Eur J Plant Pathol (2009) 124:199–218
DOI 10.1007/s10658-008-9406-y

Genetic analyses of *Pseudomonas syringae* isolates from Belgian fruit orchards reveal genetic variability and isolate-host relationships within the pathovar *syringae*, and help identify both races of the pathovar *morsprunorum*

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Received: 9 June 2008 / Accepted: 17 November 2008 / Published online: 6 December 2008

Abstract A collection of *Pseudomonas syringae* and *viridiflava* isolates was established between 1993 and 2002 from diseased organs sampled from 36 pear, plum and cherry orchards in Belgium. Among the 356 isolates investigated in this study, phytotoxin, siderophore and classical microbiology tests, as well as the genetical methods REP-, ERIC- and BOX- (collectively, rep-) and IS50-PCR, enabled identification to be made of 280 isolates as *P. syringae* pv. *syringae* (*Pss*), 41 isolates as *P. syringae* pv. *morsprunorum* (*Psm*) race 1, 12 isolates as *Psm* race 2, three isolates as *P. viridiflava* and 20 isolates as unclassified *P. syringae*. The rep-PCR methods, particularly BOX-PCR, proved to be useful for identifying the *Psm* race 1 and *Psm* race 2 isolates. The latter race was frequent on sour cherry in Belgium. Combined genetic results confirmed homogeneities in the pvs *avii*, and *morsprunorum* race 1 and race 2 and high diversity in the pv. *syringae*. In the pv. *syringae*, homogeneous genetic groups consistently found on the same hosts (pear, cherry or plum) were observed. Pathogenicity on lilac was sometimes variable among *Pss* isolates from the same genetic group; also, some *Psm* race 2 and unclassified *P. syringae* isolates were pathogenic to lilac. In the BOX analyses, four patterns included 100% of the toxic lipodepsipeptide (TLP)-producing *Pss* isolates pathogenic to lilac. Many TLP-producing *Pss* isolates non-pathogenic to lilac and the TLP-non-producing *Pss* isolates were classified differently. *Pseudomonas syringae* isolates that differed from known fruit pathogens were observed in pear, sour cherry and plum orchards in Belgium.

Keywords *Pseudomonas syringae* · Adaptation · Pathogenicity · Rep-PCR · IS50-PCR

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Introduction

Pseudomonas syringae is a heterogeneous phytopathogenic bacterial species divided into >50 pathovars (Young 1991). This pathogen is commonly encountered in fruit orchards in Wallonia in southern Belgium. *P. syringae* pv. *syringae* (*Pss*) has been reported in pear orchards in various regions of the world; *Pss* and *P. syringae* pv. *morsprunorum* (*Psm*) race 1 have been reported in plum orchards, and *Pss*, two races of *Psm* and *P. syringae* pv. *avii* have been reported in cherry orchards or plantations (Freigoun

and Crosse 1975; Yessad et al. 1992; Ménard et al. 2003; Vicente et al. 2004; Kennelly et al. 2007; Renick et al. 2008). Among these pathovars, *P. syringae* pv. *avii* is genetically homogeneous (Ménard et al. 2003) and *Psm* comprises two highly homogeneous races that are clearly distinct from each other (Ménard et al. 2003; Vicente et al. 2004; Vicente and Roberts 2007). In contrast, *Pss* is genetically highly heterogeneous (Weingart and Völksch 1997; Little et al. 1998) and a range in aggressiveness to the reference plant, lilac, was reported among strains of this pathovar (Vicente et al. 2004).

Despite the diversity existing within *P. syringae*, all the fluorescent strains of *P. syringae* and of the closely related species *Pseudomonas viridiflava*, *Pseudomonas ficuserectae*, *Pseudomonas savastanoi* and *Pseudomonas cannabina* produce the atypical pyoverdin siderophore PaA. This atypical pyoverdin has characteristics that enable distinction to be rapidly made in identification between these species and all other fluorescent *Pseudomonas* species (Bultreys et al. 2001, 2003). Strains of *Psm* race1 and *Pss* can produce phytotoxins that are recognised as important components of virulence (Bender et al. 1999) and the detection of these phytotoxins can be used in identification (Young et al. 1992). Most of the *Psm* race 1 strains produce the phytotoxin coronatine and the detection of the *cfl* gene involved in coronatine production can be used in *Psm* race 1 identification (Bereswill et al. 1994). Also, most *Pss* strains produce the toxic lipodepsipeptides (TLP) syringomycins and syringopeptins, and genetical tests based on this characteristic can be used for the diagnosis of this pathovar (Quigley et al. 1994; Bultreys and Gheysen 1999). In addition, many different methods of pathovar identification have been reported, but performing pathogenicity tests is necessary for differentiating pathogenic and non-pathogenic strains (Burkowicz and Rudolph 1994; Vicente et al. 2004). Many pathogenicity tests on different organs and hosts have been described for identifying virulent *P. syringae* strains at the pathovar level. With regard to *Pss*, a standardised procedure on lilac leaves has been described (Young 1991), but lilac tests have not always been decisive in confirming *Pss* identification (Scortichini et al. 2003; Vicente et al. 2004).

Among the various genetical methods used for characterising *P. syringae* strains, the Repetitive Extragenic Palindromic (REP)-PCR, Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, and BOX-PCR (collectively known as rep-PCR) were shown to be highly discriminating methods adapted for characterising clonal isolates. Rep-PCR and Insertion Sequences 50 (IS50)-PCR were used to classify bacterial strains between and within *P. syringae* pathovars (Louws et al. 1994; Weingart and Völksch 1997). REP-PCR showed that *P. syringae* pv. *avii*, *Psm* race 1 and *Psm* race 2 constitute tight genetic groups distinct from each other and from other *P. syringae* pathovars (Ménard et al. 2003; Vicente and Roberts 2007). In California, ERIC-PCR analyses showed that the *Pss* strains isolated from *Prunus* generated similar genetic profiles, whereas most *Pss* strains isolated from other hosts generated different and variable patterns. This led the authors to suggest a host specialisation of the stone fruit strains within *Pss* (Little et al. 1998).

The purpose of the present study was to identify and characterise a large number of *P. syringae* isolates isolated from diseased lesions in fruit orchards in Wallonia in order to determine the diversity within this species in Belgium. Within *Pss*, the combination of microbiological tests and rep-PCR and IS50-PCR results enabled a high genetic heterogeneity to be visualised, as well as the existence in this pathovar of isolate–host relationships. The information provided by a pathogenicity test on lilac leaves was also assessed. The genetic methods proved very informative in helping to identify isolates of *P. syringae* pv. *morsprunorum* race 1 and race 2 as well as unclassified *P. syringae* isolates that were also encountered in Belgian fruit orchards.

Materials and methods

Bacterial strains and identifications

The 400 *P. syringae* and *P. viridiflava* strains and isolates used in this study are listed in Tables 1 and 2. The 44 strains listed in Table 1 are strains from various origins obtained from culture collections. The 356 Belgian *Pseudomonas* isolates listed in Table 2 were isolated from diseased plants from necrotic lesions on fruits, leaves, stems, flowers or buds in 36 Belgian orchards between 1993 and 2002. The studied isolates originated mainly from pear orchards (226 isolates), but also from sweet cherry (84

Table 1 Bacterial reference strains used in the study

Pseudomonas strains	Host	Source	Country	TLP[a]	Coronatine[b]
P. syringae pv. aptata					
LMG 5059^T	Beet	LMG	USA	+	NT
LMG 5143	Beet	LMG	Unknown	+	NT
LMG 5646	Beet	LMG	New Zealand	+	NT
UPB 110	Beet	UPB	Belgium	+	NT
UPB 133	Beet	UPB	The Netherlands	+	NT
UPB 152	Beet	UPB	Switzerland	+	NT
UPB 156	Beet	UPB	Italy	+	NT
UPB 165	Beet	UPB	France	+	NT
UPB 221	Beet	UPB	Uruguay	+	NT
UPB 225	Beet	UPB	Germany	+	NT
UPB 339	Beet	UPB	Sweden	+	NT
P. syringae pv. *atrofaciens*					
LMG 5000	Wheat	LMG	Unknown	+	NT
LMG 5095^T	Wheat	LMG	New Zealand	+	NT
P. syringae pv. *avii*					
CFBP 3846^T	Wild cherry	CFBP	France	NT	NT
CFBP 3848	Wild cherry	CFBP	France	NT	NT
P. syringae pv. *morsprunorum* race 1					
CFBP 3801	*Prunus* sp.	CFBP	UK	NT	+
CFBP 3802	*Prunus avium*	CFBP	UK	NT	+
CFBP 3803	*Prunus cerasus*	CFBP	UK	NT	+
LMG 2222	Sweet cherry	LMG	UK	−	+
LMG 5461	Plum	LMG	Switzerland	NT	NT
LMG 5463	Plum	LMG	UK	NT	−
LMG 5467	*Prunus* sp.	LMG	South Africa	−	−
LMG 5468	Sweet cherry	LMG	UK	−	+
LMG 5698	Plum	LMG	France	−	−
LMG 6110	Sweet cherry	LMG	South Africa	NT	−
P. syringae pv. *morsprunorum* race 2					
CFBP 3800	Sour cherry	CFBP	UK	NT	−
LMG 5075t2^T	Plum	LMG	Unknown	−	−
P. syringae pv. *syringae*					
B301D	Pear	D.C. Gross	UK	+	−
B3A	Peach	D.C. Gross	California, USA	+	−
B457	Orange	D.C. Gross	California, USA	+	NT
CFBP 2117	Cherry	CFBP	France	+	−
CFBP 2118	Cherry	CFBP	France	+	NT
HS191	Millet	D.C. Gross	Australia	+	−
LMG 1247^T	Lilac	LMG	UK	+	−
LMG 5141	Pear	LMG	UK	+	−
LMG 5189	Plum	LMG	Switzerland	+	NT
LMG 5190	Peach	LMG	Holland	+	NT
LMG 5493	Apricot	LMG	France	+	NT
LMG 5494	Sweet cherry	LMG	Hungary	+	−
LMG 6104	Plum	LMG	South Africa	+	−
LMG 6106	Plum	LMG	South Africa	+	NT
LMG 6107	Peach	LMG	South Africa	+	NT
LMG 6108	Apricot	LMG	South Africa	+	NT
PaBF1	Wheat	A. Bultreys	Belgium	+	NT
PsM17	Corn	A. Bultreys	Belgium	+	NT
Ps268	Lemon	D.C. Gross	California, USA	+	−

