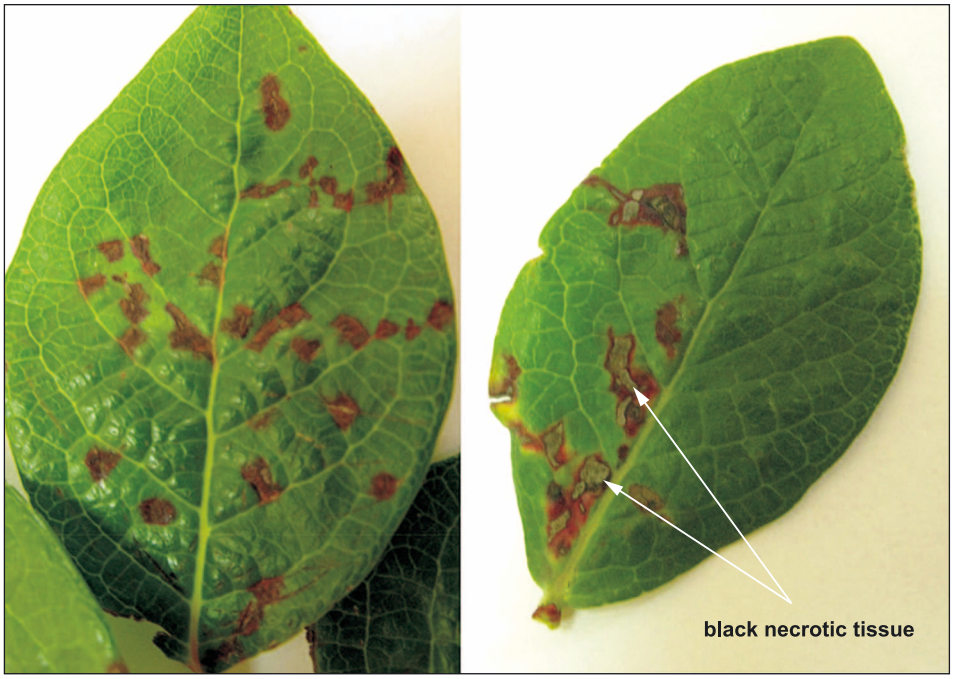

Fig. 1. Disease symptoms on blueberry leaves cv. Nelson: on the left are symptoms caused by isolates belonging to the LOPAT group Ib, on the right are symptoms caused by the isolate of the LOPAT group Ia

