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Genetic diversity reflects geographical origin of *Ralstonia solanacearum* strains isolated from plant and water sources in Spain

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Summary. The characterization and intraspecific diversity of a collection of 45 *Ralstonia solanacearum* strains isolated in Spain from different sources and geographical origins is reported. To test the influence of the site and the host on strain diversity, phenotypic and genotypic analysis were performed by a polyphasic approach. Biochemical and metabolic profiles were compared. Serological relationship was evaluated by Indirect-ELISA using polyclonal and monoclonal antibodies. For genotypic analysis, *hrpB* and *egl* DNA sequence analysis, repetitive sequences (rep-PCR), amplified fragment length polymorphism (AFLP) profiles and macrorestriction with *Xba*I followed by pulsed field gel electrophoresis (PFGE) were performed.

The biochemical and metabolic characterization, serological tests, rep-PCR typing and phylogenetic analysis showed that all analysed strains belonged to phylotype II sequevar 1 and shared homogeneous profiles. However, interesting differences among strains were found by AFLP and macrorestriction with *Xba*I followed by PFGE techniques, some profiles being related to the geographical origin of the strains. Diversity results obtained offer new insights into the biogeography of this quarantine organism and its possible sources and reservoirs in Spain and Mediterranean countries.

Keywords: Bacterial wilt · potato · soil · PFGE · AFLP

Introduction

Ralstonia solanacearum (E. F. Smith) [45], responsible for potato brown rot and bacterial wilt of Solanaceae and other hosts, is one of the most harmful bacterial pathogens and a quarantine organism in the European Union (EU) [7]. It can persist, for varying periods in soil, water, rhizosphere, plant residues or inside host plants [3,4,5,12,42], reaching aquatic niches from infested plants. *R. solanacearum* has been long considered as a species complex [22] due to its high intraspecific variability. This bacterial species was traditionally subdivided in five races based on host range and six biovars based on the utilization of different sugars [23,25]. In 2005, Fegan and Prior [21] proposed a new classification scheme, based on the phylogenetic analysis of the sequences of the 16S-23S rRNA intergenic region and the endoglucanase (*egl*)

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and *hrpB* virulence genes, dividing *R. solanacearum* strains in four phylotypes that correlated with different geographic origins [21]. In Europe, *R. solanacearum* strains isolated from potato, tomato, soil, waterways and weeds have been classified, as phylotype II, historically known as biovar 2 race 3 (b2r3), except for some biovar 1 strains isolated in 2007 in a confined area of Portugal [3,12,14,15,33,38]. However, the *R. solanacearum* species complex has recently experienced a major taxonomic revision [36] and consequently, only phylotype II strains belong to the current *R. solanacearum* species.

In the EU, since the first identification of *R. solanacearum* in Sweden in 1972 [31], several outbreaks have been reported in Northern and Western countries such as United Kingdom, The Netherlands, Belgium, France and Portugal [19] and in Mediterranean countries such as Italy, Greece and Spain [2,19,29,32]. In Spain, this pathogen was first isolated from potatoes cultivated in Canary Islands and later from the mainland [20,32]. Subsequent surveys also led to the detection of *R. solanacearum* from water in some rivers located in several regions, as well as on potato or tomato crops [12,32]. Eradication measures were taken in the detected foci following the European Directives 98/57/EC and 2006/63/EC to prevent the spread of the disease [6,8].

Typing methods that could discriminate *R. solanacearum* strains from different sources can be useful in tracing back bacterial wilt outbreaks, thereby allowing a better understanding of the epidemiology and ecology of this pathogen in Mediterranean countries and leading to the development of more effective prevention and eradication strategies [9,15,28]. Until now, only a few European studies have been focused on the characterization of the diversity within European strains of *R. solanacearum* [15,33,38,40,41]. In the first one, van der Wolf et al. [41], analysed 30 strains from Europe and 4 from outside Europe, showing that, using rep-PCR and *Xba*I digested genomic DNA followed by pulsed field gel electrophoresis (PFGE), it was possible to detect some variability among race 3 strains from France, The Netherlands and the United Kingdom. Thereafter, Timms-Wilson et al. [40] analysed 44 strains from Europe and 38 from the 'rest of the world'. They proposed the possible selection of a *R. solanacearum* "European" variant, according to results of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), fatty acid methyl esters (FAME), exopolysaccharide (EPS) production, 16S rRNA, RFLP, amplified ribosomal DNA restriction analysis (ARDRA) and sequence analysis of 16S–23S rRNA gene. More recently, Stevens and van Elsas [38] found clear genomic differences among 44 Dutch strains from different sources, including water, sediment and bittersweet plants (*Solanum dulcamara*) by using PFGE analysis of *Xba*I restricted genomic DNA. Cruz et al. [15] used a polyphasic approach to analyze *R. solanacearum* strains isolated from plants and environmental sources in Portugal. They observed higher polymorphism levels with Rep-PCR and fluorescent amplified fragment length polymorphism (FAFLP) techniques. Instead Parkinson et al. [33] used Variable-Number Tandem Repeat (VNTR) analysis for source tracing of *R. solanacearum* strains associated with English watercourses and bacterial wilt outbreaks.

