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Short communication

Identification and *in planta* detection of *Pseudomonas syringae* pv. *tomato* using PCR amplification of $hrpZ_{Pst}$

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Abstract

A rapid detection method based on PCR amplification of *Pseudomonas syringae* pv. *tomato* chromosomal sequences was developed. Primer design was based on the *P. syringae* DC3000 $hrpZ_{Pst}$ gene, which maps on a pathogenicity-associated operon of the *hrp*/*hrc* pathogenicity island.

A 532 bp product corresponding to an internal fragment of $hrpZ_{Pst}$ was amplified from 50 isolates of *P. syringae* pv. *tomato* belonging to a geographically representative collection. The amplification product was also obtained from three coronatine-deficient strains of *P. syringae* pv. *tomato*. On the other hand, PCR did not produce any such products from 100 pathogenic and symbiotic bacterial strains of the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Rhizobium* and 75 unidentified bacterial saprophytes isolated from tomato plants. The method was tested using leaf and fruit spots from naturally-infected tomato plants and asymptomatic nursery plants and artificially contaminated tomato seeds. The results confirmed the high specificity observed using pure cultures.

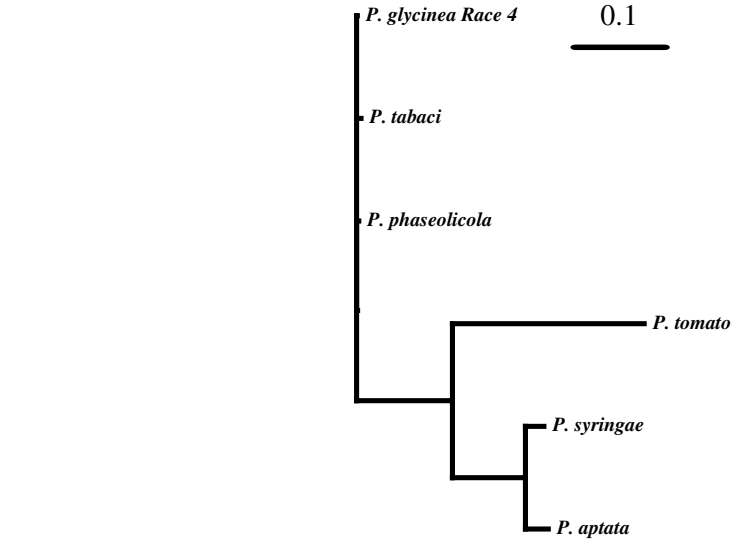
Pseudomonas syringae pv. *tomato* is the causal agent of bacterial speck of tomato plants. This bacterium produces angular necrotic spots on leaves and fruits, followed by the appearance of a chlorotic halo. The disease causes serious economic losses especially on fruits of susceptible genotypes (Zaccardelli et al., 2002). Heavy economic losses are also experienced by the tomato seed and plantlets industry under certain disease-conducive conditions (Yunis et al., 1980; Psallidas, 1988). The use of disease-free material is essential for cost effective field production. As for most bacterial diseases, freedom from pathogens can be expected through good cultivation practice, including crop and storage hygiene, and strict certification schemes *via* several routes, including indexing (Janse and Wenneker, 2002).

The molecular identification of *P. syringae* pv. *tomato* is often based on the isolation of bacteria in semiselective media (VBTar) coupled to DNA hybridization using coronatine synthesis genes as probes (Cuppels et al., 1990). Serological techniques such as indirect fluorescent antibody staining (IFAS) and enzyme-linked immunosorbent assay (ELISA) are also commonly used in our laboratory. All these approaches are laborious and time-consuming, not well suited for the high throughput screenings required for seed and plant certification programmes. PCR amplification of the *cfl* gene was reported by Bereswill et al. (1994)

for the identification and genetic diversity analysis of coronatine-producing *P. syringae* pathovars. Unluckily the diagnostic use of such an approach was limited by the observation that coronatine-deficient strains of *Pseudomonas syringae* exist in the environment (Wiebe and Campbell, 1993).