Table 1 (continued)

Pseudomonas strains	Host	Source	Country	TLP[a]	Coronatine[b]
P. viridiflava					
LMG 2352^T	Bean	LMG	Switzerland	−	NT
LMG 2353	Pear	LMG	England	NT	NT
LMG 6480	Chicory	LMG	Belgium	NT	NT

[a] Production of toxic lipodepsipeptides: +, positive; −, negative; NT, not tested. Data are from Bultreys and Gheysen (1999) or Maraite and Weyns (1997)

[b] PCR detection of the *cfl* gene involved in coronatine production: +, positive; −, negative; NT, not tested

LMG Laboratorium voor Microbiologie van Ghent, Ghent, Belgium, *CFBP* Collection Française des Bactéries Phytopathogènes, Angers, France, *UPB* Bacterial Collection of the Unit of Phytopathology, Université Catholique de Louvain, Louvain-la-Neuve, Belgium

isolates), sour cherry (21 isolates), and plum (24 isolates) orchards. Except for one Flemish orchard, all the orchards were in Wallonia in southern Belgium. The preliminary identifications of about 170 of the Belgian isolates have been previously reported (Bultreys and Gheysen 2003).

The isolates from Belgian orchards were classified by different methods over the years depending on the availability of identification tests, and based on the fact that these isolates had been isolated from cherry, plum or pear. The tests that were relevant at least at one period for classifying an isolate in *P. syringae* and related species were the fluorescence on King's B medium, the hypersensitive reaction (HR) in tobacco leaf (Klement et al. 1963), the oxidase activity, the Api 20 NE tests, which include the arginine dihydrolase test, and the pyoverdin siderophore visual and HPLC tests (Bultreys et al. 2001, 2003). The tests that were relevant at least at one period for classifying a strain in a *P. syringae* pathovar or in *P. viridiflava* are described below. The GATTa tests (G, gelatin liquefaction; A, β-glucosidase activity; T, tyrosinase activity; and Ta, tartrate use) combined with the lactate use test (Garrett et al. 1966; Latorre and Jones 1979) enabled identification of *Pss* and *Psm* race 1: + + − − + responses were expected for *Pss*, and − − + + − responses for *Psm* race 1. A positive response in the potato rot test enabled distinction of *P. viridiflava*. The detection of TLP production by a biological test and the PCR detection of the *syrD* gene involved in TLP secretion enabled identification of *Pss* (Bultreys and Gheysen 1999), whereas the PCR detection of the *cfl* gene involved in coronatine production enabled identification of *Psm* race 1 (Bereswill et al. 1994). Also, findings from this study led to the use of rep-PCR and particularly BOX-PCR (see below for protocols) to identify *Psm* race 2, by comparisons with the English reference strain CFBP 3800, and *P. syringae* pv. *avii*, by comparison with the French reference strains CFBP 3846 and CFBP 3848. Also, BOX-PCR was used to confirm identifications of *Psm* race 1 isolates determined previously using other methods.

IS50- and rep-PCR analyses

The BOX primer BOXA1R (5′-CTACGGCAAGGC-GACCTGACG-3′) was used with the BOX-PCR conditions described by Louws et al. (1994). The ERIC primers ERIC-1R (5′-ATGTAAGCTCCTGGG-GATTCAC-3′) and ERIC-2 (5′-AAGTAAGT GACTGGGGTGAGCG -3′), the REP primers REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′), and the IS50 primer (5′-CAGGACGCTACTTGTGT-3′) were used under the ERIC-PCR, REP-PCR or IS50-PCR conditions, respectively, described by Weingart and Völksch (1997). The primers were obtained from Eurogentec. Bacterial strains and isolates were streaked onto plates of nutrient agar and incubated at 28°C overnight. Approximately 10^6 cells mixed in 1% (*v*/*v*) Tween 20 were transferred to 25 µl of PCR mixture. The PCR mixtures contained: 50 pmol of each primer; 67 mM Tris–HCl (pH 8.8); 16 mM $(NH_4)_2SO_4$; 3.5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 400 µM of each deoxynucleoside triphosphate; 4 µg of bovine serum albumin; 5% dimethyl sulfoxide; and 0.75 units (REP-, ERIC-, and BOX-PCR) or 1.5 unit (IS50-PCR) *Taq* DNA polymerase (GE Healthcare). PCR was performed in a Thermal I cycler (Biorad®). The ERIC-PCR programme comprised: one cycle at 95°C for 5 min; 30 cycles at 94°C

Table 2 Characteristics and identification of the Belgian isolates from fruit trees

Year of collection[a]	Isolates[b]	Origin[c]	Characterisation and identification tests									BOX-P[m]
			F[d]	Ox[e]	Api[f]	PaA[g]	HR[h]	TLP[i]	C[j]	GATTa + Lactate[k]	Pot. rot[l]	
1993 (AB)	5 *Psm* r1 *cfl*+	Sw. ch. var. col.	+	−	D				+	− − + + −		*Psm* r1
	2 *Psm* r1 *cfl*−	Sw. ch. var. col.	+	−	D		+		−	− − + + −		*Psm* r1
	2 *Psm* r1 *cfl*+	Sw. ch. var. col.	−	−	D		+		+	− − + + −		*Psm* r1
	23 *Psm* r1 *cfl*+	Sw. ch. var. col.	−	−	D				+	− − + + −		*Psm* r1
1996 (AB)	1 *Pss* TLP+	1 pear orch.	+	−	D	+	+	+ BG		+ + − − +		1(1)
1999 (AB)	5 *Psm* r1 *cfl*+	Sw. ch. var. col.	+	−		+			+			*Psm* r1
	4 *Psm* r1 *cfl*+	Sw. ch. var. col.	−	−			+		+			*Psm* r1
	12 *Psm* r2	So. ch. var. col.	+	−	D	+		− BG	−	4 + − − − − Y	−	*Psm* r2
										2 + − ± − − Y		
										2 + − ± − + Y		
										+ − + − + Y		
										+ ± − − − Y		
										+ ± ± − − Y		
										NT		
	25 *Pss* TLP+	Sw. ch. var. col.	+	−		+		+ BG				1(20) 5(3) 6(1) 35(1)
	2 *Pss* TLP+	So. ch. var. col.	+	−		+		+ BG				1(1) 5(1)
	43 *Pss* TLP+	1 pear orch.	+	−		+		+ BG				1(42) 4(1)
	13 *Pss* TLP+	Plum var. col.	+	−		+		+ BG				1(12) 38(1)
	2 *Pss* TLP−	1 pear orch.	+	−	D	+		− BG	−	+ + − − +	−	10(2)
	3 *Pv*	So. ch. var. col.	+	−	D	+		− BG	−	2 + + + − +	+	11(3)
										+ + ± − +		
	8 *Ps*	Plum var. col.	+	−	D	+		− BG	−	+ + − − − Y	−	40(2) 41(4) 42(1) 43(1)
										3 + + − − −		
										2 + + ± − −		
										+ − − − +		
										+ + + − −		
	2 *Ps*	So. ch. var. col.	+	−	D	+		− BG	−	+ − − − − Y	−	*Psm* r2
	3 *Ps*	1 pear orch.	+	−	D	+		− BG	−	+ ± + − −	−	9(3)
										+ − − + +		
										+ + − − + Y		
2001–2002 (FL)	13 *Pss* TLP+	5 sw. ch. orch.	+	−		+		+ B				1(2) 3(9) 30(1) 37(1)
	8 *Pss* TLP+	6 so. ch. orch.	+	−		+		+ B				1(3) 3(4) 34(1)
	158 *Pss* TLP+	34 pear orch.	+	−		+		+ B				1(82) 2(45) 3(6) 4(8) 14(1) 15(1) 16(1) 18(1) 19(1) 20(1) 21(1) 22(1) 24(2) 26(2) 27(2) 28(2)

Table 2 (continued)

Year of collection[a]	Isolates[b]	Origin[c]	Characterisation and identification tests									BOX-P[m]
			F[d]	Ox[e]	Api[f]	PaA[g]	HR[h]	TLP[i]	C[j]	GATTa + Lactate[k]	Pot. rot[l]	
	3 *Pss* TLP+	3 plum orch.	+	−		+		+ B				1(2) 3(1)
	12 *Pss* TLP−	3 pear orch.	+	−	D	+		− B	−	+ + − − +	−	4(12)
	7 *Ps*	4 pear orch.	+	−	D	+		− B	−	+ + + − +	−	12(1) 23(2) 25(2) 89(1) 90(1)
										2 + − − − −		
										+ + − − − Y		
										+ + − − −		
										+ − ± − −		
										+ + ± − +		

[a] (AB), collection, characterisation and identification tests by A. Bultreys; (FL), collection by F. Legros, characterisation and identification tests by F. Legros and A. Bultreys

[b] *Psm*, *Pseudomonas syringae* pv. *morsprunorum*; r1, race 1; *cfl*+, isolates posses the *cfl* gene involved in coronatine production; *cfl*−, isolates lacking the *cfl* gene; r2, race 2; *Pss*, *Pseudomonas syringae* pv. *syringae*; TLP+, isolates producing toxic lipodepsipeptides; TLP−, non-producing isolates of toxic lipodepsipeptides; *Pv*, *Pseudomonas viridiflava*; *Ps*, *Pseudomonas syringae*

[c] Sw., sweet; ch., cherry; var., variety; col., collection; so, sour; orch., orchard. The sweet cherry, sour cherry and plum variety collections are those of the Walloon Agricultural Research Centre in Gembloux in Belgium. The other Belgian orchards are dispersed in Wallonia, but the 1996 isolate was collected in Flanders

[d] Fluorescence on King's B medium; +, positive; −, negative.