In our work, the diversity of representative strains of *R. solanacearum* isolated from different bacterial wilt outbreaks and waterways in Spain, was analysed by phenotypic and genotypic methods, including biochemical and serological tests, rep-PCR, PFGE, AFLP, and the sequence analysis of the endonuclease (*egl*) and the regulatory transcription regulator (*hrpB*) genes. Our goal was to study the molecular epidemiology of *R. solanacerum* in our environmental conditions, as a basis for designing more efficient eradication and/or control strategies for this pathogen in Spain and other countries.

Materials and methods

Bacterial identification. A selection of 45 strains of *R. solanacearum* isolated in Spain from different sources, sampling points and geographical origins was used as well as three afluid variants that spontaneously appeared in Yeast Peptone Glucose Agar (YPGA) [27] plates and were named IVIA 1546-af, IVIA 1632.2-af, and IVIA 1861-af. One reference strain, PD 2762 (or IPO 1609, from The Netherlands), was also included in some analyses for comparative purposes (Table 1). Bacterial strains were maintained at -80°C in 30% (v/v) glycerol and routinely cultured on YPGA at 29ºC for 72 h. The Spanish isolates were initially identified as *R. solanacearum* by their colonial morphology on modified SMSA agar [18] and YPGA after 48-72 h at 29ºC. Gram-staining, nutritional and enzymatic tests were performed for their identification as described in Commission Directive 2006/63/EC [8].

Presumptive *R. solanacearum* isolates were also identified by a DASI-ELISA detection kit (PlantPrint Diagnostics, Valencia, Spain) using the specific monoclonal antibody (MAb) IVIA-8B for this pathogen [11]. The isolates were further confirmed by a specific Co-PCR assay [10], phenotypic tests and by multiplex PCR [21,35]. Strains *R. solanacearum* PD 2762 and *Chryseobacterium indologenes* P 27 were included respectively as positive and negative controls.

Pathogenicity on potted tomato plants (cv Roma, 3-4 weeks old) was tested according to Council Directive 98/57/EC [6], using the reference strain PD 2762 and sterile phosphate buffered saline (PBS) (10 mM, pH 7.2) as positive and negative controls, respectively. Wilting appearance was monitored every two days and symptoms severity was recorded as indicated in Table 1. All the tests were performed at least twice on separate assays.

Phylotype determination. *R. solanacearum* strains were classified into phylotypes by multiplex PCR [21] using phylotype and *R. solanacearum* specific primers (Table 2). Phylotypes were identified based on the reported phylotype-specific PCR amplicons.

Table 1. Characteristics of *Ralstonia solanacearum* strains used in this study.

