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Short communication

Epiphytic occurrence of *Pseudomonas syringae* pv. *papulans* (Rose) in France, where blister spot has never been seen

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Abstract

Pseudomonas syringae pv. *papulans* (PSP) the causal agent of blister spot, on the apple cultivar Mutsu in the USA, Canada and Italy, has not been described in France. A study on epiphytic populations of *P. syringae* isolated from French apple orchards revealed two isolates called KA54 and E121, whose biochemical characterisation showed high similarities with PSP strains. Identical symptoms were obtained with KA54, E121 and PSP strains, after vacuum inoculation of detached immature fruits of the cultivar Fuji, and young leaves of the cultivars Fuji, Mutsu, Gala and Golden Delicious. Koch's postulate was verified. These results indicate the presence of PSP in France. Differential characterisation criteria including serological, molecular and pathogenicity tests are proposed.

Pseudomonas syringae pv. *papulans* (PSP) is the causal agent of the apple blister canker. This disease was first described in the USA (Missouri) by Rose (1916). At the end of the seventies Dhanvantari (1977), then Burr and Hurwitz (1979), described heavy damages in orchards of apple cv. Mutsu, due to PSP in Canada (Ontario) and the USA (New York and Michigan), respectively. In Europe, this disease was recorded in Italy in 1983 (Bazzi and Calzolari, 1983). The presence of this disease has not been yet reported in France.

Symptoms on apple fruit appear as small spots on the surface. As the fruits mature, these spots tend to have a vesiculous (blister) aspect, brown in colour, with a diameter of 1–5 mm. The number of such lesions on a single fruit may vary from less than 10 up to 200 (Burr and Katz, 1982; Bazzi and Calzolari, 1983). On young leaves of the cv. Mutsu, PSP induces necrotic areas along the main ribs, as well as a wilt of leaf margins (Bonn and Bedford, 1986). Dormant buds are likely to be the sites where the bacterial cells overwinter. They may also show necrotic lesions, which look like lesions caused by *P. s.* pv. *syringae* (PSS) (Burr and Katz, 1982; 1984). Data from Burr and Katz (1984) and Bedford et al. (1988) indicated epiphytic survival of PSP on buds, blossoms, leaves and fruits of the Mutsu cultivar.

In the course of a study on epiphytic populations of *Pseudomonas syringae* in apple orchards in France, two isolates were obtained which resembled PSP. We present here their phenotypical, serological, pathological and molecular characterisation. Two isolates, KA54 and E121, were obtained from apple leaves (cv. Golden Delicious) near Angers (France) in 1997 and 1999, respectively. They were compared to a collection of strains of PSP originating from the USA, Canada and Italy, including the pathotype strain 1757^{pt} (Table 1) and three strains of PSS obtained from the Collection Française de Bactéries Phytopathogènes (CFBP).

Biochemical and physiological tests were performed mainly according to Lelliott et al. (1966) and Schaad (1988). All the strains studied (PSS, PSP, KA54 and E121) produced a fluorescent pigment on King's medium B (King et al., 1954), did not produce levan on sucrose medium and were negative for

Table 1. Bacterial strains of *Pseudomonas syringae* used for phenotypical, pathological, serological and molecular tests

Strains and isolates	Source
KA54	France, *Malus sylvestris* cv. Golden Delicious, this study; 1997
E121	France, *M. sylvestris* cv. Golden Delicious, this study; 1999
PSP	
1754[pt]	Canada, *M. sylvestris* (B.N. Dhanvantari); 1973 (CFBP)
3324	Canada, *M. sylvestris* cv.Mutsu (B.N. Dhanvantari); 1968 (CFBP)
P 8810	Canada, *M. sylvestris* cv. Mutsu (W.G. Bonn)
P 8811	Canada, *M. sylvestris* cv. Mutsu (W.G. Bonn)
10759	Canada,*M. sylvestris* cv. Mutsu (W.G. Bonn)
3323	USA, *M. sylvestris* cv. Mutsu (A. Jones) (CFBP)
PSP5	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1977
PSP6	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1978
PSP7	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1981
PSP8	USA, *M. sylvestris cv.* Mutsu (T.J. Burr); 1979
PSP9	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1981
PSP19	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1980
PSP24	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1980
PSP32	USA, *M. sylvestris* cv. Mutsu (T.J. Burr); 1985
IPV-BO 1511	Italy, *M. sylvestris* cv. Mutsu (C. Bazzi); 1981 (NCPPB 3262)
Lb 107/98	Italy, *M. sylvestris* cv. Golden Delicious (L. Linder);1998
Lb 16/99	Italy, *M. sylvestris* cv. Golden Delicious (L. Linder);1999
PSS	
602	France, *M. sylvestris* (M. Ridé); 1964 (NCPPB 2775) (CFBP)
1392[T]	UK, *Syringa vulgaris* (K.A. Sabet); 1950 (NCPPB 281) (CFBP)
3077	France, *Pyrus communis* (C. Manceau); 1983 (CFBP)

