Cultivar testing, pathogenesis and quantitative distinction of live and dead cells of *E. amylovora*

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Abstract

Fire blight, caused by the Gram-negative bacterium Erwinia amylovora, *is the most devastating bacterial disease of pome fruit. In order to identify standard cultivars less susceptible to fire blight, we tested 39 old standard apple cultivars from the Lake Constance region using artificial shoot inoculation and subsequent visual rating over a period of four weeks. Since it was previously shown that there is a built up of large bacterial populations in asymptomatic tissue, we also quantified the pathogen load within shoots of Regalis® treated and untreated trees of the apple variety Jonagold. A possible explanation for the detected high bacterial load might be a high number of dead* E. amylovora *cells in the infected asymptomatic tissues. Thus, we developed methods for a Live/Dead-distinction of* E. amylovora*. The most promising method is based on the combination of Real Time PCR and FACS-analyses.*

Keywords: *E. amylovora,* live and dead cells, pathogenesis, Real Time PCR

Introduction

The most harmful bacterial disease throughout the world in economical important rosaceous plants (e.g. apple) is fire blight (Stöger *et al.* 2006). The causative agent of this disease is the Gram-negative bacterium *Erwinia amylovora* which is peritrichous flagellated and surrounded by a mucilaginous capsule. Symptoms of fire blight are the discoloration of infected twigs, leafs or blossoms, the appearance of exudate and the typical deformation of infected shoots, formally known as “sheperd’s crook”. Until now no resistance against fire blight is reported. Therefore, the search for at least less susceptible cultivars is a very important part of the fight against fire blight. To investigate the susceptibility of cultivars, Real Time PCR is a very powerful method to get an overview of the degree of infection and the quantity of the pathogen (Higuchi *et al.* 1993), and to investigate the susceptibility of cultivars. In reference to Salm and Geider (2004), we used whole bacteria extracted from shoot samples from pathogenesis-experiments. During Real Time PCR analysis of field samples we detected very high cell numbers in asymptomatic tissue. These findings suggest that there are large numbers of living and dead cells accumulated in the samples tested. This phenomenon is called latent infestation. The fact that Real Time PCR is not capable to discriminate between live and dead bacteria might lead to a significant overestimation of the effective pathogen load (Weißhaupt 2008). Based on a modified Real Time PCR and FACS-analysis we are able to clearly discriminate between live und dead cells and therefore exclude „false positive“ estimates of pathogen load.

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Material and Methods

Cultivar testing and pathogenesis:

For the testing of different cultivars and pathogenesis experiments we used freshly grafted apple trees provided by the Kompetenzzentrum Obstbau Bodensee (KOB) within the Interreg IV project „Gemeinsam gegen Feuerbrand“. All experiments were done in the greenhouse using the following parameters: 12 h light and 27 °C/15 °C day/night temperature. At the beginning of the experiments apple trees were needle-inoculated with an *E. amylovora* suspension containing 10^9 cells per ml. The experimental setup for cultivar testing was composed of 29 different apple cultivars, each represented by 10 plants. Determination of different symptoms caused by *E. amylovora* was done by visual rating four times in weekly intervals starting one week after inoculation. Absolute shoot length and length of lesion were measured.

Within the pathogenesis experiments an untreated apple cultivar was tested in comparison to a cultivar treated with the growth regulator Prohexadion-Ca (BASF). This regulator is supposed to have an influence on the course of infection (Bubán *et al.* 2004). Treatment was done 14 days and 1 day before inoculation, according to the recommendation of BASF (2009). Each time 1.6 g Regalis®-granulate were dissolved in 1000 ml water. Shoots were cut 2, 3, 4, 6 and 12 days post inoculation. Cut shoots were processed as described by Voegele *et al*. (2010). After processing the samples were analysed by Real Time PCR.

Quantification:

Real Time PCR analyses were performed as described by Voegele *et al*. (2010).

