RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY

Evaluation of four whole-plant inoculation methods to analyze the pathogenicity of *Erwinia amylovora* under quarantine conditions

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Summary. Four methods were tested to assess the fire-blight disease response on grafted pear plants. The leaves of the plants were inoculated with *Erwinia amylovora* suspensions by pricking with clamps, cutting with scissors, local infiltration, and painting a bacterial suspension onto the leaves with a paintbrush. The effects of the inoculation methods were studied in dose-time-response experiments carried out in climate chambers under quarantine conditions. A modified Gompertz model was used to analyze the disease-time relatiobbnships and provided information on the rate of infection progression (r_g) and time delay to the start of symptoms (t_0). The disease-pathogen-dose relationships were analyzed according to a hyperbolic saturation model in which the median effective dose (ED_{50}) of the pathogen and maximum disease level (y_{max}) were determined. Localized infiltration into the leaf mesophile resulted in the early (short t_0) but slow (low r_g) development of infection whereas in leaves pricked with clamps disease symptoms developed late (long t_0) but rapidly (high r_g). Paintbrush inoculation of the plants resulted in an incubation period of medium length, a moderate rate of infection progression, and low y_{max} values. In leaves inoculated with scissors, fire-blight symptoms developed early (short t_0) and rapidly (high r_g), and with the lowest ED_{50} and the highest y_{max}. [**Int Microbiol** 2008; 11(2):111-119]

Key words: *Erwinia amylovora* · dose-time-response models · fire blight · host susceptibility · median effective dose · pathogen aggressiveness · rosaceous plants

Introduction

Fire blight is caused by the bacterium *Erwinia amylovora* (Burrill) Winslow et al. and affects species of the rosaceous family, such as pear, apple, and several ornamentals, in most temperate regions of the world [43]. The disease causes great economic losses in many European and Mediterranean countries, where commercial cultivars of apple and pear are often susceptible to fire blight. Only a few European countries are free from fire blight and are thus considered to be zones pro-

tected from the disease. In northern Spain, localized outbreaks have occurred since 1995 in apple and pear orchards and ornamental nurseries but they have been controlled and eradicated [22,23].

In the European Union, fire blight is a quarantine disease (EPPO A2 list, EU Annex II/A2 quarantine pest [15]) and in protected zones ongoing efforts have focused on preventing disease entrance and spread. Studies on *E. amylovora* and other bacteria, under natural or controlled infections, have been aimed at improving disease control based on breeding for resistance [20,24], determining host susceptibility [2,5, 10,36], analyzing pathogen strain aggressiveness [6,29,31, 35], and evaluating new chemical or biological control strategies and products [7,18,30,32,33,34,38]. In areas where the disease is extensively present, these studies are performed in the field or under greenhouse conditions, but in

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protected zones they must be done under quarantine conditions and thus artificial inoculation of the pathogen to prevent its introduction and spread. For example, inoculation of the pathogen on detached plant organs has been widely used to evaluate the aggressiveness and pathogenicity of *E. amylovora* strains and to test host resistance [6,13], but this approach may not reflect the whole-plant response against the pathogen. Several whole-plant inoculation methods, such as inoculation of *E. amylovora* on flowers, young developing leaves [21,46], and wounded tissues [14,28], have been used to monitor tissue colonization and to assess pathogen aggressiveness and host resistance [3,4]. Here the problem is that the inoculation of unwounded tissues such as flowers in whole plants often results in inconsistent and very low percentages of infection. Accordingly, many studies instead use inoculations on wounded leaves or twigs, which result in more consistent and high-level infections, although the bacteria often gain access to tissues they might not normally invade [44].

In spite of the importance of appropriate inoculation methods in fire-blight studies carried out under controlled-environment conditions, quantitative comparisons of these methods are lacking. This deficit can be resolved by the determination of dose- and time-response relationships, which have been used to analyze pathogen aggressiveness in detached fruit and flower assays [6,26] but are also suitable for comparing whole-plant inoculation methods.

The aim of this work was to compare different whole-plant inoculation methods of *E. amylovora* under quarantine laboratory conditions. Two of these methods, leaf cutting with scissors and pricking with clamps, have been described in the literature while two others, painting wounds with a bacterial suspension and local infiltration, were developed and adapted here. The applicability of these methods in the evaluation of strain aggressiveness and cultivar susceptibility is discussed.

