Horticulture Innovation Australia

Final Report

Identification of the transfer pathway for E. amylovora on fruit

Dr Maria Lopez
Instituto Valenciano de Investigaciones Agrarias

Project Number: AP07051

AP07051

This project has been funded by Horticulture Innovation Australia Limited using the apple and pear industry levy and funds from the Australian Government.

ISBN 0 7341 3627 7

Published and distributed by:
Horticulture Innovation Australia Limited
Level 8, 1 Chifley Square
Sydney NSW 2000
Tel: (02) 8295 2300
Fax: (02) 8295 2399

FINAL REPORT OF THE PROJECT AP07051

"Identification of the transfer pathway for *Erwinia amylovora* on fruit"

(Milestone 109, June 2010)

For being submitted to: milestones@horticulture.com.au

Project title: Identification of the transfer pathway for *Erwinia amylovora* on fruit

Duration: 13 June 2008 – 11 June 2010

Coordinator: María M. López

Organization: Instituto Valenciano de Investigaciones Agrarias (IVIA)

Address: Ctra. Moncada-Náquera, km 4.5, 46113 Moncada – Valencia - Spain

Telephone: +34-963-424000 (Ext 70139)

Fax: +34-963-424001

E-mail: mlopez@ivia.es

Research group: Mónica Ordax (IVIA), ordax@ivia.es; Jaime E. Piquer Salcedo (IVIA), piquer_jed@gva.es; Beatriz Sabater-Muñoz (IVIA), bsabater@ivia.es; Elena G. Biosca (Universidad de Valencia), elena.biosca@uv.es; and Ester Marco-Noales (IVIA) emarco@ivia.es.

Collaborators: Tony Russell (General Manager, APAL) and Satish Wimalajeewa (Consultan Plant Pathologist).

Responsible Portfolio Manager: Kim James (HAL) (kim.james@horticulture.com.au)

GLOBAL AIM OF THE PROJECT AP07051

Identification of possible transfer pathways for *Erwinia amylovora* on fruit

The results of the project are divided in two experimental sections:

A) Looking for *Erwinia amylovora* in asymptomatic fruits from trees naturally infected by fire blight

Ordax M., Piquer-Salcedo J.E., Biosca E.G., López M.M., Marco-Noales E.

B) A new potential vector for *Erwinia amylovora* dissemination from apple fruit: the Mediterranean fruit fly *Ceratitis capitata*

Ordax M., Piquer-Salcedo J.E., Sabater-Muñoz B., Biosca E.G., López M.M., Marco-Noales E.

A) Looking for *Erwinia amylovora* in asymptomatic fruits from trees naturally infected by fire blight

Ordax[1] M., Piquer-Salcedo[1] J.E., Biosca[2] E.G., López[1] M.M., and Marco-Noales[1] E.

[1]Instituto Valenciano de Investigaciones Agrarias (IVIA), 46113 Moncada, Valencia, Spain.
[2]Universidad de Valencia (UV), 46100 Burjasot, Valencia, Spain.

1A. BACKGROUND INFORMATION

The bacterium *Erwinia amylovora* is the causal agent of fire blight, a very destructive and highly infectious disease of apple, pear and other pome fruits, causing important economic losses worldwide. Due to the ability of the pathogen to persist in nature [Thomson, 2000], and the difficulties for an efficient control [Norelli et al., 2003], the fire blight disease still remains as a serious global threat to the pome fruit industry.

E. amylovora usually infects immature fruits, but it has been also occasionally detected in mature fruits from severely infected trees [Hale *et al*., 1987; van der Zwet *et al*., 1990]. Moreover, it has been reported that *E. amylovora* can persist in inoculated apple and pear fruit calyces [Taylor *et al*., 2002; 2003; Taylor and Hale, 2003; Temple *et al*., 2007], as well as in apple pedicels [Tsukamoto *et al*., 2005a; Azegami *et al*., 2006], although only for short periods in a culturable state. However, we have recently demonstrated that *E. amylovora* is able to survive for one month in mature apple fruit calyces adopting the "viable but non-culturable" (VBNC) state [Ordax *et al*., 2009b] without causing disease symptoms. This suggests that the healthy appearance of the fruit is not always an indicative of the pathogen absence, so that asymptomatic contaminated fruit could be an unnoticed inoculum source for new fire blight infections [Taylor *et al*., 2003].

2A. OBJECT OF THIS STUDY

The aim of this study was to determine if the transfer pathway of *E. amylovora* from infected plants to fruits may be feasible in nature. To this end, several lots of different asymptomatic fruits harvested from naturally fire blight infected trees from several origins were analyzed looking for *E. amylovora*.

3A. EXPERIMENTAL ASPECTS

3A.1. Samples of asymptomatic fruits from naturally infected trees

Different lots of asymptomatic apples were harvested from trees naturally and severely infected by fire blight: (i) 306 apples of cultivar (cv) Jonagold from a large and productive Swiss orchard (Obstbau, Burgstrasse); (ii) 12 apples of cv Gala from another well-cared, but much more smaller, Swiss orchard (Oberhasli); (iii) 96 apples of cv Golden from a small Spanish orchard (León, Castilla y León). In all cases the trees were affected for first time by fire blight, in early summer in the Swiss orchards, and in late summer in the Spanish one. Jonagold apples were harvested in September 2007 and stored at low temperature under controlled atmosphere for approximately ten months. Gala (Fig. 1A, B) and Golden apples were harvested in September 2008 and immediately analyzed.

Fig. 1. Apple tree of cultivar Gala affected by fire blight (Oberhasli, Switzerland). A) A necrotic branch with brownish leaves caused by *E. amylovora*. B) The symptomatic branch in detail. Fruits did not show any symptom. (Photos kindly provided by T. Russell, September 2008).

At the harvest time of the following year (September 2009), around 250 asymptomatic azaroles were collected from *Crataegus acerolus* trees naturally affected by fire blight in Salamanca (in the north-west central area of Spain), and immediately analyzed.

3A.2. Fruit analysis

Calyces were taken out from asymptomatic mature apples with a sterile scalpel, and sampled based on the protocol of the European and Mediterranean Plant Protection Organization [EPPO, 2004]. Then, calyces were crushed two by two in 4 ml of antioxidant maceration buffer (AMB) [Gorris *et al*. 1996] in a plastic bag with a rubber hammer, and the extract was slightly shaken for 5-10 min at 26ºC. Afterwards, calyx

extracts were directly plated by triplicate on the non-selective King´s B medium (KB) [King *et al*., 1954] and on the semi-selective CCT medium [Ishimaru and Klos 1984], and incubated at 26ºC. Large Petri dishes (150 mm diameter) were used to plate a higher volume (200μl/plate) of calyx extracts, and incubation time was prolonged up to 5-7 days to allow the growth of stressed *E. amylovora* cells in case they were present. The detection limit was <1 cfu/ml of calyx extract. In addition, one aliquot of 1ml from each one of the calyx extracts was stored with 30% glycerol at –20ºC for further studies. Moreover, 1 ml of calyx extracts was enriched in 3 ml of KB and CCT liquid media at 26ºC, with slight shaking during 48 h. Thereafter, the enrichments were sampled by triplicate on KB and CCT plates, which were incubated as mentioned above.

Taking into account the negative results obtained in the analysis of the apples collected in 2008, in which only calyces were processed, the whole fruit was analyzed in the case of asymptomatic azaroles harvested in 2009. Moreover, an additional culture medium specially designed for the Recovery of Stressed *E. amylovora* Cells (RESC) was included [Ordax, 2008; Ordax *et al*. 2009a]. Other medium, Sucrose Nutrient Agar (SNA) [Lelliot, 1967], was also used in addition to CCT and RESC media. The azaroles were washed in batches of five, in 5 ml of antioxidant maceration buffer (AMB) [Gorris *et al.*, 1996] in plastic bags (12x30 cm) according to previous works [van der Zwet *et al*., 1990; Temple *et al*., 2007]. Following this, the washings were collected in sterile tubes, plated on CCT and RESC media, and enriched in CCT and RESC broths. The remaining extract was also stored at -20ºC with 30% glycerol.

Fruit extracts were analyzed by PCR after a DNA extraction protocol [Llop *et al*., 1999]. The primers which showed the best sensitivity in a previous assay with mature apple fruit tissues were selected to detect *E. amylovora* in the harvested fruits analyzed. They were Ea71 and Ea72, addressed to chromosomal fragments of the bacterium [Guilford *et al*., 1996; Taylor *et al*., 2001]. The PCR protocol was optimized to improve the amplification of the target DNA of *E. amylovora*, increasing the number of amplification cycles to 40, and the units of Taq DNA polymerase up 2U. Amplifications were performed in a thermocycler Perkin Elmer GeneAmp 9600 (Cetus Corp.). In all cases two positive controls (DNA of strains CFBP1430 and IVIA1614-1a) and four negative controls (HPLC-grade water) were included. When *E. amylovora*-like colonies appeared on plates, they were identified by PCR.

The hypersensitive reaction in tobacco leaves was used as an indicative of plant pathogenic ability of *E. amylovora*-like colonies, and the pathogenic potential was further confirmed by inoculation on detached pear shoots [EPPO, 2004].

4A. RESULTS

4A.1. Absence of *E. amylovora* in asymptomatic apples

No *E. amylovora*-like colonies were recovered on any of the KB and CCT plates streaked directly with calyx extracts (more than 600 plates). Results were also negative even after the enrichment of the extracts in KB and CCT liquid media (more than 200 enrichments). A relevant growth of several epiphytic bacteria was noticed, recovering between 3-6 colonial morphotypes on KB plates, and 1-3 on CCT agar. The bacteria *Pseudomonas* spp. and *Pantoea agglomerans*, species very common in host plants of *E. amylovora*, were commonly recovered on growth plates, reaching levels of 10^2-10^5 cfu/ml of calyx extract.

Similarly, the PCR detection of *E. amylovora* in more than 300 amplifications from DNA extracts of calyces was always negative (an example in Fig. 2). The detection limit of this protocol for calyx extract was < 1 cfu/ml [Update Report 1, AP07051]. Results were also negative in around 200 PCR from the enrichments in KB and CCT. In all cases, positive controls showed the expected amplicon of 187 bp, while negative controls were not amplified.

