Notulae Botanicae Horti Agrobotanici
Cluj-Napoca

Effectiveness of a Peptide-rich Fraction from *Xenorhabdus budapestensis* Culture against Fire Blight Disease on Apple Blossoms

Dávid VOZIK[1]*, Katalin BÉLAFI-BAKÓ[1], Mária HEVESI[2],

Erzsébet BÖSZÖRMÉNYI[3], András FODOR[4]

[1]University of Pannonia, Research Institute on Bioengineering, Membrane Technology and Energetics, Egyetem u. 10, H-8200, Veszprém, Hungary; vozikd@gmail.com (*corresponding author); bako@almos.uni-pannon.hu

[2]Corvinus University of Budapest, Department of Pomology, Ménesi út 29-43, H-1118, Budapest, Hungary; maria.hevesi@uni-corvinus.hu

[3]Semmelweis University, Department of Epidemiology, Vas u. 17, H-1088, Budapest, Hungary; boszormenyie@se-etk.hu

[4]University of Wisconsin-Madison, Department of Bacteriology, 1550 Linden Drive, Madison, Room 4526, WI 53507, USA; fodorandras@yahoo.com

Abstract

Erwinia amylovora is one of the most frequently occurred plant pathogenic bacterium. It causes necrosis and blight symptoms on host plants and it lead to considerable yield losses throughout the world on apple trees. There is no effective chemical treatment is currently available against fire blight. The purpose of the present study was to search a new, alternative control method. The evaluation of the plant protection potential of an enriched fraction of *Xenorhabdus budapestensis* cell-free conditioned media was investigated. Purified samples were tested *in vitro* and *in planta* against the phytopathogenic bacterium. A reproducible method for isolation of a peptide-rich fraction from *Xenorhabdus* cell-free conditioned media was established. The process resulted in 400 mg of dry sample prepared from three litres of *Xenorhabdus* cell culture. Significant correlation was found between the concentration of the purified preparation and the induced inactivation zones against *Erwinia amylovora* Ea1 in agar diffusion test method. The minimum inhibitory concentration and minimum bactericidal concentration of the purified fraction against Ea1 strain were 8 μg/mL and 16 μg/mL, respectively. *In planta* experiments were tested on an apple cultivar ('Watson Jonathan') susceptible to fire blight. The effective range of concentration was 62-200 μg/mL, while treatment with 300 μg/mL and larger amounts caused necrotic symptoms on the petals of flowers. Current study pointed to the effectiveness of the compounds produced by *X. budapestensis* against fire blight. The development of a commercially applicable formulation of these compounds would allow growers to effectively control fire blight in apple and pear orchards.

Keywords: agrobiologicals, antagonists, antimicrobial compounds, entomopathogenic bacteria, infection, prevention

Introduction

Bacterial infections are among the major cause of mortality and morbidity, thus can severely limit the commercial productivity of fruit trees all over the world (Dzotam *et al.*, 2015). One of the most dangerous diseases of plants in the *Rosaceae* family is the fire blight, caused by *Erwinia amylovora* that affects many crops in agriculture and horticulture especially apple and pear trees (Malnoy *et al.*, 2012). During the last decades, *E. amylovora* spread in the Mediterranean Basin and Western Europe from North-America (Bonn and van der Zwet, 2000). Appearance of the plant pathogenic bacterium in Hungary has been reported for the first time in 1995 (Hevesi, 1996). Nowadays there are almost no countries in Europe devoid of fire blight (Thomson, 2000). Previously, protection against fire blight disease was based on the use of the antibiotic streptomycin sulfate, which is restricted by the laws in numerous countries in Europe, because of its use in human public healthcare. Moreover, there are various natural isolates of streptomycin-resistant *E. amylovora* strains, mostly from the area of North-America (McManus and Jones, 1994; Norelli *et al.*, 1994; Sobiczewski *et al.*, 1991). Therefore, curative control of *E. amylovora* on apples and pears are lacking at present. Any future strategy, preventive or curative chemotherapy should be based on the application of antibacterial compounds which do not show cross resistance with any commercially used antibiotics. Recent studies demonstrate differences in sensitivity of apple cultivars to the infection caused by *E. amylovora* (Tóth *et al.*, 2013; Viljevac *et al.*, 2009). Therefore, resistance breeding programmes can offer a long-term solution, but meanwhile, effective and safe ingredients of control must be improved to prevent damage of these fruit crops.

548

Increasing antimicrobial resistance has created a critical need to search new antibiotics with novel mechanisms of action and to develop anti-infective products against the pathogens. The natural compounds produced by entomopathogenic bacteria (EPB) species seem to be one of the promising alternatives.

