Eur J Plant Pathol (2010) 126:263–277
DOI 10.1007/s10658-009-9538-8

Pathogenicity and aggressiveness in populations of *Pseudomonas syringae* from Belgian fruit orchards

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Received: 17 March 2009 / Accepted: 28 September 2009 / Published online: 21 October 2009

Abstract The pathogenicity of 99 Belgian *Pseudomonas syringae* strains representative of the genetic diversity encountered in Belgian fruit orchards was evaluated by using 17 pathogenicity tests conducted on pear, cherry, plum, lilac, sugar beet and wheat. The *P. syringae* pv. *morsprunorum* strains were pathogenic to stone fruit species but the race 1 strains possessing the *cfl* gene involved in coronatine production were pathogenic in more tests than those lacking the gene. Also, sweet cherry twigs were a better material to detect pathogenic strains of race 1 and sour cherry twigs of race 2, which accorded with race 2 presence in sour cherry orchards in Belgium. Three groups were defined in the pv. *syringae* based on pathogenicity. One group pathogenic in 71.1% of the tests and to lilac included toxic lipodesipeptide-producing (TLP+) strains. The second group pathogenic in 26.8% of the tests and non-pathogenic to lilac included TLP+ strains. The thirth group pathogenic in 9.1% of the tests and almost specifically pathogenic to pear included TLP− strains. The three groups were genetically heterogeneous. Although strain-host relationships were noted within the pv. *syringae*, *aptata* and *atrofaciens* when considering the strain origins, such relationships were not found in the pathogenicity tests, suggesting that pathogenicity tests could probably not reproduce all the aspects of the host-pathogen interactions. None of the pathogenicity tests was able to provide all the information provided by the complete study. A test on pear buds indicated that strains different from the pv. *syringae* were pathogenic to pear.

Keywords *Pseudomonas syringae* · Diversity · Pathogenicity · Pear · Cherry · Plum

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Introduction

Pseudomonas syringae is a heterogeneous phytopathogenic bacterial species that includes approximately 50 pathovars (Young 1991). In Belgium, it is a common pathogen of fruit orchards: *P. syringae* pv. *syringae* (*Pss*) and unclassified *P. syringae* were reported on pear; *Pss*, *P. syringae* pv. *morsprunorum* (*Psm*) and unclassified *P. syringae* were reported on stone fruit (Bultreys and Gheysen 2004; Gilbert et al.

2009). On wild cherry, also *P. syringae* pv. *avii* has been reported in France (Menard et al. 2003).

Pss is a genetically heterogeneous pathovar having a very broad range of plant hosts (Young 1991; Weingart and Völksch 1997; Little et al. 1998). The strains from this pathovar can show different virulence on lilac, wild cherry and pear (Scortichini et al. 2003; Vicente et al. 2004; Gilbert et al. 2009). A population of 356 Belgian isolates of *P. syringae* from fruit orchards was genetically characterised and many isolates were classified in known pathovars including *Pss* (Gilbert et al. 2009). Most of the Belgian *Pss* isolates produce the toxic lipodepsipeptides (TLP) syringomycins and syringopeptins, which are phytotoxins and virulence components that may be used in identification (Young et al. 1992; Sorensen et al. 1998; Bender et al. 1999; Bultreys and Gheysen 1999). Relationships were noted among *Pss* strains between a genetic classification based on BOX-PCR, the ability to produce TLP and pathogenicity to lilac (Gilbert et al. 2009). Four out of 17 BOX patterns (BOX-Ps) included 100% of the TLP-producing (TLP+) isolates pathogenic to lilac leaves, whereas the TLP+ isolates classified in the other BOX-Ps were always non-pathogenic to lilac leaves. The TLP non-producing (TLP−) *Pss* isolates, identified by physiological tests, could be similar by BOX-PCR to some poorly represented TLP+ isolates non-pathogenic to lilac leaves, but they showed combined genetic profiles obtained by BOX-PCR, ERIC-PCR, REP-PCR and IS50-PCR always different from those of the TLP+ *Pss* isolates. The TLP− *Pss* were only occasionally pathogenic to lilac leaves. However, the pathogenicity of all these isolates to their isolation hosts was not reported. Certain populations of TLP+ *Pss* were consistently found on the same host (pear, cherry or plum), and these isolate-host relationships did suggest the possible existence of specialised relations between *Pss* populations and their host (Gilbert et al. 2009).

The classification of the TLP+ *P. syringae* isolates is complicated because the pvs *syringae*, *aptata* (pathogenic to sugar beet) and *atrofaciens* (pathogenic to wheat glumes) share this characteristic and can be pathogenic to fruit trees in pathogenicity tests (Quigley et al. 1994). The distinction between these TLP+ pathovars is a matter of debate (Otta and English 1971; Wilkie 1973; Maraite and Weyns 1997; Gilbert et al. 2009). In France, the *P. syringae* strains pathogenic to melon cantaloupe were included in the pv. *aptata*, rather than *syringae*, because they were pathogenic to sugar beet (Morris et al. 2000).

Psm is more homogeneous than *Pss* but heterogeneity is known within this pathovar since two genetically different races (Ménard et al. 2003; Vicente et al. 2004; Vicente and Roberts 2007) were described based on physiological and pathological characteristics (Freigoun and Crosse 1975). However, *Psm* race 1 and *Psm* race 2 may not cause the same diseases in stone fruit trees. Indeed, Freigoun and Crosse (1975) showed that *Psm* race 2 was less infective through leaf scars than *Psm* race 1, but more invasive through bark parenchyma of sweet cherry 'Roundel'. Also, the necroses induced by *Psm* race 2 were generally weak on wild cherry plantlets, whereas *Psm* race 1 generally induced progressive necroses (Vicente et al. 2004). In Belgian orchards, *Psm* race 1 and *Psm* race 2 were more frequently isolated from sweet cherry and sour cherry, respectively (Bultreys and Gheysen 2004; Bultreys et al. 2007; Gilbert et al. 2009).

The lack of chemicals to fight bacterial diseases in orchards creates a need for alternative methods of disease control, including approaches based on prevention, biological control and plant resistance. These approaches require accurate knowledge on the pathogenicity of the different *P. syringae* strains encountered in fruit orchards. The objectives of this study were to evaluate the pathogenicity and aggressiveness on pear, sweet cherry, sour cherry, plum, lilac, sugar beet and wheat of 99 previously characterised strains belonging to genetically diverse populations of *P. syringae* from Belgian fruit orchards.

