A Detached Leaf Assay to Evaluate Virulence and Pathogenicity of Strains of *Pseudomonas syringae* pv. *syringae* on Pear

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ABSTRACT

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Results of the inoculation of 19 strains of *Pseudomonas syringae* pv. *syringae* on different plant material, including pear seedlings (*Pyrus communis*), detached leaf of pear seedlings, in vitro microcuttings, and detached leaf of in vitro microcuttings, pointed out the high susceptibility of in vitro microcuttings. Four groups of virulence were distinguished among the 19 strains. Virulent strains developed black and progressive lesions on leaves either detached or not. Weakly virulent strains induced limited necrotic spots around the inoculation point, only when a high inoculum concentration (10^8 cfu/ml) was used. No symptoms on any plant material were observed for avirulent strains. These results were correlated with those obtained after inoculation of immature pear fruits and by inoculation of blossoms in the orchard. The detached leaf assay described herein is a very useful laboratory test to check the virulence of a high number of strains, because symptoms appear within 48 hr after inoculation.

Bacterial blast of pear (*Pyrus communis* L.), first described in England (1), has been reported in Italy (11), France (19,24), and Spain (22) as well as in the United States (10,25), Canada (21), Chili (5), South Africa (7,20), New Zealand (9), and Australia (12). The disease probably occurs in all the temperate areas of the world.

Pseudomonas syringae pv. *syringae* Dye et al (*Pseudomonas syringae* van Hall), is known to cause a wide range of symptoms on pear, including a bud and blossom blast, which causes significant loss of fruits (6,7,23,24,27,28). Necrosis of twigs, necrotic spots on leaves, and papyraceous cankers on trunks and branches are the other common symptoms of the disease. *P. s. syringae* overwinters on pear trees between the scales of dormant buds. During the growing season, large epiphytic populations of the pathogen may be found on apparently healthy flowers, leaves, and fruits (17,19). These epiphytic populations are believed to initiate the disease under favorable conditions, but the pathogenicity of strains of *P. s. syringae* recovered from pear trees at various times of the year is unknown. The pathogenicity of *P. s. syringae* to pear is difficult to test, because only very young tissues are susceptible. Moreover, humid and cool environmental conditions are required for symptom expression. Inoculation of immature pear fruit is the most commonly used method to test pathogenicity of *P. s. syringae* on pear (3,13,26), but such fruits are available only for a short period of time each year.

Below, we describe a new test to check the virulence of strains of *P. s. syringae* recovered from pear trees. Assays with detached leaves, either from seedlings or from in vitro microcuttings, were performed.

MATERIALS AND METHODS

Bacteria. The 19 strains of *P. s. syringae*, each originally isolated from pear, were obtained from various sources (Table 1). All bacterial strains were restreaked for colony uniformity on King's medium B (15). They were identified as *P. s. syringae* according to Lelliott et al (16) and Hildebrand et al (14), although some strains differed from the type strain for production of levan on sucrose medium (strain JH407), hydrolysis of gelatin (strain 234), deoxyribonuclease activity (strains 234, 220 and CFBP 1147), and utilization of erythritol and inositol (strain 234) or lactate (strain JH407) as a sole carbon source. Moreover, the five strains isolated from Spain (EUPG strains) were able to use D(-) tartrate as a sole carbon source. All cultures were stored at −80 C in LP broth (yeast extract 7 g/L, bactopeptone 7 g/L, pH 7) supplemented with sterile glycerol (30%, v/v). Suspensions in sterile distilled water were prepared from bacterial cultures incubated at 17 C for 24 hr and adjusted to the required concentrations (i.e., 10^9, 10^8, or 10^6 cfu/ml).