Entomopathogenic bacteria like *Photorhabdus* and *Xenorhabdus* species, belonging to the bacterial family *Enterobacteriaceae*, are symbiotically associated with entomopathogenic nematodes (EPN) as *Heterorhabditis* and *Steinernema* species (Boemare *et al.*, 1993; Thomas and Poinar, 1979). These nematode-bacteria complexes can be used as biological control agents against insect pests in agriculture (Smart, 1995). The bacterial symbionts produce several important secondary non-ribosomal products (NRP) with biological activity. They include smaller organic compounds, which in cooperation with hydrolytic exo-enzymes, process the insect's cadaver into utilizable nutrients for nematodes and thereby protect it from microbial competitors. Dutky *et al.* (1964) proposed that the bacteria which live as symbionts of the EPN are able to produce compounds with antibiotic effect, as well. Some of these antimicrobial compounds – mainly secondary metabolites act either alone or synergistically, significantly contribute to the broad-scale protection of colonized cadaver under the polyxenic soil conditions, providing nearly monoxenic conditions within the colonized insect cadavers (Akhurst, 1982). Recent interest has turned toward biologically active NRP peptides and their hybrids with different organic residues. Lengyel *et al.* (2005) had previously described *X. budapestensis*, and deposited the strain in DSMZ, Germany as DSM 16342. Fuchs *et al.* (2012, 2014) working with *X. budapestensis* and found that some of these substances produced by these bacteria, especially the fabclavines show strong antibacterial activity against numerous Gram-positive and Gram-negative pathogenic bacteria as well as some fungi of agricultural importance. Fodor *et al.* (2012) also worked with this EPB species, and investigated and described the target-spectra of antimicrobial compounds produced by it, against a wide range of plant pathogenic bacteria and fungi.

The natural agents of EPB species have shown a wide range of bioactivities of medical and agricultural interest, such as antibiotic, antimycotic and insecticidal effects (McInerney *et al.*, 1991; Webster *et al.*, 2002). Several secondary metabolite molecules produced by *X. budapestensis* are antimicrobial peptides (AMP), like bicornutin-A (Böszörményi *et al.*, 2009), GP-19 and EP-20 (Xiao *et al.*, 2012). A recently discovered peptide-polyketide-polyamine hybrid family, called fabclavines with a broad-spectrum of bioactivity, produced by *X. budapestensis* DSM 16342 (Fuchs *et al.*, 2014), might be of an excellent potential. These substances have additional favorable properties, such as low molecular weight and thermal stability. In addition, targeted microorganisms are not able to induce resistance easy against AMPs (Hastings *et al.*, 2004).

The main objectives of the present study were to carry out and evaluate biological assays for investigate the potential of *X. budapestensis* DSM 16342 (EMA) for plant protection. The bioactive compounds were purified and tested also in a cleaner form. After accomplishing several *in vitro* tests we focused on the control of *E. amylovora in planta*. We applied a purified peptide-rich fraction (PF) prepared from *X. budapestensis* culture and examined its effectiveness in the protection of apple blossoms against *E. amylovora*, using a modified version of the method reported by Pusey (1999). The effect of *Xenorhabdus* products with different purity and composition on apple blossoms must also explain.

Materials and Methods

Test organisms and media
Entomopathogenic bacterium
Antagonistic bacterium strain used in this study was previous isolate from *Xenorhabdus budapestensis* DSM-16342^T (EMA), separated before deposition and has been kept in selective conditions since then. EMA was routinely cultured in Luria-Bertani Broth and modified Luria Agar (casein peptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar 17 g/L) (LB and LA, respectively) (Ausubel *et al.*, 1999) at 25 °C. The indicator plates (LBTA) were supplemented with bromothymol blue (BTB) and 2, 3, 5-triphenyltetrazolium chloride (TTC), and were used to distinguish AMP producing (phase I) and non-producing (phase II) variants (Leclerc and Boemare, 1991). Fresh single colonies derived from frozen bacterial stocks were used for each experiment. Microbiological media were obtained from Biolab Zrt., Hungary.

Plant pathogenic bacterium
Erwinia amylovora Ea1 strain was collected by M. Hevesi from an apple tree at Nyárlőrinc, Hungary (Hevesi, 1996). Ea1 has been deposited in the National Collection of Agricultural and Industrial Microorganisms (NCAIM), Hungary under the number B.01728. The test bacterium was cultured in LB liquid and on LA solid media.

Test plants
For *in planta* bioassay, blossoms of 'Watson Jonathan' apple were chosen. This variety had been previously reported to be susceptible to fire blight (van der Zwet and Beer, 1995). Plant materials were obtained from the apple collections at the Soroksár Research Station of the Department of Pomology, Corvinus University of Budapest.

