Plant medicine and veterinary potential of antimicrobial peptides produced by entomopathogenic nematode symbiotic bacteria

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INTRODUCTION

- **Antibiotics-resistance of pathogenic organisms emerged as a new challenge to plant protection, veterinary and even human clinical practice.**
- **The antibiotic multidrug-resistance can be overcome by anti-microbial compounds of totally different mode of action.**
- **Biocontrol potential of antimicrobial peptides produced by EPB), *Xenorhabdus budapestensis* (EMA) and *X. szentirmaii* (EMC) is discussed in this presentation.**

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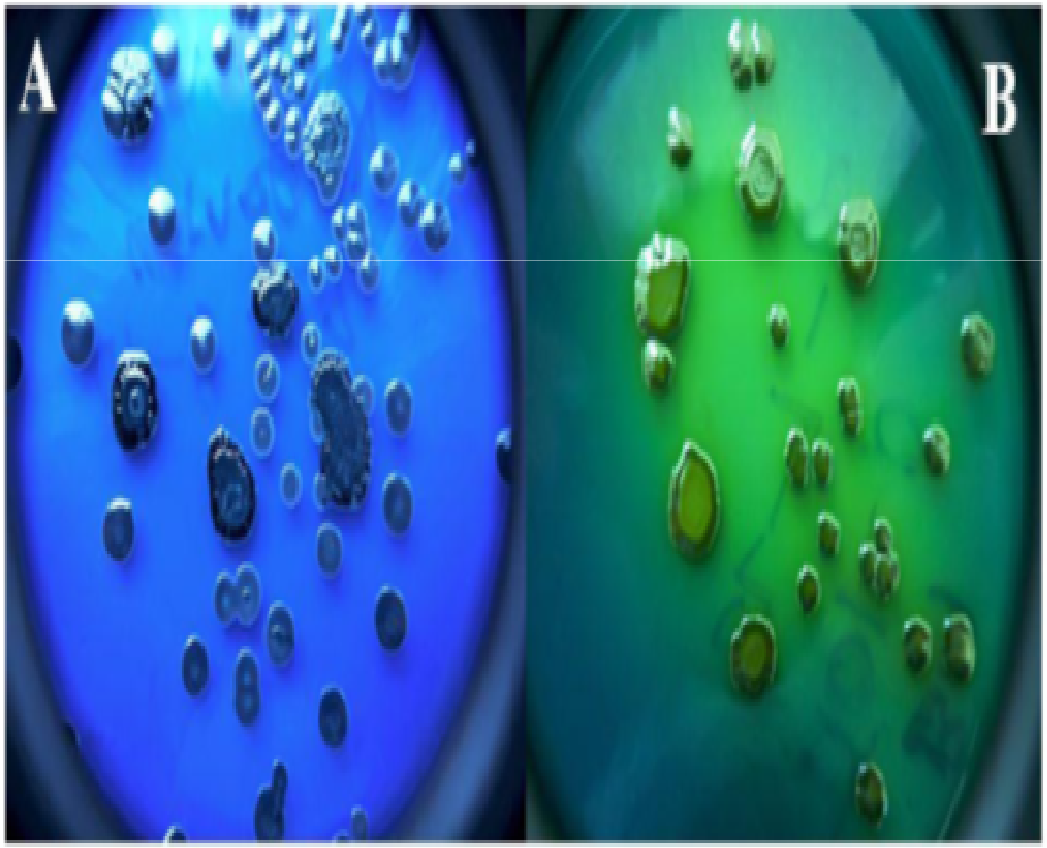
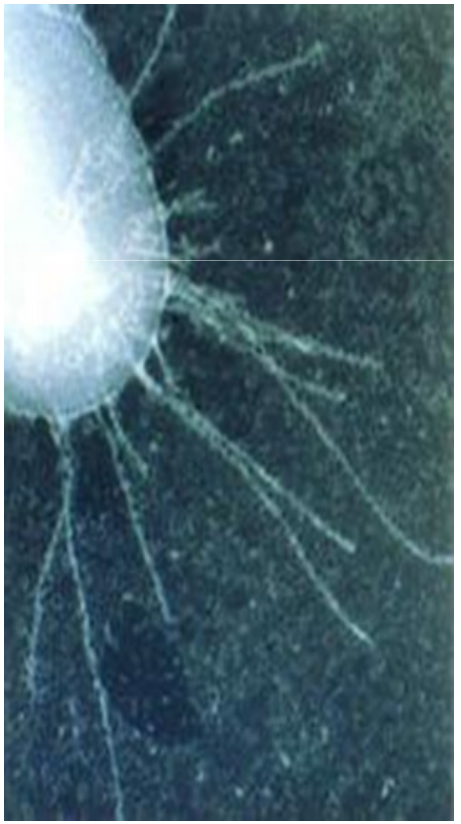
- This EPN bacterium (EPNB) is carried into the blood cavity (hemocoel) of insect hosts by a specific transmission (infective dauer juvenile, IJ) stage of the nematode.
- These bacteria could easily be isolated and cultured in the laboratory both in solid and liquid media, both in small and larger scale and test for antagonistic antimicrobial actibities.

EPNB ON LBTA INDICATOR PLATES



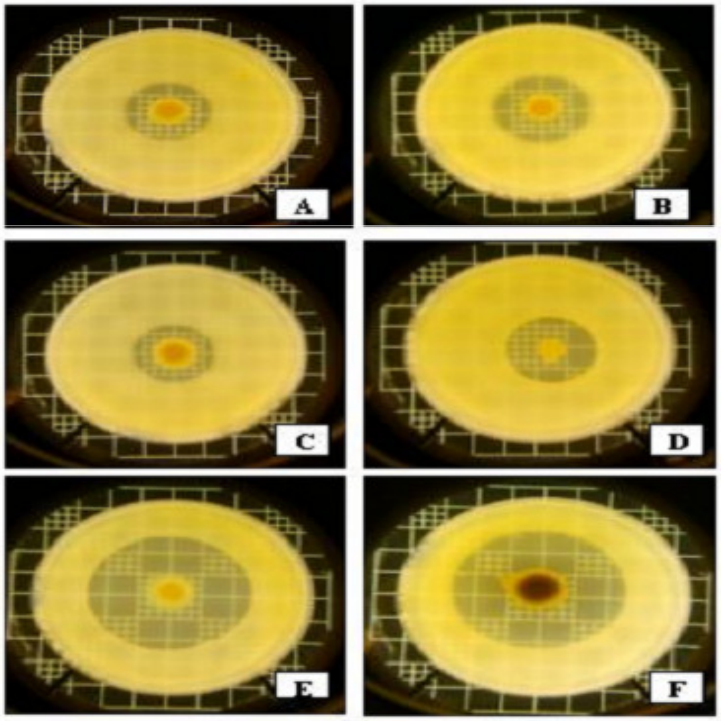


- Once there, the nematode releases the bacteria, which then express immune suppressive and virulence factors that kill insects.
- Then, within the insect cadaver, bacterial activity promotes degradation of insect tissues and deters competitors (including opportunistic non-host nematodes).
- Thus, as part of their lifecycle, EPNB obligatorily interact with and influence the physiologies of competing micro-organisms, nematode parasites, and insects, largely through the production of bioactive proteins and small molecules.

MATERIALS AND METHODS

- After detaliled comparison of EPNB species, strains and isolates from the aspect of their antagonistic activity toward different test tarhets, we found that two species, Xenorhabdus budapestensis and X. szentirmaii have outstandind antimicrobial potential.

