Alternative inoculum sources for fire blight: the potential role of fruit mummies and non-host plants

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Fire blight is the most damaging bacterial disease in apple production worldwide. Cankers and symptomless infected shoots are known as sites for the overwintering of Erwinia amylovora, subsequently providing primary inoculum for infection in the spring. In the present work, further potential sources of inoculum were investigated. Real-time PCR assays covering a 3-year-period classified 19.9% of samples taken from fruit mummies as positive. Bacterial abundance in fruit mummies during autumn, winter and spring was up to 10⁹ cells per gram of tissue and correlated well with later infection rates of blossoms. Blossoms of non-host plants growing close to infected trees were also shown to be colonized by E. amylovora and to enable epiphytic survival and propagation of bacteria. The results indicate a potential role of fruit mummies and buds in overwintering and as a source of primary inoculum for dissemination of the pathogen early in the growing season. Non-host blossoms may also serve as an inoculum source in the build-up of the pathogen population. Both aspects may contribute significantly to the epidemiology of E. amylovora. The significance of infected rootstocks as an inoculum source is also discussed. Fruit mummies might be used to determine pathogen pressure in an orchard before the beginning of the blooming period.

Keywords: epidemiology, fire blight, inoculum source, non-host plants

Introduction

Erwinia amylovora, the causative agent of fire blight, is the most devastating bacterial disease of rosaceous plants, primarily apple and pear, but also other fruit trees and ornamentals of economic importance throughout the world (Van der Zwet et al., 2012). A rapid spread of this disease has occurred in Europe during the past four decades. Dispersal of bacteria occurs by rain splash, wind and vectors such as insects and birds (Thomson, 2000). The basis of this spread is the existence of primary and secondary inoculum sources, allowing for persistence and multiplication of bacteria. Until now, only a few possible overwintering sites and inoculum sources of fire blight have been identified, despite extensive research. In the past, cankers have been regarded as the site for overwintering of E. amylovora and subsequently as the main source of inoculum for blossom and shoot infection in pome fruits in the spring (Rosen, 1929; Paulin, 1981). Shoots with infected, but symptomless, tissue have been regarded as additional overwintering sites (Crepel & Maes, 2000). Starting from active cankers, the infection of blossoms takes place, which then serve as a secondary inoculum source to infect additional blossoms (Thomson, 2000).

Around 200 plant species belonging to the family Rosaceae are considered host plants for E. amylovora, including ornamentals such as hawthorn (Crataegus sp.), rowan (Sorbus sp.), cotoneaster (Cotoneaster sp.), firethorn (Pyracantha sp.) and quince (Cydonia oblonga) (Momol & Aldwinckle, 2000). These plants may contribute to dissemination and directly provide inoculum potential for the infection of commercial pome fruit cultures. Aside from those mentioned above, further potential primary and secondary inoculum sources permitting for persistence or multiplication of E. amylovora might exist, e.g. infected fruit mummies and rootstocks of host plants, as well as blossoms of non-host plants. Rootstocks are well known to be infected with fire blight by the spread of bacteria within tissue (Van der Zwet & Van Buskirk, 1984; Momol et al., 1998), and probably by infection via suckers (Norelli et al., 2000). Despite the enormous damage that may be caused by infection of the rootstock, including the loss of whole trees (Norelli et al., 2000), little seems to be known about the spatial or seasonal variation of bacterial abundance and the potential role of infected rootstocks as a source of primary inoculum.

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Fruit mummies, small dried unripe fruits of stone and pome fruit remaining on the tree over winter, offer excellent conditions for persistence to a large range of pathogenic and non-pathogenic microorganisms, including fungi (Hong et al., 2000). Only a few previous studies have investigated the role of fruit mummies in connection to fire blight. Although the occurrence of E. amylovora in fruit mummies has been described (Anderson, 1952), a possible role of these fruit mummies in the dissemination of the bacteria and as a source of inoculum was ruled out.

Non-host plants of various taxa, including grasses (Poaceae) and plants of the families Asteraceae, Fabaceae, Ranunculaceae and Rosaceae, naturally occur in commercial orchards directly below fruit trees or surrounding the plantation. These plants might also be exposed to insect vectors, wind and rain splash, which are well-known mechanisms for the dispersal of bacteria (McManus & Jones, 1994; Thomson, 2000; Sabatini et al., 2006). Their blossoms might offer similar conditions for epiphytic survival of the bacteria as those of host plants (Hinze et al., 2015). Only a few studies have dealt with the occurrence of E. amylovora on non-host plants, especially outside the family Rosaceae. Paulin (1981) could not detect bacterial cells on weeds growing directly under infected trees, nor on corn (Zea mays, Poaceae) or Actinidia sp. (Actinidiaceae) growing close to them. However, Moltmann & Viehrig (2008) described an occurrence of E. amylovora on dandelion (Taraxacum officinale) blossoms adjacent to infected pear trees.

In this study, an improved real-time PCR approach (Hinze et al., 2015) is used to investigate the possible role of fruit mummies and buds in overwintering and as alternative inoculum sources for dissemination of E. amylovora in the spring. Furthermore, the role of symptomless non-host plants in epiphytic survival and multiplication of the bacteria, inconspicuously contributing to the epidemiology of fire blight, is investigated by analysis of field samples and in vitro experiments.

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). Enrichment of viable E. amylovora cells from environmental samples was performed in NB* (0.8% w/v nutrient broth + 5% w/v sucrose) liquid medium (Kunz, 2006) at 28°C and 250 rpm for 16 h.