Live/Dead-Distinction:

For Live/Dead-distinction the Real Time PCR method was modified by the addition of Propidium Monoazide (PMA). Since PMA binds covalently to DNA of dead cells, the amplification and quantification during Real Time PCR is restricted to DNA of living cells (Nocker *et al.* 2006; 2007). Unbound PMA was photo-inactivated with a 500 W spotlight positioned in a distance of 20 cm and 5 min exposure-time (Nocker *et al.* 2006). The modified Real Time PCR was performed with whole *E. amylovora* cells and purified plasmid DNA to determine if there are significant differences. Plasmid DNA was purified after 10 min PMA-treatment of the bacterial culture.

Another method based on intercalating dyes and labelled antibodies was also used: FACS-analyses. In this method dead bacteria were stained with Propidium Iodide (Boulos *et al.* 1999). *E. amylovora* was detected using a specific primary and a Fluorescein Isothio-cyanate-labeled secondary antibodies. This allowed the differentiation of living (green) and dead (red and green) *E. amylovora* cells.

Results

Our work on *E. amylovora* principally focussed on three main aspects: a) susceptibility of different apple standard cultivars (Cultivar Testing) b) description of the quantitative distribution of *E. amylovora* within tissue with or without symptoms (Pathogenesis) c) distinction of live and dead cells in environmental samples (Live/Dead-Distinction).

Cultivar Testing. Against the background of field experience about the susceptibility of different apple cultivars, we started to screen several old apple standard cultivars from the Lake Constance region.

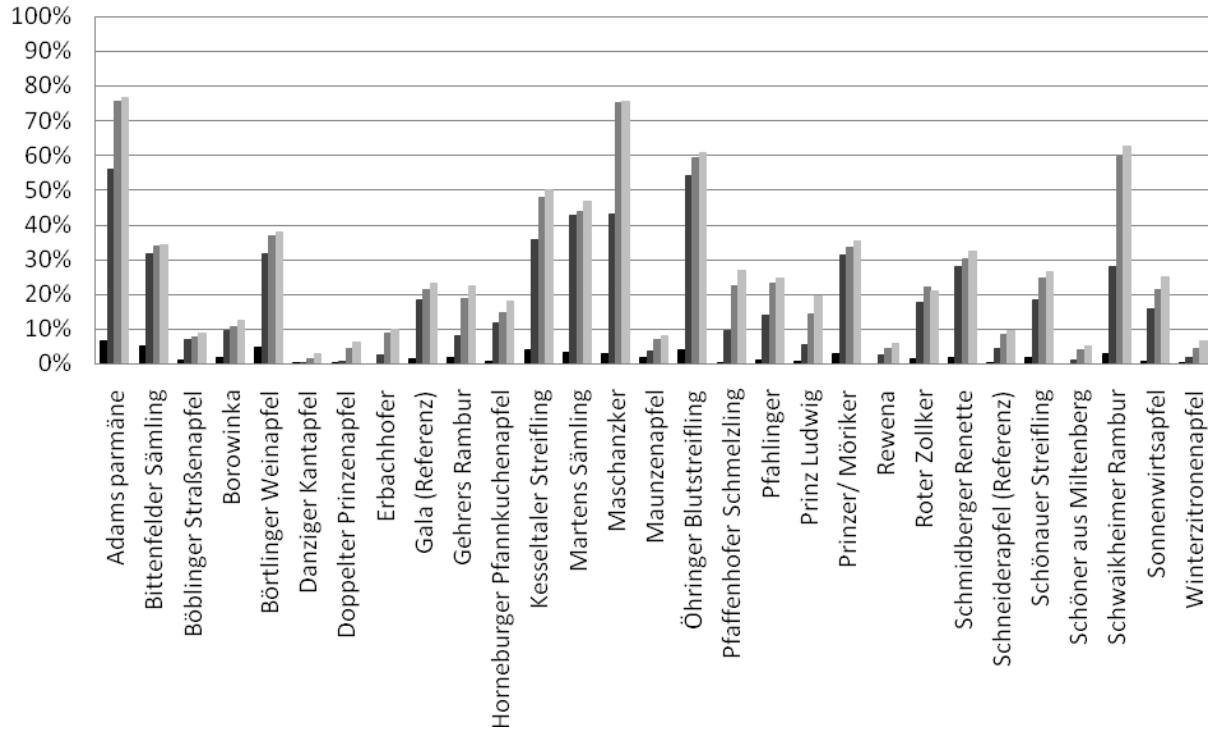

Figure 1**:** averaged relative length of lesion (%) of apple cultivars (2010)
x-axis: standard cultivars;■ 1 week post inoculation (wpi),■ 2 wpi,
■ 3 wpi,□ 4 wpi

During visual rating in 2010 we observed the highest increase in lesion-length during the second week post inoculation for most of the cultivars (Figure 1). Within the third and fourth rating only slight increases were recognised except for Adamsparmäne, Börtlinger Weinapfel, Maschanzker and Schwaikheimer Rambur.