Figure 5 Interspecific differences between *Xenorhabdus* strains tested on the *Klebsiella pneumoniae* (mastitis isolate, #696) (Photo: A., M., Fodor; 2006)



A: *X. nematophila* DSM 3370

B: *X. cabanillasii* BP

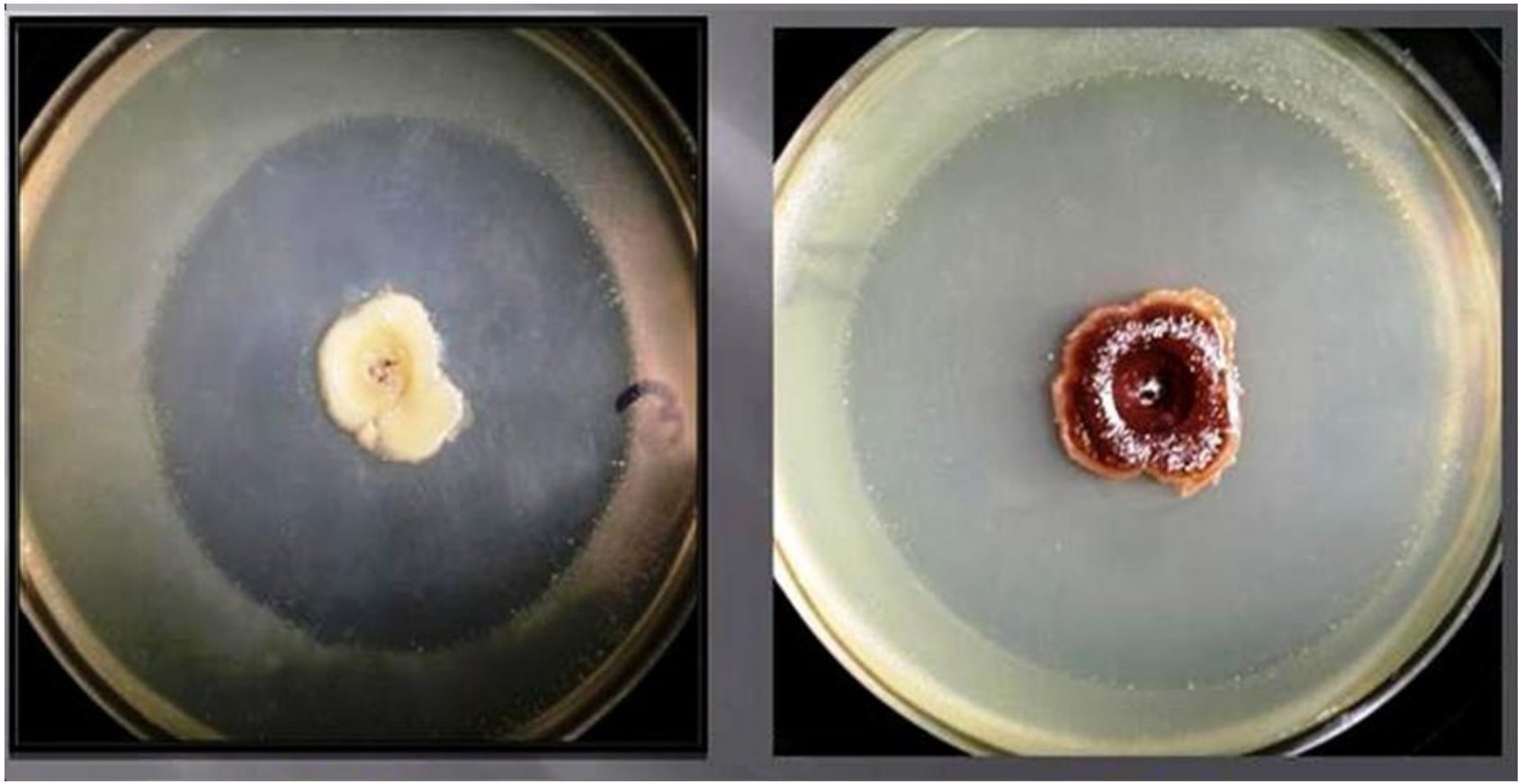
C: *X. nematophila* ATTC19061

D: *X. bovienii* NYH

E: *X. budapestensis* DSM16342^{T}

F: *X. szentirmaii* DSM16338^{T}

Figure 3 *Xenorhabdus budapestensis (left) and X. budapestensis (right)* inhibition zone on *E. coli* (Photo: Andrea Máthé-Fodor, 2010)



MATRIALS: Antimicrobial producers and test organisms

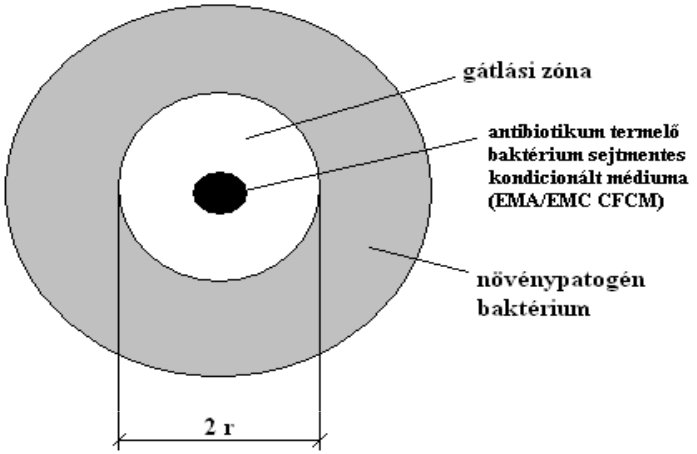
- This study focuses mainly on the bacterium *Xenorhabdus budapestensis* which is the obligate symbiont of the EPN *Steinernema bicornutum* and pathogen of insects.
- Some data on *X. szentirmaii*, a strain which had already been sequenced are also presented.

METHODS:

1. Overlay bioassay, (Furgani et al.,, 2008)

2. Agar diffusion method

- Cell-free conditioned medis (CFCM) in the center aktivitásának vizsgálata
- Size of inactivation zone, depends on:
  - Diffusin speed
  - Concentration of active compound(s)
  - Sensitivity of the test organism



Production of CFCM, (see Böszörményi et al., 2009**):**
(CFCM – cell-free conditioned media)

- Increasing of the Iscale in liquid LB media, [1]→[2]→[3]→[4]
- Centrifuge at 13000 rpm, 20 perc, RTR
- Filtration on (Millipore Stericup Filter Unit, 0.22 µm)
- Storage at , 4 ^{o}C hőmérsékleten

METHODS: BIOASSAYS USED BY US

(OVERLAY) BIOSSAY

X. budapestensis **(EMA, left),**
X. szentirmaii **(EMC, right*)***

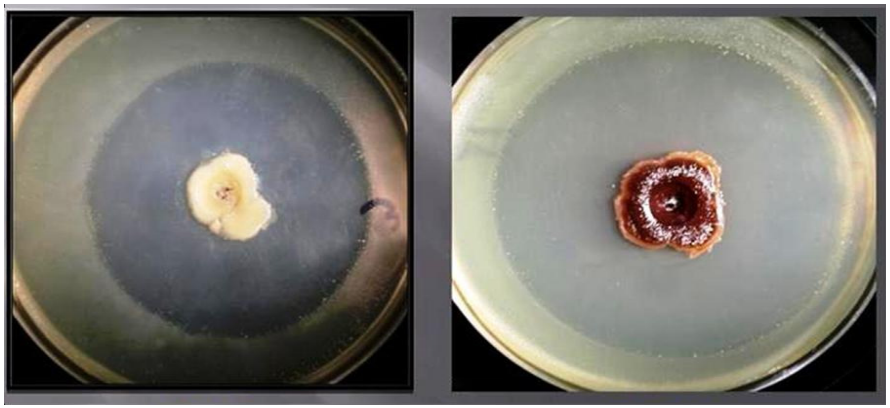


Foto: Fodorné Máthé Andrea

AGARDIFFUSION TEST:

- ***A. Curtobacterium flaccumfaciens pv. betae*** **NCAIM B 01612: EMA („Észak") EMC („dél").**
- ***B.: Xanthomonas axonopodis pv. phaseoli*** **NCAIM 1523;**
- ***C.: Dyckeya chrysanthemi*** **NCAIM B 01839;**
- ***D.: Erwinia amylovora*** **(Ea1)** ***Hevesi Mária.***

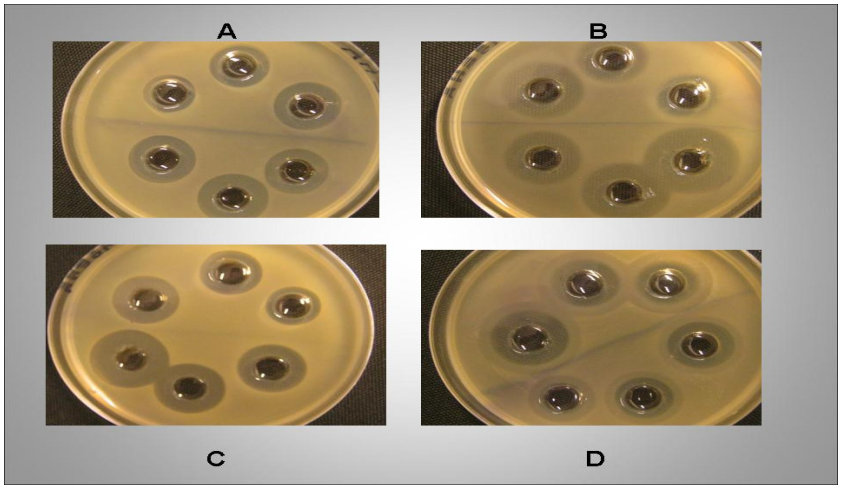


Foto: Dr. Mária Hevesi.