Sampling locations

Plant material was sampled at 16 different locations, representing 13 commercial and three experimental (e) orchards in the area around Lake Constance, Europe, during the years 2007–10. Orchard locations were spatially separated by distances between 1 km and more than 60 km. Orchards Höchst I (e) and II are located in Vorarlberg, Austria. Orchards Karsee (e), Wellmutsweiler I (e) and II, Stadel, Deggenhausertal, Uhldingen-Mühlhofen, Ludwigshafen, Wahlwies, Bodman III, Steißlingen, Öhningen, Kaltbrunn, Dingelsdorf and Litzelstetten are located in Baden-Württemberg, Germany.

At the level of different sites within an orchard, the position of non-host plants was classified either as understorey growing below infected trees (site I), within a distance of 1.5–2 m from infected trees (site II), or as accompanying plants surrounding the orchards, at a distance of a least 4 m from infected trees (site III).

Sample collection and preparation

Sample size and pretreatment of samples prior to real-time PCR analysis was dependent on the nature of the sample. For analyses of blossoms, samples were collected in 720 or 2070 mL Whirl-Pak bags (Carl Roth GmbH), and incubated in a specific volume (indicated in brackets) of autoclaved water (Millipore) for 15 min, depending on the sample size. Blossom samples consisted of 10 blossoms in the cases of Convolvulus arvensis (100 mL), Cornus sanguinea (40 mL), Rosa rugosa (100 mL), T. officinale (40 mL), and Vicia cracca (complete inflorescences; 50 mL). Twenty blossoms were combined in the cases of Anthriscus sylvestris (40 mL), Bellis perennis (20 mL), Capsella bursa-pastoris (20 mL), Fragaria × magna (20 mL), Poaceae representatives (Alopecurus pratensis, Cynosurus cristatus, Dactylis glomerata, Holcus lanatus and Phalaris arundinacea) (complete inflorescences; 40 mL), Polygonum persicaria (20 mL), Ranunculus acris (20 mL), Sambucus nigra (100 mL), Sorbus aucuparia (40 mL), Trifolium repens (20 mL), Trifolium pratense (40 mL) and Veronica officinalis (5 mL). In the case of Stellaria media, samples consisted of 40 blossoms (20 mL). Malus domestica blossoms were collected in general in samples of 100 blossoms and incubated with 2 mL of water per blossom for 15 min. Epiphytic colonization of M. domestica leaves was assayed in a similar fashion, except that processing occurred in samples of 20 leaves (40 mL). Endophytic distribution of E. amylovora in a variety of different tissues was assayed by using samples consisting of 10–20 resting buds, 10–20 fruit mummies, or three pieces of bark (in the case of rootstocks). Mechanical pulping of plant material was performed using razor blades. Chopped material was incubated with water at 30 mL per g tissue in 13 mL screw cap tubes (Sarstedt) for 15 min on an Intelli-Mixer (LTB-Labortechnik) using programme F4 and 25 rpm, or in Whirl-Pak bags for 15 min on an orbital shaker (Heidolph Instruments) at 100 rpm. From all liquid phases obtained, a 1 mL aliquot was removed and centrifuged for 5 min at 15 800 g. The supernatant was discarded and pellets were resuspended in an equal volume of water. Samples were either analysed directly or stored at −20°C.

Evaluation of fire blight symptoms

To correlate E. amylovora detection with subsequent infestation in an orchard the severity of each observed fire blight incidence was graded (Hinze et al., 2015) 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 trees infested 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.
Propagation tests of *E. amylovora* on non-host blossoms

Plant material containing flowers was collected and placed with the stem into Erlenmeyer flasks filled with water, except for *B. perennis, R. acris* and *Fragaria vesca*, which were placed in garden soil (Einheitserde P, Gebrüder Patzer GmbH). Before starting the experiment, samples were assayed for the absence of *E. amylovora*. Blossoms of *B. perennis, F. vesca, M. domestica* (positive control), *Prunus armeniaca, Prunus avium, Prunus cerasus, Prunus domestica* subsp. *domestica, P. domestica* subsp. *insititia, P. domestica* var. *syracca, Prunus persica* var. *mucipersica, R. acris, Rosa multiflora,* and *Rubus fruticosus,* were inoculated with 5 μL of a suspension of 10^6 cells mL^-1. *E. amylovora* strain Ea385 in 0.6% NaCl. Samples consisted of three blossoms and inoculation of pieces of Parafilm 0.5 × 0.5 cm, in place of blossoms, served as a negative control. Blossoms of *C. sanguinea* and *S. nigra* were inoculated with 2 μL of bacterial suspension per pseudo-umbel. Each sample consisted of eight pseudo-umbels. Blossoms of *Rosa canina* were inoculated with 15 μL of bacterial suspension and samples consisted of one blossom. Complete blossom stands of *Festuca arundinacea, Trisetum flavescens* and *T. repens* were dipped into bacterial suspensions containing 10^5 cells mL^-1. In the case of *F. arundinacea* and *T. flavescens* two blossom stands, and in the case of *T. repens* three blossom stands, were treated as one sample. Incubation occurred at 24°C and 50–70% relative humidity in the dark, except for *B. perennis, R. acris* and *F. vesca,* which were kept in a growth chamber adjusted to a cycle of 14 h light at 24°C and 10 h darkness at 14°C. Samples were taken after 0.5 and 72 h of incubation, and prepared for real-time PCR analysis as described above, except that petals of the Rosaceae representatives were removed. The experiment was conducted with at least three replicates per plant species (except *T. flavescens* with two replicates).

