EFFECT OF *BACILLUS SUBTILIS* BS 934 ANTIMICROBIAL UPON PENETRATION ABILITY, CHEMOTAXIS, MEMBRANE VOLTAGE AND ION FLUXES OF ERWINIA AMYLOVORA CELLS

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The *Bacillus subtilis* BS 934 antimicrobial has properties, which are differ it from the antimicrobials, described for other bacilli. The substance is able to change the pattern of ring formation by chemotactic cells of the *Erwinia amylovora*, the fire blight agent, and to suppress the penetration and movement of the pathogen within plant vessels and capillary tubes of chromatography paper *in vitro*. The substance caused changes in membrane voltage ($\Delta\psi$) and enhanced the K^+ ion efflux from *Erwinia amylovora* cells.

Introduction

Fire blight is a major concern of pome fruits production [10]. Genetic resistance to *Erwinia amylovora*, the causal agent of the disease, exists [23, 25], but it has not been utilized widely, because resistant cultivars have inferior marketing qualities [3]. The most effective chemical is streptomycin, however, resistance to the antibiotic has been detected in fire blight agent [13, 14, 16, 20].

Abo-El-Dahab & El-Goorani showed *in vitro* antagonism of *B. subtilis* upon fire blight agent also comparable to the antibiotic action [1]. No commercial products were developed with the use of this microorganism. Physical and chemical properties of active substance and mode of its action, particularly, on the sensitive cells energetics and ion fluxes were not studied. As bacilli are spore forming microbes, it will be easy to produce, store and apply the biocontrol formulations based on them. However, their active substances should be studied in details first.

This work was aimed to *i)* evaluate the ability of the *Bacillus subtilis* BS 934 and its antimicrobial to influence on the movement of *Erwinia amylovora* into plant vessels and chromatography paper capillary tubes and on the pathogen chemotaxis *ii)* to study the antimicrobial's effects on the *E. amylovora* cell survival, membrane voltage and K^+ ion fluxes in comparison with known membranoactive antibiotics.

Material and methods

Microbial cultures. The *B. subtilis* BS 934 strain was isolated in Ukraine. The *E. amylovora* CFBP 2312, CFBP 2582, CFBP 1430 and *E. amylovora* Ea 394, 659, 651, were from Collection of Phytopathogenic Bacteria of France, Beacouzé Cedex, and Prof. Piotr Sobiczewski, Research Institute of Pomology and Floriculture, Skierniewice, Poland, respectively.

Production of antimicrobial. The *B. subtilis* BS 934 culture filtrate concentrate (crude antimicrobial, CA) was obtained as described in [19]for the strain *B. subtilis* 107. The partially purified antimicrobial (PPA) was obtained by double sedimentation with concentrated HCl and restitutions in phosphate buffer with pH $\geq$ 7.

Penetration and chemotactic movement tests. To study the influence of the fire blight antagonist and its antimicrobial onto movement of *E. amylovora* Ea 394 within plant vessels, the split and autoclaved wooden (1 year old) 'Conference' pear shoots were placed by bottom end into 2 ml of 5% sucrose broth night *B. subtilis* BS 934 culture, or solution of its PPA at concentration 60 mg /mL, or streptomycin sulphate (Str) (Kyivmedpreparat, Kyiv, Ukraine) at concentrations 0,04 mg/mL in Tris-HCl buffer, pH 7.5 for 2 days. After drying in sterile air flow the shoots were dipped into suspension of *E. amylovora* Ea 394 at 10^9 cfu/ml for a night. All inoculations were kept at 30°C. Shoots in control were inoculated with *E. amylovora* Ea 394 suspension only. The shoots were dried sterilely to remove excess water, cut and stamped against 5% sucrose agar plates with (for suppression of *B. subtilis* BS 934) or without (for control of *B. subtilis* BS 934 movement) 1.5 g/L of lincomycin hydrochloride (Farmak, Charkiv, Ukraine). The *E. amylovora* Ea 394 formed colonies at this concentration of the antibiotic, when inoculated by appression of penetrated material against agar plate. This allowed to estimate the penetration of fire blight agent along the shoots. The penetration ability test was performed also with the use of crenate strips cut from Whatman 3MM Chr paper (Whatman International Ltd, Maidstone, U.K.) and from DIN A4 No 2043 b and No 2045 b paper muster/samples (Schleicher & Schuell, Dassel, Germany) in the same way as in the experiment with shoots.

