

SHORT COMMUNICATION

GENETIC AND PATHOGENIC DIVERSITY OF *PSEUDOMONAS SYRINGAE* pv. *SYRINGAE* ISOLATES ASSOCIATED WITH BUD NECROSIS AND LEAF SPOT OF PEAR IN A SINGLE ORCHARD

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SUMMARY

Ninety fluorescent pseudomonads obtained from pear cultivars Coscia and Tosca showing symptoms of bacterial blast were assessed by biochemical tests and pathogenicity on lemon fruits, and pear and lilac leaves. Presence of the *syrB* gene and ice-nucleation activity was also determined. In addition, genetic variability was assessed using repetitive sequence PCR with the BOX A1 primer. The patterns were analyzed with UPGMA and Dice's coefficients. Eighty-nine isolates were determined as putative *Pseudomonas syringae* pv. *syringae*. Four genomic patterns, A, B, C and D, were obtained by BOX-PCR analysis. Patterns A and C were identical to those shown by other *P. s. pv. syringae* strains previously isolated from pear in other areas. Pattern B was associated solely with leaf spot symptoms observed on cv Coscia. The *syrB* gene was present in 38.8% of the isolates, whereas ice-nucleation activity was observed in 77.7%. Isolates possessing the *syrB* gene were the most aggressive in pathogenicity tests. Different populations of *P. s. pv. syringae* appear to be involved in inducing blast symptoms on pear in central Italy.

Key words: bacterial blast, *syrB* gene, ice-nucleation activity, repetitive sequence PCR.

Pseudomonas syringae pv. *syringae* van Hall has been repeatedly found associated with blast of pear (*Pyrus communis* L.) causing economic losses in many areas of the world. The main symptoms are bud and blossom blast, necrosis of immature fruits, leaf and fruit spots, and cankers on branches and trunk. Studies have clarified the relationships between the susceptibility of pear flower buds to frost, presence of ice-nucleation active bacteria (Proebsting and Gross, 1988), and the conditions of temperature, moisture and stage of bloom development favourable for inducing blossom blast

(Whitesides and Spotts, 1991). Relationships among pathogen population levels, the quantity of ice nuclei and incidence of dormant flower bud blast (Montesinos and Vilardell, 1991) as well as the variability of *P. s. pv. syringae* strains in terms of pathogenicity, virulence and production of phytotoxic and biocidal compounds have also been studied (Gross *et al.*, 1984; Montesinos and Vilardell, 1991; Zeller *et al.*, 1997). Moreover, the susceptibility of pear cultivars to pathogen following natural (Spotts and Cervantes, 1994) or artificial infections on immature fruits or detached leaves (Moragrega *et al.*, 2003) has been evaluated. By contrast, nothing is known about the genetic diversity of *P. s. pv. syringae* strains associated with some specific symptoms of pear blast.

Assessing the population structure of 101 *P. s. pv. syringae* strains obtained from woody and herbaceous hosts using repetitive sequence PCR genomic fingerprints with the BOX A1 primer, Scortichini *et al.* (2003) found three different genomic patterns among strains isolated from pear in Greece, Italy, Spain and the UK. Studies on the genetic variability of phytopathogenic bacteria are important to elucidate possible relationships between certain populations of the pathogen and the area from where they were originally isolated as well as to assess the genetic composition of the species or pathovar to develop different specific primers to be used in diagnostics (Scortichini, 2005). The aim of this study was to assess the genetic and pathogenetic diversity of 90 *P. s. pv. syringae* isolates associated with bud necrosis and leaf spot of two pear cultivars and to compare their genomic fingerprints with those of other *P. s. pv. syringae* strains previously assessed in the same way (Scortichini *et al.*, 2003). The isolates were also assessed for pathogenicity to host and non-host plants and for presence of the *syrB* gene and ice-nucleation activity.