To approach the problem of developing a rapid and sensitive diagnostic tool for high-throughput screenings of infected tomato plants, we describe a PCR method for the amplification of an internal fragment of $hrpZ_{Pst}$. *Pseudomonas syringae* pv. *tomato hrpZ* is a chromosomal gene located in the *hrp*/*hrc* pathogenicity island, which is essential for symptom production in host plants and hypersensitive response in non-hosts (Alfano and Collmer 1997). In particular, the *hrpZ* gene encodes a class of type III secreted proteins able to elicit a hypersensitive response in tobacco and trigger systemic acquired resistance in *Arabidopsis* (He et al., 1993; Dong et al., 1999). The *hrpZ* ORF is the second in an operon encoding component of the type III secretion apparatus and its physical position is conserved among phytopathogenic pseudomonads. The role of HrpZ in virulence and pathogenicity is unknown. In *P. syringae* pv. *syringae*, non-polar deletions of *hrpZ* only slightly affect the ability of bacteria to cause HR on tobacco, but they totally abolish the ability of saprophytic bacteria expressing the *hrp* cluster in trans to elicit HR (Alfano et al., 1996). On the other hand, non-polar Δ*hrpZ::npt*II mutations did not affect bacterial growth in the phyllosphaere and virulence on bean leaves (Hirano et al., 1999). Purified HrpZ from *P. syringae* has also been shown to produce transient pores in liposomes and synthetic lipid bilayers (Lee et al., 2001), suggesting a potential role in endophytic growth and pathogenesis *via* nutrient release and delivery of type III secreted effectors. Contrary to *avr*/*hop* genes, *hrpZ* is not known to have homologs in regions of the genome unlinked from the *hrp*/*hrc* cluster and/or still undergoing horizontal transfer and therefore can be considered a genetically stable trait. Taken together, these considerations make $hrpZ_{Pst}$ an attractive target for molecular diagnostics.

Multiple alignments of the nucleotide sequences of the *hrpZ* ORFs of *P. syringae* pathovars *tomato*, *syringae*, *aptata, tabaci* and *glycinea* showed that this gene is highly conserved among pseudomonads (Figure 1a). Nevertheless, we were able to find short pathovar-specific sequences with appropriate (G + C)% content and potential for primer design (Figure 1b). Several primer pairs were tested using as the template plasmid pCPP2211, which carries the *hrpAZBCDE* operon of *P. syringae* DC3000 (Preston et al., 1995). Subsequently a limited number of *P. syringae* pathovars (not shown) were tested for specificity. The two oligonucleotide primers chosen for further analyses were MM5F (5′-GA-ACGAGCTGAAGGAAGACA-3′) and MM5R (5′-CAGCCTGGTTAGTCTGGTTA-3′), which amplify a 532-bp fragment from $hrpZ_{Pst}$. The corresponding map coordinates are shown in Figure 1b. The reaction mixture (50 μl) contained 0.4 μM of each primer, 200 μM of dNTPs, 2.5 mM of $MgCl_2$ and 1 U of *Taq*-DNA polymerase (Triple Master PCR System Kit from Eppendorf). As templates for the reactions we used, alternatively, 1 μl of crude bacterial lysate, crude extracts of necrotic bacterial lesions excised from leaves and fruits, or DNA extracts prepared from lesions, or from asymptomatic nursery plants (leaves) and artificially contaminated seeds. The crude bacterial lysates were obtained after boiling for 10 min 5 μl of bacterial suspensions at $A_{600\ nm} = 0.1$ (ca. 1×10^6 CFU) mixed with 50 μl of 50 mM NaOH. Crude extracts of leaf and fruit lesions were obtained by grinding each sample (10 lesions per sample) in a mortar with pestle upon addition of 1.5 ml antioxidant extraction buffer (Gorris et al., 1996). Homogenates were filtered through Whatman No. 1 filter paper, and a pellet was obtained by centrifugation. The same procedure was applied on asymptomatic plant material (0.2 g of leaves or seeds) artificially contaminated with decreasing doses of *P. syringae* pv. *tomato*. When indicated, DNA was extracted from the pellets using a CTAB-based protocol (Wilson, 1989). The amplification programme consisted of a denaturation at 94 °C for 5 min followed by 36 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min and soak at 4 °C. The amplicons were visualized by standard agarose gel electrophoresis and ethidium bromide staining. The identity of the amplicons were confirmed during the preliminary evaluation of different primer pairs by Southern blot hybridization using the insert of plasmid pCPP2211 (Preston et al., 1995) as a probe (data not shown).