In 2010 the cultivars tested most susceptible were: Adamsparmäne, Bittenfelder Sämling; Börtlinger Weinapfel, Kesseltaler Streifling, Martens Sämling, Maschanzker, Öhringer Blutstreifling, Prinzer/Möriker, Schmidberger Renette and Schwaikheimer Rambur.

In 2011 (Figure 2) we retested 19 cultivars, including the references Gala and Schneiderapfel, to validate our results from 2010. Additionally, we tested ten other standard cultivars. The higher increase in lesion-length during the first week post inoculation, in comparison to 2010, is due to an upgrade of the greenhouse lightning.

As shown for 2010 we also observed the highest increase in lesion-length in 2011 during the second week post inoculation.

The cultivars tested most susceptible in 2011 are: Aargauer Jubiläumsapfel, Fießers Erstling, Grüner Stettiner, Kronprinz Rudolf, Oberländer Himbeerapfel, Öhringer Blutstreifling, Pfaffenhofer Schmelzling, Prinz Ludwig and Sonnenwirtsapfel.

The cultivars tested least susceptible over two years are: Böblinger Straßenapfel, Doppelter Prinzenapfel, Glockenapfel, Rewena and Schöner aus Miltenberg.

Thus, we have successfully verified large parts of the results of 2010. We retested some cultivars, e.g. Doppelter Prinzenapfel or Winterzitronenapfel, less or least susceptible. We also retested some cultivars most susceptible in similar ranges, e.g. Öhringer Blutstreifling. However, for some cultivars conflicting results were obtained in 2011. The cultivar

Danziger Kantapfel for example was tested least susceptible in 2010 and intermediate susceptible in 2011.

