Fire blight plant protection efficacy trial with resistant apple cultivar ‘Ladina’

Vanessa Reininger¹ · Anita Schöneberg¹ · Eduard Holliger¹

Received: 15 April 2020 / Accepted: 12 December 2020 / Published online: 16 June 2021
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Abstract

Fire blight is a devastating disease of apple, pear and quince caused by the Gram-negative bacterium *Erwinia amylovora*. As antibiotics are not allowed against fire blight in Switzerland, optimization of alternative plant protection strategies is required. In this context, fire blight tolerant cultivars become of higher importance to control the disease. A plant protection product efficacy trial against fire blight with the susceptible cultivar ‘Gala Galaxy’ and the tolerant cultivar ‘Ladina’ bred at Agroscope was set up at the Agroscope Steinobstzentrum Breitenhof in 2018. Four different treatment strategies, including an untreated control, were tested on both cultivars after inoculation with *Erwinia amylovora*. ‘Ladina’ showed less fire blight blossom cluster infection in untreated and treated plants compared to ‘Gala Galaxy’. Blossom cluster infection rates following different plant protection treatments did not differ significantly from each other in ‘Gala Galaxy’ whereas they did in ‘Ladina’.

Keywords *Erwinia amylovora* · Fire blight · ‘Gala galaxy’ · ‘Ladina’ · Tolerant apple cultivar · Plant protection strategies

Introduction

Fire blight is a devastating disease affecting pome fruit production. *Erwinia amylovora* is a Gram-negative bacterium causing fire blight within the Rosaceae family on crops such as apple, pear, and quince (Vanneste 2000; Van der Zwet et al. 2012). The first fire blight incidence in Switzerland was observed in 1989 around Lake Constance on *Cotoneaster* spp. (Duffy et al. 2005) with a peak in 2007/2008 and incidence declining ever since. Owing to its distribution in Switzerland, fire blight no longer meets the criteria for regulation as a quarantine organism (exception: protected zone in the canton of Valais). In 2018, the European and Mediterranean Plant Protection Organization (EPPO) recommended regulation of the bacterium as a regulated non-quarantine pest (RNQP) on known host plant products (*Malus*, *Pyrus*, *Cydonia*, *Sorbus* etc.). These plants must still be free from fire blight if they are intended for commercial

use (agricultural fruit production, ornamental plants). With the exception of the protected zone (canton of Valais), fire blight is no longer subject to notification or control obligations. However, fire blight will still be a major concern in pip-fruits production as it is one of the most devastating bacterial diseases. As no antibiotics have been allowed in pip-fruits production in Switzerland from 2016 on, effective alternative plant protection strategies against fire blight are needed as stated by Sundin et al. (2016). Up to date there is authorized products available showing partial efficacy only (Reininger et al. 2017a, b, c; Stockwell et al. 2010). In this context, also tolerant cultivars will become of more importance to apple production (Sundin et al. 2016; Kellerhals 2017; Kellerhals et al. 2017; Silvestri and Egger 2011). One of those fire blight tolerant cultivars is ‘Ladina’, a breed from the research institute Agroscope in Wädenswil, Switzerland (Kellerhals et al. 2014; Leumann et al. 2013).

The purpose of this study was to determine efficacy of several non-antibiotic plant protection agents against fire blight on apple trees of the cultivars ‘Gala Galaxy’ and ‘Ladina’ in an outdoor, enclosed field site.

✉ Vanessa Reininger
vanessa.reininger@agroscope.admin.ch

¹ Müller-Thurgau Strasse 29, 8820 Agroscope, Wädenswil, Switzerland

Materials and methods

Experimental design

The efficacy of several plant protection treatments using antagonists was tested on potted three-year old ‘Gala Galaxy’ and ‘Ladina’ trees on rootstock ‘M9’. Trees were potted in 10 l containers, placed into an enclosed field site with a black insect-proof mesh around (1 x 2 mm) and a hail mesh on top. Trees were drip-irrigated as needed without fertilizer and looked vigorous throughout the experiment. A total of 288 potted trees were arranged in a randomized complete block design, with six replicate blocks including 36 trees per treatment (six experimental trees per block). Trees were spaced one by three meter. A seventh, untreated but inoculated ‘Gala Galaxy’ tree was placed in the middle of each block for the sole purpose of inoculation (see below) (Fig. 1). This tree was excluded from rating and sampling. In compliance with strict biosafety regulations, experiments in this enclosed field site are allowed to be inoculated with *Erwinia amylovora*.