Materials and methods

Bacterial strains

The characteristics of the strains used in this study are listed in Table 1. Ninety-nine Belgian strains were chosen in numerically important BOX-Ps and rep-IS-patterns (repIS-P, based on combined BOX-, REP-, ERIC- and IS50-PCR analyses), or were the unique member of specific repIS-Ps defined in a previous study (Gilbert et al. 2009); 21 reference strains from international culture collections were also included in the study. The selected strains were 60 TLP+ and 11

Table 1 Characteristics of Belgian and reference strains used in this study

Identification	BOX pattern[a]	repIS pattern[b]	Observed repIS-P specificity[c]	Name of reference strain or number of Belgian strains[d]	Country	Pathogenicity to lilac[c]	Pathogenicity[e]
P. syringae pv. *morsprunorum* race 1 *cfl*+	17 (52)	86 (43)	*Prunus* sp.	CFBP 3801	UK	−	+
			Sweet cherry	LMG 2222	UK	−	+
				LMG 5468	UK	−	+
				7	Belgium	−	+
P. syringae pv. *morsprunorum* race 1 *cfl*−	17 (52)	90 (1)	Plum	LMG 5461	Switzerland	−	+
		91 (1)	Plum	LMG 5698	France	−	+
		94 (2)	Sweet cherry	2	Belgium	−	+
P. syringae pv. *morsprunorum* race 2	4 (16)	87 (14)	Plum	LMG 5075^T	Unknown	−	+
			Sour cherry	9	Belgium	2 strains +	8 strains +
						7 strains −	1 strain −
P. syringae pv. *syringae* TLP+	1 (169)	1 (95)	Pear	5	Belgium	4 strains + 1 strain −	+
		3 (24)	Sweet cherry	3	Belgium	+	+
			Sour cherry	1	Belgium	+	+
		5 (10)	Plum	2	Belgium	+	+
		7 (7)	Pear	2	Belgium	1 strain + 1 strain −	+
		11 (4)	Pear	1	Belgium	−	+
		13 (4)	Pear	1	Belgium	+	+
		18 (3)	Pear	1	Belgium	+	+
		24 (2)	Pear	1	Belgium	+	+
		25 (2)	Pear	1	Belgium	+	+
		32 (2)	Sour cherry	1	Belgium	−	+
		34 (2)	Sour cherry	1	Belgium	+	+
		41 (1)	Pear	LMG 5141	UK	+	+
		47 (1)	Pear	1	Belgium	+	+
		49 (1)	Pear	1	Belgium	+	+
		62 (1)	Sweet cherry	LMG 5494	Hungary	+	+
		63 (1)	Sweet cherry	CFBP 2118	France	+	+
		75 (1)	Plum	1	Belgium	+	+
		85 (1)	Pear	1	Belgium	+	+
	2 (46)	2 (46)	Pear	B301D	UK	+	+
				3	Belgium	+	+
	3 (20)	6 (8)	Cherry	3	Belgium	+	+
		14 (3)	Pear	1	Belgium	+	+
		33 (2)	Sweet cherry	1	Belgium	+	+
		46 (1)	Pear	1	Belgium	+	+
		50 (1)	Pear	1	Belgium	+	+
		61 (1)	Sweet cherry	1	Belgium	+	+
		64 (1)	Sour cherry	1	Belgium	+	+
		65 (1)	Sour cherry	1	Belgium	+	+
		74 (1)	Plum	1	Belgium	−	+
	5 (4)	9 (4)	Sweet cherry	1	Belgium	−	+
	6 (20)	8 (6)	Pear	2	Belgium	−	+
		44 (1)	Pear	1	Belgium	−	+
		53 (1)	Pear	1	Belgium	−	+
	14 (1)	52 (1)	Pear	1	Belgium	−	+
	15 (1)	54 (1)	Pear	1	Belgium	−	+

Table 1 (continued)

Identification	BOX pattern[a]	repIS pattern[b]	Observed repIS-P specificity[c]	Name of reference strain or number of Belgian strains[d]	Country	Pathogenicity to lilac[c]	Pathogenicity[e]
	18 (1)	57 (1)	Pear	1	Belgium	−	+
	19 (1)	58 (1)	Pear	1	Belgium	−	+
	22 (1)	60 (1)	Pear	1	Belgium	−	+
	24 (2)	27 (2)	Pear	1	Belgium	−	+
	26 (2)	29 (2)	Pear	1	Belgium	−	+
	27 (2)	30 (2)	Pear	1	Belgium	−	+
	28 (2)	31 (2)	Pear	1	Belgium	−	+
	30 (1)	66 (1)	Sweet cherry	1	Belgium	+	+
	34 (1)	70 (1)	Sour cherry	1	Belgium	−	+
	35 (1)	71 (1)	Sweet cherry	1	Belgium	−	+
	39 (1)	78 (1)	Plum	LMG 6104	South Africa	−	+
P. syringae pv. *syringae* TLP−	6 (20)	4 (12)	Pear	9	Belgium	3 strains + 6 strains −	7 strains + 2 strains −
	10 (2)	19 (2)	Pear	2	Belgium	−	1 strain + 1 strain −
Unclassified *P. syringae*	4 (16)	88 (2)	Sour cherry	1	Belgium	+	+
	9 (2)	23 (2)	Pear	2	Belgium	−	1 strain + 1 strain −
	12 (1)	43 (1)	Pear	1	Belgium	+	+
	23 (2)	26 (2)	Pear	2	Belgium	−	+
	25 (2)	28 (2)	Pear	2	Belgium	1 strain + 1 strain −	+
	40 (3)	17 (3)	Plum	1	Belgium	−	−
	41 (4)	10 (4)	Plum	2	Belgium	−	−
	42 (1)	80 (1)	Plum	1	Belgium	−	+
	43 (1)	81 (1)	Plum	1	Belgium	−	+
	89 (1)	56 (1)	Pear	1	Belgium	+	+
	90 (1)	45 (1)	Pear	1	Belgium	−	+
P. syringae pv. *aptata* TLP+	60 (2)	21 (2)	Sugar beet	LMG 5646	New Zealand	NT	+
				UPB 133	Holland	NT	+
	61 (3)	22 (3)	Sugar beet	UPB 165	France	NT	+
				UPB 221	Uruguay	NT	+
				UPB 110	Belgium	NT	+
	62 (4)	12 (4)	Sugar beet	UPB 225	Germany	NT	+
				UPB 339	Sweden	NT	+
				LMG 5059^T	United States	NT	+
P. syringae pv. *atrofaciens* TLP+	57 (1)	35 (1)	Wheat	LMG 5095^T	New Zealand	NT	+
	58 (1)	36 (1)	Wheat	LMG 5000	Unknown	NT	−

[a] Patterns obtained by BOX-PCR; under brackets are the number of strains classified in each pattern in a previous study (Gilbert et al. 2009)

[b] Patterns obtained by combining REP-, ERIC-, BOX- and IS50-PCR; under brackets are the number of strains classified in each pattern in a previous study (Gilbert et al. 2009)

[c] Data from Gilbert et al. (2009); repIS-P, repIS pattern

[d] LMG, Laboratorium voor Microbiologie van Ghent, Ghent, Belgium; CFBP, Collection Française des Bactéries Phytopathogènes, Angers, France; UPB, Bacterial collection of the Unit of Phytopathology, UCL, Louvain-la-Neuve, Belgium