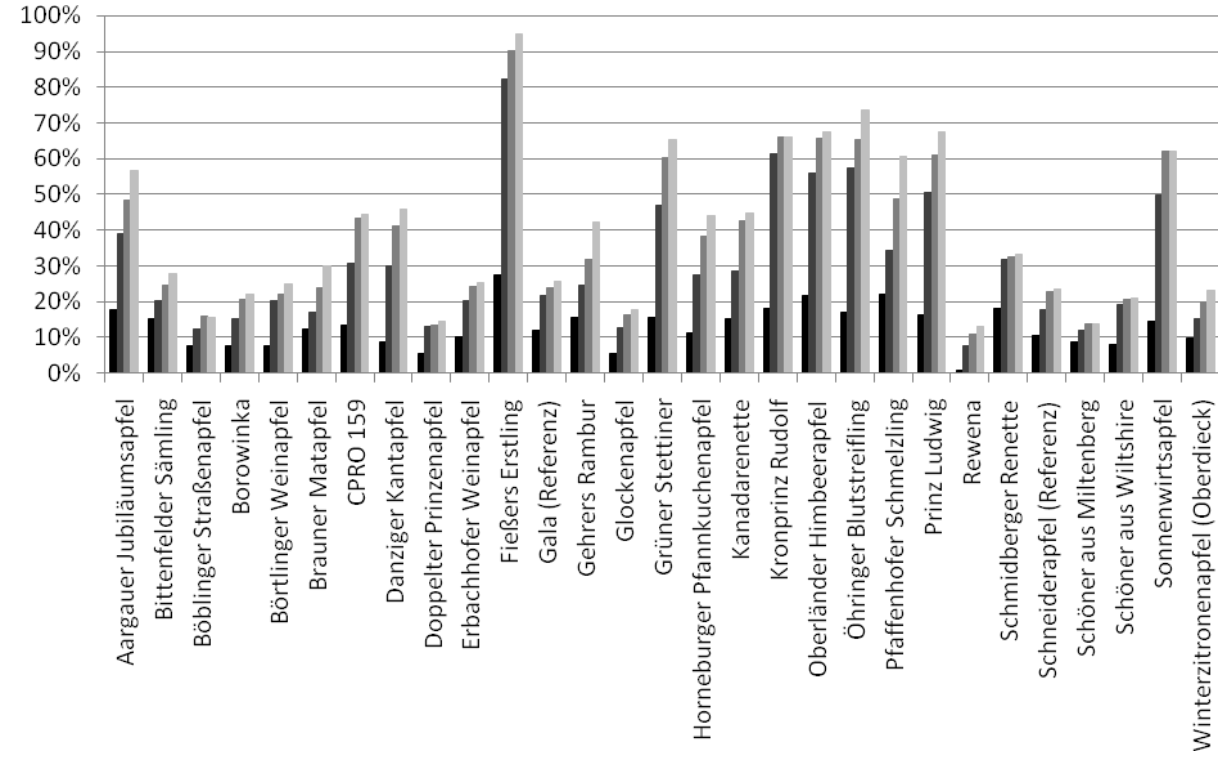

Figure 2**:** averaged relative length of lesion (%) of apple cultivars (2011)
x-axis: standard cultivars; ■ 1 week post inoculation (wpi), ■ 2 wpi, ■ 3 wpi, □ 4 wpi

Pathogenesis. As expected there was an observable difference between untreated and treated trees. Trees treated with Prohexadion-Ca showed reduced shoot growth and thickened shoots in comparison to the untreated trees.

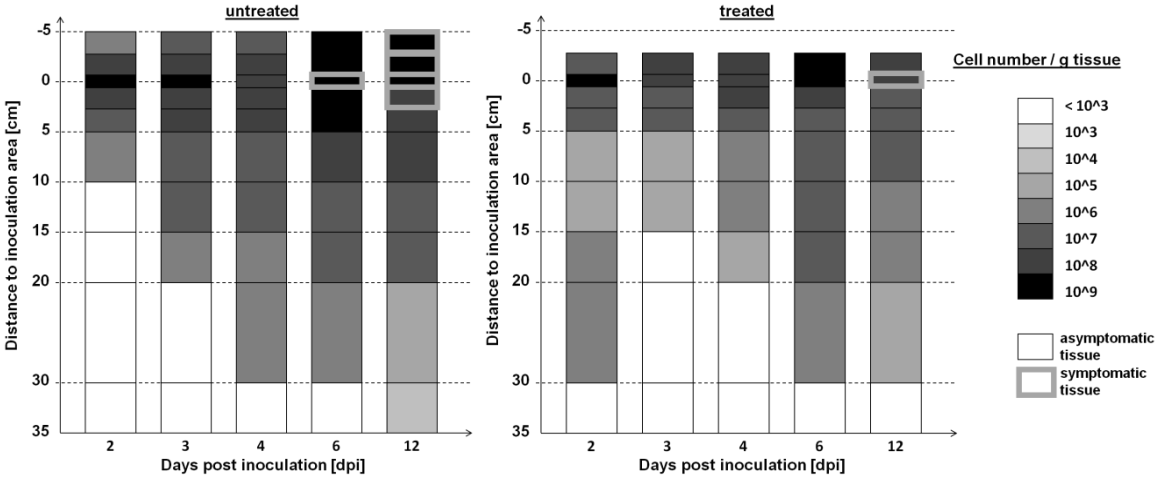

Figure 3: Spread of *E. amylovora* in young apple trees of the cultivar Jonagold after artificial shoot inoculation. 25 trees untreated/treated, 10 trees control; each time point shows the average of 5 shoots
Left: untreated apple trees, Right: Regalis® treated apple trees

With respect to symptoms, there is a remarkable influence of the treatment. In untreated trees symptoms appeared after 6 days post inoculation whereas symptoms only appeared after 12 days post inoculation in treated trees. However, cell numbers were very similar in both setups.

