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EFFECT OF *ENTEROCOCCUS DURANS* BACTERIOCIN ON BACTERIAL WILT AGENT

Abstract

Effect of a bacteriocin produced by Enterococcus durans A5-11 on growth of Ralstonia solanacearum strains and their ability to cause wilt in tomatoes was investigated. Among tested phytopathogenic strains, 33% were sensitive and inhibited as it was shown after spotting bacteriocin on Ralstonia lawns. Minimal inhibitory concentrations and sizes of lysis or inhibition zones varied depending on the strain used for study. The same differences were evidenced for the effect of the bacteriocin on Ralstonia in a liquid medium and when inoculating Lycopersicon esculentum Mill test plants. Treatment of plant roots with the bacteriocin of E. durans A5-11 simultaneously with inoculation with bacteria of a highly susceptible strain R. solanacearum 6189 resulted in diminished number of wilted tomatoes.

Key words: Ralstonia solanacearum, Enterococcus durans, bacteriocin, wilt of tomatoes.

Introduction

Ralstonia solanacearum [32] causes bacterial wilt in a wide range of plant hosts. Depending on its strain, the pathogen infects economically important species from genera *Solanaceae* (potato, tomato, pepper, tobacco, egg plants), *Musaceae* (banana), *Malvaceae* (cotton), *Zingiberaceae* (ginger, curcuma), *Euphorbiaceae* (rubber), etc. [10; 11; 14; 24].

The disease is mostly dangerous in the tropical and subtropical climatic zones with increased humidity, but the infections caused by strains adapted to cold temperatures (race 3) can occur also in temperate climate [20; 30].

As biological control agents used against bacterial wilt, avirulent *Ralstonia* [31], actinomycetes like *Streptomyces coralus* [2] and bacteria of *Pseudomonas* and *Bacillus* genera are the most known [5; 7; 9; 28]. Antagonistic bacteria have been isolated from soil like *Bacillus* spp. inhibiting growth of *R. solanacearum* from *Curcuma alismatifolia* Gagnep. [28], or from plants like *P. fluorescens* found in healthy roots of *Solanum melongena* L. [4]. Among other microorganisms, satisfactory results were

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obtained applying *Enterobacter cloacae*, *Pichia guilliermondii*, *Candida ethanolitica* [22; 23], and *Stenotrophomonas maltophilia* [19].

Lactic acid bacteria (LAB) dwell on plant surfaces and can compete therefore with the phytopathogens for nutrients and attachment sites. For instance, *Enterococcus durans* bacteriocin-producing strain was initially isolated from the carrot peel [13].

A biopreparation EM4 containing 90% of other LAB – lactobacilli and the remaining percentage of photosynthetic bacteria, fungi and yeasts – had a positive effect on humus formation in soil resulting in increased growth of test plants [12]. EM4 bacterial mixture has also shown a protection effect against some phytopathogens [3], including *Ralstonia solanacearum* [18]. These results demonstrate the potential of LAB use in agriculture for plants protection against bacterial wilt.

The objective of this work was to estimate the possibility of bacterial wilt control by metabolites of LAB *Enterococcus durans*.

Materials and methods

Ralstonia solanacearum strains used in this study were kindly provided by Dr. L.D. Varbanets: *R. solanacearum* ATCC 11696, ICMP 7859 (bv 1), 7944, 6189, 8202, 749, 4157, 8089, 7954, 758, 7986. Strains VC4, ML, TS3, HL, TX1, KL2, HD2 were kind gifts from the Institute of Agricultural Genetics (Hanoi, Viet Nam). All studied strains were stored in LB-broth with 20% glycerol at –20 °C.

Enterococcus durans A5-11 strain was isolated from Mongol yogurt in BIA-FIP laboratory of INRA, France [1; 8].

Bacteriocin from *Enterococcus durans* A5-11 strain was purified by cation-exchange chromatography, reversed phase chromatography and HPLC-chromatography with CHT-column according to Batdorj et al. [1]. Bacteriocin solutions of different concentrations (0.10–0.03 mg/ml) were stored at +4 °C and –20 °C, and were adjusted to pH 6.8–7.0 before use for test of antagonistic activity.

Initial screening for susceptible strains was performed using double-agar layer method [27]. Pre-poured 1.5% LB agar was overlaid with a soft agar (0.6% LB) containing 10% bacterial culture in an exponential phase of growth. Enterococcal bacteriocin was spotted on the upper agar surfaces (5 µl of each repeat), and Petri dishes were incubated overnight at 28–30 °C. The results of tests were obtained by observation of clear zones of bacteriocin activities. Serial dilutions were made to determine the minimal inhibitory concentrations (MIC) of bacteriocin.