(a)

(b)

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Ps. phaseolicola  466 AACCCCGCACAGTTTCCCAAACCGGACTCGGGTTCGTGGGTGAACGAACTCAAGGAAGATA 526
Ps. syringae      463 AATCCCGCACAGTTTCCCAAGCCGGACTCGGGCTCCTGGGTGAACGAACTCAAGGAAGACA 523
Ps. glycineaR4    466 AACCCCGCACAGTTTCCCAAACCGGACTCGGGTTCGTGGGTGAACGAACTCAAGGAAGATA 526
Ps. aptata        466 AATCCCGCACAGTTTCCCAAACCGGACTCGGGCTCCTGGGTGAACGAACTTAAGGAAGACA 526
Ps. tabaci            ------------------------------------------------------------
Ps. tomato        538 AACAAGGCCCAGTTCCCTACTCGGGACGGCGGCTCGTGGATGAACGAGCTGAAGGAAGACA 598


Ps. phaseolicola  988 CTGCTGGTCAACATGCTGCTGCAAAGCACTAAAAACCAGGCTG--CTGCCTGA------- 1038
Ps. syringae      976 TTGCTGGTCAGTACGCTGCTGCAAGGCACCCGCAATCAGGCTG--CAGCCTGACCGACAA 1033
Ps. glycineaR4    988 CTGCTGGTCAACATGCTGCTGCAAAGCACTAAAAACCAGGCTG--CTGCCTGA------- 1038
Ps. aptata        979 TTGCTGGTCAGTACGCTGCTGCAAGGCACCCGCAATCAGGCTG--CAGCCTGA------- 1029
Ps. tabaci        662 CGGCTGGTCAACATGCTGCTGCAAAGCACTAAAAACCAGGCTG--CTGCCTGA------- 712
Ps. tomato       1054 CAGCTGATCAATGCGCTGTTGCAAGGCACCAATAACCAGACTAACCAGGCTGTGGCCTGA 1113
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Figure 1. (a) Phylogenetic tree showing the relatedness of the nucleotide sequences of *hrpZ* genes from five *P. syringae* pathovars including *P. syringae* pv. *tomato*. DNA sequences from the NCBI nucleotide database were aligned using ClustalX and a phylogenetic tree was constructed using the neighbor-joining method and visualized with TreeView. Branches are supported by the values shown at the nodes after 1000 bootstraps. (b) Detail of the multiple sequence alignment with indicated the positions of the forward and reverse primers used in this work. Numeration of the coordinates starts at the first base of the ORF obtained from Genbank.

We used 50 *P. syringae* pv. *tomato* isolates (three of them coronatine-deficient), collected from infected tomato plants in different geographic origins (Table 1), 100 pathogenic and symbiotic bacterial isolates from the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Rhizobium* and *Mesorhizobium* available in our collections (Table 1) and 75 unidentified bacterial isolates collected from uninfected tomato plants. The infected plant material consisted of 50 leaf lesion samples and 30 fruit lesion samples collected during Spring 2002 from different tomato varieties grown in two locations of the Salerno province (Southern Italy), with both fields showing heavy infections which were presumptively attributed to *P. syringae* pv. *tomato*.

The expected pathovar-specific 532 bp amplification product was produced from all the *P. syringae* pv. *tomato* isolates, including the three coronatine-deficient isolates, but not from the other pathogenic and symbiotic bacterial isolates belonging to the genera *Pseudomonas*, *Xanthomonas*, *Erwinia*, *Rhizobium* and *Mesorhizobium* (Figure 2). Moreover, no amplicons were obtained from the 75 unidentified bacterial isolates obtained from tomato plants grown under field conditions. Finally, we always amplified the expected PCR product using crude extracts of leaf lesions collected from naturally infected tomato plants (Figure 3). The specific amplicon was also produced in 26 out of 30 crude extracts prepared with