Strain	Geographic origin [d]	Source	Phylotype/ biovar	Pathogenicity test [e]	PFGE pattern (XbaI)	AFLP cluster
Spanish [a]					*Xba*I	
IVIA 1496.1a [g]	Canary Islands (La Palma)	*S. tuberosum*	II/2	2		B
IVIA 1532.4 [h]	**Galicia**	***S. tuberosum***	**II/2**	**3**	**X7**	**SP**
IVIA 1546-f [h]	**Castile and Leon (Burgos)**	***S. tuberosum***	**II/2**	**3**	**X10**	**SP**
IVIA 1546-af [b,h]	Castile and Leon (Burgos)	*S. tuberosum*	II/2	1		SP
IVIA 1600.4.1	Canary Islands (La Palma)	*S. tuberosum*	II/2	2		
IVIA 1602.1 [h]	**Canary Islands (La Palma)**	***S. tuberosum***	**II/2**	**2**	**X9**	**SP**
IVIA 1602.10 [g,h]	Canary Islands (La Palma)	*S. tuberosum*	II/2	2		SP
IVIA 1620.1.1	**Castile and Leon (Soria)**	***S. tuberosum***	**II/2**	**2**	**X4**	**E**
IVIA 1632.2-f	**Castile and Leon (Soria)**	***S. tuberosum***	**II/2**	**2**	**X1**	**C**
IVIA 1632.2-af [b,g]	Castile and Leon (Soria)	*S. tuberosum*	II/2	5		C
IVIA 1634 [g]	Castile and Leon (Salamanca)	*S. tuberosum*	II/2	2		A
IVIA 1635	**Castile and Leon (Soria)**	***S. tuberosum***	**II/2**	**3**	**X1**	**A**
IVIA 1671	Castile and Leon (Soria)	*S. tuberosum*	II/2	2		
IVIA 1672 [i]	**Castile and Leon (Burgos)**	***S. tuberosum***	**II/2**	**4**	**X1**	**C**
IVIA 1673 [i]	Castile and Leon (Burgos)	*S. tuberosum*	II/2	5		
IVIA 1674	**Unknown**	***S. lycopersicum***	**II/2**	**2**	**X2**	**A**
IVIA 1678 [i]	Castile and Leon (Soria)	*S. tuberosum*	II/2	4		
IVIA 1692 a	**Castile and Leon (Segovia)**	***S. tuberosum***	**II/2**	**2**	**X1**	**E**
IVIA 1738.1	Unknown	*S. lycopersicum*	II/2	2		
IVIA 1760.1.1b [g]	Canary Islands (La Palma)	*S. tuberosum*	II/2	2		B
IVIA 1778.1.1	Unknown	*S. lycopersicum*	II/2	2		
IVIA 1805.1 a	Canary Islands (La Palma)	*S. tuberosum*	II/2	3		
IVIA 1861-f	Castile and Leon (Salamanca)	*S. tuberosum*	II/2	2		
IVIA 1861-af [b]	Castile and Leon (Salamanca)	*S. tuberosum*	II/2	5		
IVIA 2068.58.a	**Canary Islands (La Palma)**	***Potato roots***	**II/2**	**2**	**X2**	**D**
IVIA 2068.61.a	Canary Islands (La Palma)	Potato roots	II/2	3		
IVIA 2093.3.1	**Canary Islands (La Palma)**	***S. tuberosum***	**II/2**	**3**	**X2**	**D**
IVIA 2093.5.t1.a	Canary Islands (La Palma)	*S. tuberosum*	II/2	2		
IVIA 2158.1b	Castile and Leon (Salamanca)	*S. tuberosum*	II/2	2		
IVIA 2158.3	**Castile and Leon (Salamanca)**	***S. tuberosum***	**II/2**	**2**	**X3**	**A**
IVIA 2167.1a [h]	**Castile and Leon (Salamanca)**	**River water**	**II/2**	**2**	**nd**	**SP**
IVIA 2167.1b	**Castile and Leon (Salamanca)**	**River water**	**II/2**	**2**	**X3**	**nd**
IVIA 2167.2b [h]	**Castile and Leon (Salamanca)**	**River water**	**II/2**	**2**	**X5**	**SP**
IVIA 2297.4T2.a [g]	Canary Islands (La Palma)	Soil	II/2	2		B

(Continue in the next page)

Table 1 (Cont.). Characteristics of *Ralstonia solanacearum* strains used in this study.

Strain	Geographic origin [d]	Source	Phylotype/ biovar	Pathogenicity test [e]	PFGE pattern	AFLP cluster
IVIA 2424 h	**Basque country (Álava)**	***S. tuberosum***	**II/2**	**3**	**X8**	**SP**
IVIA 2528.1.A3	Castile and Leon (Salamanca)	River water	II/2	3		
IVIA 2528.3.A3	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2528.4.A1	Castile and Leon (Salamanca)	River water	II/2	3		
IVIA 2528.54.A2	**Castile and Leon (Salamanca)**	**River water**	**II/2**	**2**	**X6**	**A**
IVIA 2533.1.1.A1	Castile and Leon (Salamanca)	River water	II/2	3		
IVIA 2533.A2 [g,h]	Castile and Leon (Salamanca)	River water	II/2	2		SP
IVIA 2533.7.1.A3	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2550.6.A2	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2550.10.A3	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2550.20.A4	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2550.A5	Castile and Leon (Salamanca)	River water	II/2	2		
IVIA 2567.A3.3	Castile and Leon (Salamanca)	River water	II/2	3		
IVIA 2581.A1.3	Castile and Leon (Salamanca)	River water	II/2	2		
Reference [c]						
PD 2762 or IPO 1609	The Netherlands	*S. tuberosum*	II/2	2	X8	nd

[a] *R. solanacearum* strains from IVIA, Collection of Plant Pathogenic Bacteria, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada (Valencia), Spain.
[b] Afluidal variants of three fluidal Spanish *R. solanacearum* strains that spontaneously appeared in our laboratory after subculturing.
[c] *R. solanacearum* reference strain from Collection of Plant Protection Service, Wageningen, Netherlands.
[d] Unless otherwise indicated all the strains were isolated in Spain: the first term indicates the Spanish regions and the second one indicates the provinces where the pathogen was isolated.
[e] Scale of symptom severity: 1= no symptoms, 2= wilted plants within two weeks, 3= wilted plants within three weeks, 4= wilted plants in more than three weeks; 5= atypical symptoms.
[g] Strains analyzed by AFLP in addition to those marked in bold.
[h] Strains analyzed by AFLP in addition to those marked in bold that showed a specific pattern (SP).
[i] Strains afluid originally isolated as fluid.
nd: not done.

Biochemical and physiological characterization. API 20 NE, API 50 CH, ATB G-5 and API ZYM systems (BioMèrieux, Marcy-l'Etoile, France) were used for the characterization of a selection of 17 *R. solanacearum* strains (in bold in Table 1). The manufacturer's instructions were followed for all systems, with the exception of API 50 CH gallery for which the basal medium was replaced by an inorganic one [24], the incubation temperature was 29ºC and the readings were performed after 6 h for API ZYM and 48, 72 and 96 h for the remaining systems.

