Pseudomonas syringae Pathovars and Related Pathogens

Pseudomonas syringae Pathovars and Related Pathogens – Identification, Epidemiology and Genomics

Edited by

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Preface

The Conference on *Pseudomonas syringae* which started in 1973 as an informal meeting of a group of scientists working on these bacteria in Angers, France, has become more and more important with time. Many meetings have been held since then: 1984, 1987, 1991, 1995, and 2002 in Cape Sounion, Greece; Lisbon, Portugal; Florence, Italy; Berlin, Germany; and Maratea, Italy; respectively. This Conference is considered as the most important scientific forum in which recent advances in different research aspects on *Pseudomonas syringae*, a plant pathogenic bacterial species that includes a high number of pathogens (referred as pathovars) and Related Pathogens such as *Acidovorax, Burkholderia, Ralstonia,* affecting several economically important crops. The proceedings resulting from these meetings are considered as valuable sources of information related to this group of pathogens.
The interest in organising this conference regularly is reflected by the attendance of more than 80 scientists from 20 countries worldwide, who participated at the 7th International Conference on *Pseudomonas syringae* pathovars and related pathogens organized by the Institut Agronomique et Vétérinaire Hassan II in Agadir, Morocco, from 13th to 16th November 2006.
Recent advances on:

- New methods and approaches for specific and sensitive detection and identification of *Pseudomonas syringae* and *Ralstonia solanacearum*
- Ecology and epidemiology bases of *Pseudomonas syringae* that enable the development of management strategies
- Pathogenesis and determinant of pathogenicity, and in particular, mechanisms involved in virulence and virulence gene expression
- Evolution and diversity of the pseudomonads through multilocus sequence typing (MLST) analysis
- Determination of pathogens associated with new and emerging diseases
- Effect of global warming on increase and emergence of new bacterial diseases; are reported in 43 papers written by leading scientists in the respective fields.

In this volume, manuscripts of the oral presentations and posters, presented at the 7th International Conference on *Pseudomonas syringae* pathovars and related

pathogens, are combined under six section parts Identification and Detection; Epidemiology and Disease Management; Pathogenesis and Determinants of Pathogenicity; Genomics and Molecular Characterization; Taxonomy and Evolution; and New Emerging Pathogens. Each section part is introduced by a key review paper. All the papers presented in this volume have been reviewed by the editors.

I gratefully acknowledge the fruitful collaboration of the scientific and organizing committees, the financial support of several institutions and private companies and Professor M.C. Harrouni for his precious assistance during the organization of the Conference and the preparation of this book.

Professor M'Barek FATMI

Contents

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Part I
Identification and Detection

Current Technologies for *Pseudomonas* spp. and *Ralstonia solanacearum* Detection and Molecular Typing

M.M. López[1], J.M. Quesada[1], R. Penyalver[1], E.G. Biosca[2], P. Caruso[1], E. Bertolini[1], and P. Llop[1]

Abstract Standard protocols for detection of phytopathogenic *Pseudomonas* spp. and *Ralstonia solanacearum* in plant material, soil, water or other sources, often still rely on the isolation of bacterial colonies on appropriate media, and/or on the use of serological techniques. However, over the last several years, molecular techniques, mainly based on PCR methods after extraction of nucleic acids from samples, have improved enough to allow a more rapid, reliable detection of these bacteria. When maximum accuracy is required the use of multiple techniques in an integrated approach is advised. Other promising technologies like flow cytometry, electronic nose or microarrays are emerging due to the need for developing more rapid high throughput detection methods.

Molecular typing methods are very useful to study intra-specific diversity, to identify sources of inoculum and to track the spread of the infections, although phenotypic methods are also used. Several reliable molecular techniques including AFLP, BOX, ERIC and rep-PCR, IS-RFLP, MLST, and macrorestriction-PFGE are available. However there is no single technique that can be recommended and polyphasic analyses have provided to be most useful. The diversity of Spanish strains of *R. solanacearum* and *P. savastanoi* pv. *savastanoi* analysed by several techniques is discussed in detail.

Keywords PCR, diversity, AFLP, IS*53*-RFLP, PFGE, *P. savastanoi* pv. *savastanoi*

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1 Introduction

Preventive measures for an integrated control of plant pathogenic bacteria include the use of pathogen-free propagative plant material, growth of plants in soil or substrate free of pathogens, and use of pathogen-free irrigation water. The implementation of such measures requires detection methods of high sensitivity, specificity and reliability (López et al., 2005). Furthermore, phytopathogenic bacteria may remain latent and can be present in low populations, making detection in plant material difficult. This required for rapid and accurate techniques is especially important for quarantine pathogens, because of the high risk they represent.

Efficient methods to detect phytopathogenic bacteria in soil, sediments, water, sewage, agricultural samples or air, are required to assess the role of the different inoculum sources on the ecology, epidemiology and life cycle of the target plant pathogen (López et al., 2006).