RESULTS

- *Xenorhabdus budapestensis* (AF 2013), a strain with impressive antimicrobial potential.
- *X. budapestensis* culture cell free supernatant has antimicrobial activity against mastitis isolates [1], wild type and antibiotic resistant strains of the plant pathogen *Erwinia amylovora* [2],
- the eukaryotic potato pathogen *Phytophthora infestans*, [2],
- multi-drug resistant *Staphylococcus aureus* (MRSA) (Fodor and McGwire, in prep.), and closely related EPNB strains [3].

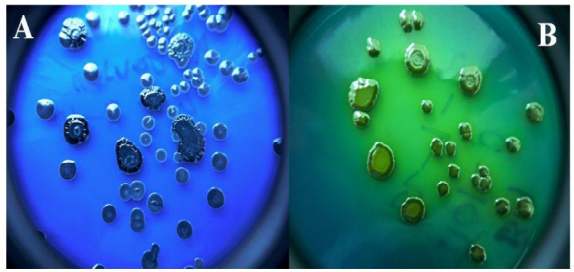
X. budapestensis culture cell free supernatant has antimicrobial activity against mastitis isolates [1],

SUCCESFUL EXPERIMENTS ON

EPB strains

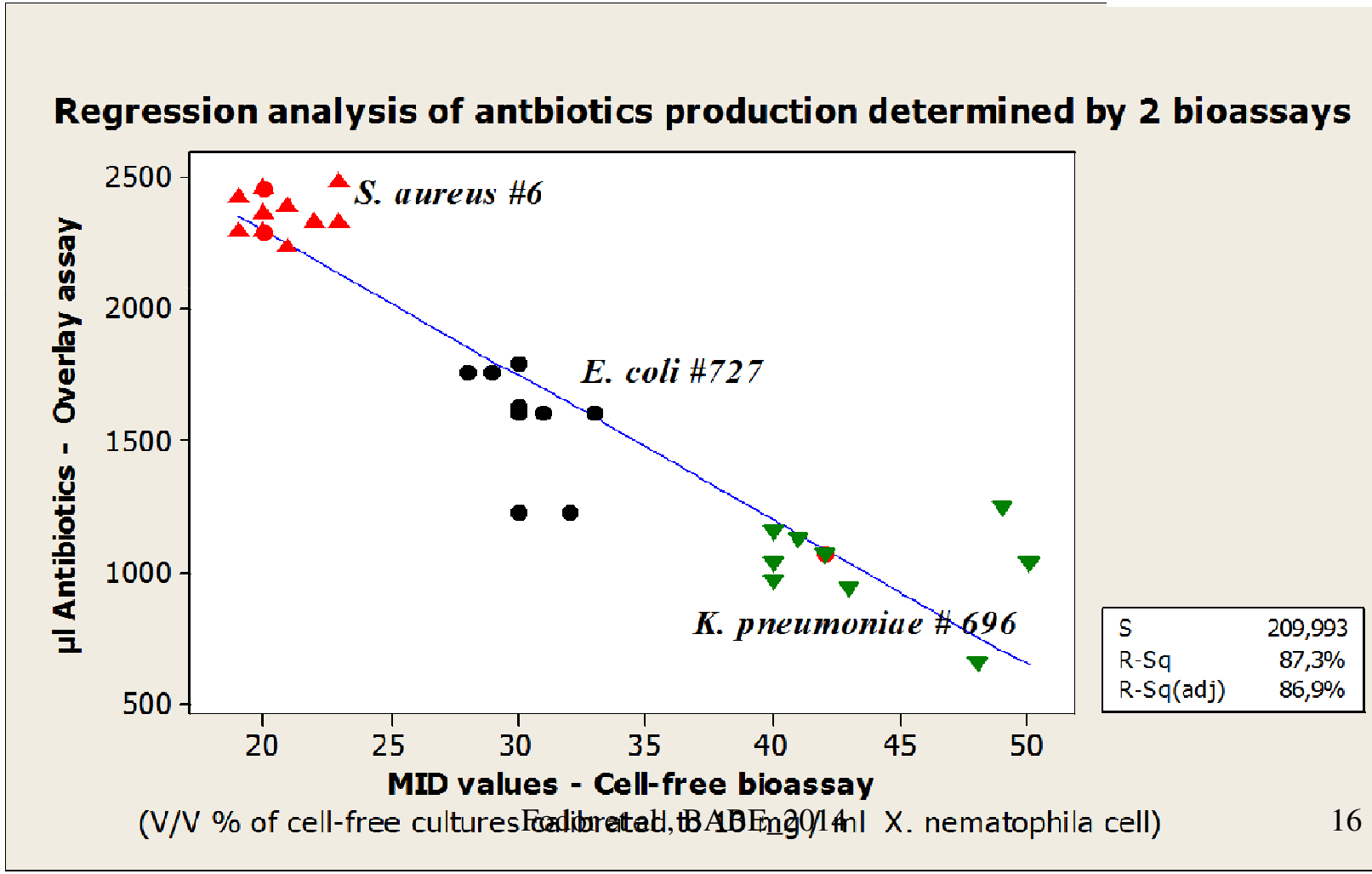
(*Lengyel et al., 2005*)

- ***X. budapestensis*** **EMA**
- ***X. szentirmaii***
- **EMC***

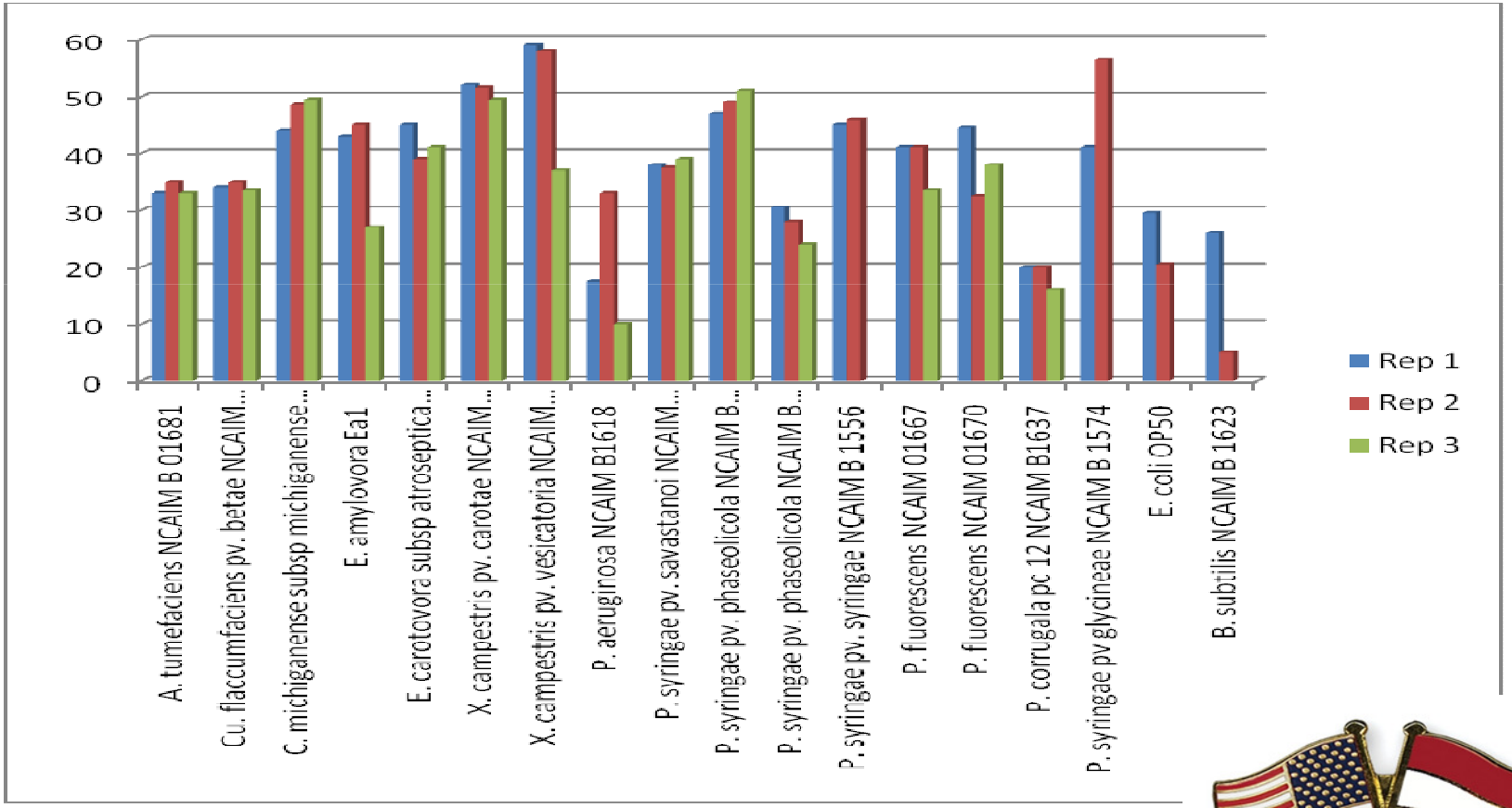


- ***Escherichia coli*** **B (OF-323);**
- ***E. coli*** **K12 (OF-319;**
- ***E. coli*** **TG1 (OF-290);**
- ***E. coli*** **TG90 (OF-630);**
- ***Salmonella enteritis*** **(OF- MA-1504)**
- ***Salmonella enteritis*** **NCAIM B 02186;**
- ***Salmonella*** *typhimurium* **NCAIM B 02212;**
- ***Campylobacter coli*** **NCAIM B 02255;**
- ***Campylobacter jejuni*** **NCAIM B 02254;**
- ***Clostridium perfrigens*** **NCAIM B 01417;**
- ***Ralstonia solanacearum*** **1226 és 879**

