METHODS AND PROTOCOLS

Development of SCAR markers for rapid and specific detection of *Pseudomonas syringae* pv. *morsprunorum* races 1 and 2, using conventional and real-time PCR

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Abstract  Specific primers were developed to detect the causal agent of stone fruit bacterial canker using conventional and real-time polymerase chain reaction (PCR) methods. PCR melting profile (PCR MP) used for analysis of diversity of *Pseudomonas syringae* strains, allowed to pinpoint the amplified fragments specific for *P. syringae* pv. *morsprunorum* race 1 (Psm1) and race 2 (Psm2), which were sequenced. Using obtained data, specific sequence characterised amplified region (SCAR) primers were designed. Conventional and real-time PCRs, using genomic DNA isolated from different bacterial strains belonging to the *Pseudomonas* genus, confirmed the specificity of selected primers. Additionally, the specificity of the selected DNA regions for Psm1 and Psm2 was confirmed by dot blot hybridisation. Conventional and real-time PCR assays enabled accurate detection of Psm1 and Psm2 in pure cultures and in plant material. For conventional PCR, the detection limits were the order of magnitude ~10⁰ cfu/reaction for Psm1 and 10¹ cfu/reaction for Psm2 in pure cultures, while in plant material were 10⁰–10¹ cfu/reaction using primers for Psm1 and 3 × 10² cfu/reaction using primers for Psm2. Real-time PCR assays with SYBR Green I showed a higher limit of detection (LOD) – 10⁰ cfu/reaction in both pure culture and in plant material for each primer pairs designed, which corresponds to 30–100 and 10–50 fg of DNA of Psm1 and Psm2, respectively. To our knowledge, this is the first PCR-based method for detection of the causal agents of bacterial canker of stone fruit trees.

Keywords Dot blot hybridisation · Stone fruit tree pathogens · PCR MP · SCAR primers · Real-time PCR

Introduction

Bacterial canker of fruit trees occurs in stone fruit growing areas all over the world (Agrios 2005). In Poland, the disease incidence on stone fruit trees orchards is observed every year with different intensity and is becoming more economically significant. Moreover, in the last vegetative seasons, bacterial canker was dangerous not only to stone fruit trees, but also to apple and pear trees. The causal agents of the disease belong to the polyphagous *Pseudomonas syringae* species, able to infect more than 180 plant species, both annual and perennial, including fruit trees, ornamental plants and vegetables. *P. syringae* affects all organs of the aboveground parts of trees (i.e. the branches and main trunk as well as buds, blossoms, leaves and fruits), which causes reduction of yield and sometimes leads to death of the trees.

*P. syringae* is composed of plant pathogens divided into 60 pathovars (Young 2010) belonging to nine genomospecies, as determined by DNA:DNA hybridisation (Gardan et al. 1999). On King’s B medium, the majority of these bacteria produce a fluorescent pigment visible under UV light (King et al. 1954). Bacteria that cause bacterial canker on stone fruit trees belong to three genomospecies (gs): gs 1—*P. syringae* pv. *syringae* (Pss); gs 2—*P. syringae* pv. *morsprunorum* race 1 (Psm1); and gs 3—*P. syringae* pv. *morsprunorum* race 2 (Psm2),
P. syringae pv. avii (Psa) and P. syringae pv. persicae (Psp) (reviewed in Bultreys and Kałużna 2010). In Poland, three taxa were already described as present: Pss, Psm1 and Psm2. Recently, the new atypical taxon including bacteria that infect only cherries (mainly sour cherry) was also found (Kałużna data not published).

The diagnostics of bacterial canker are commonly based on isolation and phenotypic characterisation of the causal agent, including pathogenicity (Bultreys and Gheysen 1999; Vicente et al. 2004). The phenotypic tests LOPAT (Lelliott et al. 1966), GATTa and L-lactate utilisation (Lattore and Jones 1979) enable the determination of morphological, physiological and biochemical features of the bacteria. These features are used for identification of species and their discrimination into pathovars and races. However, this methodology requires the implementation of a high number of often laborious and time-consuming tests. Moreover, the obtained results can sometimes be ambiguous or difficult to interpret, and they are often not sufficient for proper strain classification (Vicente et al. 2004).

Concerning serological methods, the slide agglutination test, immunofluorescence and indirect-enzyme-linked immunosorbent assay (ELISA), with the antisera produced from live whole-cell antigens, were widely adopted for routine bacterial identification. However, nowadays these methods are less frequently used for the identification of bacteria that cause bacterial canker because of frequent cross-reactions with non-pathogenic bacteria. Furthermore, serological tests do not always provide a response in distinguishing isolates of P. syringae (Vicente et al. 2004).

Molecular methods are currently the most widely adapted and are considered very useful for the identification of bacterial canker causal agents and for studying their genetic diversity. For many years, the identification of the pathogen has been based on detection of genes encoding the toxins coronatine, syringomycin and the siderophore yersiniabactin (Bereswill et al. 1994; Sorensen et al. 1998; Bultreys and Gheysen 1999). However, it should be noted that the determination of presence of genes encoding for toxin production is not reliable for identification in itself and thus cannot be the only criterion for the classification of strains. In fact, strains of Psm1 and Pss, which do not have the ability to produce coronatine or syringomycin, respectively, are quite common (Ullrich et al. 1993; Renick et al. 2008; Kałużna et al. 2010a). On the other hand, although production of the siderophore yersiniabactin is now considered a stable feature of all Psm2 strains and could be a criterion for their identification, is should be mentioned that it is not an exclusive feature of strains of Psm2, since positive amplification with primers for the irp1 gene (encoding this siderophore) was also confirmed in other pathovars of P. syringae, including the following: antirrhini, apiii, berberidis, delphinii, lachrymans, passiflorae, persicae, tomato, viburni, helianthi, tagetis and theae (Bultreys et al. 2006).

In recent years, fingerprinting methods have been widely applied for the identification and genotyping of P. syringae through the analysis of repetitive regions (i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC), BOX, Repetitive Extragenic Palindromic Elements (REP) and Insertion Sequence (IS50) sequences) (Ullrich et al. 1993; Weingart and Völksch 1997) and through PCR MP (Kałużna et al. 2010b). However, it should be taken into account that all fingerprinting methods require inclusion of the reference strains for comparison of obtained amplification patterns (Vicente and Roberts 2007; Gilbert et al. 2009), and, in the case of heterogeneous strains of Pss (Vicente et al. 2004; Renick et al. 2008; Kałużna et al. 2010a, b), it is difficult to determine affiliation of analysed strains to this taxon.

Despite the availability of different approaches for characterisation and genotyping of P. syringae, they require time-consuming and labour-intensive classical microbiological methods or complex analyses including comparison of amplification patterns and housekeeping gene sequencing. Therefore, there is still the need to develop a rapid and specific method of diagnosis that would allow the detection and identification of the causal agent of stone fruit bacterial canker (López et al. 2010). This specific, fast diagnostic system would be invaluable in the study on etiology of cankers on trunks and branches, which are similar to those caused by fungi of the genus Leucostoma (Valsa) and Monilinia, and also necrotic spots on leaves, which may be mistaken with those caused by Prunus necrotic ring spot virus or Clasterosporium carphophilum, especially late in the growing season. Moreover, the occurrence of gummosis on woody tissue often associated with bacterial infection may be related to the physiological response of the trees to damage caused by abiotic factors, such as frost, sunburn, periodic water flooding or mechanical damage, and is not due to biotic factors only (Saniewski et al. 2006).