2 Techniques Commonly Used for Detection of Phytopathogenic *Pseudomonas* and *Ralstonia*

2.1 Standard Protocols

Detection of bacteria in seeds, fruits, plants, propagative material, or any other reservoir often relies on their isolation on common or semiselective media. This is followed by colony identification by their morphological, biochemical and serological characteristics and pathogenicity assays (López et al., 2003; Saettler et al., 1995; Schaad et al., 2001). However, when analysing samples in natural conditions, isolation on agar media will not detect stressed or injured bacteria or those in the viable but non culturable state (VBNC) (Grey & Steck, 2001; Ordax et al., 2006). In state the bacterial cells are unable to multiply sufficiently even on non-selective agar medium to yield visible growth as a colony.

For detection of bacteria commercial serological kits can be very useful, but they often fail to detect the pathogen in asymptomatic tissues. Furthermore, the number of useful specific antibodies available for detection is not great (Álvarez, 2004).

The current trend for detection of bacteria in the European Union (EU) is to use integrated approaches (Álvarez, 2004; López et al., 2005) including conventional, serological, and molecular techniques and to validate the protocol in ring tests (López et al., 2006). Diagnostic protocols for detection of 23 viruses, bacteria, fungi, nematodes, and insects considered as quarantine organisms in the EU, have been set up and validated in a project financed by the "Standard, Measurements and Testing" programme of the EU. The approved protocols are available through the web page of the Central Science Laboratory (www.csl.gov.uk/prodserv/know/diagpro) and have being published by the European and Mediterranean Plant Protection Organization (EPPO) (www.eppo.org). After a similar procedure, detection and protocols for *R. solanacearum* have been published as EU Directives

(Anonymous, 1998; Anonymous, 2006) and by the EPPO (Anonymous, 2004). The official protocol of the EU for *R. solanacearum* analysis is based on the combined use of several techniques (Fig. 1). It includes a detection scheme for symptomatic potato tubers and tomato or other host plants, a detection scheme for asymptomatic

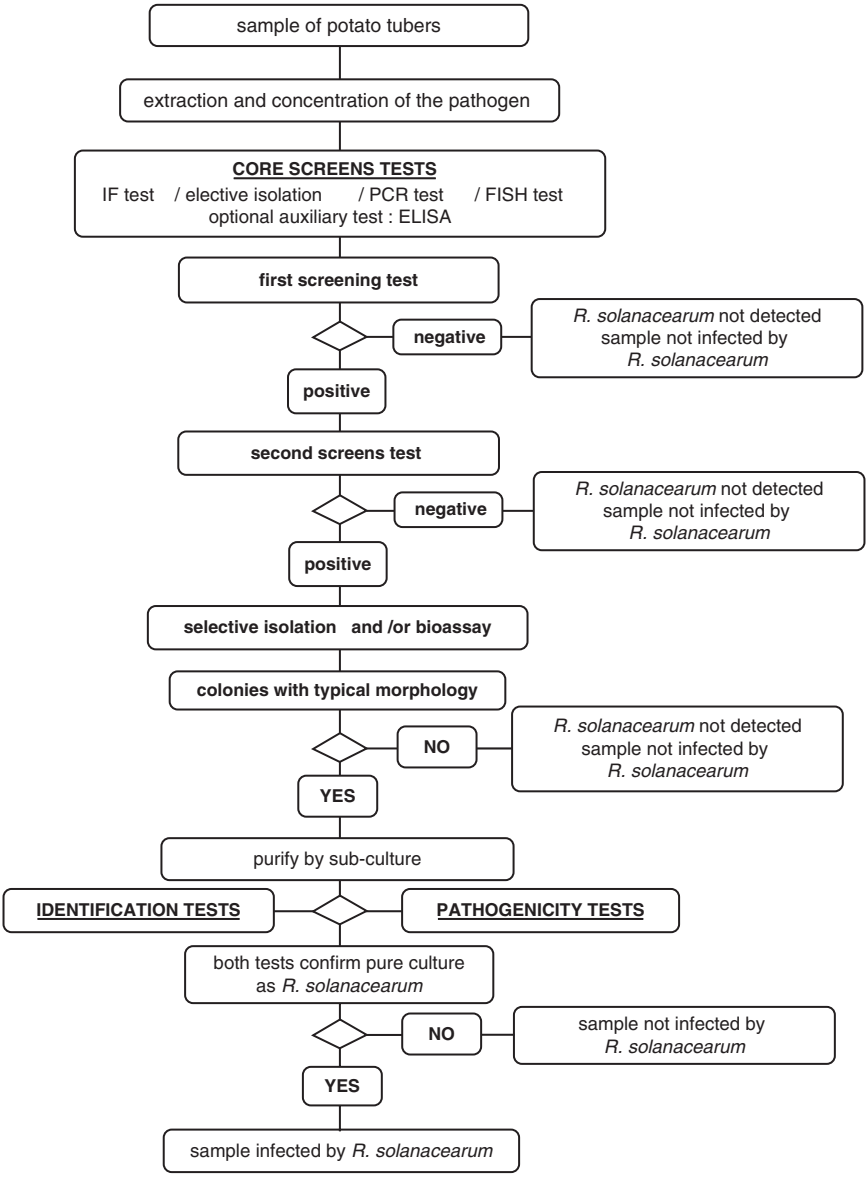

Fig. 1 Scheme for detection and identification of *Ralstonia solanacearum* in samples of asymptomatic potato tubers (Anonymous, 2006).