ANTIMICROBIAL ACTIVITIES OF *X. BUDAPESTENSIS* ON MAST ISOLATES



Inhibition diameter in mm of *Xenorhabdus budapestensis* on the phytopathogenic bacteria overlaid on LBA media plates with four replications



RESULTS OF AGAR DIFFUSION BIOASSAYS OF SOME PLANT PATHOGENIC BACTERIA

***Xenorhabdus budapestensis* (AF2013, EMA) as a potential tool of controlling *Ralstonia.* Vozik, D., J. Bélafi-Bakó, K., Hogan, J. Racsko, A. Fodor at al., 2014)**

• Abstract

- *Rastonia solanacearum* is a pathogen causes bacterial wilt in potato and other *Solanaceum* species, which has been considered as one of the most significant epidemic disease in plant medicine. The potential of using antibacterial substances from entomopathogenic nematode-symbiotic bacterium strains *Xenorhabdus budapestensis* (EMA) and *X. szentirmaii* (EMC) in *Ralstonia* control has been studied. We have elaborated reproducible methodology to quantitate (1) optimum inoculum size needed for successful *Ralstonia* infection in plant experiments; (2) the minimum phytotoxic concentration and (3) the minimal *Ralstonia* inhibiting concentration of EMA cell free conditioned medium (CFCM) *in vitro*. At the light of the results we consider the antibacterial component(s) of EMA CFCM potential tool(s) of *Ralstonia* control.
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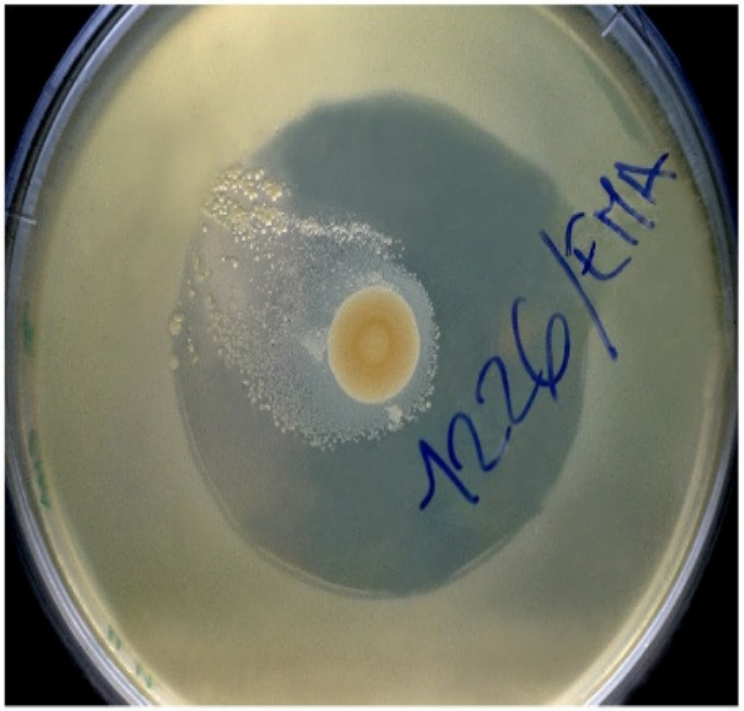
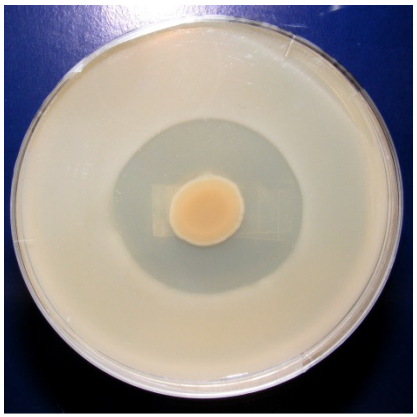
- Keywords: *X. budapestensis*, *R. solanacearum*, antimicrobial activity, antibiotic resistance, phytotoxicity



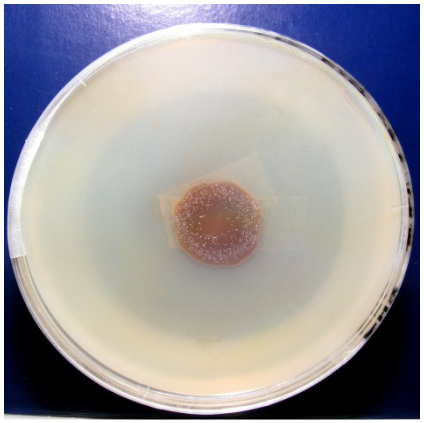
SENSITIVITY OF *Ralstonia solanacearum 1226* virulent strain on EMA CFCM substances in overlay bioassay (Photó: Dr. Mária Hevesi

RESULTS

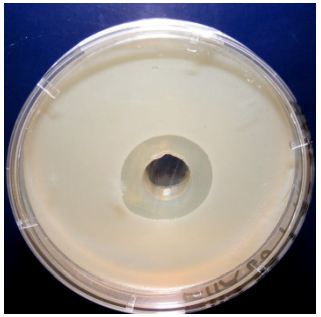
1. Overlay bioassay (Method: Furgani et al., 2008)



EMA / Rs. 1240



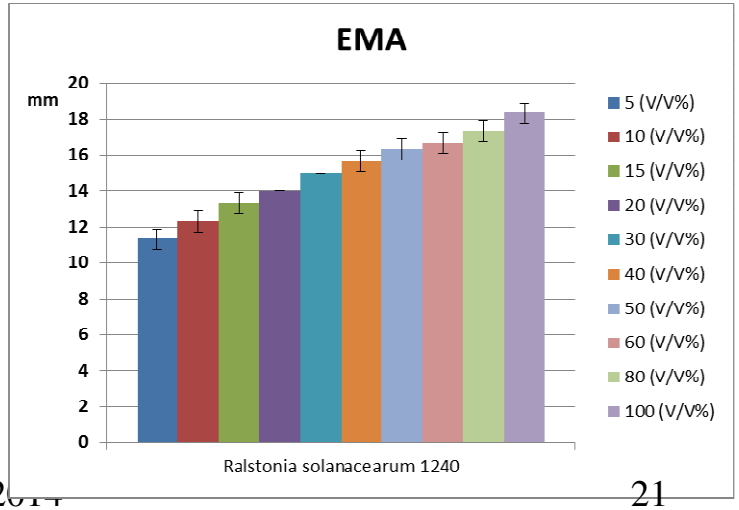
EMC / Rs. 1240



EMA (60 V/V%) / Rs. 1240 - agardiffúziós teszt

2. Agardiffusion test

EMA	EMA CFCM (V/V %)	REPLICATES diam of inactivation zone in mm			Average	s
Teszt-baktérium: Rs.1240	**5**	12	11	11	**11,3**	0,58
	10	13	12	12	**12,3**	0,58
	15	14	13	13	**13,3**	0,58
	20	14	14	14	**14,0**	0,00
	30	15	15	15	**15,0**	0,00
	40	16	16	15	**15,7**	0,58
	50	17	16	16	**16,3**	0,58
	60	17	17	16	**16,7**	0,58
	80	18	17	17	**17,3**	0,58
	100	19	18	18	**18,3**	0,58



RESULTS

3. Antibiotikum-sensitivities of *R. solanacearum* 1240

	GEN	KAN	STR	AMP	CAR	TET	CHL	NAL	RIF
Ralstonia solanacearum 1240	S	S	S	R	R	S	R	R	S

S = érzékeny
R = rezisztens

4. *In planta* infection test (*R. solanacearum* 1240 – on potato seedlings)

DILUTION:	1	0.5	0.25	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	0
O/N Rs.1240 Baktérium szuszpenzió (ml) (OD: 1,050 ; CFU/ml: $6*10^8$)	8	4	2	1				
10X hígított bakt. szuszp. (ml)					0.9			
100X hígított bakt. szuszp. (ml)						0.9	0.08	
autoklávozott csapvíz (ml)	0	4	6	9	8.1	8.1	7.92	8
Abszorbancia (λ = 620 nm)	1,050	0,585	0,306	0,126	0,014	0,005	0,002	0,000