Ideally, a novel diagnostic system would apply specific primers and the PCR technique, both conventional and real-time, making them more useful for a wide group of researchers according to available lab equipment, which allows for the detection and identification of the pathogen within a short amount of time. Additionally, such a system would undoubtedly be very useful in enforcing appropriate programmes to prevent and control disease occurrence in nurseries and orchards of stone fruit trees, especially sweet and sour cherry, where the damage is the most severe.

The aim of this study was to design and validate novel specific primers and to develop conventional and real-time PCR-based methodologies for rapid and specific detection of Psm1 and Psm2, with the aim of enhancing bacterial canker diagnostic procedures.
Materials and methods

Bacterial strains

Species and pathovar identification of previously uncharacterised *Pseudomonas* strains from our collection, obtained from stone fruit trees in Poland, was determined on the basis of phenotypic tests (i.e. Gram reaction with 3 % KOH (Suslow et al. 1982), LOPAT (Lefliott et al. 1966), GATTa and L-lactate utilisation (Lattore and Jones 1979). A total of 168 isolates were analysed. The reference strains *P. syringae* pv. *syringae*—LMG 1247, *P. syringae* pv. *morsprunorum* race 1—LMG 2222 and *P. syringae* pv. *morsprunorum* race 2—CFBP 3800 were included in all tests (Table 1). Additionally, type and non-type strains of other *P. syringae* pathovars (79) and related species (three) were included in the analysis (Table 2). The strains were kept at −75 °C in a mixture of glycerol (200 μl/ml) and phosphate-buffered saline (PBS) and streaked on King’s B medium (3.8 % *Pseudomonas* Agar F Difco, 1 % glycerol) (King et al. 1954) for routine culturing.

DNA isolation

Bacterial DNA was isolated using the method described by Aljanabi and Martinez (1997), with slight modifications described by Kaužna et al. (2012). DNA was diluted to a final concentration of 10 ng/μl and kept at −20 °C for further analysis.

PCR melting profile

A slightly modified method of PCR MP described by Masny and Plucienniczak (2003) was used. An amount of 100 ng of DNA from 23 *Pseudomonas* strains (Figs. 1 and 2) was digested with *Pst*I endonuclease (10 U/μl; Promega Corporation, Madison, WI, USA) or *Taq*I (10 U/μl; Thermoscientific, Vilnius, Lithuania) according to the manufacturer’s instructions. Digested DNA was ligated with two oligonucleotides forming an adaptor: DNA digested by *Pst*I endonuclease with a *Pst*I adaptor—5′-TGTACGAGTCTCAG-3′/5′-CTCGTAGACTGCATGAC-3′ (Waugh et al. 1997) and DNA digested by *Taq*I endonuclease with a *Taq*I adaptor—5′-GACGATGAGTCCTGAC-3′/5′-CGTGTCAGACTCATGCAG-3′ (Ajmone-Marsan et al. 1997). PCR amplification was performed separately for *Pst*I- or *Taq*I-digested DNA in a 25-μl reaction mixture containing the following: 1 μl of ligation mixture; 0.4 μl of GoTaq DNA polymerase (Promega, Madison, WI, USA) for *Pst*I and 0.4 μl of Dream Taq Green DNA Polymerase (Thermo Scientific, Vilnius, Lithuania) for *Taq*I; and 1× of appropriate Taq polymerase buffer, 0.2 mM of dNTPs and 1 μM of each primer (PstI-0—5′-GACTGCGTACATGCAG-3′ for *Pst*I-digested DNA (Waugh et al. 1997) or TaqI-0—5′-GACGATGAGTCTCCTGAC-3′ for *Taq*I-digested DNA (Ajmone-Marsan et al. 1997)). The amplification reactions were conducted in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany) with the following conditions: initial step of 72 °C for 5 min; 30 cycles at 86.5 °C for *Pst*I and 83 °C for *Taq*I for 40 s, 55 °C for 40 s and extension at 72 °C for 90 s; and final extension at 72 °C for 10 min. PCR products from each reaction and the O’GeneRuler 100-bp DNA Ladder Plus (Thermo Scientific, Vilnius, Lithuania) were separated on a 1.5 % agarose gel in 0.5× TBE buffer (0.045 M tris-boric acid, 0.001 M EDTA, pH 8.0) and electrophoresis was run at 5–7 V/cm of gel. After staining with an ethidium bromide solution (0.5 μg/ml), the obtained amplification profiles were visualised under UV light. The same conditions were used in all subsequent electrophoroses.

Selection of specific fragments

Based on the results of genetic analyses using PCR MP, DNA fragments characteristic of *Psm1* and *Psm2* strains were selected. The fragments were excised from the gel, purified with the DNA AxyPrep Gel Extraction Kit (Axygen Scientific, Inc. Union City, CA, USA) and cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The resulting ligation mixture was used to transform *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA). The cloned fragments were sequenced with universal primers M13Rev 5′-CAGGAAACAGCTATGAC-3′ and M13 (−40) 5′-GTTTTCCCAGTCAGTAC-3′ at Genomed S.A. (Warsaw, Poland). The sequences obtained were assembled using the SeqMan software package LASERGENE (DNASTAR, Madison, USA).

