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Susceptibility of European pear cultivars to *Pseudomonas syringae* pv. *syringae* using immature fruit and detached leaf assays

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Abstract

The susceptibility of thirty-three pear cultivars and two pear rootstocks to four virulent strains of *Pseudomonas syringae* pv. *syringae* was evaluated by inoculating detached immature fruits and young leaves. The four strains were similarly virulent and did not show cultivar specificity although they were isolated from different pear cultivars and exhibited different biochemical profiles. The most frequently planted pear cultivars, Conference, Abate Fetel, General Leclerc, Williams, D. Comice, El Dorado, Alexandrine, B. Anjou, Passe Crassane and the rootstock OHxF 333 were susceptible to *P. syringae* pv. *syringae*. Maximal severity values were obtained on 'Preguystar' leaves (about 90%). The rootstock Winter Nelis was less susceptible. Results with immature fruit and detached leaf assays agreed with field observations on cultivar susceptibility to bacterial blast. However, the detached leaf test gave a more accurate prediction and has the advantages that symptoms develop quickly (48 h), and leaves are available for a longer period of time than fruits. This method is proposed as a rapid and reproducible screening system of cultivar susceptibility to bacterial blast of pear.

Introduction

Pseudomonas syringae pv. *syringae* infects a wide range of deciduous fruit trees such as pear, cherry, peach and plum as well as other woody plant species (Lelliot and Stead, 1987). On pear, *P. syringae* pv. *syringae* causes bacterial blast, a disease that results in economic losses in pear production areas around the world (Barker and Grove, 1914; Wilson, 1934; McKeen, 1955; Dye, 1956; Ridé and Sutic, 1957; Ercolani, 1967; English et al., 1980; Fahy and Lloyd, 1983; Montesinos and Vilardell, 1988; Manceau et al., 1990). Symptoms are variable and depend on the organ or tissue affected and the phenology of the host plant. Papyraceous cankers appear on trunks and branches late in autumn and during winter. Necrosis of buds, blossoms, immature fruits, twigs and leaves are observed in periods of cool wet weather during bloom and post-bloom stages (Jones and Aldwincle, 1990). *P. syringae* pv. *syringae* overwinters on dormant buds (Montesinos and Vilardell, 1988), and during the growing season, large epiphytic populations of the pathogen occur on apparently healthy flowers, leaves and fruits (Luisetti and Paulin, 1972; Mansvelt and Hatting, 1986). These epiphytic populations initiate subsequent disease outbreaks (Latorre and Jones, 1979; Wimalajeewa and Flett, 1985; Ross and Hatting, 1986; Mansvelt and Hatting, 1988).

Control of bacterial blast is based on treatments with copper compounds and antibiotics (Jones and Aldwinckle, 1990), depending on the specific legal restrictions of each country. The phosphonate derivatives fosetyl-Al and potassium phosphonate have a

moderate efficacy (Moragrega et al., 1998). However, the levels of disease control are limited by the low efficacy of available products, the phytotoxicity to the plant or the resistance development of the pathogen (Andersen et al., 1991). Therefore, management of the disease has to be achieved within an integrated disease control approach, including the employment of resistant or tolerant pear cultivars. However, there is little information about the susceptibility of pear cultivars to *P. syringae* pv. *syringae*, and most data come from field observations on the most frequently grown cultivars (English et al., 1980). Little data are available on virulence of *P. syringae* pv. *syringae* isolates on pear.

Although field experiments to obtain information about genetic susceptibility of pear to bacterial blast is a good system, this approach can be affected by the availability of natural inoculum and its heterogeneous distribution, the changing environmental conditions and the phenological stage of the host plant. To prevent the uncertainty of natural inoculum availability, evaluation of susceptibility can be carried out under field conditions by means of artificial inoculation of *P. syringae* and subsequent observation of disease symptoms. However, this method is time-consuming and the results are highly influenced by weather conditions and by the method of inoculation. To avoid these problems, host resistance or tolerance to the pathogen can be determined by inoculation of plants or plant organs followed by disease expression under controlled environment conditions. *Ex vivo* methods can be used as rapid screening systems for both, determination of strain virulence and cultivar or host susceptibility, provided that they only reflect host susceptibility at the genetic level, and that results are correlated with field information to derive agronomic applications. Several reports describe methods for evaluation of pathogenicity or virulence of *P. syringae* pv. *syringae* consisting of inoculations on immature fruits (Endert and Ritchie, 1984; Gross et al., 1984), detached leaves (Yessad et al., 1992) or tissue culture (Scheck et al., 1997).

