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Incidence and distribution of '*Candidatus* Phytoplasma prunorum' and its vector *Cacopsylla pruni* in Spain: an approach to the epidemiology of the disease and the role of wild *Prunus*

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'*Candidatus* Phytoplasma prunorum' is the causal agent of the European stone fruit yellows (ESFY) disease. This phytoplasma affects wild and cultivated species of *Prunus* to different degrees, depending on their susceptibility. '*Candidatus* Phytoplasma prunorum' is present in the four regions of Spain surveyed in this study (Aragon, Catalonia, Extremadura and Valencia) with a variable incidence. Results showed that '*Ca*. Phytoplasma prunorum' was detected in all of the cultivated *Prunus* species studied, except *P. avium* and *P. dulcis*, and was widespread in Spain. The most affected species was *P. salicina*, with symptoms including early bud break and blooming, leaf curling and yellowing, collapse, and a major decrease in production. In some plots in the Baix Llobregat area of Barcelona province (Catalonia), the incidence of ESFY on *P. salicina* was as high as 80%. The insect vector, *Cacopsylla pruni*, was present in all four of the regions studied, with the highest captures in yellow sticky traps in Catalonia on *P. mahaleb* and in Extremadura in peach orchards. In Baix Llobregat, large populations of *C. pruni* were present on infected *P. mahaleb* bushes, and with high infection rates. This was a key factor in the local pathogenic cycle that caused a major ESFY outbreak in the nearby *P. salicina* orchards. In the Ebro valley (Lleida and Aragon) and Valencia, the surveys showed very low incidences of the disease and low *C. pruni* populations.

Keywords: European stone fruit yellows, Phytoplasma prunorum, *Prunus*, vector

Introduction

'*Candidatus* Phytoplasma prunorum' is the causal agent of several diseases of stone fruit trees such as apricot chlorotic leaf roll, plum leptonecrosis and peach yellows. These are collectively referred to as European stone fruit yellows (ESFY) disease, a major problem affecting stone fruit trees in Europe (Lorenz *et al*., 1994). '*Candidatus* Phytoplasma prunorum' belongs to the apple proliferation (16Sr-X ribosomal) group, and is closely related to '*Ca*. Phytoplasma mali' and '*Ca*. Phytoplasma pyri', causing apple proliferation (AP) and pear decline (PD), respectively (Seemüller & Schneider, 2004; Danet *et al*., 2011). ESFY is a quarantine disease that is widespread in Europe and the Mediterranean basin and is a limiting factor for apricot and Japanese plum tree cultivation in several major growing areas (Marcone *et al*., 2010; Cieslinska, 2011; Steffek *et al*., 2012).

European stone fruit yellows symptoms and their intensity vary considerably depending on different factors, including: *Prunus* species, variety and rootstock; age; agro-ecological conditioning; phytoplasma concentration; insect vector infection pressure; and strain virulence. The symptoms are generally characterized by physiological disorders such as short internodes, off-season growth, premature bud break, leaf rolling, chlorosis, reddening, slow or sudden decline and a loss of vigour and production (Kison & Seemüller, 2001; Marcone *et al*., 2010). The expression and intensity of the symptoms differ depending on the susceptibility of the species, from the more susceptible ones such as *Prunus salicina* and *P. armeniaca*, to the most tolerant ones such as *P. cerasifera* and *P. spinosa*, which may be infected but symptomless (Giunchedi *et al*., 1982; Jarausch *et al*., 2000; Carraro *et al*., 2002; Yvon *et al*., 2004). '*Candidatus* Phytoplasma prunorum' is transmitted by vegetative multiplication and by its insect vector *Cacopsylla pruni* (Scopoli, 1763; Carraro *et al*., 1998). *Cacopsylla pruni* is oligophagous on *Prunus* species, univoltine, and overwinters as an adult in refuge plants, mainly conifers far from the orchards (Thebaud *et al*., 2009). In late winter, depending on the climate conditions, the overwintering adults (remigrants) lay next generation eggs on *Prunus* species, where nymphs develop. In June, young adults (emigrants) move to refuge trees to overwinter. *Cacopsylla pruni* acquires the phytoplasma within 2–4 days and is infective for the rest of its life, after a latency period of approximately 3 weeks (Carraro *et al*., 2004; Labonne & Lichou, 2004; Thebaud *et al*., 2009).

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European stone fruit yellows was first reported in Spain, by Sala (1935), on Japanese plum in the Baix Llobregat area (Barcelona, Catalonia), where it appeared as a syndrome similar to those observed before in southern France and Italy (Marcone *et al.*, 2010). More recently, vegetative disorders have been observed in Japanese plum and apricot growing in Valencia, Murcia and Catalonia regions (Llácer *et al.*, 1986; Torres *et al.*, 2004). *Cacopsylla pruni* carrying '*Ca.* Phytoplasma prunorum' was first reported in Spain in 2003 (Laviña *et al.*, 2004). Given the importance of stone fruit cultivation in Spain, the destructive potential of ESFY and its geographical dispersion, there is a serious threat for Spanish stone fruit production. The aim of this study was to obtain data on the incidence, hosts, life cycles and geographical distribution of '*Ca.* Phytoplasma prunorum' and *C. pruni* in Spain. These data will be helpful in evaluating the losses and the risk of spread for different species and regions, to establish control measures and prevent the expansion of ESFY.