- Dilution: autokclaved tap water
- 2 replicates (test tubes) in each doses
- 2-3 plants / test tube
- 1-5 stages of sickness (visual observation)
- 1=healthy plant; 5= destroyed plant

DILUTION:	1	0.5	0.25	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	0
DAY 0	1	1	1	1	1	1	1	1
DAY 3	3	2	2	1	1	1	1	1
DAY 6	4	3	3	2	1	1	1	1
DAY 18	5	5	5	5	2	1	1	1

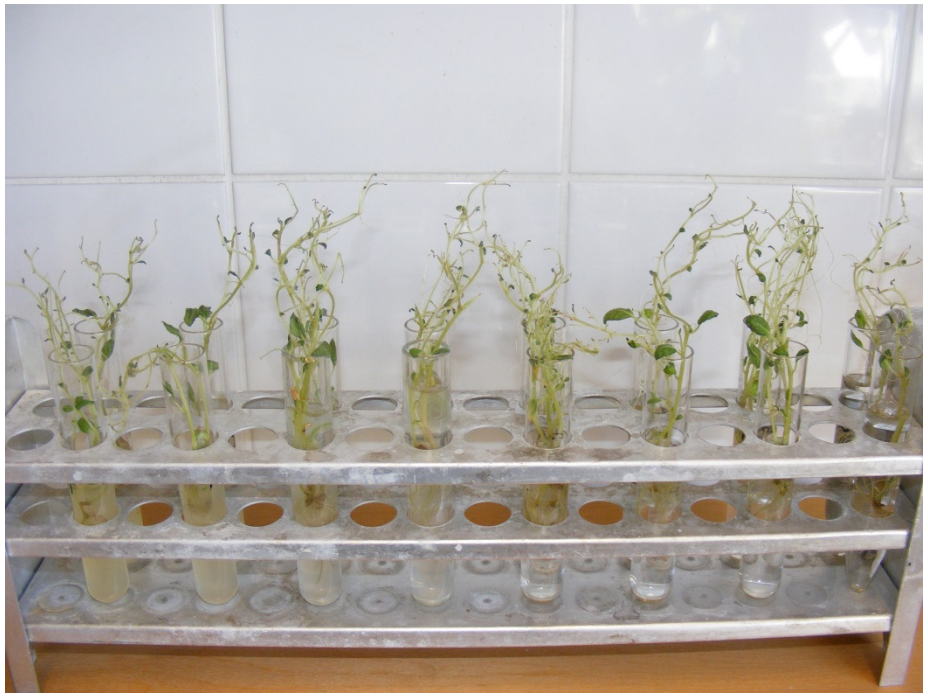
RESULTS

6. Bactericid effect of EMA CFCM on R/ solanacearum RS 1240

EMA CFCM (V/V%)	0	5	10	15	20	25
O/N Bacterium Suspension (OD: 0.866 ; CFU/ml: $4,9*10^8$)	2.0	2.0	2.0	2.0	2.0	2.0
Autoclaved tap water (ml)	6.0	5.6	5.2	4.8	4.4	4.0
EMA CFCM (ml)	0	0.4	0.8	1.2	1.6	2.0
CFU/ml (0)	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$
CFU/ml (2 óra után)	lawn	465000	250000	220000	45000	20000
CFU/ml (4 óra után)	lawn	405000	75000	60000	25000	15000
CFU/ml (6 óra után)	lawn	340000	70000	40000	16500	fertőzött
CFU/ml (8 óra után)	lawn	80000	10000	16000	4500	fertőzött
CFU/ml (24 óra után)	lawn	25000	-	-	-	fertőzött

EMA CFCM (V/V%)	30	35	40	45	50	75
O/N Bacterium Suspension (OD: 0.866 ; CFU/ml: $4,9*10^8$)	2.0	2.0	2.0	2.0	2.0	2.0
EMA CFCM (ml)	3.6	3.2	2.8	2.4	2.0	0
EMA CFCM (ml)	2.4	2.8	3.2	3.6	4.0	6.0
CFU/ml (0)	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$
CFU/ml (2 óra után)	10000	10000	-	5000	5000	5000
CFU/ml (4 óra után)	5000	-	-	-	-	-
CFU/ml (6 óra után)	2500	-	-	2500	500	-
CFU/ml (8 óra után)	-	-	-	-	-	-
CFU/ml (24 óra után)	-	-	-	-	-	-

- Dilution: with autoclaved tapwater
- Incubation> 110 rpm; 28 ^oC-on
- Plating and colony counting on LBA plates (at 2^{nd},; 4th; 6th; 8th; 24 th hrs



DAY 0

DAY 6, INFECTED

DAY 18, INFECTED

DAY 18, NOT INFECTED

DAY 0

DAY 6

DAY 18

RESULTS

5. PHYTOTOXICITY OF EMA CFCM

EMA CFCM (V/V%)	100	75	50	40	30	20	10	0
EMA CFCM (ml)	8	6.0	4.0	3.2	2.4	1.6	0.8	0
AUTOCLAVED TAP WATER (ml)	0	2.0	4.0	4.8	5.6	6.4	7.2	8

- Dilution: autokclaved tap water
- 2 replicates (test tubes) in each doses
- 2-3 plants / test tube
- 1-5 stages of sickness (visual observation)
- 1=healthy plant; 5=totally destroyed plant

EMA CFCM (V/V%)	100	75	50	40	30	20	10	0
DILUTION:	1	1	1	1	1	1	1	1
DAY 0	2	1	1	1	1	1	1	1
DAY 3	3	3	2	1	1	1	1	1
DAY 6	5	5	5	5	5	5	3	1

RESULTS

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CFU/ml (0)	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$
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EMA CFCM (ml)	3.6	3.2	2.8	2.4	2.0	0
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CFU/ml (0)	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$	$1,2*10^8$
CFU/ml (2 óra után)	10000	10000	-	5000	5000	5000
CFU/ml (4 óra után)	5000	-	-	-	-	-
CFU/ml (6 óra után)	2500	-	-	2500	500	-
CFU/ml (8 óra után)	-	-	-	-	-	-
CFU/ml (24 óra után)	-	-	-	-	-	-

- Dilution: with autoclaved tapwater
- Incubation> 110 rpm; 28 ^oC-on
- Plating and colony counting on LBA plates (at 2^{nd},; 4th; 6th; 8th; 24 th hrs

Experiments in Columbus with Dr. Bradford McGwire. Effects of CFCM of EMA, EMC és *X. innexii* on different clinical pathogens

	Biochemistry			Anti-microbial activity									
				bacteria						parasite		fungi	
CM	Heat	P-K	Centricon	MS	MR	FN	ST	EC	PA	LA	TC	CA	Tox
1	+	+	<3 Kda	+	+	+	+	+	+	+	-	+	+
2	+	-	>3 Kda	+	+	+	+	+	+	+	-	+	-
3	nd	nd	nd	+	+	+	+	+	+	+	-	+	-

Notes: CM 1-3, conditioned-medium from three different *Xenorhabdus species*; Heat, autoclaved under standard conditions; Prot K, proteinase K digestion at RT for 24 hrsthen subject to heat inactivation; Centricon, indicates fraction containing anti-bacterial activity; Tox, host toxicity determined microscopically and using MTT metabolism using J-line murine macrophages exposed to indicated CM at 37° C for 72 hrs. Microbes used: MS and MR, methicillin-sensitive and -resistant *Staphylococcus aureus*, respectively; FN, *Francisella novicida*; ST, *Salmonella typhimurium*; EC, E. coli; PA, *Pseudomonas aeruginosa*; LA, *Leishmania amazonensis*; TC, *Trypanosoma cruzi*; CA, *Candida albicans*. Activity assays were performed using microbes grown in 25-100 μL CM (Luria-Bertani based) overnight then plated on appropriate medium for CFU-reduction assays (for bacterial or fungal pathogens) or using MTT utilization assays (parasites). +, retains activity; -, devoid of activity; nd, not determined.