To prove the antagonistic action of proteinaceous enterococcal bacteriocin and to exclude the effect of other compounds in investigated solutions, the samples of bacteriocin were treated with proteinase K (Amersham) according to manufacturer's instructions.

Study of antagonistic effect was also carried out in a liquid medium. Mixtures containing cultures of phytopathogens in exponential phase of growth (50 µl), LB-broth (1 ml), and bacteriocin (500 µl) initial concentrations of 0.2 mg/ml and 0.1 mg/ml were prepared to investigate the dynamics of microbial growth measured by counting viable cells inoculated on Petri dishes with LB-agar and incubated overnight at 28 °C.

Root dipping method was performed immersing roots of *Lycopersicon esculentum* Mill. cv Odessa pearl plants at a stage of three leaves into different mixtures of bacterial suspensions and antagonistic substances [25] prepared as mentioned below. Tips of roots were preliminarily damaged to insure the penetration of *Ralstonia*. For this part of experimental work, two strains of tested *R. solanacearum* (*R. solanacearum* 6189 and *R. solanacearum* 7859) showing different results in sensitivity were used.

Positive control plant roots were dipped for 1 h into suspensions of 50% of overnight pathogen cultures (concentrations 2–4 x 10⁹ CFU/ml) and 50% of sterilized distilled water (SDW). Negative controls were soaked in SDW for the same time. 50:50% suspensions of overnight *R. solanacearum* cultures and enterococcal bacteriocin (concentration 0.03 mg/ml) were prepared and immediately used for 1 h treatments of plant roots. In such treatment, plants were exposed to pathogens and to antagonistic substance at the same time [18], but without preliminary interaction of *Ralstonia* and antagonistic substances. The control and inoculated tomatoes were planted into commercially available non-sterile nursery soil with abundance of peat (pH 5.8–6.0) and placed in a greenhouse for 2 weeks with 70–75% of humidity and 26–30 °C air temperature. Number of treated plants was 20 of each variant in each of three independent experiments. The results of inoculation were evaluated in 14 days by the following scale [16]: 1 – no visible symptoms; 2 – from 1% to 25% of plants showing wilt symptoms; 3 – from 26% to 50% of plants displaying wilt symptoms; 4 – from 51% to 75% of plants showing wilt symptoms; 5 – greater than 75% of plants with wilt symptoms or dead.

Results and discussion

LAB antagonistic activity *in vitro*. Among 18 investigated *R. solanacearum* strains, 33% showed the susceptibility to enterococcal bacteriocin resulting in clear spots on bacterial lawns. MIC of bacteriocin varied from 1.00 to 0.02 mg/ml.

To prove the suggestion that clear zones resulted from the action of proteinaceous bacteriocin, proteolysis with proteinase K was carried out athen the resulting mixture was spotted on *Ralstonia* lawn. No zone of inhibition or lysis occurred. The obtained results indicate that it was a proteinaceous substance, which caused the decline in bacterial population.

Producer strain (*E. durans* A5-11) could not cause itself the lysis zones around its colonies on *R. solanacearum* 7859 and 6189 strains.



The effect of bacteriocin on target bacteria can differ in solid and liquid media [26]. To investigate this phenomenon in case of antagonistic substance, two strains were chosen showing differences in size lysis or inhibition zones on solid media (*R. solanacearum* 6189 and *R. solanacearum* 7859).

In case of susceptible strain *R. solanacearum* 6189, decline of cell quantity occurred from the first hour of experiment (Fig. 1).