Materials and methods

Incidence of '*Ca.* Phytoplasma prunorum' on different *Prunus* species, orchards and regions

The incidence of '*Ca.* Phytoplasma prunorum' was studied in 2005, in four regions in Spain; Aragón, Catalonia, Extremadura and Valencia, in seven stone fruit production areas with different characteristics. A total of 47 plots of seven cultivated *Prunus* species were studied. Plots were chosen with the advice and collaboration of the regional phytopathological services, which identified orchards that were representative of each area and species. The incidence of '*Ca.* Phytoplasma prunorum' was evaluated in orchards of *P. avium*, *P. salicina*, *P. cerasifera*, *P. armeniaca*, *P. persica*, *P. domestica* and *P. dulcis*. The objective was to cover a wide range of hosts and agro-ecological conditions, including tolerant species and areas with few reported cases of ESFY. The incidence of '*Ca.* Phytoplasma prunorum' was surveyed in the Baix Llobregat, Ribera d'Ebre and Lleida areas of Catalonia, in the Vegas Altas and Vegas Bajas areas of Extremadura, and in Aragon and Valencia. The incidence of ESFY was initially assessed by visual inspection of 200 trees per orchard in a large central block and subsequently confirmed by PCR. To standardize variations in the expression of ESFY symptoms in different conditions, species and varieties, visual inspections were carried out in March and September. The whole symptom range was covered: phenological mismatch in March, and yellowing, curling and reddening in September.

The assessment was particularly concentrated in the Baix Llobregat area (Barcelona, Catalonia), in 22 plots. The coincidence in a small area of historic reports, a high incidence, several wild and cultivated *Prunus* spp., and a stable population of *C. pruni*, makes this area an interesting case to study. This area includes the lower valley of the Llobregat River and the coastal mountains, both with a Mediterranean climate. The valley is characterized by flat gravity-irrigated intensive orchards, devoted to cultivating vegetables and stone fruit trees. In the mountains there are small, non-irrigated orchards of *P. avium* grafted on *P. mahaleb*. Two other areas in Catalonia were studied. One in Ribera d'Ebre, in Tarragona province in the lower valley of the Ebro River, also has a Mediterranean climate and has intensive drip-irrigated orchards of *P. persica*, *P. salicina* and *P. armeniaca*. The other, Lleida, is in the plains of the central Ebro valley with a dry continental Mediterranean climate. This area is mainly dedicated to peach, apple and pear production in large, intensive drip-irrigated orchards.

The Aragon region fruit production area, as Lleida, is in the central Ebro valley, and both have the same agro-ecological conditions. The Valencia area is characterized by small, flat drip-irrigated orchards of *P. salicina* and *P. armeniaca*, with a Mediterranean climate. In Extremadura, the fruit production areas are characterized by a southern Mediterranean climate and large new, drip-irrigated, orchards of *P. salicina* and *P. persica*. In each plot, the shoot samples for phytoplasma detection were taken randomly, in September, from 10 individual trees showing symptoms of ESFY. To ensure detection, samples were taken from four branches per tree. In orchards where fewer than 10 trees had clear symptoms, samples were collected from those with symptoms and also from randomly chosen symptomless trees, to give 10 samples per plot. In plots with no symptoms, all samples were taken randomly from any tree. Samples were stored in plastic bags at 4°C prior to DNA extraction.

Cacopsylla pruni captures in *Prunus* species orchards

Cacopsylla pruni populations on different cultivated *Prunus* species were surveyed using yellow sticky traps in the four regions. The surveys were begun in the Baix Llobregat area with eight traps per plot in 2003 and 2004, four in the centre and four in the corner trees of the orchards. In following years, due to the results obtained, only the four traps in the corners were used. In 2005 and 2006, the surveys were extended to the other areas and regions. In Catalonia, affected *P. salicina* orchards were also monitored by beating. Different branches on trees were beaten 10 times and psyllids were collected and aspired from a tray. The 20×20 cm (800 cm^2) yellow sticky traps (Projar), placed at a height of 1·75 m, were replaced once every 15 days, from January to July. *Cacopsylla pruni* individuals were then removed, identified following Hodkinson & White (1979) and stored at −20°C prior to analysis. The insects were analysed individually.

Evaluation of wild hosts for '*Ca.* Phytoplasma prunorum' and *C. pruni* in Catalonia

The communities of potential wild hosts for '*Ca.* Phytoplasma prunorum' and *C. pruni* in the three stone fruit production areas of Catalonia were surveyed in 2009 and 2010. Five communities of *P. mahaleb*, four of *P. spinosa*, two of *P. cerasifera* and one of naturalized *P. domestica* were studied in the coastal mountains of the Baix Llobregat. In these mountains, wild *P. mahaleb* is relatively abundant above 100 m a.s.l., most of them naturalized rootstocks from old deserted *P. avium* orchards. There are also small communities of wild *P. spinosa* above 400 m a.s.l. The Ribera d'Ebre area (Tarragona) is surrounded by the broken 1000 m-high precoastal mountains, where a *P. mahaleb* community was studied. In the Lleida area, two plots of *P. spinosa* and three plots of naturalized *P. dulcis* near the orchards were studied. These *P. spinosa* trees are present above 500 m a.s.l. in the Pyrenean foothills, but the nearest stone fruit orchards are 30 km away. The samples of wild or naturalized, symptomless *Prunus* spp. were taken as described for the symptomless orchards. The number of samples varied with the size of the community, with a maximum of 10 individuals per plot. *Cacopsylla pruni* was captured on wild hosts by yellow sticky traps and beating the trees as described for orchards.