Preparation of Xenorhabdus cell-free media (CFCM)
X. budapestensis was maintained on LA medium and sub-cultured freshly. The AMP producing phase I has instability under normal culture conditions, therefore, glycerinated frozen stocks of the bacteria were used as a starting material for culture. In order to ensure the presence of phase I, stocks were incubated on LBTA plates at 25 °C. Single black - dark blue colonies were added separately into test tubes containing 3 mL of LB liquid medium. At the end of the log phase the cell culture was inoculated into 50 mL of LB in 500 mL flasks. 50 mL aliquots of culture grew overnight at 25 °C, then were inoculated to 250 mL of LB in 1000 mL flasks and incubated on a gyrorotatory shaker (200 rpm) at 25 °C until stationary phase. The cells were removed by centrifugation (15000×g) for 20 min at 4 °C. A supernatant was filtered through Millipore Express Plus filter (pore-sizes 0.22 μm) (Merck Millipore), to obtain a cell-free filtrate. The filtrate was stored at 4 °C until its utilization.

Purification of a peptide-rich fraction (PF) from X. budapestensis CFCM

Three litres of *X. budapestensis* cell-free media were processed for further purification. CFCM was mixed with a polymeric adsorbent Amberlite® XAD-1180 (Alfa Aesar GmbH & Co KG, Germany) in a concentration 20 g/L. The adsorbent was previously activated in distilled water in an autoclave at 121 °C for 30 min. The resin containing CFCM was incubated at 25 °C in a gyrorotatory shaker at 150 rpm for 48 h. The resin slur was removed by filtration on a glass filter and washed thoroughly with 1000 mL of sterile distilled water. Amberlite adsorbent was placed on the top of the Millipore Express Plus filter of 0.45 µm pore-sizes (Merck Millipore), washed with 500 mL of 25 % methanol, and 3×300 mL of 50 % methanol in order to remove all coloured and inactive materials. Subsequently, 3×300 mL of 80 % methanol was used to elute some additional compounds, mainly triptamine. Finally, the active compounds were eluted with 300 mL of 100% methanol (Molar Chemicals kft, Budapest, Hungary) containing 3 mL of 2N HCL. This acidic solution was neutralized (pH~6) with 2N NH4OH, and evaporated by Heidolph VV 2000 rotary vacuum evaporation system. The method resulted in 400 mg of dry, biologically active preparation.

In vitro bioassays
Antibacterial activity on solid media
Overlay bioassay was carried out as previously described (Böszörményi *et al.*, 2009; Furgani *et al.*, 2008), and was conducted to determine antibiotic activity, reifying antagonistic effect of living *Xenorhabdus* cells. Overlay test was done in quadruplicate sets of plates.

Antimicrobial activity of CFCM from *X. budapestensis* was followed by agar diffusion hole test method (Fodor *et al.*, 2012). Briefly, 300 µL of an overnight cell culture of the test bacterium (Ea1) was mixed with 5 mL soft agar (0.6 % agar), which was previously been warmed in a water bath thermostat to approximately 45 °C and then poured on the surface of LA plates. Then an 8 mm diameter hole was made in the centre of each plate with a sterile cork borer, and 200 µL volume of the antimicrobial solution was added. The plates were incubated at 25 °C for 48 h, and inactivation zones were evaluated by measuring of their diameters. Tests were performed in triplicate. Culture filtrates, diluted with sterile LB liquid media, were tested in four different concentrations (40, 60, 80, 100 % (V/V)). Effectiveness of PF from *X. budapestensis* was also tested against *E. amylovora* Ea1 at eight different concentrations (40, 62, 100, 150, 200, 300, 500, 1000 µg/mL).

Determination of bacterial toxicity in liquid media
Cell-free media of *X. budapestensis* was serially diluted in microtiter plates with LB (100, 80, 60, 50, 40, 30, 20, 10 and 0% (V/V), respectively). Each well contained 1950 µL CFCM and was supplemented with 50 µL cell suspension from an overnight culture of Ea1. The cultures were shaken (120 rpm) at 25 °C for 24

h. The cell densities were determined spectrophotometrically by Hach-Lange DR 3900 spectrophotometer, at 620 nm. Results were given by plotting OD values versus dilution of CFCM. Moreover, 50 µL samples were plated onto LA plates. MID (maximum inhibitory dilution) is defined as the highest dilution of CFCM where test bacteria were unable to grow. Three replicates of plates were used for the experiment. The highest dilution means the lowest concentration of an active substance tested. Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the PF against *E. amylovora* Ea1

