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Fire blight control in organic fruit growing – systematic investigation of the mode of action of potential control agents

Abstract

Effective control agents are needed to prevent blossom infections by the fire blight pathogen *Erwinia amylovora* in organic fruit growing. In this study 18 preparations of potential control agents were compared for their efficiency against *E. amylovora*. In shaken cultures twelve control agents inhibited the growth of *E. amylovora* completely. In this system different mechanisms of action were found. Six control agents shifted the pH of the cultures to values not suitable for bacterial growth (<5 or >8). Three agents act by copper, a known bactericide. The control agent Elot-Vis has high ethanol content, which was responsible for the high efficiency *in vitro*. On detached apple blossoms only four control agents led to a symptom reduction of more than 50%. Only two of them (Blossom-Protect fb, BPASc) exhibited a high efficiency in field trials. Our results suggest that the control agents, which led to a high efficiency in shaken cultures, are not sufficient for good performance in the field.

Introduction

Fire blight caused by *E. amylovora* is the most serious bacterial disease in apple trees. The economic importance of fire blight is considerable. During the last three decades it has spread throughout Europe. Since pruning of diseased material and other sanitation methods are not sufficient to stop the spread of the disease, efficient control agents are needed. Primary infection occurs in the blossom, where the pathogen enters through natural openings after multiplying on the stigmas. To prevent blossom infections in organic fruit growing orchards several potential control agents were evaluated.

In this study 18 preparations were tested for their ability to suppress the multiplication of *E. amylovora* in shaken cultures and to reduce the symptom development on detached blossoms. The mode of action of effective preparations has been evaluated.

Materials and methods

Test preparations: The preparations were tested at doses in accordance with the manufacturer's recommendations (Table 1). Slaked lime was purchased from Sigma Aldrich. Streptomycin sulfate (Sigma Aldrich) was used as a positive control in the laboratory experiments.

Reduction of *E. amylovora* growth in shaken cultures: *E. amylovora* strain Ea385 was subcultured on NBS-A (8 g/l nutrient broth, 50 g/l sucrose, 20 g/l agar) at 27°C. Inoculum suspensions of Ea385 were standardised by measurement of their optical density at 660 nm. $1x10^7$ cells/ml of Ea*385* were added to 25 ml NBS (8 g/l nutrient broth, 50 g/l sucrose) in a 100 ml Erlenmeyer flask containing the test preparation. Four hours and 24 h after incubation on a rotary shaker at 27°C, the Ea385 concentration in the culture was calculated by dilutions plated on Petri-dishes with NB-A (8 g/l Nutrient Broth, 20 g/l agar). If the test preparations contained *Bacillus subtilis* or *Aureobasidium pullulans* the culture was plated on MacC-A (40g/l MacConkey broth, 20g/l Agar) or NBAc-A (8 g/l nutrient broth, 0,05g/l actidione, 20 g/l agar) respectively. Preparations which changed the pH value in the NBS below 5 or above 8 were also tested in NBS-pH7 (NBS amended with 5.4 g/l KH_2PO_4 and 10.6 g/l K_2HPO_4).

Reduction of fire blight symptoms on detached blossoms: An *in vivo* test system with detached apple blossoms was established according to Pusey (10). Apple trees (`Gala`) were stored at 2°C in the dark from January to August. Every week a group of trees was transferred to the greenhouse to force them to bloom. The blossoms were cut and maintained with the pedicel submerged in 10 % sucrose in plastic racks (23°C, 100% RH). Blossoms were sprayed with a suspension of Ea385 (10^6 cfu/ml) in water until

run-off. Treatments were applied 1h after inoculation. The number of blossoms with bacterial ooze at the pedicel was counted 6 days following inoculation (Table 1).