Phytoplasma detection in plants and insects

The samples of *Prunus* plants and *C. pruni* individuals were analysed by PCR to detect phytoplasmas. Total DNA from plants was obtained by mixing 1 g of fresh leaf midribs and phloem tissue from stems together, using the phytoplasma-enrichment procedure of Ahrens & Seemüller (1992). Total DNA from insects was obtained following the procedures of Batlle *et al.* (2008) and García-Chapa *et al.* (2005). The DNA extracts were stored at −20°C. The universal primers for phytoplasma detection, P1/P7 (Deng & Hiruki, 1991; Smart *et al.*, 1996), were used during the first step and fO1/rO1 AP group-specific primers for the second (Lorenz *et al.*, 1995). The first amplification was in a total volume of 20 μL containing 5–10 ng DNA and the following mixture: 0·25 μM each universal primer, 250 μM dNTPs, 0·2 U *Taq* DNA polymerase (Promega) and 1 × Taq buffer. Two microlitres of a 1:50 dilution of the first amplification product were used for the second step, with 0·375 μM of the group-specific primers. The products were analysed by electrophoresis in a 1·5% D-1 agarose gel (Pronadisa) according to standard procedures. DNA was stained with ethidium bromide and exposed to ultraviolet light. Healthy *Prunus* and *Catharanthus roseus* seedlings were used as a negative control in PCR assays. To ensure '*Ca.* Phytoplasma prunorum' detection, an RFLP analysis of fO1/rO1 primer pair amplification products was performed with *Rsa*I and *Ssp*I enzymes following Lorenz *et al.* (1995) and García-Chapa *et al.* (2003).

Results

Incidence of '*Ca.* Phytoplasma prunorum' in orchards of stone fruit species

'*Candidatus* Phytoplasma prunorum' was detected on all of the cultivated *Prunus* species studied, except *P. avium* and *P. dulcis*, and in all four of the regions surveyed (Table 1). The species most affected and showing most symptoms was *P. salicina*, which exhibited early bud break, leaf curling, yellowing, collapse and a complete loss of the harvest. Its visual incidence ranged from 1% in Valencia to more than 80% in some plots in the Baix Llobregat area (Table 1). Two plots of *P. domestica* were studied in Baix Llobregat: Santa Coloma 6 and 7. In the Santa Coloma 6 plot, 'Reina Claudia' presented a visual incidence of 2%, confirmed by PCR on the four trees with symptoms analysed. However, PCR analysis of the infection rate on samples randomly collected from the six symptomless trees was 2+/6. In the Santa Coloma 7 plot, the traditional local variety of *P. domestica* 'Colló de Mico' was completely symptomless, but PCR analysis gave an infection rate of 4+/10. The visual incidence of *P. persica* was low, with PCR analysis of infection rates ranging from 0 to 4% on all the plots, except Encomienda 1 and 2 in the Vegas Altas area (Extremadura) where the incidence was as high as 25% (Table 1). The main symptoms observed on peach were leaf yellowing, curling and in some cases collapse. There were no symptoms and ESFY was not detected in the five *P. avium* and three *P. dulcis* plots, even in areas with high infection pressure. Three commercial plots of *P. cerasifera* were studied, one a pollinator within a highly affected *P. salicina* orchard (Olesa 4). No clear ESFY symptoms were observed on *P. cerasifera* in any of the cases, but '*Ca.* Phytoplasma prunorum' was detected by PCR in 22 of the 30 symptomless trees randomly collected in the three plots (Table 1).

European stone fruit yellows incidence and dissemination were very high in the Baix Llobregat area of Catalonia, where the phytoplasma was present in all the plots of *P. salicina*, *P. domestica*, *P. cerasifera*, *P. armeniaca* and *P. persica* surveyed. In the Ribera d'Ebre area, the incidence was more than 30% on *P. salicina* in the Miravet 1 and 2 plots. In the Lleida area, ESFY was only detected in one *P. salicina* orchard (Borges 2; Table 1). The incidence of ESFY in Valencia and Aragon was very low, ranging in both cases from 1 to 7%, even in very susceptible species. In Extremadura, ESFY was present in all except a *P. dulcis* orchard (Lobón). The visual incidence was low in the Vegas Bajas area and high in the Vegas Altas area, up to 25% in some plots (Table 1).

Cacopsylla pruni captures and '*Ca.* Phytoplasma prunorum' infection rate in *Prunus* spp. orchards

Cacopsylla pruni was captured by yellow sticky traps in all four of the regions studied, but not in all of the fruit growing areas. The beating method was not successful in *P. salicina* and *P. cerasifera* orchards in the Baix Llobregat, with zero captures, whilst it proved to be effective for wild *P. mahaleb* growing nearby. In the Ribera d'Ebre area of Tarragona (Catalonia), a peak of 1·75 individuals per trap was captured in a *P. salicina* plot (Miravet). No *C. pruni* were captured in the Lleida area (Borges and Alcarras), or in the Torres de Berrelén plot in Aragon and the Barxeta plot in Valencia. In the other plots in Aragon, the number of *C. pruni* captures was very low with a maximum of 0·75 and 0·5 individuals per trap in the Montañana and Alagón plots, respectively. Peaks of capture in the Belgida and Llutxent plots (Valencia) were 0·25 and 0·5 individuals per trap, respectively. The *C. pruni* captures in Extremadura rose to a maximum of 12 insects per trap in 2006 (Fig. 1). *Cacopsylla pruni* adults mainly appeared in the middle of February, but in warm winters, in some years and plots such as in the Baix Llobregat area in 2004, they appeared in January (Fig. 2). The *C. pruni* peaks were very variable in the date and the number of captures between years. In the *P. mahaleb* of Torrelles plot, the remigrant peak ranged from 15 March in 2004 to 15 April in 2006, and the captures ranged from 11 individuals per trap in 2003 to 3·75 in 2005. The emigrant peaks commonly appeared in early June, with captures lower than in remigrant peaks. The dates of peaks in the nearby St Vicenç and Sta Coloma plots of *P. salicina* were very similar, but the captures were lower, especially in the remigrant peaks (Figs 1, 2 & 3). In Extremadura, only one clear peak was observed between 1 March and 15 July, on 15 May in 2005 and 1 May in 2006. These peaks were of emigrant individuals, with remigrant individuals only captured in March 2005. The captures in

