Analysis of Aggressiveness of *Erwinia amylovora* Using Disease–Dose and Time Relationships

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ABSTRACT

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The aggressiveness of an extensive collection of strains of *Erwinia amylovora* was analyzed using immature fruit and detached pear flower assays under controlled environmental conditions. The analysis was performed by means of a quantitative approach based on fitting data to mathematical models that relate infection incidence to pathogen dose and time. Probit and hyperbolic saturation models were used for disease–dose relationships and provided information on the median effective dose (ED_{50}). Values of ED_{50} ranged from 10^3 to 10^6 CFU/ml (10 to 10^4 CFU per site of inoculation). A modified Gompertz model was used for disease–time relationships and provided information on the rate of infection incidence progression (r_g) and time delayed to start of the incidence

progress curve (t_0). Values of r_g ranged from near 0 to 1.90, and t_0 varied from 1.3 to more than 10 days. The more aggressive strains showed high r_g , low ED_{50} values, and short t_0 , whereas the less aggressive strains showed low r_g , high ED_{50} , and long t_0 . The aggressiveness was dependent on plant material type and pear cultivars and was significantly different between strains of *E. amylovora*. Infectivity titration and kinetic analysis of progression of incidence of infections using the immature pear test and a standardized scale are proposed for assessment of strain aggressiveness. The implications of r_g , ED_{50} , and t_0 for the epidemiology and management of fire blight are discussed, particularly the wide range of aggressiveness among strains, the degree of host specificity observed in pear isolates, the very high infective potential of this pathogen, the independent action of pathogen cells during infection, and the possible advantage of including aggressiveness parameters into fire blight risk forecasting systems.

Fire blight of rosaceous plants caused by *Erwinia amylovora* is an economically important disease that affects mainly apple and pear production and several woody ornamental plants worldwide (53,55). The disease has spread to new regions in recent years (11,19,24,52) and its control is difficult (45,53–55).

Knowledge of the variability in pathogenicity of *E. amylovora* is important for disease management and epidemiology because it may give information about the evolutionary potential of this pathogen in terms of durability of cultivar resistance and of control methods (e.g., biological control). However, there is a considerable lack of consensus among plant pathologists about terms and concepts related to pathogenicity. Pathogenicity has been defined as the overall ability of a pathogen to infect a host and cause disease (10,46). According to Shaner et al. (46), the components of pathogenicity are virulence and parasitic fitness, whereas Boss and Parlevliet (10) considered pathogenicity to be composed of virulence and aggressiveness. Some authors refer to virulence as a qualitative attribute of pathogenicity (1,10) or the capacity of a pathogen to cause disease as a result of the expression of genetic determinants or virulence factors in the host–pathogen interaction (e.g., virulent or nonvirulent). Parasitic fitness refers to the success of the pathogen within a population (e.g., reproductive fitness, dispersal capacity) (1), whereas aggressiveness is considered quantitative and refers to the intensity of disease or attack produced by a pathogen (10,46). Accordingly, with these concepts, the present study deals with aggressiveness in *E. amylovora*.

Aggressiveness can be measured in terms of the progression of infections, migration of pathogen cells from the point of inoculation, amount of pathogen inoculum needed to produce a given

effect on host, or rate of disease progression. Several studies have been performed to analyze the variability in aggressiveness among strains of *E. amylovora*. Some reports have been unable to observe significant differences in aggressiveness among strains (25,39), whereas others (17,33–36,45) have observed them. This lack of agreement may be due to the composition of the strain collection studied, but also to differences between studies in the methods of assay used to measure aggressiveness, plant material, dose of pathogen, or time of disease assessment after pathogen inoculation.

Methods of analysis used to study aggressiveness in *E. amylovora* include inoculation of the pathogen to whole plants or in vitro micropropagated plant material, and monitoring the progression of infections or the migration from the point of inoculation (8,34,35,50). However, ex vivo alternative methods, originally developed for screening large amounts of biological control agents of fire blight, such as the immature pear fruit (3) and the crab apple blossom assays (37,38), can be useful and have not been tested for assessment of aggressiveness in an extensive collection of strains.

The analysis of aggressiveness is influenced by the dose of pathogen inoculated and the time at which disease is evaluated. The effect of pathogen concentration on disease was first studied by Ercolani and Van der Plank (15,51) and, later, models to relate disease levels to the dose of pathogen, and tools to fit data and derive aggressiveness parameters (e.g., median effective dose) were developed and tested in several fungal and bacterial plant pathogens (16,20,31,42,43,47). However, disease–dose relationships have not been used to analyze aggressiveness in *E. amylovora*. Studies of aggressiveness also are affected by the time at which disease is evaluated after pathogen inoculation. Disease progress curves have been used to compare the susceptibility of apple and pear cultivars to a strain of *E. amylovora* (30) based on kinetic analysis of disease progression currently used in epidemi-

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ology (12). However, the mathematical equation used did not account for the delay in the start of the disease progress curve which often is observed in weakly virulent strains or hosts with some resistance to *E. amylovora*.

Several authors consider aggressiveness to be composed of infectiveness and invasiveness (9,46). Infectiveness can be defined as the efficiency of the pathogen inoculum in terms of development of infections and is inversely proportional to the number of pathogen cells required to cause an infection or to kill a susceptible host. It can be estimated as a median effective dose (ED₅₀) using infectivity titration experiments (31) in an approach similar to that used in insect (49) and clinical microbiology (13). Invasiveness can be defined as the capacity of a pathogen to grow and spread within the host, and it can be estimated as a rate of disease progression using kinetic analysis of infection incidence. Analysis of infectiveness and invasiveness in representative strains of *E. amylovora* may provide useful information for understanding the epidemiology and management of fire blight.