Tab. 1. Name of tested preparations, main ingredients and providers

Name	Ingredients	Provider
Cutisan	Clay mineral	Biofa AG
Kaolin Tec.	Clay mineral	Biofa AG
Phyto-Vital	Lignin derivatives	Ligmeda Consult
Quassia extract	Quassin	Biofa AG
Biplantol Erwinia	Homoeopathic	Bioplant Naturverfahren GmbH
Fungend	Essential oil of *Thymbra spicata*	Biofa AG
BioZell2000B	Essential oil of *Thymbra spicata*	Gisela Zeller
DoMoF/Lysozym	Lysozyme and milk proteins	Novaprot GmbH
Elot-Viss	Alcoholic extract of plants	Biofa AG
Mycosin	Acidic stone meal	Biofa AG
Funguran	Fungicide, Copper oxychloride	Biofa AG
Protex-Cu	Fertiliser, Copper sulfate	MAC-GmbH
Copper-Protein	Copper chloride, protein-complex	Proagro GmbH
Serenade WPO	B. subtilis QST713	GAB Consulting GmbH
Lime sulphur	Calcium polysulphide	Biofa AG
Blossom-Protect	*A. pullulans* and buffer P	Blossom-Protect
BPASc	*A. pullulans* and buffer C	Bio-Protect GmbH

Efficiency of the preparations in field experiments: The results from the laboratory studies were compared to efficiencies found in field experiments conducted from 1997 to 2004 in Germany in accordance with EPPO guideline PP1/166(3). In the test orchards one tree per lot was inoculated with the pathogen. From this tree *E. amylovora* was spread over the entire orchard by natural vectors. (2) Only the results from trees which had not been inoculated were taken into account ((5) and literature therein).

Results

Efficiency of control agents: 18 preparations were tested in shaken cultures and on detached apple blossoms for their efficiency against *E. amylovora*. The use of Cutisan (15 g/l), Kaolin Tec (15 g/l), Phyto-Vital (20ml/l), Quassia extract (2g/l), Biplantol Erwinia (2ml/l) or Fungend (0.25 ml/l) neither reduced the growth of *E. amylovora* in shaken cultures nor prevented symptom development on detached blossoms. Kaolin Tec, Biplantol Erwinia and Fungend were also tested in field experiments and did not reduce disease incidence (6).

Tab. 2 Efficiency of control agents for fire blight control in shaken cultures, on detached blossoms or in field experiments.

		Shaken culture % reduction of Ea385 after 24h (pH)	Symptom reduction on detached blossoms (%)	Field trials in Germany 1997-2004 [5]	
Preparation	Dose (%)			efficiency (%)	No.
Plantomycin	0,06			84 ± 7	9
Streptomycin sulfate	0,025	100 (7.2)	84 ± 15		
Blossom-Protect	1.2	100 (4.0)	70 ±18	72 ±10	4
BPASc	1.2	100 (3.7)	78 ± 13	83	1
Serenade WPO	1.0	100 (6.4)	46 ± 17	45±26	6
Mycosin	1.0	100 (3.8)	78 ± 11	38±18	7
Funguran	0.3	100 (6.2)	73 ± 15	-	
	0.03	100 (-)	47 ± 15	-	
Protex –Cu	0.1	100 (4.7)	34 ± 10	49	1
Copper protein	1.0	100 (6.9)	40 ± 21	-	
Slaked lime	2.0	100 (12.4)	14 ± 20	48	1
Lime sulphur	1.5	100 (9.2)	-5 ± 10	28	1
Elot-Vis	10.0	100 (7.3)	54 ± 26	19	1
DoMoF/Lysozym	2.0	100 (6.9)	-36 ± 47	-	
BioZell 2000B	0.05	100 (6.7)	7 ± 18	-	

All other preparations completely inhibited the growth of the pathogen in shaken cultures. Elot-Vis, Funguran, Mycosin, BPASc and Blossom-Protect also exhibited a high efficiency on detached blossoms (Table 2). Elot-Vis and Mycosin however showed no or only slight disease reduction in the field. Only BPASc and Blossom-Protect exhibited a high efficiency in field trials comparable to streptomycin sulfate.

