Monitoring *E. amylovora* using Real Time PCR

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Abstract

Fire blight, caused by the Gram-negative bacterium Erwinia amylovora *is the most damaging bacterial disease of pome fruit world wide. Disease outbreak is only sporadic, disease outcome, however, can be devastating. Aside from physical factors like temperature and humidity, presence of the pathogen in sufficient amounts to cause infection is a crucial aspect in abatement of disease. We have refined a real time PCR based quantification of* E. amylovora *to be used in the field on a variety of environmental samples. This method allowed us to monitor pathogen abundance during blossom during the last four years. Integration of the amount of pathogen detected into refined prediction algorithms will most certainly improve applied forecasting models. The method also allows the quantification of pathogen outside the blooming period within or on the surface of different tissue, or plant organs. This may allow a monitoring of the pathogen population throughout the year and in the long run may facilitate timely countermeasures. Results also indicate a potential role of fruit mummies in overwintering and dissemination of the pathogen early in the growing season, as well as a role of non-host flowers in the built up of pathogen potential. The method is also capable of monitoring disease progression in infected, but symptomless tissue. Results from this set of experiments might improve recommendations for good cultural practice.*

Keywords: *E. amylovora,* epidemiology, monitoring, pathogenesis, Real Time PCR

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 (Stöger et al. 2006). The typical symptoms of infected plants are the brown to black color of twigs, flowers, and leaves, the production of exudate and the typical “shepherd’s crook” of infected shoots. Proper sanitation methods like pruning of symptomatic tissue or clearing of infected trees are vital procedures to reduce the infection pressure. Important tools to predict infection conditions are computerized forecasting systems like Maryblyt (Steiner 1990), Billing´s Integrated System 95 (BIS95) (Billing 1996), or Cougarblight (Smith 1999). However, all these models only take physical factors like temperature or moisture/wetness into account, but not the actual presence of the pathogen. An important step in control of the disease depends on the fast and specific detection of the pathogen. Current approaches for the identification of fire blight include morphological, biochemical, serological, and DNA-based methods (Svircev et al. 2009). However, most of these methods are fairly time consuming, and none of these methods is quantitative. Real Time PCR is a fast and sensitive method that allows quantification of a pathogen within a few hours (Higuchi et al. 1993). Real Time PCR assays so far mostly focused on the analysis of DNA extracted from diverse samples.

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Based on the Real Time PCR approach described by Salm and Geider (2004), we have further improved the method to be used directly on whole bacteria in the field on a variety of samples. The method allows for a detailed description of fire blight epidemiology and pathogenesis as well as for an integration of results into improved predictive forecasting models.

Material and Methods

Real Time PCR analyses were performed either on a SmartCycler II (PEQLAB Biotechnologie GMBH, Erlangen) or using a CFX96 Real-Time PCR Detection System (BioRad, Munich). Manual thresholds were set to 30 rfu, and 250 rfu, respectively. As Real Time PCR chemistry the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden) together with primers P29TF and P29TR (Salm and Geider 2004) in a reaction volume of 25 µl was used. Maximum sample volume to be tested was 10 µl. The PCR protocol consisted of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of denaturation at 95 for 10 sec and annealing and extension at 60°C for 30 sec. A melt analysis followed each PCR run to verify identity and homogeneity of the amplicon.

Samples consisted of extraction or washing fluids containing intact bacteria. No DNA extraction was performed prior to PCR analysis. Absolute quantification of bacteria in samples was done by standardization with respect to serial dilutions of washed pure cultures of *E. amylovora*.

Pre-treatment of samples was dependent on the nature of the sample. For the analyses of blossoms, organs (in general 100) were collected in 2070 ml Whirl-Pak bags (Carl Roth GmbH, Karlsruhe), and incubated with 2 ml H_2O per blossom for 15 min. A 1 ml aliquot was removed and centrifuged for 1 min at 15,000 g. The supernatant was discarded and pellets resuspended in an equal volume of H_2O. Samples were either analyzed directly or stored at -20°C. Epiphytic colonization of leaves was assayed in a similar fashion, except that 10 ml of H_2O were used per gram fresh weight. Endophytic distribution of *E. amylovora* in diverse tissue was assayed by controlled crushing of the plant material using razor blades. Chopped material was incubated with H_2O at 3 ml per 100 mg tissue in 12 ml Sarsted tubes for 15 min on an Intelli-Mixer (LTF-Labortechnik, Wasserburg) using program F4 and 25 rpm. Subsequent processing was as described.