The present study assesed, under controlled environment conditions, the susceptibility of pear cultivars and rootstocks to several representative strains of *P. syringae* pv. *syringae*. *Ex vivo* methods based on pathogen inoculation on immature fruits and detached young leaves were used to compare pear cultivar susceptibility and pathogen strain virulence.

Materials and methods

Plant material

Young leaves and immature fruits were collected from the pear cultivar collection of Mas Badia Agricultural Experiment Station (Girona, Spain). A set of 33 European pear cultivars and two pear rootstocks were evaluated. Cultivars used were Abate Fetel (provided by Instituto Agrario di San Michele all Adige, Trento, Italy); Alexandrine Douillard, Beurre Anjou, Beurre Hardy, Beurre Hardenpont, Blanquilla, Bonne Louise, Doyenne du Comice, El Dorado, Epine du Mas, Flor d'hivern, General Leclerc, Grand Champion, Highland, Kaiser, Magness, Maxine, Morettini, Packham's Triumph, Passe Crassane, Pierre Corneille, President Drouard, President Heron, Red Rogne, Rocha, Rogue Red, and Star (provided by Servicio de Investigación Agraria, S.I.A., Montañana, Spain); Conference INFEL 415A (provided by D.L. SA, Davodeau Ligonniere, Angers, France); Devoe and Williams (provided by CERTIPLANT, Mollerusa, Spain); Preguystar and Super Comice (provided by Pépinières et Roserales Georges Delbard, Malicorne, France); and Starking (provided by Station d'Experimentation Arboricole P.A.C.A., Mallemort, France). Two widely used pear rootstocks: OHxF 333 and Winter Nelis (provided by S.I.A., Montañana, Spain) were also included.

Young leaves, consisting of the latest completely developed leaf within a shoot, were collected at the same phenological stage for all cultivars, corresponding to two weeks after the full bloom stage. Leaves were picked up the same day that the inoculation was performed and were stored at 4 °C under high humidity conditions until inoculation. Immature fruits were collected 6 weeks after the fruit set stage and maintained at 4 °C until inoculation.

Bacteria

Four strains of *P. syringae* pv. *syringae* isolated from pear were used (Table 1). The strains were selected because of their different cultivar and organ of origin and geographical location (Moragrega, 1997). Strains were characterized by their biochemical profile based on the use of 11 substrates including D(−)-tartrate, 2-cetoglutarate, malonate, N-caproate, pyruvate, DL-5 aminobutirate, L-cysteine, L-leucine,

glucosamine, sarcosine and DNAase (Table 1), and by their virulence on five susceptible pear cultivars.

Lyophilized bacterial cultures were grown on King's B agar plates (King et al., 1954) for 48 h at 25 °C. Loopfuls of surface colonies were resuspended in King's B broth supplemented with sterile glycerol (20% v/v) and stored at −80 °C. These stored cultures were used for preparing fresh culture plates of each strain for fruit or leaf inoculations. All inoculations of one strain in a trial were performed with inoculum obtained from the same lyophilized tube.

Bacterial suspensions prepared in sterile distilled water from cultures grown at 25 °C for 24 h and adjusted to 10^8 cfu ml^{-1} were used in leaf inoculations. Immature pear fruit inoculations were done using higher concentrations (10^9 cfu ml^{-1}).

