Plant Pathology (2016) **65**, 462–469 Doi: 10.1111/ppa.12429

Real-time PCR detection of *Erwinia amylovora* on blossoms correlates with subsequent fire blight incidence

M. Hinze[ab], L. Köhl[a†], S. Kunz[ab], S. Weißhaupt[ab], M. Ernst[ac], A. Schmid[a] and R. T. Voegele[ad*]

[a]*Lehrstuhl Phytopathologie, Fachbereich Biologie, Universität Konstanz, Universitätsstr. 10, Konstanz 78457;* [b]*Bio-Protect GmbH, Lohnerhofstr. 7, Konstanz 78467;* [c]*Büro für Biologische-Ökologische Beratung, Dr. Michael Ernst Wissenschaftliche Dienstleistungen, Postfach 10 05 62, Konstanz 78405; and* [d]*Fachgebiet Phytopathologie, Institut für Phytomedizin, Fakultät Agrarwissenschaften, Universität Hohenheim, Otto-Sander-Straße 5, Stuttgart 70599, Germany*

Fire blight is the most devastating bacterial disease of rosaceous plants. Forecasting fire blight infections is important to allow for countermeasures that reduce economic damage in pome fruit production. Current computerized forecasting models are solely based on physical factors such as temperature and moisture, but not on the actual presence of the pathogen *Erwinia amylovora*. Although the inoculum concentration is considered to be crucial for infection and disease outbreak, most current approaches used for identification of fire blight inoculum including morphological, biochemical, serological, and DNA-based methods are nonquantitative. Based on a real-time PCR approach previously published, an improved protocol to be used directly on whole bacteria in the field is described. The method allows for early detection and quantification of the pathogen prior to the occurrence of first symptoms. There is a clear correlation between bacterial abundance and subsequent disease development. However, in some cases, no disease symptoms could be observed despite a pathogen load of up to $3{\cdot}4 \times 10^6$ cells per blossom. Integration of the amount of pathogen detected into refined prediction algorithms may allow for the improvement of applied forecasting models, finally permitting a better abatement of fire blight.

Keywords: absolute quantification, CDH18, epidemiology, forecasting models, monitoring, pEA29

Introduction

Fire blight, caused by infections with the Gram-negative bacterium *Erwinia amylovora*, is the most devastating bacterial disease of rosaceous plants, with great economic importance, especially in apple and pear production (van der Zwet *et al.*, 2012). The disease was first observed in North America around 1780. It spread to Europe (UK) in 1957 and reached Germany in 1971. Baden–Württemberg in southern Germany was reached in 1981, and in the following years the pathogen spread to Germany's second largest pome fruit growing area, the Lake Constance region, and neighbouring countries Switzerland (in 1989) and Austria (in 1993).

The disease causes die-back symptoms, from brown to black colouration of twigs, flowers and leaves, and the typical 'shepherd's crook' of infected shoots, having a burned appearance, up to the death of complete trees (Norelli *et al.*, 2003). The main entry points for infection are open blossoms (Bubán *et al.*, 2003). These are colonized by bacteria transferred from primary inoculum sources by insect vectors or rain splash (Thomson, 2000). *Erwinia amylovora* multiplies epiphytically on stigmas (Thomson, 1986). Subsequently a downward movement to the hypanthium occurs, where the bacteria multiply further if high humidity is present (rain or dew), diluting the nectar to favourable sugar concentrations (Hasler & Mamming, 2002). Directed by chemotactic signals the pathogen swims to the nectarthodes (Raymundo & Ries, 1980), and finally invades the plant tissue through the nectarthodes in the hypanthium, causing infection of the plant (Bubán *et al.*, 2003).

Forecasting infection conditions for *E. amylovora* is crucial to allow for timely countermeasures to reduce the economic damage in commercial orchards (Moltmann, 2004), simultaneously keeping treatments with chemical or biological agents as low as possible. Reduction or avoidance of treatments is necessary because development of resistance against the antibiotic streptomycin has been widely recognized (Miller & Schroth, 1972). Excessive treatment with antagonistic yeasts such as *Aureobasidium pullulans*, on the other hand, may lead to

*E-mail: ralf.voegele@uni-hohenheim.de
†Present address: Plant–soil Interactions, Institute for Sustainability Sciences, Agroscope, Reckenholzstrasse 191, Zürich 8046, Switzerland

Published online 29 July 2015

Plant Pathology

enhanced russeting of fruits, depending on the individual apple cultivar (Gildemacher *et al.*, 2006). Because all strategies of chemical or biological control require an optimized application in the correct period of time (Psallidas *et al.*, 2000), forecasting models are essential to permit successful disease abatement.

Important tools to predict infection conditions include computerized forecasting systems such as Maryblyt (Steiner, 1990), Billing's Integrated System 95 (BIS95) (Billing, 1996), or Cougarblight (Smith, 1999). All these models consider physical factors such as temperature and/or moisture/wetness. Maryblyt, for example, considers the CDH18 value (CDH = critical degree hours, i.e. the accumulated sum of hours >18·3°C), with a critical level of 110, and the presence of moisture, as an indication of fire blight infection conditions during the blooming period, i.e. during the presence of open blossoms required for infection.