The strains were further characterized by using the BIOLOG-Microlog System, version 4.0 (Biolog, Inc.), as recommended by the manufacturer, except for incubating plates at 29ºC. The metabolic profiles obtained were analysed by the MicroLog 2 program (Biolog, Inc.). Strains were tested twice in separated assays using reference strain PD 2762 of *R. solanacearum* as positive control.

PCR amplification and sequence analysis. The *hrpB* and *egl* virulence-related genes were amplified based on Castillo and Greenberg [13] with primers indicated in Table 2. PCR products were purified using QIAquick PCR purification kit (Qiagen Inc.). Sequencing was performed on both strands with the same primers used for the amplification (Table 2). Base call disagreements were visually edited. Twenty-one *R. solanacearum* accessions from phylotype II strains (based on Safni et al. [36]) retrieved from the GeneBank together with sequences from this work were included in the phylogenetic analysis. It was performed with the Molecular Evolutionary Genetics Analysis (MEGA) software v.

Table 2. Primers used for amplifying, phylotyping and sequencing.

Primer	Sequence of primer (5'-3')	Reference	Concentration (μM)
Nmult:21:1F	CGTTGATGAGGCGCGCAATTT	Fegan and Prior 2005	0.24
Nmult:21:2F	CGTTGATGAGGCGCGCAATTT	Fegan and Prior 2005	0.24
Nmult:23:AF	ATTACSAGAGCAATCGAAAGATT	Fegan and Prior 2005	0.72
Nmult:22:InF	ATTGCCAAGACGAGAGAAGTA	Fegan and Prior 2005	0.24
Nmult:22:RR	TCGCTTGACCCTATAACGAGTA	Fegan and Prior 2005	0.24
Rs759	GTCGCCGTCAACTCACTTTCC	Opina et al. 1997	0.16
Rs760	GTCGCCGTCAGCAATGCGGAATCG	Opina et al. 1997	0.16
Endo F	ATGCATGCCGCTGGTCGCCGC	Castillo and Greenberg 2007	0.2
Endo R	GCGTTGCCCGGCACGAACACC	Castillo and Greenberg 2007	0.2
HrpB F	TGCCATGCTGGGAAACATCT	Castillo and Greenberg 2007	0.8
HrpB R	GGGGGCTTCGTTGAACTGC	Castillo and Greenberg 2007	0.8

6.0 [39], using neighbour joining (NJ) and maximum likelihood (ML) methods and the algorithm of Jukes and Cantor and Tamura-Nei [26], respectively, with 1000 bootstrap samplings.

Serological relationship. Four polyclonal antibodies (PAb_s) and four monoclonal antibodies (MAb_s) (Table 3), and an Indirect-Enzyme Linked Immunosorbent Assay (I-ELISA) were used according to Caruso et al. [11] in Polysorp Nunc microplates. Four wells were used per bacterial strain, assayed in duplicate. PAb_s and MAb_s work dilutions are indicated in Table 3 and homologous strain of each antisera were included as positive control, except for the IACR-PS-278 antiserum for which reference strain PD 2762 was used. *C. indologenes* was the negative control. The optical density (OD) of each strain was compared with the I-ELISA value of *R. solanacearum* homologous strains, or the strain PD 2762 (considered as 100%) and the percentage of Serological Relationship (SR) was calculated according to Alarcón et al. [1].

Rep-PCR analysis. The rep-PCR protocol employed was based on Louws et al. [30], using the primers ERIC1R, ERIC2 and BOXA1R. Products of PCR amplification were separated by 1.5% agarose gel electrophoresis on 0.5 TAE buffer for 2 h at 70 V, ethidium bromide stained and visualized under UV light. Each strain was assayed at least twice.

AFLP analysis. The AFLP analysis of 24 selected *R. solanacearum* Spanish strains (Table 1) was performed similar to Vos et al. [43] by Biopremier, Ltd. (Universidade da Lisboa, Portugal) using an AFLP Core Reagent commercial kit (Gibco BRL). DNA (250 ng) was digested with *Eco*RI and *Mse*I enzymes (2 h at 20°C) and then ligated to the respective adapters. Selective amplification was done with two primers (*Eco*RI: GACTGCGTACCAATTC; and *Mse*I–G: GATGAGTCCTGAGTAAG) complementary to the adapters, and the *Eco*RI and *Mse*I restriction sites, respectively. Differential AFLP bands were used to construct a similarity matrix using the Dice Coefficient. Cluster analysis was performed with the BioNumerics v. 4.01.

