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Research Papers

Criteria for efficient prevention of dissemination and successful eradication of *Erwinia amylovora* (the cause of fire blight) in Aragón, Spain

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Summary. *Erwinia amylovora* was detected on pome fruits in the Aragón region (North-Eastern Spain), in a ca. 5 km radius area located in the mid Jalón river (mid Ebro Valley) in the province of Zaragoza, during 2000–2003. Eight years have now passed since this pathogen was last detected, without new infections being reported in the same area. The bases for surveys and rapid eradication performed have been analyzed in detail to understand the reasons for the success in removing fireblight. The results demonstrate that intensive surveillance, risk assessment, plant analyses using accurate identification methods, and, especially, rapid total or selective eradication of infected trees in the plots have been very effective in preventing the generalized spread of fireblight and in delaying economic losses associated with this disease. Eradication and compensation to growers, estimated to cost approx. € 467,000, were clearly counterbalanced by the economic value of apple and pear production in the 2000–2003 period (approx. € 368 million). Fire blight risk-assessment, using the MARYBLYT system, showed that climatic conditions in the studied area were favourable to infections during the analyzed period (1997–2006). Molecular characterization of *E. amylovora* strains had revealed their homogeneity, suggesting that these fire blight episodes could have been caused by just one inoculum source, supporting the hypothesis that there was a unique introduction of *E. amylovora* in the studied area. Spatial spread of *E. amylovora* to trees was analyzed within six orchards, indicating an aggregated distribution model. This Spanish experience demonstrates the success of scientifically-based prevention methods that lead to the deployment of a fast and strict containment strategy, useful for other Mediterranean areas.

Key words: Surveys, risk-assessment, spatial analysis, strain characterization.

Introduction

Fire blight, caused by the bacterium *Erwinia amylovora*, is one of the most serious and destructive diseases affecting apple and pear trees, as well as other fruit trees and several ornamental plants of the *Rosaceae* family of great commercial importance. *E. amylovora*, first described in North America, has spread through European and Mediterranean countries and has has been reported in over 40 countries (Anonymous, 2007). This pathogen is considered a quarantine organism in the European Union (EU) (Anonymous, 2000) and in most South American, African and Asian countries, and in Australia. Fire blight causes severe economic losses, not only due to the wide range of commercial plant species affected

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but also to the lack of registered products that can effectively control the disease. Additionally, the prohibition of fruit exports to fire blight-free countries indirectly causes important losses (Hale *et al*., 1996). Eradication is the most effective preventive control method when a quarantine pathogen is detected for the first time in a new area and when no effective chemical or integrated control methods are available. In the EU, fire blight eradication has been attempted in some countries, but has generally failed (Bonn and van der Zwet, 2000).

The Spanish pome fruit industry is of great commercial importance. Spain is the second leading European country in pear production (473,000 t *p.a.*) and the fifth in apple production (596,000 t *p.a.*) (Eurostat, 2011). About 70% of the total Spanish pomefruit production is cultivated in and around the Ebro Valley, where Aragón is located (MAPA, 2006, 2007). Over 4,000 ha of apple and 5,000 ha of pear are grown in the Aragón region (North-Eastern Spain) (Anonymous, 2009).

Erwinia amylovora was first identified in Spain in Guipúzcoa (País Vasco) in 1995, close to the Western French border (López *et al*., 1999). New fire blight outbreaks were detected in the following years and the bacterium was identified and eradicated (or is being eradicated) according to specific EU (Anonymous, 2000) and Spanish (Anonymous, 2005a; b) legislation. One or more outbreaks have been found since then in the following Spanish regions: País Vasco, Navarra, Castilla y León, Aragón, Cataluña, Castilla-La Mancha, Madrid, La Rioja, Cantabria, Extremadura, Andalucía and Comunidad Valenciana (López *et al*., 2002; unpublished information), and growers have received economic compensation for eradication. In contrast to the other European countries, where the disease has not been eradicated, fire blight has not been widespread in Spain since 1995 (first detection) until 2010. Spain was considered a protected zone in the EU until 2011 (López *et al*., 2002).

A detailed study of a fire blight outbreak in the Aragón region, where *E. amylovora* was first detected on pome fruits in 2000, is presented. Knowledge of the factors for avoiding rapid spread of the disease, which is essential for its control, will both improve understanding of *E. amylovora* biology and epidemiology, and contribute to the design of suitable preventive strategies and eradication programs to deal with fire blight prevention in other Mediterranen areas.

Materials and methods

Surveys for *Erwinia amylovora*, analysis of suspicious samples and eradication measures

The monitoring protocol for fire blight prevention in the Aragón region, as well as in other Spanish areas, included visual inspections of host plants in orchards and nurseries and analyses of all suspicious samples as well as a variable number of symptomless samples, performed following the European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocol (Anonymous, 2004). All these actions have been coordinated by the regional Plant Protection Service, the official plant health authority in the Aragón region.

In June 2000, *E. amylovora* was first detected in a routine inspection in pome fruit orchards in the mid Jalón river area of Zaragoza, and strong eradication measures were immediately taken, according to Spanish legislation (Anonymous, 1999) and the EU Directive (Anonymous, 1997). A 5 km radius Security Area (SA), which held 1,385 ha of apple and 468 ha of pear trees, was established around the first infected plot identified (Figure 1). Exhaustive surveys were performed inside this SA, and in the first year all the orchards where *E. amylovora* was identified were eradicated, even when only one infected tree was found. Moreover, in order to establish the real fire-blight situation throughout Aragón, an extensive monitoring network was also set up to routinely inspect pear and apple orchards located outside the SA.