The ability of bioactive substance from bacilli to influence the chemotaxis was evaluated onto the medium of Adler [2], in which glucose was substituted by 10^{-4}M/L of sucrose. For chemotactical ring formation, the medium was inoculated with 0,01 mL drop of *E. amylovora* suspension at the plate periphery and kept at 30°C. The Whatman paper disks impregnated with 800 μg of PPA were placed in the center of agar plates in front of the moving band of the cells of particular *E. amylovora* strain.

Chemical and physical properties of the antimicrobial substance. PPA was extracted three times with 80% ethanol to remove activity. The extracts were dried in vacuum at 50°C on the rotary evaporator (Büchi, Flawil, Switzerland). Dried residues were extracted with ethyl acetate, then acetone and finally dissolved into 80% ethanol.

The ethyl acetate, acetone and 80% ethanol extracts of CA were spotted onto TLC plates SIL G-25 UV_{254} precoated with 0.25 mm silica gel with a fluorescent indicator (Camlab, Cambridge; Macherey-Nagel, Germany). The plates were developed in ethanol : water (2:1, v/v) and bands visualized in UV light (250-350 nm). The bands which contained antimicrobial activity were detected as zones of inhibition formed at 30°C within 2 day after covering the intact chromatograms with *E. amylovora*-seeded soft agar. Other plates were sprayed with 0.2% ninhydrin solution in 95% ethanol and heated at 110°C for 5 min to detect ninhydrin-positive substances.

Tris-HCl buffer solutions of PPA were subjected to the influence of changes in pH, temperature, and pressure. It was incubated for a night at 37°C with the following enzymes (at a pH optimal for each enzyme):- DN-ase, RN-ase, *α*-chymotrypsin, lipase (Sigma-Aldrich, St. Louis, USA), *Pseudomonas mendocina* lipase (gift from Dr. B. Surinenaite, Vilnius University), proteinase K (Boehringer Mannheim Ltd, Lewes,UK) at concentrations up to 20, 20, 5, 50, 8 and 10 mg per mL of the antimicrobial substance solution, respectively.

The molecular weight (M_r) of the antimicrobial substance was evaluated using benzoylated dialysis tubing which retains compounds with an M_r value greater than 2,000 Da. Diffusion of the antimicrobial substance through the tubing was indicated by inhibition of a lawn of sensitive bacteria seeded after the dialysis tubing removal. The CA was stored at 4°C for over 1 year to establish shelf-life.

Potential siderophore production was assessed by culturing of *B. subtilis* BS 934 onto the Oxoid nutrient agar (pH 5) with 10^{-4}

M ferric chloride per litre. The increase in size of the inhibition zone in the sensitive culture at the presence of Fe^{3+} ions is a positive reaction for siderophore presence.

Chitinolytic activity was tested onto agar plates with 2g/L of colloidal chitin from crab shells (Sigma-Aldrich, St. Louis, USA) at pH 6.6 by the method of Rodriguez-Kabana et al. [18].

The $FeCl_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, NaCl and $MnSO_4 \cdot 5H_2O$ were added to Oxoid NA separately at concentrations 0.1-1, 100, 100, 50 mM and 10 μM, respectively, as it was down by Milner et al. [15] to look if they have effect on antimicrobial substance accumulation.

The influence of the atimicrobial onto membrane voltage, ion fluxes and cell survival. The first site of interaction and one of the possible targets for deleterious action of *B. subtilis* BS 934 antimicrobial is the cell envelope. Therefore, we found it meaningful to investigate the influence of the antimicrobial on the barrier functions of sensitive cell envelopes.