Analysis of macrorestriction fragments of genomic DNA by PFGE. The 16 selected Spanish *R. solanacearum* strains (in bold in Table 1) and the strain PD 2762 were analysed by PFGE after digestion with *Xba*I restriction enzyme. Genomic DNA was obtained according to Donat et al. [17] and digested with 30 U of *Xba*I overnight. Restriction fragments were separated by electrophoresis with a CHEF DR III apparatus (Bio-Rad) in 1% (wt/vol) PFGE agarose (Bio-Rad) gels with Hepes

Table 3. PAb_s and MAb_s used in I-ELISA and theirs work dilutions

Antibody	Work dilution
PAbs IVIA-1632.2/WC	1/15000
PAbs IVIA-2762-Glu	1/30000
PAbs IVIA 1546-H	1/5000
IACR-PS-278	1/50000
MAbs IVIA 4D	1/2000
MAbs IVIA 8B	1/1500
MAbs IVIA 9G	1/4000
MAbs IVIA 9F	1/1500

buffer (3.5 mM HEPES, 3.5 mM sodium acetate, 0.35 mM EDTA [pH 8.3]) at 14ºC and 5 V/cm for 22 h as described before [17]. The molecular weight standard was Lambda DNA ladder (PFGE marker I from Bio-Rad). Each isolate was tested a minimum of two times from two DNA extractions.

Results & Discussion

All the putative *R. solanacearum* Spanish strains studied were accurately identified as belonging to this species by phenotypic tests, Co-PCR and phylotype-specific multiplex PCR while 90.9% of them were also identified by ELISA and pathogenicity tests. These discrepancies with different techniques were due to three afluid variants and three afluid *R. solanacearum* strains (Table 1). The 42 Spanish strains showing typical colonial morphology (fluid) were positive by DASI-ELISA (data not shown) and pathogenic on tomato (Table 1). However, afluid mutants were not recognized by the Mab IVIA-8B (data not shown) and not pathogenic on tomato or produced atypical symptoms (Table 1).

Regarding the biochemical characterization of representative *R. solanacearum* Spanish strains (Table 1), all isolates were sensitive to all antibiotics assayed in the ATB G-5 gallery. In the API ZYM system, six activities were detected in all assayed strains (alkaline and acid phosphatase, estearase, estearase-lipase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase). The Biolog system identified all isolates as *R. solanacearum*, which were positive for methyl-pyruvate, monomethyl succinate, cis aconitic acid, citric acid, D-galacturonic acid, D-glucuronic acid, β-hydroxybutyric acid, α-keto glutaric acid, quinic acid, D-saccharidic acid, succinic acid, bromo succinic acid, glucuronamide, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and γ-aminobutyric acid. The remaining carbon sources were not used or gave variable results. The visualization of the same profile for all the *R. solanacearum* strains in each one of these phenotypic miniaturized systems suggest their limited value for variability studies. The results with the API 20NE were quite homogeneous, being only positive for nitrate reduction and growth in glucose, gluconate, malate and citrate. In the API 50CH gallery, isolates produced acid within 72-96 h only from galactose, glucose, cellobiose, sucrose and D-fucose, except for some of them that also produced acid from D-fructose and D-mannose. These results agree with previous studies [16,44] showing their value for a preliminary identification of *R. solanacearum*.

A similar limited discriminatory capacity was shown by using PA_{bs} for serological characterization since each of antisera reacted with all tested strains (data not shown). However, the reaction of the strains from the Canary Islands and those from South America was quite similar with PAb IACR-PS-278 (data not shown), suggesting a possible South American origin of strains from La Palma (Canary Islands), that could be due to the uncontrolled exchange of latently infected potato tubers in the past. Nevertheless, the usefulness of MAb IVIA-8B [11] for specific serological detection of *R. solanacearum* was confirmed.

Phylogenetic analysis of *egl* and *hrpB* genes, showed a very low genetic variability among analysed *R. solanacearum* strains with a NJ phylogenetic tree (congruent with MP tree) dividing them into 2 clusters with low polymorphism for both genes (Fig. 1A and B). Cluster 1 with both *egl* (Fig. 1A) and *hrpB* (Fig. 1B) genes gathered all Spanish strains, showing a high level of similarity with the majority of the *R. solanacearum* (phylotype II) strains from GenBank from different parts of the world. These findings confirm previous works reporting [9,13,33,38] that partial sequencing analysis of *egl* and *hrpB* genes is unable to detect differences among phylotype II (b2r3) strains.

Attempts to differentiate *R. solanacearum* strains belonging to previous b2r3 by rep-PCR demonstrated that this is a highly homogenous biovar [37,41]. Nevertheless, these same authors discriminated two groups of biovar 2 strains from different origins. In this study, a high degree of homogeneity was observed within the Spanish strains, with only one BOX and one ERIC-PCR pattern (about 25 and 15 DNA bands, respectively) (data not shown), also shared by the Dutch strain PD 2762.