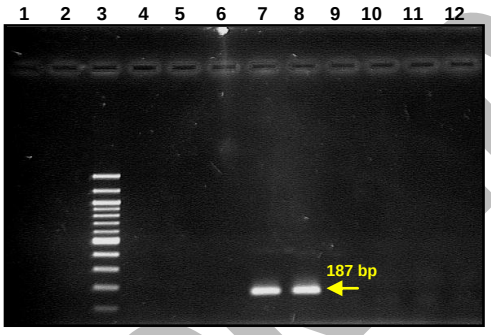

Fig. 2. Representative agarose gel of chromosomal PCR amplification for *E. amylovora* with primers Ea71 and Ea72 [Taylor *et al*., 2001] in calyx extracts of asymptomatic apples harvested from infected trees. Lanes 1-2, 4-6: calyx extracts from Golden Spanish apples; Lane 3: ladder 100 bp (Invitrogen); Lanes 7-8: positive controls (DNA from *E. amylovora* strains CFBP1430 and IVIA1614-1a); Lanes 9-12: negative controls (HPLC-grade water instead of sample).

4A.2. Presence of *E. amylovora* in asymptomatic azaroles

The analysis of 250 asymptomatic azaroles from naturally fire blight infected trees revealed the presence of *E. amylovora* in 12% of them in a viable and culturable state. Six presumptive isolates were confirmed as *E. amylovora* by colonial morphology on CCT, RESC and SNA media, as well as by PCR (Fig. 3A). In addition, the hypersensitive reaction in tobacco leaves was positive. All the isolates identified as *E. amylovora* by culture and PCR caused a complete collapse in the infiltrated tobacco tissues after only 24 h, and the typical necrosis and ooze in the leaves of pear shoots, evidencing their pathogenicity (Fig. 3B). Further, the pathogen concentration per infected azarole was quantified in levels around 10^2-10^3 cfu/fruit.

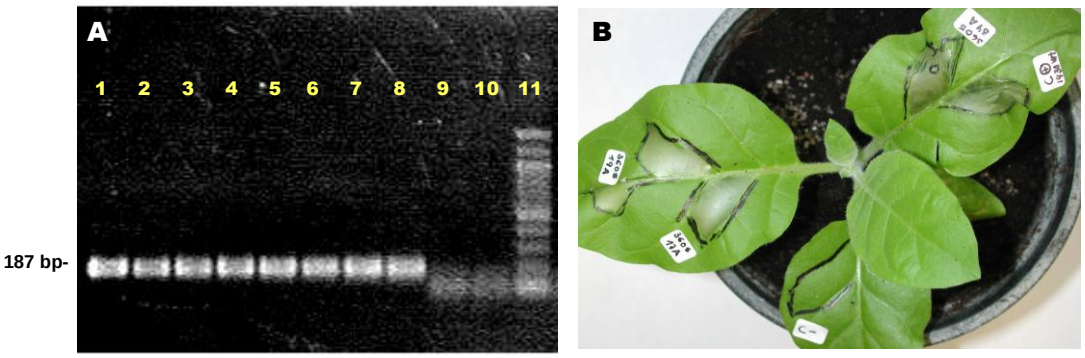

Fig. 3. Analysis of asymptomatic hawthorn fruits collected from naturally fire blight infected trees (Salamanca, Spain). A) Positive detections of *E. amylovora* by chromosomal PCR amplification [Taylor *et al*., 2001] in 6 fruit batches. Lane 1, batch nº1; Lane 2, batch nº14; Lane 3, batch nº16; Lane 4, batch nº 17; Lane 5, batch nº19; Lane 6, batch nº 34; Lanes 7-8, positive controls (DNA from strains CFBP1430 and IVIA1614-1a, respectively); Lanes 9-10: negative controls for PCR (HPLC-grade water); Lane 11, ladder 100 bp (Biolabs). B) Representative tobacco plant showing positive hypersensitive reactions produced by three *E. amylovora* isolates from hawthorn fruits, and positive and negative controls, strain CFBP1430 and PBS buffer, respectively. (Photos: J.E. Piquer-Salcedo, and M. Ordax).

The predominant native bacteria of azaroles that grew with *E. amylovora* on culture plates were also studied. Thus, two majority colonial morphotypes were always recovered in all the analyzed fruit batches. The partial sequencing of 16S ribosomal DNA of these isolates (MDC, Spain) revealed that *Pantoea agglomerans* (99.83% similarity) and *Erwinia billingiae* (99.8% similarity) were the most common epiphytic bacteria in the azaroles tested.

IMPORTANT NOTE: This information is protected and restricted because the fruits were kindly provided for confidential research purposes. Any diffusion, utilization or copy is absolutely prohibited.

5A. CONCLUDING REMARKS

The likelihood of mature fruit as a vehicle for dissemination of *E. amylovora* has been the subject of considerable research and risk assessment proceedings. However, it is concluded that there was no sufficient evidence that asymptomatic fruit could be a carrier of *E. amylovora* [WTO.org; Temple *et al*., 2007; Roberts and Sawyer, 2008]. Nevertheless, the controversy on this topic has continued in the scientific literature up to date. In fact, some recent studies have proved the possibility that mature apples could be carriers of the pathogen under *in vitro* conditions [Kimura *et al*., 2005; Txukamoto *et al*., 2005a; 2005b; Ordax *et al*., 2009b].

In this report it has been shown that more than 400 calyces from asymptomatic mature apples harvested from trees naturally infected by *E. amylovora* gave negative results for the presence of the pathogen. Our high detection limit (<1cfu/ml), and the use of big Petri dishes incubated up to one week, suggest that probably there were not culturable *E. amylovora* cells in the calyx of these apples. In addition to that, no colonies of the pathogen were recovered after enrichment in liquid media, despite their known advantage to promote bacterial growth [Biosca *et al.,* 2005; Ordax *et al*., 2006; Ordax, 2008; Ordax *et al*., 2009b]. To discard the possible existence of non-culturable cells of *E. amylovora* in these apple calyx extracts, either VBNC or dead cells, PCR technique was also performed (<1 cfu/ml calyx extract). Since PCR results were also negative, all the analysis pointed out to the apparent absence of the pathogen in the calyx sinuses of apples from naturally infected trees. However, it could be possible that *E. amylovora* was present in the latently infected fruits in other parts different from calyx, so that we decided to analyze whole fruits in the next harvest time. As in that year there were no fireblight outbreaks in Spanish apple orchards, other symptomless fruits from other naturally infected trees were harvested, over 250 azarole fruits. Interestingly, the analysis of these fruits evidenced that 12% of the collected azaroles were latently infected, remaining *E. amylovora* cells alive inside the fruits, and going unnoticed due to the absence of symptoms. Therefore, as previously pointed out under *in vitro* conditions, the healthy appearance of fruits is not always an evidence of the pathogen absence [Tsukamoto *et al*. 2005a; Azegami *et al*. 2006; Ordax *et al*., 2009b], because our results suggest that symptomless azaroles naturally infected could act as reservoirs of *E. amylovora*. Nevertheless, the fruits should come from trees severely infected by fire blight, and in any case, not all fruits harvested from an infected plant harbour the pathogen.

Besides, we have developed an appropriate methodology for the detection of *E. amylovora* in asymptomatic fruits in spite of the low pathogen doses usually present in them, which could be very useful in surveys of plants from naturally infected orchards. Thus, this integrated approach, previously validated *in vitro* with inoculated but asymptomatic mature apples, has been also shown as suitable for naturally infected symptomless fruits. This multi-approach protocol is based on that reported for *E. amylovora* detection in asymptomatic plant material [EPPO 2004], and was recently optimized for symptomless apples [Ordax *et al*., 2009b]. Then, different techniques should be simultaneously employed before discarding the presence of the pathogen: (i) plating on non-selective and selective solid culture media, (ii) enrichment in both kinds

of liquid media, (iii) PCR, and (iv) verification of the pathogenicity of the colonies recovered.

In conclusion, it has been shown that asymptomatic fruits can be naturally contaminated after a fire blight episode, at least in a certain percentage. In fact, our results of the analysis of symptomless azaroles show that latent infections in fruits can occur, as reported in other host fruits for *E. amylovora* [Schroth *et al*., 1974; van der Zwet *et al.,* 1990]. As usually in latent infections [Hayward, 1974], the pathogen populations in the analyzed azaroles were found in quite low levels (10^2-10^3 cfu/fruit). However, only one *E. amylovora* cell may develop the disease if the environmental and host conditions are favourable [Hildebrand, 1937; Cabrefiga and Montesinos, 2005]. Then, the risk of fire blight dissemination through asymptomatic but contaminated mature fruits, although low, could exit, at least, when environmental conditions are conducive for the pathogen multiplication.

B) A new potential vector for *Erwinia amylovora* dissemination from apple fruit: the Mediterranean fruit fly *Ceratitis capitata*

Ordax[1] M., Piquer-Salcedo[1] J.E., Sabater-Muñoz[1] B., Biosca[2] E.G., López[1] M.M., and Marco-Noales[1] E.

[1]Instituto Valenciano de Investigaciones Agrarias (IVIA), 46113 Moncada, Valencia, Spain.
[2]Universidad de Valencia (UV), 46100 Burjasot, Valencia, Spain.

1B. BACKGROUND INFORMATION

Fruits contaminated with *E. amylovora* could represent a vehicle for its dissemination in case that they could be in contact with susceptible hosts and/or insect vectors [Taylor *et al*., 2003]. Most of the studies on fire blight spread by insects have been focused in dissemination from flowers, so that honeybees have been considered the major vector because of their relevance as pollinating agents. However, any insect that visits infected plant material may be capable of disseminating *E. amylovora* [Schroth *et al*., 1974], and in fact, several flies and aphids have been reported as potential fire blight vectors [Ark and Thomas, 1936; Bellemann *et al*., 1994; Hildebrand *et al*., 2000; Tsukamoto *et al*., 2005b]. The presence of non-pollinating insects is also very common in fruit orchards. This is the case of the fruit flies (family *Tephritidae*), a highly successful group of great importance for agriculture because they infest and cause serious damages in fruits, seeds and even in vegetative plant tissues [White and Elson-Harris, 1992]. Several tephritids have become to be invasive species, such as the Mediterranean fruit fly *Ceratitis capitata* (medfly), one of the most destructive fruit pests worldwide.