Although the abundance of sufficient bacterial inoculum is known to be crucial for successful infection (Schroth *et al.*, 1974), the actual presence of the pathogen as a prerequisite for infection is not taken into account by any of the prediction models described above. Additionally, all currently used approaches for the identification of *E. amylovora*, including morphological, biochemical, serological (ELISA), and DNA-based methodologies (PCR, nested-PCR; Svircev *et al.*, 2009), do not allow a quantification of the pathogen. Moreover, most of these methods are fairly time-consuming. Besides phytosanitary measures such as pruning of tissue with symptoms or clearing of infected trees to reduce the infection pressure, and forecasting models based on physical factors, an important measure to control fire blight seems to be the fast and specific quantitative detection of the pathogen. Real-time PCR is a fast and sensitive method that allows quantification of a pathogen within a few hours (Higuchi *et al.*, 1993). However, so far, real-time PCR assays have mostly focused on the analysis of pure DNA extracted from a variety of samples. Based on the real-time PCR approach described by Salm & Geider (2004), the present authors have improved the method to be used directly on whole bacteria in the field on a variety of different samples (Weißhaupt *et al.*, 2015). The present study focuses on the early detection and quantification of fire blight bacteria on apple and pear blossoms in the area around Lake Constance in the northern alpine forelands of southern Germany, Switzerland and Austria, a region severely afflicted by fire blight (Moltmann, 2005). The epidemiology of this pathogen prior to its manifestation and the correlation with CDH18 value, as given by the forecasting model Maryblyt (Steiner, 1990), are investigated. Furthermore, the positive correlation of pathogen abundance and symptom development later in the growing season is tested. The overall aim of this investigation is to improve existing computer-based forecasting models, which rely solely on measuring physical factors, by adding the very important biological component.

Materials and methods

Bacterial strains and cultures

Erwinia amylovora reference strains Ea385 and Ea610 were provided by Dr Esther Moltmann, Landwirtschaftliches Technologiezentrum Augustenberg, Außenstelle Stuttgart, Germany. Reference strain Ea1430 was provided by Professor Emilio Montesinos, University of Girona, Spain.

Sampling locations

Plant material was sampled at 37 different locations, represented by commercial orchards including eight high-standard tree cultures and two experimental (e) orchards in the area around Lake Constance, during 2006 and 2010. The orchards comprised apple (29 orchards), pear (20), quince (2) and hawthorn (1) trees. Orchard locations were spatially separated by distances from 0·3 km to more than 60 km. Orchards Höchst I (e), II, III, and IV, and Koblach are located in Vorarlberg, Austria. Lindau I and II are located in Bavaria, Germany. The remaining orchards, Altenbeueren I and II, Bodman I, III, V, VI, VII and VIII, Deggenhausertal I and II, Dingelsdorf, Hepbach I and II, Hilzingen, Höri, Kaltbrunn, Leimbach I, II, and III, Litzelstetten, Ludwigshafen, Mainau, Stadel I and II, Stahringen I and II, Steißlingen, Wahlwies, Wellmutsweiler (e), Wendlingen, and Reute, are located in Baden-Württemberg, Germany.

Sample collection and preparation

Blossoms were collected and incubated with 2 mL sterile Millipore water per blossom in either 13 mL tubes (Sarstedt AG & Co.) for single blossoms, or 720 mL Whirl-Pak bags (Carl Roth GmbH) for multiple blossoms (50–100). Samples were incubated on an orbital shaker for 15 min at 150 rpm. From each sample, a 1 mL aliquot was removed and bacteria were collected by centrifugation for 5 min at 15 800 ***g*** at room temperature. The supernatant was discarded and pellets were resuspended in an equal volume of Millipore water. Samples were either analysed directly or stored at −20°C.

Real-time PCR analysis

Real-time PCR analyses were performed using a modified protocol derived from Salm & Geider (2004) using primers p29TF and p29TR as previously described (Voegele *et al.*, 2010) yielding a 112 bp amplicon. Real-time PCR analyses were performed either on a portable SmartCyclerII (PEQLAB Biotechnologie GmbH) with SmartCycler software v. 2.0d (Cepheid) using the QuantiTect SYBR Green PCR Kit (QIAGEN) (samples collected in 2006) or the QuantiFast SYBR Green PCR Kit (QIAGEN) (2007–10 samples), respectively, and a threshold set to 30 rfu (relative fluorescence units) manually. Alternatively, a Mastercycler ep realplex 2 (Eppendorf AG) with ep realplex 2 software v. 2.0.0.50 (Eppendorf AG) with a threshold set to 350 rfu (2008 samples), or a CFX96 Real-time PCR detection system (Bio-Rad) with a threshold set to 250 rfu (2009 and 2010 samples) were used. The reaction volume was 25 μL, containing a final concentration of 0·5 μM of each primer, with a maximum sample volume of 10 μL. Samples consisted of washing fluids containing intact bacteria. No DNA extraction was performed prior to PCR analysis. The PCR protocol consisted of an initial denaturation step of 5 min at 95°C; followed by 40 cycles of