[pt]Pathotype strain; [T]type strain.

cytochrome c-oxidase, calcium-pectinate hydrolysis (Prunier and Kaiser, 1961) and arginine-dihydrolase. They induced an hypersensitive reaction on tobacco leaves. The two strains belonged to group 1b of the fluorescent Pseudomonads (Lelliott et al., 1966). All produced acid from sucrose, mannitol and sorbitol, utilised DL-lactate as a carbon source but not D(−) tartrate. All strains hydrolysed aesculin. Isolates KA54 and E121, and all PSP strains did not produce proteases on gelatin or casein media (Table 2). They showed pectolytic activity, revealed by pits on polypectate gel at pH 5 (Hildebrand, 1971). All strains, except PSP5 and CFBP 1754[pt], utilised erythritol, and all except IPV-BO1511 utilised D(−) tartrate. A high degree of similarity was observed between the phenotypic characteristics of isolates KA54 and E121 and the PSP strains that were clearly distinguished from PSS strains. To the already reported differential characters between PSP and PSS, namely, absence of levane synthesis, inability to hydrolyse gelatin, we add a new physiological criterium: hydrolysis of polygalacturonate at pH 5, which is unique among the pathovars of *P. syringae* genomospecies 1 (Gardan et al., 1999).

O-serogroups were determined according to Saunier et al. (1996). Antisera were produced from rabbits, after intravenous injections of heat-killed bacterial cells. Serological relationships of lipopolysaccharides (LPS) were tested by Ouchterlony's double diffusion technique, where the antigen was a bacterial suspension adjusted to 10^{10} cells ml^{-1} in sterile distilled water. The precipitation due to the interaction of LPS-antibodies was revealed by the presence of a unique band, and indicated partial (spur) or total identity with serological references. Two new serogroups of PERSAVTOM (3 and 4) were created (Table 3). Seventeen PSP strains, including the pathotype-strain and isolates KA54 and E121, were placed in the PERSAVTOM 4 group. One PSP strain was in PERSAVTOM 3 group, and two strains joined the RIB group which recognizes strains lacking LPS sidechains (Saunier et al., 1996). One main serogroup (PERSAVTOM 4) contained most of the PSP strains. This is an additional key-character to differenciate pv. *papulans* from pv. *syringae*, since the representatives of the latter pathovar tested in this study were SYR 1 (CFBP 602), PERSAVTOM 2 (CFBP 1392[T]) and MOP 2 (CFBP 3077).

Table 2. Characteristics which differenciate KA54, E121, PSP and PSS

Characters	KA54, E121	PSP			PSS (602, 1392^{T}, 3077)
		15 Strains	2 Strains*	1 Strain**	
Levan	−	−	−	−	+
Erythritol	+	+	−	+	+
Proteolysis					
Gelatin	−	−	−	−	+
Casein	−	−	−	−	+
D(−) Tartrate	+	+	+	−	−
Polypectate pH5	+	+	+	+	−
Pathogenicity					
Pear leaves	+nr	+nr	+nr	+nr	+nr
Fuji leaves[a]	+bs	+bs	+bs	+bs	+nr
Fuji fruits[a]	+bs	+bs	+bs	+bs	−
PCR with Pap primers	+	+	+	+	−

^{pt}pathotype strain; ^{T}type strain; [a]leaf and fruit inoculated by vacuum infiltration nr: necrotic reaction; bs: blister spot; *PSP5 and 1754^{pt}; ** IPV-BO1511.

Table 3. Serological reactions of PSP strains (Ouchterlony double diffusion according to Saunier et al., 1996)

Bacterial strains	Antisera (prepared against)				O-serogroups
	196 (1670^{T})	287 (2545)	292 (2215^{pt})	294 (2348^{pt})	
P. savastanoi pv. *savastanoi* 1670^{T}	++	−	−	−	PERSAVTOM 1
P. syringae pv. *tomato* 2545	++	++	++	−	PERSAVTOM 2
P. syringae pv. *delphinii* 2215^{pt}	−	+s	++	−	DEL
P. syringae pv. *ribicola* 2348^{pt}	−	−	−	++	RIB
PSP6, P8810	−	−	−	++	RIB
PSP32	−	++	−	−	PERSAVTOM 3
PSP (16 strains)*	++	++	−	−	PERSAVTOM 4

^{T}Type strain; *including the pathotype strain CFBP 1754^{pt}; +s: precipitating band with spur.