The medfly has been extremely succesful to invade and establish in new areas mainly due to: (i) an extremely polyphagous diet, attacking nearly 400 plant species [Copeland *et al*., 2002]; (ii) a liberal host acceptance behaviour [Yuval and Hendrichs, 2000]; (iii) a rapid population growth [Duyck *et al*., 2004]; and (iv), a great tolerance for a wide range of climates [Thomas *et al*., 2007]. In addition, medfly can be spread via local sale or exportation, and fly at least 20 km, which greatly complicates the efforts to control this insect [Thomas *et al*., 2007]. Thus, medfly is now fully spread in more than 70 countries including Spain and Australia, and it is considered as a quarantine pest

throughout the world [EPPO 1981; Thomas *et al*., 2007]. As one of its favourite hosts is apple, *C. capitata* was the selected insect as a model to study the *E. amylovora* transmission from contaminated but asymptomatic mature apples.

2B. OBJECTIVE OF THIS STUDY

To determine whether the transfer of *E. amylovora* from asymptomatic infected apple fruits to healthy plant material can be possible through vector insects.

3B. EXPERIMENTAL ASPECTS

3B.1. Bacterial strains and growth conditions

Four strains of *E. amylovora* were assayed: CFBP1430 (*Crataegus oxyacantha*, France) and NCPPB2080 (*Pyrus communis*, New Zealand), and their respective GFP-transformants, 1430-GFP1 and 2080-GFP3, obtained in our laboratory.

The growth medium employed for *E. amylovora* isolation was the semi-selective CCT medium, both solid and liquid [Ishimaru and Klos, 1984]. In case of GFP-tagged strains, King´s B (KB) medium [King *et al*., 1954] supplemented with the antibiotic tetracycline (KBTc) was also used, acting the antibiotic as a marker of the GFP-plasmid presence (12.5 μg/ml of Tc for 1430-GFP1, and 5 μg/ml for 2080-GFP3). Other culture media were used to verify the morphology of *E. amylovora*-like colonies isolated throughout the experiments: sucrose nutrient agar (SNA) [Lelliot, 1967] and RESC medium [Ordax *et al*., 2009a]. In the case of solid media, the incubation conditions were 26ºC for 2-7 days. For liquid media, the conditions were 26-28ºC with a slight continuous shaking during 1-6 days according to the turbidity observed.

3B.2. *C. capitata* strain and rearing conditions

Medfly pupae were obtained from the Entomology Laboratory colony IVIA2002 [San Andrés *et al*., 2007]. Adults were maintained in a 20x20x20 (cm) perpex cage at 25 ± 4ºC, 75 ± 5 % RH and 16:8h (L:D) photoperiod in an environmental chamber (MLR-350, Sanyo). Standard food consisted in a mixture of sugar and hydrolyzed yeast (Biokar Diagnostics Co., Pantin, France) (4:1; w:w) and water. Sexually mature medflies (5-7 day-old) were used in all assays.

3B.3. Plant material

Mature Royal Gala apple fruits from organic culture were used in all acquisition experiments with *C. capitata,* and also in the transmission assays to fruit. The apples came from South America or Spain depending on the season, to avoid the long-term

storage under cold temperatures and controlled atmosphere commonly used by the fruit industry. In all cases, apples were surface disinfected with 60% commercial hypochlorite solution for 5 min, followed by 3 washings of 15 min with sterile distilled water. Then, fruits were meticulously dried one by one.

Detached young pear shoots of cv. Conference from IVIA greenhouses were used in several transmission assays, and also to verify the pathogenic potential of the isolates. They were surface disinfected with 50% alcohol for few seconds, followed by 3 washings of 10 min with sterile distilled water. After that, the shoots were left to dry at room temperature and their base introduced in sterile agar 1.5%.

Whole pear plants used for transmission experiments and in the recovery bioassays were obtained from seeds taken out from Conference pears. According to Santander *et al*. [2010b] the seeds were surface disinfected with sodium hypochlorite 3% (w/v), washed, dried, and stratified in wet silica sand at 4ºC. After 1-3 months, the seeds were transferred into autoclaved substrate (30% black peat, 30% white peat, 30% sand and 10% perlite) and grown at greenhouse conditions in an environmental chamber (MLR-350, Sanyo) for 1-2 months (stems length between 8-16 cm).

To verify the pathogenic potential and/or in the recovery through bioassays, immature Golden apples and loquats (2-4 cm diameter) were used besides the shoots. They were surface disinfected with 30% commercial hypochlorite solution for 1 min and 3 washings of 10 min, and dried in a flow laminar chamber for 1-2 h.

3B.4. Acquisition of *E. amylovora* by *C. capitata* from symptomless inoculated apples

The acquisition experiments were performed in medfly cages with two disinfected mature apples per cage (20x15x10 cm) placed in opposite positions (peduncle or calyx face up). In each apple several wounds were performed as follows: six cuts of 1-2 cm in the area of greatest diameter of the fruit, and five cuts in the surrounding area to peduncle or calyx. Each cut was inoculated placing 20 μl of *E. amylovora* suspension in PBS buffer at a final concentration around $5x10^7$-$1x10^8$ cfu/ml (Fig. 1A). Afterwards, different numbers of medflies were introduced per cage in different assays: initially 50 medflies (25 males and 25 females), or 10 (5 males and 5 females), or 5 (2 males and 3 females), or finally only one male or one female separately. During 48h these cages were maintained under favourable conditions for both *E. amylovora* and *C. capitata* (26ºC ± 2ºC, 12-hour light/dark cycle, 75-85% RH) in an environmental growth chamber (MLR-351, Sanyo) (Fig. 1B). After this acquisition period of the bacterium by the medfly, a) the inoculated apples were removed from some of the cages containing

50 medflies for the survival studies (see below in section 3B.5); and b) the insects were aspired with disinfected tubes (Fig. 1C,D) and transferred to other cages with healthy plant material for transmission assays (see section 3B.6).

Besides, in some cages with 50 medflies, the insects were analyzed for *E. amylovora* presence to verify the acquisition. Thus, groups of 3-5 medfly individuals were crushed in 2.5 ml buffer TNES [San Andres *et al*., 2007], and these direct extracts and also their dilutions down to 1/1000 were plated on solid CCT medium and enriched in 1ml CCT broth. At the same time, aliquots of 300 μl of each medlfy extract were subjected to an insect DNA extraction protocol [San Andres *et al*., 2007] before the subsequent PCR analysis for *E. amylovora* [Taylor *et al*., 2001]. The remaining insect extracts were stored at –20ºC with 30% glycerol for further studies.

All the acquisition assays were repeated independently and duplicated for each *E. amylovora* strain.

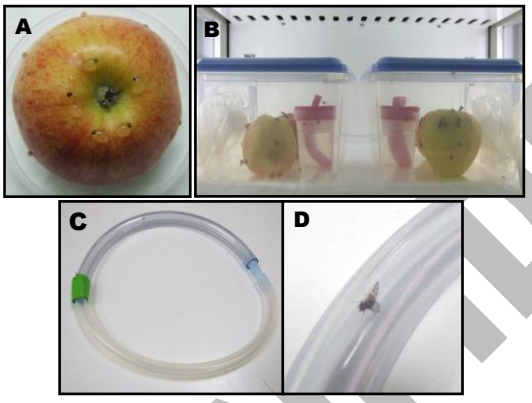

Fig.1. *E. amylovora* acquisition assays by *C. capitata* from inoculated but asymptomatic mature apples. A) Apple with drops of bacterial inoculum on equidistant cuts. B) Two representative acquisition cages at time 0. C) Suction pipe to aspirate medflies. D) Medflies aspirated remain active inside the suction pipe and ready to be transferred by expiring. (Photos: M. Ordax and J.E. Piquer-Salcedo)

3B.5. Survival of *E. amylovora* on *C. capitata*

Immediately after the acquisition stage, and after removing the inoculated apples in some of the cages with 50 medflies, we started to monitor the survival of the bacterium on the medfly throughout 28 days. Thus, *E. amylovora* populations were quantified after 7, 14, 21 and 28 days after being in contact with *C. capitata*. To maintain the medflies alive, they were fed with 10% sterile sucrose solution. Weekly, only living medflies (about 15-30 depending on the time period elapsed) were aspired to analyze the presence of *E. amylovora* by cultural and PCR methods as described above, but plating up to 1ml of medfly extract to improve the detection limit (<1 cfu/ml). *E. amylovora*-like colonies were confirmed by PCR, and their pathogenic potential was also verified by inoculation in immature apples and loquats, and in pear shoots [EPPO,

2004]. If the culturable analysis was negative but PCR was positive, the corresponding medfly extracts were subjected to recovery assays (see section 3B.8). Survival experiments were repeated independently for the four *E. amylovora* strains included in this work.

3B.6. Transmission of *E. amylovora* through *C. capitata* to mature apple fruits

The medflies which had supposedly acquired the bacterium from inoculated apples were transferred to other cages with two healthy mature apples in opposite positions. Small wounds (1-1.5 cm diameter) were performed on these apples and distributed as in the acquisition stage, but they were not inoculated. Analogously to the acquisition assays, 50, 10, 5 and only one medfly were transferred to other transmission cages with healthy apples. Throughout 5 days of the transmission period, the cages were maintained in an environmental chamber with the same conditions used for the acquisition assays. All the experiments were duplicated and repeated independently for each *E. amylovora* strain.

Once the transmission period elapsed, live medflies, eggs, and apples were analyzed to detect *E. amylovora*. Insects were processed as described above (section 3B.5) in groups of 3-5 or one by one, depending on the number of medflies per cage. Eggs were analyzed only by PCR, with a previous step at –20ºC to facilitate their crushing in 300 μl TNES buffer. DNA extraction and PCR amplification from the egg crushings were performed as for medflies.

Apples were analyzed one by one. Firstly, whole fruits were washed in 10 ml PBS buffer to detect epiphytic cells of *E. amylovora* according to previous works [van der Zwet et al., 1990; Temple *et al*., 2007]. Afterwards, flesh layers around 0.5 cm underneath each performed wound were taken out with a sterile scalpel, crushed in 2.5 ml AMB buffer [Gorris *et al*., 1996] and processed for the presence of endophytic populations of *E. amylovora*. Both, washings and flesh extracts from each one of the transmitted apples were plated by triplicate on CCT solid medium, enriched 1:1 in KB and CCT broths, and subjected to a plant DNA extraction protocol [Llop *et al.*, 1999] for subsequent PCR amplification [Taylor *et al*., 2001]. In case *E. amylovora*-like colonies were observed, they were confirmed by PCR, and their pathogenicity was demonstrated by inoculation in inmmature apples and pear shoots [EPPO, 2004]. The remaining washings and extracts were frozen with glycerol.