To evaluate antimicrobial activity of the prepared peptide-rich fraction, purified from *X. budapestensis* culture, two parameters were determined. The MIC was performed by using the macro dilution method recommended by the Clinical and Laboratory Standards Institute, USA (CLSI, 2012). The minimum bactericidal concentration (MBC) was determined as the lowest concentration of an antibacterial agent (PF) required to kill *E. amylovora* Ea1. It was determined from broth dilution minimum inhibitory concentration (MIC) tests by subculturing to agar plates that do not contain the test agent. The PF fraction was dissolved in methanol to give a concentration of 10 mg/mL and then diluted to concentrations 1000 – 1.0 µg/mL with sterile distilled water. Broth medium without PF was used as growth control. Cell suspension of Ea1 equivalent to a 0.5 McFarland standard was made as inoculum for working solutions resulted in a final inoculum of 5×10^5 CFU/mL. Tubes were incubated at 28 °C for 24 h. The MIC value is defined as the lowest concentration of the examined material that prevented visible bacterial growth. Three replicates for each concentration of the test sample were tested.

The method of Smith-Palmer *et al.* (1998) was adopted to determine MBC value. 100 µL from the MIC tubes, which showed no bacterial growth were serially diluted and plated on LA plates. The plates were incubated at 28 °C for 48 h. MBC was defined as the lowest concentration of antimicrobials where bacteria were not be able to grow on LA inoculated with 50 µL of solution. Three replicates of plates were used for each concentration of the test sample.

In planta bioassay
Effectiveness of PF on apple blossoms
Efficiency of PF obtained from *X. budapestensis* culture to control fire blight disease was determined according to the method reported by Tóth *et al.* (2013). 300 pieces of 'Watson Jonathan' flowers (at Soroksár Research Station, Hungary) were collected at "balloon state", and were placed separately in tubes, filled with 1% sucrose solution. Flowers were incubated at 23 °C and 84% relative humidity in transparent plastic boxes for 12 h. Afterwards, opened flowers were treated with aqueous solutions of PF by employing a hand-sprayer. About three hours later, the flowers were inoculated with 10 µL of Ea1 suspension with a cell density of $5×10^8$ CFU/mL by applying an automatic pipette. Ten concentrations of PF from 500 to 4 µg/mL were tested in ten different plastic boxes. Each experimental box contained 20 previously treated flowers and 5 control flowers treated with distilled water. All of them were artificially infected with Ea1 test bacterium. The control box contained 50 pieces of not infected, water-treated flowers.

After 5 days the infected flowers were cut lengthwise and evaluated based on the severity of disease symptoms using a

modified version of the rating scale recommended by Pusey (1999), where means of the values were represented as follows: 0 – no necrosis, 1 – detectable necrosis (just on the stamens), 2 – necrosis on up to half of the ovary, 3 – half to all of the ovary was affected by necrosis or spreading across the ovary to the peduncle. The blight rating concerning the flowers previously treated by the same concentration were indicated with B_f and was calculated applying the formula reported by Bertrand and Gottwald (1978): $\Sigma f_i \times n_i / n$, where f_i indicated the individual infection rate of the flowers, n_i is the frequency of either one infection rate and n is the number of flowers examined in the same way treatment.

To verify the effectiveness of the treatments the numbers of viable Ea1 cell counts on the flowers were determined. Flowers belonging to the same treatment group were ground with mortar and pestle using 100 µL added sterile distilled water per flower. Aliquots were serially diluted with sterile distilled water and performed in duplicates. 50 µL from the diluted samples were plated onto LA media with two parallel replicates, and plates were incubated at 25 °C for 48 h. The number of viable *E. amylovora* cells was determined. The CFU/flower value was calculated from the average of colony numbers on replicate plates.

Statistical analyses

The results subjected to ANOVA and Scheffé test (post hoc) were used to establish mean differences between doses of CFCM (agar diffusion test) at a significance level of $p < 0.05$.

PF obtained from *X. budapestensis* in eight different concentrations was also tested by agar diffusion method against Ea1 test bacterium. Null hypothesis was that there is a relationship between the doses of PF and the induced inactivation zones, therefore correlation of the data was investigated.

The disease symptoms (degree of the necrotic areas) of the apple blossom samplings infected with *E. amylovora* Ea1 and treated with PF from *X. budapestensis* were analysed as a fully randomized experiment and non-parametric Kruskal-Wallis test was used to discriminate between treatments. Correlation between the doses of PF-treatment and the number of viable Ea1 cells based on the isolation from apple flower samples was also examined.

All statistical analyses were performed using STATISTICA 8 (StatSoft Inc., Tulsa, OK, USA).