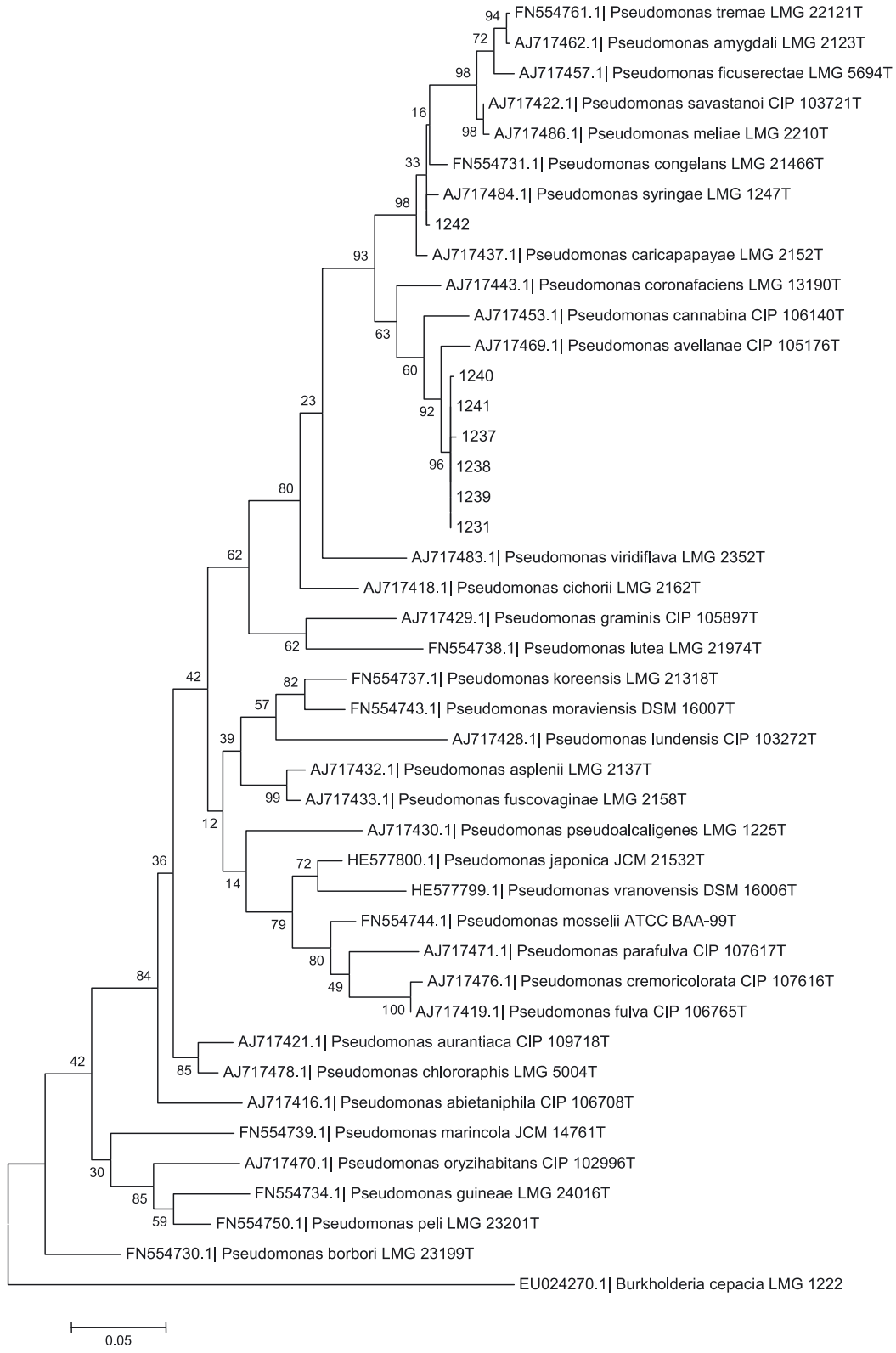

Fig. 2. Maximum likelihood tree of *Pseudomonas* isolates obtained from blueberry and the closest neighbor of other species, based on the analysis of the *rpo*B gene. Bootstrap values are indicated at each node. As an outgroup, the sequence of *B. cepacia* strain LMG 1222^T was used

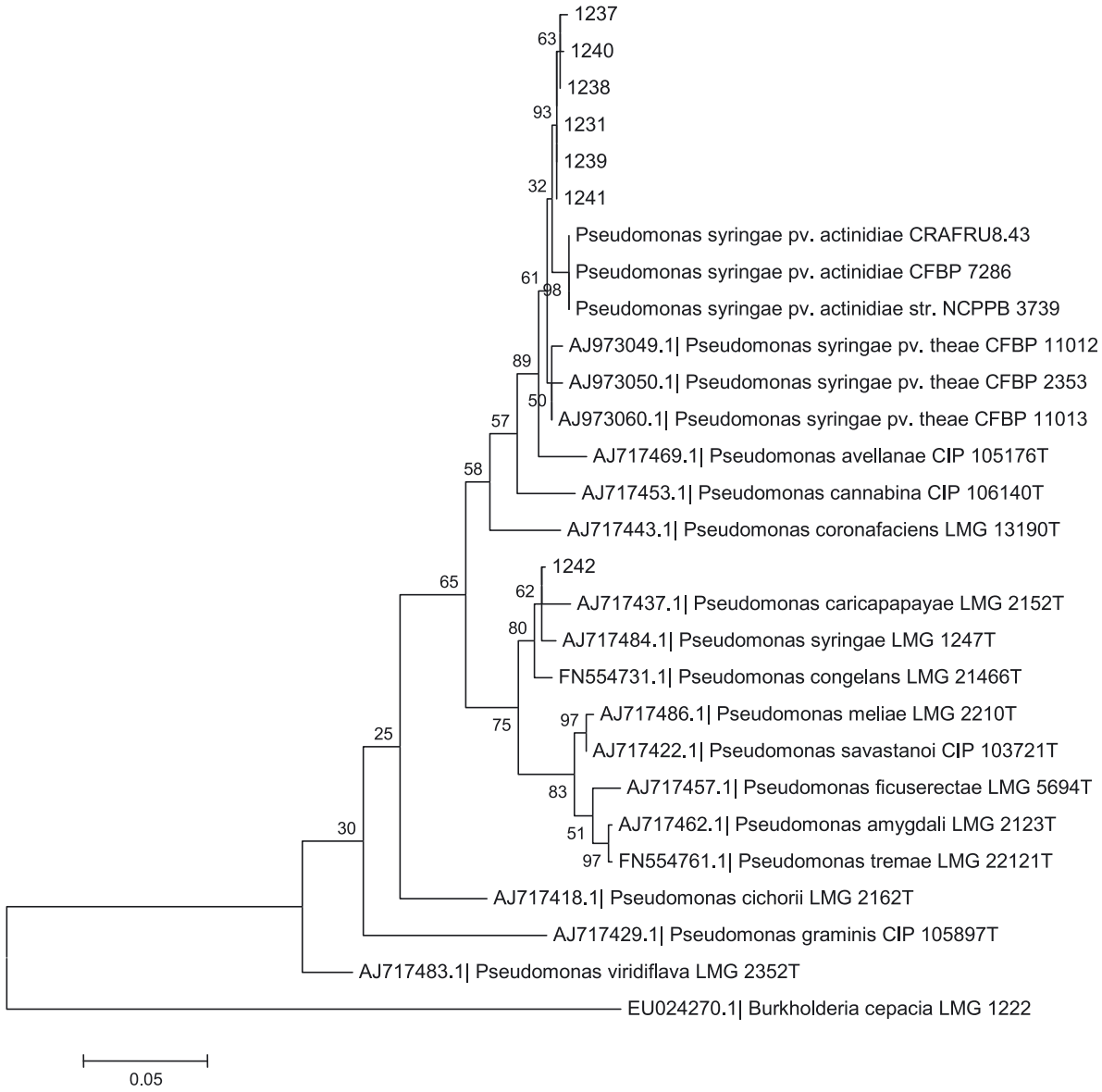

Fig. 3. Maximum likelihood tree of *Pseudomonas* isolates obtained from blueberry and the closest neighbor of other species and pathovars, based on the analysis of the *rpo*B gene. Bootstrap values are indicated at each node. As an outgroup the sequence of *B. cepacia* strain LMG 1222^T was used