Materials and methods

Plant material. Self-rooted pear plants cv. Conference (CAV clone) obtained by micropropagation (Agromillora Catalana SA, Barcelona, Spain) were used to evaluate the different inoculation methods. Strain aggressiveness was tested on self-rooted pear cv. Conference plants and apple cv. Fuji plants grafted on M-9 EMLA rootstocks (BC2 Infel 7690 clone from Certiplant, SL, Lleida, Spain). Plants were 2- to 3-years old, about 30 cm high, and were grown in 1-liter plastic containers filled with a commercial peatmoss/vermiculite/perlite potting mix (type BVU, Prodeasa, Girona, Spain). The susceptibility to fire blight of 12 ornamental species and cultivars of the rosaceous family was evaluated using the method in which leaves are cut with scissors. Plants were chilled during winter, pruned, and forced to bud in the greenhouse 2–3 months before the experiments were initiated. Actively growing plants were maintained in the greenhouse and fertilized once a week with a solution of 200 ppm N-P-K (20-10-20).

Bacterial strains. The inoculation methods were tested in *Erwinia amylovora* EPS101, isolated from an infected pear fruit in a commercial pear orchard of Lleida (Spain). Bacterial strains used in aggressiveness assay were obtained from the sources listed in Table 2. The inoculum consisted of bacterial suspensions of 24-h-old Luria Bertani (LB) [25] agar cultures grown at 25°C. Inoculum concentration was estimated by absorbance at 620 nm and adjusted to the desired concentration by ten-fold serial dilution with sterile distilled water. The inoculum was maintained at 4°C until plant inoculation.

Inoculation methods. Four inoculation methods were compared. In the first, the two youngest actively growing leaves in a shoot were transversally bisected with a scissors that had been previously dipped into a bacterial suspension [13,14]. The second method consisted of pricking a leaf with a sharp, double-toothed clamp (tooth length: 2 mm) previously dipped in bacterial suspension [14]. In the third, a hypodermic needle connected to an automatic microburette filled with the bacterial suspension was used to locally infiltrate four 20-µl inoculations into leaf tissues [27]. The fourth method consisted of using a scissors to create a wound in the tip of a leaf and then painting the wound with a paintbrush dipped into the bacterial suspension; the leaf was painted 2 h after wounding to assure infection [11].

For each method, *E. amylovora* was inoculated on the three youngest leaves in a shoot on two or three shoots per plant. Leaves pricked with clamps and infected by microinfiltration were inoculated at four sites, avoiding direct inoculation of the midvein. Each plant was considered as a replicate; in experiment 1 and 2, four and six plants were used, respectively, per treatment. Inoculated plants were sealed into wet plastic bags and incubated for 10 days at 25°C/16-h light photoperiod in a controlled-environment chamber (PGR15, Conviron, Winnipeg, Manitoba, Canada). The plants were maintained in the plastic bags throughout the incubation period. Inoculum management and inoculations were conducted inside a laminar-flow biological safety cabinet (NU-425, NuAire Inc., MN, USA) to avoid spread of the quarantine pathogen. Every 2 days, the bags were opened in the cabinet to permit air exchange. The experiment was repeated twice.

In the dose-response assay, disease was assessed 10 days after inoculation with the pathogen at 10^5, 10^6, 10^7 and 10^8 colony-forming units (CFU)/ml. In the disease-time assay, disease was assessed at 0, 3, 5, 7, and 10 days after a pathogen inoculation of 10^8 CFU/ml.

Disease assessment and data analysis. Disease was assessed according to severity indexes based on the progression of necrosis through the leaf, beginning at the inoculation point. For leaves inoculated by cutting or the paintbrush method, the indexes were: 0, no necrosis; 1, necrosis restricted to the inoculation point and the midvein; 2, necrosis affecting the midvein and the petiole; 3, necrosis expanding through the shoot; and 4, necrosis affecting the shoot and other leaves down to the shoot (modified from [14]). For pricked and locally infiltrated leaves, the severity indexes corresponded to: 0, no infection; 1, necrosis restricted to the inoculation point; 2, necrosis affecting only the infiltrated area; 3, necrosis expanding from the pricked or infiltrated area to the neighboring tissues [27]. Disease severity (S) was calculated for each plant according to the following formula:

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

where I_n is the corresponding severity index for each inoculation, N is the number of inoculations in a plant, and I_{max} is the maximum severity index value achieved.