Mode of action: The efficiency in shaken cultures was necessary but not sufficient to predict a high efficiency on detached blossoms or in the field. The following information with regard to the mode of action in shaken cultures helps to explain the discrepancy between high *in vitro* activity and bad field performance.

Some of the preparations contain substances with known bactericidal effects, which explain the high efficiency in liquid medium. BioZell2000B contains essential oil from *Thymbra spicata* which is described as a bactericide (1). Elot-Vis is a plant extract with an alcohol content of more than 90%. 10 % alcohol was also effective in the shaken culture. After evaporation of the alcohol from Elot-Vis, the remaining extract was resolved in water. This solution had no effect in shaken cultures. This indicates that the efficiency of Elot-Vis in shaken cultures can be attributed to the alcohol content. DoMoF/Lysozym consists of milk proteins and lysozyme from chickens eggs. Lysozymes have a potential as anti-microbial proteins because of their lytic activity against bacterial cell walls (11). Funguran, Protex-Cu and Copper-Protein contain copper ions as active ingredients. The bactericidal activity of the copper ion is already well documented (9) and all three copper agents completely inhibited Ea385 in the tested concentrations. Serenade WPO contains spores of *Bacillus subtilis* strain QST713 and substances produced by this strain during fermentation. After re-suspending Serenade WPO (10 g/l) the *B. subtilis* spores were separated from the supernatant. Both the spore pellet and the supernatant inhibited the growth of Ea385 in shaken cultures. This indicates that Serenade WPO acts by bactericidal substances which are contained in the formulation and by the antagonistic behaviour of the *B. subtilis* strain.

The second group of preparations shifted the pH of the medium to values not suitable for *E. amylovora*. Lime sulphur and slaked lime increased the pH of NBS (Table 2). Lime sulphur was also efficient in NBS-pH7 although the pH was maintained at 7. This indicates that a second mechanism was involved. Slaked lime increased the pH to a level above 8 even when NBS-pH7 was used. Therefore $CaCl_2$ was tested for its efficiency against Ea385 at a concentration corresponding to the Ca-concentration in 20g/l slaked lime. This $CaCl_2$-concentration (30 g/l) inhibited the growth of Ea385 completely, indicating that slaked lime acts by both, an increase of pH and by the high salt concentration. A lower concentration of 15 g/l $CaCl_2$ however was totally ineffective.

Mycosin, BPASc and Blossom-Protect decreased the pH of the medium to or below pH 4. At pH 4 Ea385 was not able to multiply. This explains the high efficiency of these preparations in shaken cultures. Mycosin or Blossom-Protect were also added to NBS-pH7. In NBS-pH7 the pH value did not decrease below 6 and the two preparations did not inhibit the growth of Ea385. This indicates that the shift in pH is the mode of action for these preparations.

Blossom-Protect consists of two components (4). For further examinations the two components were tested separately in shaken cultures (Figure). Component A showed the same results as Blossom-Protect. Component B did not lead to a reduction of Ea385 concentration after 4h, but the growth of Ea385 was completely inhibited when measured after 24h (bacteriostatic). Interestingly the Component B did not inhibit the growth of Ea385 in NBS-pH7 (Figure). This indicates that the efficiency of component B is also pH dependent. As component B decreased the pH of NBS from 6.9 to 4.9 during 24h of incubation, it is likely that *A. pullulans* acts by acidifying the medium.