3B.7. Transmission of *E. amylovora* through *C. capitata* to susceptible plant material

Populations of about 50 medflies supposedly contaminated with the bacterium after the acquisition from inoculated apples were also transferred to other cages containing susceptible plant material. The transmission assays to two kinds of plant material, detached shoots and whole plants, were carried out in two ways in independent cages: keeping the leaves intact, or cutting them with scissors previously disinfected to reach the main vein [EPPO, 2004]. Drops of 10% sterile sucrose were periodically introduced into the cages to provide a carbon source to medflies. The transmission period was variable, between 5 and 14 days, depending on the symptom evolution. The climatic conditions were the same as in the acquisition stage.

When the transmission assays were over, the transmitting medflies were analyzed by cultural techniques and PCR, as previously described in the survival assays. Plant material (either shoots or whole plants) were processed one by one. The symptomatic parts of each leaf were processed by disruption in 1-1.5 ml AMB buffer [Gorris *et al*., 1996], and after few minutes, the extracts were plated on solid media, enriched in broth and analyzed by PCR as indicated for mature apples. *E. amylovora*-like colonies were also confirmed by PCR.

3B.8. Recovery assays to regain the culturability of *E. amylovora* cells

In those cases in which *E. amylovora* colony was not recovered on solid medium after plating 1 ml of medfly extract, recovery assays were carried out to determine the possible recovery of the culturability, before discarding the presence of the pathogen. In accordance with previous works [Ordax *et al*., 2006; Ordax *et al*., 2009b; Santander *et al*., 2010a], *in vitro* and *in vivo* recovery assays of the pathogen were tested. In the first case, we performed enrichments of the extracts that could contain non-culturable *E. amylovora* cells in KB and CCT broths (1:1), and afterwards we plated the enriched extracts on solid media. The *in vivo* recovery assays were based on the use of susceptible plant material, either immature apples or loquats, pear shoots in agar or whole pear plants. In all cases the plant material was cut, 4 cuts per immmature fruit and 1 cut per young leaf (either from shoots or plants), and 10-20 μl of medfly extract were inoculated by cut. All plant material was maintained under favourable conditions in a climatic chamber, and regularly examined throughout 10-30 days depending on the development of the symptoms. A suspension of the strain CFBP1430 at 10^8 cfu/ml in PBS buffer was used as positive control, and PBS, AMB or TNES buffer as negative controls in each case.

3B.9. Fluorescence microscopy studies

Several live medflies from the survival and transmission assay cages challenged with the GFP-marked *E. amylovora* strains were captured by aspiration. Afterwards, they were visualized in fresh with a Nikon ECLIPSE E800 epifluorescence microscope. Firstly, medflies were observed intact and one by one in hollow slides, to visualizate epiphytic *E. amylovora* cells. Afterwards, medflies were individually transferred to standard slides, slightly crushed with a cover slip, and examined looking for both epiphytic and endophytic GFP-tagged *E. amylovora* cells.

4B. RESULTS

4B.1. *C. capitata* is able to acquire *E. amylovora* from inoculated mature apples

The analyses of medflies after being 48h in contact with inoculated symptomless apples showed positive results for *E. amylovora* detection by cultural and PCR methods (Fig. 2A). The pathogen concentration carried per medfly was quantified in a range around 10^4-10^6 cfu/individual. The behaviour of the medflies revealed a special attraction to the inoculated wounds, and indeed, medflies were often seen feeding on inoculums drops (Fig. 2B) and showing swollen abdomens.

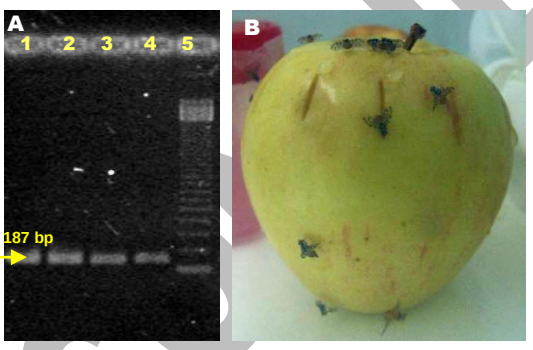

Fig.2. Acquisition of *E. amylovora* cells by *C. capitata* from inoculated mature apples. A) Representative positive detections of *E. amylovora* by chromosomal PCR amplification [Taylor *et al*., 2001] in groups of 5 medflies aspirated after 48h in contact with the apples (lane 5, ladder 200 bp, Invitrogen). B) Several medflies feeding on inoculums drops in the surrounding area to the fruit peduncle. (Photos: M. Ordax and J.E. Piquer-Salcedo).

4B.2. *E. amylovora* survives on medfly

Culturable *E. amylovora* cells, confirmed by PCR, were recovered on CCT solid medium (Fig. 3A) in populations between 10^5-10^3 cfu/medfly after 7 and 14 days, respectively, of being in contact with medflies (Fig. 3B). Their inoculation in immature apples and loquats confirmed their pathogenicity. In contrast, no *E. amylovora*-like colonies were found from medfly extracts after 21 and 28 days of contact time bacterium-fly (detection limit <1cfu/ml of medfly extract) (Fig. 3B), although PCR results were positive. To verify if *E. amylovora* had died after more than two weeks in contact

with medfly tissues, or it had lost its culturability, 21- and 28-day medfly extracts were subjected to recovery assays (see section 4B.5).

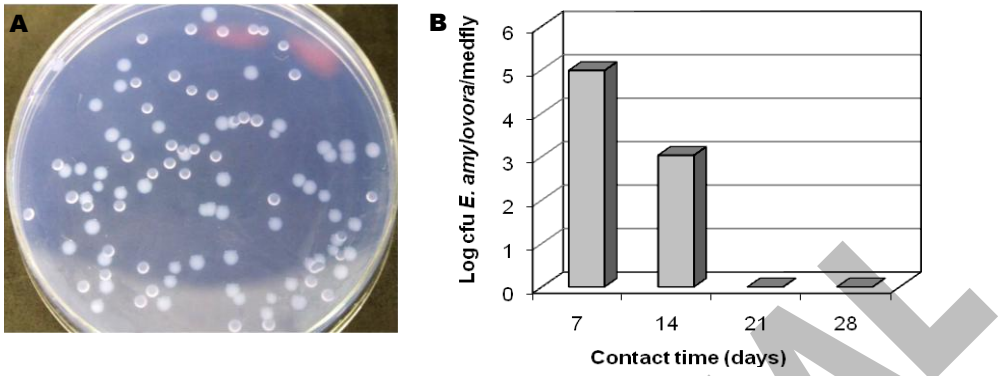

Fig.3. Survival of *E. amylovora* on medfly. A) Representative CCT plate showing *E. amylovora* colonies recovered from *C. capitata* mixed with the microbiota of the insect (dilution 10^{-3} after 48h at 26ºC). Colonial morphology of *E. amylovora* was distinguished by its typical color pale-violet with a slightly clear margin. B) Culturable cells of the bacterium were recovered only up to 14 days of being in contact with medfly tissues (detection limit <1 cfu/ml of medfly extract). (Photo A: M. Ordax and J.E. Piquer-Salcedo).

4B.3. *C. capitata* transmits *E. amylovora* to mature apples that do not develop symptoms

Contaminated medflies with *E. amylovora* cells acquired from inoculated apples were enclosed with healthy apples for 5 days. In these new cages, medflies showed a relevant attraction to the fruits, mainly to the surrounding areas of the wounds. None of the apples subjected to the transmission assays showed fire bight symptoms either on the peel on in the flesh. However, cultural (Fig. 4A, 4B) and PCR analysis (Fig. 4C) of the apples and medflies after 5 days revealed that *E. amylovora* had been transmitted to the apples. Furthermore *E. amylovora* colonies recovered from these apples were pathogenic when they were inoculated in susceptible plant material (Fig. 5A,B). Epiphytic and endophytic *E. amylovora* populations recovered from the transmitted mature apples were quantified around 10^5-10^4 and 10^4-10^3 cfu/fruit, respectively, depending on the number of transmitting insects assayed in each cage (from around 40 to only one individual). The numbers of *E. amylovora* cells carried per transmitting medfly were between 10^3-10^5 cfu, but the results of the analysis of the eggs were always negative.

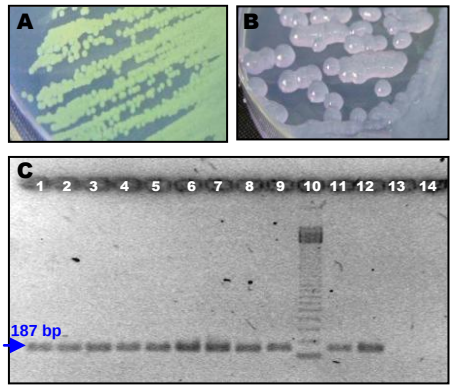

Fig. 4. *E. amylovora* transmission to mature apples. A) GFP-tagged *E. amylovora* culture from a flesh extract purified on KB-Tc medium, and B) on CCT medium. C) Representative agarose gel of chromosomal PCR [Taylor *et al*., 2001]. Lanes 1-3: extracts from apple washings; Lanes 4-6: apple flesh extracts; Lanes 7-9: medfly extracts; Lane 10: ladder 200 bp (Invitrogen); Lanes 11-12: positive controls (DNA from *E. amylovora* strain CFBP-1430); Lanes 13-14: negative controls (HPLC-grade water). (Photos: M. Ordax and J.E. Piquer-Salcedo).

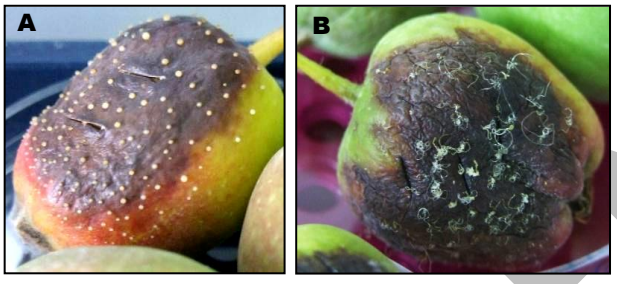

Fig.5. Typical fire blight symptoms in green apples inoculated with *E. amylovora*-like colonies recovered from a transmitted apple by *C. capitata* (at 7 dpi). Extended necrosis with exudates as drops (A), or strands (B). (Photos:M. Ordax and J.E. Piquer-Salcedo).