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 in Voegle et al. (2010) (for details see Hinze et al., 2015). Samples consisted of extraction or washing fluids containing intact bacteria. No DNA extraction was performed prior to PCR analysis. Absolute quantification of bacteria in plant samples was performed by standardization with respect to serial dilutions of pure cultures of *E. amylovora* strains Ea383, Ea610 and Ea1430. The quantification was individually adapted to the different PCR cycle systems and reaction kits used as described in Hinze et al. (2015). Each sample was measured twice and the resulting cell counts were averaged. In cases where only one analysis was positive, the sample was evaluated as positive for *E. amylovora*. The lower detection limit was one single *E. amylovora* cell per PCR reaction, i.e. 10^2 bacterial cells mL^-1. The upper limit was 10^5 cells per reaction, i.e. 10^6 cells mL^-1. Thus, dilution of more highly concentrated samples was required to ensure correct quantification. The detection limit for tissue samples was 3 × 10^3 cells per g tissue. The detection limit for host blossoms was 2 × 10^2 cells per blossom for *M. domestica* and *S. aucuparia*. For non-host blossoms the detection limit varied depending on the sample’s source, with 25 cells/blossom for *V. officinalis, 50 cells/blossom for S. media, 10^2 cells/blossom for B. perennis, C. bursa-pastoris, F. × magna, P. persicaria,* R. acris and *T. repens, 2 × 10^4 cells/blossom for A. sylvestris,* representatives of Poaceae, *P. avium* and *T. pratense, 5 × 10^2 cells/blossom for S. nigra* and *V. cracca, 10^2 cells/blossom for C. arvensis* and *R. rugosa,* and 4 × 10^3 cells/blossom for *C. sanguinea* and *T. officinale.*

Data analysis

Cell counts determined by real-time PCR were log_{10}-transformed prior to calculation of means and for statistical analysis. In the propagation experiment replicates of log_{10}-transformed population sizes were subjected to analysis by t-tests as implemented in Microsoft Excel (*P < 0.05*). Statistical analyses of positive samples originating from more than two different sites were performed using one-way analysis of variance (ANOVA). Mean separation was accomplished using Tukey’s multiple comparison test (*P < 0.05*) as implemented in GraphPad Prism (GraphPad Software). Homogeneity of variance was confirmed by Bartlett’s test. *Erwinia amylovora* occurrence (scored as 0 or 1 for negative or positive samples, respectively) at different sites and data sets with inhomogeneity of variance were analysed using the nonparametric Kruskal–Wallis procedure (*P < 0.05*). Mean separation was accomplished using Dunn’s multiple comparison test (*P < 0.05*) as implemented in GraphPad Prism.

For figures with logarithmic scale and for field data analysis, the counts from samples found to be negative were manually defined as zero. Average cell counts given in the text have been transformed back from means of replicate logarithmic values.

Results

Real-time PCR detection of *E. amylovora* in various special samples

The real-time PCR protocol described by Salm & Geider (2004) has been refined for detection of *E. amylovora* in a variety of samples without prior DNA extraction (Hinze et al., 2015). The modified protocol can be successfully used for blossom samples of host, and non-host plants, as well as bark (not shown), cankers (not shown), buds and fruit mummies from pome fruit hosts.

Detection of *E. amylovora* in resting buds

To investigate whether resting buds could play a role in overwintering of *E. amylovora* and act as primary inoculum in the area around Lake Constance a total of 84 samples (72 apple, 12 pear) were collected from six different orchards known to be infested with *E. amylovora* (Höchst I, Höchst II, Karsee, Stadel, Bodman III, Dingelsdorf). Sampling occurred during February and May 2008, i.e. before the beginning of the blooming period. Four out of 84 (4.8%) apple bud samples originating from three different orchards (Höchst I, Höchst II, Bodman III) tested positive using real-time PCR, with low cell counts between 2 × 10^3 and 3 × 10^4 cells per gram of tissue, which was close to the detection limit (data not shown). Two of these samples were proven to contain viable and culturable cells by enrichment in liquid culture.
Frequency of *E. amylovora* in fruit mummies

Single fruit mummies were analysed individually using real-time PCR to determine whether mumified fruit resting on pome fruit trees might be an overwintering site, providing a source of primary inoculum, and also at what frequency *E. amylovora* occurred in these samples. In two orchards known for fire blight occurrence (Höchst I, Höchst II) samples were collected in April, May and July 2008. Fifteen out of 90 (16.6%) single fruit mummies tested positive for *E. amylovora* (data not shown). This result indicated that, on average, one to three in 10 fruit mummies could be expected to be positive for *E. amylovora*. To ensure successful detection, further sampling consisted of batches of 10 or 20 fruit mummies. Real-time PCR analysis revealed that 107 of 539 (19.9%) such batch samples collected in a 3-year period from 2008 to 2010 were positive for *E. amylovora* (data not shown). In the year 2008 alone, 82 out of 216 (38.0%) samples collected between February and August from six orchards (Höchst I, Höchst II, Wellmutsweiler I, Stadel, Bodman III, Dingelsdorf) tested positive for *E. amylovora* (data not shown). Among the positive samples, 43.9% (36 out of 82) were proven to contain viable and culturable cells.

Seasonal variation of *E. amylovora* abundance in fruit mummies

To examine at what time during the season and to what extent *E. amylovora* was present in mumified fruits, 156 samples were taken from the orchard Höchst I over a period of 2 years. Quantification was performed by real-time PCR and revealed the presence of bacteria during all four seasons, i.e. from January (winter) until September (beginning of autumn) with average cell counts in positive samples ranging from $1.3 \times 10^4$ up to $6.3 \times 10^8$ cells per gram of tissue and detection frequencies varying between 25 and 100% in 2008 (Fig. 1). Minimum and maximum values in single samples were $2.7 \times 10^3$ and $10^9$ cells per gram of tissue, respectively (data not shown). Over the complete sampling period, fruit mummies were proven to contain viable and culturable *E. amylovora* cells (data not shown). Permanently high amounts of the pathogen were present before, during, and after apple bloom in 2008, a year of severe fire blight infestation in this region (Holliger et al., 2009; Scheer, 2009), followed by only rare cases of detection in 2009 and 2010 (Fig. 1), two years with drastically reduced fire blight incidents.