potato tubers and potato, tomato or other host plants, another for soil, water, potato, processing waste or sewage sludge, as well as techniques for identification of *R. solanacearum* strains. The only standard protocol recently published for the detection of phytopathogenic species of *Pseudomonas* is for *P. syringae* pv. *persicae* (Anonymous, 2005).

2.2 Molecular Methods

Nucleic-acids-based methods are stable, rapid and specific (López et al., 2003; Louws et al., 1999; Tenover et al., 1997; Vandamme et al., 1996) but have not yet completely replaced traditional isolation and phenotypic characterization in plant bacteriology. Most of the relatively new molecular tests are still being compared with conventional methods, validated and standardized before being accepted in official protocols (López et al., 2006).

The molecular methods most frequently used for detection and identification of plant pathogenic bacteria are amplification assays based on PCR. For their use in the analysis of plant or environmental samples, efficient DNA or RNA extraction or purification protocols are required. The preparation of samples is critical and target DNA or RNA should be purified to be available for the DNA polymerase. This aspect is crucial when detection methods are devised, but less important for bacterial identification, because the latter employs purified bacterial cells and a large amount of DNA is available. Depending on the material to be analysed the extraction method can be quite simple or more complex. The use of commercial kits has gained acceptance for detection due to the easiness of use and the avoidance of toxic reagents during the process (among others RNeasy and DNeasy Plant System, Qiagen, USA; Easy-DNA-Extraction kit, Invitrogen, USA; Nucleon plant tissue, Amersham, UK; EaZy Nucleic Acid Isolation Plant DNA kit, Omega Bio-tek, USA; Wizard Genomic DNA, Promega, USA; Extract-N-Amp Plant PCR kit, Sigma, USA) are used for different bacterial targets (López et al., 2006). Nevertheless, simple laboratory protocols have been developed reducing cost and time (Llop et al., 1999; Cubero et al., 1999; López et al., 2006). Several commercial automated systems, like ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems, USA) and QIAcube-Pure Efficiency (Qiagen, USA) allow the extraction and analysis of nucleic acids from plant samples and microorganisms, but they are not always efficient with all types of material.

In order to increase the population of the target and to avoid inhibitors, an enrichment step in the most appropriate general or semi-selective solid or liquid medium (Schaad et al., 1995, 2007; López et al., 1997, 2001) can be included before detection of *Pseudomonas* spp. and *Ralstonia solanacearum* from plant material, soil, sewage, water, etc. Afterwards, the target bacterium can be detected at a much lower level (López et al., 2001). Another alternative is to use immunomagnetic separation with magnetic beads coated with specific antibodies (De León et al., 2006; Walcott & Gitaitis, 2000).

2.2.1 Primer Design

The accuracy of nucleic acid technology for detection is based on the use of specific sequences (oligonucleotides/probes). As PCR is the most frequently used technique we will discuss here the design of primers for *Pseudomonas* spp. and *Ralstonia*, being used in the different formats of such technique. Different strategies have been developed to design PCR primers for specific pathogen detection but, in general, the DNA sequences from which the primers are designed for *Pseudomonas* and *Ralstonia* detection come from three main origins: pathogenicity/virulence genes, ribosomal genes, and plasmid genes (López et al., 2006). The pathogenicity genes used as targets can be involved in any of the several steps that lead to the development of symptoms. The ribosomal operon and the Internal Transcribed Spacer (ITS) region have been employed in several protocols (Louws et al., 1999). Genus-specific rDNA sequences of the phytobacteria are now available (Louws et al., 1999), and many diagnostic primers based on such sequences have been developed for detection of a number of plant pathogens (Louws et al., 1999). Then, this strategy employs primers from conserved regions of the 16S and 23S ribosomal genes to amplify the ITS region, that can include several tRNA genes and noncoding regions. Plasmid DNA is also widely employed in the design of primers: the plasmid genes amplified may be associated with pathogenicity, as indicated above, or be of unknown function. However a major problem with plasmids can be their possible lack of stability and their transmissibility (López et al., 2006; Louws et al., 1999).