The transmission of the pathogen to different fruit parts and the relationships with the medfly sex were further studied in cages with only one individual medfly (one male or one female repeated independently for each *E. amylovora* strain). Related with fruit parts and regardless the medfly sex, the majority of the *E. amylovora* epiphytic population was transmitted to the surrounding area to the calyx and in the area of the greatest diameter of the fruit (Fig. 6A); but the endophytic population was found mainly in the peduncle area (Fig. 6B). In contrast, when considering the fly sex, the male transmitted *E. amylovora* cells especially to the surface of the fruit, while the female did it to the surface and also into the flesh (Fig. 6A,B). The most notable differences were observed inside the distal parts of the fruit, either in the peduncle area (10^2 cfu transmitted by male in contrast with 10^5 cfu by female) or in the calyx region (10^4 cfu transmitted in total and only by the female) (Fig. 6B). This differential distribution of the pathogen on/in the mature apples depending on the medfly sex was also confirmed by PCR (Fig. 6C).

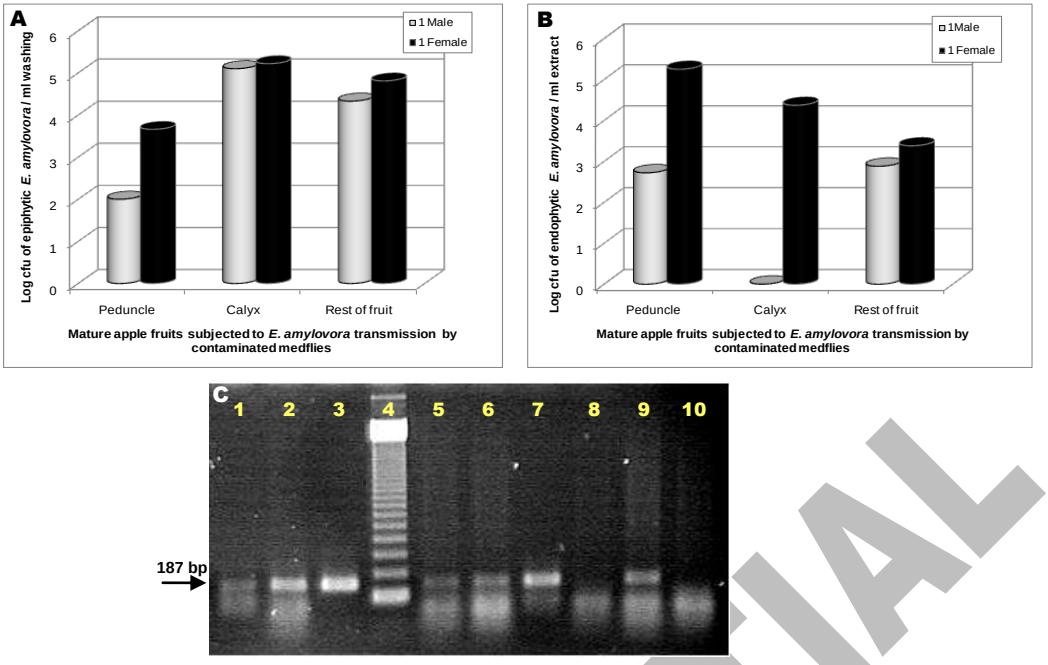

Fig.6. Transmission of *E. amylovora* cells per fruit area and medfly sex. A) Epiphytic population of the pathogen on the peel of each mature apple. B) Endophytic population in the flesh of each apple (0.5-1 cm under the peel). C) Representative agarose gel of chromosomal PCR [Taylor *et al*., 2001]. Lanes 1-2, washings of a transmitted peduncle by one male and one female, respectively; Lane 3, positive control (DNA from strain CFBP-1430); Lane 4, ladder 100 bp (Biolabs); Lanes 5-6, washings of a transmitted calyx by one male and one female, respectively; Lanes 7-8, flesh extracts from calyx transmitted by one female and one male, respectively; Lane 9, flesh extract from a peduncle transmitted by one female; Lane 10, negative control (HPLC-grade water).

4B.4. *C. capitata* can transmit *E. amylovora* to susceptible plant material which develops symptoms

In the detached pear shoots of the transmission assays (one per cage), irregular necrotic lesions of different extension (0.5-4 cm) were observed in several leaves after 5-7 days in contact with around 40 contaminated medflies (Fig. 7A,B). Some egg clusters were occasionally noticed as embedded in the leaf tissues, and surrounded by a necrotic margin (Fig. 7C). Once elapsed the transmission period, the presence of *E. amylovora* in those necrotic areas and also in the transmitting medflies was confirmed by cultural and PCR techniques (Fig. 7D, whereas the results were always negative in the egg samples. The populations of *E. amylovora* recovered from the lesions of pear leaves were quite high (10^5-10^7 cfu/necrosis) in contrast with those much lower (around 10^1 cfu/ml insect extract) in the transmitting medflies.

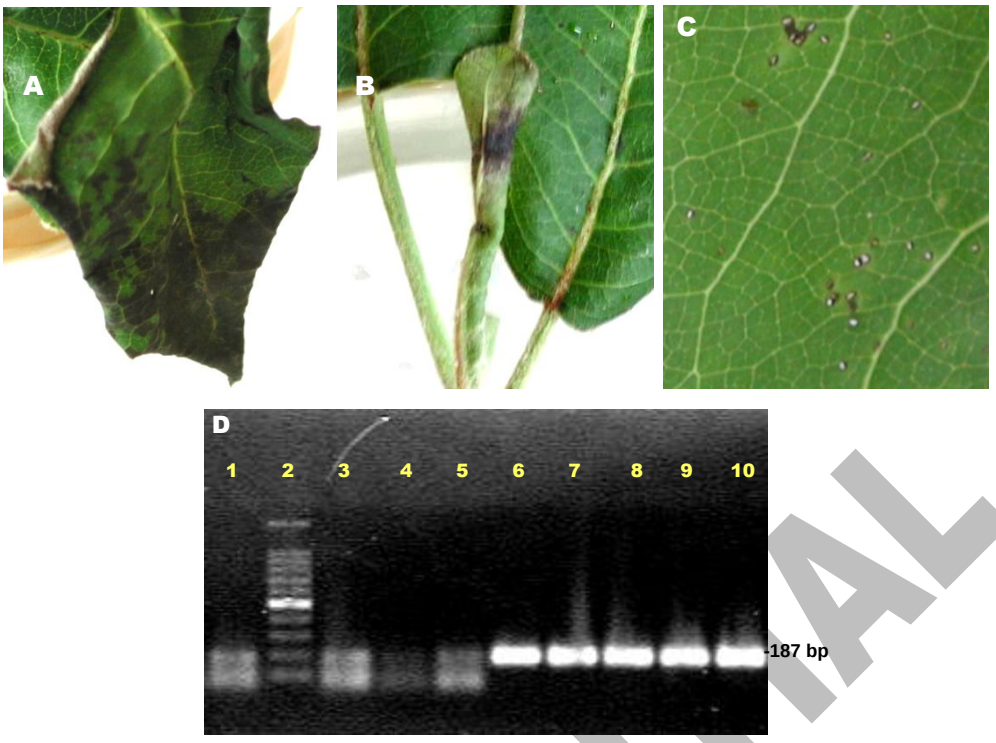

Fig. 7. Transmission of *E. amylovora* by contaminated medflies to detached young pear shoots. A,B) Necrotic lesions of different extension in leaves after 5 days in contact with the medflies. C) Some eggs embedded in the leaf tissues. D) Representative agarose gel. Lanes 1, 3-5: transmitting medflies; Lane 2, ladder 123 bp (Invitrogen); Lanes 6-9; *E. amylovora*-like colonies recovered from necrotic areas; Lane 10, positive control (DNA from *E. amylovora* strain CFBP-1430). (Photos: M. Ordax and J.E. Piquer-Salcedo).

In the assays with potted pear plants (one per cage), very slight symptoms were observed in some leaves of each plant, which showed between 1-4 black spots (around 0.2 cm diameter) after 10-14 days of transmission with around 40 contaminated medflies (Fig. 8A). The location of these spots follows a ramdom distribution, being observed in both young and adult leaves, cut or intact leaves, and in front part as well as in the back. This was in accordance with the behavior of the medflies throughout the transmission period, which no showed any special attraction to the cuts of the leaves. Some leaves were punctured by female medflies to lay their eggs, but eggs were never found in the wounds. *E. amylovora* was usually isolated in low numbers (10^1-10^2 cfu) from the black spots of each leaf (Fig. 8B), and in one case up to 10^4 cfu were recovered. The growth of microbiota from both the plant and the transmitting medflies was predominant on the CCT plates (Fig. 8B). No *E. amylovora*-like colonies grew from the medflies after 14 days of carrying the bacterium, in accordance with the survival results of the pathogen on the medfly described before (section 4B.2). PCR results for *E. amylovora* were positive in both pear leaves and transmitting medflies from all the cages assayed.

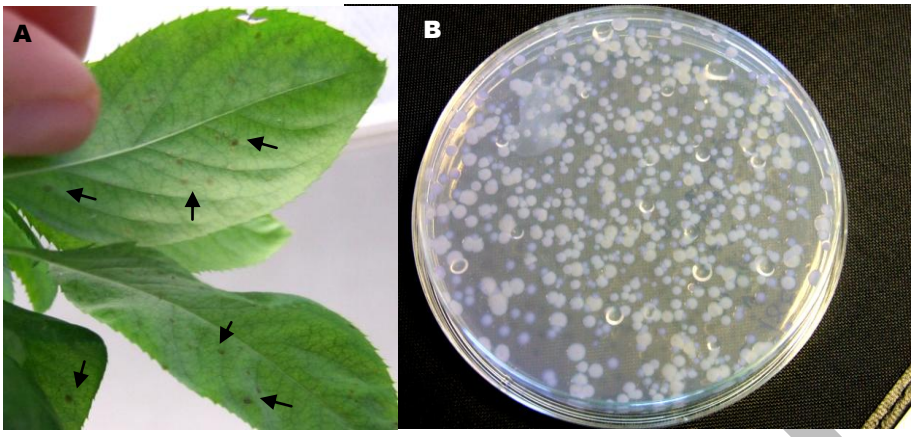

Fig. 8. Transmission of *E. amylovora* by contaminated medflies to whole pear potted plants after 14 days. A) Leaves showing black spots randomly distributed. B) *E. amylovora* colonies mixed with microbiota from both pear plants and transmitting medflies on CCT medium plated with black spot extracts from one pear leaf (48h, 26ºC). *E. amylovora* colonies could be distinguished by its typical aspect as water drops. (Photos: M. Ordax and J.E. Piquer-Salcedo).