Live/Dead-Distinction. During our efforts to distinguish between live and dead cells we were able to inhibit the amplification of dead cell DNA using PMA in Real Time PCR.

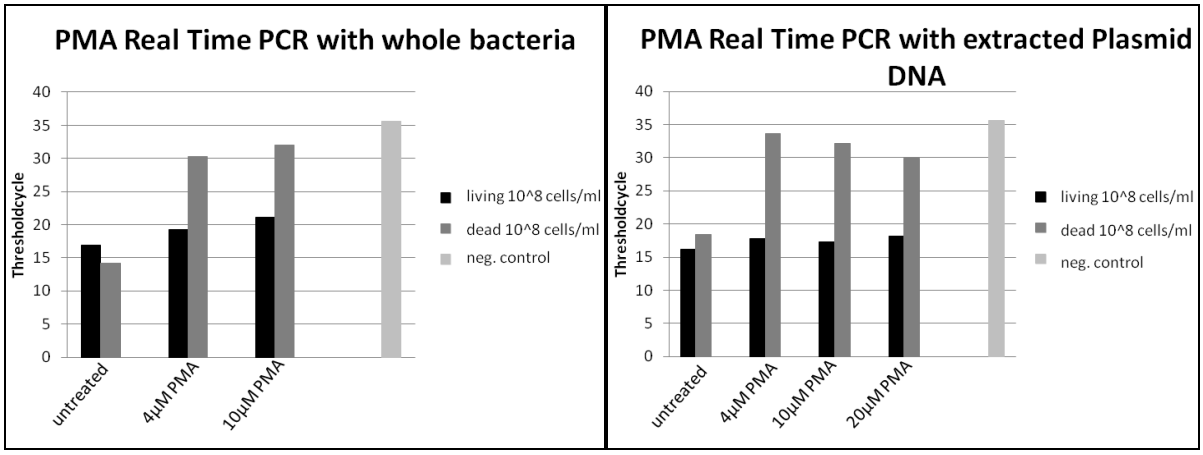

Figure 4: PMA Real Time PCR with whole bacteria and extracted Plasmid DNA
Left: living and dead whole bacteria tested untreated and treated with 4 µM and 10 µM PMA, Right: extracted Plasmid DNA of living and dead bacteria, which were untreated or treated with 4 µM, 10 µM and 20 µM PMA prior to DNA extraction

As shown in Figure 4 there is only a small difference between thresholdcycles (cts) of untreated samples. However in the treated samples there is a remarkable shift of about 12 ct values for whole bacteria and 16 to 12 ct values for extracted DNA with increasing PMA concentration. This means that the amplification of DNA arising from dead cells was inhibited by the PMA treatment. There is no difference between the 4 µM and the 10 µM PMA treatment for reactions with whole bacteria. For purified DNA best effects were achieved using a PMA concentration of 4 µM.

During the development of the FACS-analyses method for Live/Dead-Distinction we succeeded to calibrate the Flow-cytometer (FACSCalibur, BD Biosciences) for Live/Dead-staining. The calibration was done using pure cultures stained with Propidium Iodide (Boulos *et al.* 1999) and a specific primary and a Fluorescein Isothio-cyanate-labeled secondary antibody. After the calibration was done field samples were tested (Figure 5). These included a visually symptomatic Sonnenwirtsapfel (Erwinia.008) and a visually asymptomatic one (Erwinia.008.2).