The monitoring of the ion fluxes combined with the estimation of cell viability was used to evaluate the barrier properties of *E. amylovora* CFBP 2312 cell envelope and to examine the effect of *B. subtilis* BS 934 antimicrobial on *E. amylovora* outer membrane (OM) and plasma membrane (PM) permeabilities and the cell energetics. The observed effects were compared with action on sensitive cells of streptomycin sulphate (Kyivmedpreparat, Kyiv, Ukraine), the most effective chemical in fire blight control, and membranoactive compounds, the ethylenediaminetetraacetic acid (EDTA), polymyxin B (PMB) and gramicidin D (GD) (all were from Sigma-Aldrich, St. Louis, USA). The experiments were carried out at 30°C in 100 mM Tris/HCl buffer, pH 7.5, initially containing 1 μM TPP^+. For the calibration TPP^+ was added to obtain 3 μM concentration. The concentration of *E. amylovora* CFBP 2312 cells was 1.5×10^9 cells/ml. EDTA, PMB and GD were added to the final concentrations of 0.4 mM, 125 μg/ml and 5μg/ml, respectively. Streptomycin sulphate (Str) was added to the final concentrations of 0.05, 0.10, 0.25, 0.50 and 0.75 mg/ml. The crude (CA) or partially purified antimicrobial (PPA) was added to bacterial suspension to the ratio (vol/vol) 1/100, 1/50, 1/33 and 1/25 (measurements of TPP^+ accumulation) or 1/25 and 1/1 (evaluation of the citotoxicity) respectively. For estimation of viability of the cells (inserts) *E. amylovora* CFBP 2312 cells were incubated with streptomycin or antibacterial substance for 30 min at 30°C, diluted and plated. 1 mL of the *B.subtilis* BS 934 culture filtrate concentrate (crude antimicrobial, CA) contains 80 mg of PPA.

The uptake and distribution of the lipophilic cation of tetraphenylphosphonium (TPP^+), (Sigma-Aldrich, St. Louis, USA), between the cells and the external medium was monitored to assay the membrane voltage, $\Delta\psi$. The usage of TPP^+ for $\Delta\psi$ measurements requires the *E. amylovora* CFBP 2312 outer membrane permeabilization, which was achieved by treatment of the cells suspensions in 0,1M Tris-HCl - 0,01 M EDTA at 30°C for 10 min. The suspension was kept on ice until used (maximum 4 h). The suspension (50-70 μL) was added to Tris-HCl buffer in a 5-mL reaction vessel kept at 18°C and aerated on a magnetic stirrer. The K^+ and TPP^+ ions concentration were monitored by ion-selective electrodes, connected to Orion 520A pH/ISE meters. The K^+- selective electrode was from Orion Research, Inc.(model 93-19). The Ag-AgCl reference electrodes (Orion Research Inc.; model 9001-9002) were indirectly connected to the measuring vessels through an agar salt bridge. The construction of TPP^+ selective electrode has been described by Grinius et al. [8].

The nonspecific binding of TPP^+ was measured after the addition of PMB (125μg/mL) and GD (5μg/mL) to the reaction vessel.

The internal K^+ concentration was calculated from the external one, assuming that 200 Klett units (A_{540}) correspond to 10^9 cells/mL, 2 x 10^9 cells correspond to 1 mg of dry mass, and the intracellular water volume

of *E. amylovora* is approximately equal to that in *E.coli* (1.1 mL/g of dry mass [4]. The experiments were carried out at 30°C in 100 mM Tris/HCl buffer, pH 7.5. The concentration of *E. amylovora* CFBP 2312 cells was 1.5×10^9 cells/ml. Streptomycin sulphate (Str) was added to the final concentrations of 0.25 and 0.5 mg/ml. The PPA was added to bacterial suspension to the ratio 1/100 and 1/50, respectively. PMB was added to the final concentration of 125μg/ml.

The $\Delta\psi$ values were estimated from a modified Nernst equation, as described previously by [6]

Results

Penetration and chemotactic movement tests. The colonization of autoclaved shoots or chromatography paper strips by *B. subtilis* BS 934 was able to decrease the following upword movement of *E. amylovora* Ea 394 into the vessels of the material. A much stronger effect was observed for *B. subtilis* BS 934 PPA. However, streptomycin sulphate at the concentrations used was most effective in the suppression of upword movement and vessels colonization by fire blight agent bacteria (table 1, Figure 1).

When a Whatman paper disc, containing PPA from *B. subtilis* BS 934, was placed in the way of chemotactically moving *E. amylovora* cells, the antimicrobial substance caused a curving of the chemotactical band (Figure 2).

This was observed for all strains of fire blight used in this test (*E. amylovora* Ea 394, 659, 651, CFBP 2582, CFBP 1430).