The objective of the present work was to analyze aggressiveness in *E. amylovora* by estimating the ED₅₀ and the rate of progression of infection incidence (r_g) in an extensive collection of pathogen strains isolated from fire blight outbreaks in Spain and reference strains from different areas of geographical origin.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. Thirty-six *E. amylovora* strains were used in the present study (Table 1). The

strains were selected for their variety regarding different host plants and geographical origin. Twenty-five strains originally were isolated from outbreaks detected in Spain since the first introduction of fire blight in 1995. The remaining 11 strains originated from different parts of the world and were used as reference strains in this study. Strains were confirmed as *E. amylovora* based on various criteria, including their growth characteristics in several culture media (sucrose nutrient agar [7], Miller-Schroth agar [27], and MM2Cu agar [5]), double-antibody sandwich indirect enzyme-linked-immunosorbent assay (18), polymerase chain reaction (PCR) directed to pEA29 plasmid (6) and to *ams* gene (4), and pathogenicity on immature pear fruit (3). Cultures were stored in 20% glycerol at -80°C until use. Bacterial suspensions used for inoculation were obtained from cultures grown overnight at 25°C on Luria-Bertani agar. For the preparation of suspensions, the cells were scraped from the agar surface and suspended in sterile distilled water. The cell concentration was adjusted by dilution to 10⁸ CFU/ml using a previously prepared viable-absorbance relationship. Because this relationship was found not significantly different among several strains tested, a standard calibration curve was used for the 36 strains studied. The original suspension was 10-fold serially diluted in sterile distilled water to obtain concentrations of 10⁷, 10⁶, 10⁵, 10⁴, 10³, and 10² CFU/ml.

Immature pear fruit tests. The immature pear fruit assay was based on a method previously described for screening biological control agents of fire blight (3), but was modified in the present work to use whole fruit instead of pear halves. Immature pear

TABLE 1. Origin and year of isolation of *Erwinia amylovora* strains used in this work^z

Strain	Host plant	Source	Country	Year of isolation
CFBP1430	<i>Crataegus</i> sp.	CFBP	France	1972
PMV6076	<i>Crataegus</i> sp.	PMV	France	1990
Ea273	<i>Malus sylvestris</i>	CUCM	United States	1971
115.22	<i>Cydonia oblonga</i>	...	Bulgary	1989
EPS100	<i>Pyrus malus</i>	This study	Girona (Spain)	1997
EPS101	<i>Pyrus communis</i>	This study	Lleida (Spain)	1999
IVIA1614.2	<i>Pyracantha</i> sp.	IVIA	Segovia (Spain)	1996
NCPPB1734	<i>Pyrus communis</i>	NCPPB	Egypt	1965
NCPPB1819	<i>Crataegus</i> sp.	NCPPB	United States	1966
NCPPB2080	<i>P. communis</i>	NCPPB	New Zealand	1968
NCPPB311	<i>P. communis</i>	NCPPB	Canada	1952
NCPPB3159	<i>Malus sylvestris</i>	NCPPB	Netherlands	1981
NCPPB595	<i>P. communis</i>	NCPPB	England	1958
NCPPB683	<i>P. communis</i>	NCPPB	England	1959
SIA1	<i>P. communis</i>	SIA	Zaragoza (Spain)	2000
SIA13	<i>P. communis</i>	SIA	Zaragoza (Spain)	2000
SIA7	<i>P. communis</i>	SIA	Zaragoza (Spain)	2000
SV1000	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV2773	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4300	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4320	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4408	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4499	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4500	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
SV4576	<i>P. communis</i>	SV-DARP	Lleida (Spain)	1999
UPN500	<i>P. communis</i>	UPN	Guipúzcoa (Spain)	1998
UPN513	<i>P. communis</i>	UPN	Guipúzcoa (Spain)	1998
UPN514	<i>P. communis</i>	UPN	Guipúzcoa (Spain)	1998
UPN529	<i>Pyracantha</i> sp.	UPN	Navarra (Spain)	1997
UPN530	<i>Pyrus communis</i>	UPN	Navarra (Spain)	1997
UPN546	<i>Cydonia</i> sp.	UPN	Navarra (Spain)	1998
UPN562	<i>Pyracantha</i> sp.	UPN	Navarra (Spain)	1998
UPN575	<i>Pyrus malus</i>	UPN	Navarra (Spain)	1998
UPN609	<i>Sorbus</i> sp.	UPN	Navarra (Spain)	1998
UPN610	<i>Pyracantha</i> sp.	UPN	Navarra (Spain)	1998
UPN611	<i>Cotoneaster</i> sp.	UPN	Navarra (Spain)	1998

^z EPS, Escola Politècnica Superior-Universitat de Girona (Spain); SV, Servei de Protecció dels Vegetals-Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya (Spain); CUCM, Cornell University Collection of Microorganisms, Ithaca, NY (United States); NCPPB, National Collection of Plant Pathogenic Bacteria, Plant Pathogen Laboratory, Harpenden, Hertfordshire (England); CFBP, Collection Française de Bactéries Phytopathogènes, INRA Angers (France); PMV, Laboratoire de Pathologie Moléculaire et Végétale INRA/INA-PG, Paris (France); IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia (Spain); UPN, Universidad Publica de Navarra, Pamplona (Spain); and SIA, Servicio de Investigaciones Agrarias de Aragón, Zaragoza (Spain).