4B.5. Non-culturable *E. amylovora* cells from medflies are recovered after the passage through host plant tissue

Several extracts containing non-culturable *E. amylovora* cells were collected from the different experiments included in this work: a) 21- and 28-day medfly extracts from survival assays; b) 16-day extracts from transmitting medflies of transmission assays to whole pear plants (48h of acquisition period plus 14 days of transmission period).

The attempts to recover the culturability of these non-culturable *E. amylovora* cells on solid medium by a previous enrichment in KB and CCT broths was unsuccessful in all the extracts despite the use of prolonged incubation periods. In contrast, the recovery through a passage by susceptible plant material showed positive results. The use of different type of host plant material depended on its availability at the inoculation time. Immature apple and loquat fruits, as well as detached pear shoots were inoculated with extracts from contaminated medflies that carried *E. amylovora* during 21 and 28 days. Both kinds of plant material evidenced fire blight disease symptoms after 7-14 days post-inoculation (dpi), but the shoots symptoms (Fig. 9A) were more severe than those observed in green fruits (Fig. 9B), showing extensive necrosis and even ooze drops. Whole pear plants were used for the recovery of non-culturable *E. amylovora* cells from 16-day medfly extracts of transmission assays, showing noticeable necrotic lesions in the surrounding area to the cut (Fig. 10).

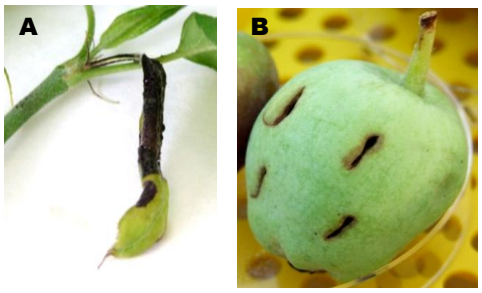

Fig. 9. Representative cases of high and low recovery levels in host plant tissues after the inoculation of non-culturable *E. amylovora* cells from medfly extracts contaminated with the bacterium during 28 days. **A**) Necrosis and ooze drops in a young pear leaf (7 dpi). **B**) Immature apple showing necrotic margins and typical invagination on the cut edges fruit (14 dpi). (Photos: M.Ordax and J.E. Piquer-Salcedo).

In general, an expected relationship between the severity of the necrotic lesions and the numbers of culturable *E. amylovora* cells reisolated was noticed. Levels of 10^3 cfu/lesion were recovered from small necrotic areas, whereas 10^7 cfu were reisolated from extensive necrosis, regardless the kind of plant material or extract inoculated. In all cases the positive controls showed severe fire blight symptoms at 5-7 dpi, and the negative controls did not show any disease sign.

Fig. 10. Necrotic lesion in a cut leaf from a whole pear plant inoculated with 16-day contaminated medfly extract from transmission assays (at 10 dpi). (Photo: M. Ordax and J.E. Piquer-Salcedo)

4B.6. Location of *E. amylovora* populations in medflies

Several medflies from survival and transmission assays contaminated with GFP-tagged *E. amylovora* cells were analyzed by fluorescence microscopy. The populations of the pathogen were mostly found as cellular groups in the ovipositor of females (7^{th}- 9^{th} abdominal segments) (Fig. 11A-C), and in the distal parts of wings in both sexes (Fig. 11D).

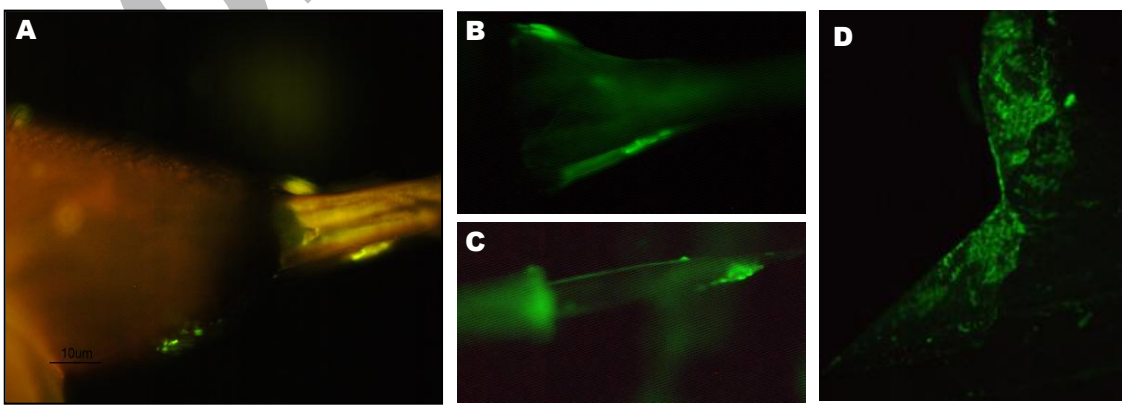

Fig.11. GFP-tagged populations of *E. amylovora* in a transmitting female medfly. A) 7th and 8th abdominal segment with GFP-tagged cells associated. B) 7th abdominal segment in detail with GFP layers on the surface. C) 9th abdominal segment showing GFP-cells on its tip. D) A wing showing spread masses of GFP cells. Photo A is shown under the filter B-2A (EX 450-490nm, DM 500nm, BA 515nm); Photos B-D, with FITC filter (EX 465-495nm, DM 505nm, BA 515-555nm). (M. Ordax and J.E. Piquer-Salcedo).

5B. CONCLUDING REMARKS

Insects are recognized as a contributing factor in the epidemiology of the fire blight disease [Emmett and Baker, 1971; Thomson, 2000]. Although the involvement of the vinegar fly (*Drosophila melanogaster*) in the fire blight spread was already reported [Ark and Thomas, 1936], the role of other well-known fruit fly, *C. capitata,* had not been previously investigated. In this work, we have demonstrated the great ability of *E. amylovora* to persist on *C. capitata* for quite prolonged time periods, and further, the transmission of this pathogen by the medfly from mature apples to different healthy plant material, showing the great potential of this insect as a vector of *E. amylovora*.

The probability that a contaminated insect transmits *E. amylovora* depends on both the pathogen dose carried by the insect and the survival time of the pathogen on the insect [Hildebrand *et al*., 2000]. We have monitored the pathogen populations surviving on medflies throughout one month, experimental period close to the average life of adult medflies in nature [Thomas *et al*. 2007]. Interestingly, *E. amylovora* showed a noticeable ability to persist on *C. capitata* in a culturable state up to 14 days of being in contact with medfly tissues, and in addition, in relatively high concentrations (from 10^5 to 10^3 cfu/medfly after 7 and 14 days). In contrast, honeybees, considered the most usual fire blight vector, can carry around 10^3 cfu/insect, but the bacterium only remains alive 48h on the insect [Alexandrova *et al.*, 2002]. Other insects are able to transport between 10^2-10^4 cfu of *E. amylovora* [Ark and Thomas, 1936; Miller and Schroth, 1972; Hildebrand *et al*., 2000; Alexandrova *et al*., 2002; Tsukamoto *et al*., 2005b], but the most prolonged survival period reported until now is up to 6 days [Ark and Thomas, 1936]. The different methodology utilized in this work to detect *E. amylovora* on the medfly could explain the differences in the survival results reported in other insects.

Remarkably, no *E. amylovora*-like colonies grew from medflies after more than 14 days, which indicated either the death or the loss of culturability of the pathogen, probably due to prolonged contact periods with medfly tissues that could represent a cause of stress to this bacterium. It is known that the recovery of culturability is often triggered by the removal of the inductor stress which caused the non-culturability response [Oliver, 1993]. The KB liquid medium has provided excellent results for recovering non-culturable *E. amylovora* cells induced by other stress conditions [Ordax *et al*., 2006; Ordax 2008; Ordax *et al.,* 2009b; Santander et al., 2010a], but surprisingly, it resulted unsuccessful when the loss of culturability was induced by the extended contact with medfly tissues. Similarly, negative results were also obtained with CCT broth. Then, the passage through host plant material could be the most appropriate

recovery method in our case, since the stress that suffered *E. amylovora* was probably caused by the stay in a non-host environment. Thus, the inoculation of the medfly extracts with non-culturable cells of the pathogen in susceptible host plant material gave successful results because the plants developed necrosis of different extension from where the pathogen was again recovered on solid medium. This pointed out that *E. amylovora*, although injured because it had lost the culturability on semiselective CCT medium [Liao and Shollenberger, 2004], remained viable and potentially pathogenic in the insect up to 28 days, at least. It is very common that non-host environments involve strong changes in a pathogen and activate a stress response [Winfield and Groisman, 2003]. Indeed, a bacterial pathogen usually has a lower or even negative growth rate outside its host [Winfield and Groisman, 2003; Ordax et al., 2006; Biosca *et al*., 2006, 2008, 2009; Santander *et al*. 2010a]. But interestingly, our results showed that *E. amylovora* managed to stay alive on *C. capitata* for nearly one month, confirming the remarkable survival of this pathogen in non-host environments [Southey and Harper, 1971; Ceroni *et al.,* 2004; Hildebrand *et al.,* 2001; Biosca *et al.,* 2006, 2008, 2009; Santander *et al*. 2010a].

Further, the transmission assays revealed that *C. capitata* efficiently acquired *E. amylovora* from inoculated but symptomless mature apples and transmit significant doses of culturable cells to other apples, not only to the peel (10^4-10^5 cfu/fruit), but also to the flesh (10^3-10^4 cfu/fruit). However, the apples were asymptomatic throughout the transmission period, probably because the difficulty of *E. amylovora* for multiplying in the ripeness stage of the fruit [Dueck, 1974; EPPO, 2004; Roberts and Sayer, 2008]. Then, it could be assumed that epiphytic and endophytic *E. amylovora* populations recovered from the apples after the transmission stage had been transferred by the medflies, revealing a considerable efficiency of *C. capitata* as a transmitting agent. In fact, in further experiments, only one individual medfly was able to transmit the pathogen outside and inside of two mature apples. In these transmission assays, both sexes contributed to the pathogen transmission to the peel, but the internal transmission was mainly due to the female, probably through the laying of eggs. It has been reported that a female medfly may lay around 20 eggs per day by puncturing the fruit up to 0.5 cm deep through her ovipositor [Thomas *et al*., 2007; Coronado-González *et al*., 2008]. In fact, some tunnels containing batches of eggs were found into the flesh of several transmitted apples. On the contrary, *E. amylovora* was never detected in the eggs, confirming that the vertical transmission of pathogens in the transmitted infections by insect vectors is quite unusual [Purcell, 1982]. The recovery of *E. amylovora* populations from the surface of the apples and also from the flesh,

suggests that there is not only a simple contact insect-pathogen-host, but also a close relationship with more complex interactions, involving reproductive and digestive processes [Cayol *et al*., 1994; Coronado-González *et al*., 2008].