Plant material. Three types of plant material were used in this experiment: 1) 8-yr-old pear trees (cv. Williams) from an experimental orchard, 2) actively growing young seedlings (8- to 10-leaf stage) cv. Fieudière obtained from seeds previously treated at 4 C for 40 days in wet conditions to break the dormancy, and 3) in vitro unrooted microcuttings of cv. Doyenné du Comice grown on agar medium according to Brisset et al (4). Seedlings of Fieudière were grown in a greenhouse in which the maximum temperature was 22 C. During winter, artificial light was supplied (for 16 hr each day) to maintain active growth of the seedlings. Microcuttings of Doyenné du Comice were grown in a growth chamber (16 hr light with 3000 lux, 26.9 μmol/m^2 s, 400–700 nm at 24 C, and 8 hr of darkness at 20 C). Tips of actively growing stems (about 1.5 cm long) were subcultured on the same medium in large test tubes 3 wk before inoculation.

Plant inoculation. Pear seedlings were inoculated with a 20 μl drop of bacterial suspension that was deposited on a fresh wound made on the midrib of the leaf. Nineteen strains of *P. s. syringae* were used and 10 leaves were inoculated per strain. After inoculation, pear seedlings were placed at 20 C in a growth chamber. Symptoms appeared within 1 wk after inoculation. The frequency of infected leaves was recorded.

The midrib of the two youngest, fully unfolded leaves of the microcuttings was punctured with teeth-nosed dissecting forceps that had been dipped into bacterial suspension (10^8 or 10^6 cfu/ml). Three replicates were performed for each strain. Inoculated plants were then placed back in the growth chamber. The proportion of blasted plants was recorded 1 wk later.

Young leaves collected from Williams trees in an orchard or from seedlings of Fieudière were dipped into a solution of sodium hypochlorite (1% active hypochlorite) for 5 min and then rinsed three times in sterile distilled water. Leaves from the microcuttings were used without disinfection. All detached leaves were inoculated by the same procedure (i.e., the midvein was cut with a scalpel and a 2-μl drop of 10^6 cfu/ml, 10^8 cfu/ml, or sterile distilled water was deposited on the wound). Ten leaves were inoculated with each strain and placed on a sterile filter paper disc located on water agar in sterile petri dishes (10 g/L agar). The petri dishes were sealed with a piece of parafilm and incubated in the growth chamber (16 hr of light at 24 C; 8 hr of darkness at 20 C). The frequency of leaves showing necrotic lesions was estimated 48 hr after inoculation.

Seven strains selected from those producing either positive or negative results in the pathogenicity tests de-

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scribed above were inoculated to seedlings of Fieudière by vacuum infiltration. Foliage of the seedlings was dipped in a 400-ml beaker containing 10^8 cfu/ml and then exposed to a vacuum (8.10^3 Pa) for 5 min. The vacuum was suddenly broken so that the bacterial suspension was drawn into the intercellular spaces of the leaves. The pear seedlings were then placed in an environmental controlled chamber set at 20 C with 16 hr of light at 3000 lux, 8 hr of darkness, and relative humidity 100%. Symptoms appeared 1 wk after inoculation. The number of leaves showing at least one lesion per plant was noted.

Immature pear fruits were inoculated with eight of 13 strains that produced positive results in the leaf assay and all the strains that were negative in that assay. The fruits, cv. Williams, were picked approximately 5 wk after petal fall and stored at 4 C for 1 wk. Before inoculation, they were washed for 10 min in a solution containing sodium hypochlorite (1% active hypochlorite) and 0.25% (v/v) of a mild detergent and rinsed three times with a large volume of sterile distilled water, and then the surface was allowed to dry at room temperature. Each fruit was punctured four times with a needle previously smeared with a bacterial colony; three pear fruits were inoculated per strain. Control fruits were inoculated with a needle dipped into sterile distilled water. The fruits were placed on a disinfected test tube rack, incubated for 3 days at 26 C in plastic boxes lined with moist paper towels, and then observed for internal and external disease symptoms.

Trees in an orchard were inoculated with two strains that were negative and two that were positive for pathogenicity in the laboratory test described above. Blossoms of cv. Williams in full bloom were gently sprayed until runoff with 10^8 cfu/ml. Twenty blossoms were sprayed for each strain. The number of blossoms showing at least one blasted flower and the proportion of blasted flowers showing symptoms within blossoms were noted 10 days after inoculation. Statistical analyses were performed using an arcsine transformation for the data expressed as percentages. Variance homogeneity was checked with a Bartlett's test (2). An ANOVA was performed and means were compared using Duncan's multiple range test (8).