[e] Oxidase test; −, isolates are oxidase negative

[f] Api 20 NE; D, test done

[g] Detection of the atypical pyoverdin PaA of *Pseudomonas syringae* and *Pseudomonas viridiflava* by HPLC (Bultreys et al. 2003); +, test performed and detection of PaA

[h] HR, hypersensitive response on tobacco leaves (Klement et al. 1963); +, test performed and HR detected

[i] Detection of toxic lipodepsipeptide (TLP) production by using the Bultreys and Gheysen (1999) procedures: B, result obtained by using the biological test; G, result obtained by using the genetical test detecting the *syrD* gene; +, positive; −, negative.

[j] Detection of the *cfl* gene involved in coronatine production by PCR; +, positive; −, negative

[k] GATTa and lactate use test results; +, positive response; −, negative response; ±, weak positive response or intermediate response. Y, possession of the *irp1* gene involved in yersiniabactin production (data not shown)

[l] Potato rot test; +, positive; −, negative

[m] Data are either the identification obtained by BOX-PCR, or a specific BOX-pattern (BOX-P) number directly followed, under brackets, by the number of isolates classified in this BOX-P

for 1 min, 52°C for 1 min, and 65°C for 8 min; and finally one cycle at 65°C for 15 min. The REP-PCR programme comprised: one cycle at 95°C for 5 min; 30 cycles at 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; and finally one cycle at 65°C for 15 min. The BOX-PCR programme comprised: one cycle at 95°C for 7 min; 30 cycles at 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min; and finally one cycle at 65°C for 15 min. The IS50-PCR programme comprised: one cycle at 93°C for 3 min; 35 cycles at 94°C for 1 min, 38°C for 1 min, and 72°C for 3.5 min; and finally one cycle at 72°C for 15 min. After PCR, 18 µl of the amplification products were separated on 1% agarose gels at 3.4 V/cm for 3 h. DNA fragments were visualised under UV illumination after staining with ethidium bromide. The analysis of every strain and isolate was repeated twice. Each amplification band was treated as a unit character and was scored as present (1) or absent (0). In the TREECON software (Van de Peer and De Wachter 1994), a distance matrix was constructed using the Link coefficient, and the Unweighted Pair-Group Method (UPGMA) with arithmetic means was used for clustering. The strength of the tree topology was assessed using the bootstrap method.

Pathogenicity tests on lilac

The pathogenicity on lilac leaves was evaluated for two Belgian isolates of *P. viridiflava* from sour cherry and for 108 Belgian isolates of *P. syringae* from cherry, pear or plum: 57 TLP+ *Pss*, 11 TLP− *Pss*, 15 unclassified *P. syringae*, 15 *Psm* race 1, and ten *Psm* race 2. In addition, the reference strains *Pss* B301D and LMG5141 from pear, *Pss* CFBP 2118 and LMG 5494 from cherry, *Pss* LMG 6104 from plum and the pathotype strain *Pss* LMG 1247 from lilac were also tested.

The protocol of the pathogenicity test on lilac was adapted from Young (1991) and Yessad-Carreau et al. (1994). Five year-old lilac plants (*Syringa vulgaris* cv. And an Ludwig sp.) were grown in the greenhouse with 16 h artificial light and the temperature maintained between 20°C and 26°C. Five fully expanded leaves were cut from shoots with eight new leaves. The leaves were dipped into a solution of sodium hypochlorite (1% active hypochlorite) for 5 min, rinsed three times in sterile osmosed water and the excess of water removed with paper. A 5 mm wound approximately 5 mm away from the lamina was made on the petiole with a scalpel. A 10 µl drop of bacterial suspension (10^8 CFU ml^{-1}) was then deposited on the wound. The inoculated leaves were placed on sterile paper filter over water agar (10 g agar l^{-1}) in sterile Petri dishes. The Petri dishes were sealed with a piece of Parafilm and incubated at 20°C for 7 days under daylight conditions. Five leaves were inoculated for each isolate and a mean progression was calculated based on the results of the five repetitions. The strains and isolates were placed in six pathogenicity classes according to their aggressiveness: class 0: no necrosis; class 1: mean necrosis limited to the cut; class 2: mean necrosis from 10 to 20 mm; class 3: mean necrosis from 21 to 30 mm; class 4: mean necrosis from 31 to 40 mm; and class 5: mean necrosis from 41 to 50 mm. Following the definition of pathogenicity to lilac leaves described by Young (1991), the strains and isolates were considered pathogenic if a progressive lesion was observed (class 2 to 5). To test the reproducibility of the results, one complete repetition with two or three representatives of each class was conducted in another period using, again, 5 leaves for each isolate.

Results

Identification

Table 2 summarises all the results of the identification of the Belgian isolates. The 1993 fluorescent isolates were classified in the phytopathogenic fluorescent *Pseudomonas* based on their fluorescence on King's B medium and on the negative responses in the oxidase test and in the arginine dihydrolase test. Comparison of the Api 20 NE results and GATTa and lactate use tests results between control *Psm* r1 strains and fluorescent and non-fluorescent Belgian isolates enabled identification of Belgian *Psm* r1. The presence of the *cfl* gene was frequent among Belgian *Psm* r1 from sweet cherry and the four fluorescent and non-fluorescent isolates tested induced HR on tobacco. The 1996 pear *Pss* isolate was also identified by Api 20 NE and GATTa and lactate use test comparisons with control *Pss* strains. It was one of the first *Pss* strains used to evaluate the reliability of using pyoverdin tests to directly affiliate an isolate to *P. syringae* and related species, and TLP tests to identify *Pss*. Pathogenicity on cherry twig of several *Psm* race 1 isolates isolated in 1993 and pathogenicity on cherry twig and pear leaf of the *Pss* isolate isolated

in 1996 was confirmed (Bultreys and Gheysen 1999, unpublished results).

The identifications of the 1999 isolates were based more on siderophore and phytotoxin-based tests (pyoverdin, TLP and coronatine). The isolates were classified according to their pyoverdin siderophore type or their HR reaction on tobacco, depending on their fluorescence in GASN solid/liquid medium (Bultreys and Gheysen 2000). The oxidase activity, the production of TLP and the PCR detection of the *syrD* and *cfl* genes were then determined. The isolates producing the atypical pyoverdin PaA and TLP (TLP+) were classified as TLP+ *Pss*. Both the fluorescent isolates producing PaA and the non-fluorescent isolates inducing HR on tobacco that possessed the *cfl* gene (*cfl*+) were classified as *cfl*+ *Psm* race 1. The isolates producing the pyoverdine PaA and inducing potato rotting were identified as *P. viridiflava.* The other isolates producing the pyoverdine PaA were analysed by classical procedures including Api 20 NE and GATTa + lactate use tests. Some isolates giving + + − − + responses were classified as TLP− *Pss*, except if they possessed the *irp1* gene involved in yersiniabactin production (data not shown) because this characteristic is related to *P. syringae* strains belonging to the genospecies 2 (pv. *phaseolicola* and *glycinea*), 3, 7 and 8, but not to the genospecies 1 including *Pss* (Bultreys et al. 2006).

The BOX-PCR data provided additional information in identification of *Psm* race 1, *Psm* race 2 and *P. syringae* pv. *avii* strains and isolates because these strains showed constant specific BOX patterns (Fig. 1). Among the unclassified Belgian isolates, 12 out of the 14 isolates from sour cherry clustered together and belonged to the same BOX-P, ERIC-P, REP-P and IS-P as the British reference strain *Psm* race 2 CFBP 3800 (Fig. 1). These isolates were therefore included in this pathovar and race (Table 2). Also, the two other remaining unclassified *P. syringae* isolates from sour cherry had the same BOX-P, but slightly different ERIC-Ps, REP-Ps and IS-Ps. Interestingly, the pathotype strain *Psm* LMG 5075 (type 2) grouped perfectly with the *Psm* race 2 strain and isolates using the BOX-, ERIC-, REP- and IS50-PCR methods and was therefore considered as a *Psm* race 2 strain (Table 1). The same observation was made with BOX-PCR, using different procedures, for the equivalent strain CFBP 2351 from the CFBP culture collection (Bultreys and Gheysen, unpublished results). Also, BOX-PCR

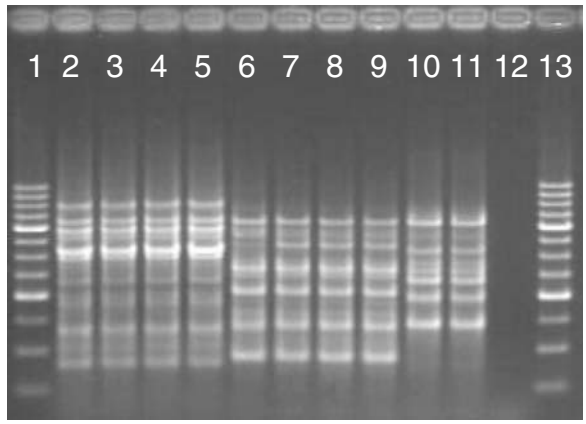


Fig. 1 BOX-PCR patterns of *Psm* race 1, *Psm* race 2, and *P. syringae* pv. *avii* isolates and strains. *Lane 1* and *13*: DNA molecular weight marker XVI (Roche). *Lane 2* to *5*: *Psm* race 1 strains and isolates LMG2222 (*cfl*+), PmC36 (*cfl*+), LMG 5463 (*cfl*−) and PmC24 (*cfl*−). *Lane 6* to *9*: *Psm* race 2 strain and isolates CFBP 3800, Pm2C80, Pm2C101 and Pm2C110. *Lane 10* and *11*: *P. syringae* pv. *avii* strains CFBP 3846 and CFBP 3848. *Lane 12*: water control

grouped all the *cfl*+ and *cfl*− *Psm* race 1 strains and isolates tested in this study in only one BOX-P (Fig. 1), which enabled the confirmation of all the previous identifications of Belgian *Psm* r1 isolates based on other characteristics (Table 2).