Results

In vitro bioassays

The results of overlay bioassay show, that the tested plant pathogenic bacterium (Ea1) proved sensitive to EMA. Mean (±standard error) inactivation zone averaged 44.75 ± 0.96 mm.

Antibacterial activity of CFCM of EMA on *E. amylovora* Ea1 was determined in four concentrations by agar diffusion tests. Data are summarized in Fig. 1. The four columns from the left to right represent the respective doses of 40, 60, 80 and 100 % (V/V) of EMA CFCM. The effect was statistically not dose dependent in all of the cases on the grounds of Scheffé test. However, there were perceptible differences between the averages of induced inhibition zones in accordance with concentration of CFCM.

PF from *X. budapestensis* was also tested for inactivation zones against *E. amylovora* Ea1. Data and the fitted line are summarized in Fig. 2. The abscissa represents the dose (µg/mL) of the PF, while the ordinate demonstrates the diameter of the induced inactivation zone in mm. Significant correlation was
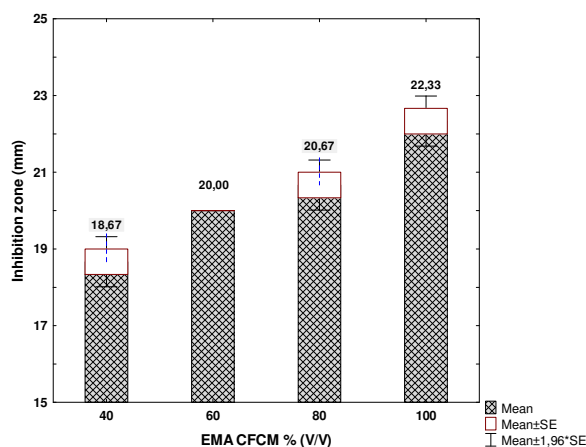

Fig. 1. Antibacterial activity of native cell-free conditioned media (CFCM) of *Xenorhabdus budapestensis* (EMA) against *Erwinia amylovora* Ea1 in agar diffusion test
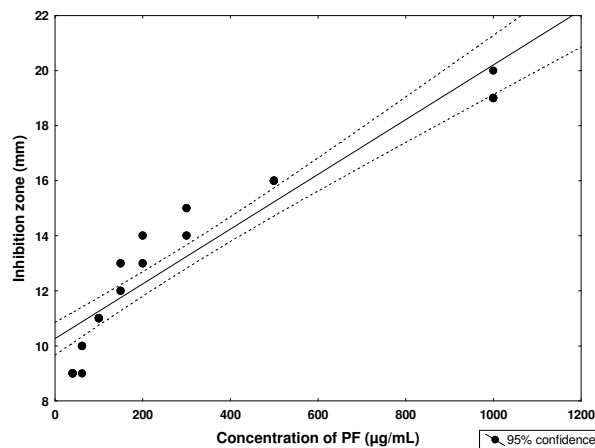

Fig. 2. Dose – effect study on the antibacterial activity of the peptide-rich fraction (PF) prepared from native cell-free conditioned media (CFCM) of *Xenorhabdus budapestensis* (EMA) on *E. amylovora* Ea1 *in vitro*
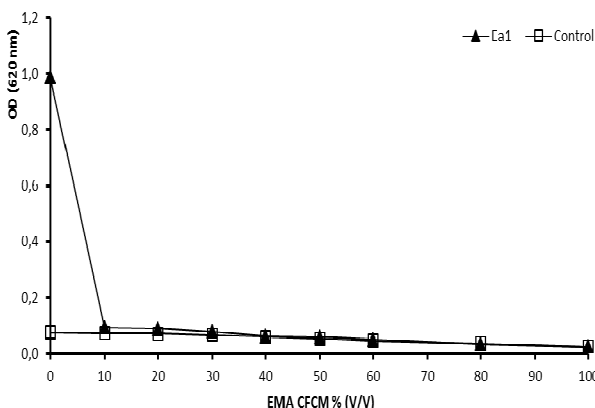