Chemical and physical properties of the antimicrobial substance.The CA decreased in activity nearly two times after 35 min of autoclaving at pH 7.5 at 2 atmospheres. It was stable at pH 1-14 for one month and exhibited no loss of antimicrobial activity when stored at 4°C for over 1 year. The solubility of antimicrobial substance increases with increasing of pH of solutions. It was dissolved several times at solutions' pH ≥ 7,5 and precipitated by the addition of concentrated HCl without decrease of activity. It was readily soluble in ethanol, but less soluble in ethyl acetate and acetone. The active substance was highly mobile (Rf 0.81) on TLC plates developed in ethanol : water (2 :1, v/v) and almost immobile in cyclohexane : ethyl acetate (1 :1, v/v). When the crude ethanol, ethyl acetate and acetone extracts were developed on TLC plates, each of them contained a band at Rf 0.81 which was active against the test-cultures. Other bands at Rf 0.76 and 0.7 detected under UV light (λ=350 nm) showed no antimicrobial activity. Bands at Rf 0.7 and Rf 0.76 were ninhydrin positive but the band at Rf 0.81 was ninhydrin negative.

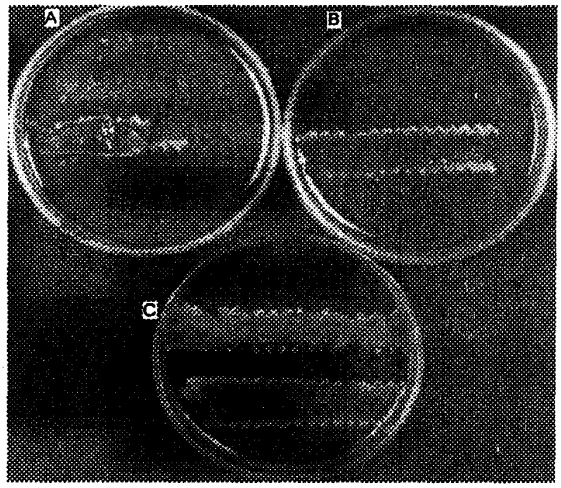

Fig. 1. *Erwinia amylovora* CFBP 2312 upword penetration in chromathography paper strips 3MM Chr
A) treated in solution of streptomycin sulphate 0.04 mg/mL in Tris-HCl buffer pH 7.5 for 2 days.
B) treated in solution of PPA of *B. subtilis* BS 934, 60 mg/mL in Tris-HCl buffer pH 7.5 for 2 days.
C) only inoculated by Erwinia amylovora CFBP 2312 (control).

The antimicrobial substance was resistant to DNase, RNase, *a*-chymotrypsin, proteinase K, lipase (SIGMA), *Ps. mendocina* lipase and able to diffuse through benzoylated dialysis tubing indicating an M_r less than 2,000 Da. The addition of 10^{-4} M ferric chloride showed no effect on the activity of antimicrobial substance.

Table 1. Penetration ability of *E. amylovora* Ea 394 in material treated and untreated with antimicrobials.

Penetrating material	***E. amylovora* Ea 394**

	penetration, mm*
Pear shoots: Autoclaved (control)[a]	
Colonized by *B. subtilis* BS 934 (92 ± 0,71)[b]	43.8 ± 1.39
Treated by PPA[c]	23.6 ± 1.03
Treated with Str[d]	16 ± 1.25
Paper strips	
3MM Chr: Autoclaved control[a]	180 ± 0,32
Colonized by *B. subtilis* BS 934 (160 ± 0.93)[b]	109 ± 0,96
Treated by PPA[c]	19.6 ± 0,51
Treated with Str[d]	15.5 ± 1.25
2043 b: Autoclaved control[a]	160.6 ± 1.5
Colonized by *B. subtilis* BS 934 (128 ± 1,21)[b]	123.6 ± 1.21
Treated by PPA[c]	19.8 ± 0.86
Treated with Str [d]	12.9 ± 1.3
2045 b: Autoclaved control[a]	160 ± 1.58
Colonized by *B. subtilis* BS 934 (161 ± 0,71)[b]	145.6 ± 2.11
Treated by PPA[c]	16.8 ± 0.37
Treated with Str[d]	11.6 ± 1

Note: * means ± standard errors, 5 replicates, [a]autoclaved for 35 min at 2 atm; [b]colonized by bacilli within 2 days; [c]deeped by one end into solution of PPA (60 mg/mL) in Tris-HCl buffer, pH 7.5 for 2 days; [d] deeped by one end into solution of streptomicin sulphate (0.04 mg/mL) in Tris-HCl buffer, pH 7.5 for 2 days. The penetration of bacilli data are presented in brackets.