Table 1. Bacterial strains tested by PCR with primers MM5 and MM6

Bacterial strains, host, country	Bacterial strains, host, country
Pseudomonas syringae pv. *tomato* IPV-BO (tomato, Italy): 1611, 2972, 2973, 2974; ISCI (tomato, Italy): 165, 166, 176, 178, 179, 180, 181, 182, 184, 186, 187, 189, 190, 195, 197, 199, 200, 201, 202, 204, 205, 206, 213, 218, 219, 221, 222, 224, 225*, 5/2A*; 78/4A*; IVIA (tomato, Spain): 117.4, 984.1, 1001.1; LNPV (tomato, France): 0.48, 6.48, 8.81, 17.41, 18.74, 18.76; OMP-BO (tomato, Italy): 407/91, 437A-1/93, 884-1/94, 443-1/96, 447-1/96, 441.	*Xanthomonas vesicatoria* IPV-BO (tomato, Italy): 2684, 2834; IVIA (Spain): 030-5 (tomato), 1779-2 and 1780-1 (pepper); LNPV (tomato, France): 4.75, 4.77, 4.79. NCPPB 422 (tomato); OMP-BO (tomato, Italy): 261-1/96, 326/92, 358/92, 512 A/95, 515 A1/95, 594 A1/95, 790 A/94, 939-1/94; OMP-PG (Italy) : 32 and 79 (pepper), 34 (unknown).
Pseudomonas syringae pv. *syringae* ISCI PSS (unknown, Italy); IPV-BO (Italy): 1326 (unknown), 2204 (pear); IPV-CT (Italy): $45S_21$ (Citrus); IVIA (Spain): 3.6 and 773-1 (pear), 1444-5 (laurel), 1507-7 (hawthorn), 1559-1 (mango); OMP-BO (Italy): 280B-1/95 (pea), 368/92 and 395/95 (apricot), 443/91 and 1006/91 (pear), 493/93 (unknown).	*Xanthomonas campestris* pv. *campestris* DAPP-PG (Italy): 248 (cabbage), 305 (cauliflower), 399 (rutabaga). IPV-CT (Italy): 62.4 (kohlrabi), 65.3 (broccoli). IPV-NA 39 (kohlrabi, Italy). ISCI (Italy): 1 (kohlrabi), 31 (broccoli), 41 (savoy), 12 and 50 (cauliflower); ISPAVE 352 (cauliflower, Italy) LMG: 568 (brusselles sprouts, UK), 8030 (cauliflower, France), 8058 (cauliflower, Holland); OMP-BO 588/90 (cabbage, Italy).
Pseudomonas syringae pv. *phaseolicola* GSPB 1549 (bean, Germany).	*Xanthomonas arboricola* pv. *pruni* NCPPB: 419 (peach, New Zealand), 923 (plum, South Africa), 1607 peach, Australia); OMP-VR 69 (peach, Italy).
Pseudomonas syringae pv. *glycinea* OMP-BO 90.1 (soybean, Italy).	*Xanthomonas transluciens* pv. *cerealis* NCPPB (USA): 1944 (bromus), 3212 (wheat).
Pseudomonas corrugata IVIA (tomato, Spain): 536.71, 542.11, 576.71, 632.2, 712.2; LNPV (tomato, France): 3.17, 3.18, 20.03; NCPPB 2450 (tomato, England); OMP-BO (tomato, Italy): 394/91, 408/91, Pc-1/96.	*Xanthomonas campestris* pv. *vitians* ISCI (Italy, lettuce): 95 a, 95 b.
Pseudomonas fluorescens IPV-CT (citrus, Italy): 47_1, 65_1; OMP-BO 90(1) (garlic, Italy); TL 3 (potato, USA).	*Xanthomonas campestris* pv. *phaseoli* IPV-BO 1921 (bean, Hungary); NCPPB 1420 (bean, Italy).
Pseudomonas viridiflava OMP-BO (Italy): 395/91 (actinidia), 401/91 (egg-plant).	*Xanthomonas campestris* pv. *pelargonii* IPV-BO 2535 (geranium, Italy).
Pseudomonas putida BK 1 (potato, USA); TS – O34 (sugar beet, USA).	*Xanthomonas campestris* pv. *juglandis* IPV-BO 796 (walnut, Italy).
Pseudomonas marginalis OMP-BO 232/96 (lettuce, Italy).	*Erwinia carotovora* OMP-BO (Italy): 161/91, 580/91 and 858/94 (potato), 512/91 (vegetable marrow).
	Rhizobium leguminosarum ISCI (Italy): F_5, F_{10} and F_{18} (fava bean), P_{32} and P_{74} (pea), F_GT_2 and F_Gb (bean). *Mesorhizobium ciceri* ISCI (Italy): C_{18} and C_{75} (chick-pea).