EMA CFCM ON SOME PLANT PATHOGEN *Erwinia amylovora* (left) and *Alternaria alternata* (right)

- ***Erwinia amylovora*** (Ea1) dose-dependence in in agar diffusion test

Alternaria **alterna** mycelium growth is i poisoned PDF agar)

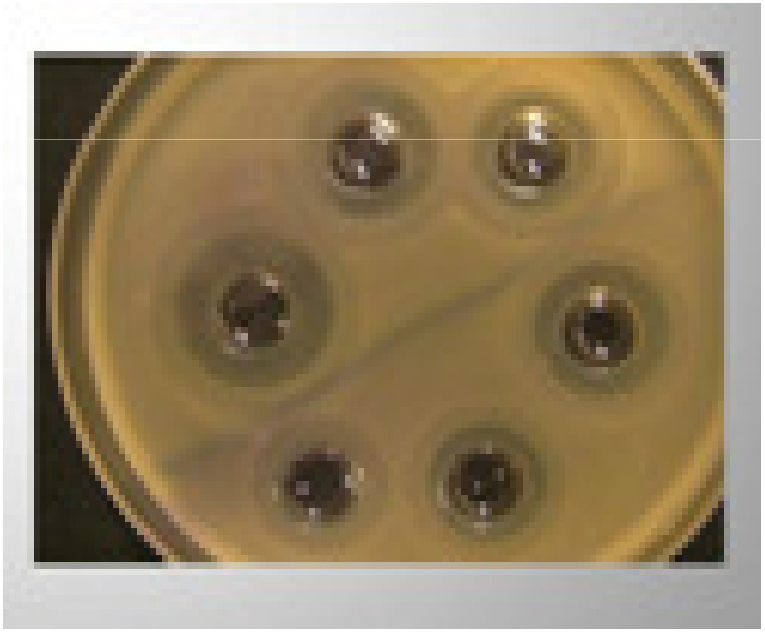


Photo: Dr. Mária Hevesi.

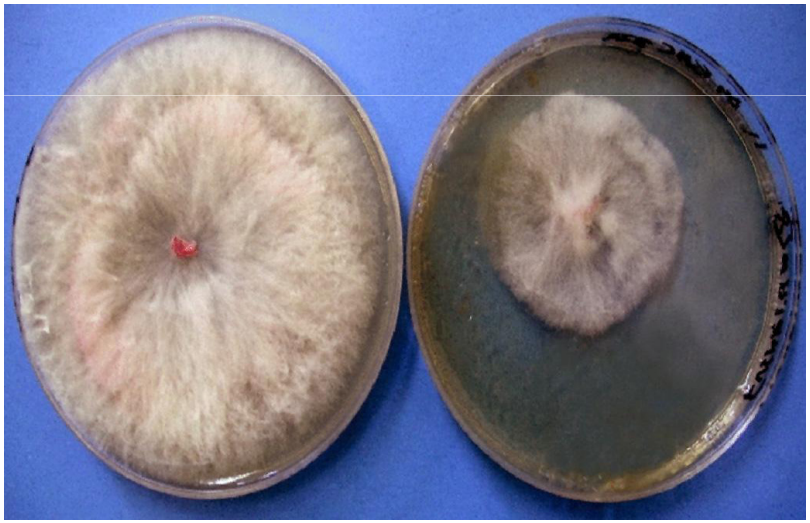
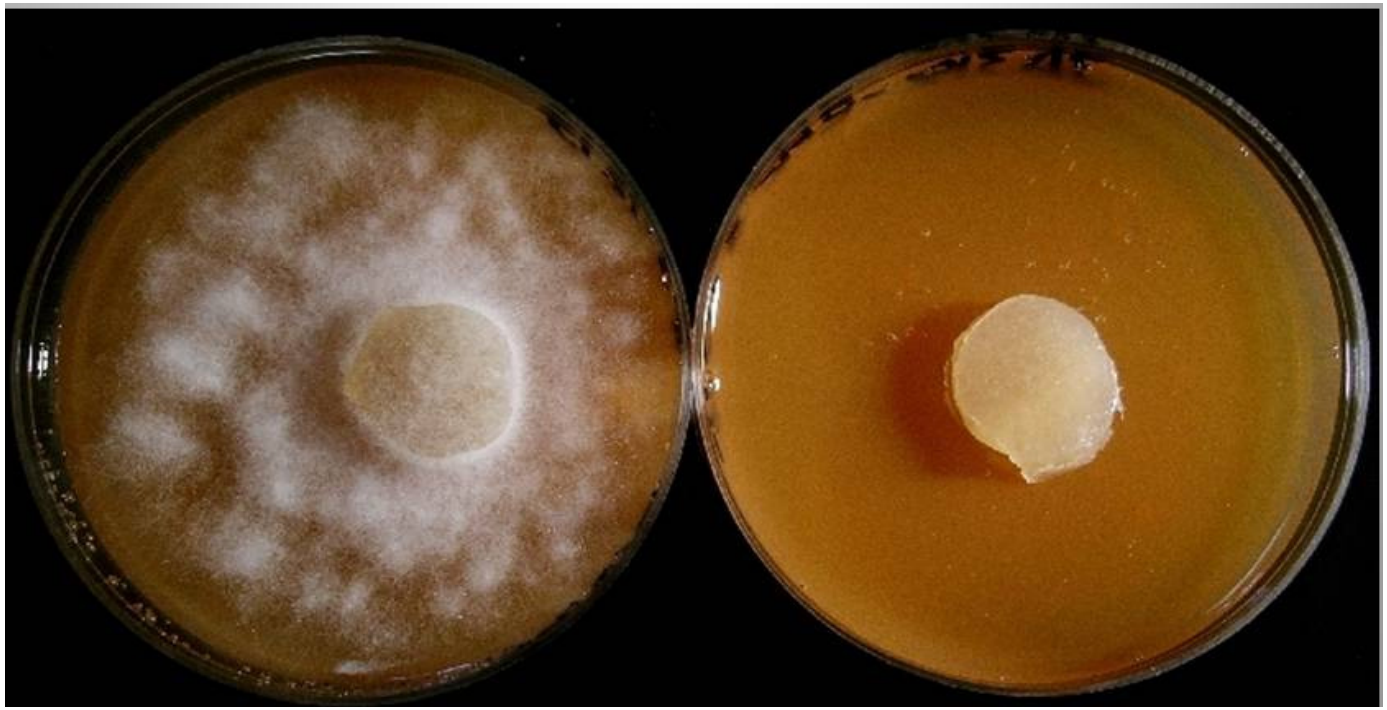


Photo: Dr. Csaba Pintér / (Zakria Favzi, MSc. Theses)

Effect of cell-free conditioned media (CFCM) of *X. szentirmaii* on the mycelial growth
Sejtmentes *X. szentirmaii* médium hatás myceliumok növekedésére



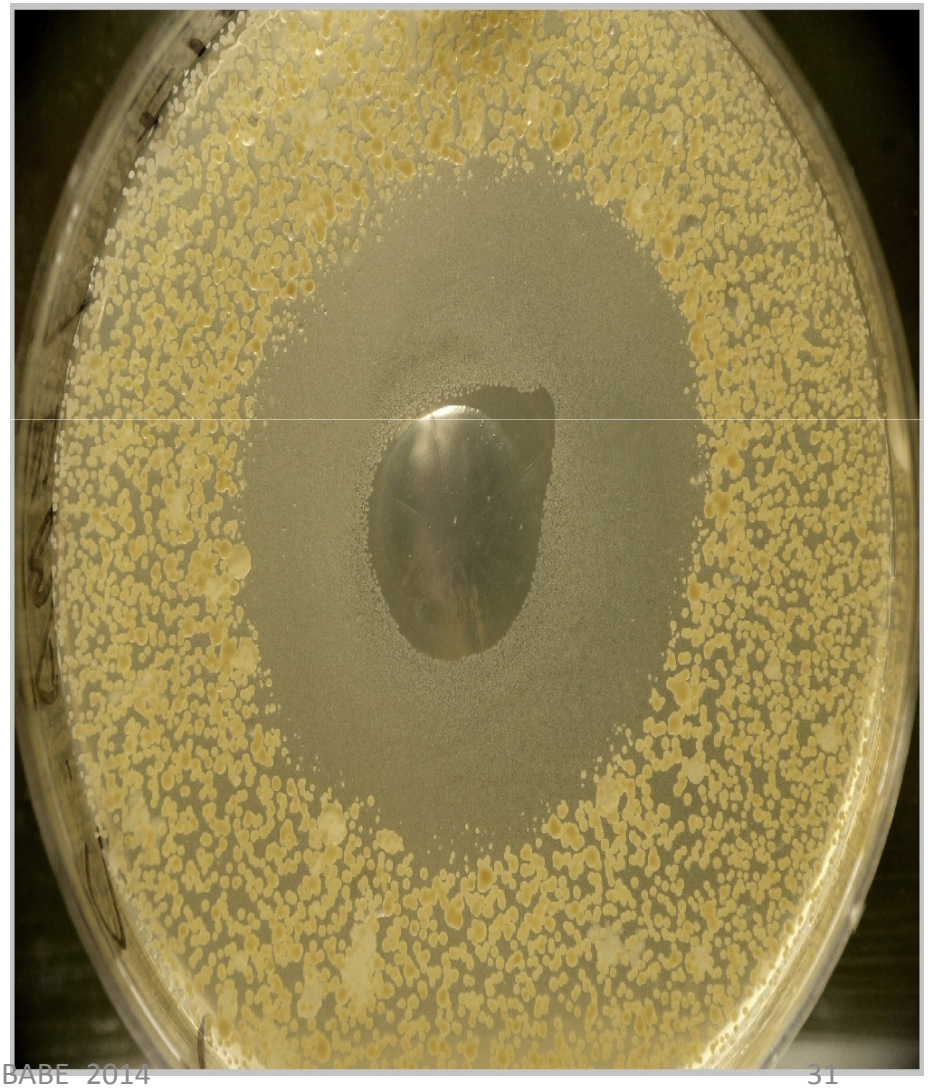
Kontrol
(Sárgarépa- agar)