AFLP using the primers combination reported by van der Wolf et al. [41] was suitable for analyzing the diversity of *R. solanacearum* European strains [3,12,14,15,33,38]. In this work, the Spanish strains generated, in several cases, AFLP fingerprints correlated with the geographical origin, regardless of the source of isolation (Fig. 2). Cluster A was formed by five strains three of which (IVIA 2528.54.A2, IVIA 2158.3, and IVIA 1634), that were isolated from the same region and province (Castile and Leon / Salamanca), while IVIA 1635 was isolated from the same region but different province (Castile and Leon / Soria) and the fifth one was of unknown origin. All the strains grouped in this cluster showed an identical pattern of 34 bands. Cluster B included three strains (IVIA 1496.1a, IVIA 1760.1.1b and IVIA 2297.4t.2a) all from La Palma (Canary Islands). These strains showed some slight differences among them. Strains IVIA 1760.1.1b and IVIA 1496.1a, both isolated from potatoes, showed a pattern of 33 bands but differed in the size of one band. Strain IVIA 2297.4t.2a, isolated from soil, showed one additional band of 122 bp that was not present in the other strains grouped in this cluster.

Cluster C grouped two strains (IVIA 1672 and 1632.2-f and the afluid variant 1632.2-af) isolated from potato from the same region but different provinces (Castile and Leon – Burgos and Soria), which presented a pattern of 35 bands. Cluster D grouped two strains IVIA 2093.3.1 and IVIA 2068.58a, both isolated from potatoes in the Canary Islands (La Palma). Cluster E was also formed by two strains (IVIA 1620.1.1 and IVIA 1692.a), both isolated from potatoes in different provinces of the same region (Castile and Leon – Soria and Segovia). Strains IVIA 1602.10 and IVIA 1602.1, both isolated from potatoes in the Canary Islands (La Palma), presented a profile quite different from the rest of tested strains. Strain IVIA 1602.10 shared

37 bands with strain IVIA 1602.1 but showed one additional band of 579 bp.

The remaining seven strains isolated from potatoes (IVIA 1532.4, IVIA 2424 and IVIA 1546-f with its variant IVIA 1546-af) and river water (IVIA 2167.1a, IVIA 2167.2b and IVIA 2533.A2) each one constituted a group with a specific pattern (Fig. 4 and indicated as SP in Table 1).

In the 5 clusters obtained after the analysis of AFLP profiles by UPGMA with the Dice coefficient (Fig. 2), some strains from Castile and Leon (clusters A, C and E) and some from the Canary Islands (clusters B and D) were grouped separated by their geographical origin, and in some cases also by the source. Interestingly, in cluster A there were strains isolated from potatoes as well as from river water a few years later from the same area (Fig. 2, Table 1), suggesting prolonged survival of the pathogen in natural water, which agrees with its the long-term survival and pathogenicity in water microcosms [5]. Similarly, in cluster B there were strains isolated from potatoes and one found in soil several years later (Fig. 2, Table 1), which points to long-term survival also in soil, in accordance to van Elsas et al. [42]. In clusters C and E two strains isolated from potato in Castile and Leon were grouped while in cluster D strains isolated from potatoes in the Canary Islands were grouped (Fig. 2, Table 1). The remaining strains isolated from potatoes and river water each one constituted a group with a specific pattern (Fig. 2 and indicated as SP in Table 1). Taken as a whole AFLP results, we assume the occurrence of several introductions of *R. solanacearum* strains in Spain. The strains isolated from the mainland could have been introduced more recently from other European countries with imported potatoes. Strains from South America or Africa (Egypt) could have also been introduced in other European countries [41], and then from there they may have been imported with seeds or ware potatoes into Spain. However, the diversity observed by AFLP was relatively small according to Poussier et al. [34]. Van der Wolf et al. [41] showed that the phylotype II strains from South America were considerably more variable than those from other countries, probably due to the Andean origin of the potato and the disease [37], in contrast to Spain, where the pathogen has only been reported since relatively recent years.

PFGE has been already demonstrated to be very useful for evaluating the genetic diversity of the bacterial wilt pathogen, including European strains [37,38,41]. In general, Spanish strains showed relatively similar patterns with *Xba*I (Fig. 3), although this enzyme yield at least ten distinct PFGE patterns (Table 1 and Fig. 3). The dominant *Xba*I profile comprised four strains isolated from potatoes in Castile and Leon, with 13 DNA fragments (pattern X1). Three strains from different sources, two of them from La Palma (Canary Islands), shared X1 pattern but with an additional fragment of approximately 100 kb (pattern X2). Two strains isolated in Salamanca (Castile and Leon) from different sources (potato and water) shared