In order to assay the expansion of bacteria in tissue shoots of young apple trees of the cultivar Jonagold were inoculated on the first leave by a cut with a scissor dipped into a solution containing 10^9 cells/ml. Disease progress was monitored visually. At different time points trees were sacrificed, fragmented into small pieces and samples analyzed as described above.

Results

We have refined the Real Time PCR set up described by Salm and Geider (2004) for the quantification of *E. amylovora* in a variety of samples without the necessity for processing of the bacteria containing samples prior to PCR analysis. Our work on *E. amylovora* during the past four years mainly focussed on three vital aspects of the disease: a) early detection of inoculum in the environment (monitoring), b) quantitative description of the pathogen on host and non-host plants and its ways of distribution (epidemiology), and c) quantitative description of the distribution of bacteria within tissue with or without symptoms (pathogenesis).

Monitoring. Over the past four years we have been using our mobile laboratory to perform on site Real Time PCR analysis of *E. amylovora* in selected orchards in the Lake Constance region, the largest pome growing region in Germany. Our methodology allows

screening and analysis of samples in less than two hours directly in the field. Representative results for the growing season 2008 are depicted in Table 1. Our results clearly show that there is a correlation between the percentage of samples tested positive and symptom development in an orchard.

Table 1: Results of Real Time PCR based flower monitoring during the growing season 2008 (positive samples/total samples).

	Germany							
location	**Bodman**				**Höri**		**Lindau**	
facility	12	10	13	11	15	16	4	3
apple	3/24	0/3	5/53	1/14	2/4	0/2	0/4	14/21
pear		0/2						
sum	3/24	0/5	5/53	1/14	2/4	0/2	0/4	14/21
positive [%]	12,5	0	9,4	7,1	50,0	0	0	66,7
symptoms [a]	+	-	+	-	++	-	-	++++

	Germany						Austria	
location	**Dingelsdorf**	**Markdorf**		**Stadel**	**Stahringen**	**Wellmutsweiler**	**Höchst**	
facility	9	7	8	6	14	5	1	2
apple	0/22	1/29	2/32	0/8	1/2	20/44	77/136	21/65
pear				1/24		1/18	6/21	
sum	0/22	1/29	2/32	1/32	1/2	21/62	83/157	21/65
positive [%]	0	3,4	6,3	3,1	50,0	33,9	52,9	32,3
symptoms [a]	-	-	+	-	+	++	++++	++

[a] - = no symptoms; + = sporadic symptoms; ++ = symptoms on more than 10 % of trees; +++ = 1 symptom per tree; ++++ = multiple symptoms per tree

What is even more important than establishing a correlation between the presence of *E. amylovora* and the development of disease symptoms is an early detection of the pathogen prior to its manifestation. Figure 1 shows that our method is capable of detecting the pathogen long before the critical CDH18 value of 110 is reached and present forecasting models would indicate the need for treatments against fire blight. This is especially true for pear.