Coronatine-deficient strains; DAPP-PG: Dipartimento di Arboricoltura e Protezione delle Piante, Perugia, Italy; GSPB: Göttinger Sammlung Phytopathogener Bakterien, Göttingen, Germany; IPV-BO: Istituto di Patologia Vegetale, Bologna, Italy; IPV-NA: Istituto di Patologia Vegetale, Portici, Italy; IPV-CT: Istituto di Patologia Vegetale, Catania, Italy; ISCI: Istituto Sperimentale per le Colture Industriali, Battipaglia, Italy; ISPaVe: Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; IVIA: Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; LMG: Laboratorium Mycrobiology Gent Culture Collection, Rijksuniversitseit, Belgium; LNPV: Laboratorie National de la Protetion des Vegetaux, Angers, France; NCPPB : National Collection of Plant Pathogenic Bacteria, York, Great Britain; OMP-BO: Osservatorio per le Malattie delle Piante, Bologna, Italy; OMP-VR: Osservatorio per le Malattie delle Piante, Verona, Italy; TL, BK and TS: strains provided from Tom Burr, USA.

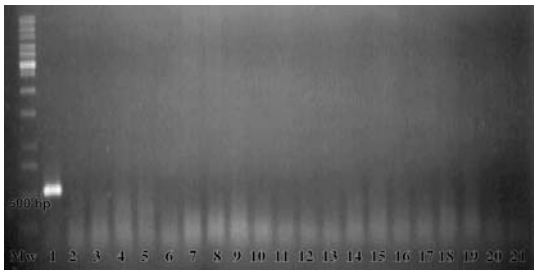

Figure 2. Gel electrophoresis analysis of PCR products amplified from pure cultures. Reactions were performed using primers MM5 and MM6 and crude cell lysates as the template. *Pseudomonas syringae* pv. t*omato* strain IPV-BO 1611 (lane 1), *Pseudomonas syringae* pv. *syringae* strain IVIA 773-1 (lane 2), *Pseudomonas syringae* pv. *phaseolicola* strain GSPB 1549 (lane 3), *Pseudomonas syringae* pv. *glycinea* strain OMP-BO 90.1 (lane 4), *Pseudomonas corrugata* strain NCPPB 2450 (lane 5), *Pseudomonas fluorescens* strain OMP-BO 90[1] (lane 6), *Pseudomonas viridiflava* strain OMP-BO 395/91 (lane 7), *Pseudomonas putida* strain BK 1 (lane 8), *Pseudomonas marginalis* strain OMP-BO 232/96 (lane 9), *Xanthomonas vesicatoria* strain NCPPB 422 (lane 10), *Xanthomonas axonopodis* pv. *vitians* strain ISCI 95a (lane 11), *Xanthomonas campestris* pv. *campestris* strain ISCI 1 (lane 12), *Xanthomonas axonopodis* pv. *phaseoli* strain NCPPB 1420 (lane 13), *Xanthomonas translucens* pv. *cerealis* strain NCPPB 1944 (lane 14), *Xanthomonas hortorum* pv. *pelargonii* strain IPV-BO 2535, *Xanthomonas arboricola* pv. *juglandis* strain IPV-BO 796 (lane 15), *Xanthomonas arboricola* pv. *pruni* strain NCPPB 419 (lane 16), *Pectobacterium carotovorum* strain OMP-BO 161/91 (lane 17), *Rhizobium leguminosarum* strains ISCI F_5 and ISCI P_{32} (lanes 18 and 19), *Mesorhizobium ciceri* strain ISCI C_{18} (lane 20). Mw is the molecular weight marker (DNA Ladder Mix M-Medical Genenco) and lane 21 is sterile water.