Other fire blight episodes were found in the following 3 years. Based on the experience acquired during 2000, surveillance and eradication strategies were discussed case by case, and modified taking into account the characteristics of each specific episode. A strategy of selective eradication of infected trees and their neighbouring trees was adopted in orchards with low inoculum levels, and where infected trees were closely clustered. The criteria for selective eradication were: number of affected trees, estimated time since infection, cultivar susceptibility, presence of host plants in surrounding areas, probable inoculum source, and dissemination factors. These selectively eradicated plantations were surveyed weekly (20 visits a year from May to October) to check the course of the disease and take decisions about the trees preservation or total eradication when new infected trees were found. The remaining plantations inside the SA were surveyed four times

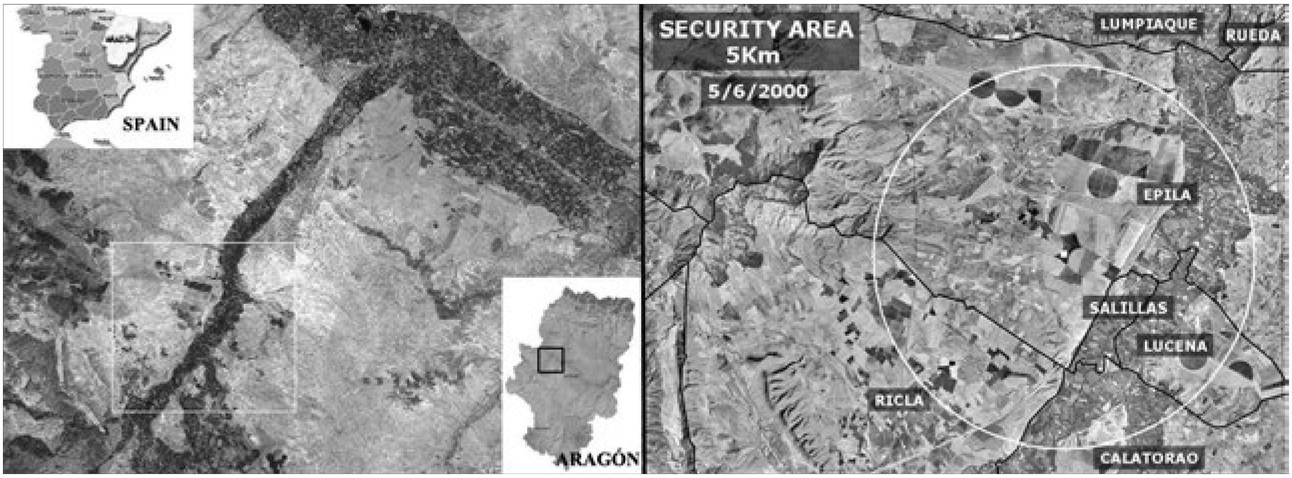

Figure 1. Jalón river area of Zaragoza (Spain) where fire blight was detected (left) (the expanded area to the right is indicated in the Aragón map at left by a square), and detail of the first infected orchard identified and 5 km radius Security Area initially established (right).

a year, whereas all the orchards with host plants outside this area were inspected once a year. Monitoring was specially focused on pear trees, since the apple cultivars grown in Aragón are considered of low susceptibility to fire blight (Montesinos and López, 1998). Ornamental host plants were also surveyed, but these were rare in this area.

Risk assessment system

The MARYBLYT risk assessment system was used to analyze fire blight risk from 1997 to 2006 (the years previous, during and subsequent to the episodes), and to relate the risk levels with the symptoms observed in some orchards. MARYBLYT (version MARYBLYT^{TM} 4.3) (Steiner and Lightner, 1996) is a comprehensive predictive system, which identifies the conditions conducive to fire blight symptoms developing (blossom, canker, shoot, and trauma blight). Criteria for blossom infections were: (i) the blossom must be open with stigmas and petals intact; (ii) the accumulation of at least 110 degree-hours above 18.3ºC from the first open bloom; (iii) more than or equal to 2.5 mm of rain on the previous day; and (iv) an average daily temperature ≥ 15.6ºC. Blossom blight symptoms will appear after 57 cumulative degree days above 12.7ºC (Steiner and Lightner, 1996). During the blooming and post-blooming period, four levels of risk are generated: low, moderate, high and infection. The model also predicts canker symptoms, which provide a source of inoculum for shoot blight, even when blossom infections do not occur.

The MARYBLYT program was run for 10 years in La Almunia and Épila, two locations in the province of Zaragoza in which the two meteorological stations were near the orchards where fire blight was detected (at ca. 2 and 10 km from these orchards, respectively). Daily maximum and minimum temperature and rainfall data were obtained from weather stations of the Agriculture and Food Department of the Aragón Government. In order to compare the data recorded during the different years, phenological pear stages such as green tip, first bloom and petal fall, all corresponded to cv. Conference. Due to the difficulty in collecting precise data about secondary bloom occurrence, this was considered to start after petal fall and end on the June 30th.

During 2002, a remote weather station (model CR10, Campbell Scientific Ltd., Leicester, UK) was installed in an orchard located in Épila (P4-3, see below) in which fire blight was detected in 2001. The objective was to relate the predictions of fire blight risk using MARYBLYT with the symptoms observed in 2002. Phenological assessment was very accurate in this orchard, including the post-blooming period. Due to the fact that fire blight was detected only on pear cv. Blanquilla in another orchard in Épila (P6-

1, see below), risk in 2003 was determined with the specific phenological stage for this cultivar instead of cv. Conference.

Erwinia amylovora spatial spread analysis

The model for *E. amylovora* spatial spread in Aragón was studied in six pear orchards in different years and fire blight episodes. The set of infected trees found in each orchard was considered as a spatial point pattern, and was analyzed to detect aggregations with regard to complete spatial randomness (Cressie, 1993; Diggle, 2003; Møller and Waagepetersen, 2004; Illian *et al*., 2008). Nearest neighbour distance was used to describe the proximity between infected trees in six orchards with over ten *E. amylovora* positive results. Distances between each symptomatic tree and its nearest infected neighbour were calculated. The average of these nearest neighbour distances was compared with the average for a hypothetical random distribution. An aggregation index was calculated to test for divergence of the observed spatial distribution from complete spatial randomness (Clark and Evans, 1954). The data were processed using the GIS software ArcGIS 9.1 (ESRI Inc., Redlands, CA, USA) and distances were calculated with *Average Nearest Neighbour* function. Measures of standard deviations from the mean were calculated using Z scores: a positive Z score indicates a greater separation between infected trees than expected in a random pattern, whereas a negative Z score denotes aggregation with smaller distances than expected. The evidence is significant when the absolute value of Z score is > 2, and is conclusive when it is > 3.