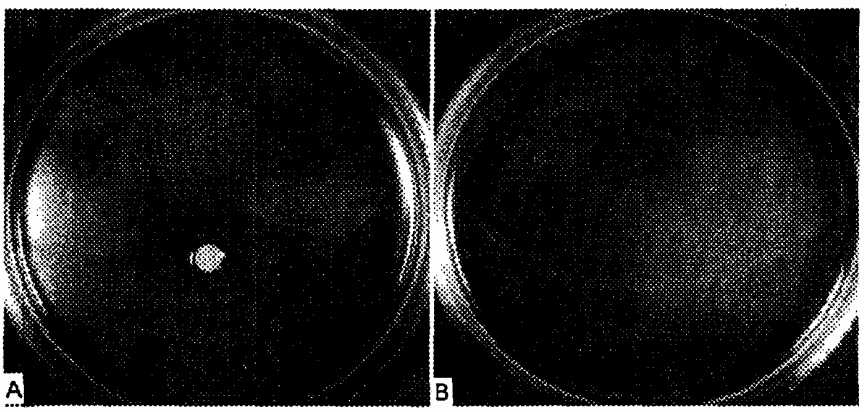

Fig. 2. Influence of *B. subtilis* BS 934 antimicrobial on *E. amylovora* CFBP 2585 chemotaxis.

A) curving of chemotactical band caused by antimicrobial diffused from the 3MM Chr paper disk impregnated by 800 μg of PPA.

B) front of chemotactically moving cells in control.

The *B. subtilis* BS 934 showed no chitinase activity (the clearance halos surrounding the bacilli colonies were not formed), when it was grown onto medium with 2 g/L of collodial chitin from the crab shells.

The addition of ferric chloride or other salts showed no effect on the activity of antimicrobial substance or its accumulation. The antimicrobial has properties which are differ from earlier described antimicrobial substances produced by bacilli [11, 15, 22].

The influence of the atimicrobial onto membrane voltage, ion fluxes and cell survival. The efflux of accumulated TPP^+ upon addition of polycationic antibiotic PMB reflects the significant depolarization of the PM, and subsequent addition of the channel-forming antibiotic GD causes the complete depolarization of the cells (Figure 3 A, curve 1). The concentrations of PMB and GD used

were bactericidal. Only partial decrease of $\Delta\psi$ was observed upon the treatment with streptomycin of EDTA-permeabilized *E. amylovora* CFBP 2312 cells (Figure 3 A, curve 2), though in the concentrations used streptomycin exhibited a profound deleterious action on the cell viability (Figure 3 A, insert). Treatment of the intact cells by CA as well as PPA from *B. subtilis* BS 934 had no influence on TPP^+ accumulation, suggesting that the substance does not enhance the OM permeability to lipophilic cations (Figure 3 B, curve 1). On the other hand, the results on *E. amylovora* CFBP 2312 cell survival showed that the OM of *E. amylovora* CFBP 2312 cells was penetrated by antimicrobial from *B. subtilis* BS 934. The addition of the CA to *E. amylovora* CFBP 2312 cells permeabilized by EDTA resulted in partial depolarization of the PM (Figure 3 B, curve 2). PMB and GD additions enabled the estimation of the remaining $\Delta\psi$, however, the depolarizing effect of the PPA was reduced (Figure 3 B, curve 3). The PPA exhibited some decrease in the bactericidal action, also, compared to the crude one (Figure 3 B, insert).