Table 1 Visual incidence and PCR detection of '*Candidatus* Phytoplasma prunorum' on different cultivated *Prunus* spp. in 2005

Plot	*Prunus* sp.[a]	Variety	Age (years)	Visual incidence (%)	PCR detection[b]
Aragon					
Santa Inés	Ps	Angeleno	9	5	6
Alagón	Ps	Sungold	15	6	8
Torres Berrelén	Ps	Pioneer	19	7	6
Catalonia					
Baix Llobregat area					
Olesa 1	Ps	606	12	63	8
Olesa 2	Ps	Fortune	12	74	10
Olesa 3	Ps	Anne Gold	12	59	9
Olesa 4	Pc	Pollinator	12	0	10
Castellbisbal	Pa	Moniqui	25	8	8
Sant Boi 1	Ps	Black Amber	15	45	8
Sant Boi 2	Pc	Valentins	50	0	4
Santa Coloma 1	Pp	Babygold	10	4	2
Santa Coloma 2	Ps	Autumn Giant	8	61	9
Santa Coloma 3	Ps	Fortune	15	82	7
Santa Coloma 4	Ps	Anne Gold	8	63	10
Santa Coloma 5	Pc	Llevadó	60	0	8
Santa Coloma 6	Pd	Claudia	25	2	6
Santa Coloma 7	Pd	Colló Mico	35	0	4
Santa Coloma 8	Pav	Burlat	30	0	0
Torrelles 1	Pav	Summit	15	0	0
Torrelles 2	Pav	Burlat	25	0	0
Torrelles 5	Pdu	Marcona	20	0	0
Begues 1	Pav	Burlat	25	0	0
Begues 2	Pav	4·70	10	0	0
Sant Vicenç 1	Ps	Black Diamond	10	76	9
Sant Vicenç 2	Ps	Pioneer	15	24	10
Ribera d'Ebre area					
Miravet 1	Ps	Angeleno	16	37	9
Miravet 2	Ps	Larry Ann	16	32	10
Benissanet 1	Pa	Moniqui	20	7	6
Benissanet 2	Pp	Snow Queen	9	1	3
Ginestar	Pa	Bulida	24	5	8
Lleida area					
Alcarràs 1	Pp	Babygold	10	1	0
Alcarràs 2	Pp	Catherine	15	0	0
Borges 1	Pp	Top Lady	8	0	0
Borges 2	Ps	Angeleno	4	15	7
Borges 3	Pdu	Texas	21	0	0
Valencia					
Belgida	Pa	Canino	11	1	3
Llutxent	Pa	Moniqui	18	2	2
Barxeta	Ps	Golden Japan	8	1	1
Extremadura					
Vegas Altas area					
Encomienda 1	Pp	Spring Crest	12	25	7
Encomienda 2	Pp	May Crest	12	23	6
Zurbarán 1	Ps	606	10	8	8
Zurbarán 2	Ps	Red Beauty	10	10	6
Zurbarán 3	Pp	Snow Queen	10	5	5
Vegas Bajas area					
Lobón	Pdu	Texas	16	0	0
Valdelacalzada 1	Ps	606	8	8	5
Valdelacalzada 2	Ps	Black Star	8	5	7
Valdelacalzada 3	Pp	Spring Crest	8	4	1

[a]Pa, *Prunus armeniaca*; Pav, *P. avium*; Pc, *P. cerasifera*; Pd, *P. domestica*; Pdu, *P. dulcis*; Pp, *P. persica*; Ps, *P. salicina*.
[b]Number positive out of 10 samples.

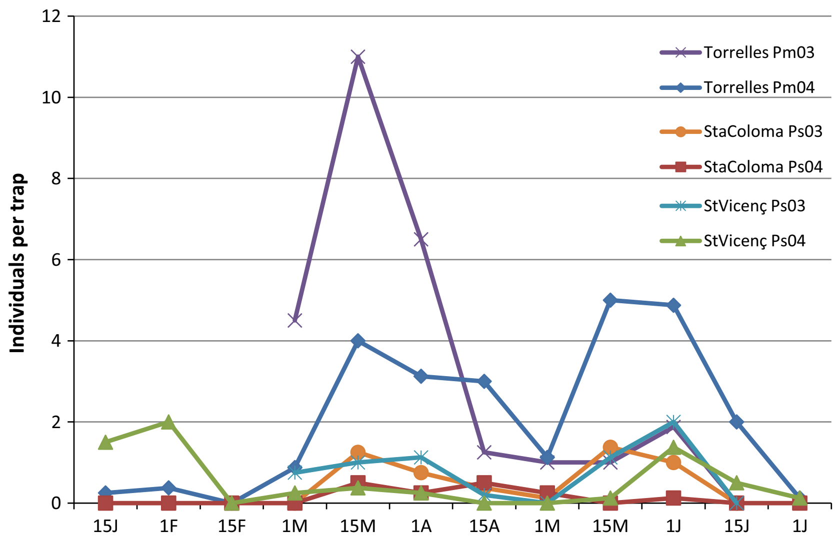

Figure 1 Evolution of the mean number of *Cacopsylla pruni* individuals captured per trap in 2003 and 2004 on wild *Prunus mahaleb* (Pm) and in *P. salicina* (Ps) orchards in Baix Llobregat area (Catalonia).