Spatial variation of *E. amylovora* abundance in fruit mummies and blossoms

Differences in *E. amylovora* presence and abundance between various locations were investigated by sampling fruit mummies and blossoms in five orchards surrounding Lake Constance in 2008 (Fig. 2). Among the five orchards, Höchst I, classified with symptoms of grade 4 (Hinze et al., 2015), showed the highest frequency of detection with 60.3% positive fruit mummies, and a slightly lower frequency of positive blossom samples at 56.6% (Fig. 2). Höchst II, separated by a distance of only 1 km, but revealing a different seasonal pattern of *E. amylovora* abundance in blossoms despite an identical CDH18 profile (Hinze et al., 2015), showed a similar overall pattern with detection frequencies of both, fruit mummies (39.1%) and blossoms (32.3%), being generally lower than those found for Höchst I. Orchard Höchst II was treated once with streptomycin showing no visible influence on the abundance of the pathogen (Hinze et al., 2015). Completely different patterns of

Figure 1  Seasonal variation of abundance of *Erwinia amylovora* in fruit mummies, quantified using real-time PCR. A set of 156 samples, each consisting of 10 or 20 fruit mummies, was collected from the orchard Höchst I, Austria, from 2008 to 2010. △ Average cell count per gram of tissue in positive samples; ■ number of positive samples; □ number of negative samples. Bars indicate standard deviation.

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relations between fruit mummies and blossoms were found in the orchards Wellmutsweiler I, Bodman III and Dingelsdorf. In Wellmutsweiler I there was a low percentage of positive fruit mummies, but one of the highest detection frequencies of *Erwinia amylovora* on blossoms. In the orchard Bodman III, no fruit mummies tested positive, but 12.5% of the blossom samples were positive, indicating that fruit mummies are not the sole source of inoculum for blossom infections. In the orchard Dingelsdorf, fruit mummies tested positive but no blossom samples were positive. This indicates that a low frequency of *Erwinia amylovora* in fruit mummies does not necessarily lead to blossom infections. Taken together, despite the variations between different sampling locations, *Erwinia amylovora* abundance in fruit mummies correlated with subsequent blossom colonization and infestation grade.

### Long-term correlation of *Erwinia amylovora* abundance in blossoms and fruit mummies

The influence of inoculum pressure, as provided by fruit mummies, on subsequent blossom infection rates and infestation, was determined on a data set originating from a total of 14 orchards in the area of Lake Constance sampled over a period of 4 years. Real-time PCR analysis revealed that after a period with high detection frequency of *Erwinia amylovora* in fruit mummies, detection frequency in blossoms was also high and subsequently led to a high percentage of orchards with infestation of at least grade 1 (Fig. 3). Correspondingly, low detection frequencies in fruit mummies led to low frequencies of *Erwinia amylovora* occurrence on blossoms, and to a low percentage of symptom development.

### Detection of *Erwinia amylovora* on non-host plants

To investigate whether *Erwinia amylovora* is not only able to colonize its large set of host plants, but might also occur on non-host plants, blossoms of different non-host plants were analysed using real-time PCR. A total of 16 species representing 13 different taxa, growing either as understorey below or at some distance from apple trees, or surrounding the orchards as accompanying plants, were examined. These included members of the families Apiaceae, Asteraceae, Brassicaceae, Caprifoliaceae, Caryophyllaceae, Convolvulaceae, Cornaceae, Fabaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae and Scrophulariaceae.

The analysis included two different types of orchards. First, orchard Karsee represented an experimental orchard with artificial inoculation of single trees to create severe fire blight infection pressure, leading to symptoms of grade 4. Non-host samples from this orchard were taken from the uninoculated area, so that any *Erwinia amylovora* detected would have been dispersed by natural mechanisms such as insect vectors, rain splash etc. The second type was represented by three commercial orchards (Höchst II, Höchst I and Wellmutsweiler I) with natural inoculum pressure. Subsequent infestation was classified as grade 2 in the case of Höchst II, and grade 4 in the cases of Höchst I and Wellmutsweiler I (Fig. 2). In 2007, eight of 12 taxa in orchard Karsee tested positive
for *E. amylovora* (Fig. 4). In the orchard Höchst II, four of seven taxa were positive (Fig. 5); these included three species on which *E. amylovora* was not detected at the Karsee location, i.e. *B. perennis*, *T. repens* and *S. nigra*.

Additional samples, including further non-host species, were taken in 2008 at Höchst I, Höchst II and Wellmutsweler I from sites neighbouring infected trees (site II), and the area surrounding the orchards (site III). Among these, one out of 40 *B. perennis* samples (site II), 1 out of 11 *Trifolium* spp. samples (site II) (data not shown), and 4 out of 12 *R. rugosa* samples (site III) (Fig. 6) were positive for *E. amylovora*. At these three naturally infested orchards the pathogen could not be detected in 13 *R. acris* (II) and three *C. sanguinea* samples (site III) (data not shown).

