Identification of pathovars and races of *Pseudomonas syringae*, the main causal agent of bacterial disease in pea in North-Central Spain, and the search for disease resistance

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Abstract A total of 298 bacterial isolates were collected from pea cultivars, landraces and breeding lines in North-Central Spain over several years. On the basis of biochemical-physiological characteristics and molecular markers, 225 of the isolates were identified as *Pseudomonas syringae*, either pv. *pisi* (110 isolates) or pv. *syringae* (112), indicating that pv. *syringae* is as frequent as pv. *pisi* as causal agent of bacterial diseases in pea. Most strains (222) were pathogenic on pea. Further race analyses of *P. syringae* pv. *pisi* strains identified race 4 (59.1% of the isolates of this pathovar), race 2 (20.0%), race 6 (11.8%), race 5 (3.6%) and race 3 (0.9%). Five isolates (4.6%) showed a not-previously described response pattern on tester pea genotypes, which suggests that an additional race 8 could be present in *P. syringae* pv. *pisi*. All the isolates of *P. syringae* pv. *syringae* were highly pathogenic when inoculated in the tester pea genotypes, and no significant pathogenic differences were observed. Simultaneous infections with *P. syringae* pv. *pisi* and pv. *syringae* in the same fields were observed, suggesting the importance of resistance to both pathovars in future commercial cultivars. The search for resistance among pea genotypes suitable for production in this part of Spain or as breeding material identified the presence of resistance genes for all *P. syringae* pv. *pisi* races except for race 6. The pea cultivars Kelvendon Wonder, Cherokee, Isard, Iceberg, Messire and Attika were found suitable sources of resistance to *P. syringae* pv. *syringae*.

Abbreviations

Ppi	*Pseudomonas syringae* pv. *pisi,* according to Vivian and Mansfield (1993)
Psy	*Pseudomonas syringae* pv. *syringae,* according to Vivian and Mansfield (1993)

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Introduction

Pea (*Pisum sativum* L.) seeds are used in a wide variety of forms. Approximately one half of the

world's production of dry peas is used to feed livestock while the remaining half is used for human consumption, mainly in developing countries. Dry mature seeds can also be processed to produce starch and protein concentrates. Pea has been the most widely produced grain legume in Spain since 2005 and its production for non-human consumption is increasing. Castilla y León, located on the North Central Spanish Meseta, with an average elevation of 750 m above sea level, is the Spanish Region with the highest pea production, with 92,380 ha devoted to this crop in 2009. The relatively cool climate in this Region requires the use of frost-tolerant cultivars and autumn sowing to obtain a satisfactory yield (Caminero 2002). In Castilla y León the pea crop is only profitable when planted in autumn and frost-tolerant cultivars are used. Early sowing allows for optimal use of water resources and avoids the drought period in the spring-summer season (Caminero 2002).

Early sowing and frost injury can increase the incidence of diseases in pea, in particular those caused by bacteria (Taylor et al. 1989; Hollaway and Bretag 1995; Reeves et al. 1996; Hollaway et al. 2007). The prevalence of these diseases represents a major constraint to the use of this crop (Carrouée 1997; Hollaway et al. 2007). Bradbury (1986) described several bacterial genera as potential agents of pea diseases, namely: Agrobacterium, Bacillus, Erwinia, Pseudomonas, and Xanthomonas. Lawyer and Chun (2001) pointed out that the world's most important bacterial pea disease is bacterial blight caused by Pseudomonas syringae pv. pisi Sackett (Ppi) (syn. P. pisi Sackett), although sporadically it can be caused by Pseudomonas syringae pv. syringae van Hall (Psy), at times with similar symptomatology making it difficult to distinguish between the two agents by visual inspection. Moreover, because the biochemical profiles of these pathovars are very similar, identification based exclusively on biochemical characteristics is uncertain. Likewise, other Pseudomonas species, such as Pseudomonas viridiflava, Pseudomonas fluorescens and some soft-rotting Pseudomonas, have been isolated from pea plants with bacterial blight symptoms. However, among these latter species, pathogenicity on pea has been proven only for P. viridiflava in New Zealand, France and Spain (Taylor and Dye 1972; Grondeau et al. 1992; Martín-Sanz et al. 2010).

Bacterial blight of pea caused by Pseudomonas syringae pv. pisi (Ppi) was first identified in 1915 in Colorado, USA (Sackett 1916), but it was subsequently found in all countries in which pea production is important (Lawyer and Chun 2001). Seven different pathogenic races have been described on the basis of the differential response after inoculation in eight pea genotypes (Bevan et al. 1995). The frequency of each race varies between regions, the predominant pea cultivars cultivated in each region is probably one of the main factors determining these differences; either way race 2 seems to be the most frequent race worldwide (Lawyer and Chun 2001). Bacterial brown spot of pea caused by Pseudomonas syringae pv. syringae (Psy) was first described in 1966 in Wisconsin, USA (Hoitink et al. 1967). The information on this species as a pea pathogen is still limited but it appears to cause significantly lower yield losses than Ppi and the incidence of this disease is associated with early sowing (Lawyer and Chun 2001). The total yield loss caused by these two pathovars is considered to be one of the limiting factors in pea cropping (Hollaway et al. 2007), with disease incidence increasing as pea production becomes greater and the practice of early sowning becomes more frequent.