Design of SCAR primers

The sequences of specific fragments for *Psm1* and *Psm2* were used to design the SCAR primers, for both conventional and real-time PCR, with the PrimerSelect programme of the LASERGENE package (DNASTAR). Different primer pairs were designed for conventional PCR (five for *Psm1* and 7 for *Psm2*) and real time PCR (four for each taxa). All primer sequences and their potential amplification reaction products were checked for homology (June 2015) to other sequences deposited in the GenBank database using the ‘blastn’ algorithm (Altschul et al. 1997). Selected primers were synthesised at Genomed S.A.
| Lp. | Strain number | Place (voivodeship/country) and year of isolation | Host-plant | Taxon based on LOPAT, GATTa/L |
|-----|---------------|-------------------------------------------------|------------|-------------------------------|
| 1.  | 58            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 2.  | 59            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 3.  | 61            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 4.  | 64            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 5.  | 65            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 6.  | 66            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 7.  | 69            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 8.  | 71            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 9.  | 72            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 10. | 73            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 11. | 74            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 12. | 75            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 13. | 76            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 14. | 78            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 15. | 80            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 16. | 81            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 17. | 82            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 18. | 83            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 19. | 86            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 20. | 87            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 21. | 88            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 22. | 89            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 23. | 90            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 24. | 91            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 25. | 93            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 26. | 94            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 27. | 95            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 28. | 96            | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 29. | 118           | Mazowieckie, PL 2007                           | Sour cherry| Atypical taxon                |
| 30. | 119           | Mazowieckie, PL 2007                           | Sour cherry| Atypical taxon                |
| 31. | 120           | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 32. | 122           | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 33. | 211           | Łódzkie, PL 2007                               | Sour cherry| Atypical taxon                |
| 34. | 271           | Silesian, PL 2007                              | Sour cherry| Atypical taxon                |
| 35. | 374           | Łódzkie, PL 2008                               | Sour cherry| Atypical taxon                |
| 36. | 439           | Łódzkie, PL 2008                               | Sour cherry| Atypical taxon                |
| 37. | 909           | Łódzkie, PL 2009                               | Sour cherry| Atypical taxon                |
| 38. | 910           | Łódzkie, PL 2009                               | Sour cherry| Atypical taxon                |
| 39. | 949           | Łódzkie, PL 2009                               | Sour cherry| Atypical taxon                |
| 40. | 963           | Lubelskie, PL 2009                             | Sweet cherry| Atypical taxon                |
| 41. | 966           | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 42. | 967           | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 43. | 968           | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 44. | 969a          | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 45. | 969b          | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 46. | 970a          | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 47. | 970b          | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| 48. | 971a          | Lubelskie, PL 2009                             | Sour cherry| Atypical taxon                |
| Lp. | Strain number | Place (voivodeship/country) and year of isolation | Host-plant | Taxon based on LOPAT, GATTa/L |
|-----|--------------|-----------------------------------------------|------------|-------------------------------|
| 49. | 971b         | Lubelskie, PL 2009                            | Sour cherry| Atypical taxon                |
| 50. | 972          | Lubelskie, PL 2009                            | Sour cherry| Atypical taxon                |
| 51. | 973          | Lubelskie, PL 2009                            | Sour cherry| Atypical taxon                |
| 52. | 981          | Lubelskie, PL 2009                            | Sour cherry| Atypical taxon                |
| 53. | 982          | Lubelskie, PL 2009                            | Sour cherry| Atypical taxon                |
| 54. | 1017         | Łódzkie, PL 2009                             | Sour cherry| Atypical taxon                |
| 55. | 1021         | Łódzkie, PL 2009                             | Sour cherry| Atypical taxon                |
| 56. | 791          | No data 2001                                 | Sour cherry| Atypical taxon                |
| 57. | 441          | Łódzkie, PL 2008                             | Plum       | Psm1                          |
| 58. | LMG 2222     | No data, UK 1958                             | *Prunus avium* | Psm1                        |
| 59. | 25b          | Łódzkie, PL 2007                             | Sweet cherry| Psm1                          |
| 60. | 28a          | Łódzkie, PL 2007                             | Sweet cherry| Psm1                          |
| 61. | 29a          | Łódzkie, PL 2007                             | Sweet cherry| Psm1                          |
| 62. | 38a          | Łódzkie, PL 2007                             | Plum       | Psm1                          |
| 63. | 98           | Łódzkie, PL 2007                             | Sweet cherry| Psm1                          |
| 64. | 100          | Łódzkie, PL 2007                             | Plum       | Psm1                          |
| 65. | 107          | Łódzkie, PL 2007                             | Plum       | Psm1                          |
| 66. | 158          | West Pomerania, PL 2007                      | Sweet cherry| Psm1                          |
| 67. | 174          | West Pomerania, PL 2007                      | Sweet cherry| Psm1                          |
| 68. | 175          | West Pomerania, PL 2007                      | Sweet cherry| Psm1                          |
| 69. | 177          | West Pomerania, PL 2007                      | Peach      | Psm1                          |
| 70. | 199          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 71. | 201          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 72. | 202          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 73. | 203          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 74. | 204          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 75. | 205          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 76. | 206          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 77. | 209          | West Pomerania, PL 2007                      | Plum       | Psm1                          |
| 78. | 213          | Świętokrzyskie, PL 2007                      | Plum       | Psm1                          |
| 79. | 214          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 80. | 215          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 81. | 216          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 82. | 217          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 83. | 218          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 84. | 219          | Kuyavian-Pomeranian, PL 2007                 | Sweet cherry| Psm1                          |
| 85. | 220          | Kuyavian-Pomeranian, PL 2007                 | Plum       | Psm1                          |
| 86. | 221          | Kuyavian-Pomeranian, PL 2007                 | Plum       | Psm1                          |
| 87. | 250          | Kuyavian-Pomeranian, PL 2007                 | Plum       | Psm1                          |
| 88. | 274          | Silesian, PL 2007                           | Plum       | Psm1                          |
| 89. | 276          | Silesian, PL 2007                           | Plum       | Psm1                          |
| 90. | 280          | Silesian, PL 2007                           | Plum       | Psm1                          |
| 91. | 283          | Silesian, PL 2007                           | Sweet cherry| Psm1                          |
| 92. | 291          | Łódzkie, PL 2007                            | Sweet cherry| Psm1                          |
| 93. | 527          | Mazowieckie, PL 2008                         | Sweet cherry| Psm1                          |
| 94. | 528          | Mazowieckie, PL 2008                         | Sweet cherry| Psm1                          |
| 95. | 671          | Lubelskie, PL 2008                           | Sweet cherry| Psm1                          |
| 96. | 1061         | Łódzkie, PL 2009                            | Plum       | Psm1                          |
| Lp. | Strain number | Place (voivodeship/country) and year of isolation | Host-plant | Taxon based on LOPAT, GATTa/L |
|-----|---------------|-------------------------------------------------|------------|-----------------------------|
| 97. | 701A          | No data, PL 2005 Sweet cherry                   | Psm1       |
| 98. | 702           | No data, PL 1994 Plum                           | Psm1       |
| 99. | 704           | No data, PL 1994 Sweet cherry                   | Psm1       |
| 100.| 710           | Lower Silesian, PL 1996 Sweet cherry            | Psm1       |
| 101.| 755           | No data, PL 1999 Plum                           | Psm1       |
| 102.| 771           | Łódzkie, PL 1999 Plum                           | Psm1       |
| 103.| 782           | No data, PL 2001 Sweet cherry                   | Psm1       |
| 104.| 787           | Mazowieckie, PL 2001 Plum                       | Psm1       |
| 105.| 788           | Łódzkie, PL 2001 Plum                           | Psm1       |
| 106.| 793           | Łódzkie, PL 2001 Plum                           | Psm1       |
| 107.| CFBP 3800     | No data, UK ND Prunus cerasus                   | Psm2       |
| 108.| 77            | Łódzkie, PL 2007 Sour cherry                    | Psm2       |
| 109.| 117           | Mazowieckie, PL 2007 Sour cherry                | Psm2       |
| 110.| 266           | Silesian, PL 2007 Sour cherry                   | Psm2       |
| 111.| 417           | Mazowieckie, PL 2008 Sour cherry                | Psm2       |
| 112.| 701           | No data, PL 1994 Sour cherry                    | Psm2       |
| 113.| 719           | Łódzkie, PL 1997 Sour cherry                    | Psm2       |
| 114.| 732           | Łódzkie, PL 1997 Sour cherry                    | Psm2       |
| 115.| 733           | Łódzkie, PL 1997 Sour cherry                    | Psm2       |
| 116.| 745           | Łódzkie, PL 1999 Sour cherry                    | Psm2       |
| 117.| 764           | Mazowieckie, PL 1999 Sour cherry                | Psm2       |
| 118.| LMG 1247      | No data, UK ND Syringa vulgaris                 | Pss        |
| 119.| 2905          | No data/PL 1978 Sour cherry                     | Pss        |
| 120.| 68            | Łódzkie, PL 2007 Plum                           | Pss        |
| 121.| 103           | Łódzkie, PL 2007 Sour cherry                    | Pss        |
| 122.| 106           | Łódzkie, PL 2007 Plum                           | Pss        |
| 123.| 109           | Łódzkie, PL 2007 Plum                           | Pss        |
| 124.| 110           | Łódzkie, PL 2007 Plum                           | Pss        |
| 125.| 112           | Łódzkie, PL 2007 Plum                           | Pss        |
| 126.| 115           | Łódzkie, PL 2007 Plum                           | Pss        |
| 127.| 141           | West Pomerania, PL 2007 Peach                   | Pss        |
| 128.| 147           | West Pomerania, PL 2007 Peach                   | Pss        |
| 129.| 165           | West Pomerania, PL 2007 Sweet cherry            | Pss        |
| 130.| 184           | West Pomerania, PL 2007 Peach                   | Pss        |
| 131.| 192           | West Pomerania, PL 2007 Plum                    | Pss        |
| 132.| 210           | Łódzkie, PL 2007 Sour cherry                    | Pss        |
| 133.| 222           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 134.| 226           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 135.| 227           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 136.| 229           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 137.| 233           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 138.| 234           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 139.| 235           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 140.| 236           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 141.| 237           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 142.| 239           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 143.| 240           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
| 144.| 242           | Kuyavian-Pomeranian, PL 2007 Plum               | Pss        |
Dot blot hybridisation