Table 1. List and origin of bacterial strains

Pseudomonas syringae pv. *syringae*	Date of isolation	Location of isolation	Organ of isolation[a]	Source[b]
CFBP1391	1959	U.K.	...[c]	CFBP
CFBP311	1962	France	...	CFBP
CFBP1762	1962	U.K.	...	CFBP
CFBP675	1965	France (Savoie)	Infected fruit	CFBP
CFBP1147	1965	France	Infected fruit	CFBP
CFBP1587	1974	France	...	CFBP
2027-37	1983	France (Angers)	Leaf (D)	CM
2027-38	1983	France (Angers)	Leaf (D)	CM
EUPG16	1987	Spain (Girona)	Bud (D)	UPC
EUPG94	1987	Spain (Girona)	Flower (D)	UPC
EUPGLL3Y	1987	Spain (Lerida)	Bud (D)	UPC
EUPGLL3H	1987	Spain (Lerida)	Leaf (D)	UPC
EUPGLL7	1987	Spain (Lerida)	Leaf (D)	UPC
JH407	1988	France (Angers)	Leaf	This work
220	1989	France (Angers)	Bud (H)	This work
221	1989	France (Angers)	Bud (H)	This work
222	1989	France (Angers)	Bud (H)	This work
234	1989	France (Angers)	Bud (H)	This work
SY1	1989	France (Angers)	Leaf (D)	This work

[a] D = from diseased orchard; H = from healthy orchard.
[b] CFBP = Collection francaise de Bactéries Phytopathogènes, INRA Angers, France. CM = C. Manceau, 1984 (18). UPC = E. Montesinos, UPC Girona, Spain.
[c] Unknown.

RESULTS

Inoculation of seedlings (Table 2). For seven of the 19 strains, black and pro-

Table 2. Pathogenicity of strains of *Pseudomonas syringae* pv. *syringae* on different pear materials[a]

	Pear seedling cv. Feudière					In vitro microcutting cv. Doyenné du Comice				
	On leaves in situ			On detached leaves		In test tube		On detached leaves		Immature pear fruit cv. Williams
Bacterial strains	10^6 cfu/ml	10^8 cfu/ml	10^9 cfu/ml	10^6 cfu/ml	10^8 cfu/ml	10^6 cfu/ml	10^8 cfu/ml	10^6 cfu/ml	10^8 cfu/ml	10^8 cfu/ml
2027-37	+	+	+	+	+	+	+	+	+	+
2027-38	+	+	+	+	+	+	+	+	+	+
CFBP1762	+	+	+	+	+	+	+	+	+	+
EUPGLL7	+	+	+	+	+	+	+	+	+	+
EUPGLL3H	+	+	+	+	+	+	+	+	+	+
EUPGLL3Y	+	+	+	+	+	+	+	+	+	+
EUPG94	+	+	+	+	+	+	+	+	+	+
CFBP1587	−	+	−	−	+	+	+	+	+	NT
CFBP1391	−	+	−	−	+	+	+	+	+	NT
CFBP675	−	+	−	−	+	+	+	+	+	NT
222	−	+	−	−	+	+	+	+	+	NT
SY1	−	NS	−	−	NS	+	+	−	NS	NT
220	−	NS	−	−	NS	+	+	−	NS	NT
221	−	NS	−	−	NS	+	+	−	NS	+
CFBP311	−	−	−	−	−	−	−	−	−	−
CFBP1147	−	−	−	−	−	−	−	−	−	−
234	−	−	−	−	−	−	−	−	−	−
JH407	−	−	−	−	−	−	−	−	−	−
EUPG16	−	−	−	−	−	−	−	−	−	−
Sterile water	−	−	−	−	−	−	−	−	−	−