Both pathovars, Psy and Ppi, are primarily transmitted by infected seeds, although Psy has a wide host range with a possible resident phase in crop or weed species (Lawyer and Chun 2001). Cold and wet weather conditions favour the spread of the disease since these bacteria mainly penetrate the host plant through lesions which can originate from frost injury. Furthermore, these bacteria can act as ice nucleation particles under cold conditions increasing frost injury effects and lesions (Hirano and Upper 2000). For instance, in France outbreaks of bacterial blight of pea have been generally described after frost or mechanical injury, while without these factors no disease was observed even though contaminated seeds were sown in the field (Samson et al. 1997). The symptomatology caused by both species, Ppi and Psy, in fields is very similar. Frequently, after frost periods, elliptical water-soaked areas, which become olive-green and finally a brown area, are observed in aerial plant parts. These lesions often encircle the stem and may extend several centimetres and infect both stipules and leaflets. Severe infection of the stem can cause plant death (Hollaway et al. 2007).

According to López and Montesinos (1996) Ppi and Psy were recently described as pea pathogens in

Spain. *Pseudomonas syringae* pv. *pisi* was first found as a pea pathogen in Spain in 1991 in experimental fields (Valladolid, Castilla y León) (Elvira-Recuenco and Taylor 2001), but only since 2002 has this bacteria been repeatedly but locally described by the Crop Protection Service in Castilla y León (J.L. Palomo, unpublished). Preliminary data indicate that the prevalence of bacterial diseases in pea has increased as the size of the pea crop increased. Considering the steady increase in the area devoted to early pea crops in Castilla y León, it is expected that the prevalence of these bacterial diseases will increase, eventually becoming a serious threat and a limiting factor for crop production.

The aims of this research were to identify the main agents of bacterial disease in pea in North-Central Spain (the highest pea-producing area in this country) and surrounding areas, to identify the variability in pathogenicity of *Psy*, to determine the most frequent pathogenic races for *Ppi*, and to find possible sources of resistance to these pathogens in high yielding and adapted genotypes.

Materials and methods

Sampling

Samples were collected from 2004 to 2008 in pea fields in Castilla y León and some adjacent Regions, namely, Navarre, the Basque Country, and the Province of Madrid. Together they represent 70% of the area planted with peas in Spain, and autumn sowing is predominant. Samples were collected from pea plants with symptoms of bacterial disease (water-soaked or brown areas on stem and leaves sometimes associated with wilt). Nearly all pea cultivars collected corresponded to dry pea used as livestock feed. Infected tissue from stems and leaves were removed, stored individually in sterile plastic bags maintained at 4°C, and processed within 24 h. Samples were surfaced sterilized with 0.5% NaOCl for 1 min, and then rinsed several times in sterilized distilled water. Plant tissues were macerated, spread on King's B agar medium (KB) (King et al. 1954) and incubated for 48 h at 24°C. Pure cultures of bacteria were obtained from all the samples by selecting single colonies. Isolates were stored in 30% glycerol at −70°C.

Biochemical identification

Isolates were identified following the methodology described by Braun-Kiewnick and Sands (2001): Gram test (KOH), Hugh–Leifson oxidation(O)/fermentation(F) test, fluorescence on KB medium, LOPAT test (Levan production, oxidase reaction, potato soft rot, arginine dihydrosilase activity, and tobacco hypersensitivity), and the use of homoserine as carbon source. The following strains were used as controls: *Pseudomonas syringae* pv. p*isi* race 4 HRI-W 895A (Horticultural Research International, Wellesbourne, U. K.) as positive control for the fluorescence, homoserine, levan and tobacco tests; *Pseudomonas cichorii* CRD*99/378 (Centro Regional de Diagnóstico, Salamanca, Spain) as positive control for the oxidase activity; *Pseudomonas fluorescens* CECT 378 (Colección Española de Cultivos Tipo, Valencia, Spain) as positive control for arginine dihydrosilase and potato soft rot; and *Erwinia rhapontici* NCPPB 3766 (National Collection of Plant Pathogenic Bacteria, York, U.K.) as positive control for the Hugh-Leifson test. Sterilized water was used as negative control for all tests. Duplicate tubes or plates were run for each test, and tests were repeated at least twice.

Molecular identification

A single colony from each isolate was used for identification. Bacteria were collected on the end of a micropipette tip and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) in polymerase reaction tubes. Polymerase chain reactions (PCR) were carried out using a primer set specific for the AN3 and AN7 markers of *Pseudomonas syringae* pv. *pisi* (Arnold et al. 1996) (Table 1) in a thermal cycler (Perkin Elmer 9600) using the following procedure: initial strand separation at 95°C (15 min), 40 cycles of 72°C (1 min), 94°C (30 s), 60°C (1 min), final extension at 72°C (5 min). The amplifications were carried out in a volume of 25 μl containing 10 ρM of each primer, 1X Buffer II Ampli*Taq* Gold (Applied Biosystems), 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$ and 1U Ampli*Taq* Gold polymerase.

Two primers which amplify a sequence of 752 bp of the *syrB* gene (Sorensen et al. 1998) were used to identify *Pseudomonas syringae* pv. *syringae* (Table 1).