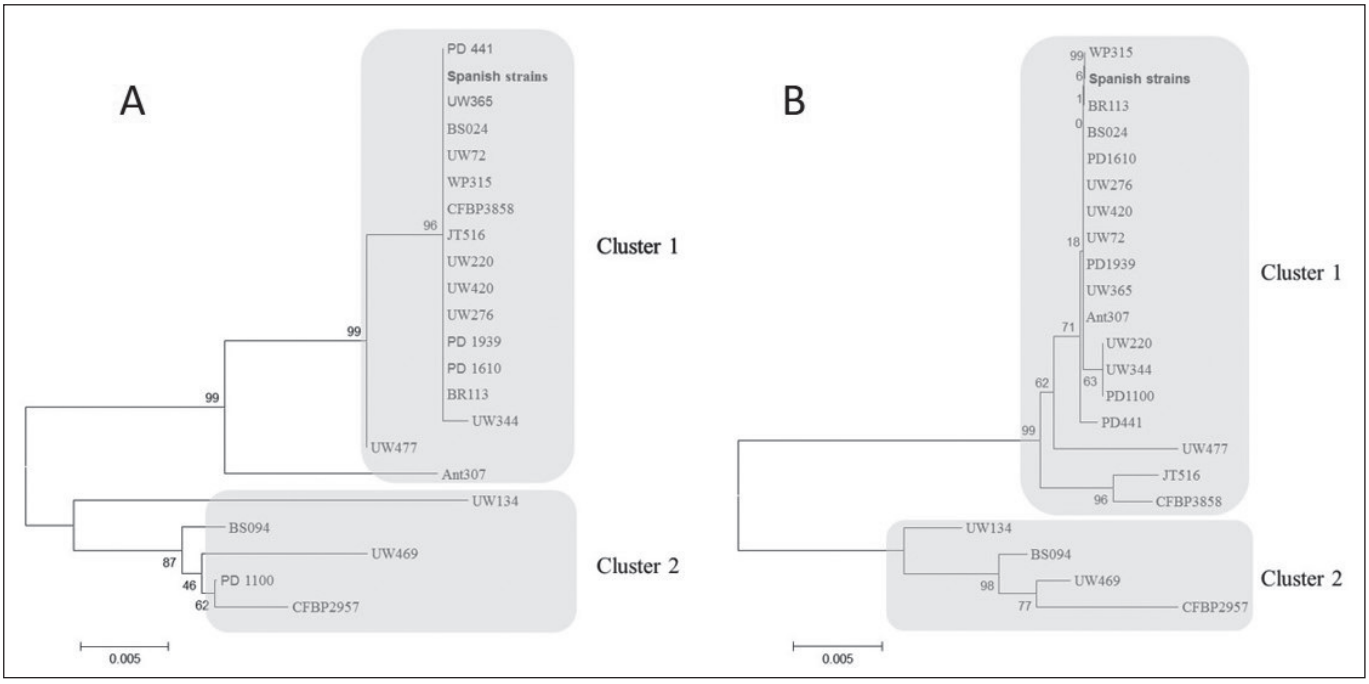

Fig. 1. NJ phylogenetic tree based on the comparison of partial *egl* (A) and *hrpB* (B) gene sequences from *R. solanacearum* '**Spanish strains'** and 21 *R. solanacearum* accession phylotype II (based on the taxonomic revision of Safni et al. [36] retrieved from the GeneBank. Values at the branches indicate percentage bootstrap support for 1000 resamplings.

Due to the high degree of similarity among all Spanish sequences derived from different sources, we indicated the Spanish-type sequence representative for *egl hrpB* genes with the term '**Spanish strains'.**