[e] Data from this study; +, strain classified in at least one pathogenicity test in a class > 1; −, strain always classified in the classes 0 or 1 in every pathogenicity test investigated; see Table 3 for detail about pathogenicity tests

TLP− *Pss*, 10 coronatine-producing (*cfl*+) *Psm* race 1, 4 coronatine non-producing (*cfl*−) *Psm* race 1, 10 *Psm* race 2, 15 unclassified *P. syringae*, 8 *P. syringae* pv. *aptata* and 2 *P. syringae* pv. *atrofaciens.*

Pathogenicity tests

Pear 'Conference', sweet cherry 'Lapins', sour cherry 'Montmorency', plum 'Reine Claude', sugar beet 'Festival' and wheat 'Elegans' were used in pathogenicity tests. In these tests, incubation conditions were chosen to resemble natural conditions encountered in Belgium during diseases. The strains were classified in 4 to 6 classes. Class 0 and 1 strains were not considered pathogenic in this study. For the pathogenic strains (classes > 1), the higher the class number, the higher the strain virulence. Bacteria were generally grown overnight at 28°C on nutrient agar medium and suspended at specific concentrations in sterile water before plant infections. Except when otherwise specified, all the strains listed in Table 1 were tested, and control plants or organs were inoculated with sterile water. For each pathogenicity test, one repetition of the complete test was performed with strains classified in each class.

Leaf and hypocotyl pathogenicity tests

The test on leaves of pear, sweet cherry, sour cherry and plum was performed on detached leaves from one year-old budding branches using the modified Yessad et al. (1992) method described for lilac leaves (Gilbert et al. 2009). Briefly, five fully expanded leaves were wounded (a 5-mm wound made with a scalpel) on the petiole at approximately 5 mm from the lamina. A 10 µl drop of bacterial suspension (10^8 cfu ml^{-1}) or water was deposited on the wound. The treated leaves were incubated in Petri dishes at 20°C for 7 days under daylight conditions. The lengths of the necroses, including the 5-mm wound, were measured. Five repetitions per strain and test were used.

To further assess the differences in virulence among TLP+ *P. syringae* pathovars, 58 TLP+ *Pss* strains and the strains of the pvs *atrofaciens* and *aptata* were evaluated for their virulence on wheat leaves and sugar beet hypocotyls. Two 8- to 10-cm long young wheat leaves were wounded three times each with a sterile needle, 5 µl of bacterial suspension (10^8 cfu ml^{-1}) were deposited on the wounds and the seedlings were incubated at 20°C for 7 days under daylight. The necroses were evaluated and repartited in 5 classes. For sugar beet, 5 3-cm long hypocotyls were inoculated with a needle prealiminary dipped in a 10^8 cfu ml^{-1} bacterial suspension. The seedlings were incubated at 20°C for 7 days under daylight. Four symptom severity classes were described.

Twig pathogenicity tests

Two kinds of tests were carried out in cherry. The inoculation of cut ends of twigs (twig E test; Sobiczewski and Jones 1992) is well adapted for *Pss* when a frost period is followed by an incubation at 15°C; on the other hand, the lateral inoculation of an exposed 'window' of cortical tissues (twig W test) followed by incubation at 15°C is more adapted for *Psm* race 1 (Bultreys and Gheysen 1999; A. Bultreys, unpublished results). These conditions are encountered in Belgium in autumn and winter, the period favourable for damage to cortical tissues. Both tests were performed on one year-old dormant twigs collected during the November-March period. The twig E test was carried out on sweet cherry and pear with the strains listed in Table 1, except those of the pvs *morsprunorum* race 1, *apata* and *atrofaciens*, and on sour cherry with 34 TLP+ *Pss*, 6 *Psm* race 2 and 10 unclassified *P. syringae*. The twig W test was carried out on sweet and sour cherry with the strains listed in Table 1, except those of the pvs *syringae*, *apata* and *atrofaciens*. Five repetitions per strain and test were used.

The twig E test protocol was that of Sobiczewski and Jones (1992) with some modifications: the collected twigs were 20 cm long and the inoculated twigs were incubated in the dark at 15°C for 7 days, −10°C for 3 days and 15°C for 10 days. The lengths of the necrosed zones starting from the cut end were measured.

For the twig W test (Bultreys and Gheysen 1999; Bultreys et al. 2008), 17 cm-long twigs were prepared as described above. A drop of 20 µl of bacterial suspension containing 10^8 cfu ml^{-1} was deposited on an exposed window of cortical tissue (5 mm wide and 20 mm long) resulting from the removing of the superficial bark. The drop was spread in the zone by using the lateral part of the inoculation tip, without direct contact between the tip and the exposed tissue.

The entire twigs were transferred to glass tubes as described above and incubated at 15°C for 30 days in the dark. Test reading consisted in visual estimation of the necrosed zone in the inoculated window.

Fruit pathogenicity tests

The strains listed in Table 1, except those of pvs *atrofaciens* and *aptata*, were tested on pear, sour cherry and sweet cherry fruits, but only two *cfl*+ and two *cfl*− *Psm* race 1 strains were tested on sour cherry. The fruits were collected 6 weeks after flowering, disinfected with 1% sodium hypochlorite and rinsed twice with sterile osmosed water. The pear and sweet cherry fruits were wounded with the ellipsoidal edge of a sterilised needle, inoculated on the wounds with 20 µl of bacterial suspension (10^8 cfu ml^{-1}) and incubated for 7 days at 24°C; in each test, two pears were wounded four times or five sweet cherries were wounded once. The sour cherry fruit test included no wounding: five fruits were soaked for 1 min in a bacterial suspension (10^8 cfu ml^{-1}) and incubated for 5 days at 25°C. The incubation conditions were adequate for symptom developments on fruits. In each test, the fruits were suspended under daylight conditions on sterile trays in closed sterile plastic boxes containing 20 ml of water agar (10 mg of agar l^{-1}). The necrotic lesions were measured. In the case of wounded fruits, the narrowest sides of the necroses resulting from the needle wounds or the diameter of the necroses resulting from strain pathogenicity were measured. For unwounded fruits, as multiple and variable necroses were observed, the diameters of the necroses were measured individually, summed and allocated into six classes.

Bud pathogenicity test

The *P. syringae* strains from pear were tested for their virulence on pear buds. Twenty cm-long twigs were collected during the November–March period and washed (see twig tests). Five buds per shoot were cut at 1 mm from the apical part and 5 µl of bacterial suspension (1.5×10^6 cfu ml^{-1}) were deposited on the cut. The twigs were transferred to glass tubes (see twig tests) and incubated in the dark at 10°C for 7 days, −10°C for 1 day, and 10°C for 6 days. These conditions reproduce temperatures encountered in winter in Belgium during bud dormancy. Disease progression from the cut was measured.