	Species	Marker	Primer sequence 5′ → 3′	Expected size	Positive races[a]
Table 1 Primers used to identify *Pseudomonas syringae* pv. *pisi* and *P. syringae* pv. *syringae*	*Ppi*	AN3	F, CACCCAGCGCATTACTAGGA	132 bp	1, 3, 4, 5, 7
			R, CCAGCACCCAGATTGAGACT		
		AN7	F, TCACTCCGAGCTCCTCACTA	272 bp	2, 3, 4, 6
			R, AACGGCGAGGGTTGTGGAAA		
Arnold et al. (1996)	*Psy*	*syrB* gene	F, CTTTCCGTGGTCTTGATGAGG	752 bp	
Sorensen et al. (1998)			R, TCGATTTTGCCGTGATGAGTC		

This primer pair amplifies the *syrB* gene fragment in the pathovars *atrofaciens*, *aptata* and *syringae* of *P. syringae*, but do not in *Pseudomonas syringae* pv. *pisi* (Sorensen et al. 1998). Pathovars *atrofaciens* and *aptata* have not been described as pea pathogens (Bradbury 1986). The amplification procedure was similar to that described above except that PCR conditions were: initial strand separation at 95°C (15 min), 35 cycles of 72°C (3 min), 94°C (30 s), 60°C (1.5 min), final extension at 72°C (10 min).

Each amplification reaction was carried out at least twice. PCR products were electrophoresed in 1.5% TBE 1X agarose gels and detected by staining with ethidium bromide (0.2 ŋg/ml). The size of the PCR products was estimated using a 100 bp fragment ladder (Biotools). In all gels, several controls were included. For the AN3 and AN7 markers the reference strains of the seven *Ppi* races (Bevan et al. 1995), and for the gene *syrB* the CFBP 1769 strain of *Psy* (isolated on pea) (Collection Française de Bactéries Phytopathogènes, Angers, France), were used as positive control, respectively. The product of a reaction tube without bacterial template was used as negative control.

Pathogenicity test

All isolates and controls were inoculated in stems and leaves of the pea cultivar Gracia following the procedure described by Elvira-Recuenco et al. (2003). Gracia is one of the most extensively grown feed pea cultivar in Spain and it is susceptible to all races of *Ppi* and to *Psy* (Martín-Sanz 2008). The isolates which produced negative results on Gracia were inoculated on the pea cv. Kelvendon Wonder, which is also susceptible to all *Ppi* races (Bevan et al. 1995). Furthermore, in order to confirm pathogenesis, the isolates were inoculated in the genotype from which they were collected, when known. Two

replicates of five plants each were used for each inoculation. From one of the plants which showed disease symptoms after these inoculations a new isolation was made to confirm Koch's postulates. The identity of the new isolates was tested by the Gram reaction, glucose metabolism in the Hugh-Leifson medium, fluorescence on KB, LOPAT test, homoserine utilization as carbon source, and the molecular tests described in the previous section.

Furthermore, the isolates were inoculated into immature lemons since *Psy* is pathogenic in this fruit but *Ppi* is not (Mazarei and Kerr 1990). Lemons were surface sterilized with ethanol 70%, rinsed in distilled sterilized water, and then sterilized entomological needles were used to inoculate each isolate by depositing 20 µl of bacterial culture (10^8 ufc/ml) on punctures made at ten sites per isolate. Inoculated fruits were kept in a humid chamber at 25°C for 7 days, and the infection response was recorded daily.

Pathogenic variability

All the isolates that were pathogenic in the test described above were tested further on the eight pea cultivars listed in Table 2 to identify the *Ppi* pathogenic races (Bevan et al. 1995) and also on the *P. abyssinicum* accession JI2202, described as resistant to *Ppi* by Elvira-Recuenco et al. (2003). The methodology described by Elvira-Recuenco et al. (2003) was used with minor modifications. Ten seedlings per pot (containing a sterilized 2:1 mixture of peat and sand) were grown to the three-leaf stage at 20°C and 14 h light. Then, they were transferred to a climatic chamber and grown at 22/18°C (day/night) with 16 h light. Humidity was maintained at 100% until 24 h after inoculation, then humidity was progressively decreased to 70%. The inoculations were carried out on stems by stabbing the main stem–stipule junction of the youngest node with an

Table 2 Pea cultivars used to identify *P. syringae* pv. *pisi* races, and pea resistance genes according to Bevan et al. (1995)

	Resistance genes[a]	Stem resistance to races
Kelvendon Wonder	None	None
Early Onward	R2	2, 5 and 7
Belinda	R3	1, 3 and 7
Hurst's Greenshaft	R4+(R6)	1, 4, 5 and 7
Partridge	R3+R4	1, 3, 4, 5 and 7
Sleaford Triumph	R2+R4+(R5)	1, 2, 4, 5 and 7
Vinco	R1+R2+R3+(R5)	1, 2, 3, 5 and 7
Fortune	R2+R3+R4	1, 2, 3, 4, 5, and 7

[a] Resistance genes not completely proven within parenthesis (Bevan et al. 1995)

entomological mounting pin containing bacteria scraped from the surface of an agar plate. Disease symptoms were scored 10 days after inoculation. In all assays, negative controls of sterilized water, and positive controls inoculated with *Ppi* type races and the *Psy* CFBP 1769 strain were included.