Pathogen inoculation

Nearly at full bloom, the middle tree in each block was sprayed with 135 ml of a 1×10^9 cfu/ml suspension of a

1:1:1 mixture of three strains of *E. amylovora* (Ea 66116, Ea 66035 and Ea L 610/03/2013) isolated from *Malus*. *E. amylovora* stains were grown on King’s B media in Petri dishes for 48 h at 28 °C in an incubator. Bacterial cells were washed off and diluted to the respective concentration using a 0.8% NaCl solution. A bumblebee population big enough to pollinate our field site was placed into the experimental site allowing pollination and inoculation of the non-inoculated trees and their newly opened flowers with *Erwinia amylovora* on the experimental trees. As flowers last for only a few days newly opened flowers continuously serve as new inoculum source. Inoculated trees showed no symptoms during flowering yet but when flower clusters were counted for disease incidence rating on May 25th those inoculated trees were in bad condition.

Treatments

The dates of applications of treatments are listed in Table 1 and the treatments for the experiment are presented in Table 2. The plant protection products LMA and BlossomProtectTM used in this study are commercially available in Switzerland via Omya and Andermatt Biocontrol, respectively. Plant protection products were applied using a motorized backpack sprayer (Birchmeier Sprühtechnik AG, Stetten, Switzerland) applying approx. 150 ml per tree (corresponds to 500 liters per hectare)

Fig. 1 Schematic illustration of our randomized complete block design showing six single replicates of Treatment I. Each of those blocks or single replicates includes an inoculated tree in the middle (illustrated in red) and six experimental trees (illustrated in green)

Table 1 Treatment protocol for the fire blight efficacy experiment in 2018. Applications were conducted after sampling for cfu determination via real-time PCR. First treatment with antagonists took place already at the day of inoculation: Blossom Protect™ and Pomavita (April 24th)

	Inoculation with <i>E. amylovora</i>	1. Application	2. Application	3. Application
Experiment I	April 24 th (1 x 10 ⁹ cfu/ml)	April 25 th	April 27 th	April 29 th

and half of the recommended amount of plant protection product to account for small crown sizes in this field experiment. Inoculated middle trees were non-treated. The first application took place three hours after inoculation of trees with *E. amylovora* (Table 1). Control trees were not treated at all.

Disease incidence rating

Flower clusters were counted at the beginning of the experiment (full bloom) to get the total for each tree. About four and a half weeks after pathogen inoculation (May 25th), when fire blight symptoms were clearly visible, infected and healthy flower clusters were counted. On average 2600 flower clusters were counted per treatment for ‘Gala Galaxy’ and 1700 flower clusters for ‘Ladina’ (36 trees per treatment). Disease incidence and control efficacy according to Abbott (Abbott 1925) were calculated from means of each block (six trees per block) using the following formulas:

i. **Percent disease [%]**

(Total number of flower clusters with fire blight symptoms / Total number of flower clusters at full bloom) × 100

ii. **Control efficacy [%] (according to Abbott(1925))**

((Percent disease on control tree – Percent disease on treated tree) / Percent disease on control) × 100

Pathogen epidemiology on flowers

Pathogen population dynamics on flowers during the experiment were investigated at four times after the inoculation using real-time PCR. The first sampling represents the baseline, i.e. the starting number of *E. amylovora* cells three hours post inoculation. The untreated control (T1 and T5) and the LMA strategy (T2 and T6) were sampled in both cultivars (see Table 2). Twenty-four flowers per sample (~ 24 till 48 h old to already show a pathogen history, means not recently opened flowers) were harvested (petals removed) from twelve replicate trees receiving the same treatment and stored at 4 °C until further processing the next morning. Per flower 1 ml PBS was added and flowers were put into an ultrasonic water bath for 4 min to transfer cells into the liquid.

For real-time PCR analysis, DNA was extracted using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) on a BioSprint 96 workstation (Qiagen, Hilden, Germany). Samples were prepared as follows: 1.5 ml-Eppendorf tubes containing 500 µl sample were centrifuged at 9,900 g for 11 min and the supernatant was discarded. The pellet was re-suspended in 300 µl RTL buffer (lysis buffer) and vortexed for 20-30 seconds. Tubes were put into an incubator-shaker at 65 °C at 1,000 rpm for 30 min and subsequently centrifuged at 6,000 g for 5 min. The BioSprint 96 was loaded according to the manufacturer’s protocol using 100 µl elution buffer instead of 200 µl and ‘BS96 DNA Plant’ was chosen as extracting program.