High-throughput specificity assays were carried out using a dot blot platform, essentially as previously described (Albuquerque et al. 2011). PCR amplicons obtained using primers Psm1-6F/6R, with template DNA from strain Psm28a (race 1), and primers Psm2-8F/8R, with Psm77 (race 2), were purified using the GFX PCR and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and labelled with digoxigenin, using the DIG-High Prime DNA labelling kit (Roche, Basel, Switzerland) in order to obtain the two tested hybridisation probes Psm1 and Psm2, respectively.

Amounts of 100 ng of heat-denatured DNA from each bacterial strain were transferred to a nylon membrane using a Bio-Dot apparatus (Bio-Rad, Hercules, USA). Hybridisation was carried out overnight at 68 °C with a final probe concentration of 100 ng/mL, and the washing and detection steps were carried out according to the DIG application manual (Roche). The chemiluminescent signal indicative of probe–target hybrids was detected using a Molecular Imager ChemiDoc XRS+ System (Bio-Rad), with all pixels below saturation point.

Conventional and real-time PCR amplifications

Amplification reactions with the two selected primer pairs, one specific for the strains of Psm1 and the second specific for Psm2, were performed in a Biometra T3000 thermocycler (Biometra, Göttingen, Germany). The reaction mixture in 15 μl of total reaction volume contained 10 ng of DNA, 0.4 U of Dream DNA Polymerase (Promega, Madison, WI, USA), 1× reaction Dream Taq Green buffer (Thermo Scientific, Vilnius, Lithuania), 0.15 mM dNTPs and 0.7 mM of each primer. The following

Table 1 (continued)

| Lp. | Strain number | Place (voivodeship/country) and year of isolation | Host-plant | Taxon based on LOPAT, GATTA/L |
|-----|---------------|-----------------------------------------------|------------|------------------------------|
| 145.| 244           | Kuyavian-Pomeranian, PL 2007                   | Plum       | Pss                          |
| 146.| 245           | Kuyavian-Pomeranian, PL 2007                   | Plum       | Pss                          |
| 147.| 247           | Kuyavian-Pomeranian, PL 2007                   | Plum       | Pss                          |
| 148.| 248           | Kuyavian-Pomeranian, PL 2007                   | Plum       | Pss                          |
| 149.| 256           | Kuyavian-Pomeranian, PL 2007                   | Plum       | Pss                          |
| 150.| 257           | Kuyavian-Pomeranian, PL 2007                   | Sour cherry| Pss                          |
| 151.| 258           | Kuyavian-Pomeranian, PL 2007                   | Sour cherry| Pss                          |
| 152.| 259           | Łódzkie, PL 2007                               | Sweet cherry| Pss                         |
| 153.| 264           | Łódzkie, PL 2007                               | Peach      | Pss                          |
| 154.| 286           | Ślęsian, PL 2007                               | Sweet cherry| Pss                         |
| 155.| 373           | Łódzkie, PL 2008                               | Sour cherry| Pss                          |
| 156.| 376           | Łódzkie, PL 2008                               | Sour cherry| Pss                          |
| 157.| 415           | Świętokrzyskie, PL 2008                         | Plum       | Pss                          |
| 158.| 420a          | Mazowieckie, PL 2008                           | Sour cherry| Pss                          |
| 159.| 435           | Mazowieckie, PL 2008                           | Sour cherry| Pss                          |
| 160.| 437           | Łódzkie, PL 2008                               | Sour cherry| Pss                          |
| 161.| 442           | Łódzkie, PL 2008                               | Plum       | Pss                          |
| 162.| 460           | Podkarpackie, PL 2008                          | Sour cherry| Pss                          |
| 163.| 663           | Lubelskie, PL 2008                             | Sour cherry| Pss                          |
| 164.| 914           | Kuyavian-Pomeranian, PL 2009                   | Sour cherry| Pss                          |
| 165.| 959           | Lubelskie, PL 2009                             | Sour cherry| Pss                          |
| 166.| 702A          | Łódzkie, PL 2005                               | Plum       | Pss                          |
| 167.| 753           | Łódzkie, PL 1999                               | Apricot    | Pss                          |
| 168.| 757           | Mazowieckie, PL 1999                           | Plum       | Pss                          |
| 169.| 760           | Mazowieckie, PL 1999                           | Sour cherry| Pss                          |
| 170.| 762           | No data, PL 1999                              | Apricot    | Pss                          |
| 171.| 763           | No data, PL 1999                              | Sour cherry| Pss                          |