Disease severity data through time were fitted to the Gompertz model [8], modified to account for the typical delay observed at the start of infection depending on the inoculation method [6] according to the equation:

$$ y = K \cdot \exp\left[-B_g \cdot \exp\left(-r_g\left(t - t_0\right)\right)\right] $$

where K is the maximum disease level, r_g is the rate of disease progression, B_g is a parameter accounting for the origin of the curve with respect to the ordinate axes, and t_0 is the incubation time. It was assumed that at 12 days the disease severity was 1. Regression and parameter estimation were determined by the non-linear-least squares procedure.

The severity values at each inoculum concentration were fitted to the hyperbolic saturation model for each inoculation method according to the equation:

$$y = y_{max} \left(\frac{x}{x + k_x} \right)$$

where y_{max} is the maximum disease severity and k_x is the half-saturation constant corresponding to the median effective dose of the pathogen (ED_{50}). It was assumed that at 10^9 CFU/ml the disease severity was 1. Regression and parameter estimation were determined by linear regression using the REG procedure of SAS (SAS System v. 8.00; SAS Institute Inc., Cary, NC).

Treatment effects on disease severity or dose-time relationship parameters were analyzed by means of repeated measures analysis of variance (ANOVA) using the general linear model (GLM) procedure of SAS. Differences among methods were analyzed using Fisher's LSD test.

Strain aggressiveness assays. Seven *E. amylovora* strains were evaluated for their aggressiveness on pear cv. Conference. The experiment was repeated using apple cv. Fuji plants. Plants were inoculated by cutting 0.5 cm from the tip of the three youngest leaves in a shoot with scissors dipped in a pathogen suspension of 1×10^8 CFU/ml. Three shoots per plant were inoculated. The plants were then introduced into wet plastic bags and maintained in a controlled-environment chamber (PGR15, Conviron) at a temperature (25°C) and photoperiod (16-h light) optimal for disease development. Fire-blight symptoms were assessed 3, 5, 7, and 10 days after inoculation and disease severity per plant was calculated from severity indexes as previously described. The experiment was repeated twice. Nine plants of each host species were inoculated with each strain in the first experiment and six plants in the second experiment. The effect of the strain on disease severity was analyzed using the GLM analysis of variance. Differences among strains were analyzed using Tukey's test.

Susceptibility assay. The susceptibility of twelve ornamental species and cultivars of the rosaceous family (*Cotoneaster dammeri* 'Major', *C. horizontalis*, *C. lacteus*, *C. salicifolius* 'Parkteppich', *C. suecicus* 'Skogholm', *Malus* × *Perpetu* 'Evereste', *Pyracantha koidzumii* × *P. coccinea* 'Wyattii' 'Mohave', *Pyracantha coccinea* 'Orange Glow', *Pyracantha coccinea* 'Teton', *Pyrus calleryana* 'Chanticleer' and *Sorbus aucuparia*) to the virulent strain EPS101 of *E. amylovora* was evaluated by the scissors inoculation method, as described above. Disease severity was assessed 14 days after inoculation according to the severity indexes and formula previously described. Five to eight plants per plant species or cultivar were evaluated. Differences among species and cultivars with respect to disease severity were analyzed using the SAS GLM procedure and Tukey's test.

Results

Comparison of inoculation methods.

All four methods resulted in the successful inoculation of *E. amylovora* suspensions onto leaves of potted plants and the development of disease symptoms in plants maintained up to 10 days in controlled-environment chambers. No symptoms of stress, such as leaf wilting or premature defoliation, were observed in plants maintained inside the plastic bags during the incubation period. Although all of the inoculated plant leaves developed progressive disease symptoms, lesion morphology varied according to the inoculation method used. Leaves inoculated by cutting or by painting a cut in the leaf with bacterial suspensions developed necrosis that started in the wound and progressed through the veins to the petiole and twig. Leaves inoculated by local infiltration or by pricking with clamps dipped in a bacterial suspension developed progressive water-soaked necrotic spots around the inoculation point that progressed through the leaf limb. The disease symptoms produced by the leaf cutting and paintbrush methods were similar to those arising from natural infections of *E. amylovora*, including the oozing of sticky droplets.