Flower pathogenicity test

The *P. syringae* strains from pear were tested for their virulence on pear flowers. The flowers were collected closed in early spring and suspended in plastic boxes (see fruit tests). Ten µl of bacterial suspension (1.5×10^6 cfu ml^{-1}) were deposited on the flower calyx. The flowers were incubated at 15°C for 7 days under daylight, conditions encountered in spring in Belgium. Test reading consisted of visual estimation of necrose progressions.

Data analysis

A hierarchical clustering method has been applied to analyse the pathogenicity test results, either to compare the strain behaviour or to compare the pathogencity tests among themselves. The single linkage method was applied on Euclidean distances used as similarity measures (Minitab software, version 14).

The results of the pathogenicity tests on pear and sweet cherry fruits, leaves and CE, on sour cherry leaves and fruits, and on plum leaves, as well as the results on lilac leaves from a previous study (Gilbert et al. 2009), were globally analysed by a multivariate analysis to evaluate the behaviour of 60 TLP+ and 11 TLP− *Pss* strains. This analysis was carried out to allow the grouping together of strains that behaved similarly in pathogenicity tests, for example possibly because of host-specificity.

Another comparison using this clustering method concerned the pathogenicity test results on leaves, fruits and twigs E of pear, sweet cherry, and sour cherry, as well as on plum and lilac leaves. The objective was to compare the pathogenicity test information and detect tests that gave additional information about strain pathogenicity and virulence. This analysis concerned 34 TLP+ *Pss* strains isolated from pear, sweet cherry and plum.

Also, as tests on buds and flowers were only performed on pear, a second comparison by the same clustering method concerned the results of all the pathogenicity tests on pear and lilac. This analysis concerned the *Pss* strains from pear: 36 TLP+ and 11 TLP− strains.

Results

Pathogenicity of Belgian strains

Table 2 and Figs. 1 and 2 present classes that were defined in pathogenicity tests according to the symptom intensities observed on the water- and *P. syringae*-inoculated plants. The classes 0 always included the values of the mean necroses observed in the water-treated control tests, but individual measures in these water-treated control tests were in class 1. The classes 0 and 1 strains were not considered pathogenic in this study because they induced no or weak symptoms not very different from those observed with the water-treated controls. In the test repetitions, class 1 strains in one test could be noted class 0 in another test. The pathogenic strains from the other classes (classes > 1) were able to induce progressive necroses. The symptom severity could vary from test to test, but the range of variation was restricted to one class: for example, some strains were classified once in class 2 and then in class 3. In case of variation, the strains were finally classified according to the mean of the length of necroses in both tests.

Table 1 shows the global pathogenicity results. Only nine strains, including eight Belgian strains, were non-pathogenic in all the tests investigated. The other strains were pathogenic (class > 1) in at least one pathogenicity test. The percentages of pathogenic strains among Belgian strains were 100% within *Psm* race 1 (nine strains tested), 88.9% within *Psm* race 2 (nine strains tested), 100% within TLP+ *Pss* (55 strains tested), 72.7% within TLP− *Pss* (11 strains tested) and 73.3% within unclassified *P. syringae* (15 strains tested). In several cases, Belgian strains genetically identical by the genetic methods considered varied in pathogenicity behaviour. This occurred within *Psm* race 2 strains, TLP− *Pss* strains, and unclassified *P. syringae* strains (Table 1). In one case,

Table 2 Description of classes in the leaf, twig E, fruit and bud pathogenicity tests

Organ tested	Pathogenicity[a]	Class	Necrose length intervals (mm) corresponding to classes[b]			
			Pear	Sweet cherry	Sour cherry	Plum
Leaf	Non-pathogenic	0	5–6	5.6	5–6	5–6
		1	6.1–10.9	6.1–10.9	6.1–10.9	6.1–10.9
	Pathogenic	2	11–20.9	11–20.9	11–20.9	11–15.9
		3	21–30.9	21–30.9	21–30.9	16–21
		4	31–41	31–41	31–41	>21 (23)
		5	>41 (50)	>41 (63)	>41 (47)	
Twig E	Non-pathogenic	0	0–5.9	0–5.9	0–5.9	
		1	6–9.9	6–10.9	6–10.9	
	Pathogenic	2	10–14.9	11–20.9	11–15.9	
		3	15–20	21–30.9	16–20.9	
		4	>20 (26)	31–41	21–30	
		5		>41 (67)	>30 (37)	
Fruit	Non-pathogenic	0	0.4–0.6	0.4–0.6		
		1	0.61–1.0	0.61–2.0		
	Pathogenic	2	1.1–2.0	2.1–5.0		
		3	2.1–3.0	5.1–7.5		
		4	3.1–4.0	7.6–10		
		5	>4 (12.8)	>10 (12)		
Bud	Non-pathogenic	0	0.0–1.0			
		1	1.1–2.0			
	Pathogenic	2	2.1–3.0			
		3	3.1–4.0			
		4	>4 (4.3)			

[a] The strains inducing no (class 0) or very limited symptoms (class 1) were not considered pathogenic in this study

[b] Under brackets are the maximal lengths observed in the respective tests

Fig. 1 Classes in the pathogenicity test on cortical windows of cherry twigs

two genetically identical Belgian unclassified *P. syringae* strains from plum were both noted as non-pathogenic. The last non-pathogenic Belgian strain was another unclassified *P. syringae* strain from plum (Table 1).

Among the Belgian TLP+ *Pss* strains, the pathogenicity to the pv. *syringae* reference host, lilac, was restricted to strains belonging to the BOX-Ps 1, 2, 3 and 30, although five strains in these BOX-Ps were non-pathogenic to lilac (Table 1). But when considering the other hosts pear, cherry and plum, all the Belgian TLP+ *Pss* strains tested showed pathogenicity capabilities. Concerning TLP− *Pss*, only three Belgian strains out of 11 were pathogenic to lilac, but eight strains showed pathogenicity on fruit trees (Table 1).

P. syringae pv. *morsprunorum*

In Table 3, the *Psm* strains are differentiated by race and ability to produce coronatine (in race 1). General observations made in this study were well apparent in this pathovar. The strain pathogenicity was influenced by the host, and by the test on a given host. In one homogeneous group, the percentage of pathogenic strains could vary from 0 to 80% on a same host depending on the organ tested (*cfl*+ *Psm* race 1 on sweet cherry). In one test, the level of virulence among pathogenic isolates could be relatively stable within one homogeneous genetic goup, as for the *cfl*+ *Psm* race 1 strains on sweet cherry twigs (mean class of 2.3±0.4), but it could also be very variable, with strains dispersed sometimes in classes 2 to 5, as in TLP+ *Pss* in repIS-P3 (BOX-P1) or in TLP− *Pss* repIS-P4 (BOX-P6) (Table 1; data not shown).