Fig. 3. Determination of the quantitative parameters of the bactericide (cytotoxic) activity of the native cell-free conditioned media (CFCM) of *X. budapestensis* (EMA) on *Erwinia amylovora* Ea1 (▲) in liquid cultures. Control (□): EMA CFCM diluted with sterile LB media and without bacteria
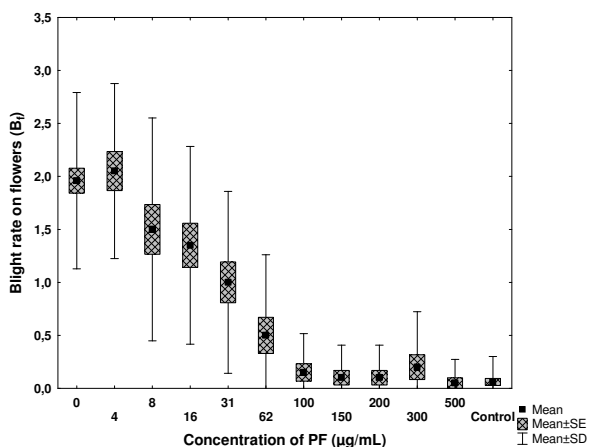
Fig. 4. Effect of preventive treatment with PF on the condition of dissected flowers subsequently infected with *Erwinia amylovora* Ea1 test bacterium. The blight rating (B$_f$) was calculated based on the formula reported by Bertrand and Gottwald (1978): $\Sigma f_i \times n_i / n$

found at a 95 % significance level. The adjusted coefficient indicates an excellent fit (r = 0.93).

Antimicrobial activity of the CFCM from EMA was measured in liquid media by the turbidimetric determination of bacterial growth following exposure to the test material in different concentrations for 24 h. The cytotoxicity curve is presented in Fig. 3. The effect was dose dependent. The effective dose of CFCM (MID value) was found as 10 % (V/V).

A MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism (Turnidge *et al.*, 2003). Diluted samples of PF extracted from EMA CFCM were tested for antibacterial activity against *E. amylovora* Ea1 strain using standard methods. MIC and MBC were found as 8 µg/mL and 16 µg/mL, respectively. It appeared that there was no considerable difference between the effective MIC and MBC values, thus the effect was bactericidal rather than bacteriostatic.

In planta bioassay

In preliminary experiments we demonstrated that undiluted cell-free culture filtrate of *X. budapestensis* is harmful to apple flowers. Treatment employing a hand-sprayer caused necrotic

symptoms on blossoms. Therefore, further purified preparation of EMA was used for *in vivo* bioassay. Although the preparation has slightly lower efficiency against *E. amylovora* based on the results of agar diffusion test method, it would contain fewer compounds, which may damage apple flowers.

Data on effectiveness of the purified peptide-rich fraction (PF) on apple blossoms are presented in Table 1. The blight rating (B$_f$) was calculated based on the formula reported by Bertrand and Gottwald (1978): $\Sigma f_i \times n_i / n$, where f_i indicated the individual infection rate of the flowers, n_i is the frequency of each infection rate and n is the number of flowers examined in the same way treatment. The development of fire blight symptoms on flowers with different treatments was compared based on a scale of 0 to 3, so parametric tests cannot be used for the statistical analysis. Therefore, the overall comparison of the treatments was made by non-parametric Kruskal-Wallis test. The results on Fig. 4 show, that the preventive treatment of dissected flowers with 62 µg/mL, or larger dose of PF significantly reduced the frequency of infection rate (f$_i$) after the artificial infection with *E. amylovora* Ea1 compared to negative control (treated with sterile distilled water), and was significantly not different from the positive control (not infected with Ea1). Treatment with 300 µg/mL and higher dose caused necrotic symptoms on the petals of apple flower samples.

Data concerning average number of Ea1 cells were based on the results of colony forming units of Ea1 bacteria on LA media extracted from flowers and plated in replicate following incubation for 48 h. Significant correlation was found between the doses of PF-treatment based on the number of isolated viable Ea1 cells from apple flower samples. However, the adjusted coefficient was fairly small (r = -0.56), indicating that this method has highly scattered. Nevertheless, the procedure is capable of demonstrating considerable differences in changes of number of viable Ea1 cells on flowers.

Discussion

Böszörményi *et al.* (2009) proved the efficacy of *X. budapestensis* cell-free culture filtrates in reduction of the fire blight symptoms in both laboratory and greenhouse experiments. This study was designed to control only vegetative shoot infection. CFCM from *Xenorhabdus* have never been investigated as a treatment for the blossoms of apple trees.

Primary blossoms are particularly important, because the fire blight lesions are initiated as the blossom infections and can directly

Table 1. Effectiveness of the purified peptide-rich fraction (PF) on apple blossoms