Leaf and fruit inoculations

Before inoculation, leaves were dipped in a solution of sodium hypochlorite (1% active hypochlorite) for 5 min, rinsed three times in sterile distilled water and excess water removed with a filter paper. Detached leaves were inoculated with a 20 µl drop of the bacterial suspension deposited on a fresh wound made with a scalpel on the midrib of the leaf (Yessad et al., 1992). Inoculated leaves were placed on a sterile filter paper over water agar (10 g agar l^{-1}) in sterile 15 cm long squared Petri dishes. The Petri dishes were sealed with a piece of Parafilm® and incubated in a controlled environment chamber.

Fruits were disinfected (as above) and punctured with a sterile needle previously immersed in bacterial suspension. Three inoculations were performed on each fruit. The inoculated fruits were placed on disinfected test tube racks and introduced into plastic boxes lined with moist paper towel. Inoculated leaves and fruits were incubated at 24 °C, 16 h light and 18 °C, 8 h darkness, for 3–5 days in a controlled environment chamber (Conviron PGR15, Manitoba, Canada).

Experimental design

For strain virulence experiments, immature fruits and detached young leaves of five susceptible pear cultivars (Conference, Abate Fetel, Passe Crassane, Rocha and Williams) were inoculated. Three replicates of nine fruits or leaves per replicate were inoculated with each strain. As negative controls, nine leaves of each cultivar were inoculated with sterile distilled water and nine additional leaves with the saprophytic bacterium *P. fluorescens* EPSB4. The immature fruit test was performed once, while the detached leaf assay was done twice.

Pear cultivar susceptibility was determined with both the immature fruit and the detached leaf assays. Immature fruits from 22 pear cultivars were inoculated with strain CFBP3077. Detached young leaves from 33 pear cultivars and two pear rootstocks were inoculated with strains CFBP3077, EPS94, EPSMV4 and EPSLL3Y. Since the four strains are similarly highly virulent, each strain inoculation experiment was considered as a replicate. Each experiment consisted of three replicates of nine fruits or leaves per replicate. The same non-inoculated or saprophyte-inoculated controls described for strain virulence experiments were used.

Strain virulence and cultivar susceptibility experiments were designed as a split-plot with several factors (strain, cultivar, type of plant material and trial, depending on the experiment).

Assessment of infection severity and statistical analysis

Five severity index levels (I) were established in order to quantify the intensity of infections on fruits and leaves. The severity index ranged from 0 to 4 depending on the absence or presence of infection and its intensity according to the following scale: 0, no infection; 1, necrosis limited to the inoculation point; 2, necrosis affecting the inoculation point and the leaf midvein or presence of a necrotic area of less than 5-mm diameter in fruits; 3, necrosis expanding through the midvein and additional veins in leaves or necrotic area of 5–10-mm diameter on fruits; and 4, necrosis of more than 50% leaf surface or necrotic area higher than 10-mm diameter on fruits). Severity (S) was calculated for each treatment replicate (composed by 9 fruits or 9 leaves) according to the following formula:

$$S = \frac{\sum_{n=1}^{N} I_n}{N \cdot I_{\max}} \times 100$$

where I_n is the corresponding severity index, N is the number of inoculated leaves or fruits per replicate and $I_{\max}$ is the maximum severity index (corresponding to 4).

Analysis of variance (ANOVA) was performed with the severity values (S) per replicate using a General Linear Model (GLM) procedure (8th version, SAS

Institute Inc., Cary, N.C., U.S.A). The residuals of the data sets met the assumption of normality without any transformation. Homogeneity of variances was determined with Bartlett's test.

Results

Strain description and virulence

Differences were observed in metabolic profile among *P. syringae* pv. *syringae* strains (Table 1). Spanish strains were not able to use 2-cetoglutarate, malonate, DL-5 aminobutirate and sarcosine, while the French strain was positive for all of them. Malonate and sarcosine were only used by strain CFBP3077, pyruvate only by strain EPSMV4, and L-cysteine only by strain EPSLL3Y.

Symptoms on inoculated detached leaves were observed 48 h after inoculation and on immature fruits the symptoms were observed from 4 to 5 days after inoculation. No symptoms developed upon inoculation with the saprophyte *P. fluorescens* EPSB4, nor with sterile water. Strains CFBP3077, EPS94, EPSMV4 and EPSLL3Y caused necrosis progressing from the inoculation point on fruits and leaves of all tested pear cultivars.