The *Psm* race 1 strains showed a host specificity to cherry and plum in our pathogenicity tests. The *cfl*+ strains, isolated mainly from sweet cherry (Table 1), were pathogenic to sweet cherry twigs and fruits, sour cherry leaves, fruits and twigs, and plum leaves (Table 3). They were more virulent than the *cfl*− *Psm* race 1 srains (from plum and sweet cherry; Table 1) that were non-pathogenic to sour cherry and plum leaves and to sour cherry fruits. Also, the percentage of pathogenic *cfl*− strains on sour cherry twigs was only 25% in comparison to 70% for the *cfl*+ strains. The test on sweet cherry twigs W gave the most comparable results with 80% (*cfl*+) and 75% (*cfl*−) pathogenic strains. The percentages of pathogenic strains were also high on sweet cherry fruits (80% of *cfl*+ and 50% of *cfl*− strains), but the necroses on fruits induced by pathogenic strains were generally limited (mean

Fig. 2 Symptoms on detached pear flowers after inoculation with *P. syringae* strains. **a** Class 0; **b** Class 2; **c** Class 3

Table 3 Pathogenicity tests results for described pathovars

Pathogenicity tests		Name[a]							
		Psm race 1 *cfl*+ BOX pattern[b]	*Psm* race 1 *cfl*−	*Psm* race 2	*Pss* TLP+	*Pss* TLP+	*Pss* TLP−	*Ps* pv. *aptata*	*Ps* pv. *atrofaciens*
		17	17	4	1, 2, 3, 30	5, 6, 14, 15, 18, 19, 22, 24, 26, 27, 28, 34, 35, 39	6, 10	59, 60, 61, 62	57, 58
		Results in the respective pathogenicity tests[c]							
Pear	Leaf	0% (10)	0% (4)	0% (10)	86% (43) 2.7±0.9	58.8% (17) 3±0	0% (11)	50% (8) 2.5±0.9	0% (2)
	Fruit	0% (10)	0% (4)	0% (10)	55.8% (43) 3±0.8	0% (17)	9.1% (11) 4		
	Flower				82.6% (23) 3.6±1.3	30.8% (13) 2.8±0.8	45.5% (11) 3.6±1		
	Bud				73.9% (23) 2.3±0.6	61.5% (13) 2.4±0.5	18.2% (11) 3±0		
	Twig E			0% (10)	55.8% (43) 2.3±0.6	17.6% (17) 2±0	0% (11)		
Sweet cherry	Leaf	0% (10)	0% (4)	20% (10) 3±0	86% (43) 3.5±1.2	17.6% (17) 2±0	0% (11)	12.5% (8) 2	0% (2)
	Fruit	80% (10) 2±0	50% (4) 2±0	20% (10) 2.5±0.5	95.3% (43) 3.4±1.0	88.2 % (17) 2.9±1.2	0% (11)		
	Twig E			20% (10) 2±0	79.1% (27) 3±0.9	17.6% (17) 2±0	0% (11)		
	Twig W	80 % (10) 2.3±0.4	75% (4) 2.3±0.5	30% (10) 2±0					
Sour cherry	Leaf	40% (10) 2.3±0.4	0% (4)	30% (10) 2±0	81.4% (43) 2.7±1	47.1% (17) 2±0	0% (11)	25% (8) 2±0	0% (2)
	Fruit	50% (2) 2±0	0% (2)	10% (10) 2	48.8% (43) 3.2±1.2	0% (17)	9.1% (11) 2		
	Twig E			0% (6)	77.8% (43) 3.2±1.1	50% (8) 2.5±0.5			
	Twig W	70% (10) 2.6±0.7	25% (4) 2	80% (10) 2.4±0.7					
Plum leaf		30% (10) 2.3±0.5	0% (4)	20% (10) 3.5±0.5	53.5% (43) 2.4±0.6	11.8% (17) 2±0	0% (11)	12.5% (8) 3	0% (2)
Beet hypocotyls					63.4% (41) 2.2±0.4	29.4% (17) 2.8±0.4		100% (8) 3±0	0% (2)
Wheat leaf					46.3% (41) 2.4±0.6	35.3% (17) 2.8±0.8		25% (8) 2±0	50% (2) 3
Lilac leaf		0% (10)	0% (4)	20% (10) 3.5±0.5	88.4% (43) 3.4±1.1	0% (17)	27.3% (11) 2.7±0.5		

[a] *Psm*, *P. syringae* pv. *morsprunorum*; *Pss*, *P. syringae* pv. *syringae*; *Ps*, *P. syringae*; *cfl*+ and *cfl*−, presence of the *cfl* gene involved in coronatine production or not, respectively (Gilbert et al. 2009); TLP+ and TLP−, ability to produce toxic lipodepsipeptides or not, respectively (Gilbert et al. 2009)

[b] Genetic classification based on BOX-PCR (Gilbert et al. 2009)

[c] For each group of strains and each test, the data on the first line are the percentages of pathogenic strains classified in classes > 1 and, under brackets, the number of strains tested; the data on the second line are either the mean class and the standard deviation for the pathogenic strains in the group, or the class of the unique pathogenic strain in the group

diameters of lesions between 2.2 and 4.2 mm). The strains non-pathogenic on sweet cherry fruits were not the same than those non-pathogenic on sweet cherry twigs W.

The *Psm* race 2 strains showed less specificity to cherry and plum than race 1 strains. They could also be pathogenic to lilac and to sweet cherry leaves (Tables 1 and 3). The most susceptible organ was,

however, the twig W of sour cherry with 80% pathogenic strains, compared to only 30% in the twig W test of sweet cherry (Table 3).

P. syringae pv. *syringae* and related pathovars

The Belgian *Pss* strains were classified in the pv. *syringae* based on either TLP tests (TLP+ *Pss* strains) or physiological tests (TLP− *Pss* strains) (Gilbert et al. 2009). A surprising observation was that the isolate-host relationships reported among TLP+ *Pss* strains (Table 1) were not apparent in the pathogenicity tests. The BOX-P1 includes repIS-Ps whose strains were always specifically isolated from specific hosts in Belgium: pear for the repIS-Ps 1 and 2 (141 strains); cherry for the repIS-Ps 3 and 6 (32 strains), and plum for the repIS-P5 (10 strains) (Table 1). But, we observed that the 17 Belgian strains from these patterns included in the pathogenicity tests were indifferently pathogenic to pear, sweet cherry, sour cherry and plum (data not shown). This was apparent in the clusters observed in the multivariate statistical analysis applied to strains (Fig. 3). Two main groups were formed by the global analysis of the aggressiveness of the TLP+ and TLP− *Pss* strains. The pathogenicity group (PG) 1 was formed of TLP+ strains from cherry, pear and plum, without a clear specific relationship with the isolation hosts. On the other hand, clear relationships were observed with TLP production, the genetic classification and the pathogenicity to lilac: PG1 was exclusively constituted of the TLP+ strains pathogenic to lilac, which all belong to the BOX-Ps 1, 2, 3 and 30 (Table 1). PG2 included the four strains from BOX-P1 and the single strain from BOX-P3 that were non-pathogenic to lilac, the other TLP+ *Pss* strains, classified in 14 BOX-Ps and non-pathogenic to lilac, and the TLP− *Pss*, pathogenic to lilac or not and classified in 2 BOX-Ps (Fig. 3; Table 1). Despite a numerical dominance of pear strains, the strains belonging to the PG2 undergroups PG2-1, PG2-2 and PG2-3 did