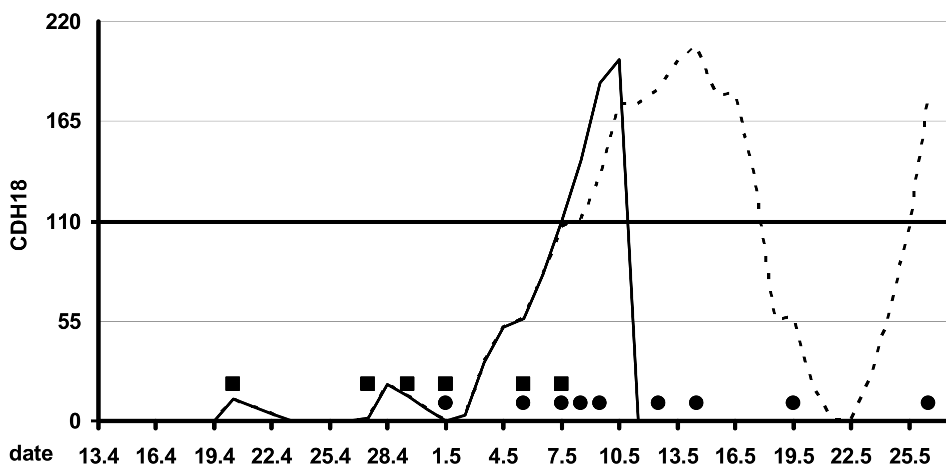

Figure 1: CDH18 values for pear (▬) and apple (▪▪▪) for the year 2008 in the orchard Höchst (1), Austria. ■ positive samples on pear, ● positive samples on apple.

Our results over the four year sampling period clearly indicate the suitability of the method to detect *E. amylovora* early during bloom. It is our goal to incorporate quantification of the pathogen into predictive forecast models in order to improve abatement of fire blight.

Epidemiology. Another important aspect of our work is to determine the capacity of *E. amylovora* to persist and multiply on different host and non-host plants. Our results indicate that the bacterium is capable not only to persist, but to multiply in blossoms of several non-host species (Figure 2).

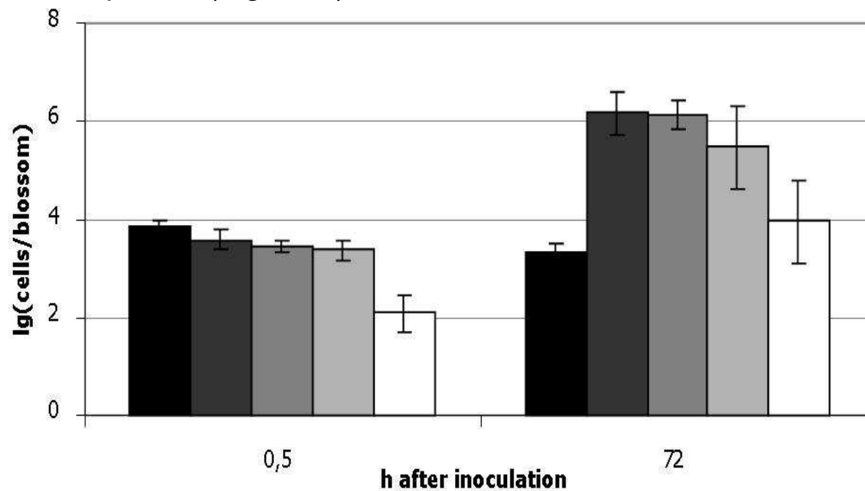

Figure 2: Propagation of *E. amylovora* in blossoms of non-host plants. ■ negative control (parafilm), ■ *Prunus avium,* ■ *Fragaria vesca,* ■ *Bellis perennis,* □ *Sambucus nigra*.

While no symptoms occurred on these blossoms, such a propagation of *E. amylovora* on non-host plants early in the growing season could contribute significantly to the build up of inoculum endangering susceptible host cultivars in the process.

Bacteria could also be detected on the surface of leaves long after bloom. These epiphytic bacteria might pose a serious risk for shoot infections (Figure 3).