fruit were collected from a commercial orchard in early June at the 6-week stage from fruit set, and kept in the dark at 0 to 4°C. The fruit were used before 1 month of storage to avoid significant physiological changes affecting the assay. Before inoculation, the fruit were surface disinfected by immersion for 1 min in a diluted solution of sodium hypochlorite (1% active chlorine), washed two times in distilled water, and left under air flow in a sterile cabinet to remove excess water. Each fruit was wounded four times on opposite sides with a needle (approximately 2 mm in diameter and 5 mm in depth). Thereafter, 10 µl of a suspension of *E. amylovora* at the suitable concentration was deposited in each wound. Inoculated fruit were placed on polystyrene tray packs which were introduced in plastic boxes and incubated at 21°C in a controlled environment chamber. Wounds were considered to be infected when either drops of bacterial exudates or necrosis appeared in and around the point of inoculation of the pathogen.

Detached pear flower tests. The detached pear flower assay was based on the crab apple blossom assay described by Pusey (37,38) for screening biological control agents against fire blight. Detached pear branches, 2 years old, bearing 7 to 15 dormant flower buds were taken from a commercial orchard during winter. Individual pear flowers were obtained from pear branches forced to bloom in an environmental chamber following the procedure described by Montesinos and Vilardell (32). The open blossoms were detached from branches and the individual flowers were maintained with the cut peduncle submerged in 1 ml of 10% sucrose solution in a single, 1.5-ml plastic Eppendorf vial. Vials were placed in plastic racks and flowers were inoculated by deposition on the hypanthium of 10 µl of a pathogen suspension at the corresponding concentration. Then, racks were placed in plastic boxes, covered with plastic bags, and incubated at 21°C under moist conditions and 16 h of fluorescent light and 8 h of darkness. Flowers were considered to be infected when necrosis was observed in the hypanthium.

Infectivity titration assays. Plant material consisting of detached flowers or fruit of cvs. Conference, Passe Crassane, or Doyenne du Comice were inoculated with strains of *E. amylovora* (EPS100, NCPPB311, IVIA1614.2, NCPPB595, UPN529, UPN611, and EPS101) at 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , and 10^8 CFU/ml. Due to the multiplicity of factors, the study was split into two experiments. In experiment 1, the same *E. amylovora* strain, EPS101, was inoculated in two plant materials and cultivars (Doyenne du Comice and Conference). In experiment 2, immature pear fruit of Passe Crassane cultivar were inoculated with seven *E. amylovora* strains. The experimental design consisted of three repetitions of six fruit per repetition (4 wounds per fruit and a total of 24 wounds per repetition) or three repetitions of eight flowers per repetition, for each strain, plant material, and cultivar combination. The incidence of infection of individual wounds in immature pear fruit and on the hypanthium of flowers was assessed at 5 days after inoculation.

The mean proportion of infected wounds on flowers per repetition at each pathogen dose was fit to the probit and hyperbolic saturation models (31) for each strain, pear cultivar, and plant material combination. The equation of the probit model which relates incidence of infections (y) to the concentration of pathogen (x) is

$$y = \Phi \left[\frac{(\log_{10}(x) - \lambda)}{\tau} \right] \quad (1)$$

where Φ denotes the cumulative distribution function for the standard normal, λ is a parameter equivalent to the ED_{50} , and τ is the efficiency of the pathogen.

The equation corresponding to the hyperbolic saturation model is

$$y = y_{\max} \frac{x}{(x + K_x)} \quad (2)$$

where $y_{\max}$ is the maximum disease incidence and K_x is the half saturation constant corresponding to the ED_{50} of the pathogen.

Regression and parameter estimation were performed by a nonlinear least squares method using the NLIN procedure of the PC-Statistical Analysis System (version 8.2; SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed for each of the two experiments using the GLM procedure of the SAS. Means were separated by the Waller-Duncan test ($P \leq 0.05$).

Kinetics of infection incidence. The aggressiveness of the 36 strains of *E. amylovora* described in Table 1 was evaluated on immature pear fruit using progression curves of infection incidence. The experiment was performed twice. In the first trial, the strains were inoculated at 10^3 CFU/ml whereas, in the second experiment, inoculations were performed at 10^5 CFU/ml. The experimental design consisted of three repetitions of six fruit per repetition for each strain and concentration. Aggressiveness was evaluated on the basis of the incidence of infections that was assessed at 3, 4, 5, 6, 7, 8, 9, and 10 days after the pathogen inoculation.

Infection incidence data at different times were fit to the Gompertz model (12). However, a modification of the original model was used to account for the typical delay observed in the start of infections depending on the strain. The equation of the modified Gompertz model is

$$y = K - \exp(-B_g \cdot \exp[-r_g(t - t_0)]) \quad (3)$$

where K is the maximum disease level or curve asymptote, 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 axis, and t_0 is the time delayed to start of the disease progress curve.

Regression and parameter estimation was performed by a nonlinear least squares method using the NLIN procedure of the SAS. To improve convergence to the solution, the parameter K was determined directly from curves as the incidence at the end of the incubation period and was fixed in the equation.

ANOVA was performed with the estimated parameters and means were separated by the Tukey's test at $P \leq 0.05$. The analysis was done with the GLM procedure of the SAS.