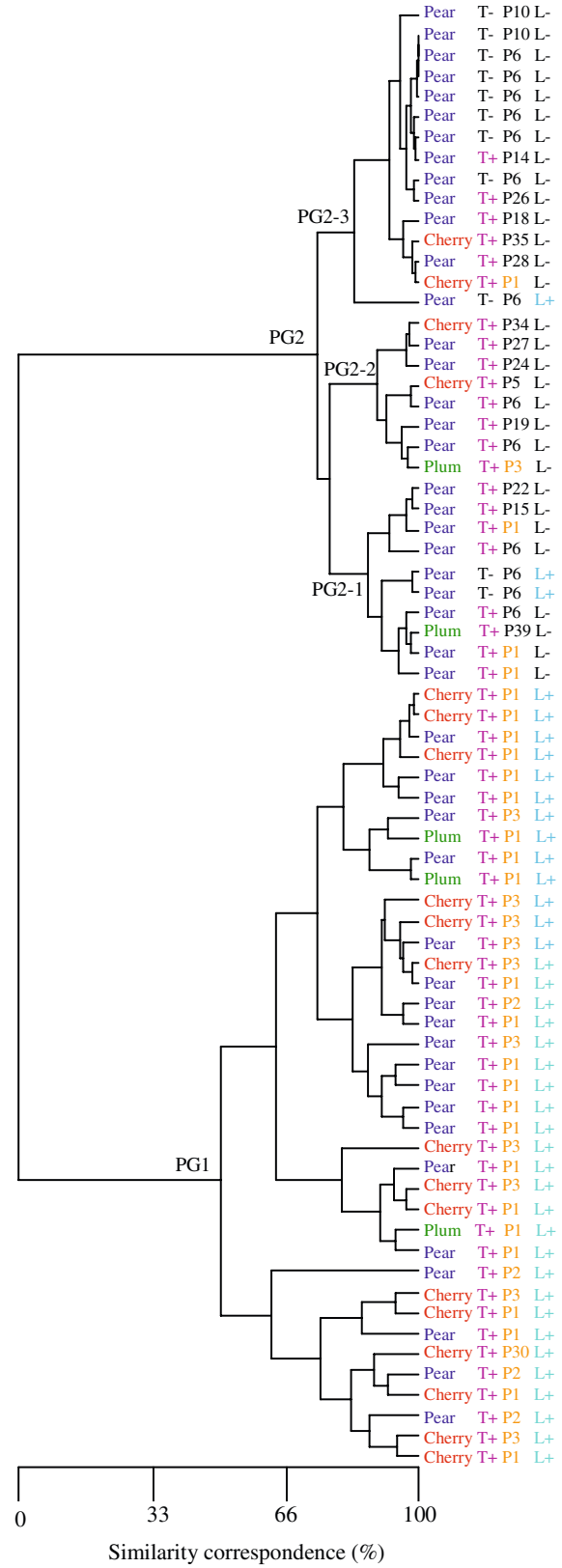

Fig. 3 Similarity correspondence in pathogenicity tests for 71 *Pss* strains. The similarity correpondence values are the Euclidean distance measures between strains (in %). For each strain, data are the colour-coded isolation host (*blue* for pear, *red* for cherry, *green* for plum), the ability to produce TLP (T+; *purple-coded*) or not (T-), the BOX-P (P; *yellow-coded* for BOX-Ps 1, 2, 3 and 30) and the ability to induce disease in lilac (L+; *light blue-coded*) or not (L-)

not show perfect host specificity to pear. The undergroup PG2-3 contained nine out of the 11 TLP− *Pss* strains tested.

The presentation of the *Pss* strains in Table 3 derives from these observations: the TLP+ strains from BOX-Ps 1, 2, 3 and 30 (almost all classified in PG1; 88.4% pathogenic to lilac leaves) were differenciated from the TLP+ strains from other BOX-Ps (all classified in PG2; always non-pathogenic to lilac leaves) and from the TLP− strains (all classified in PG2). It appeared that the TLP+ strains belonging to the BOX-Ps 1, 2, 3 and 30 were pathogenic to a broader spectrum of hosts and organs on fruit tree species: they were pathogenic in 71.1 (± 20.8) % of the tests, compared to only 26.8 (± 14.1) % in the TLP+ strains from the other BOX-Ps. In BOX-Ps 1, 2, 3 and 30, the percentages of pathogenic strains in each test was always very near or superior to 50%. Pear leaves and flowers, sweet cherry leaves and fruits and sour cherry leaves were the most susceptible organs with 86%, 82.6%, 86%, 95.3% and 81.4% pathogenic strains, respectively.

The TLP+ *Pss* strains from the 14 other BOX-Ps belonging to PG2 were always non-pathogenic on pear and sour cherry fruits, but pear leaves and buds and sweet cherry fruits remained susceptible organs with 58.8%, 61.5% and 88.2% pathogenic strains, respectively. Also, the pathogenic strains in this group could be similarly virulent to those of BOX-Ps 1, 2, 3 and 30 in specific tests such as pear leaves (mean class of 3 versus 2.7) and buds (mean class of 2.4 versus 2.3).

The TLP− *Pss* strains were all isolated from pear in Belgium and they showed a host specificity essentially restricted to pear. But they could occasionally be pathogenic to lilac leaves (27.3%, with a mean class of 2.7±0.5) and sweet cheery fruits (9.1%). They generally behaved differently from the TLP+ strains and were pathogenic in only 9.1 (± 7.5) % of the tests. They were occasionaly pathogenic to pear fruits (9.1%), flowers (45.5%) and buds (18.2%). Pear flowers were the most susceptible organs. Interestingly, the level of virulence of the pathogenic strains on pear was rather high, with mean classes of 4, 3.6 and 3 on fruit, flower and bud, respectively (Table 3).

Strains of the related TLP+ pvs *aptata* and *atrofaciens* were tested on fruit tree leaves and on their hosts, sugar beet and wheat, to determine their ability to induce disease (Table 3). The *P. syringae* pv. *aptata* strains could be pathogenic on pear (50%), sweet and sour cherry (25%), plum (12.5%) and wheat (25%) leaves, but they were always pathogenic to sugar beet hypocotyls. One of the strains of *P. syringae* pv. *atrofaciens* tested was non-pathogenic (LMG 5000; Table 1) and the other was only pathogenic to wheat. On the other hand, many TLP+ *Pss* strains from fruit trees belonging to the BOX-Ps 1, 2, 3 and 30 could induce disease on sugar beet (63.4%) and wheat (46.3%). The percentages were less important among the other TLP+ *Pss* strains (29.4% and 35.5%), but these latter strains showed comparable to higher virulence with a mean class of 2.8, versus 2.2, on sugar beet and 2.8, versus 2.4, on wheat.