LOPAT—levan production from sucrose (L), presence of oxidase (O), ability to cause rot on potato tubers (P; pectolytic activity), presence of arginine dihydrolase (A), hypersensitive reaction (HR) on tobacco plants; GATTA—gelatine hydrolysis (G), aesculin hydrolysis (A, activity of the β-glucosidase), tyrosinase activity (T), utilisation of tartrate (Ta); test of L-lactate utilisation (L); PL Poland, UK United Kingdom
Table 2  Results of specificity of designed primers in reactions with DNA of different pathovars of *Pseudomonas syringae* and other *Pseudomonas* species tested

| Pathovar of *P. syringae* | Strain number* | Host                | Origin-place/year of isolation | PCR result with primers | Reference/source |
|---------------------------|----------------|---------------------|--------------------------------|-------------------------|-----------------|
| *aceris*                  | CFBP 2339<sup>PT</sup> | Acer sp.            | 1961                           | ** CFBP***              |                 |
| *actinidiae*              | CFBP 4909<sup>PT</sup> | Actinidia deliciosa | Japan/1984                      | –                       | CFBP            |
|                           | MAFF 302135     | Actinidia argute    | Japan/1987                      | –                       | MAFF            |
|                           | MAFF 302145     | Actinidia deliciosa | Japan/1988                      | –                       | MAFF            |
|                           | MAFF 613005     | Actinidia deliciosa | Japan/1986                      | –                       | MAFF            |
| *aesculi*                 | CFBP 2894<sup>PT</sup> | Aesculus indica    | India/1980                      | –                       | CFBP            |
|                           | 6617            | Aesculus hippocastanum | UK/2006                      | –                       | R. W. Jackson  |
|                           | 2250            | Aesculus hippocastanum | UK/2008                      | –                       | (UK)            |
|                           | H3              | Aesculus hippocastanum | Germany/2007                  | –                       | Schmidt et al., |
|                           | H4              | Aesculus hippocastanum | Germany/2007                  | –                       | 2008            |
|                           | 2190            | Aesculus hippocastanum | UK                     | –                       |                 |
| *antirrhini*              | CFBP 1620<sup>PT</sup> | Antirrhinum majus  | UK/1956                         | –                       | CFBP            |
| *apii*                    | CFBP 2103<sup>PT</sup> | Apium graveolens   | USA/1942                        | –                       | CFBP            |
|                           | BS 426          | Petroselinum crispum | USA/2003                      | –                       | Bull et al. 2011|
|                           | BS 463          | Flat-leaf parsley   | USA/2002                        | –                       |                 |
| *aptata*                  | CFBP 1617<sup>PT</sup> | Beta vulgaris      | USA/1959                        | –                       | CFBP            |
| *atrofaciens*             | CFBP 2213<sup>PT</sup> | Triticum aestivum  | New Zealand/1968                | –                       | CFBP            |
| *atropurpurea*            | CFBP 2340<sup>PT</sup> | Lolium multiflorum | ND/1967                         | –                       | CFBP            |
|                           | 1304            |                     |                                 | –                       | K.Geider        |
|                           |                 |                     |                                 |                         | (Germany)       |
| *avii*                    | CFBP 3846<sup>PT</sup> | Prunus avium       | France/1991                     | –                       | CFBP            |
| *berberidis*              | CFBP 1727<sup>PT</sup> | Berberis sp.       | New Zealand/1972                | –                       | CFBP            |
| *broussonetiae*           | CFBP 5140<sup>PT</sup> | Broussonetia kazinoki Sieb. X Broussonetia papryrif a Vent. | Japan/1980                | –                       | CFBP            |
|                           | MAFF 810038     | Broussonetia kazinoki Sieb. | Japan/1996                | –                       | MAFF            |
|                           | MAFF 810044     | Broussonetia kazinoki Sieb. | Japan/1996                | –                       | MAFF            |
| *castaneae*               | CFBP 4217<sup>PT</sup> | Castanea crenata   | Japan/1977                      | –                       | CFBP            |
| *cerasicola*              | CFBP 6109<sup>PT</sup> | Prunus X yedoensis | Japan/1995                      | –                       | CFBP            |
| *ciccaronei*              | CFBP 2342<sup>PT</sup> | Ceratonia siligua  | Italy/1942                      | –                       | CFBP            |
| *coriandricola*           | CFBP 5010<sup>PT</sup> | Coriandrum sativum | Germany/1990                    | –                       | CFBP            |
|                           | BS 456          | Curled-leaf parsley | USA/2003                       | –                       | Carolee T. Bull |
|                           | BS 462          | Flat-leaf parsley   | USA/2002                        | –                       | (USA)           |
| Species         | Accession | Host Species                      | Location | Notes     | Host Code |
|-----------------|-----------|-----------------------------------|----------|-----------|-----------|
| coronafaciens   | CFBP 2216 | *Avena sativa*                    | UK/1958  | –         | CFBP      |
| cunninghamiae   | CFBP 4218 | *Cunninghamia lanceolata*         | China/1995| –         | CFBP      |
| daphniphylli    | CFBP 4219 | *Daphniphyllum teigmanni*        | Japan/1981| –         | CFBP      |
| delphinii       | CFBP 2215 | *Delphinium sp.*                  | New Zealand/1957| –         | CFBP      |
| dendropanacis   | CFBP 3226 | *Dendropanax trifidus*            | Japan/1979| –         | CFBP      |
| dysoxyli        | CFBP 2356 | *Dysoxylum spectabile*            | New Zealand/1949| –         | CFBP      |
| eriobotryae     | CFBP 2343 | *Eriobotrya japonica*             | USA/1970  | –         | CFBP      |
| garcae          | CFBP 1634 | *Coffea arabica*                  | Brasil/1958| –         | CFBP      |
| helianthi       | CFBP 2067 | *Helianthus annuus*               | Mexico/ND | –         | CFBP      |
| hibisci         | CFBP 2895 | *Hibiscus rosa-sinensis*          | USA/1984  | –         | CFBP      |
| japonica        | MAFF 301159 | *Triticum aestivum* (L.) Thell. | Japan/ND  | –         | MAFF      |
| lapsa           | CFBP 1731 | *Zea sp.*                         | ND/1968  | –         | CFBP      |
| lachrymans      | CFBP 6463 | *Cucumis sativus*                 | Hungary/1958| –         | CFBP      |
| maculicola      | LMG 5071  | *Brassica oleracea*               | New Zealand/1965| –         | LMG       |
| mellea          | CFBP 2344 | *Nicotiana tabacum*               | Japan/1968| –         | CFBP      |
| mori            | CFBP 1642 | *Morus alba*                      | Hungary/1958| –         | CFBP      |
| morsprunorum    | CFBP 2351 | *Prunus domestica*                | USA/1931  | – (Psm1)  | CFBP      |
|                 |           |                                   |          | + (Psm2)  | M. Hevesi (Hungary) |
|                 |           |                                   |          | – (Psm1)  | J.D. Janse (Netherlands) |
|                 |           |                                   |          | + (Psm1)  | LMG       |
|                 |           |                                   |          | – (Psm2)  | CFBP      |
|                 |           |                                   |          | – (Psm1)  | MAFF      |
|                 |           |                                   |          | + (Psm1)  | MAFF      |
| myricae         | CFBP 2897 | *Myrica rubra*                    | Japan/1978| –         | CFBP      |
|                 | MAFF 302457 | *Myrica rubra Sieb. et Zucc.* | Japan/ND  | –         | MAFF      |
|                 | MAFF 302944 | *Myrica rubra Sieb. et Zucc.* | Japan/ND  | –         | MAFF      |
| oryzae          | CFBP 3228 | *Oryza sativa*                    | Japan/1983| –         | CFBP      |
| papulans        | CFBP 1754 | *Malus sylvestris*                | Canada/1973| –         | CFBP      |
| passiflorae     | CFBP 2346 | *Passiflora edulis*               | New Zealand/1962| –         | CFBP      |
| persicae        | LMG 5184  | *Prunus persica*                  | France/1974| –         | LMG       |
| philadelphii    | CFBP 2898 | *Philadelphus coronarius*         | UK/1985   | –         | CFBP      |
| photiniaae      | CFBP 2899 | *Photinia glabra*                 | Japan/1976| –         | CFBP      |
| pisi            | CFBP 2105 | *Pisum sativum*                   | New Zealand/1969| –         | CFBP      |
|                 |           |                                   |          |           | M. Hevesi |
Experimentally determined amplification conditions were used: initial denaturation at 94 °C for 4 min; 30 cycles at 94 °C for 45 s, 55–62 °C for 45 s for primers Psm1-6F and Psm1-6R (for detection of Psm1 strains) and 50–58 °C for 45 s for primers Psm2-8F and Psm2-8R (for detection of Psm2 strains) and 72 °C for 1 min; and final extension at 72 °C for 10 min. The resulting PCR products were separated by electrophoresis on 1.5 % agarose gels as described above.