Other sources of primers can be anonymous DNA, obtained through molecular analysis by different techniques, as random amplified polymorphic DNA (RAPDs) for *Pseudomonas corrugata* (Catara et al., 2000). Genomic subtraction, a powerful non-sequencing approach to find genetic differences between bacterial strains (Agron et al., 2002; Mills et al., 1997), can identify nearly all major sequence differences between two closely related bacteria and has been used to design specific probes to identify *R. solanacearum* (Seal et al., 1992).

The list of available primers for the detection of *Pseudomonas* species is increasing and often several sets of primers to several targets of the same pathogen are available (Table 1). At least 24 different primers pairs had been designed to detect *R. solanacearum* (Arahal et al., 2004). An important feature to take into account is the reliability of the information available in the sequence databases from which to perform the design of specific primers for detection. Unfortunately, often times published primers have discrepancies with the sequences to which they should match (Arahal et al., 2004).

2.2.2 Classical PCR, Nested PCR, Co-Operational PCR and Multiplex PCR

Classical PCR protocols have been developed for the many important plant pathogenic bacteria (López et al., 2003; Álvarez, 2004). They normally show a relatively good sensitivity for bacterial detection in plant material (10^3–10^4 bacterial cells/ml

Table 1 PCR protocols for detection or identification of several *Pseudomonas* species

Species	Target DNA	Number of sets of primers designed
Pseudomonas spp.	16S rRNA	1 (1998)
P. avellanae	*hrp W*	1(2002)
P. corrugata	Unknown	1 (2000)
P. syringae	IS*50,* Tabtoxin	1 (1997), 1 (1998)
pv. *actinidiae*	*arg K*	1 (1997), 1 (2002)
pv. *atropurpurea*	Coronatine, *cfl*	1 (1996), 1 (1995)
pv. *phaseolicola*	Phaseolotoxin, *Tox*-, arg K	5 (1991–1996), 1 (2006) 2 (1997), 1 (2006), 1 (2007)
pv. *papulans*	*hrp L*	1 (2002), 1 (2006)
pv. *pisi*	RAPD	1 (1996)
pv. *syringae*	*syr B, syr D*	1 (1998), 1 (2003)
pv. *tagetis*	Tagetitoxin	1 (2004)
pv. *tolaasi*	Tolaasin	1 (2004)
pv. *tomato*	*hrp 2*	1 (2005)
pv. *cannabina*	*efe*	1 (1997)

extract) and good specificity. For both reasons there is an increasing number of laboratories that include them in routine detection, after a DNA extraction step. Some sensitivity problems associated with classical PCR have been overcome by using nested PCR which can detect about 1–10^2 bacterial cells/ml extract (Prosen et al., 1993). The process is based on two consecutive rounds of amplification but when it is performed in different tubes it increases the risk of contamination, especially when such method is used on large scale (López et al., 2006). To avoid it, the single tube nested PCR has been proposed for *P. savastanoi* pv. *savastanoi* (Bertolini et al., 2003b).

The co-operational amplification proposed for sensitive and specific detection of some viruses, has been applied to *R. solanacearum* (Caruso et al., 2003). The process can be performed easily in a simple reaction based on the simultaneous action of three or four primers. When coupled with colorimetric detection, the sensitivity is about 1–10 bacterial cells/ml extract. However, the low final volume of reagents can increase the susceptibility to inhibitors in the sample, requiring also an initial DNA extraction or an enrichment step.

Multiplex amplification is based on the use of a PCR mix with different compatible primers, specific to different targets. The use of a pair of common primers to amplify different targets is not advised because the reaction will be displaced to the most abundant target. Another technique, multiplex nested PCR in a single reaction, combines the advantages of the multiplex with the sensitivity, specificity and reliability of the nested, but it needs an accurate design of compatible primers. It can be used for the simultaneous detection of several pathogens (viral RNA and bacterial or fungal DNA targets) in a single analysis. Nevertheless, only in one case has this technology been carried out in a single reaction for including specific detection of four viruses (*Cucumber mosaic virus*, *Cherry leaf roll virus*, *Strawberry latent ring spot virus* and *Arabis mosaic virus*) and the bacterium *P. savastanoi* pv.

savastanoi in olive plant material, using 20 compatible primers (Bertolini et al., 2003a) and it is proposed as an analysis for certification purposes. Multiplex nested-PCR saves time and reagents because it can be performed in a single reaction. The sensitivity achieved for detection of *P. savastanoi* pv. *savastanoi* by multiplex nested RT-PCR was similar to the sensitivity reached by applying the monospecific nested PCR after an enrichment step, which demonstrated to be 100-fold more sensitive than conventional PCR (Penyalver et al., 2000; Bertolini et al., 2003b). This multiplex nested RT-PCR has been coupled with colorimetric detection increasing the sensitivity and facilitating the interpretation of results (Bertolini et al., 2003a).