Overall, 12 species representing 10 taxa growing either as understorey or accompanying plants, tested positive for *E. amylovora* in at least one of the orchards, four of them from orchards with natural inoculum pressure (Fig. 5). In a summarized data set comprising 293 blossom samples originating from all non-host species occurring at the four orchards, a total of 19.5% of the samples tested positive for *E. amylovora* (data not shown). General colonization frequencies were: Poaceae representatives 42.5% (40 samples total), *R. acris* 32.7% (55), *R. rugosa* 37.5% (24), *Trifolium* spp. 12.1% (33), and *B. perennis* 3.4% (59). Individual colonization frequencies ranged from 5-6 up to 68%, depending on collection site and species (Fig. 4). The average cell count in positive samples ranged from $1 \times 10^2$ (Fig. 4) up to $6 \times 10^4$ cells per blossom (Fig. 5). Cell counts in positive non-host samples averaged $1.6 \times 10^3$ cells per blossom, approximately 60 times lower than the average counts of $9.3 \times 10^4$ cells in positive apple blossoms (Fig. 4).

Spatial variation of *E. amylovora* abundance on non-host plants

Spatial variation of the abundance of *E. amylovora* on non-host plants was considered at two levels: first, at the level of region, represented by orchard locations spatially...
separated from each other, and secondly, at the level of different sites within an orchard according to distance from infected trees.

At the regional level, it is noteworthy that among the four taxa (*Bellis*, *Taraxacum*, *Trifolium* and *Sambucus*) sampled in 2007 at Karsee and Höchst II, separated by 33 km, there was no non-host species that tested positive at both locations (Figs 4 & 5), revealing striking spatial differences. To investigate *E. amylovora* occurrence on a non-host species growing at several locations, *B. perennis* blossom samples were collected in 2008 from three orchards of the same type, i.e. Höchst I, Höchst II and Wellmutsweiler I, representing locations separated by only 1 km in the case of Höchst I and Höchst II, and by 20 km in the case of Wellmutsweiler I. At Höchst I, one of 20 samples tested positive for *E. amylovora*, at Höchst II, none of six, and at Wellmutsweiler I, none of 14 (data not shown). Thus, for *B. perennis*, generally colonized by *E. amylovora* at a very low level in 2008, there were only slight spatial differences between the different orchards.

At the level of different sites within an orchard, in orchard Karsee, a general decline of colonization frequency with increasing distance from infested trees was observed, but a similar decline was not observed for cell counts of positive samples (Fig. 4). In orchard Höchst II, no clear tendency was observed, with detection frequency and cell counts of site III being higher than those of site II (Fig. 5). However, it should be noted that the non-host plants at the different sites were not the same.

Using a summarized data set comprising all non-host plant species, four orchards and both orchard types, with a total of 293 samples (including data from Figs 4 & 5), *E. amylovora* colonization frequencies and cell counts were analysed for site I, site II or site III. For site I, represented by 70 samples comprising six species, an overall detection frequency of 47% and an average cell count in positive samples of \(7 \times 10^2\) cells per blossom were observed. Site II, represented by 182 samples comprising seven species, was characterized by a frequency of 7% and an average cell count of \(1 \times 10^3\) cells per blossom. For site III, represented by 41 samples comprising five species, a detection frequency of 24% and an average cell count of \(3.5 \times 10^4\) cells per blossom was observed (Fig. 6). Thus, the colonization frequencies of site II and III were generally lower than that of site I, but a slight tendency of cell counts to increase with distance from infected apple trees seems to exist. To determine whether the differences observed between the site types were significant, statistical analysis was performed,
comparing the log₁₀-transformed cell counts in positive samples and the presence/absence of *E. amylovora* scored as 1/0. Log₁₀-transformed cell counts of positive samples were compared by ANOVA followed by Tukey’s multiple comparison test and showed significant differences for site I versus site III and site II versus site III (*P* < 0.001). Comparison between sites of the presence/absence of *E. amylovora* was carried out by a Kruskal–Wallis ANOVA and subsequent Dunn’s multiple comparison test and significant differences were proved to exist between all three sites with a high level of significance for site I versus II and I versus III (*P* < 0.001), and a lower level of significance for site II versus III (*P* < 0.01).

Analysis was performed in more detail for two frequently colonized taxa, *R. acris* and representatives of Poaceae (comprising *A. pratensis*, *C. cristatus*, *D. glomerata*, *H. lanatus* and *P. arundinacea*), each growing in the Karsee location at site types I and II. For each species, the analysis revealed significant differences in the occurrence of *E. amylovora* with respect to position (*t*-test with *P* < 0.05; data not shown). The individuals growing below infected trees were generally much more frequently colonized by *E. amylovora* than those growing 1.5–2 m from the trees (Figs 4 & 7a). In contrast, cell counts of positive samples (Figs 4 & 7b) did not show any significant difference between the site types I and II for these two species (*t*-test with *P* < 0.05).

**Seasonal variation of *E. amylovora* abundance on non-host plants**

Non-host species exhibiting either a high percentage of positive samples or high average cell counts were analysed in more detail to reveal at which time point during or after apple bloom bacteria were present. In general, continuous detection of *E. amylovora* over a long period of time was possible on blossoms of the taxa *Ranunculus*, Poaceae and *Rosa* (Figs 7a,b & 8). In the orchard Karsee (with main blooming period of apple trees being from 20 April to 1 May), *E. amylovora* could be detected on *R. acris* during May, i.e. during and shortly after apple bloom, while on representatives of the Poaceae detection was possible until the end of June (Fig. 7a,b). In the naturally infested orchard Höchst II *E. amylovora* was detected after apple bloom on *B. peregrinus*, *Trifolium* spp. and *R. rugosa* from late May until the end of June. Detection frequencies and cell counts were generally lower on non-host samples compared to apple leaves, which served as the positive control. In the course of time there was an overall decline of detection frequency of *E. amylovora*, reaching zero on the non-host.
host samples 2–3 weeks before the host samples (data not shown). On *R. rugosa*, detection was possible until August, indicating bacterial persistence long after bloom of the host plants and a complete disappearance of bacteria even on host leaves. In the present study *R. rugosa*, growing at the border (i.e. site III) of the orchard, was the sole non-host species on which bacteria were observed for several years in succession at a single site (Fig. 8). Average cell counts on the *Rosa* blossoms were around 17-fold higher than the epiphytic occurrence of *E. amylovora* on host leaves in 2007 (Fig. 5), and only approximately a factor of nine lower than those on host blossoms in 2008 (see Fig. 3a in Hinze *et al.*, 2015). On *B. perennis*, *E. amylovora* could not be detected in 2008 at Höchst II (data not shown) despite its detection in the preceding year (Fig. 5).