[a] Results were obtained from inoculation by wounding the limb and the midrib of leaves. + = 40–100% of inoculated leaves or fruits show progressing lesions as shown on Fig. 1B. NS = At least one leaf showing a necrotic spot limited to the inoculation point as shown on Fig. 1E; − = no reaction on any inoculated leaves. NT = not tested.

gressive lesions started to develop in the veins and in the lamina of the inoculated leaf within a few days, even at the lowest inoculum concentration (10^6 cfu/ml). With four strains, such results were obtained only with the highest inoculated concentration (10^8 cfu/ml) (Fig. 1B and D). Three strains produced only a necrotic spot limited to the inoculation point (Fig. 1E), and five strains were not pathogenic, i.e., no symptoms were observed at either inoculum concentration (Fig. 1F). No significant differences were observed between in situ and detached leaves.

Three (2027.37, 2027.38, and CFBP 1762) among the seven strains inoculated by vacuum infiltration led to the development of lamina necrosis and finally to the desiccation of all leaves within 7 days after inoculation (Fig. 1A). With one strain (CFBP 1587), leaf desiccation occurred only with the highest inoculum concentration. Strains CFBP 311, CFBP 675, and JH 407 induced no symptom development.

Inoculation of in vitro microcuttings (Table 2). Progressive lesions developed on microcuttings that were inoculated on the leaf in a test tube, with the 14 strains that were positive on seedlings as mentioned above. Use of both inoculum concentrations led to similar results. On detached leaves such symptoms were observed only with 11 strains (Fig. 1G); the last three strains caused necrotic spots but only with the highest inoculum concentration. The five strains that were nonpathogenic on seedlings failed to cause symptoms on the microcuttings.