Strains KA54 and E121 gave a specific 240 bp long DNA amplification fragment after PCR amplification with primers specific to PSP designed by genomic comparison (Kerkoud et al., in preparation). The same signal was obtained with PSP strains reisolated from pathogenicity tests.

Pathogenicity tests (as described below) were performed with KA54 and E121, PSP strains and PSS strains prepared from 24 h cultures on King's medium B at 25 °C. Sterile distilled water was used as control. All inoculated plant materials were incubated in a growth chamber (25 °C, 8 h darkness a day) for 4 weeks and were examined periodically for symptom development.

In the first type of experiments, wound inoculations were performed, with bacterial suspensions adjusted to 10^{8} cfu ml^{-1}, on plantlets of apple and pear (from open-pollinated Golden Delicious cultivar, and Kirschensaller cultivar, respectively), on cuts through the midrib of fully expanded young leaves, (Le Lézec and Paulin, 1984). No reaction was observed on apple leaves, but limited necrosis were obtained with PSP (CFBP 1754^{pt}, CFBP 3323 and KA54) and PSS (CFBP 602, CFBP 1392^{T} and CFBP 3077) on pear leaves. Lilac (*Syringa vulgaris*) seedlings, inoculated by infiltration with a syringe of the bacterial suspension into the petiole, showed symptoms only when inoculated with the three PSS strains.

In the second type of experiments, detached immature apple fruits were injected with bacterial suspensions (KA54 and E121 isolates, PSP strains: CFBP 1754^{pt} and CFBP 3323) prepared as for wound inoculation, by syringe injection in fruit flesh. Before injection, fruits were surface sterilised in 1 per cent