For classification of strains into different levels of aggressiveness, the following procedure was used. For each experiment and strain, a four-level index was computed. In experiment 1, for parameter r_g , the levels of aggressiveness were: 1, nonvirulent or very low ($0 \leq r_g < 0.4$); 2, low ($0.4 \leq r_g < 0.8$); 3, medium ($0.8 \leq r_g < 1.2$); and 4, high ($1.2 \leq r_g < 1.5$). For parameter t_0 , the levels of aggressiveness were: 1, nonvirulent or very low ($7.5 < t_0 \leq 10$); 2, low ($5 < t_0 \leq 7.5$); 3, medium ($2.5 < t_0 \leq 5$); and 4, high ($0 < t_0 \leq 2.5$). For K parameter, the levels of aggressiveness were: 1, nonvirulent or very low ($0 \leq K < 25$); 2, low ($25 \leq K < 50$); 3, medium ($50 \leq K < 75$); and 4, high ($K \geq 75$). For experiment 2, an analogous rescaling method was used. A combined index of aggressiveness also was computed for each strain as the sum of the aggressiveness levels obtained using each parameter. Finally, a four-level scale of combined index of aggressiveness (CI) was calculated as follows: 1, nonvirulent or very low ($3 \geq CI$); 2, low ($3 < CI \leq 6$); 3, medium ($6 < CI \leq 9$); and 4, high ($9 < CI \leq 12$). The Kuskall-Wallis rank order test was used to compare the classification of strains according to the single parameter index and the combined index of aggressiveness in the two experiments performed.

RESULTS

Infectivity titration assays. Several patterns of the effect of pathogen concentration on infection incidence at 5 days in immature pear fruit and flowers in a set of strains in different plant materials are shown in Figure 1.

The aggressiveness of strains in different plant materials according to λ (ED_{50}) and τ for the probit model, and K_x (ED_{50}) and $y_{\max}$ for the hyperbolic saturation model, is shown in Table 2. Both models adequately fit the data sets for 9 of 11 strain-plant

material combinations studied, on the basis of the mean square error (MSE) and the asymptotic standard errors for the estimated parameters.

The values of ED_{50} estimated by both models were similar, ranging from 2.78×10^6 to 7.08×10^5 CFU/ml for the hyperbolic saturation model and from 2.59 to 6.12 $\log_{10}$ CFU/ml for the probit model. In the less aggressive strains EPS100 and NCPPB311, it was impossible to fit data to models because only the highest concentrations of pathogen gave some infected fruit.

Fig. 1. Infectivity titration of strains of *Erwinia amylovora* in immature pear fruit of cv. Passe Crassane (EPS101, ●; UPN529, ■; and NCCPB595, ○) and flowers of cv. Conference (EPS101, ▲). Curves represent predictions according to the hyperbolic saturation model (equation 2) using the estimated parameters shown in Table 2. Lines indicate the confidence interval for the mean.

However, a rough estimation of ED_{50} was $\approx 10^7$ CFU/ml for EPS100 and $>10^8$ CFU/ml for NCPPB311.

In experiment 1, plant material (flower and immature pear fruit) and cultivar type (Doyenne du Comice and Conference) significantly affected aggressiveness (ED_{50}) of strain EPS101 ($F = 128.8$ and 8.7, $P < 0.001$ and 0.001, respectively, using the probit model; and $F = 70.7$ and 12. 1, $P < 0.001$ and 0.001, respectively, using the hyperbolic saturation model). In experiment 2, the effect of the strain of *E. amylovora* on aggressiveness was significant ($F = 10.6$, $P < 0.001$ for the probit model and $F = 8.0$, $P < 0.001$ for the hyperbolic saturation model). Flowers were more susceptible to *E. amylovora* infections (low ED_{50}) than immature pear fruit (high ED_{50}). Cv. Doyenne du Comice was the more susceptible among cultivars tested. Strains EPS101, UPN611, and UPN529 were more aggressive (exhibited similar and low ED_{50}) according to both models than strain IVIA1614.2, which was intermediate (different between models), and NCCPB595 was less aggressive.

Kinetics of progression of infection incidence. The progression of disease incidence in wounded immature pear fruit inoculated with different strains of *E. amylovora* is shown in Figure 2. The four strains shown represent different patterns of progression of infection incidence from low (UPN610) and medium (IVIA1614.2 and NCCPB1819) to high rate of progression (EPS101), and for the time of start of the progress curve.

The aggressiveness of the 36 strains of *E. amylovora* listed in Table 1 was studied in relation to the progression of the incidence of infected wounds in immature Passe Crassane pear fruit (Table 3). Aggressiveness was measured according to the estimated parameters (r_g , t_0 , and K) obtained by fitting infection incidence progression data to the modified Gompertz model. Two experiments were performed at different doses of pathogen. Significant differences were observed among strains at 10^3 CFU/ml (experiment 1) in r_g ($F = 44.4$, $P < 0.001$), t_0 ($F = 334.5$, $P < 0.001$), and K ($F = 114.5$, $P < 0.001$), and at 10^5 CFU/ml (experiment 2) in r_g

TABLE 2. Infectivity titration of strains of *Erwinia amylovora* in different ex vivo plant material and estimated parameters of the probit and hyperbolic saturation models that relate the incidence of infections with the pathogen concentrations^z