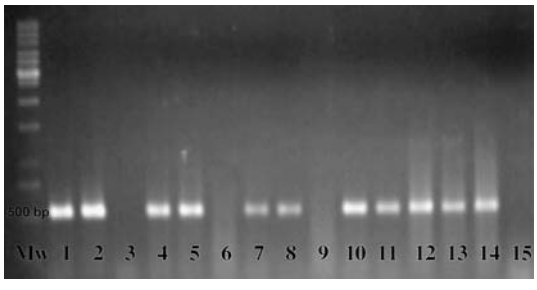

Figure 3. Gel electrophoresis analysis of PCR products amplified from infected plant material. MW = molecular weight marker (DNA Ladder Mix M-Medical Genenco); lanes 1, 2: lysates of *Pseudomonas syringae* pv. *tomato* strains LNPV 7.62 and IVIA 117.4 (positive controls); lane 3: water (negative control); lanes 4, 5, 7, 8: crude extracts of leaf spots from experimentally inoculated tomato plants; lanes 6, 9: healthy leaves; lanes 10–14: crude extracts of leaf spots from naturally infected tomato plants; lane 15 = healthy leaves from the field.

fruit lesions. The higher content in polyphenols and other secondary metabolites may account for the lower sensitivity of the method when applied directly on such crude extracts (Henson and French, 1993). This explanation is consistent with a 100% sensitivity achieved when fruit lesions were analyzed after DNA purification (Figure 3). Standard controls were performed, and in all cases amplicons were not obtained either from double sterile distilled water or from samples of healthy leaves and fruits (Figure 3).

Identification by classic bacteriological tests (namely levan production, Kovacs oxidase test, arginine dehydrolase, ice nucleation, HR on tobacco, phytopathogenetic tests on tomato plants and effector genes analyses) (Braun-Kiewnick and Sands, 2001; Zaccardelli et al., 2003) of putative *P. syringae* colonies confirmed in all cases the presence of *P. syringae* pv. *tomato* cells in the infected samples analyzed by PCR. Titration experiments showed a detection threshold of 200 cells (determined as CFU on KB-agar) per PCR reaction tube when suspensions from pure cultures were used. When asymptomatic tomato plant materials which had been experimentally contaminated were used, detection threshold was 2.5×10^5 and 2.5×10^6 cells (CFU) per gram of leaves and seeds respectively. The detection threshold obtained with asymptomatic leaf tissue is comparable with or lower than that obtained using a nonradioactive DNA hybridization probe from the coronatine gene (Cuppels et al., 1990). Nevertheless, our PCR protocol is less laborious and expensive and more reliable because it permits the detection of coronatine-deficient isolates of *P.s.tomato*.

These results show that this PCR protocol is suitable for specific detection of *P. syringae* pv. *tomato* in pure culture and in symptomatic and asymptomatic tomato plant materials. The high specificity and low sensitivity threshold of the method should allow its use for screening of tomato seeds and plantlets during certification. In particular, using this PCR protocol, the absence of detection of *P. syringae* pv. *syringae*, a bacterium that occasionally cause similar disease symptoms on tomato, avoids confusions in the certification of tomato plant materials (Denny, 1988). Target concentration of the samples and alternative downstream analysis approaches involving amplicon detection by methods other than ethidium bromide staining (Merighi et al., 2000) could be

devised in order to increase sensitivity thresholds with field material.