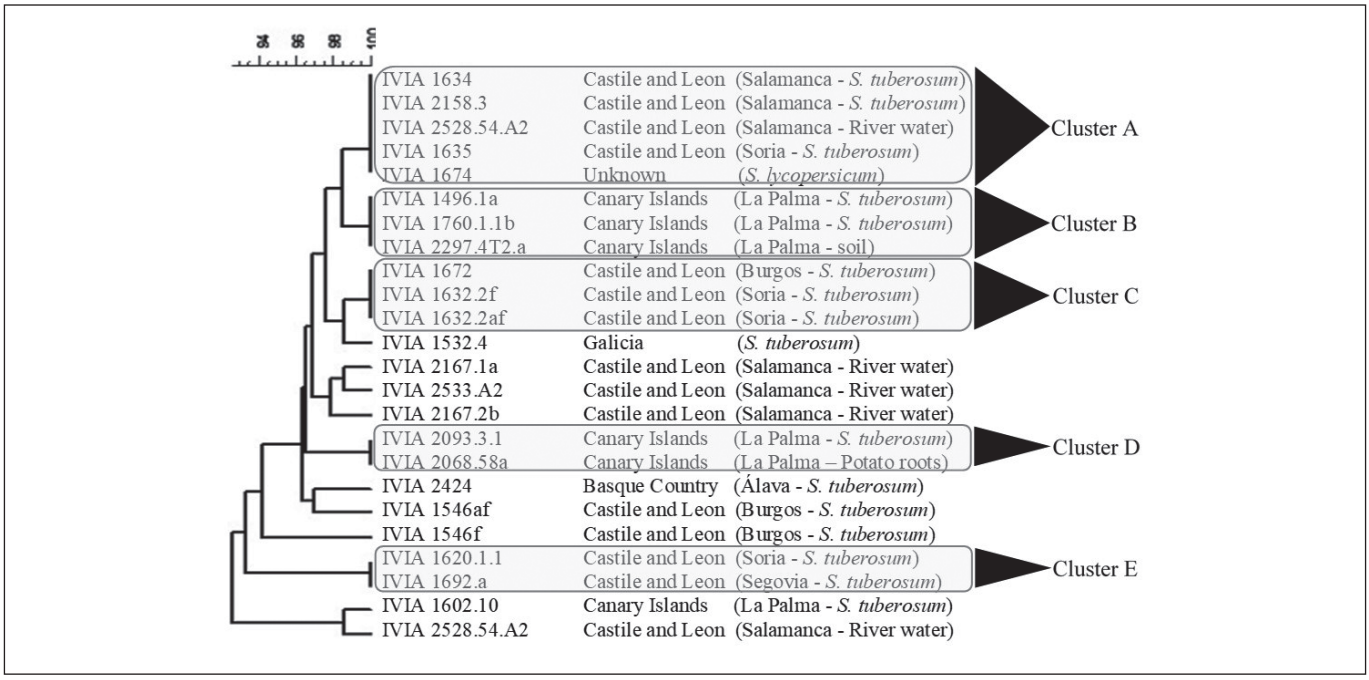

Fig. 2. Dendrogram showing the relationship between *R. solanacearum* Spanish strains based on AFLP analysis using *Eco*RI/*Mse*I enzymes, and the pair of primers *Eco*RI/*Mse*I-G. Correlation coefficients were calculated and cluster analysis was achieved by UPGMA.

the pattern X2 except for missing one fragment of about 72 kb (pattern X3). The rest of the patterns were different for each of the remaining strains and were named X4 to X10 (Table 1) but shared about ten DNA fragments with the dominant profile. Then, in some cases, the intraspecific diversity detected was related to the Spanish region. It is interesting that one Spanish strain IVIA 2424 isolated from potatoes in Álava (Northeastern Spain) exhibited the same pattern (X8) as the reference strain from The Netherlands (data not shown) also isolated from potatoes but some years before. Moreover, this pattern and another one (X10) from a potato strain from Burgos (Northern Spain) are very similar to two profiles previously described in other *R. solanaceraum* biovar 2 Dutch strains [38,41]. Other patterns were new for the European strains, one of them (X2) observed in one strain from potatoes isolated in La Palma (Canary Islands) was very similar to a pattern previously reported for a *R. solanaceraum* strain from Kenya [37]. Our main conclusion is that some of the Spanish foci of bacterial wilt, at least in the North of Spain, could be related with the import of contaminated potatoes from other European countries where the pathogen was detected earlier than in Spain.

The *R. solanacearum* groups obtained after the PFGE analysis were different to those obtained by AFLP, but these discrepancies could be due to the different molecular basis of the two techniques. However, both PFGE and AFLP were able to discriminate several clonal lines among Spanish strains of *R. solanacearum* and some were related to the geographical origin of strains. Our results support the hypothesis that several clones

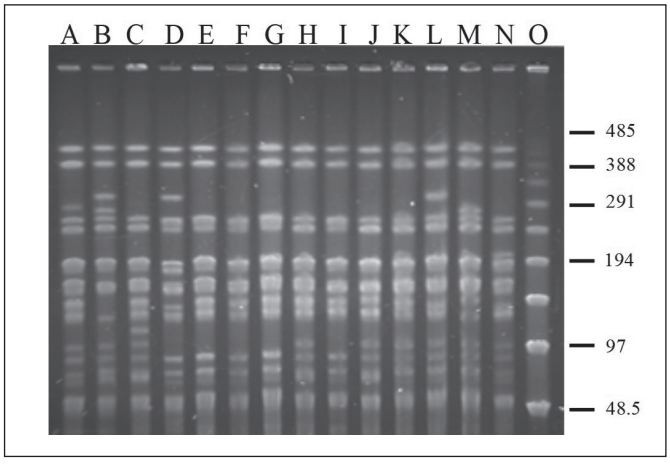

Fig. 3. PFGE banding patterns of Spanish *R. solanacearum* strains digested with *Xba*I: patterns of 14 Spanish isolates. Lanes: A, IVIA 1532.4 (X7); B, IVIA 1546-f (X10); C, IVIA1602.1 (X9); D, IVIA 1620.1.1 (X4); E, IVIA 1632.2-f (X1); F, IVIA 1635 (X1); G, IVIA 1672 (X1); H, IVIA 1674 (X2); I, IVIA 1692.a (X1); J, IVIA 2093.3.1 (X2); K, IVIA 2158.3 (X3); L, IVIA 2167.2b (X5); M, IVIA 2424 (X8); N, IVIA 2528.54.A2 (X6) and O, PFGE marker I from Bio-Rad.

of the pathogen have been introduced into Spain as previously suggested by van der Wolf et al. [41] for other European strains.

Diversity results obtained in this work offer new insights into the biogeography of this quarantine organism and its possible sources and reservoirs in Spain and probably in other Mediter-

ranean countries and improves the knowledge on its ecology and epidemiology that are the bases for designing more effective preventative measures and eradication strategies.

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