Unclassified *P. syringae*

The 15 unclassified *P. syringae* strains were isolated from pear (nine strains), plum (five strains) and sour cherry (one strain); 11 strains were pathogenic in at least one pathogenicity test (Table 1). Except for two strains from pear pathogenic in 40 and 33.3% of the tests, the pathogenic strains were pathogenic in a limited number of pathogenicity tests (21.4 to 6.7%). The nine pear strains were allocated in 6 repIS-Ps (Table 1) and showed variable characteristics in pathogenicity tests. Three strains were pathogenic to lilac and eight were pathogenic in at least one pathogenicity test. Seven strains were pathogenic to at least one pear organ and six to pear buds. Five strains also showed occasional pathogenicity to cherry. The five plum strains were dispersed in 4 repIS-Ps and three strains were non-pathogenic in this study (Table 1). The two pathogenic strains were only slightly pathogenic (class 2) on pear twigs and were non-pathogenic in the only test performed on plum (plum leaf). The plum strains were, however, mainly isolated from plum buds and cortical tissues of branches. The strain from sour cherry was only pathogenic on sweet cherry twigs and lilac leaves.

Similarity correspondence among pathogenicity tests

The similarity correspondence between pathogenicity tests on different organs and hosts allows evaluation of the variability of results between the tests. It allowed the detection of pathogenicity tests that gave different information, either because they were less

adequate to induce disease, as observed with the test on pear twigs, or because they enabled the detection of strain virulences undetected by other tests, as observed with the tests on pear flowers and buds. The similarity correspondence analysis was used in this study to investigate if some strains of *Pss* could have a more specific relation to one host or organ and to determine if it was useful to perform several pathogenicity tests. Among 34 TLP+ *Pss* strains, it appeared in Fig. 4 that although some correspondence existed among tests, none gave exactly the same information. The tests on lilac leaves, sweet cherry twig ends and leaves, pear leaves and fruits and sour cherry leaves gave similarities near 85%, but similarities of 65% were also observed. The sweet cherry fruit test enabled detection of the pathogenicity behaviour of 93% of the pathogenic TLP+ *Pss* strains, compared to only 63.3% for the lilac leaf test. Indeed, these tests are well differentiated in Fig. 4. When a clustering analysis was applied only to pear strains and pear and lilac tests, it appeared that the tests on pear flowers and buds were those that classified the strains most differently from the tests on pear and lilac leaves (Fig. 5). In this study, the strains from pear that were able to cause damage to pear buds were found in the TLP+ *Pss* strains from BOX-P 1, 2, 3 and 30, in the other TLP+ *Pss*, in the TLP− *Pss*, and in unclassified *P. syringae*. This enabled the detection of more strains than if only a leaf test on pear or lilac had been considered.

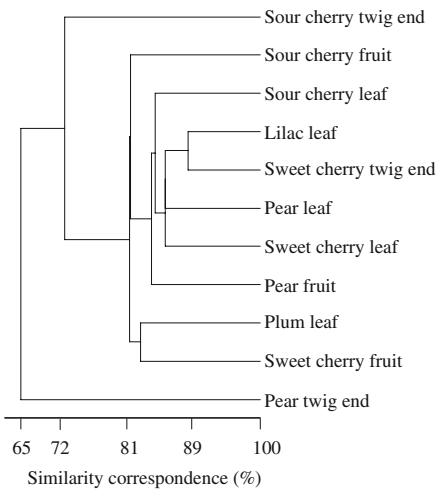

Fig. 4 Similarity correspondences between 11 pathogenicity tests on pear, sweet cherry, sour cherry, plum and lilac based on the results of 34 TLP+ *Pss* strains from pear, sweet cherry and plum. The similarity correpondence values are the Euclidean distance measures between tests (in %)

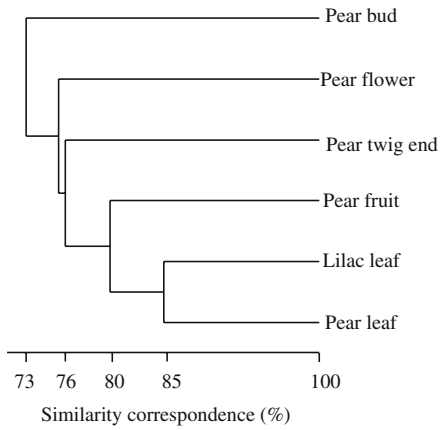

Fig. 5 Similarity correspondences between six pathogenicity tests on pear and lilac based on the results of 36 TLP+ and 11 TLP− *Pss* strains from pear. The similarity correpondence values are the Euclidean distance measures between tests (in %)

Discussion

This study highlighted the importance of considering the isolation hosts and multiple pathogenicity tests to accurately determine the pathogenicity of the Belgian *P. syringae* strains from orchards. Indeed, few strains appeared to be non-pathogenic when considering all the tests and no test would have enabled us to obtain all the information provided by the complete study. This probably reflects the complexity of *P. syringae* and the various conditions and lifestyles this bacterium encounters in orchards. Only one cultivar was used per culture in this study and additional information would possibly be obtained by multiplying the cultivars and the test procedures. Even within one test, different symptoms were observed: on sweet cherry 'Lapins' fruits, *Psm* race 1 strains induced non-sunken discoloured lesions generally <3 mm diam, as noted by Garrett et al. (1966) and Burkowicz and Rudolph (1994), but TLP+ *Pss* generally induced larger black sunken lesions (data not shown).

Pseudomonas syringae causes damages on pear buds, flowers, leaves and fruits; only in very cold winter conditions can cankers on trunks and branches be observed (Wilson 1936; Crosse 1966; Jones and Aldwinkle 1990). In Belgium and in other countries, economically important diseases are blossom blast

and bud deads during winter (Clara 1932; Wormald 1946; Parker and Burkholder 1950; Deckers and Daemen 1991; Montesinos and Vilardell 1991). One practical interest of the multiple pathogenicity analyses was clear in pear where the flower and bud tests gave additional information compared to the more classical leaf and fruit tests. Interesting information was that both TLP+ and TLP− *Pss* strains could be pathogenic on pear flowers and that unclassified *P. syringae* could cause disease on pear buds. This indicated that strains other than *Pss* can be pathogenic on pear and that they should not be disregarded in the problem of dead bud. Unclassified *P. syringae* strains were also encountered on plum but they were poor pathogens in this study. However, the limited number of tests conducted on plum (one leaf test) could perhaps explain these results as most of the strains originated from dead buds.