For isolation, bud and leaf samples of pear cultivars 'Coscia' and 'Tosca' showing necrosis were collected in spring from a mature pear orchard in the province of Rome. Approximately 10% of trees showed typical symptoms. The plant material was processed within one hour of sampling. Small (1-2 mm) pieces of tissue were crushed in sterile mortars containing 5 ml of sterile saline (0.85% NaCl in distilled water). Serial ten-fold dilutions in tubes were also prepared. Aliquots of 0.1 ml

of the suspensions were spread on Petri dishes containing medium B of King *et al.* (1954). The plates were incubated for 48 h at 25-27°C. Fluorescent colonies were isolated on nutrient agar (NA) prior to identification. For fluorescent isolates, the following biochemical tests were performed according to Lelliott and Stead (1987): levan production, presence of oxidase, soft rot activity on potato slices, presence of arginine dehydrolase, hypersensitivity reaction in tobacco leaves (LOPAT tests), metabolism of glucose, presence of tyrosinase, hydrolysis of aesculin and arbutin. In addition, with isolates belonging to LOPAT test group Ia, pathogenicity tests were also done on lemon fruits and pear and lilac plants. With 24-h-old cultures grown on NA, suspensions in sterile saline, photometrically adjusted to $1-2 \times 10^7$ cfu/ml, were prepared. Lemon (*Citrus limon* (L.) Burm.) fruits were surface-sterilized with sodium hypochlorite solution, rinsed with sterile distilled water, and inoculated by puncturing the surface with a sterile needle and placing 10 µl of the bacterial suspension on the wound. Pathogenic reactions were assessed seven days after inoculation. For each isolate, ten sites involv-

ing two fruits were inoculated. In addition, pot-grown plants of pear and lilac (*Syringa vulgaris* L.) were inoculated with a 20 µl drop of bacterial suspension deposited on a fresh wound made on the leaf midrib. For each isolate, ten different leaves were inoculated. The inoculations were done in spring, some days after the isolation, in open-field conditions. Pathogenic reactions were assessed 14 days after inoculation according to Yessad-Carreau *et al.* (1994). Ice-nucleation activity of the isolates was determined using the procedure of Lindow (1990).

Possible presence in the putative *P. s. pv. syringae* isolates of the *syxB* gene coding for production of cyclic lipodepsinonapeptides was determined by PCR using primers B1 (5'-CTTTCCGTGGTCTTGATGAGG-3') and B2 (5'-TCGATTTTGCCGTGATGAGTC-3') (Eurogentec, Seraing, Belgium), amplifying a 752 bp fragment. The PCR procedure of Sorensen *et al.* (1998) was followed. In addition, aliquots of the PCR products were precipitated with three volumes of ethanol and digested with *SalI* restriction enzyme (Promega, Madison, WI, USA) (Sorensen *et al.*, 1998) following manufacturers' instructions.

Putative *P. s. pv. syringae* isolates were also assessed using repetitive sequence PCR. For total DNA preparations the technique of Smith *et al.* (1995) was used. A single colony of each strain grown for 48 h on NA was suspended in sterile saline and centrifuged at 12,000 g for 2 min. The supernatant was discarded and the pellet suspended in sterile saline at an optical density corresponding to $1-2 \times 10^8$ cfu/ml. The suspension was heated in boiling water for 10 min, and then stored at -20°C. The repetitive sequence PCR (rep-PCR) method used was that of Louws *et al.* (1994). The BOX A1 primer was synthesized by Eurogentec (Seraing, Belgium). Amplifications were performed in a MJ Research (Watertown, MS, USA) PTC programmable thermocycler in 25 µl reaction volumes containing 200 µM deoxynucleoside triphosphate, 2 mM MgCl₂, primer at 60 pmol, 1.0 U *Taq* polymerase and 4 µl template DNA preparation. The PCR mixture was overlaid with 25 µl mineral oil. Thermal cycling was carried out as described by Louws *et al.* (1994): initial denaturation at 95°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 65°C for 8 min, a single final extension step at 65°C for 16 min, and final soak at