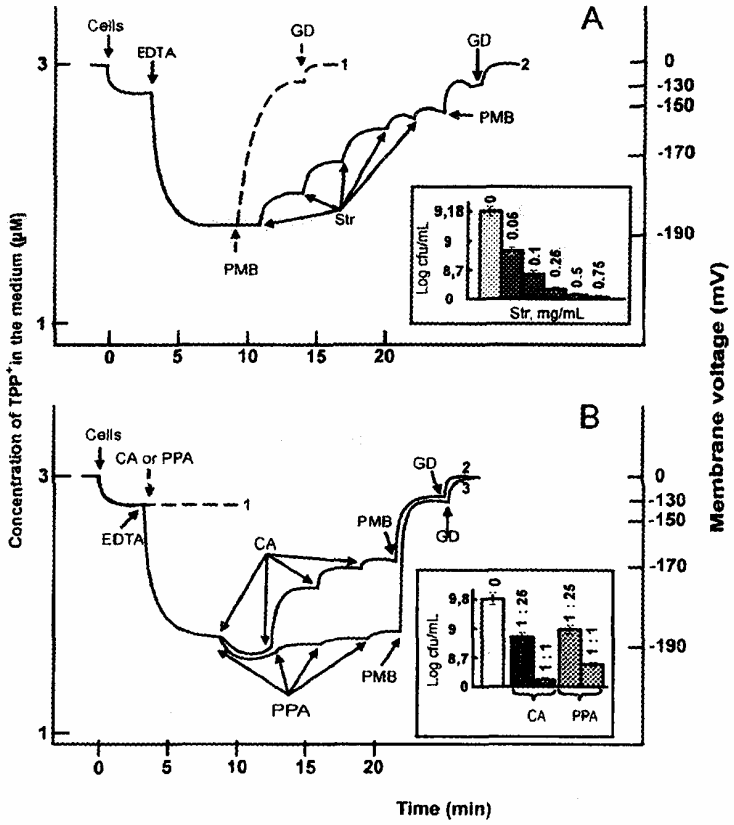

Fig. 3. Effects of antimicrobials on membrane voltage and viability of *E. amylovora* CFBP 2312 cells.

The measurements of K^+ ion fluxes revealed the substantial difference between the action of the antimicrobial substance tested and streptomycin. A slow and irreversible efflux of K^+ was observed upon the addition of the PPA (Figure 4, curve 2) (the measurements with CA were not performed, because of rather high K^+ content in it). Streptomycin, however, did not cause any fluxes of K^+ under experimental conditions (Figure 4, curve 1). The PMB addition finally dissipated the K^+ gradient (Figure 4, curves 1 and 2).

Discussion

The influence of the compound onto chemotactic cells movement suggested its reppelent properties. The penetration test demonstrated stronger influence of streptomycin on *E. amylovora* movement into vessels, than influence of *B. subtilis* BS 934 colonization and its antimicrobial. It shoud be taken to account, however, that PPA from the bacilli was used and its activity may be increased after better purification.

The intact *E. amylovora* CFBP 2312 cells bind a rather low amount of TPP^+ due to the low permeability of the cell envelope to this lipophilic cation. The OM of Gram-negative bacteria is an asymmetric bilayer [24]. As the Gram-negative microbe, *E. amylovora* has the inner layer of the OM consisting of phospholipids and the outer layer of lipopolysaccharide (LPS). The latter forms in Gram-negative bacteria an effective permeability barrier against lipophilic compounds [21]. Permeabilization of the OM of *E. amylovora* CFBP 2312 by EDTA resulted in the enhanced influx of TPP^+ into the cells. These data are consistent with previous studies of Gram-negative bacteria [12], suggesting that EDTA removes by chelation divalent cations from their binding sites in LPS. This results in the release of a significant amount of LPS from the outer leaflet of the OM, which is substituted by patches of phospholipids. The latter act as channels for diffusion of lipophilic compounds, including TPP^+. The TPP^+ acquires the possibility to distribute between the cell cytosol and the external medium according to the membrane voltage ($\Delta\psi$).

$\Delta\psi$ is one of the main indicators of the energetic state of bacterial cells [5]. On the other hand, the analysis of TPP^+ uptake by bacterial cells is a simple but informative way to estimate the OM permeability [6].

The results obtained indicate that antimicrobial substance produced by *B. subtilis* BS 934 exerts a slight membranotropic action on *E. amylovora* CFBP 2312. This action is different from the membranotropic effects of the aminoglycosidic antibiotic streptomycin, a chelator EDTA, a policationic antibiotic PMB and an ion-channel forming antibiotic GD.