2.2.3 Real-Time PCR

Classical PCR-based methods have good sensitivity and specificity but do not provide quantitative data and requires agarose gel electrophoresis and hybridisation as the confirming endpoint analysis. On the contrary, real-time (RT) PCR allows amplification, detection, monitoring and quantitation in a single step by employing several chemistries which are used to detect PCR products as they accumulate within a closed reaction vessel during the reaction. It has been proposed for detection of *R. solanacearum* (Weller et al., 2000; Ozakman & Schaad, 2003) and for *P. savastanoi* pv. *phaseolicola* (Schaad & Frederic, 2002; Schaad et al., 2007) among other plant pathogenic bacteria and it is expected that protocols for the most common *Pseudomonas* will be developed in the near future.

Moreover, the advantages this technology offers is leading to more quickly and accurate detection protocols. In addition, the identification of a pathogen in imported material may cause problems at the point of introduction, especially for perishable commodities because the time needed for sending the sample to a specialized laboratory represents a delay to take the appropriate measures. This can be solved with portable real-time PCR instruments (RAPID. system, Idaho Technology, Utah, USA; Smart Cycler, Cepheid, USA), that allow a rapid on-site diagnosis (Schaad et al., 2003).

A comparative evaluation of several described PCR protocols for their sensitivity in detection of *R. solanacearum performed in our laboratory* is shown in Table 2. The different protocols, based in the use of several sets of primers reached sensitivity levels from 10^{-1} to 10^{3} cfu/ml in pure cultures and from 10 to 10^{3} cfu/ml in plant material, after DNA extraction following Llop et al. (1999) or using commercial kits.

2.3 Flow Cytometry

Flow cytometry (Davey & Kell, 1996) is a technique for rapid identification of cells or other particles as they pass individually through a sensor in a liquid stream, providing quantitative and sensitive detection in few hours. Bacterial cells are

Table 2 Comparison of sensitivity of conventional and real-time PCR protocols for *R. solanacearum* detection (López et al., unpublished data)

		Sensitivity	
Reference of the protocol	PCR type	Pure culture (cfu/ml)	Plant material (cfu/ml)
Caruso et al. (2003)	Co-PCR	10^{-1}	10
Seal et al. (1993)	OLI 1/Y2	10^3	10^4
Boudazin et al. (1999)	OLI 1/2	10^3	10^2
Weller et al. (2000)	Real-time	10^2	10^3
Ozakman & Schaad (2003)	Bio real-time	ND[a]	10

[a] ND Not determined

identified by fluorescent dyes conjugated to specific antibodies and detected electronically using a fluorescence-activated cell sorter, which measures several cellular parameters based on light scatter and fluorescence. Multiparameter analysis includes cell sizing, fluorescence imaging and gating out, or elimination of unwanted background associated with dead cells and debris. Flow cytometry has excellent potential as a research tool and possibility for routine use in seed health testing and other fields (López et al., 2003). The cost for instrumentation is currently a major disadvantage that will be solved when less expensive models become available.

2.4 Electronic Nose

Sensor systems for the easy detection of *R. solanacearum* potato tubers have been developed recently (Stinton et al., 2006). The system operates through an electronic nose containing a set of sensors selected for their sensitivity to marker volatile organic compounds and the resulting data are captured, displayed and recorded by computer. The electronic nose appears promising and this technology would be applicable to the detection of statutory organisms by plant health and seed inspectors (de Lacy Costello et al., 2006) but in addition it could be very useful for accurate identification of pure cultures.

2.5 Microarray Technology

Although the potential of the microarray technology in the detection and diagnosis of plant diseases is great, the practical development of these applications is still under progress and few are available for diagnosis of plant pathogens (Schoen et al., 2002, 2003; Fessehaie et al., 2003). Despite the slow development of the microarray technology for detection of plant pathogenic bacteria, it shows some potential features that make it a very promising tool. The use of thousands of probes at the same time allows the possibility of detection and differentiation of several pathogens in

only one analysis. Nevertheless, the need for a previous PCR reaction, the low level of sensitivity achieved, and the high cost of the equipment makes this technique still far from being used for routine analysis of plant pathogens (López et al., 2006) but it could be very useful for accurate identification of pure cultures.

3 Molecular Typing of Phytopathogenic *Pseudomonas* and *Ralstonia*

There are many techniques available for molecular typing of different species of plant pathogenic bacteria. Table 3 summarises the most frequently utilised and their efficiency for identification and diversity in species of both genus. Perhaps the most promising is Multilocus Sequence Typing (MLST). MLST studies are based on sequences from housekeeping genes located on the bacterial core genome, and the method has been proposed for sensitive and reproducible typing of bacterial strains. It allows the assessment of the relative contribution of mutations and recombinations in the evolution of a species and determines the clonal relationships between strains. MLST analysis of a wide range of *P. syringae* strains found that it was a clonal species (Sarkar & Guttman, 2004), and when applied to *R. solanacearum* 18 sequence types were described (Danial et al., 2006).