Table 2 Treatments and their products with blossom cluster infection and efficacy data for the fire blight experiment 2018. Treatments were conducted three times after inoculation with *E. amylovora* (see Table 1). Different letters behind efficacy values indicate statistically

significant differences due to the treatments according to Tukey’s HSD ($p \leq 0.05$). * Amount of product for three-year-old potted trees per hectare. ** This amount corresponds to the active ingredient

ID	Cultivar	Product	Active ingredient	Amount of Product*	Blossom cluster infection [%] +/- SE	Efficacy [%]
T 1	Gala	Untreated Control	-	-	14.3 ± 1.3	
T 2	Gala	LMA	Potassium-aluminum-sulfate (80%)	10 kg	11.9 ± 1.2	16.9 (a)
T 3	Gala	Blossom Protect™	<i>Aureobasidium pullulans</i> (5 × 10 ⁹ cfu/g)	6 kg	7.7 ± 0.8	46 (a)
T 4	Gala	Pomavita	<i>Pantoea agglomerans</i>	1.5 g/6L**	10.9 ± 1.6	22.6 (a)
T 5	Ladina	Untreated Control	-	-	6.4 ± 1.1	
T 6	Ladina	LMA	Potassium-aluminum-sulfate (80%)	10 kg	0.6 ± 0.2	94.8 (a)
T 7	Ladina	Blossom Protect™	<i>Aureobasidium pullulans</i> (5 × 10 ⁹ cfu/g)	6 kg	0.3 ± 0.2	97.8 (a)
T 8	Ladina	Pomavita	<i>Pantoea agglomerans</i>	1.5 g/6L**	1.8 ± 0.3	87.3 (b)

The *amsC* gene, which is involved in the synthesis of the polysaccharide amylovoran, is supposed to be unique to *E. amylovora* (Bugert and Geider 1995; Bereswill et al. 1995). Thus, we used those very specific *Ams* primers developed by Pirc et al. (2009) for our real-time PCR analysis (Table 3). Each reaction contained 20 µl composed as follows: 2 µl ddH₂O, 10 µl TaqMan® Environmental Master mix 2.0 (Applied Biosystems by life technologies, Warrington, UK), 1.8 µl primer *Ams* 116F and *Ams* 189R each (10 µM) (Sigma-Aldrich, Buchs, Switzerland), 0.4 µl TaqMan® probe *Ams* 141T (10 µM) (Sigma-Aldrich, Buchs, Switzerland) and 4 µl DNA. Universal cycling conditions were used [2 min at 50°C, 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C] and samples run in duplicates on a ViiA™ 7 Real-time PCR machine (Applied Biosystems, Carlsbad, USA). A standard curve with known amounts of DNA run on each plate in triplicates to quantify DNA in our samples. Mean amplification efficiency of real-time PCR was 95%.

Statistical analysis

Statistically significant differences were calculated running a linear mixed effects model (lme) using sqrt-transformed or non-transformed data for every single tree, followed by a

Table 3 Primer and TaqMan® probe for real-time PCR amplification of the *amsC* gene in *E. amylovora* (Pirc et al. 2009)

Name	Sequence (5′ – 3′)	Amplicon length
<i>Ams</i> 116F	TCC CAC ATA CTG TGA ATC ATC CA	74 bp
<i>Ams</i> 189R	GGG TAT TTG CGC TAA TTT TAT TCG	
<i>Ams</i> 141T	FAM-CCA GAA TCT GGC CCG CGT ATA CCG-TAMRA	

Tukey's HSD Test for pairwise comparisons between treatments in the statistics software R (R Core Team 2014). In the lme model, efficacy was tested in dependence of block treatment and cultivar. For real-time PCR data a pairwise t-test was run for each sampling day.

Results

Disease incidence

Experimental conditions in April/May 2018 at the Agroscope experimental fire blight field site in Wintersingen, BL were conducive for *E. amylovora* infection (Fig. 2). Blossom cluster infection in the untreated 'Gala Galaxy' control

Fig. 2 Weather conditions and EIP-values (Epiphytic Infection Potential) during the experiment in 2018 in Wintersingen, Switzerland. The Maryblt prediction model was used and the EIP-value threshold predicting an infection event was set to 110. No infection days were predicted for the experiment. Timing of artificial inoculation of the

middle trees with *E. amylovora* is symbolized by the red arrow; green arrows symbolize timing of post-inoculation treatments with plant protection products. Antagonists were applied the first time at the day of inoculation

Fig. 3 Boxplots showing disease incidence [%] in both cultivars ‘Gala Galaxy’ and ‘Ladina’ according to different plant protection treatments. Each box consists of 36 single trees, therefore those boxplots represent raw data. Efficacy values were calculated according to Abbott. Infestations correspondss to blossom cluster infection. The boxplots are drawn according to Crawley (2007): The bold horizontal line shows the median. The bottom and top of the box show the 25th and 75th percentiles, respectively. The vertical dashed lines (‘whiskers’) show either the maximum value or 1.5 times the interquartile range of the data, whichever is the smaller

was 14.3% whereas it was 6.4% in ‘Ladina’ (see Table 2 and Fig. 3). The European and Mediterranean Plant Protection Organization (EPPO) standard 1/166(3) blossom cluster infection rates (min. 5% fire blight blossom cluster infection in the untreated control) were met in this experiment for both cultivars (Bulletin 2013). Fire blight blossom cluster infection in treatments with plant protection products was significantly lower compared to the control both in ‘Gala Galaxy’ ($p < 0.001$) and in ‘Ladina’ ($p < 0.001$). There were no significant differences in terms of efficacy

between treatments in ‘Gala Galaxy’ but for ‘Ladina’, as the antagonist product Pomavita showed a significantly lower efficacy than LMA and Blossom ProtectTM. Efficacy values for ‘Ladina’ were relatively high ranging from 87.3% to 97.8% compared to a maximum of 46% in ‘Gala Galaxy’ (Table 2 and Fig. 3). The most effective plant protection product for both cultivars was Blossom ProtectTM. Due to natural experimental conditions, variation between replicates was high, especially for lower efficacy values, resulting in a rather high variance.