Finally, among the 356 Belgian isolates analysed in this study, 14 pear isolates were identified as TLP− *Pss* and 266 isolates were identified as TLP+ *Pss*, of which 202 had been isolated from pear, 44 from sweet cherry, 16 from plum and four from sour cherry. Thirty-nine isolates from sweet cherry were identified as *cfl*+ *Psm* race 1, and only two isolates as *cfl*− *Psm* race 1. Twelve isolates from sour cherry were identified as *Psm* race 2 and the two resting *P. syringae* isolates from sour cherry were genetically very close to this pathovar and race. The potato test identified three *P. viridiflava* isolates from sour cherry. Ten isolates from pear and eight from plum remained unclassified in *P. syringae.*

ERIC-, REP-, BOX- and IS50-PCR analyses

Among the methods investigated to evaluate variability within the 400 reference and Belgian *P. syringae* strains and isolates analysed, REP-PCR was the most discriminating method and generated 63 patterns. BOX-PCR was the least discriminating method and

generated 43 patterns. ERIC- and IS50-PCR were intermediate with 59 and 58 patterns, respectively. The fragment lengths ranged from 150 to 6,000 bp. The highest diversity was observed among the *Pss* isolates (TLP+ and TLP−), with between 36 and 51 patterns, depending on the method. The *Psm* race 1 strains and isolates (*cfl*+ and *cfl*−) were genetically more homogeneous. The *cfl*+ *Psm* race 1 strains and isolates were grouped in only one ERIC, REP, BOX and IS50 pattern, independent of country of origin. The *cfl*− *Psm* race 1 strains and isolates generated one BOX pattern (BOX-P), two related REP patterns (REP-Ps), three related IS50 patterns (IS-Ps) and five related ERIC patterns (ERIC-Ps). For each method, these patterns differed from each other by only one or two bands. They also differed by only one or two bands from the corresponding pattern of the *cfl*+ *Psm* race 1 isolates. BOX-PCR was the least discriminating method: it grouped all the *cfl*+ and *cfl*− *Psm* race 1 strains and isolates tested in only one BOX-P (Fig. 1). The two strains of *P. syringae* pv. *avii* investigated gave identical results using ERIC-, REP-, BOX- and IS50-PCR; they were clearly different from all the other pathovars tested (Fig. 1).

Among the TLP+ *Pss*, the plum, pear and cherry strains and isolates clustered differently from the reference strains isolated from apricot, peach, cereals, lilac, lemon and orange. A high diversity was observed among the Belgian and reference TLP+ *Pss* strains and isolates isolated from pear, plum and cherry: 41 patterns were generated by REP-PCR, 26 by BOX-PCR, 35 by ERIC-PCR and 38 by IS50-PCR. Some patterns were frequently observed, whereas 8–13% of the patterns, depending on the method, included only one isolate. In the frequently observed patterns, the genetic groupings differed depending on the method (Table 3). For example, the ERIC-P number 5 (ERIC-P5) included 13 isolates isolated from plum and 32 isolates isolated from cherry, but these plum and cherry isolates belonged to different patterns when analysed using REP-PCR (REP-P10 and REP-P5) or IS50-PCR (IS50-P9 and IS50-P3). With BOX-PCR, they were grouped mainly in BOX-P1 with isolates from pear (Table 3). The TLP− *Pss* isolates generally clustered differently from TLP+ isolates, except with BOX-PCR, the least discriminating method, where 86% of TLP− *Pss* isolates clustered in BOX-P6, with some rare TLP+ isolates from pear (Table 3).

Combined ERIC-, REP-, BOX- and IS50-PCR analyses

As the genetic groupings differed depending on the method used, a combination of the rep- and IS50-PCR results was produced. The results are presented in Figs. 2 and 3 and the resulting combined patterns were named repIS-Ps. The information given by rep- and IS-PCR was very fine because very closely related strains could be differentiated. But, distant strains did have little in common in these analyses. As a result, the dendrograms in Figs 2 and 3 give little useful relative classification information for distant strains and the bootstrap values are particularly low in the left part of these dendrograms.

Figure 2 compares TLP+ reference strains and Belgian isolates belonging to *Pss*, *P. syringae* pv. *aptata* and *P. syringae* pv. *atrofaciens*, the Belgian TLP− *Pss* isolates, the Belgian unclassified *P. syringae* isolates, and the Belgian isolates and reference strains of *P. viridiflava*. It shows that there was high variability among the strains and isolates analysed. Two relatively well supported clusters were specific to the tested TLP+ strains of the pvs *atrofaciens* and *aptata*, respectively. Some weak genetic heterogeneity was observed among the TLP+ strains within the pvs *atrofaciens* and *aptata*, but there was no overlapping between these pathovars and the TLP+ *Pss* strains and isolates. The percentage of similitude between strains of pvs *aptata* and *atrofaciens* and the closest *Pss* strains and isolates was <30%. A great diversity was observed among the *Pss* strains and isolates. The high number of related patterns probably explained the low bootstrap values observed in the tree, which often excluded definition of well supported clusters. Several groups based on a genetic similarity of >50% were however apparent, as was a tendency of the isolates from the same host to group together. The Belgian isolates from fruit orchards were genetically not similar (<40% similarity) to the reference *Pss* strains isolated from other crops (apricot, peach, wheat, corn, millet, lilac, lemon, orange) shown in brown in Fig. 2. The three Belgian isolates of *P. viridiflava* from sour cherry classified in repIS-P20 were also distant from the *P. viridiflava* reference strains isolated from bean, pear and chicory classified in repIS-P38, 39 and 40 (Fig. 2; Table 1).

The TLP+ *Pss* from pear, plum and cherry were genetically highly variable and formed 59 repIS-Ps

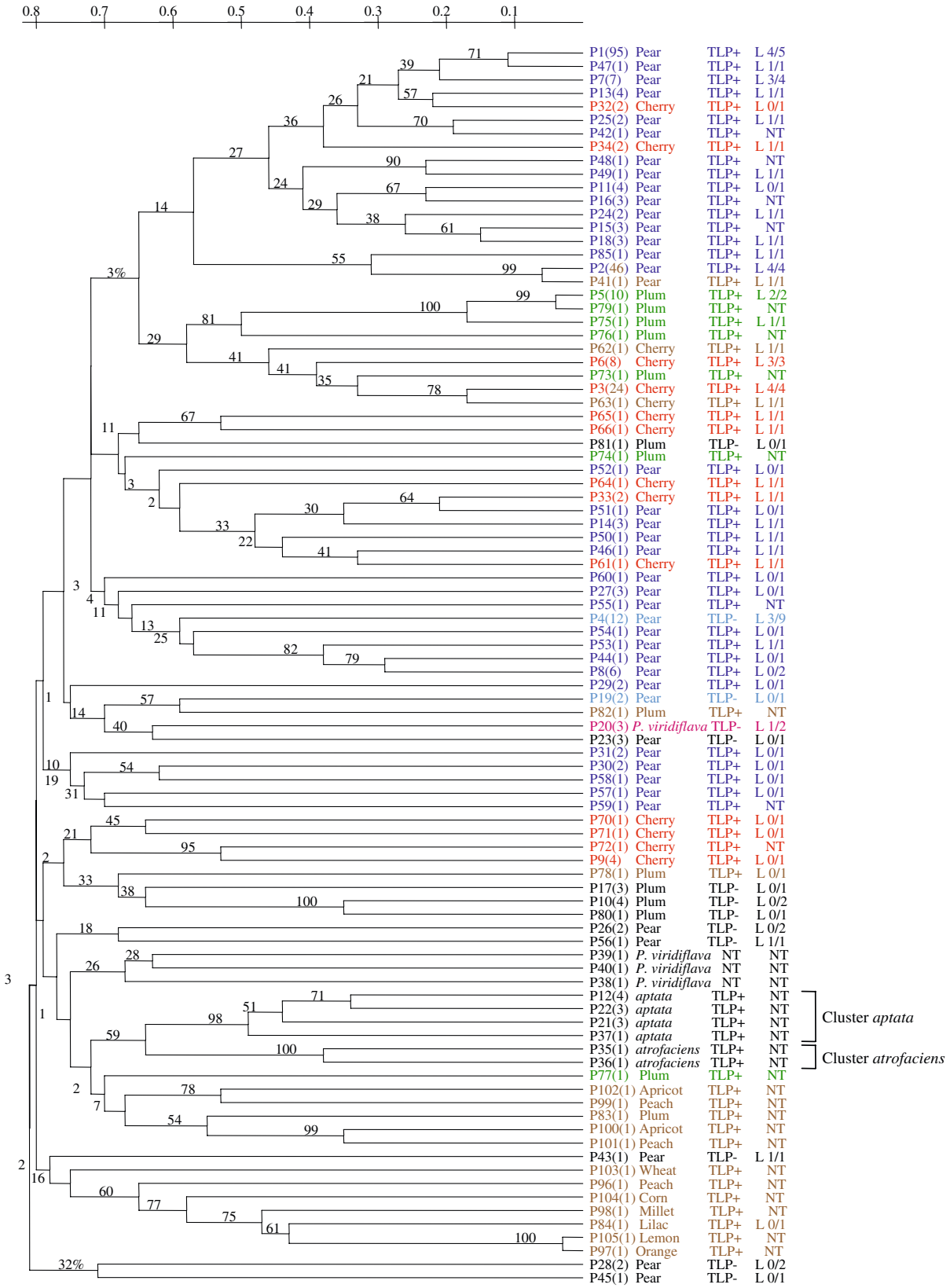
Table 3 Numerical distribution in rep- and IS50-patterns of *Pss* isolates from fruit trees