Sejtmentes (25 V/V%)
EMC-fermentlé

(Fotó: Pintér, Cs., 2011

EREDMÉNYEK - 2

- **A *Clostridium perfringens*** NCAIM 1417 **törzse rárétegzéses és agardiffúziós tesztekben egyaránt érzékeny a**
- ***X. budapestensis* és a**
- ***X. szentirmai* antibakteriális aktivitásával szemben**.
- **Fotó: *X. budapestensis*** agar-difffúziós tesztben ***C. perfringens* NCAIM 1417** ellen. **Dr. Pintér Csaba**



DISCUSSION

- Our goal has been to exploit the potential of the active antmicrobial compounds of X. budapestensis against *plant and animal pathogens*.
- One of the discovered very active microbial compound in *X. budapestensis* is the hexapeptid Bicornutin A
- Let us make a short comparison with our finding with the literature of AMPs.

Antimicrobial peptides – a general discussion

- Organisms of different taxa produce antimicrobial peptides. Their general biological role is self-defense.

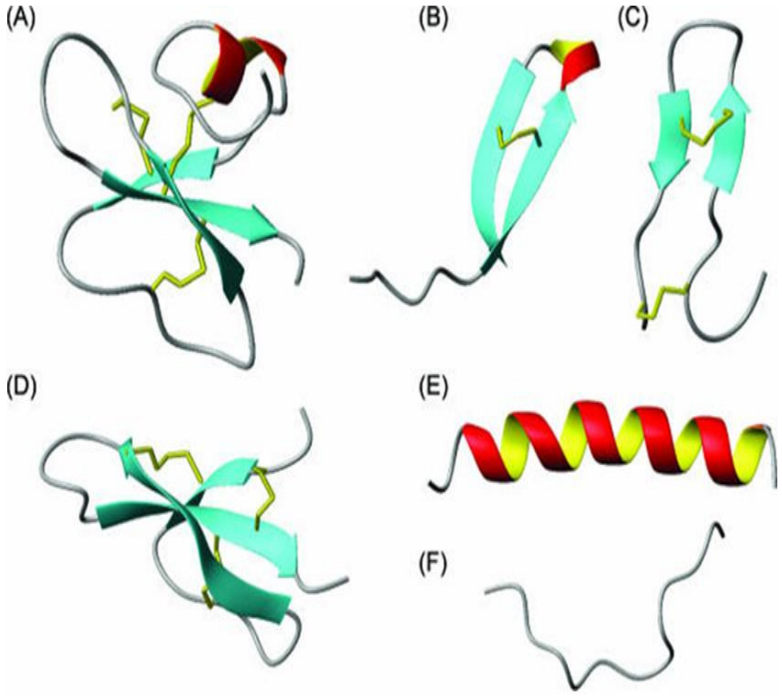
- Each peptide of known antibacterial activity (AMP) is of cationic (positively charged) and amphipathic nature. It is also true for most antiviral, anti-parasitic and antifungal peptides.

- The targets of these peptides as well as of their mode of actions are rather different.

- There are great differences between their targetspectra of different AMPs.

Molecular structures of antimicrobial peptides

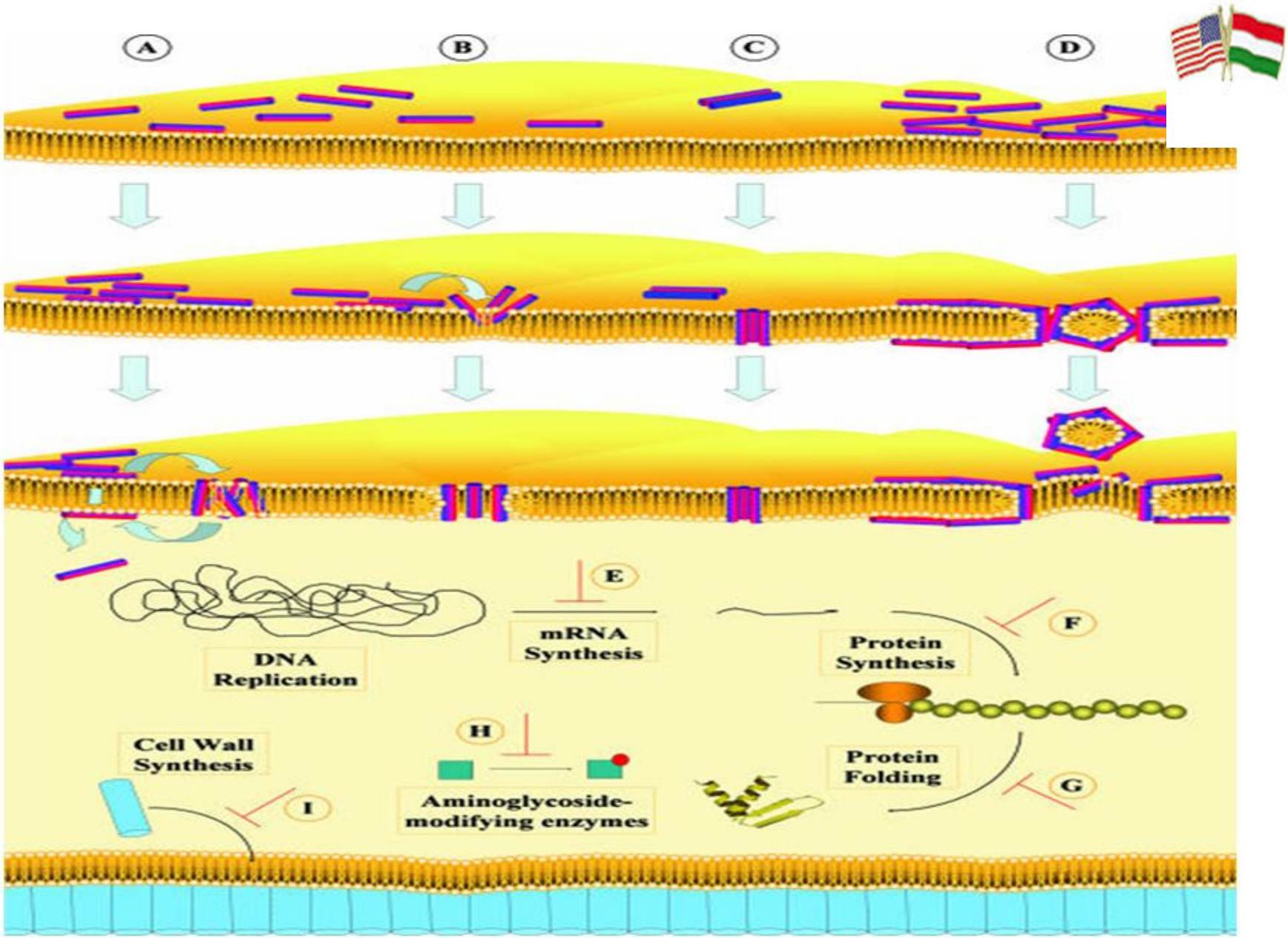
Jenssen, et al. *Clin Microbiol Rev*. (2006)19:491-511.



(A) Human β-defensin-2 (PDB code 1FQQ)
(B) (B) Loop-like structure of thanatin (PDB code 8TFV) ;
(C) (C) β-laminal structure of polyphemusin (PDB code 1RKK)
(D) (D) Rabbit defensin-1 (PDB code 1EWS) 165); [65]
(E) (E) α-helical structure of magainin-2 (PDB code 2MAG) (76);
(F) (F) Relaxed structure of indolicidin (PDB code 1G89) [66] . The disulfide bridges are yellow. The pictures were created by a Mol Mol 2K.1 [67] graphic program.