Plant material, pear cultivar	Strain	Probit model			Hyperbolic saturation model		
		λ	τ	MSE	K_x	$y_{\max}$	MSE
Experiment 1							
Pear flowers							
Doyenne du Comice	EPS101	2.66 a (0.09)	0.97 (0.12)	0.0019	5.00×10^2 a (0.61×10^2)	0.98 (0.01)	0.0019
Conference	EPS101	2.59 a (0.21)	1.23 (0.22)	0.0095	2.78×10^2 a (0.81×10^2)	0.91 (0.02)	0.0662
Immature pear fruit							
Conference	EPS101	3.84 b (0.10)	1.73 (0.16)	0.0043	3.76×10^3 b (0.90×10^3)	0.89 (0.03)	0.0121
Doyenne du Comice	EPS101	4.59 c (0.10)	1.26 (0.15)	0.0064	4.03×10^4 c (0.94×10^4)	0.96 (0.04)	0.0133
Experiment 2							
Immature pear fruit							
Passe Crassane	EPS101	4.78 a (0.09)	1.01 (0.13)	0.0070	6.19×10^4 ab (1.07×10^4)	0.96 (0.03)	0.0081
	UPN611	4.77 a (0.07)	0.75 (0.09)	0.0059	5.19×10^4 ab (0.96×10^4)	0.97 (0.025)	0.0052
	UPN529	4.94 a (0.14)	1.43 (0.20)	0.0109	3.31×10^4 a (0.90×10^4)	0.86 (0.03)	0.0096
	IVIA1614.2	5.12 a (0.05)	0.88 (0.08)	0.0522	1.13×10^5 b (0.14×10^5)	0.98 (0.02)	0.0030
	NCP595	6.12 b (0.09)	0.97 (0.13)	0.0072	7.08×10^5 c (2.85×10^5)	0.98 (0.04)	0.0084

^z Median effective dose of the pathogen (CFU/ml) = ED_{50} . Model parameters according to equations 1 and 2 are λ ($\log_{10}ED_{50}$), τ (efficiency of the pathogen), K_x (ED_{50}), and $y_{\max}$ (maximum disease proportion). Models were fit to pooled data consisting of three repetitions of seven pathogen concentrations (21 data points). Values shown are estimated parameters for the pooled data. The asymptotic standard errors for the parameter estimates are given in parentheses. MSE = mean square error of the model. Statistical comparisons were performed using model parameters estimated separately for each of the three repetitions corresponding to combinations of plant material, pear cultivar and pathogen strain. Values within the same column followed by different letters are significantly different ($P \leq 0.05$) according to the Waller-Duncan test.

($F = 21.2$, $P < 0.001$), t_g ($F = 105.9$, $P < 0.001$), and K ($F = 27.3$, $P < 0.001$).

At the lower pathogen dose (experiment 1), a higher variability of the parameters was observed than at the higher dose (experi-

Fig. 2. Kinetics of disease progression in immature pear fruit of cv. Passe Crassane upon inoculation with strains EPS101 (□), IVIA1614.2 (■), NCPPB1819 (●), and UPN610 (▲) of *Erwinia amylovora*. Inoculations were performed at 10^5 CFU/ml. Curves represent predictions of the modified Gompertz model (equation 3) according to the parameters shown in experiment 2 in Table 3. Lines indicate the confidence interval for the mean.

ment 2), and probably was due to the low inoculum concentration used. Globally, t_0 was shorter and r_g was higher in the second experiment, whereas the values of K were similar. Also, some strains showed considerable differences in r_g values between experiments (e.g., EPS101, NCPPB595, and SIA7), whereas others (e.g., UPN611, IVIA1614.2, and Ea273) have similar values in both experiments.

Values of r_g varied from 0 to 1.59 at the lower pathogen dose and from 0 to 1.90 at the higher dose. In both experiments, r_g was distributed gradually and overlapped in many strains, but was clearly separated for the more and less aggressive strains. Generally, the more aggressive strains had high r_g values and short t_0 , whereas the less aggressive strains showed low r_g and K values and large t_0 .

A classification of the aggressiveness of the strains was performed using the single-parameter index (r_g , t_0 , or K) and the combined index as described previously. According to the Kruskal-Wallis rank order test, there were no significant differences in the classification of the strains between experiments 1 and 2 for r_0 ($\chi^2 = 1.07$, $P = 0.30$) and K ($\chi^2 = 1.41$, $P = 0.24$). However, there were differences between both experiments for t_0 ($\chi^2 = 9.78$, $P = 0.002$). Upon using the combined index, there were no significant differences in the classification of strains between experiments according to the Kruskal-Wallis test ($\chi^2 = 0.95$, $P = 0.33$).

The results of the global classification obtained are presented in the last column of Table 3. The reference strains from other sources, such as CFBP1430, NCPPB3159, CUCM273, and

TABLE 3. Aggressiveness of strains of *Erwinia amylovora* in immature Passe Crassane pear fruit according to the kinetics of disease progression^y