denaturation at 94°C for 20 s, annealing at 52°C for 20 s, and extension at 72°C for 20 s (QuantiTect SYBR Green PCR kit), or 40 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s (QuantiFast SYBR Green PCR kit). A melt analysis followed each PCR run to verify identity and homogeneity of the amplicon (Valasek & Repa, 2005). The melting point of the amplicon was 84 ± 0·5°C. Absolute quantification of bacteria in plant samples was performed by standardization with respect to serial decimal dilutions of washed pure cultures of *E. amylovora* strains Ea385, Ea610 and Ea1430. At least four serial dilutions of at least one strain, always including Ea385, were combined for the calculation of each standard curve. Great care was taken that standard curves produced on the different PCR machines and using different chemistries were congruent. Concentrations of the bacterial cell suspensions used for the standard curves were determined by correlation with the optical density (OD) at 600 nm, given by the equation [cells $mL^{-1} = OD_{600} \times 3{\cdot}3 \times 10^9$]. PCR efficiency ($E$) was determined by the equation $E = 10^{(-1/\text{slope})}$ (Wilkening & Bader, 2004). The equation used for quantification of bacteria was [bacterial cells/10 μL sample volume = $10^{(-0{\cdot}303 \times \text{Ct-value} + 11{\cdot}9204)}$] ($C_t$ = threshold cycle). The lower detection limit was one single cell per PCR reaction, i.e. a suspension with 10^2 bacterial cells mL^{-1}. The lower limit for reliable quantification was determined as 10 cells per reaction, i.e. a suspension with 10^3 cells mL^{-1}, and the upper limit was 10^5 cells per reaction, i.e. a suspension with 10^7 cells mL^{-1}. Dilutions of more highly concentrated samples were made to ensure correct quantification.

Evaluation of fire blight symptoms

To correlate *E. amylovora* detection with subsequent disease development in an orchard the severity of observed fire blight incidences was graded as follows: grade 0, no symptoms visible; grade 1, single blossom clusters with symptoms in an orchard on <10% of trees; grade 2, ≥10% of all trees in an orchard showing symptoms; grade 3, symptoms appearing on every tree in an orchard; grade 4, multiple blossom clusters with symptoms on every tree.

Data analysis

The values of population sizes of *E. amylovora* determined by real-time PCR on or within a plant organ were $\log_{10}$ transformed, prior to calculation of means and statistical analysis. For figures with a logarithmic scale and for field data analysis, the counts from samples detected to be negative were manually defined as value zero. Average cell counts given in the text have been transformed back from means of replicate logarithmic values.

Results

Properties of a modified real-time PCR system for *E. amylovora* detection

The real-time PCR protocol described by Salm & Geider (2004) has been refined for *E. amylovora* detection in a variety of samples without prior DNA extraction (Voegele *et al.*, 2010). The modified protocol was successfully applied to blossoms from different host plants, i.e. *Malus domestica*, *Pyrus communis*, *Cydonia oblongata* and *Crataegus* sp., for blossoms of various non-host plant species (Weißhaupt *et al.*, 2015), bark (not shown), cankers (not shown), buds and fruit mummies (Weißhaupt *et al.*, 2015).

The standards used for absolute quantification yielded an equation of [$C_t = -3{\cdot}2177 \times \log_{10}$ (cells/reaction) + 38·976] (C_t = threshold cycle), with a coefficient of determination $R^2 = 0{\cdot}98$. The obtained coefficient of determination underlines the suitability of the standard curve for quantification purposes, especially considering that data were obtained on different PCR machines as well as using different chemistries. Based on the standard curve, the PCR efficiency of the system was determined to be 2·05. Thus, the standard curve described above indicated optimal amplification efficiency of this real-time PCR protocol. No inhibition of PCR by plant exudates or other components in water washed from blossoms (for screening for epiphytic bacteria) was observed (data not shown).

Frequency of *E. amylovora* in single blossoms

To determine the frequency of *E. amylovora* colonization in blossoms, single apple blossoms from two orchards in the Lake Constance region were analysed by real-time PCR in May 2008. In the orchard Höchst I, 41 of 50 blossoms (82%) of the cultivar Summerred tested positive, with cell counts ranging from $1{\cdot}2 \times 10^3$ to $8{\cdot}0 \times 10^7$ cells per blossom. Sixteen days later, 2·3% of the blossom clusters showed symptoms. In the orchard Wahlwies two of 48 blossoms (4·2%) of the cultivar Boskop tested positive, with cell counts ranging from $6{\cdot}0 \times 10^3$ to $3{\cdot}9 \times 10^4$ cells per blossom. At this site only sporadic fire blight incidents occurred, classified as grade 1. These results confirmed the hypothesis that the analysis of a small number of samples, consisting of 100 pooled blossoms, would be sufficient to obtain reliable data on the presence of *E. amylovora* in an orchard.

Correlation of *E. amylovora* abundance in blossoms and subsequent fire blight incidence

To examine whether the level of blossom colonization by *E. amylovora* correlated with the subsequent degree of fire blight incidence, 37 different orchards without any treatment against *E. amylovora* were examined, from 2006 to 2010, using real-time PCR analysis. This resulted in 95 year × orchard combinations (51 apple; 40 pear; 4 others, i.e. quince/hawthorn), defined as 'cases'. In 29 cases (7 apple; 20 pear; 2 others) (30·5%) *E. amylovora* was detected with maximum amounts of up to $3{\cdot}4 \times 10^6$ cells per blossom, but no symptoms occurred (Fig. 1). In the case of pear blossoms, the samples that tested positive could not be correlated to any symptom development. However, it should be noted that, during pear bloom in these sampling years, Maryblyt (Steiner, 1990) did not calculate that conditions were suitable for infection in this region. In 12 cases (11 apple; 0 pear; 1 other) (12·6%) *E. amylovora* was detected with up to $5{\cdot}2 \times 10^7$ cells per blossom, resulting in development of symptoms with severity generally

increasing with cell count. Seven of these fire blight incidents were classified as grade 1, two cases as grade 2, and three cases as the most serious grade, 4. In 54 cases (33 apple; 20 pear; 1 other) (56·8%) *E. amylovora* was not detected and no symptoms occurred, i.e. incidents were classified as grade 0 (Fig. 1). Below a cell number of approximately 1000 bacteria per blossom, no, or only sporadic, damage occurred (Fig. 1). This indicates a putative threshold, i.e. a specific amount of pathogen necessary for symptom development.