PCR	Patterns	TLP+[a]				TLP–[a]
		Pear	Sweet cherry	Sour cherry	Plum	Pear
ERIC	ERIC-P4	119 (58%)	1 (2%)	1 (10%)	0	0
	ERIC-P3	49 (24%)	3 (7%)	2 (20%)	0	0
	ERIC-P5	0	29 (70%)	3 (30%)	13 (68%)	0
	ERIC-P30	0	0	0	0	12 (86%)
	Other ERIC-Ps	36 (18%)	8 (21%)	4 (40%)	6 (32%)	2 (14%)
REP	REP-P2	114 (56%)	0	0	0	0
	REP-P1	50 (24.5%)	1 (2%)	0	1 (5%)	0
	REP-P5	3 (1.5%)	29(71%)	3 (30%)	0	0
	REP-P10	0	0	0	11 (58%)	0
	REP-P6	0	0	0	0	12 (86%)
	Other REP-Ps	37 (18%)	11 (27%)	7 (70%)	7 (37%)	2 (14%)
BOX	BOX-P1	128 (63%)	25 (61%)	4 (40%)	14 (74%)	0
	BOX-P2	45 (22%)	0	0	0	0
	BOX-P3	6 (3%)	9 (22%)	4 (40%)	1 (5%)	0
	BOX-P4	8 (4%)	0	0	0	12 (86%)
	Other BOX-Ps	17 (8%)	7 (17%)	2 (20%)	4 (21%)	2 (14%)
IS50	IS50-P1	111 (54%)	3 (7%)	3 (30%)	0	0
	IS50-P2	53 (26%)	0	0	0	0
	IS50-P3	6 (3%)	29 (71%)	3 (30%)	1 (5%)	0
	IS50-P9	0	0	0	12 (63%)	0
	IS50-P8	0	0	0	0	12 (86%)
	Other IS50-Ps	34 (17%)	9 (22%)	4 (40%)	6 (32%)	2 (14%)

[a] The data are, for each culture, the number of isolates and, in brackets, their corresponding frequency expressed as a percentage of the corresponding host

(Fig. 2). However, some patterns were clearly more frequent than others: the pear TLP+ *Pss* isolates and strains were classified in 34 repIS-Ps, but 69% belonged to repIS-P1 or repIS-P2; the cherry TLP+ *Pss* isolates and strains were classified in 15 repIS-Ps, but 63% belonged to repIS-P3 or repIS-P6; and the plum TLP+ *Pss* isolates and strains were classified in ten repIS-Ps, but 52% belonged to repIS-P5. Isolate–host relationships were apparent between the TLP+ *Pss* isolates and their hosts of isolation (Fig. 2): no *Pss* isolates from one host (cherry, plum or pear) had exactly the same profile as *Pss* isolates from another host. For example, none of the 95 isolates belonging to repIS-P1, or the 46 isolates belonging to repIS-P2, was isolated from a host other than pear. The repIS-P3 and repIS-P6 contained only 24 and eight isolates from cherry, respectively. The repIS-P5 contained only ten isolates from plum. The repIS-Ps including the Belgian isolates were not specific to Belgium. Indeed, although some investigated foreign strains had specific patterns (Fig. 2 and Table 4), the two pear strains isolated in 1959 in UK B301D and LMG 5141 belonged to the pear-specific repIS-P2, including many Belgian pear isolates, and the closely related repIS-P41, respectively. Also, the CFBP 2117 and CFBP 2118 strains isolated in 1979 from cherry in France belonged to the cherry-specific

Fig. 2 Dendrogram of the genetic relatedness of the repIS-Ps of the *Pss* strains and isolates and distribution of the unclassified *P. syringae* isolates. For each line, the information is: the repIS-P number, the number of strains and isolates in the corresponding pattern (*under brackets*), the host, the capacity to produce TLP and the pathogenicity on lilac leaves (*L*) expressed as the number of strains and isolates pathogenic on lilac leaves related to the number of strains and isolates tested in the pattern. The *scale* indicates the degree of genetic dissimilarity between strains and isolates. The *coloured patterns* refer to Belgian *Pss* isolates: TLP+ isolates from pear (*blue*), TLP− isolates from pear (*light blue*), TLP+ isolates from cherry (*red*), TLP+ isolates from plum (*green*). The Belgian *P. viridiflava* isolates are coloured in *purple* and the *Pss* reference strains in *brown*. In repIS-P2, and -P3 the number under brackets is in *brown* because these patterns include one *Pss* reference strain. The other patterns are either unclassified *P. syringae* isolates or strains from specified pathovars or species. The *numbers* on the *branches* are the bootstrap values


0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1
P1(95) Pear TLP+ L 4/5
P47(1) Pear TLP+ L 1/1
P7(7) Pear TLP+ L 3/4
P13(4) Pear TLP+ L 1/1
P32(2) Cherry TLP+ L 0/1
P25(2) Pear TLP+ L 1/1
P42(1) Pear TLP+ NT
P34(2) Cherry TLP+ L 1/1
P48(1) Pear TLP+ NT
P49(1) Pear TLP+ L 1/1
P11(4) Pear TLP+ L 0/1
P16(3) Pear TLP+ NT
P24(2) Pear TLP+ L 1/1
P15(3) Pear TLP+ NT
P18(3) Pear TLP+ L 1/1
P85(1) Pear TLP+ L 1/1
P2(46) Pear TLP+ L 4/4
P41(1) Pear TLP+ L 1/1
P5(10) Plum TLP+ L 2/2
P79(1) Plum TLP+ NT
P75(1) Plum TLP+ L 1/1
P76(1) Plum TLP+ NT
P62(1) Cherry TLP+ L 1/1
P6(8) Cherry TLP+ L 3/3
P73(1) Plum TLP+ NT
P3(24) Cherry TLP+ L 4/4
P63(1) Cherry TLP+ L 1/1
P65(1) Cherry TLP+ L 1/1
P66(1) Cherry TLP+ L 1/1
P81(1) Plum TLP- L 0/1
P74(1) Plum TLP+ NT
P52(1) Pear TLP+ L 0/1
P64(1) Cherry TLP+ L 1/1
P33(2) Cherry TLP+ L 1/1
P51(1) Pear TLP+ L 0/1
P14(3) Pear TLP+ L 1/1
P50(1) Pear TLP+ L 1/1
P46(1) Pear TLP+ L 1/1
P61(1) Cherry TLP+ L 1/1
P60(1) Pear TLP+ L 0/1
P27(3) Pear TLP+ L 0/1
P55(1) Pear TLP+ NT
P4(12) Pear TLP- L 3/9
P54(1) Pear TLP+ L 0/1
P53(1) Pear TLP+ L 1/1
P44(1) Pear TLP+ L 0/1
P8(6) Pear TLP+ L 0/2
P29(2) Pear TLP+ L 0/1
P19(2) Pear TLP- L 0/1
P82(1) Plum TLP+ NT
P20(3) P. viridiflava TLP- L 1/2
P23(3) Pear TLP- L 0/1
P31(2) Pear TLP+ L 0/1
P30(2) Pear TLP+ L 0/1
P58(1) Pear TLP+ L 0/1
P57(1) Pear TLP+ L 0/1
P59(1) Pear TLP+ NT
P70(1) Cherry TLP+ L 0/1
P71(1) Cherry TLP+ L 0/1
P72(1) Cherry TLP+ NT
P9(4) Cherry TLP+ L 0/1
P78(1) Plum TLP+ L 0/1
P17(3) Plum TLP- L 0/1
P10(4) Plum TLP- L 0/2
P80(1) Plum TLP- L 0/1
P26(2) Pear TLP- L 0/2
P56(1) Pear TLP- L 1/1
P39(1) P. viridiflava NT NT
P40(1) P. viridiflava NT NT
P38(1) P. viridiflava NT NT
P12(4) aptata TLP+ NT
P22(3) aptata TLP+ NT
P21(3) aptata TLP+ NT
P37(1) aptata TLP+ NT
P35(1) atrofaciens TLP+ NT
P36(1) atrofaciens TLP+ NT
P77(1) Plum TLP+ NT
P102(1) Apricot TLP+ NT
P99(1) Peach TLP+ NT
P83(1) Plum TLP+ NT
P100(1) Apricot TLP+ NT
P101(1) Peach TLP+ NT
P43(1) Pear TLP- L 1/1
P103(1) Wheat TLP+ NT
P96(1) Peach TLP+ NT
P104(1) Corn TLP+ NT
P98(1) Millet TLP+ NT
P84(1) Lilac TLP+ L 0/1
P105(1) Lemon TLP+ NT
P97(1) Orange TLP+ NT
P28(2) Pear TLP- L 0/2
P45(1) Pear TLP- L 0/1
Cluster aptata
Cluster atrofaciens

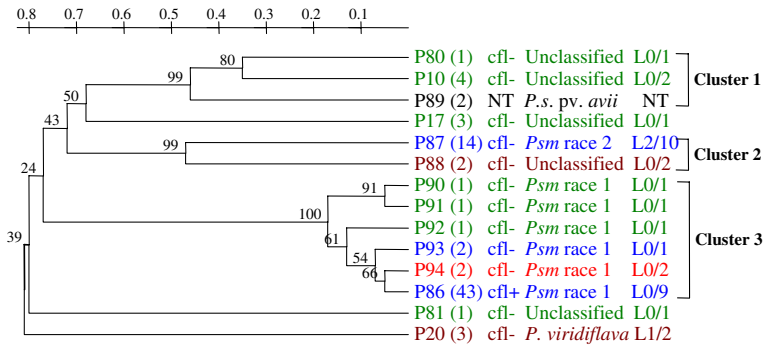


Fig. 3 Dendrogram of the genetic relatedness of the repIS-Ps of the *Psm* race 1, *Psm* race 2, *P. syringae* pv. *avii* and the unclassified *P. syringae* isolates and strains from plum or cherry. For each line, the information is: the repIS-P number, the number of strains and isolates in the corresponding pattern (*under brackets*), the capacity to produce coronatine, the identification, and the observed pathogenicity on lilac leaves (*L*) expressed as the number of strains and isolates pathogenic on lilac leaves related to the number of the strains and isolates tested in the pattern. The *scale* indicates the degree of genetic dissimilarity between strains and isolates. The *coloured patterns* include strains and isolates isolated from sweet cherry and *Prunus* (*blue*), from plum (*green*), from sweet cherry (*red*), from sour cherry (*dark red*) and from wild cherry (*black*). The *numbers* on the *branches* are the bootstrap values

repIS-P3, including many Belgian cherry isolates, and the closely related repIS-P63, respectively.