Strain	Experiment 1			Experiment 2			Combined class ^z
	r_g	t_0	K	r_g	t_0	K	
SV4408	1.49 a-c	4.07 e-h	100 a	1.90 a	2.04 h-m	100 a	4/4
SV2773	1.42 a-d	4.35 de	100 a	1.70 a-b	2.86 h-k	100 a	4/4
SV4300	1.46 a-c	4.00 e-i	100 a	1.61 a-c	3.06 g-i	100 a	4/4
UPN500	1.52 ab	4.31 d-f	100 a	1.56 a-d	2.87 h-k	100 a	4/4
UPN513	1.36 a-e	3.19 g-l	100 a	1.56 a-d	1.58 lm	100 a	4/4
SV4320	1.59 a	4.21 d-g	100 a	1.54 a-d	3.07 g-i	100 a	4/4
SV4499	1.16 a-g	3.41 e-l	100 a	1.41 a-e	2.84 h-k	100 a	4/4
SV4576	1.51 ab	2.92 i-l	100 a	1.49 a-d	1.74 k-m	100 a	4/4
UPN530	0.99 c-h	4.11 e-h	100 a	1.57 a-d	2.97 h-j	100 a	4/4
EPS101	0.84 f-h	3.51 e-k	96 a	1.46 a-d	1.86 j-m	100 a	4/4
CFBP1430	1.05 b-h	2.61 j-l	100 a	1.44 a-e	1.29 m	100 a	4/4
UPN514	1.15 a-g	2.38 l	100 a	1.35 b-f	2.21 h-m	100 a	4/4
NCPPB3159	1.11 a-g	4.39 de	100 a	1.31 b-g	3.21 f-h	100 a	4/4
UPN611	1.25 a-f	3.17 g-l	100 a	1.23 b-h	2.06 h-m	100 a	4/4
Ea273	1.09 a-g	4.10 e-h	92 a	1.13 c-h	1.96 i-m	100 a	4/4
SIA1	0.83 f-h	4.51 de	100 a	1.13 c-h	1.73 k-m	100 a	4/4
IVIA1614.2	0.91 e-h	3.04 h-l	100 a	1.03 d-h	1.99 i-m	100 a	4/4
NCPPB1734	0.95 d-h	4.19 d-g	88 ab	0.85 f-i	2.37 h-m	88 a-c	4/4
SV4500	0.74 gh	4.38 de	67 bc	1.13 c-h	2.13 h-m	100 a	3/4
NCPPB2080	0.85 e-h	5.29 cd	67 bc	1.12 c-h	3.03 g-j	100 a	3/4
SV1000	0.55 hi	3.23 f-l	92 a	1.12 c-h	2.73 h-l	100 a	3/4
NCPPB683	0.86 e-h	3.67 e-j	100 a	0.88 e-i	3.14 g-i	100 a	4/3
UPN529	0.71 g-h	4.35 de	21 d	1.51 a-d	2.69 h-l	100 a	2/4
NCPPB1819	0.81 f-h	5.81 c	59 c	0.81 f-i	4.40 ef	67 de	3/3
SIA7	0.00 j	>10.00 a	0 d	0.81 f-i	4.17 e-g	58 ef	1/3
SIA13	0.00 j	>10.00 a	17 d	0.76 g-j	4.72 de	71 de	1/3
115.22	0.00 j	>10.00 a	13 d	0.69 h-j	2.29 h-m	79 b-d	1/3
NCPPB595	0.57 hi	7.95 b	5 d	1.05 c-h	6.01 c	46 fg	1/2
UPN575	0.00 j	>10.00 a	0 d	0.81 f-i	4.69 de	33 g	1/2
EPS100	0.18 ij	6.00 c	21 d	0.24 jk	5.62 cd	29 g	1/1
UPN609	0.00 j	>10.00 a	0 d	0.42 i-k	7.25 b	4 h	1/1
UPN610	0.00 j	>10.00 a	0 d	0.40 i-k	7.43 b	4 h	1/1
UPN562	0.00 j	>10.00 a	0 d	0.39 i-k	7.64 b	29 g	1/1
UPN546	0.00 j	>10.00 a	0 d	0.38 i-k	7.61 b	4 h	1/1
NCPPB311	0.00 j	>10.00 a	0 d	0.00 k	>10.00 a	0 h	1/1
PMV6076	0.00 j	>10.00 a	0 d	0.00 k	>10.00 a	0 h	1/1

^y Parameters r_g , t_0 , and K were obtained by fitting disease incidence progression data to the modified Gompertz model given in equation 3. Results for the two experiments performed are shown. Means within the same column followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Experiments 1 and 2 were performed at an initial pathogen concentration of 10^3 and 10^5 CFU/ml, respectively.

^z Obtained upon rescaling each parameter from 1 to 4 and calculating a global index $r_g + t_0 + K$. First and second numbers correspond to experiments 1 and 2, respectively.

NCPB1734, showed high aggressiveness. Strains NCPB2080, NCPB683, NCPB1819, and NCPB595 showed moderate aggressiveness and strain 115.22 showed low aggressiveness. Strain NCPB311 and the mutant PMV6076 were nonvirulent. The Spanish strains were distributed in the four classes of aggressiveness, but were more frequent in the highly aggressive group (56%).

Relationships between r_g , ED_{50} , and t_0 . Only 11 data points were included in these analyses, resulting from the combinations of strain, plant material, and cultivar that were analyzed with both infectivity titration and kinetics of progression of infection incidence. An inverse relationship was observed between r_g and ED_{50} , and a direct relationship was observed between t_0 and ED_{50} (Fig. 3). Also, a multiple linear regression analysis indicated a relationship between the three parameters ($ED_{50} = 6.31 + 0.174t_0 - 2.49r_g$; $R^2 = 0.84$). Therefore, a clear partitioning of the aggressiveness into infectiveness and invasiveness was not possible because of the correlation between the three parameters.

DISCUSSION

This study found differences in aggressiveness among an extensive collection of strains of *E. amylovora* composed of isolates from Spanish outbreaks of fire blight and reference strains from different geographical origins. This was, in part, because of the wide range of origin of the strains, dose of pathogen, plant material type, time of incubation, and accurate control of environmental conditions during the assays.