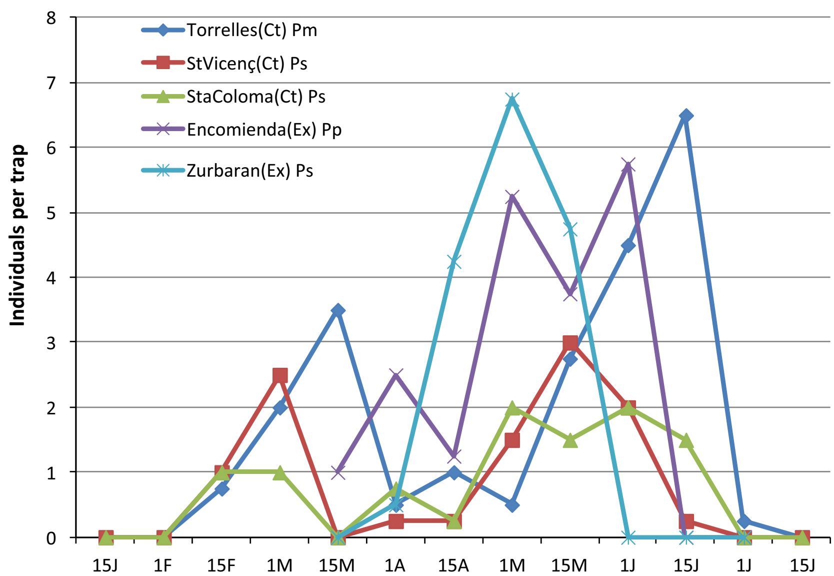

Figure 2 Evolution of the mean number of *Cacopsylla pruni* individuals captured per trap in 2005 in the Baix Llobregat (Catalonia, Ct) and Vegas Altas (Extremadura, Ex) areas of Spain on wild *Prunus mahaleb* (Pm), *P. salicina* (Ps) and *P. persica* (Pp).

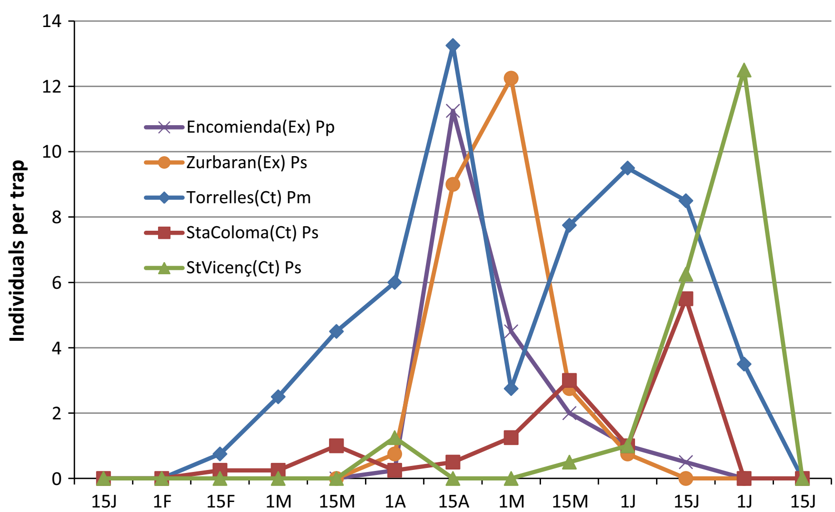

Figure 3 Evolution of the mean number of *Cacopsylla pruni* individuals captured per trap in 2006 in the Baix Llobregat (Catalonia, Ct) and Vegas Altas (Extremadura, Ex) areas of Spain on wild *Prunus mahaleb* (Pm), *P. salicina* (Ps) and *P. persica* (Pp).

plots and regions with very low populations only differed slightly, but it was difficult to track the peaks due to the low level of captures. In the two *P. salicina* plots in the Baix Llobregat, there were differences between the corners and the central part of the orchard, with captures in central traps being very low (Fig. 4). '*Candidatus* Phytoplasma prunorum' was detected in *C. pruni* in all of the regions studied. In the Ribera d'Ebre (Miravet), '*Ca.* Phytoplasma prunorum' was detected in around 7% of the insects. In Aragon, Valencia and Extremadura, '*Ca.* Phytoplasma prunorum' was detected in 4, 5 and 9% of the insects, respectively. There were no significant differences

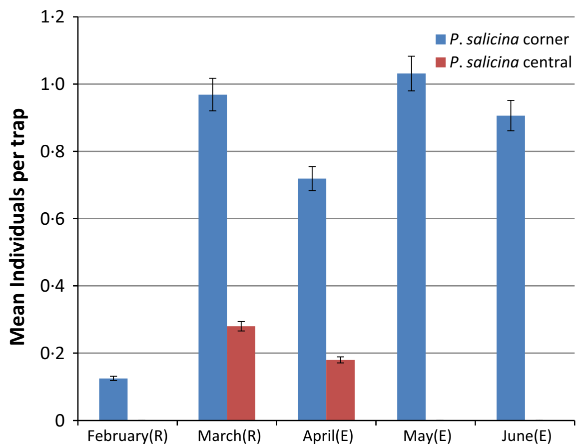

Figure 4 Mean number of *Cacopsylla pruni* individuals captured per trap in the central part and in the corners of two *Prunus salicina* orchards (St Vicenç and Sta Coloma plots) in 2003 and 2004 in Baix Llobregat area (Catalonia, Spain). Remigrants (R); emigrants (E).

in infection rates between insects captured on different species in the same area. Analysing the *C. pruni* infection rate throughout the season in Baix Llobregat (Catalonia), the highest level of carriers corresponded to remigrants in late winter (March), with infection rates of around 40%. In the new generations of young emigrant adults (June), the infection rate fell to approximately 10% (Fig. 5).