A more detailed analysis was carried out by estimating the K function for the six point patterns: the K function was defined for each distance *d* as the expected number of further events within distance *d* of an arbitrary event divided by the mean number of events per unit area. The data were analyzed with the statistical software R (R Development Core Team, 2004) using the *splancs* package (Rowlingson and Diggle, 1993; Bivand and Gebhardt, 2000). The K function was estimated with the *khat* function. Each estimated K curve was compared with the theoretical K function using simulated envelopes (Bailey and Gatrell, 1995; Diggle, 2003) calculated with the *kenv.csr* function. For each K function 1,000 simulations were obtained to determine upper and lower 99.9% confidence bounds. An estimated K curve over the confidence bounds indicates an aggregation of the infected trees because there is an excess of short distance between them.

Molecular characterization of *Erwinia amylovora* strains

Strains of *E. amylovora* were isolated from the first infected trees in each of the six fire-blight episodes registered in Aragón. From them, nine strains were selected for comparative analysis and characterization. These strains were identified and characterized according to the EPPO standards (Anonymous, 2004). Besides, simultaneous screening for the presence of the pEA29 plasmid and the recently identified conjugative *E. amylovora* plasmid of ca. 70 kb, named pEI70, was performed by duplex PCR (Llop *et al*., 2008). Furthermore, minisatellite-primed-polymerase chain reaction (MSP-PCR), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) were also utilised. MSP-PCR analysis was applied using primer M13 based on the core sequence of phage M13, and RAPD analyses using four primers (OPC-15, OPC-19, 1281 and 1290), and both were performed according to Donat *et al*. (2007). AFLP analyses were performed by Biopremier, Ltd (Universidade da Lisboa, Portugal), according to Vos *et al*. (1995) with modifications (Donat *et al*., 2007), using *Eco*RI/*Mse*I enzymes and the two pairs of primers E3/M1 and E00/M01-g (Rico *et al*., 2004). Cluster analysis of the fingerprints obtained was carried out following the unweighted pair group method with arithmetic mean (UPGMA) and Dice similarity coefficient (Sneath and Sokal, 1973). To evaluate the clustering method, cophenetic correlation was applied. The software used for all cluster analyses was BioNumerics from Applied Maths.

Results

Surveys for *Erwinia amylovora*, analyses of suspicious samples and eradication measures

The surveys and sample analyses performed in Aragón are summarized in Table 1. Inspections of pome fruit plantations first detected fire blight in June 2000, in the mid Jalón river area of Zaragoza, which was over 100 km from any previous foci of the disease detected in Spain (Figure 1). After the first

Table 1. Fire blight surveys and sample analyses performed in Aragón (Spain).

Year	No. inspectors	Surveyed area (ha) and species	No. analyses
1996	2	40 apple	37
1997	2	53 apple	85
1998	2	67 apple	110
1999	8	1.601 pear	303
2000	28	1,404 pear (SA)[a] 1,932 apple (SA) 1,445 pear	318 (SA) 179
2001	2 (SA) 22	712 pear (SA) 4,632 pear	485 (SA) 58
2002	4 (SA) 20	1,404 pear (SA) 4,758 pear	1,246 (SA) 15
2003	6 (SA) 12	2,383 pear (SA) 2,484 pear	1,042 (SA) 9
2004	6 (SA) 8	2,447 pear (SA) 1,820 pear	192 (SA) 17
2005	6 (SA) 8	2,301 pear (SA) 1,782 pear	118 (SA) 6
2006	6 (SA) 8	2,068 pear (SA) 2,809 pear	150 (SA) 14
2007	6 (SA) 8	2,132 pear (SA) 3,118 pear	119 (SA) 236
2008	6 (SA) 8	2,309 pear (SA) 2,830 pear	15 (SA) 146
2009	6 (SA) 8	2,223 pear (SA) 2,738 pear	12 (SA) 140
2010	6 (SA) 8	2,114 pear (SA) 2,236 pear	15 (SA) 117

[a] SA: Security Area (includes 1,385 ha of apple and 486 ha of pear trees). Unless indicated, data are referred to outside the Security Area.

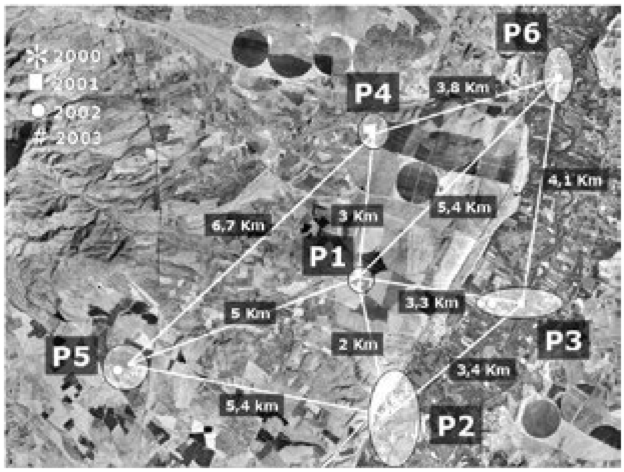

Figure 2. Location of the six areas where fire blight episodes were detected in pome fruits in Zaragoza (Spain) between years 2000 to 2003. Distances among each area as well as affected orchards inside each area (indicated by symbols) are shown.

year, and after the negative results for new foci of *E. amylovora* outside the SA, the number of inspectors surveying orchards outside the above indicated SA gradually decreased in the period 2000–2003 (from 22 to eight inspectors). Conversely, inspectors inside this area increased(from tow to six) (Table 1). About 5,000 ha of pome fruits were inspected annually.