Modeling data sets was necessary to summarize differences in aggressiveness among strains because the dose of pathogen and time of incubation had an effect on disease levels (measurement of the proportion of infected flowers or fruit). The system consisted of a procedure of nonlinear fit to mathematical equations relating incidence of infection to dose of pathogen (probit and hyperbolic saturation models) and time of incubation upon pathogen inoculation (modified Gompertz model). The models fit well to the data, providing a tool to determine parameters related to aggressiveness such as ED_{50} , time delayed to start of the infection progress curve (t_0), and rate of progression of incidence of infections (r_g).

Values of ED_{50} have not been reported previously for *E. amylovora*. However, an approximate estimation for comparison purposes can be obtained from data provided in some reports. Vanneste (54,55) showed that the proportion of immature pear fruit infected upon 3 days of inoculation with a strain of *E. amylovora* approached 1 when using pathogen concentrations ranging from 10^4 to 10^5 CFU/ml, suggesting that ED_{50} was lower than 10^4 CFU/ml under these experimental conditions. Other reports reflected the low susceptibility of the plant material or the low aggressiveness of the strain used. For example, we estimated values of ED_{50} at $\approx 10^8$ CFU/ml for apple plantlets inoculated in vitro with strain E4001A (33,34) and shoots of hawthorn in the greenhouse inoculated with strain Ea519 (56). These data are in agreement with values of ED_{50} ranging from 10^2 to 10^8 CFU/ml observed in the present study from infectivity titration experiments.

Values of r_g , either directly reported or calculated using data from other works, are in agreement with our findings from near 0 to 1.90 day^{-1} . An r_g of 2.22 day^{-1} in immature pear fruit was calculated from raw data of Vanneste (54,55) in accordance with our results for the most aggressive strains inoculated in the most susceptible plant material. Values of r_g ranging from 0.10 to 0.43 day^{-1} were calculated from raw data on strain E4001A inoculated to in vitro apple plants of cv. Novole (33,34), and for a strain inoculated in pear seedlings of cv. Durondeau in the greenhouse (17). Also, in greenhouse trials using pear (cvs. Kirchensaller and Doyenne du Comice) and apple seedlings (cv. Golden Delicious), values of r_g ranged from 0.14 to 0.29 day^{-1} (L. Ruz and J. Cabrefiga, unpublished data). However, lower values of r_g of

0.03 day^{-1} have been reported in field trials in pear and apple shoots of six cultivars (30). Therefore, differences in r_g between reports can be attributed to the different susceptibility of the plant material used, the environmental conditions (greenhouse or field trials), and the aggressiveness of the strain used. However, in our study, these differences in r_g are due to strain variability because the plant material and environmental conditions were fixed.

From the infectivity titration experiments, a minimum infective dose (MID) can be calculated for each strain. A threshold value of 0.2 (mean of the confidence intervals for the disease proportion data) can be considered as significantly different from zero. Therefore, the minimum dose of pathogen causing a disease level higher than 0.2 can be considered as the MID. Accordingly, MID values from 10^2 (detached flowers) to 10^5 CFU/ml (immature fruit) can be calculated easily from data of Table 2. Taking into account the inoculation volume used ($10 \mu\text{l}$), the MID can be estimated from 1 to 1,000 CFU per inoculation site. This means that, under highly favorable conditions, as much as one viable cell of *E. amylovora* is enough to develop an infection. This is of particular importance when taking into account the fact that the concentration of viable bacteria in the typical ooze produced in infected plant tissues in the field under certain conditions can account for 10^9 to 10^{10} CFU/drop (J. Cabrefiga, data not shown). Under field conditions, this inoculum can be distributed by several abiotic (thunderstorms or wind aerosols) or biotic (insects) agents, and has been considered important in disease spread (21,53,55). The low MID of the pathogen and the high inoculum potential of ooze illustrates the enormous capacity for spread of fire blight. Another implication of the low MID for this pathogen concerns the methods of detection and analysis of *E. amylovora*. The level

Fig. 3. Relationships between the rate of disease progression (r_g) and time to start of the disease progression curve (t_0) at 10^3 CFU/ml with median effective dose (ED_{50}) at 5 days from inoculation for strains of *Erwinia amylovora*. The symbols correspond to immature pear fruit of cvs. Passe Crassane strains UPN611 ($\diamond$), IVIA1614.2 ($\blacklozenge$), EPS101 ($+$), UPN529 ($\blacktriangledown$), NCPB595 (∇), EPS100 ($\blacksquare$), and NCPB311 ($\square$); Doyenne du Comice strain EPS101 ($\bullet$); and Conference (strain EPS101 ($\circ$); and detached pear flowers of Doyenne du Comice strain EPS101 ($\blacktriangle$) and Conference ($\triangle$).

of detection typically used in assays needs to be improved, especially in propagation plant material from nurseries, in order to prevent delivery of symptomless plants harboring incipient infections or infective population levels below the assessed detection level ($\approx 10^3$ CFU/g fresh weight or susceptible entry site).

The efficiency parameter (τ) provided by the probit model can be related to the degree of independence of action of the pathogen cells during infection. Peto (42) demonstrated mathematically that τ should be below or ≈ 2 if pathogen cells are acting independently during infection. This hypothesis was confirmed in laboratory and field experiments with *Pseudomonas syringae* on several hosts (15,42). Fitting the probit model to infectivity titration data of *E. amylovora* leads to τ values which are consistent with the hypothesis of independent action of pathogen cells on pear (τ from 0.75 to 1.73). Furthermore, this result suggests that the value itself may be an indication of the degree of independence.