The TLP− *Pss* isolates clustered differently from the TLP+ *Pss* isolates (Fig. 2). Out of 14 TLP− *Pss* isolates from pear, 12 clustered in repIS-P4. The two other isolates were genetically different and clustered together in repIS-P19.

Figure 3 compares the *cfl*+ and *cfl*− *Psm* race 1 strains and isolates, the *Psm* race 2 strains and isolates, the Belgian unclassified *P. syringae* isolates isolated from *Prunus* sp., and the *P. syringae* pv. *avii* strains. This dendrogram had better bootstrap values and it showed that three clusters were formed. Cluster 1 included the five unclassified *P. syringae* isolates from plum, as well as the two *P. syringae* pv. *avii* reference strains CFBP 3846 and CFBP 3848. However, the two pv. *avii* strains from wild cherry had completely identical patterns, different from those of the Belgian isolates from plum. Cluster 2 grouped repIS-P87 and repIS-P88. RepIS-P87 contained the British *Psm* race 2 CFBP 3880 strain, 12 Belgian *Psm* race 2 isolates and the *Psm* race 2 pathotype strain LMG 5075 (Fig. 3). RepIS-P88 contained the two previously evoked unclassified *P. syringae* isolates from sour cherry. Cluster 3 grouped the *cfl*+ and *cfl*− *Psm* race 1 strains and isolates. It contained six closely related repIS-Ps: repIS-P86, including 43 *cfl*+ *Psm* race 1 strains and isolates, and five other repIS-Ps, including all the *cfl*− *Psm* race 1 strains and isolates tested (Fig. 3). Other rare unclassified *P.*

Table 4 Numerical distribution in repIS-patterns of reference *Pss* strains from fruit trees

Strain	Country	Isolation year	Host	repIS-P[a]	Related Belgian isolates
CFBP 2117	France	1979	Cherry	P3	23 cherry isolates
CFBP 2118	France	1979	Cherry	P63	Close to repIS-P3 including 23 cherry isolates
LMG 5494	Hungary	1958	Cherry	P62	None
LMG 5189	Switzerland	1965	Plum	P82	None
LMG 6106	South Africa	1984	Plum	P83	None
LMG 6104	South Africa	1984	Plum	P78	None
B301D	UK	1959	Pear	P2	45 pear isolates
LMG 5141	UK	1959	Pear	P41	Close to repIS-P2 including 45 pear isolates

LMG Laboratorium voor Microbiologie van Ghent, Ghent, Belgium, *CFBP* Collection Française des Bactéries Phytopathogènes, Angers, France

[a] See Fig. 2

syringae isolates from *Prunus* sp. were distant from *Pss*, *Psm* race 1, *Psm* race 2 and *P. syringae* pv. *avii* (Figs. 2 and 3).

Pathogenicity tests on lilac leaves

The observed lengths of necroses on lilac leaves ranged from 0 to 46 mm. Examples of symptoms are shown in Fig. 4. A general presentation of the results is shown in Table 5 and the pathogenicity results related to the repIS-P are also shown in Figs. 2 and 3. In the observations, weakly pathogenic isolates were sometimes non-pathogenic on some leaves. However, as limited necrosis had been observed in at least one of the leaves, they were placed in class 1. The test was repeated in time with representative isolates from all pathogenicity classes, but non-pathogenic isolates were always non-pathogenic. With pathogenic isolates, the symptom severity sometimes varied from one group of tests to the other. However, the range of variation was always restricted to one pathogenicity class. For estimating variation, the isolates were classified according to the mean of the lengths of the necroses in both tests (ten inoculated leaves).

Among the 62 TLP+ *Pss* strain and isolates tested, only 40 (65%) induced progressive necroses and were pathogenic to lilac leaves. But the repartition of the pathogenic isolates to lilac was not homogeneous among the different repIS-Ps. Indeed, 20 of the 22 isolates tested (90.9%) belonging to the main patterns repIS-P1, repIS-P2, repIS-P3, repIS-P5, repIS-P6 and repIS-P7 shown in Fig. 2 were pathogenic to lilac. Surprisingly, however, there could be heterogeneity within repIS-Ps in relation to their pathogenicity on lilac. For example, one of the five isolates tested belonging to repIS-P1 was non-pathogenic to lilac, although it could not be differentiated genetically from other isolates in the same repIS-P. Many isolates belonging to the little-represented repIS-Ps were non-pathogenic to lilac. The repartition of the TLP+ *Pss* pathogenic to lilac leaves was analysed by the less discriminating BOX-PCR method: 100% of the pathogenic TLP+ *Pss* isolates were grouped in the three main patterns BOX-P1, BOX-P2 and,BOX-P3, as well as in BOX-P30, with aggressiveness ranging from class 2 to class 5 (Table 5; Fig. 5). It was interesting to note that 88.6%, 85.7% and 93.7% of the Belgian TLP+ *Pss* isolates from pear, cherry and plum, respectively, belonged to one of these four BOX patterns. Surprisingly, however, within the three main BOX-Ps isolates belonging to repIS-P11, 32 and 51 induced no necroses on lilac leaves (Fig. 6). Figure 6 showed that two of the three main BOX-Ps (BOX-P1 and BOX-P2) were genetically close to each other, with 15% differentiation (Fig. 6). However, BOX-P3 and BOX-P30 were genetically very different. The *Pss* pathotype strain LMG 1247 induced no necroses on lilac leaves under the test conditions.

With regard to the TLP− *Pss* isolates, three of the 11 tested were pathogenic to lilac leaves (Table 5). These three isolates belonged to repIS-P4, with aggressiveness ranging from class 2 to class 3. None of the 15 *cfl*+ or *cfl*− *Psm* race 1 isolates tested induced necroses on lilac leaves (Table 5). However, among the ten *Psm* race 2 isolates tested belonging to repIS-P87 (Fig. 3), two were pathogenic to lilac leaves (Table 5) and were grouped in the pathogenicity class 2.

Two of the eight unclassified *P. syringae* isolates from pear tested were pathogenic to lilac leaves (Table 5). They were very different genetically from the TLP+ or the TLP− *Pss* isolates, but were highly aggressive (class 4). The tested unclassified *P.*

Fig. 4 Example of symptoms on detached lilac leaves after inoculation with *P. syringae* isolates. **a** non-pathogenic isolate; **b** virulent isolate; **c** highly virulent isolate

Table 5 Pathogenicity to lilac of *P. syringae* and *P. viridiflava* isolates and strains from fruit trees

Group of isolates and strains	Pathogenic isolates and strains related to tested isolates and strains
Pss TLP+ of BOX-P 1, 2, 3 and 30	40/45
Other *Pss* TLP+	0/17
Pss TLP−	3/11
Psm race 1	0/15
Psm race 2	2/10
P. viridiflava	1/2
Unclassified *P. syringae*	2/15

syringae isolates from sour cherry (two isolates) and plum (five isolates) were non-pathogenic to lilac leaves. Of the two *P. viridiflava* isolates tested belonging to repIS-P20, one was pathogenic to lilac leaves and was classified in the pathogenicity class 2 (Table 5).

Discussion

With regard to the pathogen occurrence in Belgium, the detection of *Pss* isolates in pear, sweet cherry, plum and sour cherry orchards in the country agreed with the findings of other studies (Yessad et al. 1992; Burkowicz and Rudolph 1994; Vicente et al. 2004).

In Belgium, the *Psm* race 1 isolates have been found in sweet cherry and sour cherry (Bultreys and Gheysen 2003, 2004; Bultreys et al. 2007). In other countries, *Psm* race 1 has also been reported in plum orchards (Garrett et al. 1966; Burkowicz and Rudolph 1994; Vicente et al. 2004). Most but not all the Belgian *Psm* race 1 isolates possessed the *cfl* gene involved in coronatine production.

Psm race 2 was found only occasionally in Belgium in sweet cherry (Bultreys and Gheysen 2003, 2004; Bultreys et al. 2007). These results contrast with the UK, where many strains were isolated from cv. Roundel and to a lesser extend from cv. Napoleon (Freigoun and Crosse 1975; Vicente et al. 2004). In contrast, 12 Belgian sour cherry isolates of the 21 tested were identified as *Psm* race 2. *Psm* race 2 was described on sweet cherry (*Prunus avium*; Freigoun and Crosse 1975), and it was also detected on wild cherry (*Prunus avium*; Vicente et al. 2004). In this study and previous reports (Bultreys and Gheysen 2004; Bultreys et al. 2007), it appears as a frequent pathogen of sour cherry (*Prunus cerasus*). In another study, *Psm* was reported as the cause of bacterial canker of sour cherry in Michigan, USA, but the study and the identification methods (GATTa tests) were orientated towards *Psm* race 1 only (Latorre and Jones 1979).