Resistance to *Ppi* and *Psy* among pea cultivars

Forty-nine pea cultivars which showed bacterial blight symptoms in the field during the collection periods, other feed pea cultivars widely used in Spain, and high-yielding breeding lines adapted to Spanish agro-ecological conditions were evaluated under controlled conditions for their resistance to *Ppi* and *Psy* in stems. The methodology used was the one described in the previous section, and previously used

in other studies to evaluate resistance to pea bacterial blight (Taylor et al. 1989; Bevan et al. 1995; Hollaway and Bretag 1995; Elvira-Recuenco and Taylor 2001; Elvira-Recuenco et al. 2003). A total of ten seedlings per cultivar or breeding line were evaluated per strain in two replicates. The *Ppi* races used are listed in Table 3. In addition the highly virulent isolate CFBP 1769 of *Psy* (Martín-Sanz 2008) was also used. The evaluation was carried out as follows. For *P. syringae* pv. *pisi* (Elvira-Recuenco et al. 2003) a susceptible reaction was a clear water-soaked area surrounding the inoculation point; a resistant reaction was a localized necrotic area surrounding the inoculation point (hypersensitive response), and partial resistance was a localized necrotic area within water-soaked areas. For *P. syringae* pv. *syringae* (Martín-Sanz 2008) a highly

Table 3 *P. syringae* pv. *pisi* and *P. syringae* pv. *syrinage* strains used in the inoculation experiments

Collection number[a]	Pathovar	Race	Avirulence genes[b]	Origin				Other collection numbers
				Cultivar	Country	Year	Identified by	
CFBP 1769	*syringae*			Unknown	New Zealand	1970	J.D. Taylor	NCPPB 2691
HRI-W 299 A	*pisi*	1	1, 3, 4, (6)	Rondo	New Zealand	1970	J.D. Taylor	CFBP 3196
HRI-W 202	*pisi*	2	2	Unknown	U.S.A.	1944	W.R. Burkholder	CFBP 3195
HRI-W 870 A	*pisi*	3	3	Martus (seed)	U.S.A.	1975	J.D. Taylor	CFBP 2704
HRI-W 895A	*pisi*	4	4	Martus (seed)	U.S.A.	1975	J.D. Taylor	CFBP 2706
HRI-W 974B	*pisi*	5	2, 4, (5), (6)	Puget (seed)	U.S.A.	1978	J.D. Taylor	CFBP 2707
HRI-W 1704B	*pisi*	6	None	Stehgolt (seed)	France	1986	K.P. Higgins	CFBP 2709
HRI-W 2491A	*pisi*	7	2, 3, 4	Unknown	Australia	1976	J. Bevan	CFBP 4271

[a] CFBP: Collection Française de Bactéries Phytopathogènes, Angers, France; HRI-W: Horticulture Research International, Wellesbourne, Warwickshire, U.K.; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK

[b] Avirulence genes not completely proved within parenthesis (Bevan et al. 1995)

resistant reaction (0) consisted of small localized necrosis in the infection point, a resistant reaction (1) consisted of necrosis spots less than 1 cm in diameter, a moderately resistant reaction (2) showed necrotic lesions at the inoculation point between 1 and 2 cm in diameter, a susceptible reaction (3) consisted of dark brown necrotic depressed lesions more than 2 cm long and reaching the next node in the apical direction, and a highly susceptible reaction (4) was similar to reaction type 3 but lesions were more than 3 cm long with a clear extension into the apical part of the plant.

Results

Identification of bacterial isolates

A total of 298 isolates were obtained from field-grown pea plants with disease symptoms. Most isolates (276) were collected in the Castilla y León Region, while the remaining samples were collected in the surrounding regions of Navarre, Madrid and the Basque Country. The plant material collected corresponded to cultivars, landraces and breeding lines that represented most of the pea feed cultivars grown in the main pea producing areas in Spain.

The isolates were preliminarily characterized by their biochemical-physiological responses. Most of them (225, 77.5%) showed characteristics which fitted the expected pattern for *P. syringae* (Table 4). Among

Table 4 Reaction of *Pseudomonas syringae* isolates in biochemical tests and preliminary identification

Test	Reaction[a]	
Gram	−	−
Hugh-Leifson	O	O
Fluorescence in KB	+/−	+
Levano	+	+
Oxidase	−	−
Pectolysis on potato	−	−
Arginine dihydrolase	−	−
Tobacco hypersensitivity	+	+
Homoserine	+	−
Number of isolates	201 (67.45%)	24 (8.05%)
Preliminary identification	*Ppi*	*Psy*

[a] O = oxidation, F = fermentation, + = positive, − = negative

them, 201 isolates (67.45%) were capable of metabolizing homoserine, and thus, according to Braun-Kiewnick and Sands (2001) were preliminarily identified as *P. syringae* pv. *pisi*. The homoserine negative isolates were preliminarily identified as *P. syringae* pv. *syringae*. The remaining isolates did not fit the expected response patterns of *P. syringae* pathovars, nor were pathogenic on susceptible pea tester cultivars, except for three isolates which were further identified as *P. viridiflava* (Martín-Sanz et al. 2010).