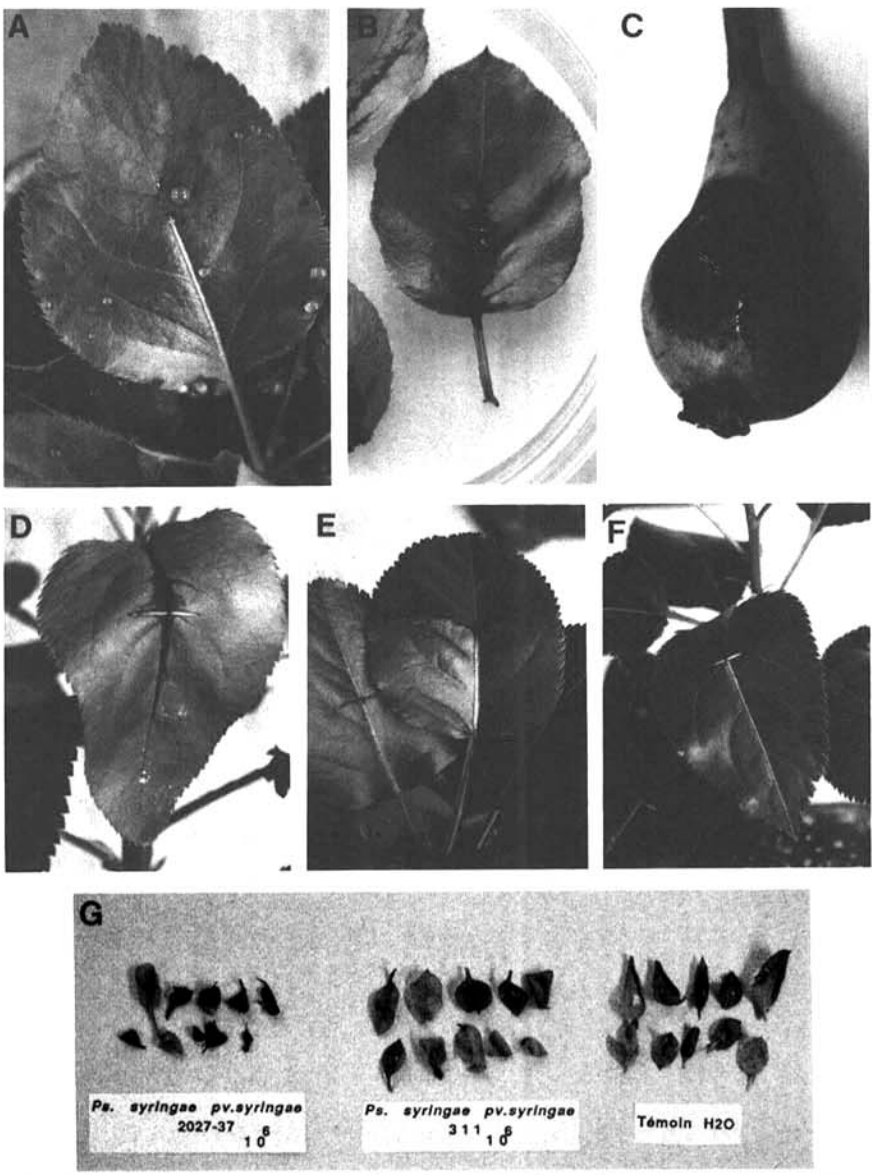

Fig. 1. Symptoms on pear after inoculation with strains of *Pseudomonas syringae* pv. *syringae*: **(A)** Leaf from seedling inoculated by vacuum infiltration, **(B)** detached leaf of pear seedling inoculated with 10^8 cfu/ml, **(C)** immature fruit, **(D)** leaf from seedling inoculated with 10^8 cfu/ml of pathogenic strain, **(E)** leaf from seedling inoculated with 10^8 cfu/ml of slightly virulent strain (necrosis limited to puncture point), **(F)** leaf from seedling inoculated with avirulent strain, and **(G)** detached leaves of in vitro microcuttings.

Inoculation on immature pear fruit (Table 2). Among the 13 tested strains, only the eight that were previously pathogenic in the leaf assay caused black and sunken progressive lesions (Fig. 1C). No internal or external discoloration occurred after injection of sterile distilled water or of any suspension of the last five strains.

Flower inoculation in the orchard (Table 3). Lesions on calyx cups or blast of entire flowers occurred 10 days after inoculation on 95% of blossoms inoculated with strain 2027.37 or CFBP 1762 and on 35% of blossoms inoculated with strain CFBP 311. However, strain JH 407 did not induce symptoms and, apparently, is not pathogenic in pear.

The pathogenicity tests performed in the laboratory with wounded leaves (detached or not from seedlings or microcuttings) allowed us to separate the 19 strains into four groups. The first group contains seven strains that were pathogenic on seedlings, microcuttings at all inoculum concentrations, and immature fruits. Two of these (2027.37 and CFBP 1762) caused a severe blossom blast in inoculated trees in the orchard. The second group included four strains that did not cause, at the lowest inoculum concentration (10^6 cfu/ml), symptoms on leaves of seedlings but did cause a necrosis on microcuttings. Three strains in the third group caused typical symptoms in intact microcuttings with both inoculum concentrations, but with seedlings or detached leaves from the microcuttings, a necrotic spot limited to the inoculation point formed only after inoculation with the higher inoculum concentration. When injected in immature fruit, one of these strains (221) caused typical lesions. The fourth group included five strains that did not cause any symptom in plant material: strains CFBP 311 and JH 407 belonged to this group. When sprayed in orchard, strain JH 407 did not cause any blossom blast, whereas 35% of blossom blast developed among flowers sprayed with CFBP 311. However, these symptoms could be attributable to natural inoculum.

Table 3. Disease incidence on pear flowers (cv. Williams) in the orchard 10 days after inoculation with a bacterial suspension (10^8 cfu/ml) of four strains of *Pseudomonas syringae* pv. *syringae*

Bacterial strains	Proportion of blossoms with symptoms[y]	Means % of diseased flowers[z]
2027-37	18/19 a	40 a
CFBP1762	19/20 a	42 a
CFBP311	7/20 b	21 b
JH407	0/20	

[y] Means followed by the same letter do not differ significantly ($P \leq 0.05$), according to Duncan's multiple range test.