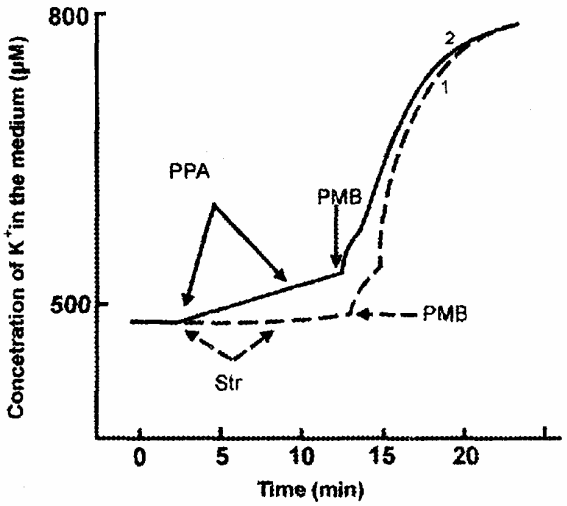

Fig. 4. Effects of antimicrobials on K^+ efflux across the envelope of *E. amylovora* CFBP 2312 cells

$\Delta\psi$ is stringently coupled with H^+ as well as other ion gradients. K^+ gradient plays one of the main roles in bacterial cells [5]. Regardless of bactericidal concentrations used, the streptomycin showed no effect on K^+ ion efflux. The K^+ losses in PPA-treated cells suggest it is one of possible modes of action of the *B. subtilis* BS 934 antimicrobial. The mode of action of the active substance and its targets in sensitive cells should be studied in more detail.

However, the information concerning the envelope permeability and membrane energetics of *E. amylovora* CFBP 2312 could contribute to better understanding of the barrier functions of cells of this species. Hopely, the results of this work may provide an approach for characterization of the action of newly found antimicrobial substances on fire blight bacteria and thus could be applied for the development of improved methods of their control.

The barrier functions of cell envelopes were characterized in such representatives of *Enterobacteriaceae* as *E. coli* and *S. typhimurium* [6, 8, 9, 17, 24]. This study is the first attempt to characterize the envelope permeability and membrane energization of *E. amylovora*, the plant pathogenic member of the family.

Acknowledgements

We are grateful to Prof. V. Smirnov, a member of NAS of Ukraine, and Prof. S. Reznik (both are from IMV, Kyiv, Ukraine) for help in identification of *Bacillus* isolate, Professors J.-P. Paulin (INRA, Angers, France) and J. Beloumi (CFBP, France), Prof. P. Sobiczewski (RIPF, Skierniewice, Poland), for the kindly supplied microbes.

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ВПЛИВ АНТИМІКРОБНОЇ РЕЧОВИНИ *BACILLUS SUBTILIS* BS 934 НА ПРОНИКНУ ЗДАТНІСТЬ, ХЕМОТАКСИС, МЕМБРАННИЙ ВОЛЬТАЖ ТА РУХ ІОНІВ КЛІТИН *ERWINIA AMYLOVORA*

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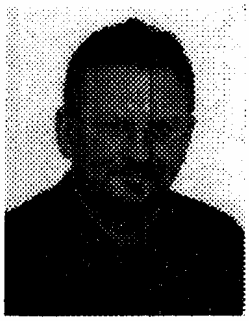
Антимікробна речовина *Bacillus subtilis* BS 934 має властивості відмінні від властивостей антимікробних речовин, описаних у бацил раніше. Вона порушує форму хемотактичного кільця клітин *Erwinia amylovora*, збудника опіку плодових, і пригнічує проникнення та рух цього патогена у судинах рослини та капілярах хроматографічного паперу *in vitro*. Речовина спричинює зміни мембранного вольтажу ($\Delta\psi$), впливаючи на проникність клітинної мембрани для іонів, зокрема, викликає вихід іонів K^+ з клітин *Erwinia amylovora*.



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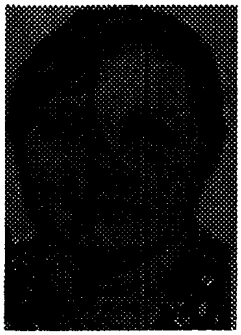

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