All isolates were tested in PCR to amplify DNA sequences specific to *Ppi* or *Psy*. Among the 225 isolates classified as *P. syringae* (either pv. *pisi* or pv. *syringae*), 222 isolates had a single amplifiable marker band; amplified DNA of 110 isolates was characteristic of the AN3 (132 bp) or AN7 (272 bp) *Ppi* marker. All these isolates were homoserine positive. The 99 isolates with the amplifiable AN7 marker also showed fluorescence under UV light in KB medium, while the 11 with the amplifiable AN3 marker did not show any fluorescence. From 112 out of the 225 isolates, a 752 bp band corresponding to the *syrB* gene absent in *Pseudomonas syringae* pv. p*isi* could be amplified by PCR (Sorensen et al. 1998). Among these latter isolates, 91 were homoserine positive and 21 homoserine negative (Table 5). From the three remaining isolates initially classified as *P. syringae* and all the isolates with other biochemical profiles, and identified as other species than *P. syringae*, none of the three marker bands could be amplified by PCR. Some examples of AN3, AN7 and *syrB* amplifications are shown in Fig. 1.

Pathogenicity tests

All the 222 isolates from which any of the marker bands could be amplified by PCR also showed disease symptoms when inoculated in the cultivar Gracia. The isolates from which either the AN3 or AN7 marker (110) could be amplified, also generated the typical water-soaked lesions similar to the ones produced by the *Ppi* control race strains (Fig. 2). The isolates from which the *syrB* gene marker (112) could be amplified, generated brown necrotic lesions in the stems and leaves associated with apical death, similar to the symptoms generated by the *Psy* control strain (Fig. 3). This agreement in pea pathogenicity and molecular characteristics attributed to *Psy* led us to consider all the isolates which amplified the *syrB*

Table 5 Summary of the identification of the 225 *Pseudomonas syringae* isolates

	Homoserine response	Fluorescence	Amplification of:			Pea disease symptoms	Disease on lemon	Number of isolates	Totals
			SyrB	AN3[b]	AN7[b]				
P. syringae pv. *syringae*	−	+	+	−	−	Brown-necrotic	+	21	
	+	+	+	−	−	Brown-necrotic	+	91	112
P. syringae pv. *pisi*[a]	+	−	−	+	−	Water-soaked	−	11	
	+	+	−	−	+	Water-soaked	−	99	110
P. syringae unclassified	−	+	−	−	−	No symptoms	−	3	3

[a] *Ppi* isolates were identified as race 2, 22; race 3, 1; race 4, 65; race 5, 4; race 6, 13; putative race 8, 5

[b] *Ppi* races 5 and 3 were AN3 +/fluorescence -; races 2 and 6 were AN7 +/fluorescence +; seven isolates of race 4 were AN3+/fluorescence -, and 58 AN7+/fluorescence +; race 8 AN7+/fluorescence +

gene marker as *Pseudomonas syringae* pv. *syringae*, irrespective of their response to homoserine. Furthermore, all the isolates from which the *syrB* gene marker was amplified, generated the same necrotic spots in immature lemons as the *Psy* control strain, while the isolates from which this marker could not be amplified, were not pathogenic in lemon as were also the *Ppi* control race strains. The remaining 3 isolates were non-pathogenic on either Gracia or Kelvendon Wonder and were not included in further tests. The isolates which were pathogenic in Gracia were tested in the pea material (cultivar, landrace,

Fig. 1 DNA fragments from *P. syringae* isolates and collection strains. 1, size marker 100 bp ladder de (Biotools); 2–6, fragments amplified with the AN7 primers; 7–11, fragments amplified with AN3 primers; 12–20, fragments amplified with *syrB* primers. In the following list the isolates are preceded by the letter P and the race in which they were included is indicated between parentheses. *P. syringae* pv. *pisi*, 2, P134 (2); 3, P123 (6); 4, P119 (4); 5 CFBP4766 (6); 6, CFBP4485 (2); 7, P68 (4); 8 P89 (5); 9, P214 (4); 10, CFBP1688 (1); 11, CFBP4768 (7); *P. syringae* pv. *syringae*, 12, P59; 13, P94; 14, P125; 15, P141; 16, P262; 17, SITA960; 18, CFBP1392; 19, NCPPB3505; 20, NCPPB3509

breeding line) from which they were isolated, and they were able to develop disease symptoms in all cases. New bacterial samples were isolated from these infected genotypes, and all these re-isolates showed the same biochemical and molecular characteristics as the corresponding original isolate, thus fulfilling Koch's postulates.

The isolates which induced blight symptoms (water-soaked areas) in the cultivar Gracia were inoculated into the set of pea cultivars used to identify the *Ppi* pathogenic races (Bevan et al. 1995). This test allowed for the attribution of 105 isolates out of 110 to a specific *Ppi* race: 59.1% (65) isolates were identified as race 4, 20.0% (22) as race 2, 11.8% (13) as race 6, 3.6% (4) as race 5, and 0.9% (1) as race 3. The other five isolates (4.6%) showed a response pattern not previously described; they were incapable of developing disease in Fortune, Sleaford Triumph

Fig. 2 Susceptible response of Kelvendon Wonder to *P. syringae* pv. *pisi* showing a typical water-soaked area

Fig. 3 Apical death caused by *P. syringae* pv. *syringae* in the pea cultivar Fortune

and the accession JI2202 of *Pisum abyssinicum* and capable in all other cultivars. According to this new response pattern, these five isolates should be included in a new pathogenic race of *P. syringae* pv. *pisi*, which following the serial numeration would be race 8. Table 5 summarizes the identification results.