Study of '*Ca.* Phytoplasma prunorum' and *C. pruni* on wild *Prunus* species

The wild *Prunus* species were surveyed for '*Ca.* Phytoplasma prunorum' and *C. pruni* in three stone fruit producing areas in Catalonia. The highest captures in all the study were on a wild *P. mahaleb* plot in the Baix Llobregat area (Barcelona), with a maximum mean up to 13 insects per trap in 2006 (Fig. 1). No symptoms were observed on any wild or naturalized *P. mahaleb*, *P. spinosa*, *P. cerasifera*, *P. dulcis* and *P. domestica* sampled. In the Lleida area, wild *P. spinosa* communities studied were not infected, and no *C. pruni* was captured by beating or yellow sticky traps. In this area, there was no *C. pruni* capture or phytoplasma detection in analyses of naturalized *P. dulcis* near the plots. In the Ribera d'Ebre area (Tarragona), a small community of wild *P. mahaleb* had the same negative results (Table 2). In the coastal mountains of Barcelona, '*Ca.* Phytoplasma prunorum' and *C. pruni* were found on different wild hosts. In Cabrils plots, some *C. pruni* individuals were captured by beating on *P. spinosa*, but no '*Ca.* Phytoplasma prunorum' were detected in either plants or insects (Table 2). In Begues plots, the *P. cerasifera*, *P. spinosa* and *P. mahaleb* communities found were very

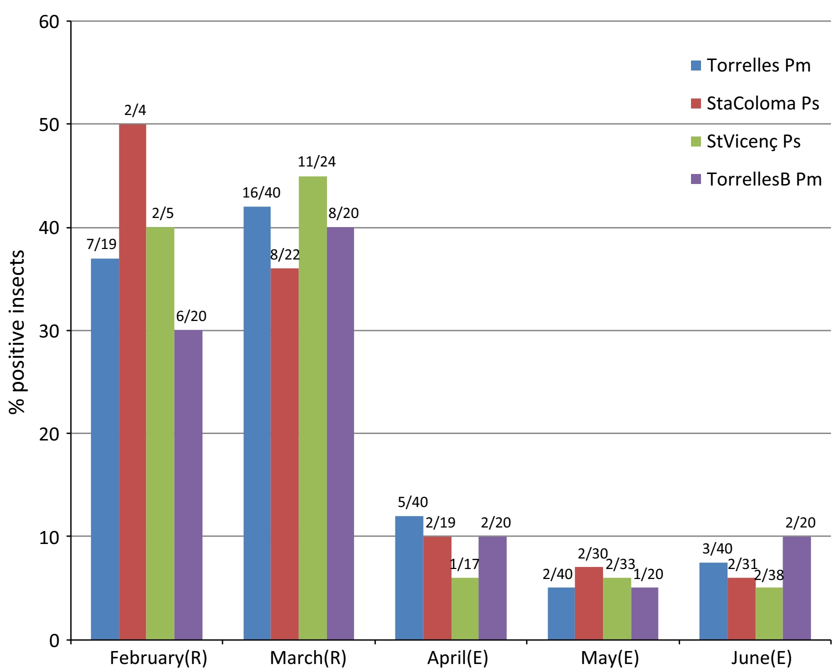

Figure 5 Monthly percentage of *Cacopsylla pruni* individuals carrying '*Candidatus* Phytoplasma prunorum' in orchards of *Prunus salicinia* (Ps) and wild *P. mahaleb* (Pm) in the Baix Llobregat area (Catalonia, Spain) in 2005 and 2006. Remigrants (R); emigrants (E). All captured by yellow sticky traps except Torrelles B, captured by beating.

Table 2 '*Candidatus* Phytoplasma prunorum' infection rate and *Cacopsylla pruni* captures in different wild symptomless *Prunus* spp. communities in Catalonia in 2009 and 2010

Province	Plot	*Prunus* sp.	*C. pruni* capture method	PCR detection[a]	
				Trees	Insects
Barcelona	St Vicenç	*P. domestica*	Beating	2/2	No captures
	Torrelles 1	*P. mahaleb*	Yellow/beating	7/10	15/69
	Torrelles 2	*P. mahaleb*	Yellow/beating	4/8	8/44
	Torrelles 3	*P. mahaleb*	Yellow/beating	3/5	9/25
	Sta Coloma	*P. cerasifera*	Beating	2/3	No captures
	Begues 1	*P. spinosa*	Yellow/beating	0/3	0/4
	Begues 2	*P. spinosa*	Beating	0/4	No captures
	Begues 3	*P. cerasifera*	Beating	0/3	No captures
	Begues 4	*P. mahaleb*	Beating	0/2	No captures
	Cabrils 1	*P. spinosa*	Beating	0/5	No captures
	Cabrils 2	*P. spinosa*	Beating	0/4	0/2
Tarragona	Cardó 1	*P. mahaleb*	Beating	0/3	No captures
Lleida	Balldellou	*P. spinosa*	Beating	0/2	No captures
	Baells	*P. spinosa*	Yellow/beating	0/6	No captures
	Baells	*P. dulcis*	Yellow/beating	0/7	No captures
	Alcarrás	*P. dulcis*	Yellow/beating	0/4	No captures
	Borges	*P. dulcis*	Beating	0/11	No captures

[a]PCR analysis with fO1/rO1 primers. Results are number of positive results/total number analysed.

small and scattered. A few *C. pruni* individuals were found on these *P. spinosa*, but no '*Ca*. Phytoplasma prunorum' was detected on either plants or insects. In Torrelles sampling points, there was a high '*Ca*. Phytoplasma prunorum' infection rate in the communities of *P. mahaleb*, and major *C. pruni* infected populations, showing a high hosting capacity. Here, the phytoplasma was detected in 14 out of 23 *P. mahaleb* tested and in two naturalized *P. domestica* (Table 2).