Disease progression over time depended on the inoculum dose and the inoculation method. Due to the significant differences in the results obtained in the two independent experiments (F = 2.82, $P = 0.10$), statistical analyses were done with separated data. Severity values corresponding to 10^8 CFU/ml dose were fitted as a function of time using the modified Gompertz model (Fig. 1) in order to analyze the effect of the inoculation method on the incubation period (t_0) and on the rate of disease progression (r_g). Significant differences were observed between the inoculation methods for both parameters (exp. 1: r_g F = 3.7, $P = 0.05$, and t_0 F = 28.8, $P < 0.01$; exp. 2: r_g F = 11.5, $P < 0.01$, and t_0 F = 67.7, $P < 0.01$) (Table 1). Local infiltration resulted in the shortest incubation periods (exp. 1 $t_0 = 0.66$ days and exp. 2 $t_0 = 0.80$ days). This method also produced the lowest rate of disease progression ($r_g = 0.28$ and 0.25, respectively) (Fig.1). Inoculation with clamps yielded the longest incubation periods ($t_0 = 4.00$ and 3.41 days for experiments 1 and 2, respectively) and the highest rates of disease progression ($r_g = 0.88$ and 0.49, respectively) (Table 1). The incubation periods were short in plants inoculated by leaf cutting ($t_0 = 1.38$ and 1.05 days, respectively) and disease progression rates were high ($r_g = 0.57$ and 0.47, respectively). In paintbrush-inoculated plants, the incubation periods were of medium length ($t_0 = 3.29$ and 1.99 days, respectively) and moderate r_g values ($r_g = 0.53$ and 0.32, respectively).

The effect of inoculum concentration on disease severity was studied for each inoculation method. Severity values at each inoculum concentration were fitted to the hyperbolic model for each method (Fig. 2). Table 1 shows the y_{max} and k_x values estimated for each inoculation method and each experiment. The effect of inoculation method on maximum disease severity (y_{max}) was not significant in experiment 1 (F = 1.7, $P = 0.23$) but significant in experiment 2 (F = 4.7, $P = 0.01$). Inoculation by leaf cutting and the clamp method resulted in the highest y_{max} values in both experiments (Table 1), whereas the values obtained with the paintbrush and local infiltration methods were lower.

Table 1. Effect of inoculation method on rate of disease symptom progression (r_g), time delay to the start of symptoms (t_0), maximum disease (y_{max}), and median effective pathogen dose (K_x) assessed using disease-dose and -time relationships in cv. Conference pear plants inoculated with *E. amylovora* EPS101

Leaf inoculation method[*]	Experiment 1								Experiment 2							
	Dose-response[¶]				Symptoms progression[¶]				Dose-response				Symptoms progression			
	y_{max}		K_x ($\times 10^5$)		r_g (day^{-1})		t_0 (day)		y_{max}		K_x ($\times 10^5$)		r_g (day^{-1})		t_0 (day)	
Pricking with clamps	0.87		7.73	a	0.88	a	4.00	a	0.77	a	5.87	a	0.49	a	3.41	a
Cutting with scissors	0.89		2.01	b	0.57	ab	1.38	b	0.84	a	2.28	b	0.47	a	1.05	c
Painting wounds	0.68		5.91	a	0.53	ab	3.29	a	0.64	b	5.40	a	0.32	b	1.99	b
Local infiltration	0.75		4.16	ab	0.28	b	0.66	b	0.68	b	4.46	a	0.25	b	0.80	c

[*]The youngest leaves in potted plants were inoculated with bacterial suspensions at different inoculum doses (10^5–10^8 CFU/ml) and incubated under optimal conditions for disease development. Disease severity was recorded 3, 5, 7, and 10 days after inoculation.
[¶]Letters r_g, t_0, y_{max} and K_x are model parameters according to equations 2 and 3 (see text). Parameter estimates were obtained by nonlinar (modified Gompertz model) or linear (hyperbolic saturation model) regression of data from two replicate experiments designed to model the equations. Parameter values within the same column followed by different letters are significantly different ($P \leq 0.05$) according to Fisher's least significant difference test (LSD).