Once demonstrated the *E. amylovora* transmission from contaminated to healthy mature apples through *C. capitata*, we aimed to go further by assaying the transmission to susceptible host plant material, such as detached young pear shoots and whole pear potted plants. In both cases the transmission was noticed visually because necrosis of different extension appeared in the leaves. The severity of the necrotic lesions was quite different between detached shoots and whole plants, being much more severe in the shoots. This could be related with the plant defense response against pathogens, more effective as whole-plant response than in detached plant organs [Ruz *et al*., 2008]. In relation with the severity of the symptoms, high numbers of *E. amylovora* cells were recovered from the extensive lesions in the leaves from the shoots (10^5-10^7 cfu/necrosis), whereas low concentrations were found in the black spots of the pear plants (10^1-10^2 cfu/spot), either in intact or cut leaves.

The microscopic analysis allowed to deep into the knowledge of the interaction between *E. amylovora* and *C. capitata*. The presence of GFP-cells in the ovipositor of transmitting medflies was in agreement with the detection of *E. amylovora* cells inside the flesh of the mature apples after the transmission. According to several studies on fruit fly-bacteria interactions, the internal transmission of the pathogen into a fruit is mainly caused by the ovipositor, through the laying of eggs and/or its fecal contamination [Behar *et al*., 2008]. The presence of GFP masses on the wings of both sexes is probably related with the position adopted by the medflies when they lay the eggs, feed or defecate, making possible the contact of the distal part of the wings with the fruit or the leaf. This could explain, at least in part, the involvement of both males and females in the transmission of *E. amylovora*.

The phytosanitary risk of *E. amylovora* dissemination by *C. capitata* is increased by the fact that this fruit fly is able to transmit the pathogen in a full-active state to different healthy plant material, not only to fruits. Thus, the bacterium was directly isolated after the transmission to mature apples, but also from detached pear shoots and whole pear plants. These results, coupled with the noticeable carrier capacity of *E. amylovora* per medfly, and the exceptional ability of the pathogen to remain alive on this insect, show up the great potential of *C. capitata* as a fire blight vector, which was unknown up to date. This work provides new epidemiological information not only in the dissemination of *E. amylovora* through insects, but also in the role of medfly as a disease vector, since fruit flies have seldom been referred as vectors of plant or human diseases

[Janisiewicz *et al*., 1999; Cayol *et al*. 1994; Sela *et al*., 2005]. As far as we know, the medfly has been reported as a plant disease vector only in the case of the fruit-decaying fungus *Rhizopus stolonifer* [Cayol *et al*. 1994].

In conclusion, our results show a new scenario in the life cycle of *E. amylovora*. First, latently infected mature fruits in nature could act as an unnoticed reservoir of the pathogen (A part of this report). Secondly, in B part, the transmission experiments with the medfly point out that the contaminated fruit could be a vehicle for *E. amylovora* dissemination, at least if insect vectors are present, host plants are in the surrounding area, and the climatic conditions are favourable. Therefore, two kind of potential carriers of *E. amylovora* arise from our results, mature fruit (passive) and medfly (active), suggesting that rigorous protection and sanitation measurements are required to achieve an efficient control of the quarantine organism *E. amylovora*.

Acknowledgements. Work fully funded by HAL through APAL. M. Ordax and J.E. Piquer-Salcedo are deeply thankful to HAL/APAL for their research and technician contracts, respectively. E. Marco-Noales wishes to thank Ministerio de Innovación y Ciencia of Spain (Program INIA/CC.AA) and European Social Fund (ESF) for her research contract. We thank to C.I. Salcedo her technical assistance. Authors especially thank Tony Russell (General Manager, APAL) and Satish C. Wimalajeewa (Consultant Plant Pathologist) their support and supervision labor throughout the project period.

6. GENERAL SUMMARY

The bacterium *Erwinia amylovora* is the causal agent of fire blight, a devastating and widespread disease affecting fruits of great commercial importance such as apple and pear. Despite intensive efforts, fire blight still remains a serious threat for agriculture due to its difficult control and the ability of this pathogen to persist in nature.

E. amylovora usually infects immature fruits causing necrosis and exudates, but it has also been detected occasionally in asymptomatic mature fruits from trees severely affected by fire blight. Then, mature fruit could act as an unnoticed carrier of the pathogen. In this work, the analysis of a large number of asymptomatic apples and azaroles harvested from trees naturally and severely infected by fire blight has confirmed that the transfer of *E. amylovora* from the infected plant to fruits can be possible under certain conditions. Thus, although *E. amylovora* was not detected in any of the apples analyzed, the pathogen was isolated in 12% of the harvested azaroles despite their healthy appearance. Therefore, the mature fruit could occasionally act as a reservoir of *E. amylovora* cells, at least in the case of plants with high inoculum doses and under climatic conditions conducive to the pathogen multiplication.

Furthermore, contaminated fruits could act as a vehicle for *E. amylovora* dissemination in case they becomes in contact with insect vectors and/or susceptible hosts. To study this transfer pathway, several transmission assays were carried out under *in vitro* conditions using the Mediterranean fruit fly (medfly) *Ceratitis capitata*, one of the most damaging fruit pests worldwide. Our results revealed that medflies can acquire *E. amylovora* from contaminated apples, and then transmitting it to healthy apples. Even only one individiual medfly was able to transmit the pathogen to both outside and inside of mature apples (at 10^6 and 10^3 cfu, respectively), which did not develop any disease symptom. Further, contaminated medflies also transmitted *E. amylovora* to detached pear shoots and whole pear plants, inducing fire blight symptoms but with different severity. The shoots showed extensive necrosis in leaves after 5-7 days of transmission, being recovered *E. amylovora* at levels of 10^5-10^7 cfu/lesion, whereas the plants showed only black spots after 14 days, and with a low pathogen dose (10^1-10^2 cfu/spot).

Overall, mature fruits from areas with high inoculum doses of *E. amylovora* could carry the pathogen regardless of their healthy appearance, being possible that these fruits could act as an unnoticed vehicle for the dissemination of this pathogen when insect vectors and susceptible host plants are present. This is also the first report on *C. capitata* as a potential insect vector for *E. amylovora* dissemination.

7. LITERATURE CITED

Alexandrova, M., Cimini, B., Bazzi, C., Carpana, E., Massi, S., and Sabatini, A.G. (2002) The role of honeybees in spreading *Erwinia amylovora*. Acta Horticulturae 590: 55-60.

Ark, P.A., and Thomas, H.E. (1936) Persistence of *Erwinia amylovora* in certain insects. Phytopathology 26: 375-381.

Azegami, K., Tsukamoto, T., Matsuura, T., Inoue, Y., Uematsu, H., Ohara, T., Mizuno, A., Yoshida, K., Bessho, H., Sato, S., Kimura, S. and Goto, M. (2006) *Erwinia amylovora* can pass through the abscission layer of fruit-bearing twigs and invade apple fruit during fruit maduration. Journal of General Plant Pathology 72: 43-45.

Behar, A., Jurkevitch, E. and Yuval, B. (2008) Bringing back the fruit into fruit fly-bacteria interactions. Molecular Ecology 17: 1375-1386.

Bellemann, P., Bereswill, S., Berger, S., and Geider, K. (1994) Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. International Journal of Biological Macromolecules 6: 290-296.

Biosca, E. G., Caruso, P., Bertolini E., Álvarez, B., Palomo, J. L., Gorris, M. T. and López, M. M. (2005) Improved detection of *Ralstonia solanacearum* in culturable and VBNC state from water samples at low temperatures, p. 501–506. *In* C. Allen, P. Prior, and A. C. Hayward (ed.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex. APS Press, St. Paul, Minnesota, USA.

Biosca, E.G., Marco-Noales, E., Ordax, M. and López, M.M. (2006) Long-term starvation-survival of *Erwinia amylovora* in natural water. Acta Horticulturae 704: 107-112.

Biosca, E.G., Santander, R.D., Ordax, M., Marco-Noales, E., Águila, B., Flores, A. and López, M.M. (2009) Survival of *Erwinia amylovora* in rain water at low temperatures, p. 88-91. *In* Current Research Topics in Applied Microbiology and Microbial Biotechnology. World Scientific Publishing Co. Pte. Ltd, Singapore.

Biosca, E.G., Santander, R.D., Ordax, M., Marco-Noales, E. and López, M.M. (2008) *Erwinia amylovora* survives in natural water. Acta Horticulturae 793: 83-87.

Cabrefiga, J., and Montesinos, E. (2005). Analysis of aggressiveness of *Erwinia amylovora* using disease–dose and time relationships. Phytopathology 95:1430-1437.

Cayol, J.P., Causse, R., Louis, C., and Barthes, J. (1994) Medfly *Ceratitis capitata* Wiedemann (Dipt., Trypetidae) as a rot vector in laboratory conditions. Journal of Applied Entomology 117: 338-343.

Ceroni, P., Minardi, P., Babini, V., Traversa, F., and Mazzucchi, U. (2004) Survival of *Erwinia amylovora* on pears and on fruit containers in cold storage and outdoors. EPPO Bulletin 34:109-115.

Coronado-González, P.A., Vijaysegaran, S. and Robinson, A.S. (2008) Functional morphology of the mouthparts of the adult Mediterranean fruti fly, *Ceratitis capitata*. Journal of Insect Science 8:73.

Copeland R.S., Wharton R.A., Luke Q. and De Meyer M. (2002) Indigenous hosts of *Ceratitis capitata* (Diptera:Tephritidae) in Kenya. Annals of the Entomological Society of America 95: 672-694.

Dueck, J. (1974) Survival of *Erwinia amylovora* in association with mature apple fruit. Canadian Journal of Plant Science 54: 349-351.

Duyck, P., David, P. and Quilici, S. (2004) .A review of relationships between interspecific competition and invasions in fruit flies (Diptera: Tephritidae). Ecological Entomology 29:511–520.

Emmett, B.J. and Baker, L.A.E. (1971) Insect transmission of fireblight. Plant Pathology 20: 41-45.