Real-time PCR with SYBR Green I was conducted in the Bio-Rad CFX96 with SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, USA). The reaction mixture in 20 µl of total volume contained 1× reaction SYBR
Green Supermix and 0.5 mM of each of the following primers: Psm1-1F-RT/Psm1-1R-RT for Psm1 and Psm2-1F-RT/Psm2-1R-RT for Psm2. Bacterial DNA was used as a template (10 ng per PCR reaction). No-template reactions were used as negative controls. The PCR programme was started from one cycle of denaturation at 98 °C for 130 s, followed by 35 cycles at 95 °C for 10 s and then 60 °C for 15 s, finished by a melting curve analysis for verification of the specificity of amplification in real-time PCR products. Progressive denaturation of products was carried out at a rising temperature, starting from 65 °C and continuing to 95 °C, with 0.5 °C of increment for 5 s each.

Specificity of designed primers and their usefulness in detection in plant material

In the first stage of this part of the study, the specificity of the two designed primer pairs was determined with PCR using DNA from all strains of Psm1, Psm2 and Pss as well as strains of atypical taxa (Table 1). In the second stage, the primers were tested with DNA from other P. syringae pathovars and related species (Table 2).

In order to assess the suitability of the designed primers for the detection of Psm1 and Psm2 strains in plant material, several leaves, shoots and fruits of sweet cherry, sour cherry and plum were collected. Amounts of 100 mg of crushed/cut plant tissue of each organ were placed in 1.9 ml of PBS buffer. For each type of tissue (organ) and host plant, two tubes were prepared (18 tubes in total). One hundred microlitres of bacterial suspension (10^5 cfu/ml) of the Psm1 reference strain (LMG 2222) or the Psm2 reference strain (CFBP 3800) were added to nine of the samples (one of each organ and of each plant). One hundred microlitres of sterile water were added to the remaining nine samples, which were tested to verify the purity of the plant material. After 1 h of shaking incubation at 26 °C, 1 ml of washing liquid separate from each of all 18 samples was centrifuged; the resulting pellet was suspended in 100 μl of TE buffer, and the DNA was isolated using a Genomic Mini DNA Extraction Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s instructions. The sensitivity of gDNA detection was checked using 2-fold serial dilutions of gDNA isolated (11 ng to ~11 fg per PCR reaction for Psm1 and 14 ng to ~14 fg per PCR reaction for Psm2) using the method described by Aljanabi and Martinez (1997), with slight modifications described by Kalužna et al. (2012). The PCR efficiency was calculated from the slope of the standard curve generated for each run in the following equation $E = 10^{(-1/slope)}$ where $E \approx 2$ and corresponds to 100 % efficiency (Ramakers et al. 2003).

Results

Phenotypic characterisation

All 168 isolates have been classified into species P. syringae LOPAT group Ia. GATTa and L-lactate utilisation tests allowed further discrimination of pathovars and races: 49 isolates were identified as P. syringae pv. morsprunorum race 1 (Psm1), 10 as race 2 of this pathovar, 53 as pathovar syringae (Pss) and 56 as belonging to atypical taxa, having most of the features of Pss without, however, the ability of esculine hydrolysis (lack of β-glucosidase activity) (Table 1).

PCR MP

To select specific fragments of the taxon, the PCR MP method was applied using DNA from different strains of P. syringae (Figs. 1 and 2; Table 1). The obtained PCR MP patterns corresponded to phenotypically determined pathovars and races. Similar electrophoretic patterns were obtained for races within pathovar morsprunorum, confirming their homogeneity; however, different patterns were observed for strains belonging to pathovar syringae. For Psm1 and Psm2, the products that were specific and were shared between all strains of each taxa were selected, cloned and sequenced. Two products specific for Psm1 (after digestion by Psrl) had sizes of 1,208 and 1,128 bp, while the unique amplification product (after digestion by TaqI) for strains of Psm2 was 781 bp long. No specific and unique band was found for strains of Pss.
**Fig. 1** Electrophoretic patterns obtained after polymerase chain reaction melting profile (PCR MP) of fluorescent Pseudomonads with primer Pst1:

Lane 1—M—marker 100-bp ladder (Genoplast, Rokocin, Poland); pathovar *morsprunorum* race 1 isolates: 2—LMG 2222, 3—702, 4—710, 5—755, 6—787, 7—782, 8—793, 9—701A; pv. *morsprunorum* race 2 isolates: 10—CFBP 3800, 11—719, 12—733, 13—732, 14—745, 15—764, 16—701; pv. *syringae* isolates: 17—LMG 1247, 18—2905, 19—760; 20—762, 21—702A, 22—757, 23—753, 24—763, 25—M—marker 100-bp PCR Molecular Ruler (Bio-Rad, Hercules, USA)

**Fig. 2** Electrophoretic patterns obtained after polymerase chain reaction melting profile (PCR MP) of fluorescent Pseudomonads with primer Taq1:

Lane 1—M—marker 100-bp ladder (Genoplast, Rokocin, Poland); pathovar *morsprunorum* race 1 isolates: 2—LMG 2222, 3—25b, 4—28a, 5—107, 6—201, 7—701A, 8—755, 9—771; pv. *morsprunorum* race 2 isolates: 10—CFBP 3800, 11—77, 12—701, 13—732, 14—733, 15—745, 16—764; pv. *syringae* isolates: 17—LMG 1247, 18—2905, 19—68; 20—110, 21—141, 22—286, 23—415, 24—763, 25—M—marker 100-bp PCR Molecular Ruler (Bio-Rad, Hercules, USA)
Design of SCAR primers

The nucleotide sequences obtained for the \( Psm_1 \) and \( Psm_2 \) fragments were used to design different SCAR primers. After validation, the most specific primers for conventional and real-time PCR were selected (Table 3). A BLAST analysis of selected primer sequences showed no similarity to any bacterial sequences in GenBank.