The effect of inoculation method on median effective dose (k_x) was significant (F = 6.5, $P < 0.01$ in exp. 1, and F = 7.6, $P < 0.01$ in exp. 2). Inoculation with scissors gave the lowest median effective dose, and the values differed significantly from those obtained with the other methods. Paintbrush and local infiltration yielded intermediate k_x values, and clamp
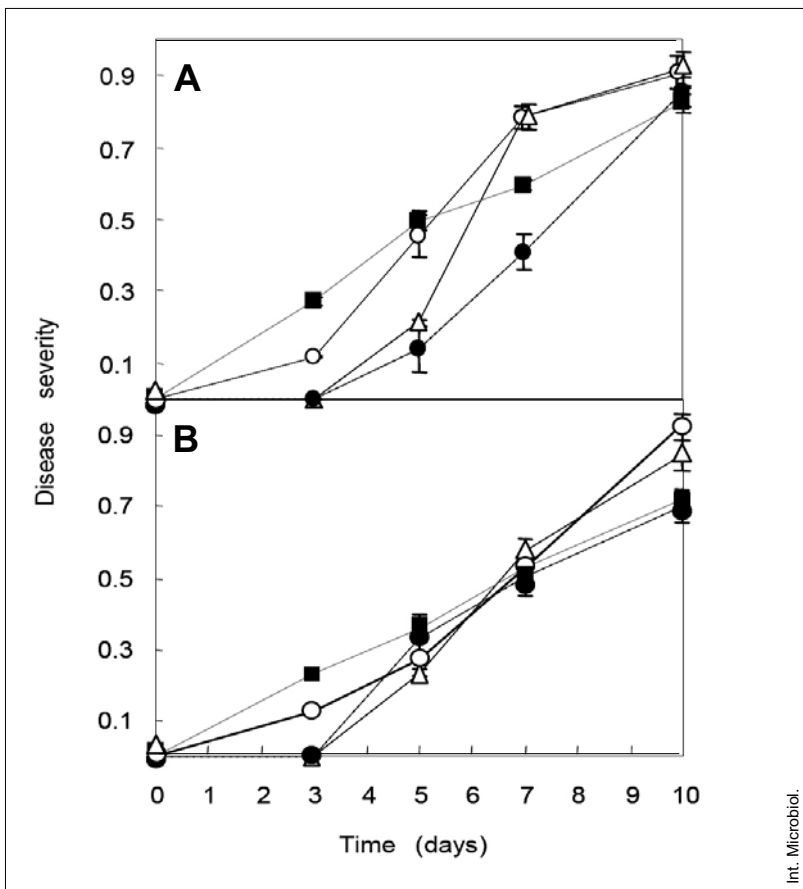
Fig. 1. Disease progression in cv. Conference pear plants inoculated with suspensions of *E. amylovora* EPS101 (10^8 CFU/ml) by the following methods: clamps (open triangle); scissors (open circle); localized microinfiltration (black square); and paintbrush (black circle). (**A**) Experiment 1; (**B**) experiment 2. Values are the mean of four or six replicates for experiment 1 and 2, respectively. Error bars correspond to the standard error of the mean.