EPPO, European and Mediterranean Plant Protection Organization (1981) Data sheets on quarantine organisms No. 105, *Ceratitis capitata*. Bulletin OEPP/EPPO Bulletin 11 (1).

EPPO, European and Mediterranean Plant Protection Organization (2004) Diagnostic protocols for regulated pests: *Erwinia amylovora*. *In* EPPO Standards PM 7/20 (1) ed. López, M.M., Keck, M., Llop, P., Gorris, M.T., Peñalver, J., Donat, V. and Cambra, M. EPPO Bulletin 34:159-171.

Gorris, M.T., Cambra, M., Lecomte, P., Llop, P., Chartier, R., Paulin, J.P. and López, M.M. (1996) A sensitive and specific detection of *Erwinia amylovora* based on ELISA-DASI enrichment method with monoclonal antibodies. Acta Horticulturae 411: 41-46.

Guilford, P.J., Taylor, R.K., Clark, R.G., Hale, C.N. and Forster, R.L.S. (1996) PCR-based techniques for the detection of *Erwinia amylovora*. Acta Horticulturae 411: 53-56.

Hale, C.N., McRae, E.M. and Thomson, S.V. (1987) Occurrence of *Erwinia amylovora* on apple fruit in New Zealand. Acta Horticulturae 217: 33–40.

Hayward, A.C. (1974) Latent infections by bacteria. Annual Review of Phytopathology 12: 87-97.

Hildebrand, E.M. (1937) Infectivity of the fire blight organism. Phytopathology 95: 1430-1437.

Hildebrand, M., Dickler, E., and Geider, K. (2000) Ocurrence of *Erwinia amylovora* on insects in a fire blight orchard. Journal of Phytopathology 148: 251-256.

Hildebrand, M., Tebbe, C.C., and Geider, K. (2001) Survival studies with the fire blight pathogen *Erwinia amylovora* in soil and in a soil-inhabiting insect. Journal of Phytopathology 149: 635-639.

Ishimaru, C. and Klos, E.J. (1984) New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74: 1342-1345.

Janisiewicz, W. J., Conway, W. S., Brown, M. W., Sapers, G. M., Fratamico, P. and Buchanan, R. L. (1999) Fate of *Escherichia coli* O157:H7 on fresh cut apple tissue and its potential for transmission by fruit flies. Applied and Environmental Microbiology 65:1-5.

Kimura, S., Katsumata, H., Saito, S., Tsukamoto, T., Mizuno, A., Yoshida, K., Suyama, K., and Goto, M. (2005) World Trade Organization Japan—Measures affecting the importation of apples. Exhibit JPN-10.

King, E.O., Ward, M. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. Journal of Laboratory Clinical Medicine 44: 401-407.

Lelliot, R.A. (1967) The diagnosis of fire blight (*Erwinia amylovora*) and some diseases caused by *Pseudomonas syringae*. EPPO Bulletin 45: 27-34

Liao, C., and Shollenberger, L.M. (2004) Enumeration, resuscitation, and infectivity of the sublethally injured *Erwinia* cells induced by mild acid treatment. Phytopathology 94: 76-81.

Llop, P., Caruso, P., Cubero, J., Morente, C., and López, M.M. (1999). A simple extraction procedure for efficient routine detection of pathogenic bacteria in plant material by polymerase chain reaction. Journal of Microbiological Methods 37: 23-31.

Miller, T.D., and Schroth, M.N. (1972) Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62: 1175-1182.

Norelli, J.L., Jones, A.L. and Aldwinckle, H.S. (2003) Fire blight management in the twenty-first century. Plant Disease 87: 756-765.

Oliver, J.D. (1993) Formation of viable but nonculturable cells, p. 239-272. In S. Kjelleberg (ed.), Starvation in bacteria. Plenum Press, New York, USA.

Ordax, M. (2008) Supervivencia de *Erwinia amylovora* en condiciones de estrés: influencia de la presencia de cobre y la limitación de nutrientes. PhD thesis. Universidad Politécnica de Valencia, Spain.

Ordax, M., Biosca, E.G., López, M.M. and Marco-Noales, E. (2009a) The addition of copper sulphate to a non-selective medium improves the recovery of plant associated bacteria: *Erwinia amylovora* as a model, p. 92-96. *In* A. Mendez-Vilas (ed.), Current Research Topics in Applied Microbiology and Microbial Biotechnology. World Scientific Publishing Co. Pte. Ltd, Singapore.

Ordax, M., Biosca, E.G., Wimalajeewa, S.C., López, M.M. and Marco-Noales, E. (2009b) Survival of *Erwinia amylovora* in mature apple fruit calyces through the viable but non-culturable (VBNC) state. Journal of Applied Microbiology 107: 106-116.

Ordax, M., Marco-Noales, E., López, M.M. and Biosca, E.G. (2006) Survival strategy of *Erwinia amylovora* against copper: induction of the viable-but-nonculturable state. Applied and Environmental Microbiology 72: 3482-3488.

Purcell, A.H. (1982) Insect vector relationships with prokaryotic plant pathogens. Annual Review of Phytopathology 20: 97-417.

Roberts, R.G. and Sawyer, A.J. (2008) An updated pest risk assessment for spread of *Erwinia amylovora* and fire blight via commercial apple fruit. Crop Protection 27: 362-368.

Ruz, L., Moragrega, C., and Montesinos, E. (2008) Evaluation of four whole-plant inoculation methods to analyze the pathogenicity of *Erwinia amylovora* under quarantine conditions. International Microbiology 11: 111-119.

San Andres, V., Urbaneja, A., Sabater-Muñoz, B., and Castañera, P. (2007) A novel molecular approach to assess mating success of sterile *Ceratitis capitata* (Diptera: Tephritidae) males in sterile insect technique programs. Journal of Economic Entomology 100:1444-1449

Santander, R.D., Catalá-Senent, J., Marco-Noales, E., Biosca E.G. (2010a) *In vivo* recovery of *Erwinia amylovora* viable but non culturable cells in pear plantlets. 12th International Workshop on Fire Blight. Warsaw, Poland.

Santander, R.D., Marco-Noales, E., Ordax, M., Biosca E.G. (2010b) Colonization and migration abilities of *Erwinia amylovora* in host plants inoculated by irrigation, (in press). Microorganisms in industry and environment. From scientific and industrial research to consumer products. World Scientific Publishing Co. Pte. Ltd, Singapore.

Schroth, M.N., Thomson, S.V. and Hildebrand, D.C. 1974. Epidemiology and control of fire bligh. Annual Review of Phytopathology 12: 389-412.

Sela S., Nestel, D., Pinto, R., Nemny-Lavy, E., and Bar-Joseph, M. (2005) Mediterranean fruit fly as a potential vector of bacterial pathogens. Applied and Environmental Microbiology 71: 4052-4056.

Southey, R.F.W., and Harper, G.J. (1971) The survival of *Erwinia amylovora* in airborne particles: tests in the laboratory and in the open air. Journal of Applied Bacteriology 34: 547-556.

Taylor, R.K., Guilford, P.J., Clark, R.G., Hale, C.N. and Forster, R.L.S. (2001) Detection of *Erwinia amylovora* in plant material using novel polymerase chain reaction (PCR) primers. New Zealand Journal of Crop and Horticultural Science 29: 35-43.

Taylor, R.K. and Hale, C.N. (2003) Cold storage affects survival and growth of *Erwinia amylovora* on the calyx of apple. Letters in Applied Microbiology 37: 340-343.

Taylor, R.K., Hale, C.N., Gunson, F.A. and Marshall, J.W. (2003) Survival of the fire blight pathogen, *Erwinia amylovora*, in calyxes of apple fruit discarded in an orchard. Crop Protection 22: 603-608.

Taylor, R.K., Hale, C.N. and Marshall, J.W. (2002) The viability and persistence of *Erwinia amylovora* in apples discarded in an orchard environment. Acta Horticulturae 590: 153-155.

Temple, T.N., Stockwell, V.O., Lawrence Pusey, P. and Johnson, K.B. (2007) Evaluation of likelihood of co-occurrence of *Erwinia amylovora* with mature fruit of winter pear. Phytopathology 97: 1263-1273.

Thomas, M.C., Heppner, J.B., Wooddruff, R.E., Weems, H.V., Steck, G.J., and Fasulo, T.R. (2007) Mediterranean fruit fly *Ceratitis capitata* (Wiedemann). EENY-214 (IN371). Institute of Food and Agricultural Sciences, University of Florida, USA. http://creatures.ifas.ufl.edu

Thomson, S.V. (2000) Epidemiology of fire blight, p. 9-36. *In* J.L. Vanneste (ed.), Fire Blight: the disease and its causative agent, *Erwinia amylovora*. CABI Publishing, Wallingford, United Kingdom.

Tsukamoto, T., Azegami, K., Matsuura, T., Ohara, T., Inoue, Y., Mizuno, A., Yoshida, K., Bessho, H., Kimura, S. and Goto, M. (2005a) Infection frequency of mature apple fruit with *Erwinia amylovora* deposited on pedicels and its survival in the fruit stored at low temperature. Journal of General Plant Pathology 71: 296-301.

Tsukamoto, T., Uematsu, H., Mizuno, A., and Kimura, S. (2005b) Transmission of *Erwinia amylovora* from blighted mature apple fruit to host plants via flies. Research Bulletin of the Plant Protection Service Japan 41: 65-70.

Update Report 1, project AP07051. (2008) Attempts to detect *Erwinia amylovora* in calyces of symptomless mature apple fruits harvested from orchards naturally affected by fire blight.

van der Zwet, T., Thomson, S.V., Covey, R.P. and Bonn, W.G. (1990) Populations of *Erwinia amylovora* on external and internal apple fruit tissues. Plant Disease 74: 711–716.

White, I. M., and Helson-Harris, M. M. (1992). Fruit flies of economic significance: their identification and bionomics. ACIAR, CAB. Redwood Press, Londres. 601 pp.

Winfield, M.D., and Groisman, E.A. (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. Applied and Environmental Microbiology 69: 3687-3694.

World Trade Organization, WTO, Dispute Settlement Reports, http://onlinebookshop.wto.org

Yuval, B., and Hendrichs, J. (2000). Behavior of flies in the genus *Ceratitis*, p. 429-456. *In* M. Aluja and A. Norrbom (ed.), Fruit flies (Tephritidae): phylogeny and evolution of behavior. CRC Press, Boca Raton, Florida, USA.