The four strains behaved similarly virulent in each cultivar (Conference, Abate Fetel, Rocha, Williams and Passe Crassane) and severity values ranged from 45% to 85%, depending on the cultivar. ANOVA indicated no effect of strain ($P = 0.7645$) nor of type of plant material (leaf or fruit) ($P = 0.8981$) on severity, but a highly significant effect of pear cultivar ($P < 0.001$) and leaf experiment replicate ($P < 0.001$) was observed.

Cultivar susceptibility

The ANOVA for the susceptibility of the 35 pear cultivars to the four *P. syringae* pv. *syringae* strains using the detached leaf assay showed a significant effect of cultivar ($P < 0.001$), strain ($P < 0.001$) and interaction ($P < 0.001$). However, when strains were compared using a contrast test, no differences were observed between EPSLL3Y and CFBP3077 ($P = 0.0874$) and between EPSMV4 and EPS94 ($P = 0.2080$). The results from the immature fruit assay showed a highly significant effect of pear cultivar ($P < 0.001$) on severity.

An ANOVA was performed using only severity values from cultivars in which both, immature fruits and detached leaves were inoculated with strain CFBP3077. Results showed no effect of the type of plant material used ($P = 0.05$), but a significant effect of the interaction of cultivar and the type of plant material ($P < 0.001$). Therefore, the susceptibility of cultivars depends on the type of plant material used. However, changes in cultivar rank from fruit and leaf assays were minor. Only cv. General Leclerc ranked very different in immature fruits than in leaf inoculations, showing higher severity levels (100%) on fruits (Figures 1 and 2).

Immature fruits of 22 pear cultivars inoculated with strain CFBP3077 showed different levels of infection depending on the cultivar (Figure 1). In most cultivars progressive necrosis were induced by this strain. Fruits from cvs. General Leclerc, B. Hardy and Conference developed necrotic areas higher than 10-mm diameter in more than 70% inoculations and severity values were higher than 85%. 'Passe Crassane', 'Rogue Red', and 'Super Comice' immature fruits showed necrotic spots of 5–10-mm diameter in more than 50% inoculations and severity values for these cultivars were 67% and 75%, respectively. Fruits from the other cultivars developed progressive necrotic spots smaller than 5-mm diameter in most inoculations, and severity values were lower than 60%. None of the tested cultivars showed necrosis limited at the inoculation point, so severity values for all cultivars were higher than 30%.

Detached leaves from the 35 cultivars showed different levels of infection and a wide range of susceptibility (severity values ranging from 19% to 92%) among pear cultivars inoculated with strains CFBP3077, EPS94, EPSMV4 and EPSLL3Y (Figure 2). Maximal severity values were obtained on 'Preguystar' leaves (about 90%), which developed necrosis that expanded through the veins and, for some strains, through the leaf surface. In 26 of the 35 cultivars, most strains showed severity values from 50% to 80%, with necrosis expanding through the main and secondary leaf veins. This type of leaf necrosis was observed in cultivars Abate Fetel, Beurre Hardy, Doyenne du Comice, Conference, Grand Champion, General Leclerc, Williams, and Pierre Corneille, and the rootstock OHxF 333. Additionally, 5 of the 35 cultivars (Star, Maxine, Alexandrine Douillard, Passe Crassane and Beurre Anjou) leaf infections were moderate with severity values ranging from 30% to 50%. In these cultivars, necrosis expanded through the main vein or

Table 1. Origin and biochemical profiles of *P. syringae* pv. *syringae* strains used in the present work

Strain	Location of isolation	Host plant and organ	Specific biochemical profile										
			D(−)-tartrate	2-cetoglutarate	Malonate	N-caproate	Pyruvate	DL-5 aminobutirate	L-cysteine	L-leucine	Glucosamine	Sarcosine	DNAase
EPSMV4[1]	Girona Spain	*Pyrus communis* (General Leclerc) necrotic flower	−	−	−	−	+	−	−	−	−	−	+
EPS94[1]	Girona Spain	*Pyrus communis* (Passe Crassane) necrotic pistil	−	−	−	−	−	−	−	+	−	−	−
EPSLL3Y[1]	Lleida Spain	*Pyrus communis* (Conference) necrotic bud	−	−	−	+	−	−	+	+	−	−	+
CFBP3077[2]	France	*Pyrus communis* necrotic leaf	−	+	+	+	−	+	−	+	+	+	+

Source of strain:
[1]INTEA. University of Girona, Spain.
[2]Collection Française de Bactéries Phytopathogènes. INRA, Angers, France.