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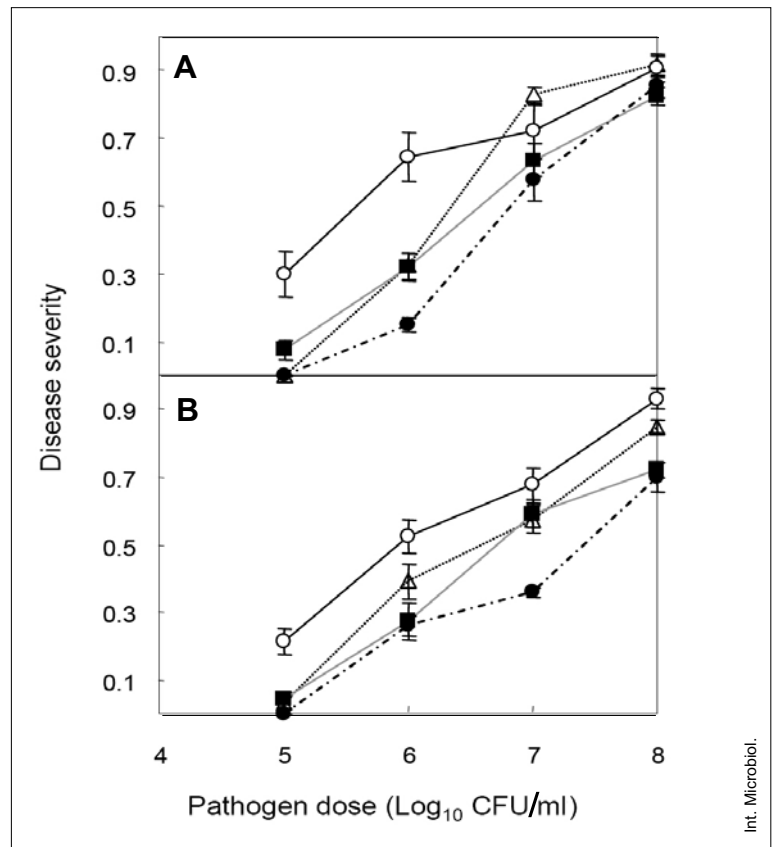
Fig. 2. Infectivity titration 10 days after *E. amylovora* inoculation of cv. Conference pear plants methods by the following methods: clamps (open triangle); scissors (open circle); localized microinfiltration (black square); and paintbrush (black circle). Severity values are the mean of four or six replicates for experiment (**A**) and (**B**), respectively. Error bars correspond to the standard error of the mean.

Table 2. Aggressiveness of *E. amylovora* strains on pear cv. Conference and apple cv. Fuji plants, as assessed with the leaf-cutting inoculation method

Strain[*]	Origin[¶]	Disease severity (%)[§]							
		Experiment 1				Experiment 2			
		Pear		Apple		Pear		Apple	
CFBP1430	*Crataegus* sp. (France)	90.7	a	60.7	a	82.4	a	52.8	a
EPS101	*Pyrus communis* (Spain)	84.3	ab	64.5	a	81.9	a	62.8	a
OMP-BO1185	*Pyrus communis* (Italy)	84.1	ab	54.8	a	80.2	a	51.7	a
CUCM273	*Malus* sp. (USA)	65.7	b	69.6	a	73.0	a	64.7	a
NCPPB3159	*Malus sylvestris* (Netherlands)	63.0	b	54.5	a	57.0	b	51.0	a
EPS100	*Pyrus malus* (Spain)	26.1	c	8.3	b	33.3	c	17.7	b
NCPPB311	*Pyrus communis* (Canada)	0.9	d	3.7	b	2.2	d	0.0	b

[*]Strains were inoculated by cutting the youngest leaves of potted pear or apple plants with scissors dipped into bacterial suspensions (10^8 CFU/ml). Plants were incubated in plastic bags under optimal conditions for disease development.

[¶]CFBP: Collection Française de Bactéries Phytopathogènes, INRA, Angers, France; CUCM: Cornell University Collection of Microorganisms, Ithaca, USA; EPS: Escola Politècnica Superior, Universitat de Girona, Girona, Spain; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; OMP-BO: Osservatorio per le Malattie delle Piante, Bologna, Italy.

[§]Disease severity was recorded 10 days after bacterial inoculation. Values correspond to the mean of nine replicates for experiment 1 and six replicates for experiment 2. Means within the same column followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test.

Table 3. Susceptibility of ornamental rosaceous species to *E. amylovora* EPS101, as determined by the leaf-cutting inoculation method.