P. syringae pv. *morpsunorum*

The *Psm* race 1 and race 2 strains confirmed in this study their known pathogenicity on stone fruit species (Garrett et al. 1966; Roos and Hattingh 1987; Burkowicz and Rudolph 1994). In *Psm* race 1, the *cfl*+ strains producing the phytotoxin coronatin were more often noted pathogenic than the *cfl*− strains. Also, the test on twig W of sweet cherry was the most adapted to detect pathogenic strains among both *cfl*+ and *cfl*− strains, with 80 and 75% pathogenic strains respectively. However, the reason why a weak proportion of genetically identical strains originating from diseased cortical tissues was non-pathogenic on cortical tissues remains an intriguing open question. On the other hand, these strains showed the usual relatively low pathogenicity of *Psm* race 1 strains on sweet cherry fruits.

Concerning *Psm* race 2, some strains were able to cause disease on a different host, lilac. They were clearly less pathogenic to sweet cherry fruits (20%) than the *cfl*+ and *cfl*− *Psm* race 1 strains (80% and 50%). The test on twig W of sour cherry 'Montmorency' was the best suited to detect the pathogenic *Psm* race 2 strains: 80% compared to 30% on twig W of sweet cherry 'Lapins'. Most *Psm* race 2 strains were isolated from sour cherry in Belgium (Bultreys and Gheysen 2004; Bultreys et al. 2007; Gilbert et al. 2009) and these observations could be related. Also, a higher virulence of *Psm* race 1 strains to shoots of sweet cherry 'Black Tartarian' compared to *Psm* race 2 strains was observed by Roos and Hattingh (1987). However, *Psm* race 2 strains were found on sweet cherry 'Roundel' and 'Napoleon' as well as on wild cherry (Freigoun and Crosse 1975; Vicente et al. 2004) and cultivar-related results could therefore not be excluded. Further studies on cultivars of sweet, sour and wild cherry should help us to understand the range of aggressiveness of the two races of *Psm*.

Pseudomonas syringae pv. *syringae*

Pss appeared as a pathovar with a wide host range. At least three groups differing in pathogenicity were defined among the Belgian strains. A first group included TLP+ *Pss* strains pathogenic in 71.1% of the tests, generally pathogenic to lilac leaves, and belonging to the BOX-Ps 1, 2, 3 and 30 (Table 3) and mainly to PG1 (Fig. 3). It represented 83% of the *Pss* strains in Belgian fruit orchards and was composed of all the dominant genetic groups specifically detected on pear, cherry and plum in Belgium (Gilbert et al. 2009). A second group included the TLP+ *Pss* strains belonging to the other BOX-Ps and to PG2 (Table 3; Fig. 3). These strains were pathogenic in only 26.8% of the tests. They were highly heterogeneous genetically and constituted small distant genetic groups occasionally detected in Belgium. They were always non-pathogenic to lilac leaves but could be clearly virulent in a limited number of tests on fruit trees. The third group included the TLP− *Pss* strains belonging to the BOX-Ps 6 and 10 and to PG2 (Table 3; Fig. 3). They were isolated from pear, pathogenic in only 9.1% of the tests and almost specifically pathogenic to pear flowers and buds. Whether all these strains should be kept in the pv. *syringae* is an open question. One problem if a differentiation was decided would be that none of the groups is genetically homogeneous. Ranges of symptom severity between and within groups formed by rep-PCR profiles were also observed in England and Italy concerning *Pss* strains from woody or herbaceous host plants (Scortichini et al. 2003; Vicente and Roberts 2007).

One surprising point was that although the Belgian TLP+ *Pss* strains from the repIS-Ps 1 and 2 originated from pear, those from the repIS-Ps 3 and 6 originated from cherry, and those from the repIS-Ps 5 originated from plum; the 17 representatives

from these repIS-Ps tested in the pathogenicity tests were indifferently pathogenic to pear, cherry and plum. Also, although the TLP+ strains of the pvs *aptata* and *atrofaciens* were genetically distinct from the TLP+ *Pss* strains from fruit trees (Table 1; Gilbert et al. 2009), strains of pv. *aptata* were pathogenic on fruit trees in pathogenicity tests and, conversely, strains from Belgian orchards were pathogenic on sugar beet and on wheat, although corresponding strains have never been found naturally on these hosts. One explanation would be that isolating more strains from these hosts would finally enable us to find typical *aptata* and *atrofaciens* strains on fruit trees, and conversely. Another explanation would be that pathogenicity tests conducted in laboratory conditions do not reproduce all the aspects of the life-cycle of *P. syringae* on a host, for example: the ability to grow from a very small initial population to a population able to induce disease, or the ability to live a complete year-cycle on a host, or the ability to compete with the natural populations of microorganisms on a host. In a pathogenicity test, the inoculum is generally put on wounded tissue and that could mask important aspects of the ecology of a strain and of strain-specificity with its host.

The previous point would influence the method of naming strains producing TLP isolated from fruit trees. Indeed, the distinction between the TLP+ pvs *syringae*, *aptata* and *atrofaciens* has been much disputed (Otta and English 1971; Wilkie 1973; Quigley et al. 1994; Maraite and Weyns 1997; Bultreys and Gheysen 1999). The question is: should the observed pathogenicity of an isolate on sugar beet or wheat in a pathogenicity test be decisive in classifying the isolate in the pv. *aptata* or *atrofaciens*, rather than *syringae*? Such an approach has been done concerning the bacterial disease of melon caused in France by *P. syringae* pv. *aptata* because the pathogen was also pathogenic to sugar beet (Morris et al. 2000). We consider in our case that the genetic differences observed up to now between real *aptata* and *atrofaciens* strains from sugar beet and wheat and the strains from Belgian orchards (Gilbert et al. 2009) are presently a sufficient argument to keep the Belgian TLP+ strains in the pv. *syringae*.

The tests on pear fruits and pear leaves were often used for evaluating the aggressiveness of *Pss* strains (Yessad et al. 1992; Moragrega et al. 2003; Scortichini et al. 2003). In this study, the most efficient test to detect pathogenic TLP+ *Pss* strain was that on sweet cherry fruits (percentage of pathogenic strains of 95.3% in the BOX-Ps 1, 2, 3 and 30 and 88.2% in the other BOX-Ps), followed by the test on pear leaves (86% and 58.8%), and less strains were pathogenic to pear fruits (55.8% and 0%). However, the tests on sweet cherry fruits and pear leaves were totally ineffective to detect pathogenic TLP− *Pss* strains (percentage of pathogenic strains of 0%) and the tests on pear flowers and buds were very useful to detect additional pathogenic TLP+ and TLP− *Pss* strains.

Acknowledgements The work was supported by the Walloon Agricultural Research Centre, Ministry of the Walloon Region (A.B., V.P.), and by the 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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