Sixteen further orchards were treated with either streptomycin or Blossom Protect, both known for high efficiency against *E. amylovora* (Kunz *et al.*, 2011). These were also examined from 2006 to 2010, resulting in 27 year × orchard combinations (25 apple, 2 pear). In 11 cases, apple blossoms tested positive without any subsequent fire blight incidents. Two further cases resulted in symptoms classified as grade 1 or 2, respectively. In a third case, a fire blight incident of grade 1 was observed, without prior detection in apple blossoms. In the 5-year-study, the latter was the only case of a fire blight incident without any previous detection of *E. amylovora* by real-time PCR.

Annual and spatial variation of *E. amylovora* abundance in correlation with CDH18

To examine at which time in the course of bloom *E. amylovora* is present, a more detailed analysis was performed on four of the 12 cases of non-treated orchards that developed symptoms after testing positive for *E. amylovora* (Fig. 1, open diamonds), representing different grades of infestation, and one of the 27 cases of treated orchards (grade 2; Figs 2 & 3). The analysis focused on the variation of bacterial abundance in blossoms and putative infection conditions given as CDH18 by the forecasting model Maryblyt (Steiner, 1990). In all five cases, local CDH18 reached values above 170 in the course of bloom, and thus clearly exceeded the critical limit for infection of 110. In 2008, variation in pathogen abundance in a spatial context was examined in three orchards, representing three different locations, one being far-distant (Stahringen I) and the other two in close proximity (Höchst I and II). In the orchard Stahringen I, *E. amylovora* was detected on the cultivar Gala at a level of $1{\cdot}8 \times 10^4$ cells per blossom, three days before infection conditions were recorded (Fig. 2a). Two sam-

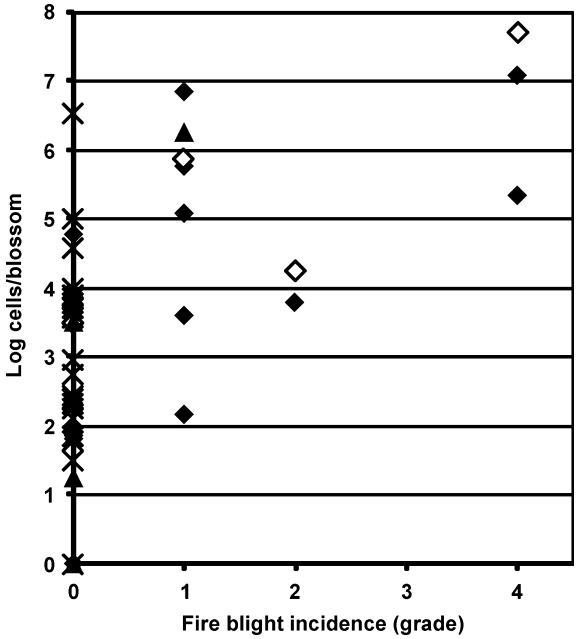

Figure 1 Correlation of *Erwinia amylovora* abundance in blossoms and subsequent fire blight incidence. Summary of 95 cases (51 apple, 40 pear and 4 others, i.e. quince/hawthorn) in 37 different orchards in the area around Lake Constance from 2006 to 2010. The ordinate values indicate the maximum cell count per blossom determined by real-time PCR. ♦ apple blossoms, ◊apple blossoms (selected cases described in more detail in the section 'Annual and spatial variation of *E. amylovora* abundance in correlation with CDH18'), × pear blossoms, ▲ others (quince/hawthorn).

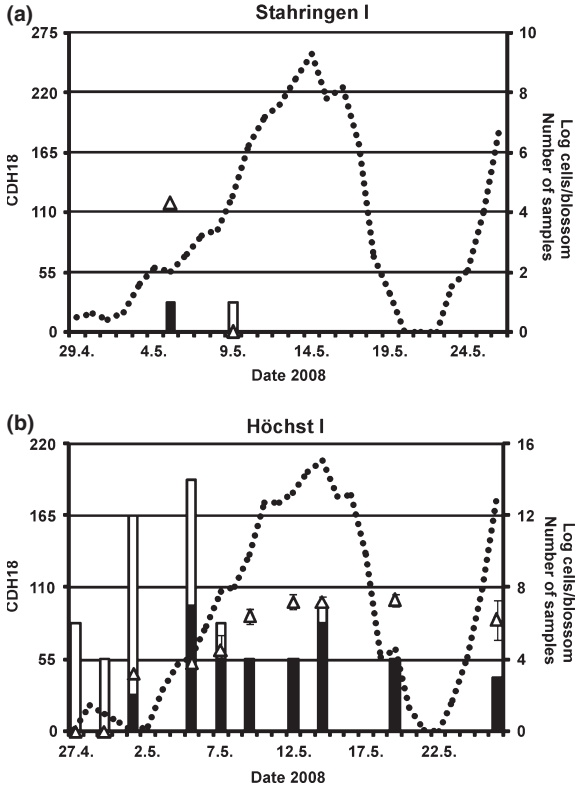

Figure 2 Seasonal variation of *Erwinia amylovora* abundance in apple blossoms in the area of Lake Constance and its correlation with CDH18 values (CDH = critical degree hours, i.e. the accumulated sum of hours >18·3°C). (a): orchard Stahringen I, (b): orchard Höchst I. ••• CDH18, △ average cell count per blossom in samples testing positive, ■ number of samples tested positive, □ total number of samples. Bars indicate standard deviations.