No *P. syringae* pv. *avii* isolate was detected in Belgium in this study, but wild cherry plantations were not investigated. However, *P. syringae* isolates other than *Pss*, *Psm* race 1, *Psm* race 2 and *P. syringae* pv. *avii* were found in pear, plum and sour cherry orchards in Belgium. The study of the ability of these unclassified *P. syringae* isolates to induce disease is being undertaken.

Pseudomonas syringae pv. *syringae*

Many isolates were named *Pss* in the present study based on their presence in diseased tissues in pear, cherry and plum orchards, on the possession of the

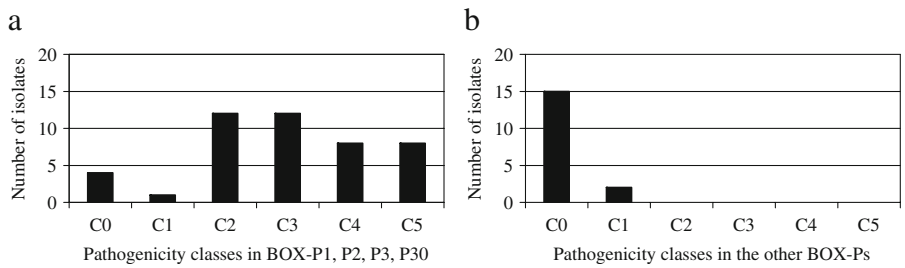


Fig. 5 Class repartition of TLP+ *Pss* isolates according to their aggressiveness to lilac leaves. **a** TLP+ *Pss* isolates belonging to BOX-P1, BOX-P2, BOX-P3 and BOX-P30. **b** TLP+ *Pss* isolates belonging to the other BOX-Ps. *Class 0*: non-pathogenic isolates; *class 1*: necrosis limited to the cut; *class 2*: necrosis from 10 to 20 mm; *class 3*: necrosis from 21 to 30 mm; necrosis from 31 to 40 mm; and *class 5*: necrosis from 41 to 50 mm

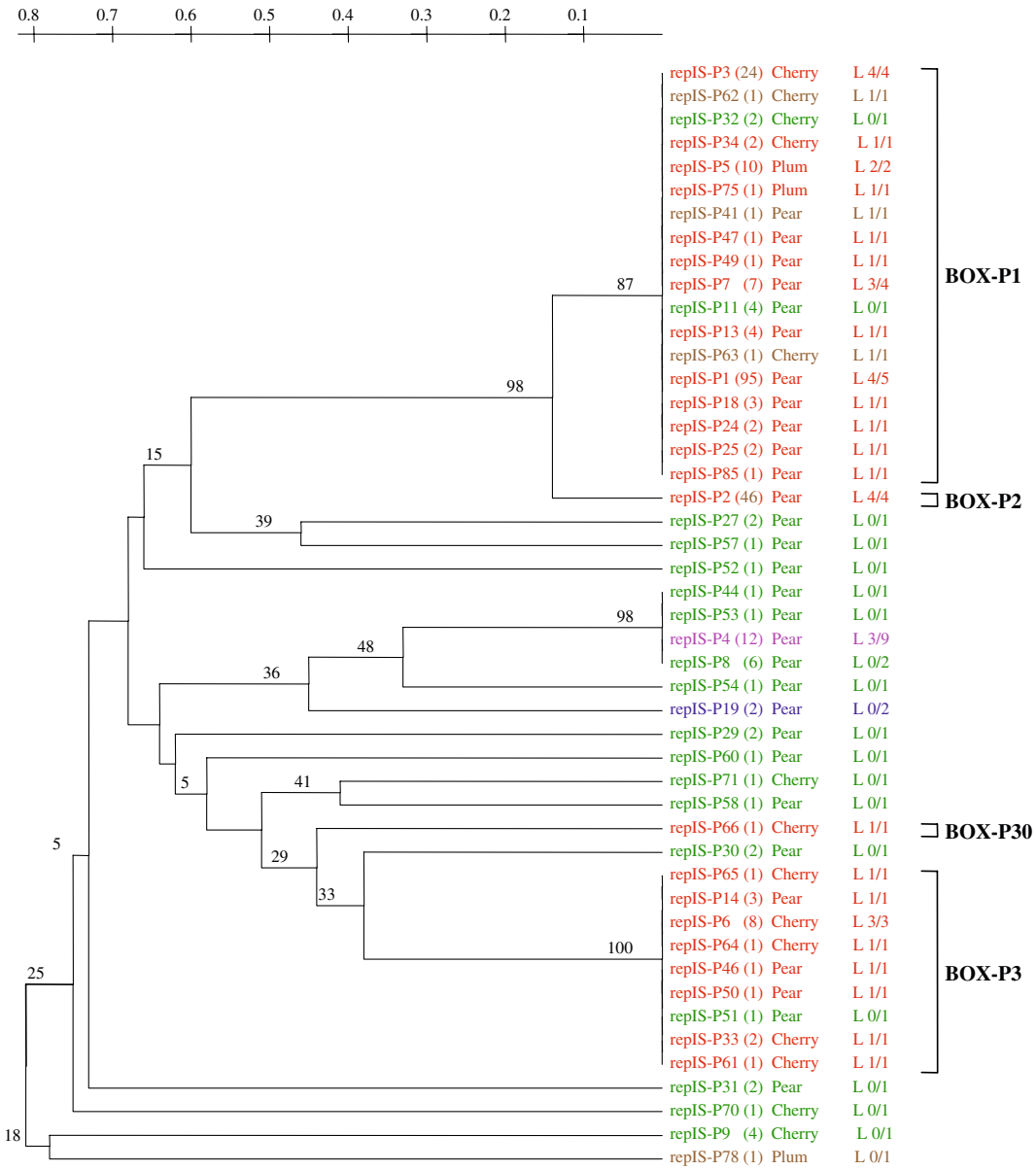


Fig. 6 Dendrogram of the genetic relatedness of the repIS-Ps between TLP+ and TLP− *Pss* isolates and strains. The genetic analysis method used was BOX-PCR. For each line, the following information is given: the repIS-P; the number of isolates and strains in the corresponding pattern (*under brackets*), the host and the observed pathogenicity to lilac leaves (*L*): presented as the number of isolates and strains pathogenic to lilac leaves related to the number of isolates and strains tested in the pattern. The colour codes are: *red* for TLP+ patterns pathogenic to lilac leaves; *green* for TLP+ pattern non-pathogenic; *pink* for TLP− pattern pathogenic to lilac leaves; *blue* for TLP− pattern non-pathogenic; and *brown* for TLP+ *Pss* reference strains. In repIS-P2, and -P3 the number under brackets is in *brown* because the patterns include one reference strain. The *numbers* on the *branches* are the bootstrap values. The *scale* at the *top* indicates the degree of genetic dissimilarity between isolates and strains

atypic pyoverdin Pa A and on the production of TLPs. However, TLPs are also produced by the pvs *aptata* and *atrofaciens* in *P. syringae* (Bultreys and Gheysen 1999). Both pathovars were shown to be pathogenic to fruit trees in laboratory assays (Quigley et al. 1994). However, there should be no confusion with these two pathovars because the TLP+ *P. syringae* isolates isolated from Belgian fruit orchards had

different rep-Ps and IS50-Ps than the tested *P. syringae* pv. *aptata* and *P. syringae* pv. *atrofaciens* control strains. In addition, the respective strains of pvs *aptata* and *atrofaciens* grouped together in clearly distinct genetic clusters. Whereas the detection of *Pss* by TLP-related tests proved to be very powerful, 5% of the isolates finally classified in *Pss* in this study did not produce TLP. These isolates were identified using the GATTa and lactic acid use tests. It has already been shown by Vicente and Roberts (2007) that some *Psm* race 2 strains could give the same GATTa tests results (+ + − −) as *Pss* strains. In addition, the results from the GATTa tests for the other pathovars of *P. syringae* (>50 known pathovars) are largely unknown and therefore it is possible that other *P. syringae* strains could give the same GATTa results as *Pss* strains. All the Belgian TLP− *Pss* isolates were isolated from pear. Most clustered together in a BOX-P comprising poorly represented TLP+ isolates. Interestingly, some TLP− isolates in this BOX-P were pathogenic to lilac, but not the TLP+ isolates. Scortichini et al. (2003) have already shown that some TLP+ and TLP− *Pss* strains could share the same BOX-P. In the present study, however, the combined repIS-Ps differentiated all the TLP+ *Pss* isolates from the TLP− *Pss* isolates. In addition, the two repIS-Ps containing the TLP− *Pss* isolates were genetically distant from the main repIS-Ps formed by the TLP+ *Pss* isolates.

The analysis of the repIS-Ps indicated a high genetic heterogeneity among the TLP+ *Pss* isolates from cherry, pear and plum in Belgium. Such heterogeneities had also been observed in other countries (Little et al. 1998; Weingart and Völksch 1997). This analysis, however, showed that specific repIS-Ps contained numerous genetically similar isolates from the same host (repIS-P1 and repIS-P2 from pear; repIS-P3 and repIS-P6 from cherry; repIS-P5 from plum). The pathogenicity of the isolates belonging to these repIS-Ps is clear since 17 out of the 18 strains tested were pathogenic to lilac. A global pathogenicity study on multiple hosts is in process and these isolates are also pathogenic to their respective isolation hosts (Gilbert and Bultreys, unpublished results). Also, the isolates belonging to repIS-P3 and repIS-P2 were genetically identical or very similar to reference strains from France (CFBP 2117 and CFBP 2118) and UK (B301D; Table 4) received in 1997 from J. P. Prunier and L. Gardan and from D. C. Gross as highly virulent strains on cherry and pear, respectively. In contrast, other repIS-Ps contained only one isolate. The existence of both isolates belonging to dominant repIS-Ps and to less frequent repIS-Ps on a same host suggests a better ecological adaptation of the isolates belonging to the dominant repIS-Ps to the host, or to diseased tissues of the host (the isolation places), or both.