Fig. 4 Pathogen epidemiology on flowers according to real-time PCR analysis. The boxplots are drawn according to Crawley (2007): The bold horizontal line shows the median. The bottom and top of the box show the 25th and 75th percentiles, respectively. The vertical dashed lines ('whiskers') show either the maximum value or 1.5 times the interquartile range of the data, whichever is the smaller. Blue = untreated control on 'Gala Galaxy', lavender = LMA on 'Gala Galaxy', pink = untreated control on 'Ladina' and rose = LMA on 'Ladina'

Pathogen epidemiology on flowers

Baseline values on April 24th were not homogenous due to high variation between replicates, even though no plant protection treatment took place yet but three hours after center trees were inoculated with the pathogen mixture. Cell numbers increased after inoculation and control trees had higher cell numbers than LMA treated trees. 'Ladina' showed higher cell numbers than 'Gala Galaxy' except for the last day of sampling (Fig. 4). In general, cell numbers went up at the second sampling day to drop again thereafter. Only one significant difference in pairwise comparisons could be detected on day two between the control and LMA treatment in 'Gala Galaxy' ($p = 0.019$).

Discussion

Even though inoculum density of *Erwinia amylovora* was very high, but still comparable with other studies using lower cell loads in combination with direct inoculation of blossoms (Pusey et al. 2011; Reininger et al. 2017a, b, c), blossom cluster infection values were in a reasonable and comparable range. Experimental conditions were favorable for fire blight development but temperatures were not extremely high

which allowed for a natural setup. No infection day, which means an EIP above 110, was observed during treatment and sampling of apple blossoms, but thereafter (see Fig. 2).

As outlined before, new methods for fire blight management are needed (Sundin et al. 2016) and therefore the integration of fire blight resistant cultivars seems to be a promising and important approach (Kellerhals et al. 2017; Leumann et al. 2013). We combined the highly susceptible cultivar 'Gala Galaxy' and the resistant cultivar 'Ladina' with antagonistic and conventional fire blight treatments. 'Ladina' showed less blossom cluster infection in the untreated control even though inoculum density was the same for both cultivars.

In terms of treatments, we found lower cell loads on treated plants and accordingly less blossom cluster infection compared to the control, regarded for each cultivar separately. This observation of less blossom cluster infection having lower amounts of cells per blossom has been confirmed before (Hinze et al. 2016). But on the other hand, we found more cells in 'Ladina' compared to 'Gala Galaxy' even though blossom cluster infection was lower in 'Ladina'. This fact might be due to a possible mechanism in 'Ladina' where blossoms will be separated from the plant or dropped prior to infection. While evaluating blossom cluster infection we found blossoms which seemed to be infected

but were dropped by the tree. BlossomProtect™ containing the yeast *Aureobasidium pullulans* and Pomavita the bacterium *Pantoea agglomerans* showed the same efficacy on ‘Gala Galaxy’. On the contrary, BlossomProtect™ showed significantly higher efficacy on ‘Ladina’ in comparison to the antagonistic bacterium *Pantoea agglomerans* being the active ingredient in Pomavita. This might be due to high variation of efficacy in ‘Gala Galaxy’. Antagonists might be carried over by bumblebees to other blossoms as other plant protection products as well. In our case the yeast or bacterial antagonistic cell load was not evaluated in the other treatments but as blossom cluster infections were highest in control plants compared to other treatments, this effect could not have been very strong if at all (Fig. 3). Additionally, the entire experiment would have been effected the same way as all trees were placed into the same containment.

In general, efficacy in ‘Ladina’ showed little variation and very high values. Therefore, the combination of a resistant apple cultivar with plant protection treatments seems to be a promising strategy, which needs to be further tested.

Acknowledgements Our thank goes to all project partners of “Together against Fire Blight”, namely the Federal Office for Agriculture (FOAG), Swissfruit and the Canton of Aargau for their funding. Additionally, we would like to thank all project partners of the project HERAKLES Plus for funding: the CAVO Foundation, IP-SUISSE, the cantons of Aargau, Berne, Lucerne, St. Gallen, Thurgau and Zurich.

Funding Open Access funding provided by Agroscope

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