Dot blot hybridisation

The dot blot results confirmed the high specificity of the selected markers towards the target pathogens. Using probe Psm1, positive hybridisation results (dark dots) were observed with all tested Psm1 strains, and no unspecific hybridisation was observed with DNA from any non-Psm1 pseudomonads. Similarly, probe Psm2 was exclusively specific for the tested Psm2 strains. Additionally, the hybridisation results showed that the selected DNA regions were present in all their respective target strains, confirming their stability (Fig. 3).

Specificity of designed primers and usefulness in detection in plant material

The PCR assays using DNA from all tested \( P. syringae \) strains including reference strains (Table 1), as well as DNA from strains of other species within the \( Pseudomonas \) genus (Table 2), showed that all the designed primers were specific for their respective taxa. PCR assays using primers Psm1-6F/6R and Psm1-1F-RT/1R-RT, specific for Psm1, successfully amplified the expected PCR products 793 bp (Fig. 4) and 101 bp (Fig. 5), respectively, using DNA from all strains of Psm1. No amplification was observed when DNA from strains identified as Psm2 or Pss and strains of atypical taxa were used. Amplification using primers Psm2-8F/8R and Psm2-1F-RT/1R-RT, designed for detection of Psm2, was achieved with DNA from all strains of Psm2, resulting in PCR products of expected lengths of 410 bp (Fig. 6) and 104 bp, respectively. No increase in fluorescence was observed with DNA from Psm1 or Pss and strains of atypical taxa. The melting curves of the reaction products obtained from real-time PCR revealed a single peak with a melting temperature of 80 °C or 77 °C for Psm1 and Psm2, respectively. Also, neither unexpected nor additional peaks in the product melting curves were observed, which clearly excluded possibilities or tendency of the primers to form dimers. Moreover, none of the four tested primer pairs amplified the DNA of 79 strains of other pathovars of \( P. syringae \) and other species (Table 2).

The usefulness of the designed primers for detection of Psm1 and Psm2 strains in plant material was assessed with PCR assays using DNA extracted from a mixture of plant tissues and a suspension of target bacteria. The results confirmed the specificity of selected primer-pairs since positive amplification was achieved in mingled samples, while no nonspecific amplification was observed in samples without bacteria addition. Additionally, these assays showed that the proposed PCR detection methodology was not affected by potential inhibitors present in plant samples.

Limit of detection of \( P. syringae \) pv. morsprunorum for conventional and real-time PCR

Both tested primer pairs designed for conventional PCR allowed for the detection of \( 10^0 \) cfu/reaction of Psm1 and \( 10^1 \) Psm2 in pure culture. Regarding the presence of bacteria in different organs of sweet and sour cherries, it was possible to detect \( 10^0 \) and \( 10^1 \) cfu/reaction for sweet cherry leaves and shoots, respectively, using the Psm1-specific primers and \( 10^2 \) cfu/reaction for sour cherry leaves and shoots using the Psm2-

| Table 3 | Primers specific for strains of Psm1 and Psm2 |
|---------|-----------------------------------------------|
| Primer name | Primer sequence | \( T_m \) | Product length |
| **Conventional PCR** | | | |
| Psm1-6F | 5′-TGTTCCCGGCCATCCAATA-3′ | 51.1 °C | 793 bp |
| Psm1-6R | 5′-ATCCGCATCAGTCAAAATAGTCAT-3′ | 52.3 °C | |
| Psm2-8F | 5′-CTTTTATAGATGGTAGGTTTGTGTA-3′ | 50.6 °C | 410 bp |
| Psm2-8R | 5′-ACTTTCGGA TTCA TCGTTTTCTA-3′ | 49.2 °C | |
| **Real-time PCR** | | | |
| Psm1-1F-RT | 5′-TCCCCGCCATCAAATCTTAC-3′ | 57.1 °C | 101 bp |
| Psm1-1R-RT | 5′-ACGGTCATCGTGCTCTGTTCA-3′ | 51.1 °C | |
| Psm2-1F-RT | 5′-GGTTGGCTTCTTTTCCTAG-3′ | 48 °C | 104 bp |
| Psm2-1R-RT | 5′-ATTGCATTACTTTTGTGC-3′ | 46.5 °C | |

F forward primer, R reverse primer, RT real-time, \( T_m \) melting temperature
specific primers. The sensitivity (LOD, limit of detection) of the detection in the conventional PCR assay was ~4 pg for Psm1 strain 199 and ~5 pg for Psm2 strain 745 when aliquots of serial 2-fold dilutions of purified DNA were used which corresponds to the order of magnitude ~10^1–10^2 cfu/reaction.

Both tested primer pairs designed for Psm1 and Psm2 strains using real-time PCR allowed the detection of 10^6 cfu/reaction of Psm1 or Psm2 in pure culture and in plant material. Only the expected products and a single peak with melting temperature were obtained. Standard curves using template DNA from bacterial suspensions, DNA from plant material with additions of bacterial suspensions and bacterial gDNA showed high amplification efficiency and linearity of the data (Table 4). An exception occurred for the products obtained from shoots of sweet cherry with additions of bacterial suspensions of Psm1. Although linearity was quite good, the noted efficiency of 83 % was not in the range considered acceptable (90–110 %). Moreover, the efficiency obtained for the mixture of shoots of sour cherry and Psm2 suspension when testing with primers for Psm2 was also lower compared to DNA template from sour cherry leaves and bacterial suspension alone. The sensitivity (LOD) of the detection in the real-time PCR assay when using gDNA ranged from ~30 to 100 fg for Psm1 strain 199 and ~10 to 50 fg for Psm2 strain 745 when 1.0-μl aliquots of serial 2-fold dilutions of
purified DNA were used which corresponds to the order
of magnitude \( \sim 10^0 \) cfu/reaction (Table 4).

### Discussion

In this study, the methods and tools enabling the rapid and highly specific identification and detection of bacterial canker causal agent \textit{P. syringae pv. morsprunorum} races 1 and 2 were developed. The methods based on the use of specific primers designed for conventional and real-time PCR allow in routine testing for omitting the application of often time-consuming methods of classical microbiology, fingerprinting methods or housekeeping gene sequence analysis used until now by other authors (Vicente and Roberts 2007; Gilbert et al. 2009). Of course in critical cases (i.e. first reports, claims, etc.) these other methods are still indispensable. Our newly developed
methods and tools are very useful and invaluable in both epidemiological studies and in development of protection programmes for stone fruits against bacterial canker.

Using the genetic fingerprinting PCR MP method, we demonstrated the diversity of *P. syringae* strains, which was very important in the selection of specific DNA fragments for two races of *P. syringae pv. morsprunorum*. Based on the obtained nucleotide sequences of these fragments, *Psm1* and *Psm2*-specific SCAR primers were designed. The specificity of the designed primers for *Psm* and amplified regions was confirmed by BLAST, since the fragments did not show (at present) any significant similarity hits within the NCBI database. Due to the high electrophoretic profile heterogeneity obtained for *Pss* strains arising from their high genetic diversity confirmed already by other authors (Vicente and Roberts 2007; Gilbert et al. 2009; Kalużna et al. 2010a, b), it was not possible to find a common DNA fragment for all strains belonging to this taxon.