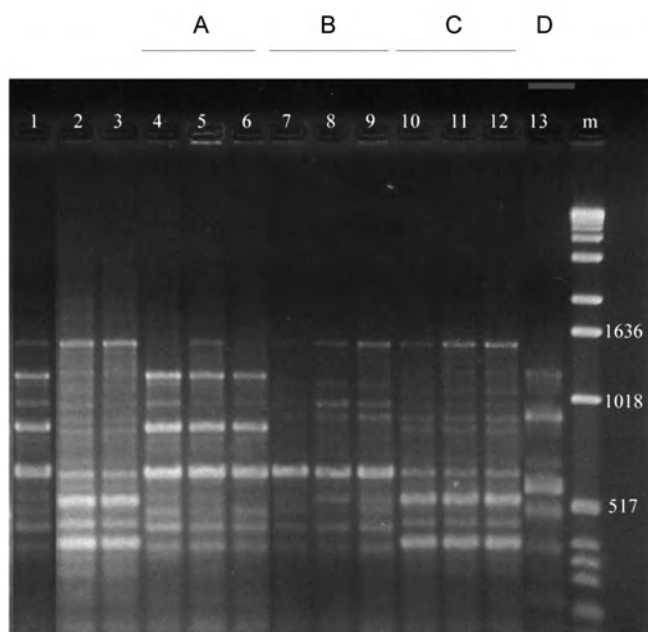


Fig. 1. Fingerprints obtained with repetitive sequence PCR and BOX A1 primer of representative *Pseudomonas syringae* pv. *syringae* isolates and a deviating isolate (pattern D) from pear cultivars 'Coscia' and 'Tosca' showing bud and leaf necrosis. Letters A-D indicate the different patterns (see also Fig. 2). m: molecular size marker in base pairs (1 Kb-ladder; Gibco-BRL). Lane 1: *P. s. pv. syringae* ISF P1 (pattern 7 of Scortichini *et al.*, 2003); lane 2: *P. s. pv. syringae* ISF P3 (pattern 11 of Scortichini *et al.*, 2003); lane 3: *P. s. pv. syringae* BPIC 692 (pattern 11 of Scortichini *et al.*, 2003); lanes 4, 5, 6: pattern A of *P. s. pv. syringae* isolates from 'Coscia' and 'Tosca' buds and leaves; lanes 7, 8, 9: pattern B of *P. s. pv. syringae* isolates from 'Coscia'; lanes 10, 11, 12: pattern C of *P. s. pv. syringae* isolates from 'Coscia' buds and 'Tosca' leaves; lane 13: pattern D shown by one isolate from a 'Coscia' bud.

Table 1. Genetic patterns indicated by letters, obtained with repetitive sequence PCR and BOX A1 primer of 90 fluorescent isolates from two pear cultivars showing bud and leaf necrosis. Number of isolates between brackets.

	"Coscia"	"Tosca"
Bud	A (18), C (5), D (1)	A (17)
Leaf	B (25)	A (12), C (12)

Table 2. Characterization of 90 fluorescent isolates of patterns A, B, C and D, obtained from two pear cultivars showing bud and leaf necrosis. The pathogenicity test scores refer to results shown by at least 75% of the isolates.

Pattern n° isolates	Lemon fruit	Pear leaves	Lilac leaves	<i>syxB</i>	Ice-nucleation
A (47)	+	+	++	+ (8), -(39)	+ (45), - (2)
B (25)	+	+	++	+ (9), -(16)	+ (21), - (4)
C (17)	+++	+++	++	+ (17)	+ (4), -(13)
D (1)	+++	+++	+	+	-

+, lesion enlargement on 60% of inoculated sites.

++, lesion enlargement on 70% of inoculated sites.