Pre-treatment	Evaluation					
Concentration of EMA PF (µg/mL)	Infection rate (f$_i$)				Bf	Average number of Ea1 cells (CFU/flower)
	0	**1**	**2**	**3**		
	Frequency of infection rate (n$_i$)					
0	4	6	28	12	1.96	1.0×10^7
4	1	3	10	6	2.05	8.4×10^6
8	3	9	3	5	1.50	6.2×10^6
16	3	10	4	3	1.35	5.4×10^6
31	6	9	4	1	1.00	2.8×10^6
62	13	4	3	0	0.50	6.5×10^5
100	17	3	0	0	0.15	1.1×10^5
150	18	2	0	0	0.10	5.0×10^4
200	18	2	0	0	0.10	6.3×10^4
300	17	2	1	0	0.20	1.4×10^5
500	19	1	0	0	0.05	3.8×10^4
Control	47	3	0	0	0.06	0

reduce yields, kill major limbs spreading toward, and supply inoculum for shoot infection and secondary blossom infection (Beer and Opgenorth, 1976). Furthermore, crop spraying against plant pathogenic bacteria is usually carried out during the bloom period of fruit-trees. Thus, spraying materials designed to prevent the infection must be able to protect from plant pathogens without damage of blossoms.

According to the evaluation of the *in planta* bioassay on artificially infected apple blossoms, purified preparation from EMA can provide a protective effect against the development of fire blight. Small populations of *E. amylovora* ($<10^5$ CFU) associated with a treated flower are unlikely to be sufficient to induce symptoms. Preventive treatment with PF can significantly reduce the number of viable *E. amylovora* cells based on the data from the *in vivo* bioassay and the results of isolation of the bacteria from extracts of flowers.

The peptide-rich fraction prepared from *X. budapestensis* culture containing the antimicrobial compounds is a promising biological preparation suitable to reduce the fire blight symptoms when applied under controlled conditions prior to infection on apple blossoms.

Conclusions

For summary, it is confirmed that the secondary metabolites from the species *X. budapestensis* has a potential to control fire blight disease under controlled conditions. These studies represent a good basis for the performance of reliable field tests. Furthermore, entomopathogenic bacterium *X. budapestensis* produce compounds with a wide range of bioactivities and can provide novel active ingredients to improve new, effective agrobiologicals for plant protection.

Acknowledgements

References

Akhurst RJ (1982). Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Journal of General Microbiology 128:3061-3065.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1999). Short Protocols in Molecular Biology, 4th Edition. A compendium of methods from current protocols in molecular biology. John Wiley & Sons, New York, USA.

Beer SV, Opgenorth DC (1976). *Erwinia amylovora* on fire blight canker surfaces and blossoms in relation to disease occurrence. Phytopathology 66:317-322.

Bertrand PF, Gottwald TR (1978). Evaluating fungicides for pear disease control. In: Zehr EI (Ed). Methods for evaluating plant fungicides, nematicides and bactericides. St Paul, Minnesota USA pp 179-181.

Boemare NE, Akhurst RJ, Mourant RG (1993). DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a Proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International Journal of Systematic Bacteriology 43:249-255.

Bonn WG, van der Zwet T (2000). Distribution and economic importance of fire blight. In: Vanneste JL (Ed). Fire Blight, the Disease and Its Causative Agent *Erwinia amylovora*. CAB International, Wallingford, UK pp 37-53.

Böszörményi E, Érsek T, Fodor A, Fodor AM, Földes LSz, Hevesi M, Hogan JS, Katona Z, Klein MG, Kormány A, Pekár S, Szentirmai A, Sztaricskai F, Taylor RAJ (2009). Isolation and activity of *Xenorhabdus* antimicrobial compounds against the plant pathogens *Erwinia amylovora* and *Phytophthora nicotianae*. Journal of Applied Microbiology 107:746-759.

CLSI (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Ninth Edition. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.

Dutky SR, Thompson JV, Cantwell GE (1964). A technique for the mass propagation of the DD-136 nematode. Journal of Insect Pathology 6:417-422.

Dzotam JK, Touani FK, Kuete V (2015). Antibacterial activities of the methanol extracts of *Canarium schweinfurthii* and four other Cameroonian dietary plants against multi-drug resistant Gram-negative bacteria. Saudi Journal of Biological Sciences doi: 10.1016/j.sjbs.2015.06.006.

Fodor A, Varga I, Hevesi M, Máthé-Fodor A, Racsko J, Hogan JA (2012). Novel Anti-Microbial Peptides of *Xenorhabdus* Origin against Multidrug Resistant Plant Pathogens. In: Bobbarala V (Ed). Biochemistry, Genetics and Molecular Biology - A Search for Antibacterial Agents. InTech pp 147-196.

Fuchs SW, Sachs CC, Kegler C, Nollmann FI, Karas M, Bode HB (2012). Neutral loss fragmentation pattern based screening for arginine-rich natural products in *Xenorhabdus* and *Photorhabdus*. Analytical Chemistry 84(16):6948-6955.

Fuchs SW, Grundmann F, Kurz M, Kaiser M, Bode HB (2014). Fabclavines: Bioactive Peptide-Polyketide-Polyamino Hybrids from *Xenorhabdus*. Chembiochem 15:512-516.