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References

Alfano JR, Bauer DW, Milos TM and Collmer A (1996) Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. Molecular Microbiology 19: 715–728

Alfano JR and Collmer A (1997) The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. Journal of Bacteriology 179: 5655–5662

Bereswill S, Bugert P, Volksch B, Ullrich M, Bender CL and Geider K (1994) Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. Applied and Environmental Microbiology 60: 2924–2930

Braun-Kiewnick A and Sands DC (2001) Pseudomonas. In: Schaad NW, Jones JB and Chun W (eds) Laboratory Guide for Identification of Plant Pathogenic Bacteria (pp 84–120) APS Press, St. Paul, MN, USA

Cuppels DA, Moore RA and Morris VL (1990) Construction and use of a nonradioactive DNA hybridization probe for detection of *Pseudomonas syringae* pv. *tomato* on tomato plants. Applied and Environmental Microbiology 56: 1743–1749

Denny TP (1988) Differentiation of *Pseudomonas syringae* pv. *tomato* from *P.s. syringae* with a DNA hybridization probe. Phytopathology 78: 1186–1193

Dong H, Delaney TP, Bauer DW and Beer SV (1999) Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the NIM1 gene. Plant Journal 20: 207–215

Gorris MT, Cambra M, López MM, Lecomte P, Chartier R and Paulin JP (1996) A sensitive and specific detection of *Erwinia amylovora* based on ELISA-DASI enrichment method with monoclonal antibodies. Acta Horticulture 411: 41–45

He SY, Huang HC and Collmer A (1993) *Pseudomonas syringae* pv. *syringae* harpinPss: a protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73: 1255–1266

Henson JM and French R (1993) The polymerase chain reaction and plant disease diagnosis. Annual Review of Phytopathology 31: 81–109

Hirano SS, Charkowski AO, Collmer A, Willis DK and Upper CD (1999) Role of the Hrp type III protein secretion system in growth of *Pseudomonas syringae* pv. *syringae* B728a on host plants in the field. Proceedings of the National Academy of Science of the USA 96: 9851–9856

Janse JD and Wenneker M (2002) Possibilities of avoidance and control of bacterial plant diseases when using pathogen-tested (certified) or – treated planting material. Plant Pathology 51: 523–536

Lee J, Klusener B, Tsiamis G, Lee J, Klusener B, Stevens C, Neyt C, Tampakaki AP, Panopoulos NJ, Noller J, Weiler EW, Cornelis GR, Mansfield JW and Nurnberger T (2001) HrpZ (Psph) from the plant pathogen *Pseudomonas syringae* pv. *phaseolicola* binds to lipid bilayers and forms an ion-conducting pore in vitro. Proceedings of the National Academy of Science of the USA 98: 289–294

Merighi M, Sandrini A, Landini S. Ghini S, Girotti S, Malaguti S and Bazzi C (2000) Chemiluminescent and colorimetric detection of *Erwinia amylovora* by immunoenzymatic determination of PCR amplicons from plasmid pEA29. Plant Disease 84: 49–54

Preston G, Huang HC, He SY and Collmer A (1995) The HrpZ proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea* and *tomato* are encoded by an operon containing *Yersina ysc* homologs and elicit the hypersensitive response in tomato but not soybean. Molecular Plant Microbe Interactions 8: 717–731

Psallidas PG (1988) *Pseudomonas syringae* pv. *tomato* (Okabe) Young. In: Smith IM, Dunez J and Lelliot RA (eds) European Handbook of Plant Diseases (pp 153-154) Blackwell Scientific Publications, Oxford

Wiebe WL and Campbell RN (1993) Characterization of *Pseudomonas syringae* pv. *maculicola* and comparison with *Pseudomonas syringae* pv. *tomato*. Plant Disease 77: 414–419

Wilson K (1989) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kinston RE (eds) Current Protocols in Molecular Biology. Vol. 1 (pp 2.4.1.–2.4.5) John Wiley & Sons, New York, USA

Yunis H, Bashan Y and Okon Y (1980) Weather dependence, yield losses and control of bacterial speck of tomato caused by *Pseudomonas tomato*. Plant Disease 64: 937–939

Zaccardelli M, Parisi M and Giordano I (2002) Susceptibility of tomato genotypes to *Pseudomonas syringae* pv. *tomato* in the field conditions. Journal of Plant Pathology 84: 200

Zaccardelli M, Vinatzer BA, Greenberg JT, Parisi M, Giordano I (2003) Susceptibility of resistant tomato genotypes to *Pseudomonas syringae* pv. *tomato*. Journal of Plant Pathology 85: 299