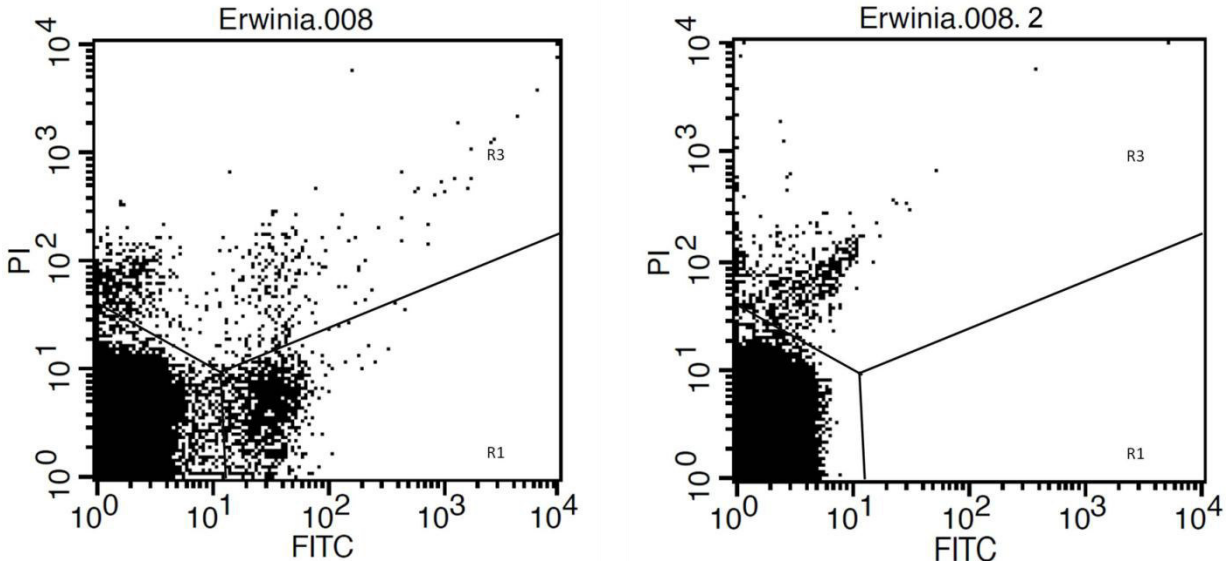

Figure 5: Environmental samples measured by flow cytometry.
Left: symptomatic apple tree, the number of detected bacteria (5510) is composed of 89 % foreign bacteria, 9 % live (R1) and 2 % dead (R3) *E. amylovora*.
Right: asymptomatic apple tree, *E. amylovora* not detectable.

Comparing both diagrams in Figure 5 one notices that the most hits/bacteria are positioned in the lower left corner. This is caused by the fact that these bacteria are unstained and therefore represent “other living bacteria” (not *E. amylovora)*. Green stained and therefore living *E. amylovora* would be positioned in the lower right corner (R1). In the symptomatic sample (Erwinia.008) 9 % bacteria stained green, no living *E. amylovora* cells were found in asymptomatic sample (Erwinia.008.2). Dead and therefore red stained bacteria would be positioned in the upper left corner and green-red stained (dead *E.amylovora*) in the upper right corner (R3). Thus, in the symptomatic sample there were all together 89 % foreign bacteria detected and 2 % dead *E. amylovora*. Contrary to this there were no *E. amylovora* detected at all in the asymptomatic sample.

These results show that we are able to detect *E. amylovora* by FACS-analyses and additionally to distinguish between different bacteria as well as living and dead cells.

Discussion

The cultivars tested less and least susceptible during our cultivar testing will be analysed by Real Time PCR to get an idea of the pathogen spread inside shoots. Probably the trees appear only less susceptible while being highly infested. In parallel, an analysis of physiological and metabolic differences between less and highly susceptible apple cultivars will be forced initiated to determine the reason for less susceptibility.

Comparing the results obtained in 2010 and 2011, several re-tested cultivars showed different behaviour. Some tested less susceptible in 2010 but intermediate or highly susceptible when re-tested in 2011 and vice versa.

In order to get a stable reproducible test-setup, the reason for these differences has to be identified. Probably the differences observed, are due to the upgrade of the greenhouse lightning.

In the pathogenesis experiments we could show, that there is no crucial difference between cell numbers in Regalis® treated trees compared to untreated. We will proceed with analysing trees from cultivar testing. Physiological and metabolic analyses of cultivars treated with Regalis® in comparison to untreated ones will also be performed.

As shown in the results of Live/Dead-Distinction we are able to nearly suppress the complete signal of dead cell DNA. This method in combination with Flow-cytometry gives us a tool at hand to analyse latent infections on their pathogenic potential because Flow-cytometry was found to provide highly reproducible, statistically safeguard data. With respect to this fact and a possible automation of the Flow-cytometry protocol this method can also be used for high throughput analyses of environmental samples.

Acknowledgements

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