Furgani G, Böszörményi E, Fodor A, Fodor AM, Forst S, Hogan J, Katona Z, Klein MG (2008). *Xenorhabdus* antibiotics: a comparative analysis and potential utility for controlling mastitis caused by bacteria. Journal of Applied Microbiology 104:745-758.

Hastings P, Rosenberg SM, Slack A (2004). Antibiotic-induced lateral transfer of antibiotic resistance. Trends in Microbiology 12:401-404.

Hevesi M (1996). Appearance of fire blight in Hungary. Plant Protection 32:225-228 (in Hungarian).

Leclerc MC, Boemare NE (1991). Plasmids and Phase Variation in *Xenorhabdus* spp. Applied and Environmental Microbiology 57:2598-2601.

Lengyel K, Lang E, Fodor A, Szállás E, Schumann P, Stackebrandt E (2005). Description of four novel species of *Xenorhabdus*, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. Nov., *Xenorhabdus ehlersii* sp. Nov., *Xenorhabdus innexi* sp. Nov., and *Xenorhabdus szentirmaii* sp. Nov. Systematic and Applied Microbiology 28:115-122.

Malnoy M, Martens S, Norelli JL, Barny MA, Sundin GW, Smits TH, Duffy B (2012). Fire blight: applied genomic insights of the pathogen and host. Annual Review of Phytopathology 50:475-494.

McInerney BV, Gregson RP, Lacey M, Akhurst RJ, Lyons GR, Rhodes SH, Smith DRJ, Lutz ME, White AH (1991). Biologically active metabolites from *Xenorhabdus* spp. part 1. Dithiopyrrolone derivatives with antibiotics activity. Journal of Natural Products 54:774-784.

McInerney BV, Taylor WC, Lacey MJ, Akhurst RJ, Gregson RP (1991). Biologically active metabolites from *Xenorhabdus* spp. part 2. Benzopyran-1-one derivatives with gastroprotective activity. Journal of Natural Products 54:785–795.

McManus PS, Jones AL (1994). Epidemiology and genetic analysis of streptomycin-resistant *Erwinia amylovora* from Michigan and evaluation of oxytetracycline for control. Phytopathology 84:627-633.

Norelli JL, Burr TJ, Lo Cicero AM, Gilbert MT, Katz BH (1991). Homologous streptomycin resistance gene present among diverse Gram-negative bacteria in New York State apple orchards. Applied and Environmental Microbiology 57:486-491.

Pusey PL (1999). Water relations and infection by *Erwinia amylovora* based on crab apple blossom model. Acta Horticulturae 489:521-524.

Smart GC (1995). Entomopathogenic nematodes for the biological control of insects. Journal of Nematology 27:529-553.

Smith-Palmer A, Stewart J, Fyfe L (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Applied Microbiology 26:118-122.

Sobiczewski P, Chiou C-S, Jones AL (1991). Streptomycin-resistant epiphytic bacteria with homologous DNA for streptomycin resistance in Michigan apple orchards. Plant Disease 75:1110-1113.

Thomas GM, Poinar GO (1979). *Xenorhabdus* gen. nov., a genus of entomopathogenic, nematophilic bacteria of the family Enterobacteriaceae. International Journal of Systematic Bacteriology 29:352-360.

Tóth M, Ficzek G, Király I, Honty K, Hevesi M (2013). Evaluation of old Carpathian apple cultivars as genetic resources of resistance to fire blight (*Erwinia amylovora*). Trees 27:597-605.

Turnidge JD, Ferraro MJ, Jorgensen JH (2003). Susceptibility Test Methods: General Considerations. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (Eds). Manual of Clinical Microbiology. American Society of Clinical Microbiology, Washington, USA 1103 p.

van der Zwet T, Beer SV (1995). Fire blight - its nature, prevention and control. A practical guide to integrated disease management. USDA. Agriculture Information Bulletin 631:97.

Viljevac M, Dugalic K, Stolfa I, Dermic E, Cvjetkovic B, Sudar R, Kovacevic J, Cesar V, Lepedus H, Jurkovic Z (2009). Biochemical Basis of Apple Leaf Resistance to *Erwinia amylovora* Infection. Food Technology and Biotechnology 47(3):281-287.

Webster JM, Chen G, Hu K, Li J (2002). Bacterial metabolites. In: Entomopathogenic Nematology. CABI Publishing, USA pp 99-115.

Xiao Y, Meng F, Qiu D, Yang X (2012). Two novel antimicrobial peptides purified from the symbiotic bacteria *Xenorhabdus budapestensis* NMC-10. Peptides 35:253-260.