Between 2000 and 2003, *E. amylovora* was identified in 22 orchards from four municipalities (Épila, Salillas, Calatorao and Ricla), all located inside the initially established 5 km radius SA. According to their location, six fire-blight infected areas or episodes were established (references P1 to P6 in Table 2). These included a variable number of orchards (one to ten), separated by a distance of less than 500 m. The distance among orchards of different episodes was at least 2 km (Figure 2). Few infected trees were detected in all plantations, suggesting low infection levels and recent introduction; furthermore, initial-stage infection symptoms were always observed. Regarding hosts, nearly all *E. amylovora* detections corresponded to pear trees and most of these were a minimum of 8 years old. Conversely, the bacterium was identified on only four Golden Delicious apple trees, which were more than 20 years old, and only in two orchards located in the midst of infected pear tree plantations (Table 2). Between 2000–2003, a yearly average of 773 suspicious and asymptomatic samples was analyzed from pome fruits orchards inside the SA and 65 samples were taken from plots located outside this area (Table 1). In the 2004–2010 period, the annual average number of samples analyzed were 89 from inside, and 96 from outside the SA. In all cases, the identity of the bacterial isolates was confirmed according to the EPPO standards (Anonymous, 2004).

In 2000–2003, 15 orchards were eradicated but their total area was only of 29.8 ha. Only one of these

Table 2. Orchards identified in mid Jalón river area (mid Ebro Valley) of Zaragoza (Spain) with trees affected by fire blight in 2000-2003, type of eradication adopted and estimated cost of eradication and compensations to growers.

Year	Orchard reference	Species / cultivar	Age (years)	Detection date	Surface[a] (ha)	No. infected trees	Adopted measures (year)					Eradication and compensation to growers cost (€)
							2000	2001	2002	2003	2004	
2000	P1-1[b]	Pear / Conference	13-14	05/06/00	8,2	128	E					
	P2-1[c]	Pear / Conference	8	15/06/00	0,7	27	E					
	P3-1[d]	Pear / Blanquilla	11	15/06/00	1,0	3	E					
	P2-2	Pear / Conference	11	16/06/00	0,2	2	E					
	P2-3	Pear / Conference	12	16/06/00	1,2	2	E					
	P2-4	Pear / Conference	15	19/06/00	0,8	4	E					
	P2-5	Pear / Blanquilla	8	19/06/00	1,1	38	E					
	P2-6	Pear / Conference	8	19/06/00	3,1	4	E					
	P2-7	Apple / Golden Delicious	>20	05/07/00	0,2	1	E					
	P2-8	Apple / Golden Delicious	>20	05/07/00	1,3	3	E					
	P2-9	Pear / Conference	15	18/07/00	1,3	36	E					
	P2-10	Pear / Blanquilla	16	29/11/00	0,5	1	E					
	Total (2000)											*326,000*
2001	P3-2	Pear / Blanquilla	19	09/04/01	1,6	9		SE	(-)	(-)	(-)	
	P4-1[e]	Pear / Abate Fetel	6	23/07/01	3,9	153		E				
	P4-2	Pear / Red Crimson	12	24/07/01	3,1	74		E				
	P4-3	Pear / Williams	12	24/07/01	10,8	30		SE	(-)	(-)	(-)	
	Total (2001)											92,000
2002	P3-3	Pear / Conference	5	05/02/02	1,7	1			SE	(-)	(-)	
	P5-1[f]	Pear / Blanquilla	14	19/06/02 - 02/07/03[h]	6,2	24			SE	SE	(-)	
	P6-1[g]	Pear / Blanquilla	10	13/08/02 - 04/06/03[h]	3,2	15			SE	E		
	Total (2002)											0
2003	P6-2	Pear / Blanquilla	8	20/05/03	0,6	1				SE	(-)	
	P6-3	Pear / Blanquilla	>20	23/05/03	2,8	2				SE	(-)	
	P5-2	Pear / Conference	9	15/07/03	5,8	1				SE	(-)	
	Total (2003)											49,000
Over total												467,000
Fire blight situation has not changed since 2003 up to date												

[a] Number of trees/ha range between 1,000 and 575; E, total eradication of the plot; SE, selective eradication; (-), weekly inspections without new detections.
[b] Two *E. amylovora* strains studied from orchard P1-1 (CITA Ea 31 and CITA Ea 36).
[c] Two *E. amylovora* strains studied from orchard P2-1 (CITA Ea 38 and CITA Ea 39).
[d] One *E. amylovora* strain studied from orchard P3-1 (CITA Ea 41).

orchards (P6-1) (Table 2) corresponded to an orchard in which infected trees had initially been selectively eradicated in 2002, but in 2003 the whole orchard was removed because newly infected trees were detected. However, another seven orchards (29.5 ha) were maintained following selective eradication and no new *E. amylovora* cases have been detected to date (8 to 10 years later). Since 2003, no new cases of fire blight have been detected in the mid Jalón river area of Zaragoza (Cambra *et al*., 2004, 2005; Palacio-Bielsa *et al*., 2008). The total estimated cost of the eradication program was € 467,000 during those 4 years (Table 2).

Risk assessment

The annual variation in the number of potential infection days during the blooming period in 1997–2006, according to MARYBLYT, is presented in Table 3. This shows that blossom infections were not predicted for any year because the infection days were zero. However, in 1997 and 2004 there was 1 day with predicted high risk; in 1999 and 2002, between 2 and 3; in 2000, zero; in 2001, 5, and in 2006, 8 days. Additionally, in 1999, 2001, 2002 and 2006, the epiphytic infection potential (EIP) was greater than 150, indicating that a large number of infections were likely to occur, whereas in 1997, 1998, 2000, 2003, 2004 and 2005 was less than 150 (Table 3). When the risk was determined during the post-blooming period, the number of days with infection risk was very high (from 10 to 22 days) in 1997, 1998, 1999, 2000, 2005 and 2006, while fewer infections days were predicted (from 2 to 6 days) in the other years (2001, 2002, 2003 and 2004).