Other molecular typing methods have demonstrated their efficiency for intra-specific diversity studies in *Pseudomonas* spp. and *R. solanacearum*, but there is no universally advised technique neither for diversity studies nor for molecular typing. We briefly describe here, in two examples, the results obtained when analysing the molecular diversity of Spanish strains of *R. solanacearum* and a collection of *P. savastanoi* pv. *savastanoi* strains isolated from olive trees.

3.1 Diversity of R. solanacearum

Ralstonia solanacearum, responsible of bacterial wilt, is one of the most important bacterial diseases of crops in the world. The bacterium was first detected in potato in Spain in 1995 and, since then, several outbreaks have been identified in different Spanish regions but rapid eradication measures following European Directive 98/57/EC (Anonymous, 1998) have been effective to prevent the spread of the disease.

R. solanacearum represents a heterogeneous group of bacteria (Fegan & Prior, 2005). The species was subdivided into five races based on host range and six biovars based upon carbohydrate utilization patterns. The potato pathogen belongs to race 3, biovar 2. There is no relationship between races and biovars (Hayward et al., 1990).

PCR restriction fragment length polymorphism analysis (PCR-RFLP), amplified fragment length polymorphism (AFLP), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), fatty acid methyl esters (FAME) analysis, 16S

Table 3 Some techniques employed for molecular characterization of *Pseudomonas* spp. and *R. solanacearum*

Technique[a]	Identification	Diversity	Remarks
16s rDNA	+++	+	Automated, discrimination at genus level
RFLP	–	++	Difficult, reliable
RAPDs	–	++	Simple, intermediate reliability
Rep-PCR	++	++	Simple, variable reliability
Ribotyping	++	+	Simple, variable reliability
PCR-RFLP	+++	++	Simple, low discrimination at strain level
SSR	–	++	Simple, variable reliability
AFLP	–	+++	Difficult, reliable, discrimination at strain level
PFGE	+	+++	Difficult, reliable, discrimination at strain level
MLST	++	+++	Simple, reliable, discrimination at species level
MLEE	–	++	Difficult
HMA-ITS	–	+++	Difficult, reliable, discrimination at infraspecific level

–: not appropriate, in general

+: sometimes useful

++: generally useful

+++: very useful

[a] Abbreviations: RFLP, restriction fragment-length polymorphism; RAPDs, random amplified polymorphic DNA analyses; Rep-PCR, repetitive PCR fingerprinting; PCR-RFLP, PCR restriction fragment length polymorphism analysis; SSR, short sequence repeats; AFLP, amplified fragment length polymorphism; PFGE, pulsed field gel electrophoresis; MLST, multilocus sequence typing; MLEE, multilocus enzyme profiles; HMA-ITS, heteroduplex mobility assay of internal transcribed spacer

rRNA, gene sequencing, RFLP, amplified ribosomal DNA restriction analysis (ARDRA), macrorestriction pulse field gel electrophoresis (PFGE) and sequence analysis of 16S–23S rRNA, indicate a possible selection of a *R. solanacearum* "European" variant (Timms-Wilson et al., 2001). To date, all potato strains isolated in Europe have been identified as race 3, biovar 2. However, only two studies have focused on the characterization of European strains (Van der Wolf, 1998; Timms-Wilson et al., 2001) showing that some of these techniques are able to discriminate the variability inside European strains of race 3, biovar 2.

The genetic diversity of 44 representative strains of *R. solanacearum* biovar 2 isolated from 1995 to 2002 from different sources in Spain, and reference strains from The Netherlands and USA, have been analysed by phenotypic and genotypic methods, including biochemical and serological characterization, repetitive PCR fingerprinting (Rep-PCR), macrorestriction followed by PFGE and AFLP (Caruso, 2005). This represents the first study of the characteristics of a large collection of Spanish strains of *R. solanacearum*.

Ten PFGE patterns were obtained after *Xba* I digestion and five patterns after using *Spe* I and, in general, the strains showing the same PFGE pattern shared their

region of origin. Clustering analysis of the AFLP profiles yielded five clusters and in four of them, strains were also grouped according to their source and origin, but in the remaining one, strains from different geographical origins were also grouped. Figure 2 shows the combined analysis of all these results. It supports the hypothesis that several clones of the pathogen have been introduced into Spain, as previously was suggested by several authors for other European countries (Van der Wolf, 1998; Timms-Wilson et al., 2001).

3.2 Diversity of P. savastanoi *pv.* savastanoi

Studies on the genetic diversity of *P. savastanoi* (Psv) strains have focused in the relationships among strains isolated from different hosts. However, studies on the genetic diversity of a worldwide collection of *P. savastanoi* pv. *savastanoi* isolated from olive are lacking. Only Scortichini et al. (2004) studied the genetic structure of an Italian collection of Psv strains isolated from different provinces and olive cultivars by repetitive PCR, using short interspersed elements. They observed 20 patterns among the 360 Italian strains with an overall similarity of 81%, with no apparent grouping.