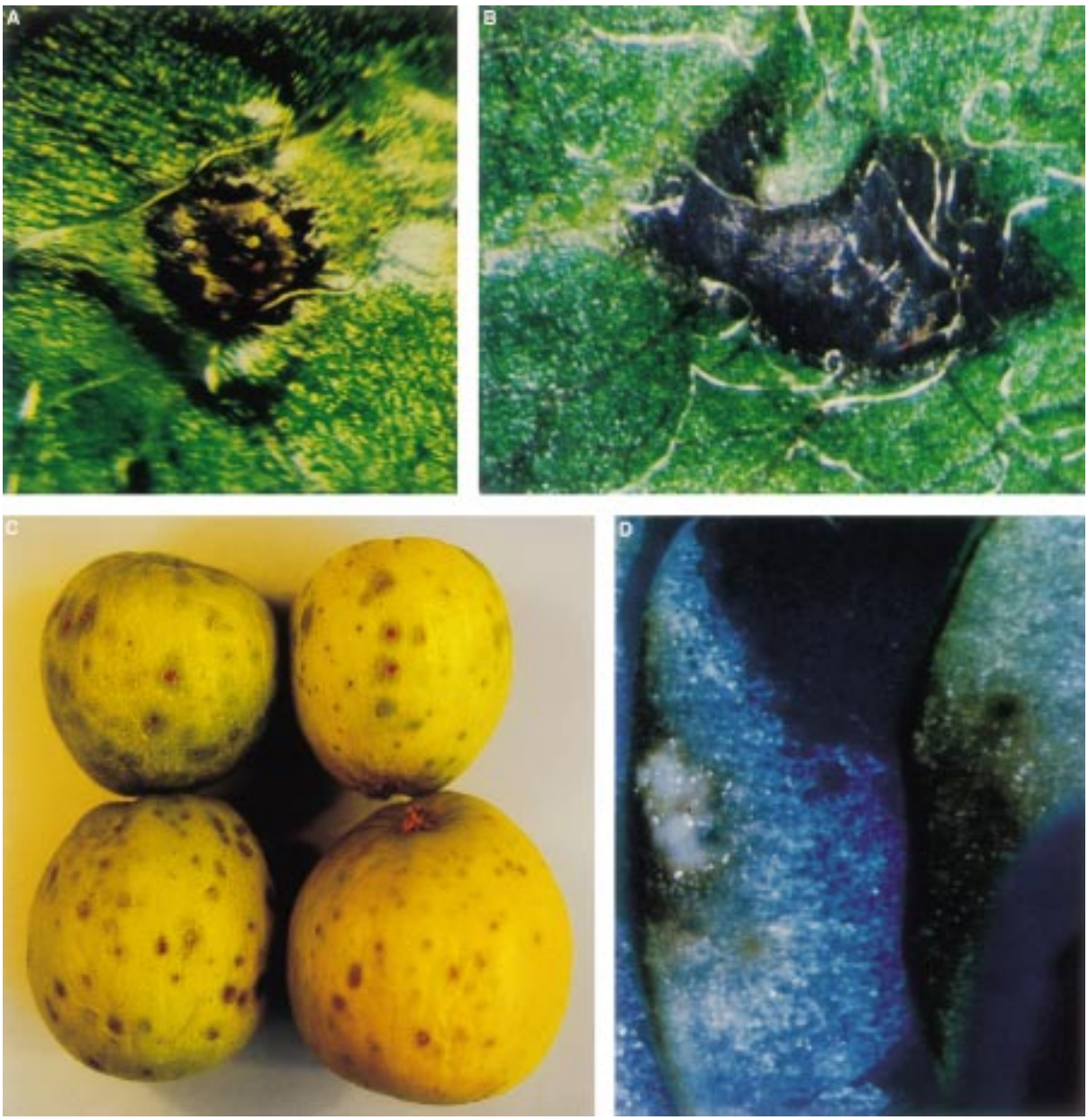

Figure 1. (A) Brown and circular reaction obtained in leaf inoculated with KA54 isolate by vacuum infiltration, typical of blister spot. (B) Black and irregular hypersensitive reaction obtained with PSS strain CFBP 3077 by vacuum infiltration. (C) Symptoms of blister spot obtained by vacuum infiltration on apple fruits of cv. Golden Delicious with (left up) KA54 isolate, (right up) E121 isolate, (left down) PSP strain CFBP 1754^{pt} and (right down) PSP strain CFBP 3323. (D) Close up (65×) section of apple fruits after vacuum infiltration with (left) PSP strains showing bacterial development and (right) PSS strains showing restricted necrotic reaction at the penetration site.

sodium hypochlorite for 5 min, washed four times in sterile distilled water and dried with sterile blotter paper. After inoculation, no symptoms were observed on the fruits of the different apple cultivars used (Fuji, Golden Delicious, Gala and Braeburn). Finally, detached immature apple fruits and attached young leaves of apple were vacuum infiltrated with bacterial suspensions adjusted to 10^7 cfu ml^{-1}.

The lesions obtained on young leaves of the apple cultivars Mutsu, Fuji, Golden Delicious and Gala began as chlorotic spots which later evolved into brown blisters. Some of the blisters showed a hole in the centre of the lesion (Figure 1A). Conversely, with PSS strains, local necrotic HR type lesions were obtained (Figure 1B). Identical symptoms on fruits from cultivars Fuji, Golden Delicious, and Braeburn, were obtained with the isolates KA54, E121, and with all the PSP strains (Figure 1C). The obtained symptoms were typical of blister spot. Fruit lesions caused by strains of PSP (CFBP 1754^{pt} and CFBP 3323) and KA54 and E121 isolates were clearly different from the hypersensitive necrosis produced by PSS. A section of fruit lesions showed, under magnifying glass examination, the extension of the bacterial invasion of PSP within the plant tissue. Conversely, with PSS strains (CFBP 602, CFBP 1392^{T} and CFBP 3077), a lesion restricted to the inoculation site was observed (Figure 1D). Isolation from these lesions, and subsequent reinoculation on suitable plant material allowed the verification of Koch's postulate. Wound inoculation of detached leaves and fruits did not allow the production of typical blister canker symptoms. This is in agreement with data from Burr and Hurwitz (1979), and Bazzi and Calzolari (1983). Therefore the technique of leaf and fruit infiltration appears to be a new pathogenicity test specific to PSP.

Taken together, the phenotypical characters, serological features and pathogenicity responses, showed that isolates KA54 and E121 were actually PSP strains. The specific Pap primers confirmed these results, and could be used to facilitate the identification of PSP in further epidemiological studies. This proves that PSP is present in France, although the corresponding disease 'blister canker' has never been reported. The occurrence of the causal agent may be explained by its aptitude to epiphytic survival. Burr and Hurwitz (1981) showed that PSP may survive as an epiphyte on healthy leaves of Golden Delicious trees. Furthermore, Burr and Katz (1984) showed that symptomless buds of apple may be colonised by both PSP and PSS. Epiphytic populations of PSP developed in France in 1997, and were found again in 1999 in the same orchard. How were they introduced? Bazzi and Calzolari (1987) reported that, in another area of the Venosta Valley (Italy), fruit infections were observed in a young orchard of Smoothee apple trees ('Golden Delicious' standard clone) imported from Northern Europe, and suggested that propagation material may play an important epidemiological role in spreading the pathogen.

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