Ornamental species*	Disease severity (%)¶	
Pyracantha coccinea 'Orange Glow'	100.0	a
Cotoneaster horizontalis	99.7	a
C. salicifolius 'Parkteppich'	99.2	a
C. suecicus 'Skogholm'	98.9	a
C. dammeri 'Major'	90.0	a
C. lacteus	87.0	a
Pyracantha koidzumii × P. coccinea 'Wyattii' 'Mohave'	63.8	b
Malus × Perpetu 'Evereste'	42.3	c
Pyracantha coccinea 'Teton'	22.1	d
Pyrus calleryana 'Chanticleer'	0.0	e
Sorbus aucuparia	0.0	e

*Potted plants were inoculated with *E. amylovora* EPS101 by cutting young leaves with scissors dipped into bacterial suspensions (10^8 CFU/ml). Plants were incubated in plastic bags under optimal conditions for disease development.
¶Disease severity was assessed 14 days after inoculation. Values are the mean of five to eight replicates. Means followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test.

inoculation the highest k_x values. Therefore, on the basis of disease progression and dose-response assays, inoculation with scissors was considered to be the best method to inoculate leaves on whole plants.

Evaluation of the leaf cutting method with respect to strain aggressiveness and host susceptibility. Scissors inoculation of *E. amylovora* on potted pear and apple plants was used to compare the aggressiveness of *E. amylovora* strains of different origins. Disease symptoms in leaves progressed across the main veins, then into the petiole, and finally through the stem, with the leaves turning brown in apple and black in pear. Under the assay conditions, ooze droplets exuded from blighted shoots in all strains tested. Significant differences in disease levels were observed among several of the strains on each host plant (Table 2). Severity values ranged from 0.9 to 90.7%, depending on the bacterial strain and the host species.

The scissors method of inoculation was also evaluated on several rosaceous species using the aggressive strain *E. amylovora* EPS101. In all plant species, black necrosis was observed, except in *Cotoneaster horizontalis* and *Malus × Perpetu* 'Evereste', which showed yellow-orange discoloration around foci of dark brown and brown necrosis, respectively. The differences in disease severity among host species were significant (F = 154.8, $P < 0.01$) (Table 3).

Discussion

Detached organs are the most suitable plant material to inoculate quarantine-level pathogens [1,12,13] as they permit the manipulation of material under accurate, biologically safe conditions. However, to study plant responses to pathogen infection or many plant-pathogen interactions, the whole plant must be used. For fire-blight studies, young pear or rosaceous potted plants offer an intermediate approach between in vitro microcuttings and pear trees, and can be manipulated under laboratory conditions. In this work, four whole-plant inoculation methods were evaluated on the basis of their capacity to induce *E. amylovora* infections, disease progression, and the development of symptoms under quarantine laboratory conditions. *E. amylovora* normally penetrates through wounds or natural openings, but preferentially enters through the nectarthodes present in the nectarial cup of the flowers [39, 40, 44]. Here, the plant leaves were wounded before or simultaneous with bacterial inoculation in order to facilitate penetration of the bacteria and infection of host tissues [5,9,11,14,16,17,37,41]. The four tested methods were chosen among those described in the literature for inoculation of *E. amylovora* or other plant pathogenic bacteria and have been used in plant-pathogen interaction studies [14,27]. They were selected on the basis of the different ways

that pathogens inoculate host tissues and were adapted to our laboratory conditions.

The four methods produced *E. amylovora* infections and symptoms of developing disease under quarantine conditions. The differences in disease progression, symptom morphology, and final disease severity, depending on the inoculation method, were in agreement with the results of other studies [3,14,29]. An inoculum concentration of at least 10^6 CFU/ml and an incubation period of 7–10 days appeared to be critical to the development of disease symptoms; maximal disease severity was obtained 10 days after inoculation of 10^7 CFU/ml pathogen suspensions in all methods except the paintbrush method, which required 10^8 CFU/ml. All four methods tested can also be used under nonquarantine conditions or in the greenhouse; however, if the inoculated plants are not incubated in wet plastic bags, incubation period and disease progression may differ from the results obtained in this study.

The inoculation methods were compared quantitatively by fitting the data to mathematical models relating disease severity to inoculum concentration and time, as described by Cabrefiga and Montesinos [6]. The hyperbolic saturation model provided information on the median effective dose (ED_{50}) and maximal disease (y_{max}). The modified Gompertz model yielded the rate of disease severity progression (r_g) and the progress curve describing the time delay to the start of disease (t_0). ED_{50} values obtained for each method could be related to the path of pathogen introduction into plant tissues. The minimum ED_{50} was obtained with the scissors method, probably because this approach assures direct access of the pathogen to the plant vascular system, and, compared to the other methods, fewer bacterial cells are needed to reach the same level of disease severity. The highest ED_{50} was obtained with the clamp puncture method, probably due to the reduced leaf area that initially interacts with the pathogen inoculum. Symptom progression was most rapid in pear leaves subjected to local infiltration (consistently lowest t_0 in both experimental replicates), but the final disease severity (y_{max}) and the disease progression rate (r_g) were lower than achieved with the other methods.