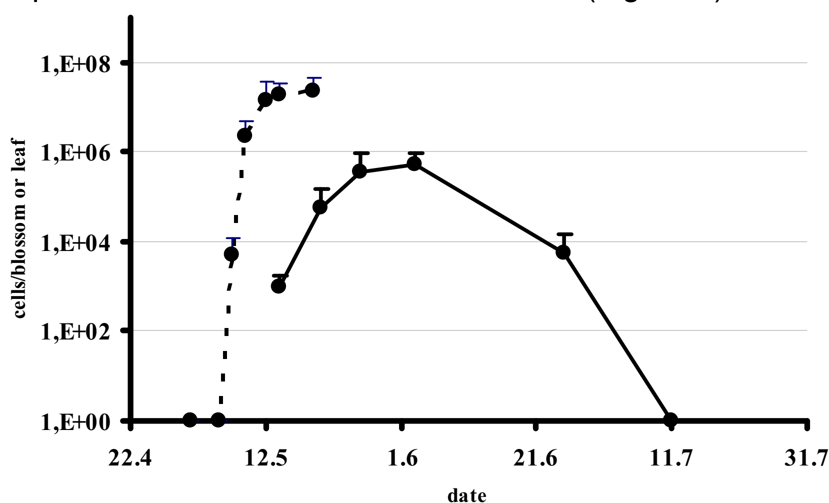

Figure 3: Quantification of *E. amylovora* in blossoms and on the surface of leaves in the orchard Höchst (1), Austria in 2008. ▪▪▪ in blossoms, ▬ on leaves.

Significant numbers of bacteria were also detected in fruit mummies (Figure 4). Aside from cankers, fruit mummies remaining on the tree or on the ground might also pose a noteworthy source of inoculum early in the growing season. Bacteria might easily be transferred from them to blossoms in their vicinity by insect vectors or rain splash.

The number of bacteria detected epiphytically on apple leaves and the number of bacteria detected in fruit mummies show a high degree of correlation with the amount of bacteria detected in blossoms (Figure 5).

Therefore, monitoring of fruit mummies might be a valuable alternative to the monitoring of blossoms in order to determine the fire blight hazard for an orchard. Fruit mummy monitoring would allow a risk assessment month before the onset of bloom.

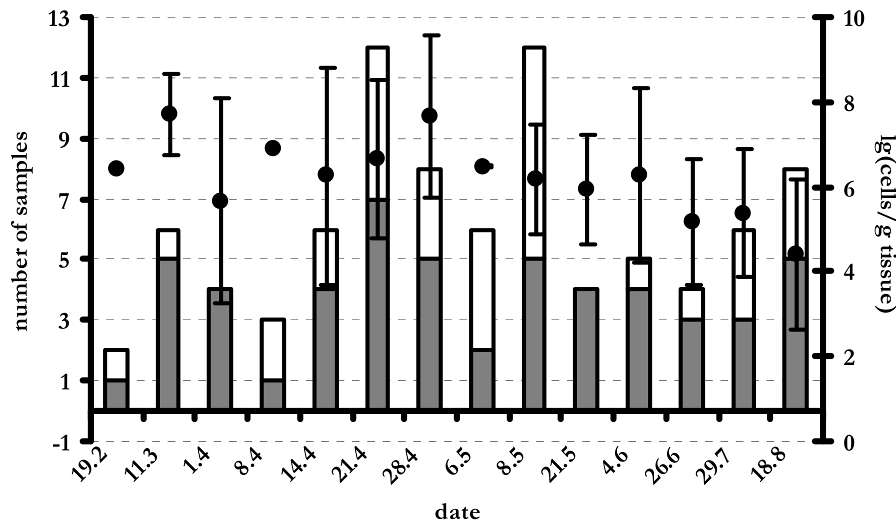

Figure 4: Quantification of *E. amylovora* in fruit mummies of the cultivar Elstar in the orchard Höchst (1), Austria in 2008. ☐ total number of samples, ■ number of samples tested positive, ● mean number of cells detected in the positive samples +/- standard error.