The existence of phytopathogenic isolates belonging to different dominant repIS-Ps on different hosts (pear, cherry and plum) is very interesting. One possible explanation could be that these clonal lineages reflect the effective dissemination of the pathogen with the planting material, or the occurrence of an effective dissemination of bacteria among trees of the same species because of pruning tools, for instance. However, the Belgian isolates isolated in 1999 in Table 2 were all isolated from the orchards of the Walloon Agricultural Research Centre in Gembloux and the pear orchard is directly near the cherry variety collection. Close proximity between cherry and pear orchards was also observed in other locations in Wallonia. It is difficult to imagine that exchange of strains would never have occurred under these conditions. Also, we tested in this study two reference *Pss* strains isolated in 1959 from pear in the UK and both were similar or identical to repIS-P2 containing 45 Belgian isolates from pear isolated in 2001 and 2002 in various pear orchards (Tables 2 and 4). The UK strain belonging to repIS-P2 is the well known phytopathogenic strain B301D, which has been the most studied *Pss* strain regarding TLP production (Bender et al. 1999). Moreover, we also tested in this study two phytopathogenic reference *Pss* strains isolated in 1979 from cherry in France and both were similar or identical to repIS-P3 containing 23 Belgian isolates from cherry (Tables 2 and 4). This is intriguing because it is very difficult to imagine that no exchange of strains would have occurred over such long periods. All this suggests that clonal populations of *Pss* could be specifically adapted to certain cultures (pear, cherry, and plum) in Belgium. Such specialisation was suggested by Little et al. (1998) with regard to strains isolated from *Prunus* in California, compared with strains from other hosts. Nevertheless, in this study a distinction could be made between Belgian *Pss* isolates isolated from pear, cherry and plum.

Interestingly, several clonal populations were detected on a same host, indicating, as discussed by Sarkar et al. (2006), that there could be different ways by which *P. syringae* can adapt to the same host.

Interestingly, the TLP+ *Pss* strains from South Africa and Switzerland were genetically different. This could suggest a distinct evolution of *Pss* in Belgium, France and the UK compared with other regions of the world. Little et al. (1998) also suggested a distinct evolution between North American and western European *Pss* strains.

The pathogenicity tests on lilac leaves gave interesting results. Although most TLP+ *Pss* isolates from the main repIS-Ps induced progressive necrotic lesions on lilac, some isolates from these patterns were non-pathogenic to lilac. The study therefore indicated that isolates with identical genetic profiles using rep- and IS50-PCR can differ in their pathogenicity to lilac. Scheck et al. (1997) had already shown that TLP+ *Pss* could be non-pathogenic to lilac. In addition, some English *Pss* strains from the same rep-PCR-based genetic groups varied in their pathogenicity to lilac (Vicente and Roberts 2007). These data suggest that negative responses in a lilac pathogenicity test should be interpreted with caution. More surprisingly, two *Psm* race 2 isolates, two Belgian unclassified *P. syringae* isolates and one *P. viridiflava* isolate were pathogenic to lilac leaves in this study. These isolates were TLP-, belonged to repIS-Ps clearly distinct from the *Pss* patterns, and could not be identified as *Pss* by physiological tests. It appears, therefore, that a positive result in a lilac pathogenicity assay should also be viewed with caution. Scortichini et al. (2003) also showed that lilac leaf and petiole inoculation assays were important, but not decisive, in confirming *Pss* identification.

In this study, four BOX patterns contained 100% of the TLP+ *Pss* isolates pathogenic to lilac and 88.4% of the Belgian TLP+ *Pss* isolates belonged to these BOX-Ps. Two of these patterns were close to each other, but the two others were genetically very different. Besides, 11.6% of the Belgian TLP+ *P. syringae* isolates from fruit orchards were non-pathogenic to lilac, were not found frequently in orchards and, as in the case of reference *Pss* strains from other hosts, were dispersed in BOX-Ps that were clearly different from the four BOX-Ps pathogenic to lilac. As in the case of TLP− *Pss* isolates, it is questionable whether they should be named *Pss*. However, the exclusion of these isolates from *Pss* should be done with caution. *Pss* is known to be genetically heterogeneous (Weingart and Völksch 1997; Little et al. 1998), to attack many different hosts (Young 1991) and to be dispersed in various ways (Morris et al. 2007), so it is possible that *Pss* strains not specifically adapted to attack pear, cherry or plum, but possibly pathogenic on other hosts could occasionally be encountered as epiphyte strains in Belgian orchards due to aerial, dust or rain propagation. In addition, it cannot be assumed that the isolates non-pathogenic to lilac will not be pathogenic to the host from which they have been isolated. In a currently global pathogenicity study currently underway we verified that 88.2% of the TLP+ isolates that were non-pathogenic in our lilac pathogenicity test were pathogenic in at least one other pathogenicity test on another host, and that 96.4% of the tested Belgian *Pss* isolates producing TLP were pathogenic in at least one test (Gilbert and Bultreys, unpublished results).

Young et al. (1992) considered, in a review on taxonomy of plant pathogenic bacteria, that the production of syringomycin by *Pss* formed part of the circumscription of the pv. *syringae* and that this character as well as other determinative tests can be used in identification. They considered that avirulent strains could even be placed in a pathovar if an appropriate identification test had been used. Indeed, pathogenicity tests should not be seen as the only method of allocating stains to a pathovar, but as the basis of circumscription of a pathovar (Young et al. 1992). For all these reasons, we believe that it is presently more appropriate to keep the *P. syringae* isolates non-pathogenic to lilac but producing TLP or giving the appropriate GATTa responses in the pv. *syringae*, rather than to name these isolates *P. syringae*. It is, however, clear that the pv. *syringae* is heterogeneous in different characteristics and that additional classification work would be useful. Taken together, the results of this study show that the use of different methods to identify *Pss* can lead to different conclusions and that it is still not clear what strains should be included in this pathovar.

Pseudomonas syringae pv. *morsprunorum*

REP, BOX, ERIC and IS50 analyses generated distinct profiles for Belgian isolates of the two races of *Psm,* confirming earlier observations in the UK and France (Ménard et al. 2003; Vicente and Roberts 2007). The BOX analysis was a good method for identifying *Psm* race 1 and race 2 because clearly related profiles were obtained for all members of each

race. In addition, no Belgian *Psm* race 2 isolate was a coronatine producer, whereas the *Psm* race 1 isolates were mainly coronatine producers. Also, all the *Psm* race 2 isolates possessed the yersiniabactin gene *irp1*, but not the *Psm* race 1 isolates (Bultreys et al. 2006; Bultreys and Gheysen, unpublished results). Vicente et al. (2004) reported differences in biochemical, physiological and serological tests between *Psm* race 1 and race 2. *Psm* race 1 and race 2 belong to two distinct genomospecies when analysed by DNA–DNA hybridisation (Ménard et al. 2003). Freigoun and Crosse (1975) showed that the two races represented two distinct physiological and pathological groups. In Belgium, *Psm* race 2 strains were isolated mainly from sour cherry (*P. cerasus*) and *Psm* race 1 strains mainly from sweet cherry (*P. avium*; Bultreys and Gheysen 2004). In the UK both races were reported on sweet cherry and wild cherry (Freigoun and Crosse 1975; Vicente et al. 2004), but there is no information about the occurrence of *Psm* race 2 on sour cherry. All these elements suggest that these two races are clearly distinct pathogens that attack the same hosts, maybe at different frequencies, and keeping them in a same pathovar probably needs to be further evaluated.

Interestingly, it was noticed in this study that the pathotype strain *Psm* LMG 5075 (t2) had the same genetic profile as *Psm* race 2 strains and is therefore a *Psm* race 2 rather than a *Psm* race 1 strain. This was also noticed with the equivalent strain CFBP 2351, which had previously been shown to belong to the genomospecies 3, as *Psm* race 2 strains, rather than 2, as the *Psm* race 1 strains (Gardan et al. 1999; Ménard et al. 2003). According to the LMG culture collection information, this strain was isolated by H. Wormald from plum and is probably a representative from his original study in UK on *P. morsprunorum* (Wormald 1932). In his work, Wormald (1932) noticed strains that were gelatine hydrolysis-positive and others that were negative. This was noted by Freigoun and Crosse (1975) as a distinctive character between the two races of *Psm* in the UK. The disease symptoms described by Wormald are clearly related to the symptoms generally attributed to race 1 of the pathogen (Garrett et al. 1966). One explanation could be that Wormald (1932) encountered both types of strains, as discussed by Freigoun and Crosse (1975), but that a *Psm* race 2 strain was finally chosen as the pathotype strain of *P. morsprunorum*, although it was not the best representative of the most damaging strains causing the disease he described.

In conclusion, the present study shows the high diversity existing among *P. syringae* isolates from Walloon fruit orchards, and that combining rep and IS50 analyses is a good way of assessing this diversity. It shows isolate–host relationships that could possibly reflect specialisation of *Pss* isolates on their host, and indicates that, beside *Pss* and both races of *Psm*, unclassified *P. syringae* strains are present in Belgian pear, sour cherry and plum orchards. BOX-PCR proved useful for identifying *Psm* race 1 and race 2 and indicated that four BOX patterns contained 100% of the TLP + *Pss* isolates pathogenic on lilac. The study also indicates that some *Pss* isolates with common genetic profiles can vary in pathogenicity on lilac and that isolates that are clearly not *Pss* isolates can be pathogenic on lilac leaves.

Acknowledgments We thank M. Malice, C. Decock and M. Amalfi for help in the interpretation of the dendrograms. The work was supported by the Ministry of the Walloon Region (A.B.) and by grants 2631 (A.B., F.L.), 2654, S 6081, D31-1080, and D31-1124 (A.B., V.G.) from the Ministry of the Walloon Region, Head Office of Agriculture.

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