**Propagation of *E. amylovora* on blossoms of non-host plants**

To determine whether the observed occurrence of *E. amylovora* on blossoms of non-host plants indicated persistence or even allowed for an active multiplication of bacteria, an assay with detached blossoms of diverse host and non-host species was carried out. This revealed that the bacterium not only persisted, but also could multiply in blossoms of several non-host plant species (Table 1). On blossoms of 11 of 18 non-host

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**Figure 7** Seasonal variation of *Erwinia amylovora* abundance on blossoms of non-host plants with respect to their location in an orchard with artificial inoculation, detected using real-time PCR. Summary of 42 *Ranunculus acris* blossom samples, 40 Poaceae blossom stand samples and 49 host blossom samples including young fruit and late blossoms, collected in orchard Karsee during and after apple bloom in 2007. Poaceae comprised *Alopecurus pratensis*, *Cynosurus cristatus*, *Dactylis glomerata*, *Holcus lanatus* and *Phalaris arundinacea*. (a) Percentage of samples tested positive, (b) average cell count per blossom in positive samples. — *Ranunculus acris* below infected trees, —— *R. acris* 1.5 to 2 m distant from infected trees, — Poaceae below infected trees, —— Poaceae 1.5 to 2 m distant from infected trees, —— apple blossoms (positive control). Each data point generally consists of 2–4 samples analysed individually. Bars indicate standard deviations.
plants, *E. amylovora* propagation occurred in at least one assay. This included stone fruit blossoms such as sweet cherry, sour cherry, plum, prune and nectarine, and other rosaceous fruit such as strawberry and blackberry. Understorey and accompanying plants of *B. perennis*, *S. nigra* and *R. multiflora*, occurring in or around orchards, proved to be a suitable substrate for *E. amylovora*. In one case, bacterial multiplication on *R. multiflora* even exceeded that on the positive control, blossoms of the apple cultivar Gala. On *R. canina* no multiplication occurred, despite *R. canina* (Bastas et al., 2013) being shown to be a host for *E. amylovora*. According to the present field data *R. rugosa* was one of the most highly colonized species showing high cell counts, colonization frequency and several-year succession (Figs 5 & 8). In general, on blossoms of non-host species showing significant multiplication in at least one case, the average cell number increased from $2 \times 10^3$ to $1.1 \times 10^5$ cells per blossom, i.e. increased to more than 50-fold within 72 h. Average cell counts on the host blossoms increased from $10^3$ to $1.6 \times 10^6$ cells per blossom, i.e. more than 1600-fold within the same time frame.

**Discussion**

Fire blight is an extremely aggressive disease of great economic importance. Despite more than 100 years of research, open questions remain concerning inoculum sources and sites of persistence. Previous studies have dealt with cankers (Rosen, 1929; Paulin, 1981) and symptomless shoots (Crepel & Maes, 2000) serving as overwintering sites and sources for inoculum in the spring. The present study has focused on alternative inoculum sources provided by fruit mummies, resting buds and non-host plants. Additionally, the use of fruit mummies for early fire blight diagnostics and prognosis was investigated.

A role for fruit mummies in the dissemination of fire blight bacteria has been discounted in research so far (Anderson, 1952; Goodman, 1954). In general, fruit mummies are known as reservoirs for a large diversity of microorganisms (Hong et al., 2000; Van Leeuwen et al., 2002) and to be suitable for persistence of microorganisms (Knoche et al., 2000). Pome fruit varieties exhibit wide variations in the extent of fruit mummy formation. Three main mechanisms of mummification seem to exist: first, the formation of fruit mummies because of a lack of or insufficient formation of the abscission layer (Knoche et al., 2000); secondly, growing fruits may be infected by a variety of pathogens including *E. amylovora* (Thomson, 2000); and thirdly, instead of fire blight bacteria invading the shoot, infected blossoms may continue to differentiate into young fruits, and subsequently dry out. Some pome fruit varieties show a low tendency to form fruit mummies, but in years with severe fire blight infestation a tendency for enhanced mummification is observed and in most cases these fruit mummies test positive for *E. amylovora* (S. Kunz, unpublished data). Studies observing a migration of bacteria through the abscission layer (Azegami et al., 2006) and within tissue of the fruit (Jock et al., 2005) give additional indications of the ability of bacteria to actively invade existing fruit mummies.