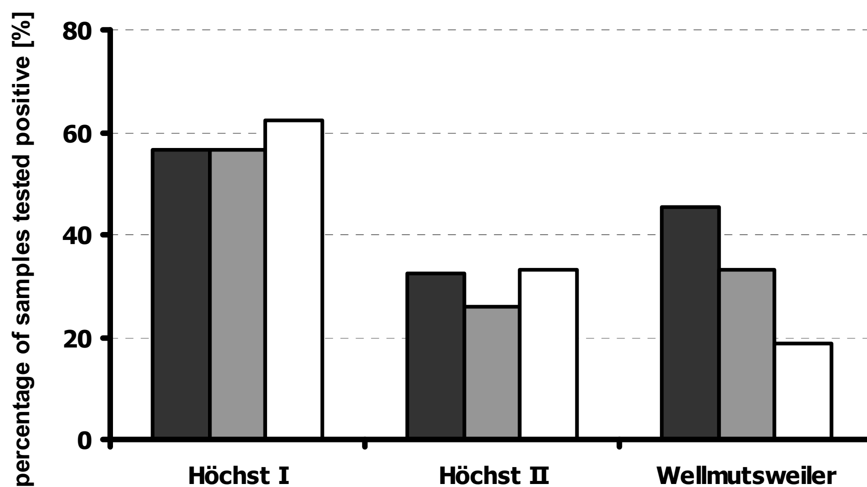

Figure 5: Correlation of detection of *E. amylovora* in blossoms ■, on apple leaves ■, or in fruit mummies ☐ for three different locations in 2008.

A comparison of blooming period, CDH18, pathogen abundance and the degree of infestation during the years 2008 and 2009 might provide an explanation for the differences in fire blight severity in these years (Figure 6). For both years an increase in bacteria correlated with an increase in CDH18, as expected. However, in 2008 (Figure 6a) much higher numbers were reached compared to 2009 (Figure 6b). The main difference between the two years which showed a degree of infestation of 20.5 % for 2008, and less than 0.01 % for 2009, is the percentage of open flowers during the period of time in which the pathogen amount increased dramatically. While in 2008 there were still a significant

amount of new blossoms available when the CDH18 crossed the critical mark of 110 (and the pathogen was detected for the first time (08.05.2008)), in 2009 almost all blossoms were already open When the CDH18 hit the critical mark of 110 (08.05.2009).

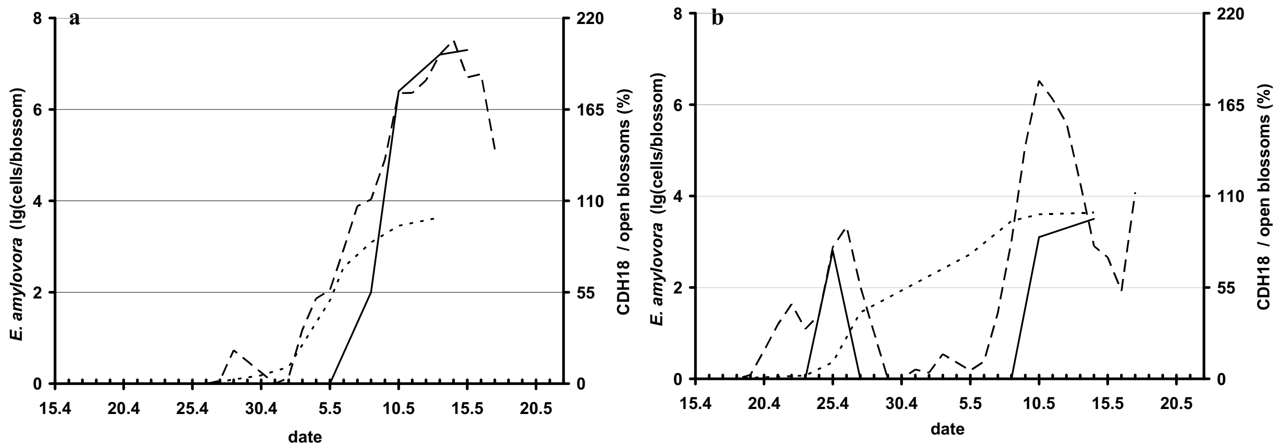

Figure 6: Correlation of amount of *E. amylovora* in blossoms ━ , CDH18 ━ ━, and percentage open blossoms ▪▪▪ for the years 2008 (a) and 2009 (b) in the orchard Höchst (1), Austria.