Local infiltration directly introduces bacterial cells into leaf mesophyll, such that symptoms quickly appear in the infiltrated leaf area; however, bacteria colonize the neighboring tissues slowly if they do not reach the midvein. Leaf cutting and clamp puncture had high and similar disease progression rates, but the disease progression curve started earlier in plants inoculated with the scissors method, probably due to direct contact of the bacterial suspension with the midvein [16,29,37]. In fact, it has been reported that inoculation into the interveinal region of a leaf more efficiently exposes the pathogen to the plant cell [47]; however, this is not as quantitative as the vein assay since inoculations into the interveinal region can fail to result in visible pathogen invasion—a problem that is even more pronounced when the bacterial suspension is applied to the leaf surface [3,4]. Inoculation by painting a wound with a paintbrush impregnated with the bacterial suspension produced the lowest disease severity levels, but high ED_{50} and moderate r_g values. The delay between wounding and bacterial inoculation can affect the ability of bacteria to infect wounded tissues, since plant defenses are quickly induced in injured tissues [45]. Indeed, the incidence of blight decreases as the interval between injury and inoculation increases, although residual susceptibility remains after 48 h [11].

On the basis of these results, *E. amylovora* pathogenicity studies are best carried out through the inoculation of bacteria by leaf cutting with bacteria-impregnated scissors because high disease severity levels at optimal conditions (high inoculum concentration, virulent strain, susceptible host) can be reached rapidly and disease symptoms correspond to those observed in natural infections, including disease progression through the leaf midvein, petiole, and twig as well as ooze production [4]. Additionally, this method is useful to evaluate pathogen strain aggressiveness and host susceptibility. Different levels of virulence were found among strains of *E. amylovora* inoculated on cv. Conference pear plants and cv. Fuji apple plants. Although pear plants were more susceptible to infection than apple plants, the virulence of each strain was consistent in both host species and experimental replicates.

Based on our results on whole plants in which the leaves were cut with scissors, strains CFBP1430 and CUCM273 were classified as highly aggressive and strain NCPPB311 as poorly aggressive, which agreed with previous reports using other methods to inoculate whole plants or detached organs [6,19,29,31]. Also, the results on host susceptibility, determined in the present work with the scissors method, agree with nursery and field observations. The most commonly infected ornamental host plants are species of *Crataegus* and *Cotoneaster* [42]. Most *Cotoneaster* and *Pyracantha* species are susceptible to fire blight; likewise, disease severity reached high levels under our laboratory conditions. Disease severity in *Pyracantha coccinea* 'Teton', which is considered quite resistant [Bobev SG, Baeyen S, Crepel C, Maes M (2004) First report of fire blight caused by *Erwinia amylovora* on *Pyracantha coccinea* in Bulgaria. Plant Dis 88:427-427 (Abstract)], was low, while, in our experiments, *Pyrus calleryana* 'Chanticleer' and *Sorbus aucuparia* did not develop disease symptoms. These species have been reported to be resistant to fire blight [20].

Although the scissors method appears to be the most suitable, the other methods should not be discarded in studies of *E. amylovora*-host plant interactions. Local infiltration and clamp puncture allow several inoculations to be made in a leaf. Additionally, in local infiltration the volume of pathogen suspension infiltrated into the leaf is known and pathogen cells interact with plant cells even if the wound area is small. Infiltration also can be used in pathogen population dynamics and inoculum dose-response studies. However, although disease progression in microinfiltrated leaves is initially fast, final severity levels are low and progressive water-soaked necrotic spots remain confined to the leaf limbus. The paintbrush method permits a delay between wounding and pathogen inoculation and thus simulates natural infections after host tissue injury. This method therefore can be used to evaluate strategies for applying chemical control products and in studies of the mode of action of those products.

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