including phenotypic and genetic analysis were undertaken. Phenotypic tests showed that all selected isolates were: Gram-negative, induced hypersensitivity reactions on tobacco leaves cv. Samsun, were negative for the oxidase test, arginine dihydrolase and did not produce pectolytic enzymes. Only one of them produced levan from sucrose. On this basis, one isolate IO 1242 was included into group Ia – the species of *P. syringae*, whereas IO 1231, IO 1237, IO 1238, IO 1239, IO 1240 and IO 1241 were included into group Ib containing *P. s.* subsp. *savastanoi* and *P. delphini* according to Lelliott *et al*. (1966). The isolate IO 1242 was obtained from the leaves with brown spots with black necrotic tissue in the centre. The other six isolates belonging to the Ib group, originated from samples with brown rust spots without black necrotic tissue (Fig. 1). DNA of isolates amplified with primers Ps-for and Ps-rev, gave a characteristic product of 990 bp, which confirmed that all isolates belonged to the genus *Pseudomonas* (Widmer *et al*. 1998).

The pathogenicity test performed on young plants of blueberry cv. Nelson confirmed that these isolates caused similar necrotic spots on the inoculation site as were observed for natural infections. Isolate IO 1242, however was more virulent. After inoculation, the disease symptoms appeared more quickly. From the diseased tissue, the bacteria with a similar colony morphology as those used for the inoculation were isolated. Phenotypic tests and PCR were done to identify the re-isolates .

Analysis of the 16S rRNA gene sequence showed that the six LOPAT Ib isolates had the highest similarity with *P. avellanae* (98.5–99.4%), the causal agent of bacterial canker and decline of hazelnut (*Corylus avellana* L.) (Janse *et al*. 1997), and that isolate IO 1242 matched with *P. s.* pv. *syringae* (99.5% sequence similarity).

Sequence analysis of the *rpo*B gene, which belongs to the group of housekeeping genes recently recommended in the determination of new bacterial species, confirmed the results of the 16S rDNA sequence analysis as shown

on figure 2. Moreover, phylogenetic analysis of the 16S rDNA and *rpo*B sequences of blueberry isolates, showed that the six LOPAT group Ib isolates are distinct from all known taxa within the *Pseudomonas* genus. An additional dendrogram using sequences of strains belonging to pathovars of *P. syringae,* showed a closer similarity of these strains to *P. s.* pv. *actinidiae* (*Psa*) causing bacterial canker disease of kiwifruit and pv. *theae,* the causal agent of bacterial shoot blight on tea plants, than to *P. avellanae* (Fig. 3). Isolate IO 1242 was found to group with *P. s.* pv. *syringae*.

Reactions carried out with two primer pairs specific for *P. avellane and P. s.* pv. *actinidiae,* gave positive results for the group of six strains with both primer sets. It is known from the literature (Scortichini and Marchesi 2002), that primers which are supposed to be specific for *P. avellanae* also amplify DNA of *P. s.* pv. *actinidiae* and pv. *theae*. Similarly, in the case of primers for *Psa*, it was reported that DNA of bacteria of pv. *theae* was also amplified (Rees-George *et al*. 2010). Therefore, more taxonomic research on these isolates has to be performed for reliable classification and identification.

The disease symptoms listed in the USA and British Columbia, Canada (http://blueberries.msu.edu/uploads/files/Bacterial canker.pdf; Dicarlo and Punja 2012) are mainly brown cankers on shoots from the apex of the shoot and extending further (only occasionally spots on the leaves). In our case, only symptoms on the leaves were observed. In the case of *P. antropogonis* (Kobayashi *et al*. 1995), the leaf spot similar to the symptoms observed in our study, was found sporadically on a few varieties but not on cv. Nelson. The distinct symptom – marginal leaf scorch (burn) of the older leaves, was observed for the disease caused by *Xylella fastidiosa* (Chang *et al*. 2009; Janse and Obradovic 2010).

In conclusion, the results obtained allowed us to identify and characterise seven pathogenic *Pseudomonas* isolates from blueberry as belonging to *Pseudomonas* LOPAT group Ia (1 isolate) and Ib (6 isolates). All of them caused leaf spot disease which was previously unknown in Poland. The one isolate IO 1242, in the Ia group, was classified to *P. s.* pv. *syringae* but the taxonomic position of the Ib group isolates should be studied in the future.

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