[z] Diseased flowers per blossom among blossoms showing symptoms.

DISCUSSION

The inoculation of small plants grown under controlled conditions led to a rapid response. Each of the types of inoculation led to the same differentiation of strains according to their virulence on pear. Vacuum infiltration or the wounding of the midvein of leaves and addition of a cell suspension most consistently resulted in symptoms. Necrotic lesions along the vein observed in the leaf assay (Fig. 1) are not exactly typical field symptoms of bacterial blast of pear, but the progressive increase in the necrosis observed in most cases differentiated it clearly from a hypersensitive reaction. Common symptoms caused by *P. s. syringae* in leaves are necrotic spots in the limb frequently surrounded by a narrow, reddish halo. However, the inoculation of wounded midribs has to be considered as valid in assessing pathogenicity since its results were, for most of the strains included in this study, correlated with those observed after inoculation on immature fruit. This method has been traditionally used for checking pathogenicity of a strain of *P. s. syringae* from pear (3). Detached leaves appear to be much more convenient to use because fresh leaves are available throughout the growing season and can be obtained from seedlings grown in the greenhouse during the winter.

With four selected strains, results of the leaf assay were correlated with those obtained by field inoculation. However, one strain, CFBP 311, was not pathogenic in leaves but seemed to cause limited symptoms on blossoms in the orchard. This could be because of either the weak virulence of a strain that cannot express symptoms on plant material other than flowers or because of disease caused by a virulent resident strain occurring in the orchard. The negative results obtained with strain CFBP 311 inoculated to the very susceptible microcuttings is evidence that the strain was nonpathogenic and that the blossom blast in the orchard originated from natural infections. Microcuttings (cv. Doyenné du Comice) were more susceptible to *P. s. syringae* than pear seedlings (cv. Fieudière), because inoculation at the lower concentration of weakly virulent strains caused symptoms on the microcuttings but not on the seedlings. Three strains (220, 221, SY1), which caused very weak reactions on pear seedlings and immature pear fruits, caused a typical necrotic lesion on these microcuttings, even at low inoculum concentrations. This high susceptibility might be explained by the continuous growth of the very young plants in the test tubes. Furthermore, the difference in susceptibility to *P. s. syringae* between seedlings cv. Fieudière and microcuttings of Doyenné du Comice was probably attributable to the type of culture and not to the cultivar, because the inoculation of detached leaves of several cultivars (Passe Crassane, Conference, Williams, Beurré Hardy) did not show any differences (*unpublished data*). Thus, microcuttings could also be a suitable material for pathogenicity test of strains of *P. s. syringae*. The high susceptibility of microcuttings grown in tubes to *Erwinia amylovora* was also described by Brisset et al (4).

Variations in virulence among the strains of *P. s. syringae* were pointed out. A part of the tested strains came from the CFBP (Collection Française de Bactéries Phytopatogènes) and were kept lyophilized for more than 30 yr. This conservation method does not seem to affect pathogenicity of bacteria since the CFBP strains were distributed in all groups of virulence, as were the fresh isolates. The test described in this work could be used easily to check the pathogenicity of strains before storage.

No phenotypic characters were correlated with virulence in the bacterial strains. Except EUPG16, all new strains isolated from an orchard with symptoms (2027-37, 2027-38, and EUPG strains) caused the most severe symptoms on all plant materials. In contrast, the strains recovered from the buds or leaf surfaces of healthy trees were weakly virulent or avirulent. Thus, the epiphytic population on healthy trees might be composed mostly of weakly virulent or avirulent strains of the pathovar *P. s. syringae*. In the diseased orchard, the frequency of virulent cells within the epiphytic population might be much higher than in the healthy one.

We conclude that the detached leaf assay in petri dishes is the easiest and most reliable test for determining the pathogenicity of *P. s. syringae* on pear. It could be especially convenient for testing numerous strains in epidemiological studies or in screening the pathogenicity of colonies after mutagenesis. It could be replaced, but less conveniently, by the inoculation of microcuttings grown in test tubes.

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