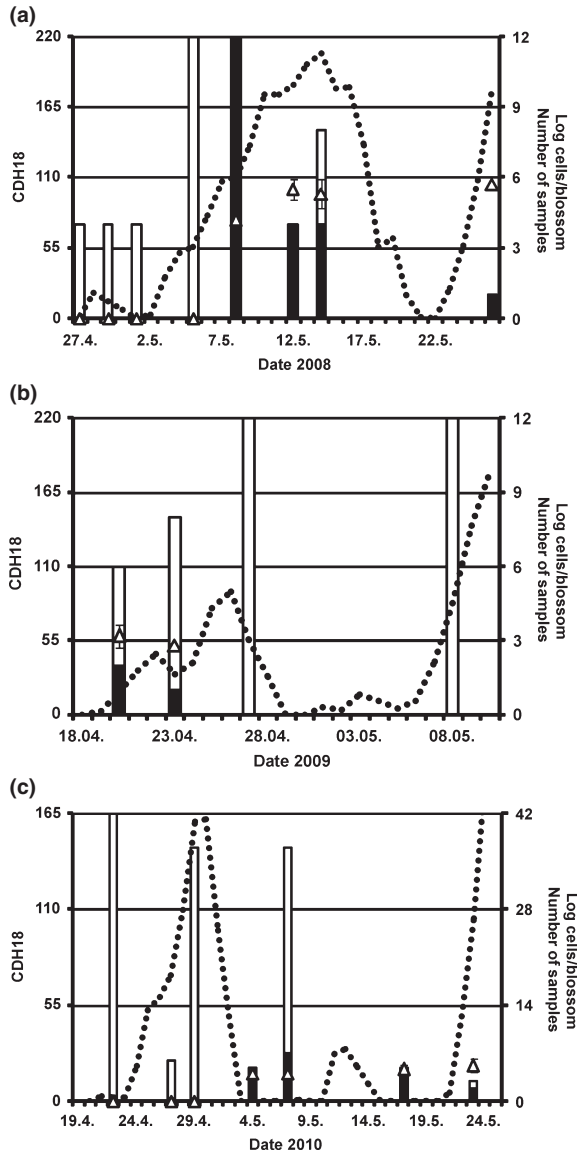

Figure 3 Seasonal variation of *Erwinia amylovora* abundance in apple blossoms in the orchard Höchst II in 2008–10, and its correlation with CDH18 (CDH = critical degree hours, i.e. the accumulated sum of hours >18·3°C). ••• CDH18, △ average cell count per blossom in samples testing positive, ■ number of samples tested positive, □ total number of samples. Bars indicate standard deviations.

ples – only one of them testing positive – were sufficient to predict a fire blight incident, which was subsequently classified as grade 2 (Fig. 1). The orchard Höchst I was classified with symptoms of grade 4 (Fig. 1). In this orchard the amount of bacteria detected rose with increasing CDH18 values and the first detection occurred 7 days before the CDH18 reached 110 (Fig. 2b). Bacterial abundance reached a maximum of $5{\cdot}2 \times 10^7$ cells per blossom (Fig. 1), and the highest average value recorded was 2×10^7 cells per blossom (Fig. 2b). In the orchard Höchst II in 2008 *E. amylovora* could be detected at the day infection conditions were fulfilled (Fig. 3a). Despite treatment with streptomycin on 11 May, bacterial abundance reached up to 5×10^5 cells per blossom, leading to infections of grade 2.

Despite a distance of more than 60 km between Stahringen I and the two Höchst orchards, the three cases analysed in 2008 show a largely similar CDH18 profile, with all maximum values exceeding 200. In Stahringen I, a generally lower bacterial abundance than in the Höchst orchards was observed, while Höchst I and II, separated by a distance of only 1 km, showed appearance of *E. amylovora* at different times, i.e. long before (Höchst I; Fig. 2b), or on the day infection conditions occurred (Höchst II; Fig. 3a). The Höchst II orchard was also analysed in the years 2009 and 2010.

Several-year seasonal variation of *E. amylovora* abundance and correlation with CDH18

To investigate whether differences in the appearance of *E. amylovora* in a given location might exist in the context of a longer period of time, one single orchard, Höchst II, was analysed in the years 2008 to 2010. In 2008, this orchard was treated once with streptomycin on 11 May. Despite this treatment, subsequent bacterial abundance was at a high level, with a maximum of up to 5×10^5 cells per blossom, leading to infections of grade 2. Despite multiple sampling, *E. amylovora* could be detected on the day infection conditions were fulfilled, but then persisted permanently at a high level of at least 2×10^5 cells per blossom (Fig. 3a). In 2009, *E. amylovora* was detected with a maximum average of only $1{\cdot}5 \times 10^3$ cells per blossom; no treatment was carried out and no fire blight incident occurred (grade 0). The pathogen was detected 18 days before the CDH18 exceeded the critical limit of 110, at the end of the blooming period (9 May to 10 May), but was no longer detected when this critical limit was reached (Fig. 3b). In 2010, *E. amylovora* was not detected at the beginning of bloom, before or during heavy infection conditions (29 April to 2 May). No treatments were carried out. However, after this initial period of infection conditions, a permanent level of *E. amylovora*, with an average of at least $1{\cdot}2 \times 10^4$ cells per blossom, was detected (Fig. 3c). This level persisted in an intermediate phase with very low CDH18 values. At the end of the blooming period another period with infection conditions occurred. However, only some late blossoms were affected, resulting in infections classified as grade 1.