The isolates from which the *syrB* marker of *Psy* could be amplified and which generated brown necrotic spots in the cultivar Gracia developed disease symptoms in all tester pea cultivars and in the *P. abyssinicum* accession, except in Kelvendon Wonder. Approximately in 90% of the inoculated susceptible plants the disease developed during the experiment time to the level of stem apical death, irrespective of the pea genotype tested. Thus, there were no appreciable differences in the pathogenic response to all these isolates and pea tester materials. In Kelvendon Wonder the lesions were restricted to the inoculation point; thus, this cultivar can be considered as resistant to *Psy*.

Response of pea materials to infection by *Ppi* and *Psy*

Table 6 shows the results obtained after the inoculation of 49 cultivars and breeding lines of pea with the seven races of *Ppi* and the strain CFBP 1769 of *Psy*. With regard to *Ppi*, none of the pea materials showed resistance to all races, although three cultivars (Cherokee, Corallo, Lincoln) were resistant to six of the races (no resistance to race 6 was found), and five cultivars were susceptible to all races (10.2%). The resistance-susceptibility response to the races was used to deduce the presence of specific resistance

genes according to the gene-for-gene relationships described by Bevan et al. (1995) for pea-*Ppi*. Almost half of the pea genotypes tested (47%) were resistant to race 1, 3 and 7 and they had the R3 gene. Overall 30.6% of the genotypes had the combination R2+R3 (resistance to races 1, 2, 3, 5 and 7), 2% the R2+R4 (resistance to races 1, 2, 4, 5 and 7), 4.1% the R3+R4 (resistance to races 1, 3, 4, 5 and 7), and 6.1% the R2+R3+R4 (resistance to races 1, 2, 3, 4, 5 and 7). Thus, all resistant genotypes had the R3 gene. In relation to *Psy*, the most frequent response was that of moderate resistance (level 2) shown by 22 genotypes (44.9%); another 22 genotypes were susceptible or highly susceptible (levels 3 and 4), while only five genotypes (cultivars Messire, Attika, Cherokee, Iceberg and Isard) were highly resistant or resistant (levels 0 and 1, respectively).

Discussion

One of the aims of the research was to identify the causal agents of the bacterial diseases observed in pea fields in Castilla y León and surrounding areas in Spain. Most of the 298 bacterial isolates collected from pea plants were pathogenic when inoculated in pea tester varieties susceptible to *P. syringae*. Among the 222 pathogenic isolates 50.5% were identified as *Psy* and 49.5% as *Ppi*. The occurrence of *Psy* as causal agent of pea diseases has been considered less important than the occurrence of *Ppi*, and *Psy* infections seemed to be restricted to autumn-winter sowing of peas (Lawyer and Chun 2001). In most of the studies to identify the causal agents of bacterial diseases, *Psy* was not mentioned (Taylor et al. 1989; Hollaway and Bretag 1995; Reeves et al. 1996; Cirvilleri et al. 1998). However, in the early1970's *Psy* had already been described as the main disease agent for some years in a few early-sown pea fields in New Zealand and in the USA (Butler and Fenwick 1970; Taylor and Dye 1972). Recently, both *Ppi* and *Psy* have been found in early-sown pea fields in New South Wales and Victoria, Australia (Hollaway et al. 2007). *Ppi* and *Psy* have seldom been described as pea pathogens in Spain and *Ppi* was first found as a pea pathogen in 1991 (Elvira-Recuenco and Taylor 2001; López and Montesinos 1996). According to data of the Crop Protection Service the incidence of bacterial diseases has dramatically increased in North-

Table 6 Response of pea cultivars to inoculation with reference strains of *P. syringae* pv. *pisi* (*Ppi*) and *P. syringae* pv. *syringae* (*Psy*), and bacteria isolated from field-grown pea samples