In orchard P4-3 (Table 2), in which fire blight was detected and 30 trees were eradicated in 2001, no infection days were predicted during the blooming period in 2002 and a high risk level was predicted on 5 days. The maximum value for the EIP was 400. During the post-blooming period infection was predicted on 6 days, and a high risk level on 27 days (data not shown).

In orchard P6-1 (Table 2), fire blight symptoms were observed in cv. Blanquilla pear during 2003 and there was a clear relationship between the days when canker margin symptoms were predicted (27 April, specially about shoot blight symptom predictions, 20 May), and the symptoms actually observed in this orchard (Figure 3). The symptoms were first observed on 25 April and subsequently on 13, 15, 19, 20 and 23 May. In a first approach, only the 15 symptomatic trees were selectively eradicated, but when new symptoms appeared on 6 June, the whole orchard was destroyed. During the blooming period no infections were predicted.

Erwinia amylovora spatial spread analysis

Nearest neighbour analysis of the six orchards analyzed (with references P2-5, P2-9, P4-2, P4-3, P5-1

Table 3. Annual variation in number of days with different levels of fire blight risk according to MARYBLYT system during blooming period in pear cv. Conference, at La Almunia and Épila orchards in Zaragoza (Spain). Epiphytic infection potential is also presented.

Risk[y]	La Almunia		Épila							
	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006
Low	7	6	9	9	6	8	12	5	9	3
Moderate	7	7	3	6	4	4	3	8	5	4
High	1	0	3	0	5	2	0	1	0	8
Infection days	0	0	0	0	0	0	0	0	0	0
EIP[z]	145	67	230	109	315	182	28	133	69	194

[y] Blossom blight risk infection was determined in funtion of four factors and the minimum requirements needed to start an infection were: open blossoms, epiphytic infection potential ≥100, rain and average daily temperature≥15.6. The final level risk was obtained in function of the number of factors that reached the minimun requirements: low 1; moderate 2; high, 3 and infection 4.

[z] Epiphytic infection potential (EIP) is an index for infection risk and the highest EIP value recorded in that season is presented. EIP<100 few or no infections are likely to occur; EIP of 100–150 is low but enough to support an epidemic of blossom blight; EIP>150 indicates that large numbers of infections are likely should a wetting event occur.

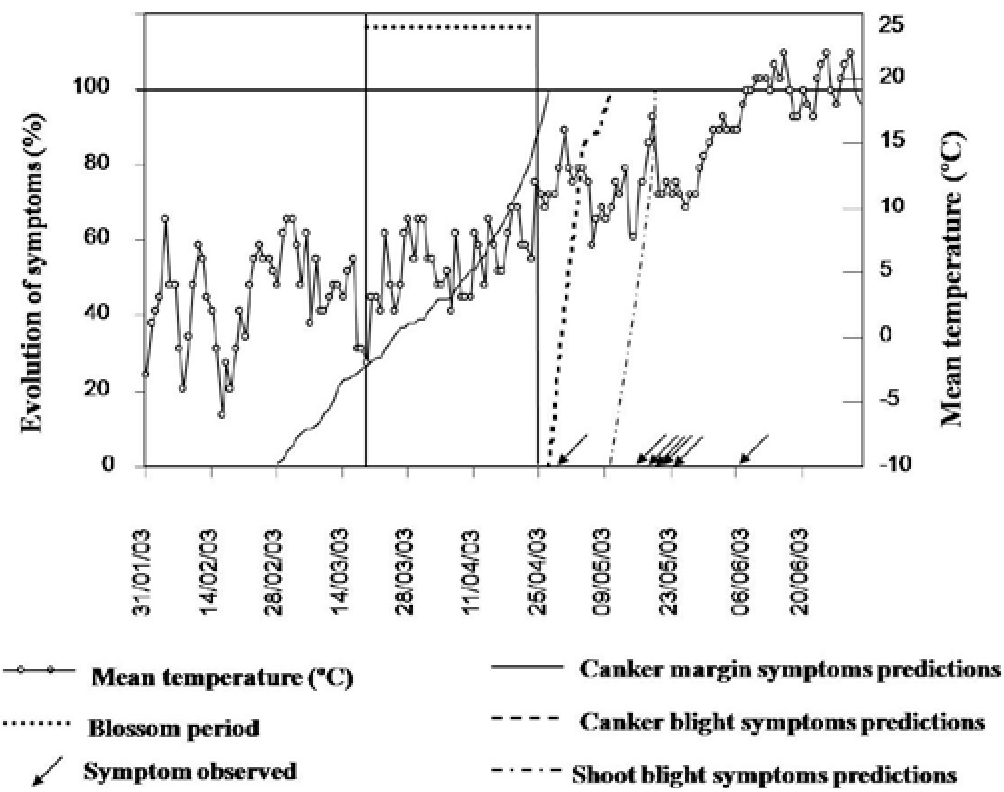

Figure 3. Fire blight risk according to MARYBLYT model in orchard P6-1 during 2003 on pear cv. Blanquilla. The mean temperature, blossom period and dates when the symptoms were observed are presented. Predicted evolution of infections on canker and shoots corresponds to oblique lines that start when the infection begins and end when the symptoms appear, corresponding to 100% of symptom evolution (horizontal bar).

Table 4. Nearest neighbour distances between infected trees in six orchards with fire blight affected trees in Zaragoza (Spain).