Discussion

This study presents extensive data on the hosts and vectors of '*Ca*. Phytoplasma prunorum' in Spain, demonstrating that it is widespread in all of the regions studied. While part of the data in this study is 10 years old, it still represents the general situation in the different areas. The regional phytopathological surveillance programmes for quarantine diseases, carried out recently, showed that the ESFY geographic distribution and incidence presented is still valid. '*Ca*. Phytoplasma prunorum' continues to be mainly present in the Baix Llobregat in Catalonia and Vegas Altas in Extremadura. In the other areas there are sporadic cases and very low incidences. These results confirm the many and continuous reports which date back to the beginning of the twentieth century. Sala reported, in 1935, early shoot growth on *P. salicina* in the Baix Llobregat area. In Valencia in the 1970s and 1980s there were some outbreaks on *P. salicina* and *P. armeniaca*: nowadays cases are isolated (Sanchez-Capuchino & Forner, 1973; Llácer *et al*., 1986). In the last decade, recurrent ESFY outbreaks and *C. pruni* have been reported in the Baix Llobregat area, conditioning *P. salicina* cultivation (Laviña *et al*., 2004; Torres *et al*., 2004). *Prunus dulcis* and *P. avium* were not infected, even in high-infection pressure areas such as Baix Llobregat (Catalonia), and confirm these species as poor hosts. *Prunus mahaleb* and *P. cerasifera* had high infection rates without symptoms. *Prunus armeniaca* and *P. salicina* were the most affected, showing clear symptoms and great damage. In general, there was a low incidence in *P. persica*, except in Extremadura, where incidence was high. These results are similar to those obtained in previous studies (Giunchedi *et al*., 1982; Jarausch *et al*., 2001, 2008).

There is disagreement between visual incidence and PCR detection rate that could be due to the condition of the trees when the samples were taken. This is the case for the *P. salicina* and *P. armeniaca* plots of Sta Inés, Torres de Berrelen and Benissanet 1, with clear symptoms of phenological mismatch in spring. These samples had to be taken in September from collapsed trees due to the low number of trees with symptoms available. On *P. persica*, some trees without symptoms were also collapsed, but this low correlation could be due to confusion with other biotic and abiotic factors. On *P. persica*, other factors could not be discriminated due to the lack of specific symptoms showing phenological mismatch in this species. This is the case for the Extremadura peach plots, where the real '*Ca*. Phytoplasma prunorum' incidence was probably slightly lower. The incidence and damage were high in Baix Llobregat (Catalonia) and Vegas Altas (Extremadura) areas, in agreement with the highest *C. pruni* captures. The low incidence in Valencia, Aragon and Lleida is associated with very low *C. pruni* populations and confirmed the limited '*Ca*. Phytoplasma prunorum' presence in these areas, even on very susceptible species such as *P. salicina* and *P. armeniaca*. The low ESFY incidence and *C. pruni* captures are probably related to a lack of potential wild hosts and overwintering refugee plants in the plains. The results show an association between high ESFY incidence areas, large *C. pruni* populations and availability of wild hosts. In the Baix Llobregat area, results pointed to *P. mahaleb* as a wild symptomless host, with a high infection rate and capacity to host *C. pruni*. The *P. spinosa* infection rate was lower, as in other countries (Yvon *et al.*, 2004; Maier *et al.*, 2013). The infected *P. mahaleb* plots of Torrelles are located only 2 km from the high incidence *P. salicina* plots in Sta Coloma and St Vicenç. Although *P. mahaleb* could play an important role in local pathological cycles, studies of this species in Italy have revealed very low infection rates and capacities to host this vector (Carraro *et al.*, 2002). *Prunus mahaleb* was not considered a common natural host for '*Ca*. Phytoplasma prunorum' (Carraro *et al.*, 2004), but sporadic cases of '*Ca*. Phytoplasma prunorum' have been reported in regions of Italy such as Trentino, even showing symptoms (Pignatta *et al.*, 2008). These differences between the hosting capacity of *P. mahaleb* communities could be due to the different *C. pruni* biotypes in Spain (Peccoud *et al.*, 2013) or to the genetic differences between the *P. mahaleb* communities. *Prunus mahaleb* was infected in the three plots of Torrelles, but tested negative in Begues and Cardó, showing an irregular hosting behaviour. It is important to point out that some *P. mahaleb* are naturalized rootstocks that could belong to different varieties. In Vegas Altas there is a high incidence, but no large wild *Prunus* communities within 50 km. It could be that *C. pruni* may have been hosted here by rare, small and distant communities of *P. spinosa* or by cultivated *Prunus* spp. Another hypothesis is that the perennial relict endemic *P. lusitanica*, 50 km from the Vegas Altas area in the Guadalupe Mountains, could be the host. These *P. lusitanica* communities, now under study, are larger and closer than other wild *Prunus* spp. Previous studies have reported that other perennial *Prunus* species, such as *P. laurocerasus*, are poor hosts for *C. pruni* and '*Ca*. Phytoplasma prunorum', but they have been experimentally infected (Carraro *et al.*, 2004).