Pathogenesis. A third vital aspect of our work on fire blight is the appraisal of bacteria spreading in host tissue without causing visible symptoms. For this purpose young apple shoots of the cultivar Jonagold were artificially inoculated. Shoots were examined visually for symptoms and *E. amylovora* quantified by Real Time PCR (Figure 7).

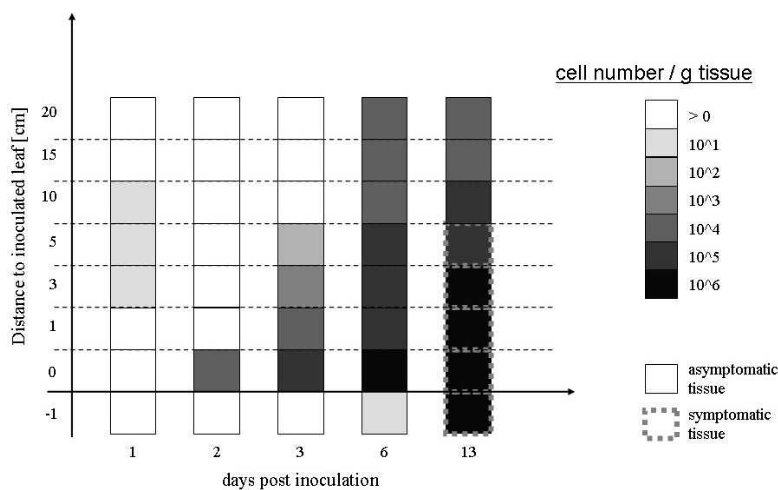

Figure 7: Symptomless spread of *E. amylovora* in young apple trees of the cultivar Jonagold after artificial shoot inoculation.

Our results show that significant amounts of bacteria, up to 10^6 cells per gram of tissue, could be detected in samples exhibiting no visible symptoms. The pathogen seems to be able to migrate and multiply within the shoot and can be detected in a distance of 20 cm six days after inoculation. Symptoms became visible only after 13 days and lacked far behind pathogen detection. Spread of bacteria down the shoot seems to proceed faster than progress towards the shoot tip.

Discussion

Since 2006 we have monitored the abundance of *E. amylovora* in a set of commercial orchards in the Lake Constance region. Our on site, no sample processing Real Time PCR approach allows the reliable detection of bacteria down to concentrations of 200 cells/blossom in less than two hours. Real Time PCR based monitoring shows a good correlation with symptom development. Moreover, our methodology allows for an early assessment of the risk of fire blight. This method thus is far superior to other rapid test like the Agristrip (Bioreba AG, Reinach, Switzerland). An integration of the quantitative compilation of pathogen abundance into advancements of the Maryblyt predictive computerized forecasting system is in progress.

To our knowledge there has been no study regarding the epiphytic development of *E. amylovora* on non-host plants. The fact that *E. amylovora* seems to be able to propagate in blossoms of several non-host plants suggests that these plants, too, should be monitored with respect to their role as potential inoculum source.

Results from our analyses of epiphytic persistence of E. amylovora corroborate earlier findings that apparently no multiplication of bacteria takes place on leaf surfaces (Thomson et al. 1975; Maas Geesteranus and de Vries 1984; Ockey and Thomson 2006). On the other hand, bacteria seem to persist for a prolonged period of time on the surface of leaves, and thus pose a significant threat to shoot infections after wounding.

Previous studies have negated a role of fruit mummies in the dissemination of fire blight bacteria (Anderson 1952; Goodman 1954). Our results clearly indicate a prominent role of these structures for early dispersion of the pathogen. At the same time, these structures may be used to assess the risk of fire blight at a very early stage during the growing season.

Finally, our results regarding the endophytic spread of bacteria without causing symptoms should result in adjusted recommendations regarding good cultural practice.

It seems highly unlikely that we will be able to completely eradicate *E. amylovora*, therefore, a further broadening of our knowledge on the epidemiology and pathogenesis of the bacterium, together with a vigilant monitoring procedure seems to be qualified to enable prudent disease management.

Acknowledgements

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 „Feuerbrand“ to RTV.

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