STRUCTURE – ACTIVITY RELATIONS

- The molecular basis of the mode of action of antimicrobial peptides is based on non-specific structural changes in the target membranes followed by rapture.
- The mode of action of AMP has largely been determined by the membrane structure of the target cells.
- At present 5 models of mode of action has unambiguously confirmed while others are hypothetical


A
B
C
D
E
mRNA Synthesis
DNA Replication
F
Protein Synthesis
H
Aminoglycoside-modifying enzymes
Cell Wall Synthesis
I
Protein Folding
G

MODE OF ACTION OF AMPs

- The target cell membrane (yellow) is a lipid double layer.
- The peptide molecules are labeled as little cylinders.
- Hydrophilic parts of the molecules are labeled as red, while the hydrophobic ones are labeled as blue.
- The peptide-glucose membrane components are labeled as purple cylinders.
- Models A-D described the consequences of the changes in the permeability of the target cell membranes.
- Models E-I show the effects on the biosynthesis and structural rearrangements (protein folding, for instance) of the macromolecules in the target cells

OUR RESULTS AT THE LIGHT OF THE LITERATURE

- In the last years have been focusing on EPB virulence factors of EPB [16-23] with special attention to exploit the antimicrobial potential of two EPB (*Xenorhabdus budapestensis* and *X. szentirmaii* [24] species identified by us and use them for controlling plant pathogenic purposes).
- Gram positive pathogens, such as *Staphylococcus aureus* and *Clostridium perfringens* pathogens proved even more sensitive to EPB antimicrobials than the Gram-negative ones (Fodor et al., in preparation).

OUR RESULTS AT THE LIGHT OF THE LITERATURE

- There are not many publications related to EPB antimicrobial peptides could be found in the literature. This provides an advantage but also a great challenge and needs an inventive approach and scientific creativity.

- The fact that the Helix BioMedix Company has intensively working on hexapeptide (putative) drugs in order to use them in lipid dense environment such as blood sera against bacterial and fungal pathogens. Several of theses hexa-peptides are in pre-clinical stage

THE ANTIMICROBIAL COMPOUNDS OF *X. BUDAPESTENSIS* ARE ANTIMICROBIAL PEPTIDES

- In our previous experiments in the last decade these compounds proved active both in Gram-positive and Gram-negative bacterium pathogens (*Clostridium perfringens*, multi-resistant pathogenic *E. coli* and *Salmonella* strains as well as in eukaryotic pathogens such as *Eimeria tenella* (Dr. Klaus Teichmann et al., Biomin, unpublished), *Alternaria alternata, Phytophthora infestans* (Fodor et al., in preparation).
- In the nature the bacterial partner of the entomopathogenic nematode (EPN) / bacterium (EPNB) symbiotic complexes produces antimicrobial peptides (AP) to protect the monoxenic EPN/EPB system in the cadaver in polyxenic (soil) conditions.

FINAL CONCLUSIONS

- We described several important plant and veterinary pathogenic organisms, (belonging to Gram (+) and (-) bacterium, oomycetal, fungal, and Protista species) susceptible to the native cell-free conditioned media (CFCM) of *X. budapestensis in vitro*.
- We discovered a different activity of CFCM on closely related species [31]
- Whether the AMPs would or would not be developed to plant protective products capable of controlling the most harmful eukaryotic and prokaryotic plant, - and animal pathogens, and overcome multiple antibiotics resistance will be decided at the end of the project.

BICORNUTIN A: A NATURAL COMPOUND OF STRONG ANTIMICROBIAL ACTIVITY

- **One active component (bicornutin A,) produced by *X. budapestensis* had been identified [26-28] by us. Other researchers also found antibacterial peptides in *X. budapestensis* [30] and others in *X. szentirmaii* [31].**

- **Each AMP published so far has a larger molecular weight and more amino-acid (AA) residua than the Bicornutin A (discovered by us). The recent interest toward antimicrobial sexta-peptides (see below) however, indicates the perspectives of our project proposal.**
- **Many antimicrobials are synthesized through the action of non-ribosomal peptide synthetases (NRPS) with modular structures. We intend to dicover the the genes responsible its biosynthesis**

POTENTIAL TO OVERCOME ANTI-BIOTICS POLY-RESITANCE

- The cell-free conditioned culture media (CFCM) of both species exerted cytotoxic activity on to mastitis isolates [25], prokaryotic *(E. amylovora)* and on eukaryotic (*Phytophthora sp.*) plant pathogens [26], coliform Gram-negative pathogens of veterinary importance independently of their antibiotics resistant profiles (Fodor et al., in preparation).
- The wild type and antibiotics resistant variants of the target species (*E. emylovora, E. Coli, Salmonella, Agrobacterium*) are equally sensitive to them [26]. They proved poly-resistant pathogens (*S. aureus* MRSA) (Fodor and McGwire, in preparation).

PLANT PROTECTION POTENTIAL

We propose further efforts toward developing application technology of EPB antimicrobial peptides against

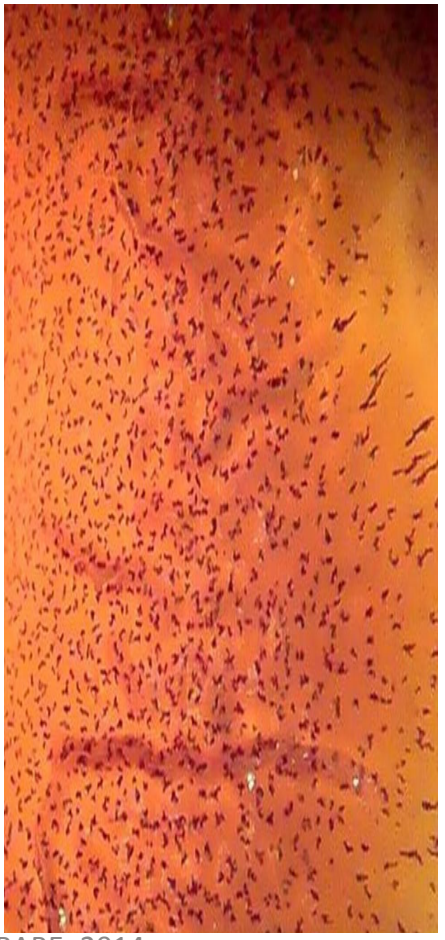
- **Fire blight (*Erwinia amylovora*)**
- **Potato blight *(Phytophthora infestans*);**
- **Plant diseases caused by**
- ***Clavibacter*,**
- ***Curtobacter*,**
- ***Xanthomonas* and**
- ***Ralstonia* species.**

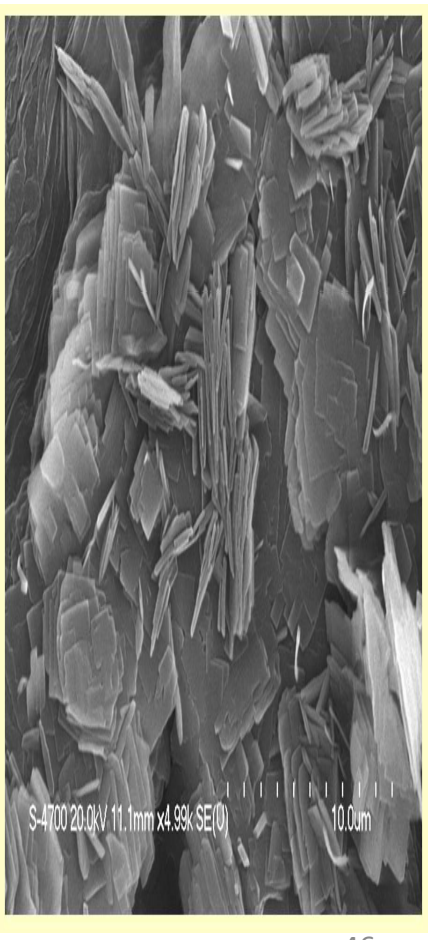
VETERINARY POTENTIAL

- As for veterinary application, we found that all studied strains of **independently of their resistance to other antibiotics**
- ***Aeromonas hydrophila***
- ***Bacillus cereus***
- ***Corynebacterium pseudotuberculosis***
- ***E. coli***
- ***Salmonella***
- ***Listeria monocytagenes***
- ***Pasteurella multicida***
- ***Rhodococcus equi***
- ***Streptococcus equi***
- ***Staphylococccus areus***
- ***Bordatella bronchoseptica***
- ***Klebsiella pneumoniae –***
- **Proved sensitive to CFCM (BicornutinA) of *X. budapestensis*.**

God as an artist: polyiodinin exo-crystals produced by *X. szentirmaii*

- ***X. szentirmaii*** **colony surfacs felszín (Foto: Dr. Pintér Csaba) and exocrystal (Fotó: F. Máthé Andrea)**





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- Ohio State University, Department of Animal Sciences, Wooster OH 44691 USA
- University of Pannonia, Georgikon Faculty, Keszthely and Veszprém, Hungary
- Szent István University, Department of Microbiology, Budapest, Hungary