Cultivar	Status[a]	Resistant to *Ppi* races	Deduced resistance genes to *Ppi*	Resistance level to *Psy*	Pathogens isolated[b]
Gracia	C.V.	None	None	2	2, 4, *Psy*
Shawnee	C.V.	None	None	2	*Psy*
A910231	B.L.	None	None	3	*Psy*
Pursan	C.V.	None	None	3	2, 4
Guifredo	C.V.	None	None	4	4
Attika	C.V.	1, 3, 7	R3	0	4, 5, *Psy*
Bacara	C.V.	1, 3, 7	R3	2	4
Blizzard	C.V.	1, 3, 7	R3	2	4
Burbia	B.L.	1, 3, 7	R3	2	*Psy*
Cartouche	C.V.	1, 3, 7	R3	2	4, *Psy*
Cheyenne	C.V.	1, 3, 7	R3	2	4, *Psy*
Chico	C.V.	1, 3, 7	R3	2	*Psy*
Enduro	C.V.	1, 3, 7	R3	2	4
Ideal	C.V.	1, 3, 7	R3	2	2, 4, 5, 6, *Psy*
PM190	B.L.	1, 3, 7	R3	2	4
PM197	B. L.	1, 3, 7	R3	2	4
Aravalle	B. L.	1, 3, 7	R3	3	2, 4
Athos	C.V.	1, 3, 7	R3	3	2
Azur	C.V.	1, 3, 7	R3	3	2
GP-4600	B.L.	1, 3, 7	R3	3	*Psy*
Lobos	B.L.	1, 3, 7	R3	3	*Psy*
Lumina	C.V.	1, 3, 7	R3	3	4
PM227	B.L.	1, 3, 7	R3	3	2, *Psy*
PM88	B.L.	1, 3, 7	R3	3	4
Sidney	C.V.	1, 3, 7	R3	3	2
Luna	C.V.	1, 3, 7	R3	4	2, 4, *Psy*
Pepone	C.V.	1, 3, 7	R3	4	*Psy*
Speleo	C.V.	1, 3, 7	R3	4	4
Mesire	C.V.	1, 2, 3, 5, 7	R2+R3	0	4, *Psy*
Iceberg	C.V.	1, 2, 3, 5, 7	R2+R3	1	4, *Psy*
Isard	C.V	1, 2, 3, 5, 7	R2+R3	1	4
Alliance	C.V.	1, 2, 3, 5, 7	R2+R3	2	*Psy*
Beckii	C.V.	1, 2, 3, 5, 7	R2+R3	2	4, 8
GP-4206	B. L.	1, 2, 3, 5, 7	R2+R3	2	6
Lucy	C.V.	1, 2, 3, 5, 7	R2+R3	2	4, *Psy*
PM05	B.L.	1, 2, 3, 5, 7	R2+R3	2	*Psy*
Rafale	C.V.	1, 2, 3, 5, 7	R2+R3	2	4, 6, 8, *Psy*
Ucero	C.V.	1, 2, 3, 5, 7	R2+R3	2	4, *Psy*
Apache	C.V.	1, 2, 3, 5, 7	R2+R3	3	4
Ballet	C.V.	1, 2, 3, 5, 7	R2+R3	3	4
Talanda	B.L.	1, 2, 3, 5, 7	R2+R3	3	4, 6, 8
GA96H40	L.R.	1, 2, 3, 5, 7	R2+R3	4	4
Pawnee	C.V.	1, 2, 3, 5, 7	R2+R3	4	*Psy*

Table 6 (continued)

Cultivar	Statusa	Resistant to *Ppi* races	Deduced resistance genes to *Ppi*	Resistance level to *Psy*	Pathogens isolatedb
BGE 037894	C.V.	1, 3, 4, 5, 7	R3+R4	2	*Psy*
Dove	C.V.	1, 3, 4, 5, 7	R3+R4	3	*Psy*
Forrimax	C.V.	1, 2, 4, 5, 7	R2+R4	4	*Psy*
Cherokee	C.V.	1, 2, 3, 4, 5, 7	R2+R3+R4	0	4, *Psy*
Corallo	C.V.	1, 2, 3, 4, 5, 7	R2+R3+R4	2	4
Lincoln	C.V.	1, 2, 3, 4, 5, 7	R2+R3+R4	2	*Psy*

[a] C.V.: Commercial Cultivar; B.L.: Breeding line; L.R.: Landrace

[b] Numbers indicated the pathogenic race of *Ppi*

Central Spain, especially in the Castilla y León Region and surrounding areas, associated with the increase of both crop acreage and the practice of early sowing (J.L. Palomo, unpublished).

Thus, *P. syringae* is clearly the predominant agent of bacterial disease on pea plants associated with an early sowing date in late autumn-early winter in Castilla y León, with both pv. *syringae* and pv. *pisi* being almost equally present. There are no previous reports in which *Psy* had been found in such high proportion among isolates from pea fields. These results point to the fact that, at least under some circumstances or some regions, *Psy* can be as important as *Ppi* in inciting pea diseases. This information is important because the breeding efforts for resistance to bacterial diseases in most countries have focused solely on *Ppi*. Thus, the knowledge on resistance to *Psy* and its heredity in pea is scarce.

Molecular markers have been used to identify *P. syringae* pathovars and races pathogenic on legume and other plant species (Arnold et al. 1996; Sorensen et al. 1998; González et al. 2003; Scortichini et al. 2003) and the advantages and limitations of molecular characterization in identification of plant pathogenic bacteria have been reviewed (Alvarez 2004). The identification of *Psy* and *Ppi* was first carried out on the basis of biochemical characteristics. A classical way to differentiate these two pathovars was the use of homoserine as C source, described as positive for *Ppi* and negative for *Psy* (Hildebrand 1972). However, further data indicated that between 15% and 25% of the isolates identified as *Psy* were homoserine positive (Mazarei and Kerr 1990; Grondeau et al. 1992; Braun-Kiewnick and Sands 2001). The percentage of homoserine positive *Psy* isolates found in

this study was much greater, 81.25%. Thus, this biochemical characteristic is not a useful criterion to distinguish between *Psy* and *Ppi*. On the other hand, all isolates finally classified as *Psy* had the PCR amplifiable *syrB* marker and generated disease symptoms (brown necrotic areas) different from *Ppi* symptoms (water-soaked areas) under growth chamber conditions. The genes *syrB* and *syrD*, which are related to the production and transport of bacterial toxins and found in a single copy in the *Psy* genome, are found in most of the *Psy* isolates from different plant species (Quigley and Gross 1994; Sorensen et al. 1998; Scortichini et al. 2003). Our results support that at least *syrB* is a good marker for identifying *Psy*, although there are some *Psy* pathogenic isolates that do not amplify *syrB* (Scortichini et al. 2003). In order to ascertain whether the high frequency of homoserine positive *Psy* isolates and the high frequency of *Psy* as a causal agent of pea disease in North-Central Spain are associated, further tests will be necessary.