Orchards	P2-5	P2-9	P4-2	P4-3	P5-1	P6-1
Number of infected trees	38	36	74	30	24	15
Observed mean distance (m)	5.64	5.66	7.20	19.27	10.72	10.86
Expected mean distance (m)	8.27	8.61	8.86	29.23	23.07	11.75
Nearest neighbour ratio	0.68	0.66	0.81	0.66	0.46	0.92
Z score	-3.75	-3.94	-3.08	-3.57	-5.02	-0.56

and P6-1 in Table 2) showed aggregation of infected trees. The observed and expected mean distances for these orchards are shown in Table 4. The average distance was always less than the average for a hypothetical random distribution, and provided nearest neighbour ratios of less than 1. These results showed that the spatial distribution of the infected trees was more aggregated than a random sample of trees. The Z scores were conclusive, except for orchard P6-1. The estimated K function for each orchard confirmed

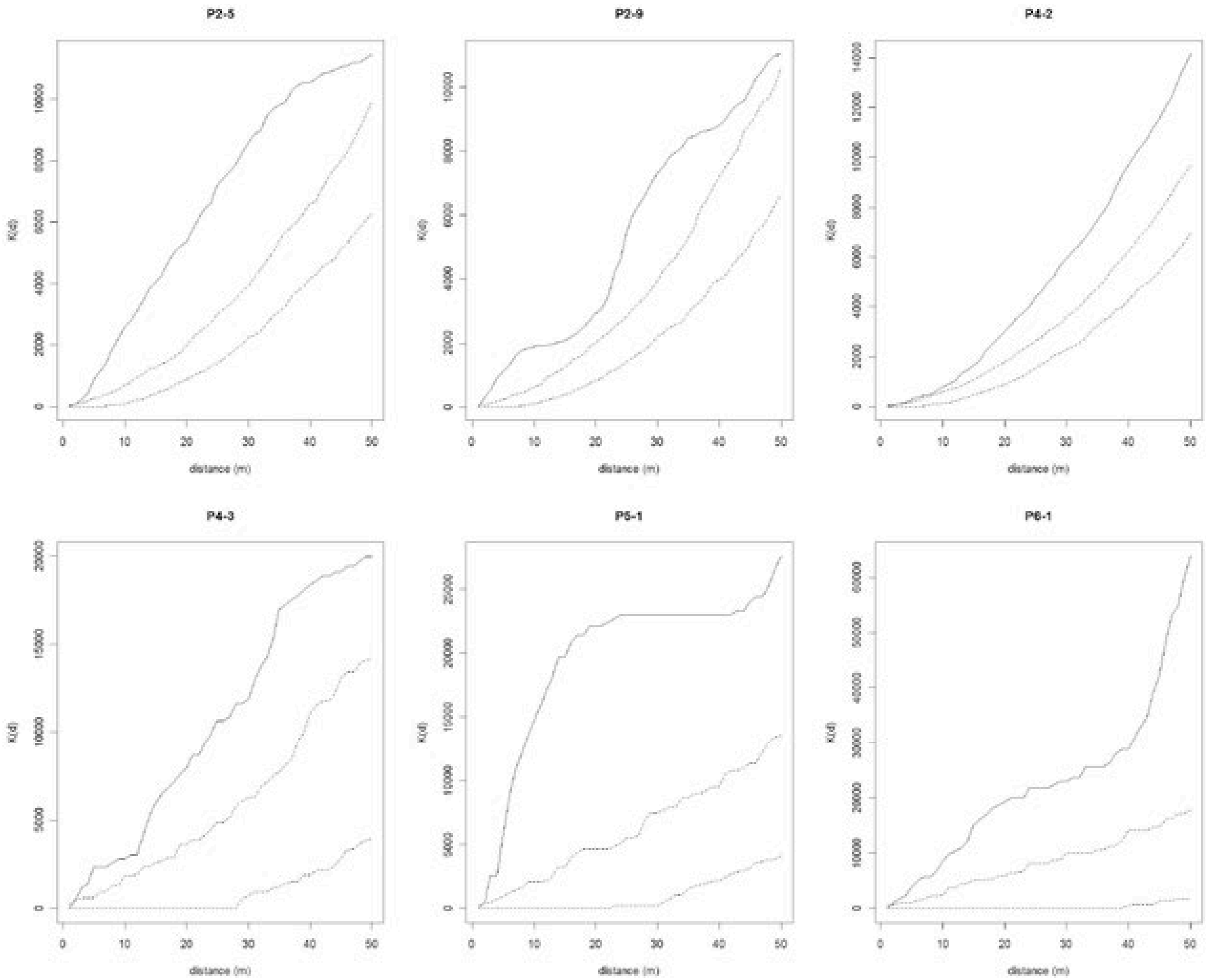

Figure 4. Analysis of estimated K function (solid line) and simulated confidence bounds (dotted lines) for each of the six fire blight affected orchards.

the aggregated nature. Figure 4 shows the observed characteristic jointly with the simulated envelopes for each of the six orchards. The K function of the infected trees fell above the range of simulated confidence bounds for a complete spatial randomness process, indicating significant clustering.

Molecular characterization of *Erwinia amylovora* strains

Duplex PCR analysis of *E. amylovora* strains yielded the single 800-bp size band expected for the pEA29 plasmid fragment, whereas no amplification was obtained for pEI70 plasmid (data not shown), confirming that the strains from this area only harboured the pE29 plasmid. In MSP-PCR and RAPD analyses, all nine strains from Aragón clustered into a very homogeneous group with identical fingerprints (Figure 5). Similar results were obtained by AFLP analyses with two pairs of primers (Figure 6). In the combined dendrogram of all the techniques, the studied strains grouped together at 100% similarity with Dice coefficient, since they were indistinguishable by all the analyses performed (Figure 6).