The *C. pruni* dynamics gave two population peaks, corresponding to remigrant and emigrant individuals, confirming the behaviour observed for *C. pruni* in other European countries (Labonne & Lichou, 2004; Jarausch *et al.*, 2008). In the Baix Llobregat area, the higher captures of *C. pruni* on wild *P. mahaleb* than in *P. salicina* orchards were probably due to host species preferences of *C. pruni* and to the insecticide treatments used on the commercial plots. While insecticide treatments do not kill all *C. pruni* in commercial orchards, they probably reduce the population to levels difficult to track, but enough to produce very high incidences. The results here showed that *C. pruni* fed and developed on wild hosts outside the commercial plots, although untreated or deserted orchards could also play an important role (Thebaud *et al.*, 2006, 2009). Yellow sticky traps were a good method for trapping *C. pruni* in commercial orchards with low populations. Zero captures were obtained when the striking method was used on commercial plots of *P. salicina* and *P. cerasifera* in the Baix Llobregat area. The capture peaks were variable in dates and number of captures from year to year. Comparing the captures between *P. mahaleb* and *P. salicina*, the dates of the two peaks were similar, but the remigrants peak was generally greater than the emigrants peak on *P. mahaleb*, whilst the opposite was the case on *P. salicina*.

Comparing the vector dynamics between Extremadura and Catalonia, there was a tendency for the emigrant peak to appear 1 month earlier in Extremadura. This cannot be fully confirmed, because winter trapping in Extremadura was not successful due to variable circumstances, and the remigrant peak was not clearly observed. The remigrant peak probably appeared in February, 1 month earlier than in Catalonia, as for the emigrant peak, but the loss of the traps means this cannot be confirmed. It is possible that the remigrant captures in the first peak were very low. The results gave the percentage of remigrant individuals as carriers of the phytoplasma in the Baix Llobregat at around 40%, in contrast to an average of less than 10% of young emigrants from April to June. This was probably due to the remigrant individuals being most infected because they had had more time to incubate the phytoplasma. This is supported by the transmission efficiency of the remigrants being much higher than for emigrants, suggesting that *C. pruni* completes latency in its secondary overwintering hosts (Thebaud *et al.*, 2009). These infection rates are higher than in other countries, but it should be taken into consideration that the present data was obtained by nested-PCR in contrast with other studies (Jarausch *et al.*, 2001, 2008; Carraro *et al.*, 2004). ESFY epidemics and damage in Spain can be divided into two major patterns: low and high impact. Low impact epidemics can be found in Valencia, Aragon and Lleida (Catalonia), characterized by generally low incidence and *C. pruni* populations. In general, there are no major hosts for the vector or '*Ca*. Phytoplasma prunorum' within 50 km of the orchards. High impact epidemics occur in the Baix Llobregat area of Catalonia and are associated with high incidence and levels of damage in the orchards; this area is characterized by populations of infected wild *P. mahaleb* hosting large populations of infective *C. pruni*. These findings for *P. mahaleb* (high '*Ca*. Phytoplasma prunorum' infection rates and high *C. pruni* hosting capacities) combined with the poor results for *P. spinosa*, suggest a major role for *P. mahaleb* in the ESFY cycle in this area.

Historical ESFY reports, the long history of stone fruit cultivation, and the tolerance of traditional varieties and wild *Prunus spp.* in the Baix Llobregat area, indicate coadaptation between '*Ca*. Phytoplasma prunorum', the autochthonous *Prunus* species/varieties and *C. pruni*. For this reason, the introduction of such a very sensitive species as *P. salicina* unleashed a major epidemic and economic loss. The identification of '*Ca*. Phytoplasma prunorum' on isolated wild or naturalized individuals of *P. domestica* and *P. cerasifera* indicates that these species could play a secondary role in the epidemiology. Based on these results, *P. mahaleb* communities could hold the key for the control of the disease in the Baix Llobregat area. Here, the main action to fight against ESFY has been substituting *P. salicina* with the tolerant *P. cerasifera* and *P. domestica*, remaining productive despite infection. Other control actions undertaken have been the elimination of deserted *Prunus* spp. near the orchards and insecticide treatments before flowering directed at remigrant *C. pruni*. However, these measures could not be fully implemented because of the impossibility of completely removing wild *Prunus* spp. and the insecticide safety margin for flowering required for bee protection.

In conclusion, these data show that ESFY is widely distributed in Spain, but generally with low incidences. It is important to take into consideration that Spain is in the southern border region of ESFY and any climate change will move the limit of distribution of the wild and cultivated *Prunus* spp., and therefore *C. pruni* and '*Ca*. Phytoplasma prunorum' distribution. In fact, the lower incidence and spread in Valencia found in this study compared to the 1980s (Llácer *et al.*, 1986) is probably due to this climate change. It could be that this area is the southern limit for ESFY in Mediterranean climate zones, but more complete studies are required to establish this limit in continental climate areas such as Extremadura. This border effect, the climate change and the adaptation of vectors and plants, are very important factors for predicting future risks. Large-scale outbreaks and their duration should highlight the possibility of destructive episodes in Spain. Future studies of ESFY should focus on characterization of the pathological cycle that led to the outbreak on peach trees in Extremadura, and the development of better control strategies for ESFY and its vector *C. pruni*.

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