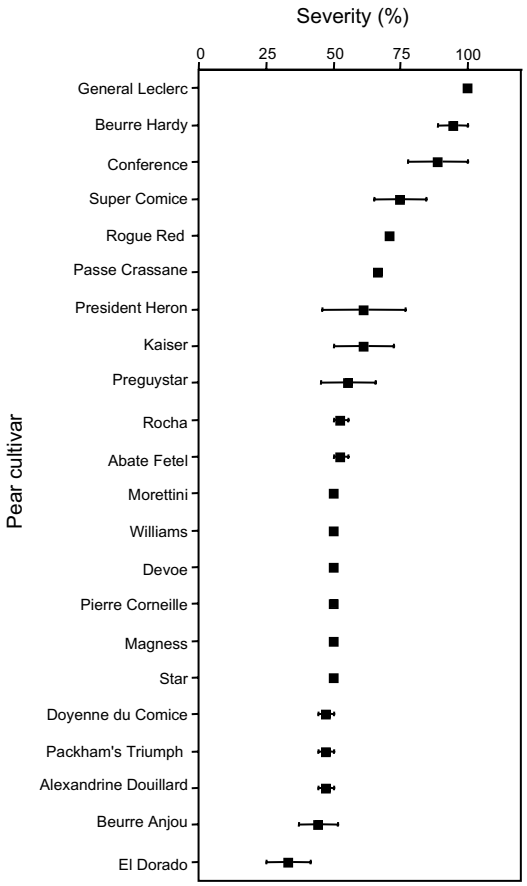

Figure 1. Severity of infections (%) caused by *P. syringae* pv. *syringae* CFBP3077 on immature fruits from 22 European pear cultivars. Severity values correspond to the mean of three replicates of nine fruits per replicate. Error bars represent the mean standard error.

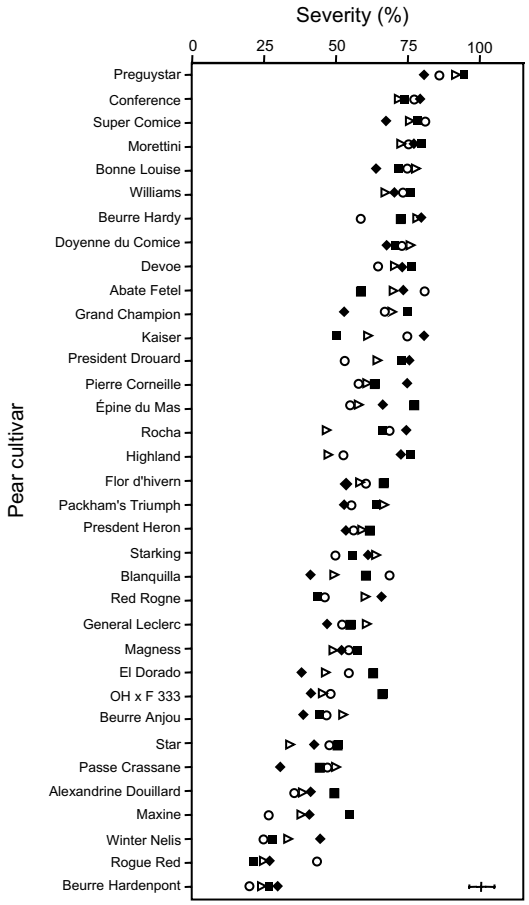

Figure 2. Severity of infections (%) caused by *P. syringae* pv. *syringae* CFBP3077 (■), EPS94 (○), EPSMV4 (▷) and EPSLL3Y (♦) on detached leaves from 33 European pear cultivars and 2 pear rootstocks. Severity values correspond to the mean of three replicates of nine fruits per replicate. Mean standard error of all data is represented on the right bottom corner. Cultivars are ranked according to the mean severity value of the four strains.

remained limited to the inoculation point. A group of less susceptible cultivars was composed of 'Beurre Hardenpont', 'Rogue Red', and the rootstock Winter Nelis, in which necrosis remained limited to the inoculation point or no necrotic tissue was observed.