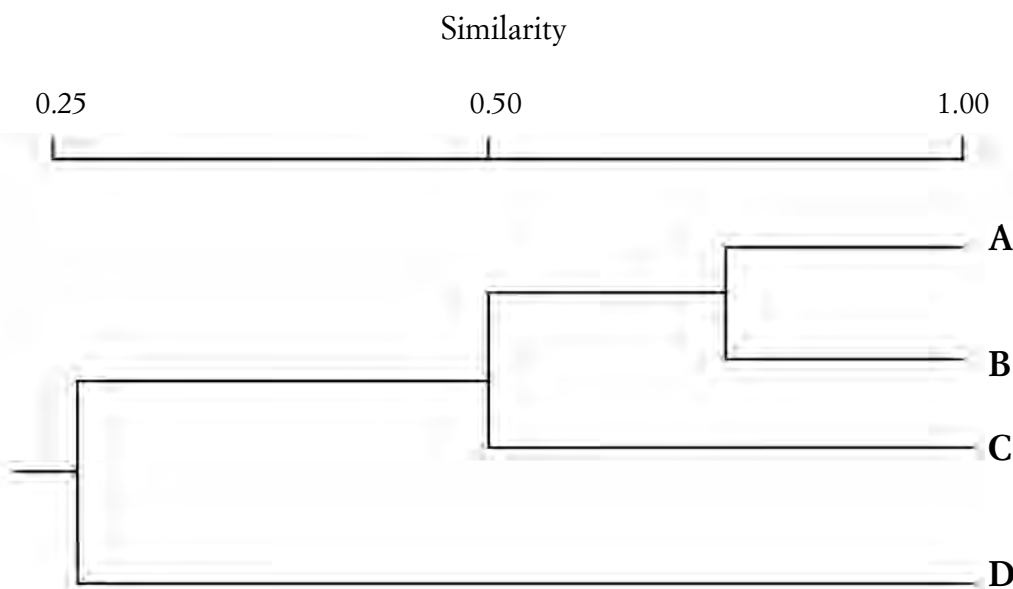
+++, lesion enlargement on more than 90% of inoculated sites.

4°C. The amplifications were performed in duplicate. PCR products were separated by gel electrophoresis on 1.5% agarose (Seakem, Rockland, ME, USA) in 1 x TAE buffer at 5 V/cm over 5 h, stained with ethidium bromide, visualized under a Spectroline UV transilluminator (Spectronic Corporation, Westburg, NY, USA) and photographed with Polaroid film type 55. Banding patterns obtained after repetitive-PCR were used to build up a binary matrix which was compared by cluster analysis using the UPGMA (Unweighted Pair-Group Method using Averages) method and Dice's coefficient (Dice, 1945). NTSYS software PC version, 2.11j (Exeter Software, New York, NY, USA) was used. Dendrograms were constructed with the tree display option (TREE). A cophenetic value matrix was calculated using the COPH option and compared with the original similarity matrix using the MXCOMP option to test the goodness-of-fit of the cluster analysis.

The fluorescent colonies obtained after 48 h of incu-

bation on KB were tested biochemically as described above. Ninety isolates belonging to LOPAT group Ia were obtained: 41 from 'Tosca' (24 from leaves and 17 from buds) and 49 from 'Coscia' (25 from leaves and 24 from buds) (Table 1). All isolates were levan-positive, oxidase-negative, potato soft rot-negative, arginine dihydrolase-negative, and tobacco hypersensitivity-positive. Moreover, all isolates showed oxidative metabolism of glucose, hydrolyzed aesculin and arbutin and liquefied gelatin but did not show tyrosinase activity. These isolates were considered as putative *P. s. pv. syringae* and were further assessed.

Repetitive sequence PCR using BOX A1 primer yielded reproducible bands ranging in size from 200 to 1.600 bp. For the UPGMA analysis a total of 14 clearly resolved bands were scored. A cophenetic value of > 0.90 was determined for the similarity matrix indicating a high goodness-of-fit for the cluster analysis. A representative gel is shown in Fig. 1.

**Fig. 2.** Dendrogram of genetic relationships of the BOX-PCR fingerprint patterns generated by 90 fluorescent isolates from pear cultivars 'Coscia' and 'Tosca' showing bud and leaf necrosis. Cluster analysis was performed using UPGMA and Dice's coefficients. Patterns A, B and C represent 89 isolates.