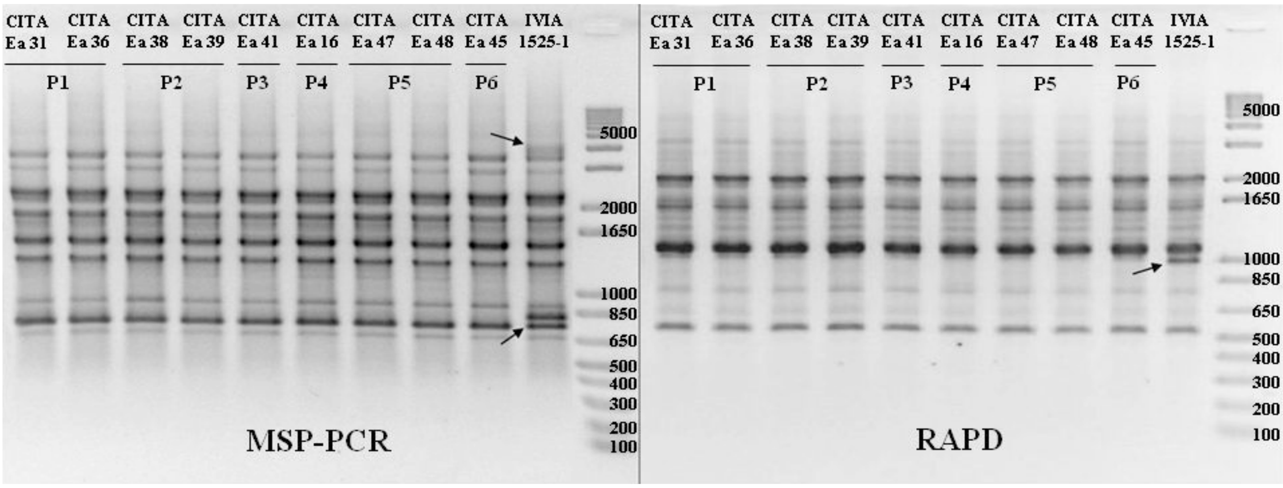

Figure 5. DNA banding pattern obtained for nine *Erwinia amylovora* strains from the six fire blight areas in Zaragoza (Spain) (P1 to P6) by MS-PCR fingerprints obtained with primer M13 (left), and RAPD fingerprints obtained with primer OPC-19 (right). Arrows indicate additional bands obtained from the *E. amylovora* strain IVIA 1525-1. Nine CITA strains were isolated from Zaragoza (Spain) and strain IVIA 1525-1 originated from País Vasco (Spain).

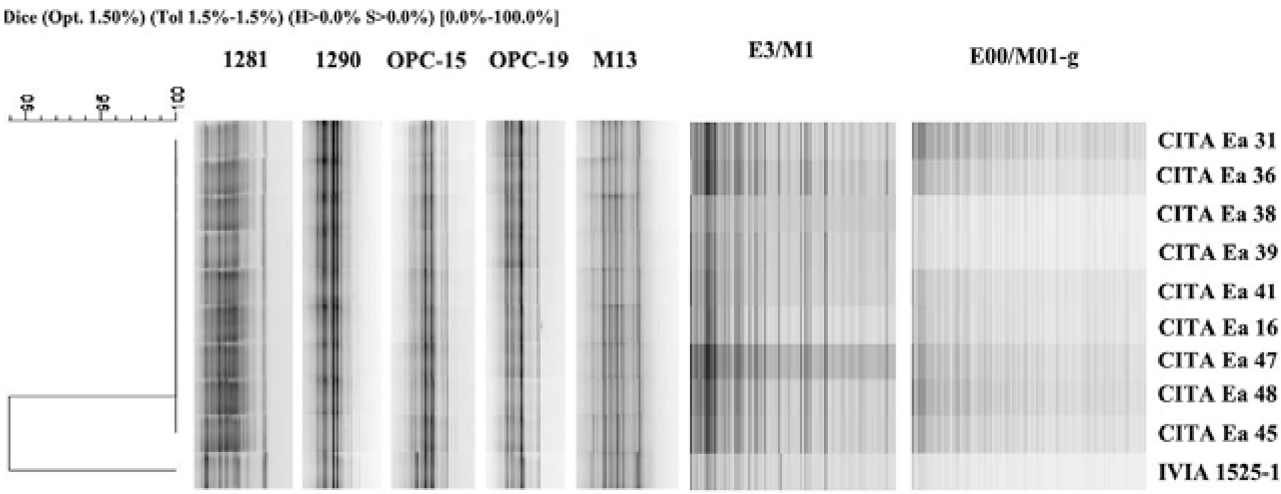

Figure 6. Combined dendrogram of the RAPD analyses (primers 1281, 1290, OPC-15 and OPC-19), MS-PCR (primer M13) and AFLP (E3/M1 and E00/M01-g primers pairs) applied to nine *Erwinia amylovora* Aragón strains. Nine CITA strains were isolated from Zaragoza (Spain) and strain IVIA 1525-1 originated from another Spanish area.

Discussion

Practical criteria for detecting primary fire blight outbreaks and avoiding spread of the disease in a representative Spanish region are detailed in this study. These included intensive surveys, risk assessment, accurate plant analysis and rapid general or selective eradication of trees in the orchards, with compensation to the growers. Detailed studies of spatial distribution of affected trees in some orchards and *E. amylovora* strain characterization contributed to improving the understanding of fire blight biology and epidemiology in the particular growing and environmmental conditions.

Fire blight was first detected on pome fruits in mid Jalón river area (mid Ebro Valley) in the Aragón region in 2000, with new events of the disease being identified in subsequent years until 2003. As soon as *E. amylovora* was identified, the number of surveys was increased and rapid eradication measures were applied resulting in no new detections recorded until 2011. Eradication as well as compensation to growers were supported by national laws (Anonymous, 1999). The role of the regional Plant Protection Service in the co-ordination of fire blight containment strategy has been crucial to the successful eradication of the disease. Inspections have facilitated early detection, because only the initial-stage symptoms of the disease were always observed, in most cases even before being found by the growers, and a variable but low proportion of infected trees was found in all the orchards. Furthermore, selective eradication of infected trees alone has been successful, if performed immediately after detecting the first symptoms and followed by exhaustive surveys. This was demonstrated by the fact that *E. amylovora* has not been detected in selectively eradicated orchards up to date, 8 to 10 years later. The reduction in the number of infected orchards and trees observed between 2000 and 2003 is noteworthy, and indicates a progressive decrease in the available bacterial inoculum. This was confirmed by exhaustive *E. amylovora* detection analyses performed in orchards after the eradication, with negative results (data not shown).