Discussion

Symptoms on detached leaves were similar to those described by Yessad et al. (1992), who assessed pathogenicity of several strains and mutants of *P. syringae* pv. *syringae* on pear. Unfortunately, in the present work, the total number of cultivars evaluated by immature fruit test was less than that tested in the detached leaf assay. Although severity values were similar for most cultivars in fruit and leaf assays, a wider range of severity was obtained on detached leaf test (19–92%) than on immature fruit test (33–100%). Highest levels of severity obtained in the fruit assay could be due to the highly concentrated bacterial suspension (up to 10^9 cfu ml^{-1}) needed to express the infection symptoms. As pointed out (Yessad et al., 1992), detached young leaves appear to be more convenient for inoculations of *P. syringae* pv. *syringae* than immature fruits because young leaves are available through the growing season and also can be obtained from seedlings

or micropropagated plants grown in the greenhouse during winter. The detached leaf test has the additional advantage that symptom development is quicker (48 h) than in immature fruits (5 days).

No cultivar specificity was observed in terms of being resistant or susceptible, among the four virulent strains of *P. syringae* pv. *syringae* despite they were isolated from different pear cultivars. This fact contrasts with the race specificity of host cultivar observed in other pathovars of *P. syringae* as pv. *pisi* (Taylor, 1972; Hadwiger and Webster, 1984; Elvira-Recuenco and Taylor, 2001) or pv. *glycinea* (Fett and Jones, 1982).

In spite of the absence of cultivar specificity, the pear cultivars and the rootstock tested varied widely in susceptibility of fruit and leaf to the four highly virulent strains of *P. syringae* pv. *syringae*. Although none of cultivars can be considered resistant, cvs. Hardenpont and Rogue Red developed the lowest levels of infection. Most frequently planted and commercially accepted pear cultivars such as Conference, Abate Fetel, General Leclerc, Williams, D. Comice, El Dorado, Alexandrine, B. Anjou, Passe Crassane and OHxF 333, were found to be highly to moderately susceptible to *P. syringae* pv. *syringae* in the detached leaf and immature fruit tests.

Although no field evaluation of cultivar susceptibility to bacterial blast has been carried out by artificial inoculations of pathogen, some data from observations in commercial or experimental orchards from different countries are available. These data agree with the susceptibility range and ranking determined in the present study. In Oregon (USA) severe symptoms were observed on wood of 'Old Home'. Severe blossom blight was reported on cvs. Packam's Triumph, El Dorado and Anjou and less severe blossom blight was observed on Comice (Pscheidt, 2001). In Italy, severe infections were reported on cvs. Abate Fetel and Passe Crassane, while symptoms on 'Williams' and 'Conference' were less evident except that for wood infection, which was severe (Camele and Iacobellis, 1998). Pome fruit rootstocks M.4 and M.9 have also been reported as susceptible to *P. syringae* pv. *syringae* after artificial inoculation of nursery plants in Canada (Sholberg and Quamme, 1999).

Although results from both assays (immature fruit and detached leaf) agree with field observations on cultivar susceptibility to bacterial blast, the detached leaf assay has the advantage of rapid symptom development (48 h). In addition, leaves are available for a longer period of time than fruits.

In the present work, we provide preliminary information on virulence of four strains of *P. syringae* pv. *syringae* on pear: no races of pathogen were observed among this group of selected strains. The wide range of susceptibility observed among cultivars and the possible absence of races of pathogen may have several implications in the epidemiology of bacterial blast of pear and should be taken into account in disease management and breeding programs.

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