Commonly used methods for designing SCAR primers include rep-PCR (repetitive PCR) (Sangdee et al. 2013), randomly amplified polymorphic DNA (RAPD) (Liu et al. 2012; Cheng et al. 2015), amplified fragment length polymorphism (AFLP) (Zhang et al. 2012), PCR with universal rice primers (URP-PCR) (Lim et al. 2009) and intersimple sequence repeat (ISSR) (Giag Merlera et al. 2015). Although the PCR MP method was described so far as helpful in the study of genetic diversity of bacteria and yeast (Leibner-Ciszak et al. 2010; Kalużna et al. 2010b, 2014; Zasada et al. 2014), it has not been previously reported to be used for the selection of SCAR markers. In this work, the PCR MP is for the first time used for the design of SCAR primers specific for detection of plant pathogenic bacteria.

The results obtained in this study showed that the designed SCAR primers can be applied for specific, direct detection of strains belonging to *Psm1* or *Psm2*, both in pure culture and infected plant material. Their specificity was confirmed by PCR, using DNA from several *Pseudomonas* spp. strains, which showed that positive amplification occurred only with DNA of the targeted taxa strains. This is especially significant in the case of strains of atypical taxa and pathovars of *P. syringae* (i.e. *pv. syringae* and *pv. avii*, which also infect cherry (Ménard et al. 2003; Renike et al. 2008)) to exclude that symptoms are connected to another taxa/pathogen or to abiotic factors. Importantly, when testing the developed primers in conventional PCR, using DNA isolated from a mixture of plant material and bacteria of *Psm1* or *Psm2*, the suppression of amplification by potential plant inhibitors like polyphenols and pesticide residues, as reported by Płużawska et al. (1997), was not found. Additionally, for DNA from the asymptomatic plant material without addition of bacterial DNA, no positive amplification was observed. This means that the designed primers did not react with DNA of potential bacteria naturally inhabiting the plant material, which is essential to prevent false-positive diagnostic results. However, in the case of real-time PCR, which is the more sensitive method, some effects of plant material were noted. Although standard curves using different template DNA showed the high amplification efficiency and linearity of the data for the majority of DNA tested, for shoots of sweet cherry with additions of bacterial suspensions the efficiency was below the range considered acceptable, indicating higher dilution of those templates than expected. Also, a decrease of efficiency (Table 4) in the case of sour cherry shoots was observed. The results therefore may indicate the influence of shoots for more sensitive real-time PCR reactions.

### Table 4

| Template                                | E (%)\(^a\) | R2 \(^b\) | Slope \(^c\) | Y = int \(^d\) |
|-----------------------------------------|-------------|-----------|-------------|----------------|
| *Psm1* (DNA from bacterial suspension) | 103         | 0.998     | -3.252      | 35.445         |
| *Psm1*+sweet cherry leaves              | 99.7        | 0.965     | -3.28       | 36.551         |
| *Psm1*+sweet cherry shoots              | 83.0        | 0.989     | -3.810      | 43.932         |
| *Psm1* gDNA                             | 99.2        | 0.997     | -3.342      | 18.425         |
| *Psm2* (DNA from bacterial suspension) | 99.8        | 0.995     | -3.326      | 33.093         |
| *Psm2*+sour cherry leaves               | 99.3        | 0.999     | -3.338      | 32.451         |
| *Psm2*+sour cherry shoots               | 91.4        | 0.994     | -3.548      | 35.130         |
| *Psm2* g DNA                            | 99.2        | 0.991     | -3.342      | 17.805         |

\(^a\) E = PCR efficiency; ideally the efficiency should be 100 %, meaning that for each cycle the amount of product doubles; high/acceptable amplification efficiency (90–110 %). Efficiency = 10\(^{-\text{R2}}\) \times \text{slope} + 1

\(^b\) R2 is a measure of data linearity amongst technical replicates of serial dilutions; indicates how good one value is in predicting another; R2 = 1 is perfect

\(^c\) The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100 % as possible, equivalent to a slope of $-3.32$

\(^d\) Y = int represents the value of Ct where the curve crosses the y-axis
The designing of primers for both systems, conventional and real-time PCR, makes the developed diagnosis system more accessible to a wider group of researchers, as many laboratories do not have access to special equipment or specialised personnel to perform the real-time PCR or have less funds. However, as described, the real-time PCR procedure is much faster (whole reaction with melting curve analysis is about 1 h from the beginning with SsoAdvanced SYBR Green Supermix); it allows the use of DNA quickly extracted from pure culture by the boiling method, without loss of detection resolution, and also excludes additional time-consuming post-PCR processes (i.e. agarose gel electrophoresis). Therefore, using this technique, it is possible to obtain a very fast response about the causal agent of the disease. However, it should be noted that this system is highly sensitive and that false-positive results can occur. The risk of false-positive results due to cross-contamination during preparation of the PCR can be minimised by using negative controls and high discipline during work (e.g., application of tips with filters during the DNA isolation step). Additionally, positive results obtained during those of the final PCR cycles should be treated as suspect only, for which additional, more detailed investigations should be conducted. Moreover, during all the assays the melting curve analysis is recommended to exclude nonspecific amplicons (as a consequence of which are visible in each run as the rest of the analysed specific ones). Dot blot hybridisation confirmed that the two selected DNA regions were highly specific for their target genomospecies and stable amongst all tested isolates of either Psm1 or Psm2, which is essential for preventing false-positive and false-negative results, respectively as much as possible.

In summary, when compared with so-far available methods for identification and differentiation of causal agents of stone fruit bacterial canker based on phenotypic characters, fingerprinting methods or MLST, the use of pathovar-specific primers allowed for greatly shortening the time required for diagnosis, while highly increasing assay accuracy and lowering detection limit. Moreover, this PCR-based method is relatively simple and inexpensive, and it does not require the time-consuming step of pre-incubation on microbiological media (Schaad et al. 1995). Even in the presence of potential inhibitors present in plant material, which can affect the limit of detection, we could detect 1 and 3×10^2 cfu/reaction using primers specific for Psm1 and Psm2 in conventional PCR. A similar detection sensitivity in conventional PCR was obtained by other authors in their identification systems for other phytopathogens (Catara et al. 2000; Kerkoud et al. 2002; Biondi et al. 2013). The sensitivity of real-time PCR was higher than in the case of conventional ones, as 1 cfu/reaction was detected when different templates were used. This is especially important in the case of naturally infected material in the presence of a small amount of pathogen DNA, which be detected in a very short time. The limit of detection when using gDNA was in the range from ~4–5 pg in conventional and ~10–100 fg in real-time PCR for both taxa, which are similar to results obtained for P. syringae pv. actinidiae (Gallelli et al. 2014) and Clavibacter michiganensis subsp. sepedonicus (Cho et al. 2015). The high sensitivity of the developed assay (obtained in our hands) will be invaluable for detecting the target bacteria in the early latent period of the disease, allowing growers to undertake appropriate prevention or protection programmes.

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Compliance with ethical standards
Conflict of interest The authors of the paper declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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