Discussion

Despite the fact that the importance of inoculum dose for development of fire blight symptoms has been known for years (Schroth *et al.*, 1974), tools for detection of *E. amylovora* have been restricted to nonquantitative methods. These comprise morphological, biochemical, serological (ELISA), and DNA-based methods (i.e. PCR, nested-PCR; Svircev *et al.*, 2009). Some of them are suit-

able for rapid on-site tests such as the Agristrip (BIOREBA AG), an antibody-based test.

To overcome the limitations for quantification and simultaneously allow for on-site examination and large-scale monitoring for the presence of *E. amylovora*, the authors have improved the real-time PCR approach introduced by Salm & Geider (2004); the procedure now allows the use of whole bacteria without prior sample processing (Voegele *et al.*, 2010) on a variety of different plant tissues including non-host plants (Weißhaupt *et al.*, 2015). Using a mobile laboratory with a portable SmartCycler II system, the method allows a quantitative screening of *E. amylovora* and on-site analysis of samples in less than 2 h directly in the field (Voegele *et al.*, 2010).

In these and the present studies (Weißhaupt *et al.*, 2015), an 'absolute' quantification by means of standard curves (Larionov *et al.*, 2005) has been used, yielding correlations with an R^2 value of 0·98. In previous studies (Larionov *et al.*, 2005) a value of at least 0·99 was considered necessary; however, these studies referred to triplicate single serial dilutions of DNA, with lower dilution factors, in contrast to the investigations of the current authors in which whole bacteria, decimal dilution steps and a combination of four to seven serial dilutions were used to produce one standard curve. Thus, the lower R^2 values can be considered sufficient for reliable quantification.

The detection limit of this real-time PCR approach was 10^2 bacterial cells mL^{-1}, which is comparable with, or even superior to, other systems where detection limits range from 10^2 to 5×10^4 cells mL^{-1} (Salm & Geider, 2004; Gottsberger, 2010). The assay of Salm & Geider (2004), origin of the present refined protocol, also allows for quantification, but is less sensitive, with a limit of 5×10^4 cells mL^{-1}. The serological ELISA-DASI (Gorris *et al.*, 1996) has a detection limit of 10–100 cells mL^{-1}, but is nonquantitative. Thus, the nested PCR by Llop *et al.* (2000) remains the most sensitive detection method, with a lower limit of less than one single cell mL^{-1}. However, it consists of a highly complicated two-step system without the possibility for quantification.

The target sequence used for the PCR approach of Salm & Geider (2004), and also employed in the present studies, is located on plasmid pEA29, which is almost ubiquitous in *E. amylovora* strains (Laurent *et al.*, 1989). This plasmid is considered to be essential for pathogenesis (Falkenstein *et al.*, 1989) and is, therefore, a target commonly used for the detection of *E. amylovora* (Llop *et al.*, 2000; Salm & Geider, 2004). However, some pathogenic *E. amylovora* strains that do not carry this plasmid are known to exist (Mohammadi *et al.*, 2009). Such strains would only be detected by PCR assays targeting *E. amylovora* chromosomal DNA (Gottsberger, 2010). However, except for the real-time PCR assay of Gottsberger (2010), these assays are nonquantitative. The method of Gottsberger (2010) is based on relative quantification and consists of a more complex protocol including DNA extraction with phenol-chloroform-isoamyl alcohol or boiling in phosphate buffer and subsequent shock-freezing. This is in contrast to the pEA29-based approach of the present study, which uses whole bacteria without sample processing; this remains the sole PCR assay that combines high sensitivity and the capability for quantification and fast analysis. Therefore, it can be considered suitable for practical application, e.g. large-scale monitoring, providing rapid decision aids and opening up new possibilities for fire blight management. The total number of *E. amylovora* strains without plasmid pEA29 amounts to at most seven globally. Generally, such strains are considered as rare (Mohammadi *et al.*, 2009); in southern Germany one of 123 strains (0·8%) was detected that lacked plasmid pEA29 (Mohammadi *et al.*, 2009). Taken together, in the area around Lake Constance in southern Germany and Austria, where the present studies were carried out, these strains represent a negligible minority, relatively unimportant in comparison to the advantages of this new rapid monitoring system based on plasmid pEA29.

Like all molecular assays that are cultivation-independent, this real-time PCR approach cannot distinguish between living, dormant and dead bacterial cells. This problem can be addressed using an ethidium monoazide (EMA) PCR method. This procedure excludes free DNA or that of dead cells from amplification (Rudi *et al.*, 2005). However, the EMA-PCR method has not yet been successfully adapted for the real-time PCR procedure described here (S. Weißhaupt, unpublished data).

To develop an adequate and efficient sampling strategy for large-scale monitoring of *E. amylovora*, the frequency of *E. amylovora* colonization in single apple blossoms was detected and the amount of samples necessary to obtain reliable data for practical prognosis was determined. The results indicate that two samples (Fig. 2a), each consisting of 100 pooled blossoms, are sufficient to obtain reliable data on the presence of *E. amylovora* in an orchard and to predict fire blight incidents. In this study there was only one case of a fire blight incident without previous detection of *E. amylovora*, representing 0·8% of all cases. Symptoms in this case were detected on shoots not on blossom clusters, indicating that symptoms were not caused by blossom infections and that the pathogen was not present during bloom. Alternatively, this result may be due to the presence of a strain lacking plasmid pEA29.