Arnold et al. (1996) described the AN3 and AN7 markers obtained from RAPD markers as specific for *Ppi*. Among our isolates, the AN3 marker (132 pb, see Table 1) was amplified from races 3, 4, and 5, while AN7 (272 pb) was amplified from races 2, 4, 6, and the putative new race 8, but from none of the *Psy* isolates or from any reference strain of other *Pseudomonas* species. As Arnold et al. (1996) have shown, there is no correlation between the DNA marker amplified and race or fluorescence phenotype. Thus, for instance, in the Arnold et al. (1996) study and in our study *Ppi* race 4 isolates were fluorescent or non-fluorescent and amplified either AN3 or AN7.

Pea tester varieties were used to identify the different *Ppi* races among the isolates. The predom-

inant race was race 4 which represented 59.1% of the *Ppi* isolates, followed by race 2 (20.0%), race 6 (11.8%), race 5 (3.6%) and race 3 (0.9%). Five isolates showed a response pattern which was not previously described suggesting that an additional race 8 could be present in *Ppi*. These five isolates amplified AN7 and showed fluorescence in KB. Previous data in Spain are limited to the description of eight isolates classified as race 4 and another eight to race 6 (Elvira-Recuenco and Taylor 2001). Taylor et al. (1989) found that race 2 was predominant (74.6%) in a world collection of 146 isolates, followed by race 4 (10.9%), race 6 (7.5%), and race 1 (5.48 %), while races 3 and 5 were each represented by a single isolate. Similar results were found among isolates from pea seeds in Great Britain (Reeves et al. 1996). In Sicily (Italy) the only race found was race 6 (Cirvilleri et al. 1998). In Australia race 3 was found to be predominant (64%) among 65 isolates (race 6, 31%; race 2, 5%) and these results were most likely due to the fact that the predominant pea cultivars were susceptible to races 3 and 6 (Hollaway and Bretag 1995). Our results could be due to a similar response since a minority (12.2%) of pea cultivars and lines found in Castilla y León are resistant to race 4 and none to race 6 (Table 6). The frequency distribution of *Ppi* pathogen races has been correlated to the presence of resistance genes in the most frequently cultivated pea cultivars in a particular region or county (Hollaway et al. 2007). Our results in North-Central Spain partially agreed with this hypothesis, for instance, the low frequency of *Ppi* race 3 agrees with the presence of R3 gene in the majority of the pea cultivars grown. However, the relatively low frequency of race 6 contrasts with the absence of resistance genes to this race in the cultivated materials. Furthermore, race 6 was present in pea materials in Spain before 2001 (Elvira-Recuenco and Taylor 2001). On the other hand, race 4 has been described as highly virulent (Elvira-Recuenco et al. 2003) which agrees with the high frequency of this race among our isolates and with the fact that this pathogen was isolated even from two cultivars (Cherokee and Corallo) with the corresponding resistance gene (Table 6). This result suggests that this resistance gene does not confer complete resistance under field conditions. In fact, in experimental field plots inoculated with race 4, the most common response observed in pea genotypes resis-

tant to this race was partial resistance (Martin-Sanz, unpublished).

The isolates identified as *Psy* were highly pathogenic in all pea tester varieties with the exception of Kelvendon Wonder. Approximately 90% of the pea plants showed apical death at the end of the observation time, while in Kelvendon Wonder the necrosis was localized near the infection point. Thus this cultivar can be considered as resistant to *Psy*. No previous description of resistance in pea to this pathovar has been found, except in two cultivars for human consumption, namely "Elf" and "Champion from England" (Lawyer and Chun 2001)

The search for resistance among the pea materials cultivated in Spain and potentially useful in breeding programs was carried out by inoculating these pea cultivars with type race strains of *Ppi* and with *Psy*. Only five out of 49 pea genotypes showed a satisfactory level of resistance (levels 0 and 1) against *Psy* in growth chamber experiments. Isard was found to be the most promising source of resistance since this cultivar showed resistance under both growth chamber and field conditions, and for two consecutive years in natural infection field trials. The other resistant cultivars showed some disease symptoms under more variable environmental field conditions, thus their resistance can only considered to be incomplete. The search for resistance sources for *Ppi* races revealed that the cultivars Cherokee, Corallo or Lincoln are suitable materials since they carry resistance genes (R2+R3+R4) which give resistance to all *Ppi* races including the new race 8 (data not shown), except to race 6. The inheritance of these genes has been described as dominant monogenic (Bevan et al. 1995; Hunter et al. 2001) which eases the breeding process. *Pisum abyssinicum* is also a very promising source of race nonspecific resistance (Elvira-Recuenco et al. 2003) including race 6 and also additional resistance to the aggressive race 4, although the type of inheritance of this resistance from *P. abyssinicum* is not fully known, and its potential resistance has not yet been tested under field conditions in Spain.

Due to the steady increase in pea production in Spain and the increase in early-sowing in the main production areas like the Castilla y León Region, it is very likely that the incidence of bacterial disease will increase. In fact, in the last two seasons of this study the effect of bacterial pathogens in pea was clearly the most important constraint of the crop. Resistant

cultivars could play an important role in reducing the losses caused by these pathogens and for this reason crossings to include them in the breeding program of the ITACyL have already started.

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