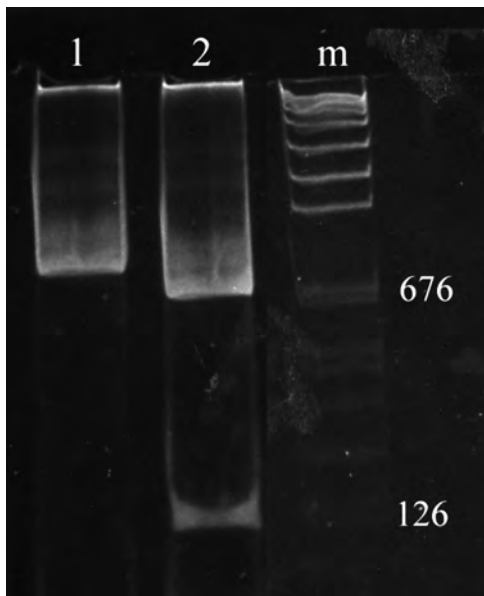


Fig. 3. Acrylamide gel showing RFLP analysis of the *syrB* gene using *SalI* restriction enzyme. m: molecular size marker in base pairs (pGem, Promega). Lane 1: *P. s. pv. syringae* B 301 showing the non-restricted 752 bp band of the *syrB* gene (group 1 of Sorensen *et al.*, 1988); lane 2: *P. s. pv. syringae* ISF CF1 showing, upon restriction, two bands of 131 and 621 bp (group 2 of Sorensen *et al.*, 1988). All the *P. s. pv. syringae* isolates of the present study possessing the *syrB* gene belong to group 2 of Sorensen *et al.*, 1988.

UPGMA analysis showed that the isolates fell into four different groups, A, B, C, D (Table 1 and Fig. 2). Pattern A was commonest and it was shown by 47 isolates obtained from necrotic lesions on buds of both cultivars and from leaf spots on 'Tosca'. Pattern B was observed only for the 25 isolates from necrotic leaves of 'Coscia'. Only one isolate was found for pattern D, which showed 25% similarity with the other isolates (Fig. 2). Comparison of these patterns with previous ones obtained with BOX A1 101 *P. s. pv. syringae* strains isolated from woody and herbaceous hosts (Scortichini *et al.*, 2003), showed that pattern A was the same as pattern 7, and pattern C the same as pattern 11 (Fig. 1) both including strains from pear.

Patterns B and D seem different from patterns previously found (Scortichini *et al.*, 2003). Interestingly, pattern 1, including three *P. s. pv. syringae* strains from pear (Scortichini *et al.*, 2003), was not found in the present study. The pathogenicity test results and presence of the *syrB* gene and ice-nucleation activity are shown in Table 2. C and D isolates were seen to be more aggressive than A and B on lemon fruits and pear leaves, whereas A, B and C were more aggressive than D on lilac leaves. Thirty-five isolates out of 90 had the *syrB* gene that, upon the restriction with *SalI* enzyme, showed a single pattern corresponding to group 2 of Sorensen *et al.* (1998), characterized by the presence of

two bands of 131 and 621 bp (Fig. 3). Interestingly, all the C isolates which were very aggressive on all plants tested, had the *syrB* gene. By contrast, most A and B isolates less virulent on lemon fruit and pear leaves, did not possess the *syrB* gene (Table 2).

Seventy isolates out of 90, showed ice-nucleation activity. Most of the A and B isolates were ice-nucleation positive, whereas only four of 17 C isolates were positive; the D was negative.

Collectively, the results indicate that all the 89 A, B and C isolates would belong to *P. s. pv. syringae*. The D isolate of pattern might be a deviating *P. s. pv. syringae* or another unidentified taxon.

This study confirms that considerable genetic variability exists within the *P. s. pv. syringae* complex, and is also present when the pathogen(s) attacks solely one host plant, *Pyrus communis* in this case. In fact, we found four different genomic BOX-PCR patterns and two of them, A and C, seem identical to two of the three patterns shown by 11 other *P. s. pv. syringae* strains from pear previously assessed in the same way (Scortichini *et al.*, 2003). Remarkably, the C isolates showed an identical pattern to a strain, BPIC 692, isolated in Greece on 1962. This would indicate that some populations of the pathogen might be strictly associated with pear. We also found two new patterns and one of them, B, seems associated with the leaf spot symptom on cv Coscia. This study also confirms previous findings indicating that not all *P. s. pv. syringae* strains isolated from pear trees showing symptoms of bacterial blast show ice-nucleation activity (Gross *et al.*, 1984; Montesinos and Vilarde, 1991) or have the *syrB* gene (Zeller *et al.*, 1997). However, isolates possessing the *syrB* gene seem to be more aggressive in host tests than strains without *syrB*. As previously observed (Gross *et al.*, 1984), the *P. s. pv. syringae* isolates did not show pathogenicity restricted only to pear. However, the specific role that any single bacterial lineage of *P. s. pv. syringae* plays during infection of pear buds and leaves is still not established.

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