A collection of 62 strains of Psv isolated from olive knots (from which 44 were isolated in Spain) were examined for the distribution, variation in positions and copy number of the IS*53* insertion element, originally described in an oleander strain (Soby et al., 1993). Southern hybridization analysis revealed that the genetic

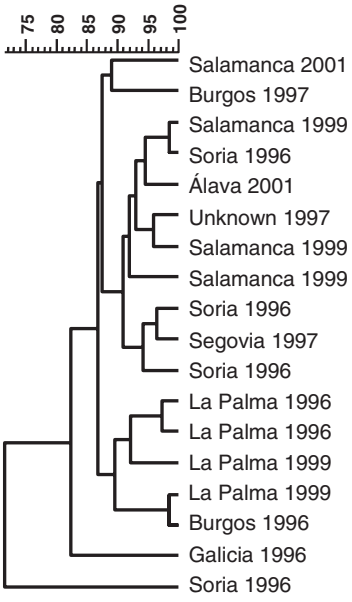

Fig. 2 Dendrogram based on combined results after macrorestriction with *Xba* I and *Spe* I followed by PFGE and AFLP, of *Ralstonia solanacearum* strains from different Spanish areas (Caruso, 2005). Average taken from experiments using Pearson correlation (PFGE), Dice coefficient (AFLP) and UPGMA clustering

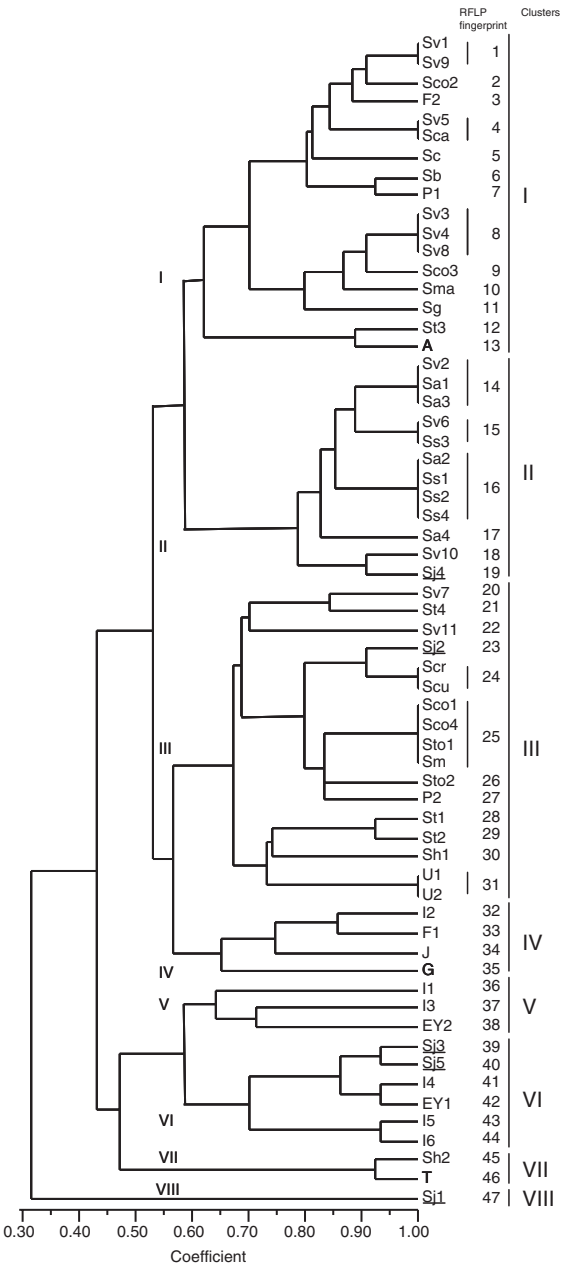

Fig. 3 Dendrogram based on RFLP fingerprints of IS*53* element of *P. savastanoi* pv. *savastanoi* strains from olive. Spanish strains are those beginning by S and the following letter indicates the province. The others are reference strains from other countries (Quesada, 2007)

element IS*53* was present in multiple copies in all analysed strains isolated from olive knots. Copy number of IS*53* elements ranged from four up to ten. Southern hybridization analysis of plasmid DNA using IS*53* as a probe revealed that this genetic element is present in the chromosomal replicon in the six olive strains

analysed, instead of in plasmids, as it was first described in the original strain. Although Psv strains displayed a remarkably high degree of IS*53* restriction fragment length polymorphism, transposition of this element was not detected in Psv olive strains grown either *in vitro* for up to 390 generations (Quesada, 2007). The genetic diversity of a worldwide collection of Psv strains based on 47 different IS*53*-RFLP fingerprints and UPGMA analysis allowed clustering all strains into eight groups with a similarity of 60% (Fig. 3).