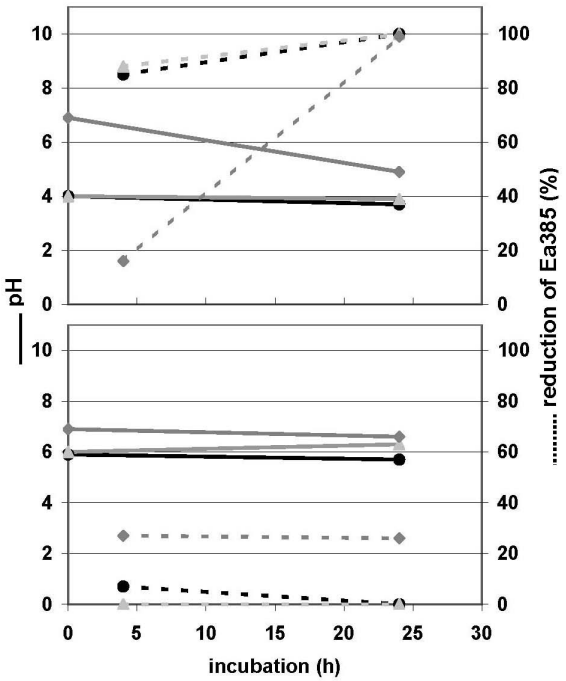

Figure Change in pH and reduction of Ea385 concentration in shaken cultures by Blossom-Protect (●), Component A of Blossom-Protect (▲) and Component B of Blossom-Protect (♦) in NBS (above) or in NBSpH7 (below)

Discussion

Many control agents are under discussion to prevent blossom infections by the fire blight pathogen *Erwinia amylovora*. In a systematic evaluation 18 preparations were tested in different test systems in comparison to streptomycin sulfate. Twelve preparations inhibited *E. amylovora in vitro*, demonstrating their bacteriostatic or bactericidal potential. However, only two of them, Blossom-Protect and BPASc, have a high efficiency in field experiments (5). This shows that the *in vitro* activity is a necessary criterion but not sufficient to predict a high effectiveness on detached blossoms or in the field.

The mode of action was evaluated for the preparations with high *in vitro* activity. Some of them contain known bactericidal substances, which explains the high efficiency *in vitro*. Higher doses of the active ingredient are needed for a high efficiency in the field than in *in vitro* experiments because of dilution effects on the plant surface. For example 0.3 g/l or 3 g/l Funguran inhibited Ea385 in shaken cultures completely. On detached blossoms the efficiency of Funguran increased from 47% to 73% when the dose was increased from 0.3 g/l to 3 g/l. Streptomycin sulfate inhibits *E. amylovora in vitro* at doses of 1 mg/l (8). In the field a dose of 123 mg/l is recommended. Therefore the dose of an active ingredient should be 10 to 100 times higher in the field than its lowest dose efficient *in vitro*.

None of the preparations with bactericidal ingredients tested in this study can be sprayed in the field in such high doses. Copper is phytotoxic at high concentrations (9). The others have already been tested at high concentrations (e.g. Elot-Vis 100 ml/l; DoMoF/Lysozyme 20g/l), or are too expensive to use 10 fold higher concentrations.

The use of antagonists, able to produce antibiotics on the plant surface, could be a possibility to bring high concentrations of active ingredients to the stigma. Once the antagonist has been sprayed on the flower it should produce its antibiotic and thus prevent growth of *E. amylovora*. Serenade WPO contains spores of *B. subtilis* QST713 and it contains antibiotics produced by the antagonist during the fermentation process. The fact that Serenade WPO has only a moderate efficiency on detached blossoms and in the field (5) indicates that the *B. subtilis* spores need too much time for germination and subsequent antibiotic production on the blossom surface.

All preparations which act by altering the pH of the medium have the same problem as the bactericides. It is easy to alter the pH in the spray solution but after spraying it on the blossom the pH will be neutralised due to dilution or reaction with e.g. atmospheric CO_2, or substances on the blossom. Blossom-Protect acts by reducing the pH to approximately 4 and has two mechanisms to prevent

neutralisation. Component A is a strong buffer which decreases the pH on the surface immediately after application and *A. pullulans* is able to hold this low pH during growth on blossom surfaces.

The two preparations BPASc and Blossom-Protect showed the best results on detached blossoms and in field experiments. Therefore the yeast preparations are promising tools for fire blight control in organic apple growing. Both preparations contain blastospores of *A. pullulans*. Despite reports that *A. pullulans* causes fruit russet (7,12), no increase in russeted fruits was found in field trials after the application of Blossom-Protect, carried out over nearly 20 ha by organic farmers in 2004 and 2005 (3).

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