The present investigation reveals the presence of *E. amylovora* in fruit mummies during all four seasons.
Table 1: Propagation of Erwinia amylovora on blossoms of non-host plants.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stigma type</th>
<th>Change</th>
<th>Cells/blossom (log₁₀ ± SD)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruit species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strawberry (Fragaria vesca)</td>
<td>+</td>
<td>3.45±0.48</td>
<td>4.72±0.92</td>
<td>0.070</td>
</tr>
<tr>
<td>Apricot (Prunus armeniaca)</td>
<td>Wet</td>
<td>4.66±0.54</td>
<td>4.97±0.49</td>
<td>0.362</td>
</tr>
<tr>
<td>Nectarine (Prunus persica var. nucipersica)</td>
<td>+</td>
<td>3.65±0.38</td>
<td>4.58±0.28</td>
<td>0.001</td>
</tr>
<tr>
<td>Plum (Prunus domestica subsp. insitita)</td>
<td>+</td>
<td>4.56±0.30</td>
<td>5.77±0.03</td>
<td>0.004</td>
</tr>
<tr>
<td>Prune (P. domestica subsp. domestica)</td>
<td>+</td>
<td>4.12±0.25</td>
<td>5.43±0.41</td>
<td>0.005</td>
</tr>
<tr>
<td>Mirabelle (P. domestica var. syriaca)</td>
<td>Wet</td>
<td>3.27±0.11</td>
<td>3.59±0.26</td>
<td>0.084</td>
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<tr>
<td>Sweet cherry (Prunus avium)</td>
<td>+</td>
<td>3.56±0.22</td>
<td>5.25±0.24</td>
<td>0.001</td>
</tr>
<tr>
<td>Sour cherry (Prunus cerasus)</td>
<td>Wet</td>
<td>3.17±0.42</td>
<td>4.84±1.06</td>
<td>0.021</td>
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<tr>
<td>Blackberry (Rubus fruticosus)</td>
<td>Dry</td>
<td>2.93±0.64</td>
<td>3.16±0.53</td>
<td>0.419</td>
</tr>
<tr>
<td><strong>Understorey species</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bellis perennis</td>
<td>Dry</td>
<td>3.38±0.21</td>
<td>5.71±0.65</td>
<td>0.018</td>
</tr>
<tr>
<td>Ranunculus acris</td>
<td>Dry</td>
<td>3.45±0.21</td>
<td>4.20±0.79</td>
<td>0.067</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>Wet</td>
<td>3.68±0.18</td>
<td>3.28±0.27</td>
<td>0.026</td>
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<tr>
<td>Festuca arundinacea</td>
<td>Dry</td>
<td>3.55±0.22</td>
<td>3.82±0.41</td>
<td>0.399</td>
</tr>
<tr>
<td>Trisetum flavescens</td>
<td>Dry</td>
<td>3.06±0.35</td>
<td>2.89±0.02</td>
<td>0.395</td>
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<td><strong>Accompanying plants</strong></td>
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<tr>
<td>Cornus sanguinea</td>
<td>Dry</td>
<td>3.50±0.17</td>
<td>3.32±0.43</td>
<td>0.378</td>
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<td>Sambucus nigra</td>
<td>Dry</td>
<td>3.12±0.54</td>
<td>3.75±0.77</td>
<td>0.234</td>
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<tr>
<td>Rosa multiflora</td>
<td>Dry</td>
<td>2.09±0.38</td>
<td>3.95±0.84</td>
<td>0.002</td>
</tr>
<tr>
<td>Rosa canina</td>
<td>Dry</td>
<td>2.89±0.13</td>
<td>6.97±0.17</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (Parafilm)</td>
<td></td>
<td>3.91±0.13</td>
<td>3.29±0.12</td>
<td>0.005</td>
</tr>
<tr>
<td>Positive (apple blossoms)</td>
<td>Wet</td>
<td>3.99±0.39</td>
<td>6.21±0.30</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Growth on blossoms was quantified using real-time PCR in samples of one to three blossoms, eight pseudo-umbels or two blossom stands, depending on the species used. At least three samples were analysed per species and point of time.

*Type of stigma according to Heslop-Harrison et al. (1975) and Heslop-Harrison & Shivanna (1977).

†Significant propagation (+) or decrease (−) of bacterial cell number (t-test, p = 0.05).

‡Result of a t-test assessing the variance between the two analyses.

(Fig. 1), with viable and culturable cells being present (data not shown); thus, these structures are suitable for long-time survival of bacteria. Erwinia amylovora occurrence in fruit mummies has previously been observed (Anderson, 1952; Goodman, 1954) and long-time survival of bacteria has also been shown (Tsukamoto et al., 2005). In most cases during the present study, the amount of bacteria detected in fruit mummies showed a high correlation with the amount of bacteria detected in blossoms and subsequent occurrence of fire blight (Figs 2 & 3). Fruit mummies might, therefore, present a noteworthy source of inoculum early in the growing season, especially as the high cell counts detected in these structures are comparable to those of cankers (data not shown). Bacteria might easily be transferred to blossoms in the vicinity of fruit mummies by insect vectors or rain splash. Thus, the results clearly show a prominent role for these structures in early dispersal of the pathogen, indicating that fruit mummies should be removed in cases of positive detection. At the same time, monitoring of these structures may be used to assess the risk of fire blight (Hinze et al., 2015). Fruit mummy monitoring would allow risk assessment at a very early stage during the growing season.
Resting buds also tested positive for *E. amylovora* and could serve as an additional overwintering site. Despite the low percentage of occurrence (4.8%), resting buds should not be underestimated with respect to their importance as an early source of primary inoculum. Bud infection might result from a latent infection and systemic spread of bacteria within the vascular system, leading to early colonization of blossoms.

The role of rootstocks as a third site for overwintering and persistence of *E. amylovora* has been neglected up to now. Rootstock infection by *E. amylovora* has already been described by several studies (Gowda & Goodman, 1970; Norelli et al., 2003). Little seems to be known about the seasonal and spatial variation of bacterial abundance at such sites. In the orchard Bodman III, *E. amylovora* was detected in highly susceptible M9 rootstocks early in 2008, with a subsequent decline of cell counts (data not shown). At the same time, evidence for dynamic changes in its spatial distribution was obtained (S. Kunz, unpublished data). The infection of rootstocks in this orchard resulted in only a few bud and blossom infections, with no fruit mummies testing positive (Fig. 2), and trees were still viable 6 years later showing no, or very few, infections (S. Kunz, unpublished data). Thus, the role of rootstocks in subsequent blossom infections and as a potential source for primary inoculum seems to be less important than fruit mummies or resting buds.