Comparison of *E. amylovora* abundance in apple blossoms and fire blight incidence showed a good correlation between cell counts and symptom development. However, the results also show that epiphytic colonization of blossoms, even with high cell counts, is possible without later symptom development. This seems especially true for pear, and is in accordance with the fact that no infection conditions occurred during pear bloom in this region during the present study. Below a maximum cell count of 1000 bacterial cells per blossom no, or only sporadic, damage may occur (see also Kunz *et al.*, 2012), i.e. this amount of pathogen seems to be necessary for later symptom development. Thus, this indicates a puta-

tive threshold, which has relevance for fire blight prognosis in non-treated orchards and also confirms earlier findings on the importance of the level of inoculum for an outbreak of fire blight (Schroth *et al.*, 1974). In previous studies, significant fire blight incidences, higher than grade 1, were observed only after the pathogen was present at amounts of more than 5000 cells per blossom (Kunz *et al.*, 2012). In the present investigation, only one case below the 1000-cell-limit was found, compared with 11 cases above. It would be highly desirable to have more data for consolidation of a universally valid threshold. It should be stressed that only cell counts detected before a CDH18 of 110 (the critical threshold of conditions for infection) were taken into account in the present study. In the case of rapid multiplication of bacteria under optimal conditions, a cell count of 1000 bacteria per blossom may easily rise quickly to 5000 and thus provide sufficient inoculum for later severe infestations of grades higher than 1. In laboratory experiments with apple blossoms (Weißhaupt *et al.*, 2015) a propagation rate of about 0·15 per hour, i.e. a generation time of about 6·7 h was observed. In orchards with treatment against fire blight, either using streptomycin or the antagonistic yeast-based Blossom Protect, a pattern similar to that in the non-treated orchards was observed. While these fire blight incidents were less severe (grade 1 and 2 instead of grades up to 4), due to the high efficacy known for these agents, their frequency was almost identical to that of non-treated ones with three incidents in 27 cases (11·1% treated versus 12·6% non-treated).

The real-time PCR assay used in the present investigation also permitted a detailed analysis of seasonal variations in the course of blooming. Thus, the inoculum pressure was determined in relation to the CDH18 value calculated by Maryblyt (Steiner, 1990), revealing the dynamics of the bacterial population on apple blossoms in correlation with weather conditions. The presence and quantity of *E. amylovora* prior to disease manifestation and long before the CDH18 value reached the critical mark of 110 were shown. In 2008, despite CDH18 curves being very similar between orchards, striking spatial differences in bacterial population development were observed. This may have been a result of different apple varieties or differences in the percentage of open blossoms and blossom age, but perhaps also a result of different types and amounts of inoculum available (Weißhaupt *et al.*, 2015). Examination of a selected orchard known to be infested with fire blight over a period of several years revealed differences in the time of appearance of bacteria prior to, or even only after, the CDH18 value exceeded 110, with successive variation in symptom development and disease severity. These observations were probably due to differences in the percentage of open blossoms, as previously described for another orchard (Voegele *et al.*, 2010).

Available models for the forecasting of fire blight, such as Maryblyt (Steiner, 1990), Billing's Integrated System 95 (BIS95) (Billing, 1996), or Cougarblight (Smith, 1999) consider physical factors such as temperature and/or moisture/wetness. However, the presence of the pathogen at a sufficient quantity (Schroth *et al.*, 1974) as a prerequisite for infection is not taken into account. Monitoring of *E. amylovora* during bloom, in combination with the presumed threshold abundance of 1000 cells per blossom, could give additional input to the decision process of when, and how often, control agents should be applied (Kunz *et al.*, 2012). As long as the pathogen is not detectable by real-time PCR, the application of control agents can be postponed (Kunz *et al.*, 2012), and detectable cell counts below the threshold do not indicate countermeasures. This is true even in the case of higher cell counts if physical data indicate conditions unfavourable for *E. amylovora* infection (Kunz *et al.*, 2012). Thus, the forecasting models may now be improved by an additional biological component. An integration of the quantification of pathogen abundance into the Maryblyt forecasting system is in progress.

Taken together, the improved real-time PCR approach, with its combination of a fast and simultaneously quantitative method, has proven over a period of several years to be a practicable and reliable method for large-scale monitoring of *E. amylovora*. The insights into the dynamics of bacterial blossom colonization and multiplication during the course of bloom give additional input for fire blight prognosis and the integration of this biological component as an improvement of physically based forecasting models finally opens up new vistas for a better abatement of the devastating plant disease fire blight.

Acknowledgements

The authors are grateful to Professor K. Mendgen for support and scientific discussions. This work was supported by grants provided by the Bundesanstalt für Landwirtschaft und Ernährung (04HS008 and 06HS037) and the European Union within the Interreg IV project 'Gemeinsam gegen Feuerbrand' to R.T.V. The authors also thank Dr E. Moltmann, LTZ Augustenberg, Germany, and Professor E. Montesinos, University of Girona, Spain, for providing *E. amylovora* reference strains.

References

Billing E, 1996. BIS95, an improved approach to fire blight risk assessment. *Acta Horticulturae* **411**, 121–6.

Bubán T, Orosz-Kovács Z, Farkas A, 2003. The nectary as the primary site of infection by *Erwinia amylovora* (Burr.) Winslow *et al.*: a mini review. *Plant Systematics and Evolution* **238**, 183–94.

Falkenstein H, Zeller W, Geider K, 1989. The 29 kb plasmid, common in strains of *Erwinia amylovora*, modulates development of fireblight symptoms. *Journal of General Microbiology* **135**, 2643–50.

Gildemacher P, Heijne B, Silvestri M *et al.*, 2006. Interactions between yeasts, fungicides and apple fruit russeting. *FEMS Yeast Research* **6**, 1149–56.