In the two experiments performed at different pathogen doses in the study of the kinetics of incidence of infection in immature pear fruit, the effect of dose on r_g was pronounced in some strains whereas, in others, it was not significant. This differential effect is related to τ , the efficiency parameter of the probit-dose relationship, which is an indication of the degree of independence of cells during the infection process. This hypothesis is supported by the fact that there is a significant direct correlation between the percentage of increase in r_g upon dose increase and the τ values ($R^2 = 0.784$, $P < 0.01$) for the six strains shown in Table 3. Thus, strains with low values of τ (e.g., UPN611; high degree of independency) showed smaller or no changes in r_g upon increase of dose, whereas strains with higher values of τ (e.g., UPN529) showed significant changes.

The values of r_g and t_0 presented in this work also provide valuable kinetic information on potential disease development in the field. In spite of the variability observed due to strain differences, mean values of r_g of 1.2 day^{-1} and of t_0 of 3.52 days can be estimated. These values agreed with the frequent observation of symptoms 3 to 4 days after risk warning by fire blight forecasting models (e.g., Maryblit), and also with the duration of fire blight epidemics from detection of first symptoms to maximal disease levels under optimal conditions. However, most fire blight prediction models actually are based on the effect of temperature according to data on in vitro cultures of *E. amylovora* (44). The tools provided in the present work would help in calculation of r_g and t_0 in host plants other than pear and under different environmental conditions (e.g., temperature), and these values would be useful to improve fire blight forecasting models.

The combined index obtained using r_g , t_0 , and K was useful for classifying strains according to aggressiveness. This is the first time that several reference strains of different geographical and host origin have been tested in the same assay. However, the results of the present work for strains Ea273 and CFBP1430, classified as highly aggressive, agreed with previous reports (22,34–48).

Most of the highly aggressive strains on pear originally were isolated from pear, whereas the nonvirulent or less aggressive

strains were isolated from different hosts. This observation supports the hypothesis of a certain degree of host specificity among pear isolates based on analysis of aggressiveness, in accordance with a certain host species-specificity described for some strains by other authors (2,33). However, it contrasts with the very low variability at genetic and phenotypic level observed in this pathogen (19,22,28,29,41). Carbon source utilization profiles (22) and repetitive DNA and ribotyping PCR (26), random amplified polymorphic DNA fragment analysis (28), and amplified 16S-23S ribosomal DNA restriction enzyme analysis (29) have enabled the ability to distinguish strains isolated from fruit trees (Maloideae) from strains isolated from *Rubus* spp. Studies using molecular techniques capable of detecting high genetic variability, such as inverse sequence-tagged repeat (40) or amplified fragment length polymorphism analysis (41), have revealed polymorphisms among Spanish isolates of *E. amylovora*, though the resulting clusters were not correlated with host, year, or country of isolation. However, a more thorough study should be performed using the tools provided here to analyze aggressiveness in combination with genetic analysis to confirm this hypothesis.

A clear-cut partitioning of aggressiveness into infectiveness and invasiveness based on ED_{50} , t_0 , and r_g cannot be done because of the significant correlation observed between these parameters. However, the information obtained by the combination of ED_{50} , r_g , and t_0 , can be used as a more complete quantitative tool for measurement of aggressiveness of a given strain. A standardized scale on a basis of three levels is proposed, consisting of highly aggressive (low ED_{50} , short t_0 , and high r_g), moderately aggressive (medium ED_{50} , t_0 , and r_g), and low aggressive (high ED_{50} , long t_0 , and low r_g). Because the parameters are correlated, any of them can be used. Therefore, the immature pear test, for its simplicity, is proposed as a standardized test under the conditions of assay described in the present work. The range of values for each parameter in relation to aggressiveness is indicated in Table 4. These ranges have been calculated on the basis of the results of ED_{50} for the five strains tested in Passe Crassane fruit (Table 2) and r_g and t_0 at the high dose of inoculation (Table 3). In cases where different plant material or cultivars are used, the scale can be adjusted simply by including highly aggressive (e.g., EPS101) and very low-aggressive (e.g., NCPB595) strains as controls.

The wide range of aggressiveness between strains observed using the methods described here, and the fact that results were affected by plant material type and cultivar, are in agreement with several studies using different methods. Previous reports have described variability in susceptibility to fire blight among hosts, cultivars, and plant material type (8,14,23,35,36,50) and in aggressiveness (named as virulence) among strains of *E. amylovora* (17,25,34). Others have studied the interaction between host and pathogen on aggressiveness (33). However, none of them used quantitative methods accounting for the effect of dose and time in terms of ED_{50} , t_0 , and r_g .

Finally, the methods proposed and tested in the present study were proven useful to describe aggressiveness in *E. amylovora*, but it is expected they also would be fruitful in studies involving other pathosystems.

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TABLE 4. Suggested scale for the classification of strains of *Erwinia amylovora* according to the parameters obtained from infectivity titration and kinetics of infection incidence progression in the immature Passe Crassane pear fruit test

Parameter ^z	Classification of aggressiveness		
	High	Medium	Low
Log(ED_{50}) (CFU/ml)	<5	5–6	>6
t_0 (day)	<3	3–6	>6
r_g (day^{-1})	>1.2	0.6–1.2	<0.6

^z Median effective dose of the pathogen (CFU/ml) = ED_{50} , rate of infection incidence progression = r_g , and time delayed to start of the incidence progress curve = t_0 .

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