Two groups (II and VIII) were composed by only Spanish strains. One of these groups (VIII), with only one strain from Jaén province, was separated from the rest of strains. Only nine out of 47 distinguishable RFLP fingerprints were shared by more than one strain (1, 4, 8, 14, 15, 16, 24, 25, 31) suggesting a common origin for all these strains. The genetic diversity within strains from Spanish provinces was quite similar to that between provinces, suggesting a lack of any geographical differentiation. Additionally, the Spanish strains were not distinguishable from strains from other sources. Due to the widespread distribution on Psv, its stability *in vitro* and *in vivo*, and the high degree of polymorphism generated, IS*53*-RFLP typing is considered a suitable marker for epidemiological and ecological studies.

4 Conclusions

Developing detection methods is a never-ending process and the detection of plant pathogenic *Pseudomonas* and *Ralstonia* is moving from conventional methods to the use of molecular techniques included in an integrated approach, that is required for maximum accuracy (Álvarez, 2004; López et al., 2005, 2006). Molecular detection is now widely used based on classical and/or PCR or Real-time PCR, but purification of nucleic acids from samples is generally required and should be optimised. PCR and especially real-time PCR are the methods of choice for rapid and accurate diagnosis of plant pathogenic bacteria, but conventional methods as IF are still widely used.

There is a lack of standard protocols for detection of plant pathogenic species and pathovars of most *Pseudomonas*, but there is an official EU protocol for detection of *R. solanacearum*.

The increased number of studies published on the diversity of some *Pseudomonas* species and *R. solanacearum* have shown that for each bacterial model, different techniques must be analysed in preliminary studies, to evaluate their respective value for the purpose of the study. It is expected that with the decrease in the costs of sequencing of total bacterial genomes, molecular techniques will replace the current analysis of diversity and will facilitate molecular typing.

The advances in genomics, proteomics and metabolomics have not had remarkable repercussions yet on developing more reliable detection and identification methods, but their impact should be important in the next few years. This could be due to the fact that developing diagnosis methods is considered of minor scientific interest. However, with the recent access to complete genome sequences,

and the microarray possibilities, the function of gene products will soon be determined and their role in bacterial cells will be understood. This could lead to new research issues, especially in the field of diagnosis and hopefully innovative methods will facilitate early detection of bacterial diseases. However, the success of the practical use of these methods will depend on the heterogeneity of the target bacteria, the functional significance of the identified genes in the progression of the disease and the expression of the genes at the different steps of the interaction between the bacteria and the plant hosts.

The development of protocols of higher sensitivity and specificity for detection of plant pathogenic *Pseudomonas* and *Ralstonia* will have a positive effect in the sanitary status of their hosts, reducing the need for pesticide treatments, protecting ecosystems and enhancing the quality of the food and the environment.

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Siderophore Uses in *Pseudomonas syringae* Identification

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Abstract The diversifying evolution observed in pyoverdin genes and the presence of numerous siderophore membrane receptors indicates that iron-supplying systems are important for fitness of the fluorescent pseudomonads. It is useful for a bacterium to produce a siderophore unusable by others and siderophores, or siderophore genes, that are specific to certain bacteria have been selected during evolution; they can therefore be used in identification. Pyoverdins are the principal siderophores of fluorescent pseudomonads and they contain a variable peptide chain. Because of the evolution in this peptide chain, the pyoverdins of *Pseudomonas cichorii* and *P. syringae*, and related species, are differentiable by visual and spectrophotometrical analysis from pyoverdins produced by species belonging to the saprophytic fluorescent *Pseudomonas* group. The common pyoverdin of *P. syringae* and related species *P. viridiflava* and *P. ficuserectae* has been shown to be specific. This characteristic can therefore be used for presumptive identification of these species by HPLC analysis of their pyoverdin. This significantly reduces the number of identification tests required. Additionally, these approaches worked well to rapidly discriminate among fluorescent strains in early stages of isolation, and to identify strains of *P. viridiflava* and of *P. syringae* pathogenic on fruit and horse-chestnut trees. *P. syringae* pathovars *antirrhini*, *apii*, *avii*, *berberidis*, *delphinii*, *lachrymans*, *passiflorae*, *persicae*, *tomato*, *maculicola*, *viburni*, *helianthi*, *tagetis*, *theae* and *morsprunorum* race 2 (genospecies 3, 7 or 8), as well as *P. phaseolicola* and *P. glycinea* (genospecies 2) possess an *irp1* gene involved in the production of the yersiniabactin siderophore. However, as yersiniabactin production is not systematically observed by HPLC, an *irp1*-based PCR test was used for these pathovars.

Keywords Siderophore, pyoverdin, yersiniabactin, *Pseudomonas syringae*, identification

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