The rapid destruction of infected trees allows total eradication of the pathogen in practice. This study leads to the conclusion that fire blight dissemination can be successfully controlled, at least for several years, if appropriate measures are adopted as soon as disease events are first identified. This experience in Aragón is an excellent example of successful fire blight eradication. Eradication and compensation to growers, at an estimated cost of about € 467,000, was clearly counterbalanced by the economic value of apple and pear production in the 2000–2003 period (around € 368 million).

In previous studies, weather-based fire blight risk maps of North-Eastern Spain were drawn up using Geographic Information Systems with data over 5–10 years from more than 60 weather stations using the Billing's Revised System (BRS), Billing's Integrated System (BIS) and Powell models (Llorente *et al*., 2002; Ruz, 2003). The maps identified regions with high risk of fire blight around the Ebro Valley, where Aragón is located. In the present study, a more detailed analysis has been performed: MARYBLYT results indicated that during the blooming period there were no days with infection risk, but that in 1997, 1999, 2001, 2002, 2004 and 2006 some high risk days were forecast. On the other hand, when the risk of the disease was analyzed during the post-blooming period, a number of days with high risk of infection were also observed. These results may partially explain why during 2000, 2001, 2002 and 2003 the disease was detected in some orchards, because once the inoculum was available, the climatic conditions and secondary blossom were also favourable for infections to develop in these years. In 2006 the predicted level of infection risk was also high, but in spite of these favourable climatic conditions no disease was detected. This suggests that the inoculum of the pathogen was not available, indicating that the eradication measures taken in the previous years were appropriate.

The EIP index is a measure of the *E. amylovora* population level and of inoculum potential. So the relationship between the high EIP values in 1999, 2001 and 2002 and the high risk during the blooming period or infection risk during the post-blooming period may explain the disease symptoms observed from 2000 to 2003. As indicated above, the fact that no disease was detected in 2006, although risk levels and EIP were similar to the latter years, may be due to the dramatic decrease in inoculum levels due to the eradication measures taken in previous years.

MARYBLYT was also evaluated during 2002 in orchard P4-3, where a fire blight event was detected the previous year and where 30 infected trees were selectively eradicated. Moreover, this orchard was located close to other affected orchards in which a total of 7 ha were destroyed. However, no disease was observed in 2002 although high risk was predicted on 5 days during blooming and 5 days post-blooming. Similar results were obtained in a previous study performed on the same orchard using MARYBLYT, BIS95 and Cougarblight models (Ruz, 2003).

When MARYBLYT was used to assess the risk in orchard P6-1 during 2003, the close relationship between the dates when symptoms were predicted to occur and the actual dates when symptoms were observed would indicate that the MARYBLYT predictions were very accurate. However, the fact that no infections were predicted during blooming suggests that cankers could have played an important role as a reservoir of inoculum in this orchard.

The origin of the disease in Aragón has not been accurately identified. However, it is very unlikely that the inoculum came from previous fire blight outbreaks in other Spanish regions, because they were more than 100 km away and apparently no plant material was introduced from them. The bacterium was almost always detected on several cultivars of pear, a host generally more susceptible than apple, and the initial fire-blight symptoms observed indicated that they had been infected recently although the trees were more than 5 years old.

As the origin of the disease could not be determined, it was not possible to perform a spread analysis of *E. amylovora* considering the fire blight events in Aragón as a whole. Thus, spatial analyses were made for each independent orchard, but we also could not study the relationships among the infected orchards. The average nearest neighbour distance and the K function provided complementary analyses that distinguish clumped patterns from random dispersion. These two methods were closely related since distances between infection areas were employed to measure spatial proximity in the pattern. However, the K function showed the proportion of pairs of events in each distance, providing both one statistic and a characteristic covering the complete range of distances. The K function, also known as second-order analysis, was the best available technique for this type of study (Dale, 1999). As expected, *E. amylovora* spatial spread analysis showed aggregation of infected trees inside orchards. Results were conclusive, except for nearest neighbour analysis Z score of orchard P6-1, due to the low number of infected trees (only 15) compared with 24 to 74 in the other plots studied. Spatial *E. amylovora* distribution models allowed the tracking of short distance pathogen dispersal among neighbouring trees inside orchards. This may improve the efficacy of visual surveys for fire blight symptoms, since aggregation implies a greater likelihood of finding other infected trees among the neighbours than in more distant trees. Temporal *E. amylovora* spread analysis inside each orchard could not be performed, since infected trees were immediately destroyed, with the exception of orchard P5-1, in which infected trees were removed selectively.

Molecular characterization of the strains from the different orchards has supported the hypothesis that only one introduction of *E. amylovora* occurred in the studied area. In other studies, MSP-PCR, RAPD and AFLP analyses have provided information about relationships between strains from different Spanish foci, with AFLP being the technique most discriminative (Rico *et al*., 2004; Donat *et al*., 2007). Nevertheless, intraspecific genetic diversity has not been found among *E. amylovora* strains in the present study, showing identical patterns regardless of the fire blight infected orchard, host or year of isolation considered. Such molecular homogeneity strongly suggests clonal strains from a common inoculum source for the six fire blight episodes detected in Aragón, which constitute a single focus of the disease (Cambra *et al*., 2004, 2005; Palacio-Bielsa *et al*., 2008).

In conclusion, the described fire blight containment strategy adopted in Aragón has so far proved to be effective. This represents a unique example of efficient long-term eradication of the disease, which has not been achieved successfully in other European countries. Consequently, these results are likely to be significant for those countries (or areas) where fire blight is not still present. Results demonstrate that eradication is possible, and that, although it is time-consuming and expensive, costs can be counterbalanced by income from pome fruit output. This is in accordance with a prediction model for an hypothetical fire blight outbreak in an Australian pome fruit growing district (Rodoni *et al*., 2006). The present study also presents detailed and complete information on fire blight dissemination and eradication, likely to be usfeful for designing more efficient surveys and eradication programs for preventing general spread of this disease and for improving the understanding of fire blight biology and epidemiology in Mediterranean countries.

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