All three potential overwintering sites, i.e. fruit mummies, resting buds and rootstocks, were shown to contain viable and culturable *E. amylovora* cells. The frequency was lower than cell counts determined by real-time PCR, but real-time PCR does not distinguish between living, dormant and dead cells. Attempts were made to overcome this discrepancy by employing the ethidium monoazide method (Luo et al., 2008), but this methodology has not yet delivered satisfactory results (S. Weißhaupt, unpublished data). Another explanation for the observed differences may be the existence of viable but non-cultur-able (VBNC) cells, which have been described for *E. amylovora* (Ordax et al., 2006). These VBNC cells cannot be cultured but might be important for the epidemiology of the fire blight pathogen.

Another aspect of the investigation was monitoring the occurrence and development of *E. amylovora* on non-host plants serving as possible external (secondary) sources of inoculum. Only a few studies have dealt with the occurrence of *E. amylovora* on non-host plants; Paulin (1981) could not detect *E. amylovora* on weeds, corn, or *Actinidia sp.*; Moltmann & Viehrig (2008), on the other hand, described an occurrence of *E. amylovora* on dandelion, which has been corroborated by the present study (Fig. 4). Further reports describe detection of *E. amylovora* on symptomless sweet cherry blossoms (Moltmann & Viehrig, 2008), and its isolation from plum tissue with symptoms (Mohan & Thomson, 1996; Vanneste et al., 2002), and strawberry (Atanasova et al., 2005), all of which are members of the Rosaceae but hitherto not classified as host plants. There has been no study regarding the epiphytic development of *E. amylovora* on non-host plants and their potential role in dissemination of fire blight bacteria. Thus, for the first time this study considers *E. amylovora* occurrence and development on non-host plants in a larger context with regard to taxonomy, seasonal, spatial and physiological aspects.

The results reveal the occurrence of the fire blight pathogen on a large taxonomic diversity of non-host plants naturally accompanying or surrounding orchards (Figs 4, 5, 7a,b & 8). Thus, the occurrence of *E. amylovora* is not restricted to the large spectrum of known host plants. Cell counts and detection frequencies of *E. amylovora* on non-host blossoms were generally lower than on host plants (Figs 4 & 7a,b), with the exception of *R. rugosa*, which showed high cell counts (Fig. 8). This work raises the question of whether *Rosa* should be described as an additional host species; this has been indicated in previous studies of *Rosa* spp. with some *E. amylovora* strains, such as the ‘Fairfax’ rose (*Rosa* sp.) (Rosen & Groves, 1928), the potato rose (*R. rugosa*) (Vanneste et al., 2002), and the dog rosehip (*R. canina*) (Bastas et al., 2013).

Striking spatial variations of *E. amylovora* colonization of non-host plants were observed at regional and site levels. The variation between different orchards (e.g. Figs 4 & 5) indicates that most non-host species are not stably colonized and that their colonization may vary from one location to another. The results of occurrence of *E. amylovora* as a function of distance from infected trees (referred to as site I, II or III) reveal no simple relationship between occurrence of fire blight and distance of the non-host plant from infected trees. For two frequently colonized taxa, *R. acris* and representatives of the Poaceae (Figs 4 & 7a), as well as for non-host plants as a whole (Fig. 4), it was shown that the frequency of colonization declined with increasing distance from infected trees. However, there was no significant overall decline of cell counts (Figs 4, 5, 6 & 7b). This may have been due to the many different plant species in the study and the fact that not all were found in all locations. Despite these limitations, there seems to be good evidence that the occurrence of *E. amylovora* is not restricted to the direct environment of infested host plants. Further research is necessary to investigate if, and to what extent, the pathogen also occurs on non-host plants even more distant from the orchards.

Furthermore, the results indicate that the bacterium is capable not only of persistence, but also of multiplication on blossoms of several non-host species (Table 1). This occurred regardless of the stigma type. It has been assumed that *E. amylovora* would establish and propagate only on wet-stigma blossoms (Johnson & Stockwell, 1998); however, the present results clearly show that successful propagation also occurs on blossoms of dry-stigma type plants. Bacterial multiplication on non-host blossoms occurred at an average rate that was 30-fold lower than on the positive control (Table 1). Changes in numbers of bacteria (increase or decrease) were
significant in most cases of rosaceous plants, including non-host species, for which occurrence or isolation from necrotic tissue had been described (Mohan & Thomson, 1996; Vanneste et al., 2002). The multiplication rate in the positive control of the present investigation (1600-fold within 72 h) was much higher than propagation under field conditions (Fig. 7b, see also Hinze et al., 2015 and Figs 2b & 3a therein).

The results indicate a significant role of non-host flowers in the build-up of the pathogen population. Although no symptoms occurred on blossoms, a propagation of E. amylovora on non-host plants early in the growing season could contribute significantly to the build-up of inoculum. Additionally, non-host species with successful blooming periods could provide propagation opportunities for the pathogen beyond the pome fruit blooming period and thus extend the period of infection hazard.

In summary, new insights and a further broadening of the knowledge of the epidemiology of fire blight have been obtained by identifying hitherto unknown sites for persistence, i.e. fruit mummies and resting buds. Both provide additional primary inoculum sources in addition to the previously known sources of cankers and infected shoots. Furthermore, examination of its non-host ecology has enabled identification of new and inconspicuous ways of distribution and propagation of this invasive pathogen, as provided by blossoms of non-host plants. Thus, it can be concluded that to fully understand the epidemiology of fire blight, the non-host plant environment must be considered in addition to the host plant range.

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