Gorris MT, Cambra M, Llop P *et al.*, 1996. A sensitive and specific detection of *Erwinia amylovora* based on the ELISA-DASI enrichment method with monoclonal antibodies. *Acta Horticulturae* **411**, 41–6.

Gottsberger RA, 2010. Development and evaluation of a real-time PCR assay targeting chromosomal DNA of *Erwinia amylovora*. *Letters in Applied Microbiology* **51**, 285–92.

Hasler T, Mamming L, 2002. Population dynamics of *Erwinia amylovora* on different blossom elements of pear and apple. *Acta Horticulturae* **590**, 181–4.

Higuchi R, Fockler C, Dollinger G, Watson R, 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology* **11**, 1026–30.

Kunz S, Schmitt A, Haug P, 2011. Development of strategies for fire blight control in organic fruit growing. *Acta Horticulturae* **896**, 431–6.

Kunz S, Schmitt A, Haug P, 2012. Summary of an eight year research project on fire blight control. In: Fördergemeinschaft Ökologischer Obstbau e.V. (FÖKO), ed. *15th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing*. Filderstadt, Germany: Vervielfältigungen F. u. T. Mueller-Bader, 146–52.

Larionov A, Krause A, Miller W, 2005. A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **6**, 62.

Laurent J, Barny M-A, Kotoujansky A, Dufriche P, Vanneste JL, 1989. Characterization of a ubiquitous plasmid in *Erwinia amylovora*. *Molecular Plant–Microbe Interactions* **2**, 160–4.

Llop P, Bonaterra A, Peñalver J, López MM, 2000. Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Applied and Environmental Microbiology* **66**, 2071–8.

Miller T, Schroth M, 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. *Phytopathology* **62**, 1175–82.

Mohammadi M, Moltmann E, Zeller W, Geider K, 2009. Characterisation of naturally occurring *Erwinia amylovora* strains lacking the common plasmid pEA29 and their detection with real-time PCR. *European Journal of Plant Pathology* **124**, 293–302.

Moltmann E, 2004. 10 Jahre Erfahrungen mit der Feuerbrandprognose in Baden-Württemberg (1994–2003). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **56**, 13–6.

Moltmann E 2005. Feuerbrandbekämpfung in Baden-Württemberg. [https://www.landwirtschaft-bw.info/pb/site/lel/get/documents/MLR.LEL/PB5Documents/recht/pdf/6/6·4_Moltmann_Feuerbrandbek%C3%A4mpfung.pdf]. Accessed 19 June 2015.

Norelli JL, Jones AL, Aldwinckle HS, 2003. Fire blight management in the twenty-first century: using new technologies that enhance host resistance in apple. *Plant Disease* **87**, 756–65.

Psallidas PG, Tsiantos J, Vanneste J, 2000. Chemical control of fire blight. In: Vanneste JL, ed. *Fire Blight: The Disease and its Causative Agent,* Erwinia amylovora. Wallingford, UK: CABI Publishing, 199–234.

Raymundo AK, Ries SM, 1980. Chemotaxis of *Erwinia amylovora*. *Phytopathology* **70**, 1066–9.

Rudi K, Moen B, Drømtorp SM, Holck AL, 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Applied and Environmental Microbiology* **71**, 1018–24.

Salm H, Geider K, 2004. Real-time PCR for detection and quantification of *Erwinia amylovora*, the causal agent of fireblight. *Plant Pathology* **53**, 602–10.

Schroth M, Thomson S, Hildebrand D, Moller W, 1974. Epidemiology and control of fire blight. *Annual Review of Phytopathology* **12**, 389–412.

Smith TJ, 1999. Report on the development and use of Cougarblight 98C – a situation-specific fire blight risk assessment model for apple and pear. *Acta Horticulturae* **489**, 429–36.

Steiner PW, 1990. Predicting apple blossom infections by *Erwinia amylovora* using the MARYBLYT model. *Acta Horticulturae* **273**, 139–48.

Svircev A, Kim W-S, Lehman S, Castle A, 2009. *Erwinia amylovora*: modern methods for detection and differentiation. *Methods in Molecular Biology* **508**, 115–29.

Thomson S, 1986. The role of the stigma in fire blight infections. *Phytopathology* **76**, 476–82.

Thomson SV, 2000. Epidemiology of fire blight. In: Vanneste JL, ed. *Fire Blight: The Disease and its Causative Agent,* Erwinia amylovora. Wallingford, UK: CABI Publishing, 9–36.

Valasek MA, Repa JJ, 2005. The power of real-time PCR. *Advances in Physiology Education* **29**, 151–9.

Voegele R, Kunz S, Olbrecht L *et al.*, 2010. Monitoring *E. amylovora* using real time PCR. In: Fördergemeinschaft Ökologischer Obstbau e.V. (FÖKO), ed. *14th International Conference on Cultivation Technique and Phytopathological Problems in Organic Fruit-Growing*. Filderstadt, Germany: Vervielfältigungen F. u. T. Mueller-Bader, 110–7.

Weißhaupt S, Köhl L, Kunz S, *et al.*, 2015. Alternative inoculum sources for fire blight: the potential role of fruit mummies and non-host plants. *Plant Pathology*. doi:10.1111/ppa.12431.

Wilkening S, Bader A, 2004. Quantitative real-time polymerase chain reaction: methodical analysis and mathematical model. *Journal of Biomolecular Techniques* **15**, 107–11.

van der Zwet T, Orolaza-Halbrendt N, Zeller W, 2012. *Fire Blight: History, Biology, and Management*. St Paul, MN, USA: APS Press.