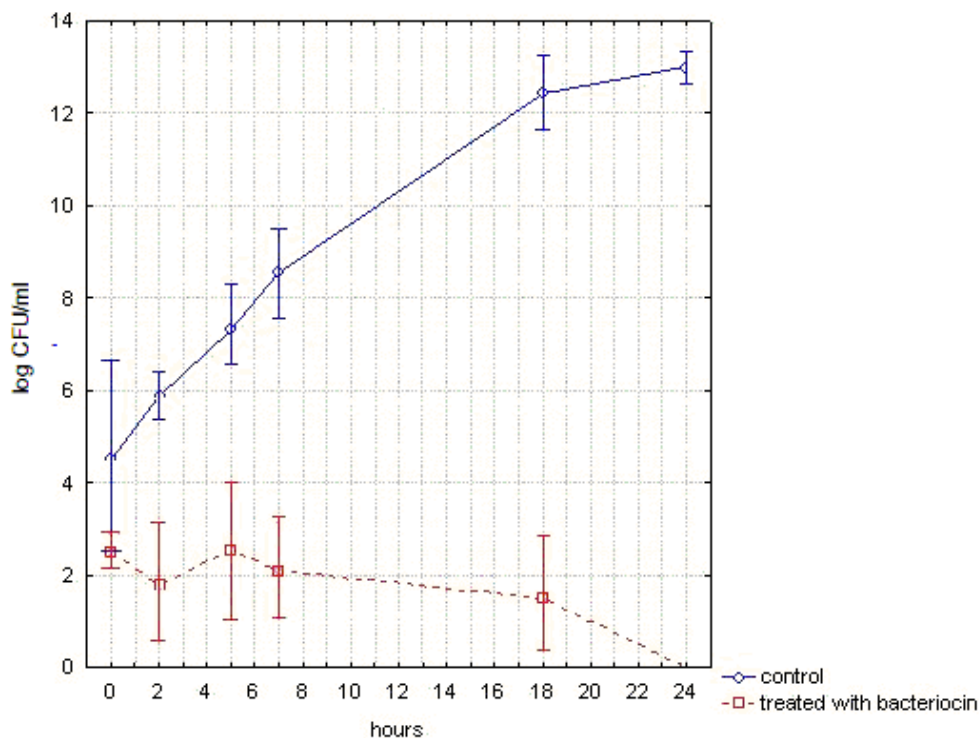


Fig. 1. Growth of *R. solanacearum* 6189 in LB broth in presence of *E. durans* A5-11 bacteriocin (concentration 0.03 mg/ml). Error bars show standard deviation.

After 24 h of incubation no viable cells of *Ralstonia* were recovered from the suspension. Indeed, bacteriocins produced by enterococci are known for their lytic activities [21]. Additionally, the clearing of mixture of “target bacteria – bacteriocin” after first 2 h of incubation as was observed spectrophotometrically (Fig. 2), indicates that the effect of this antagonistic compound on *Ralstonia* is lytic and not inhibitory.

In case of less susceptible strain *R. solanacearum* 7859, slight differences in viable cell quantities were observed during exponential phase (Fig. 3) due to decrease of its growth rate but at the end, after 24 h of cultivation, similar yields of cells in control and treated samples were obtained indicating the retarding effect of bacteriocin on the *Ralstonia* strain cell growth.

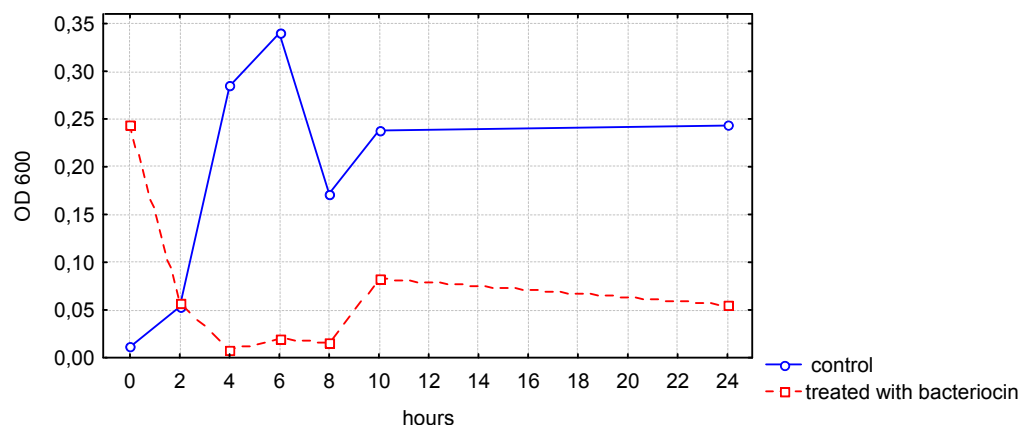


Fig. 2. Effect of *E. durans* A5-11 bacteriocin (final concentration in a mixture 0.06 mg/ml) on the growth of *Ralstonia solanacearum* 6189 in LB broth as measured spectrophotometrically.

This excludes the existence of lytic properties of studied bacteriocin A narrow, strain-restricted susceptibility of Gram-negative microorganisms to bacteriocins of LAB has been already described [6; 15; 17]. Susceptibility of *R. solanacearum* seems to be strain-specific too and it needs to be investigated with the vast diversity of target and producing strains.

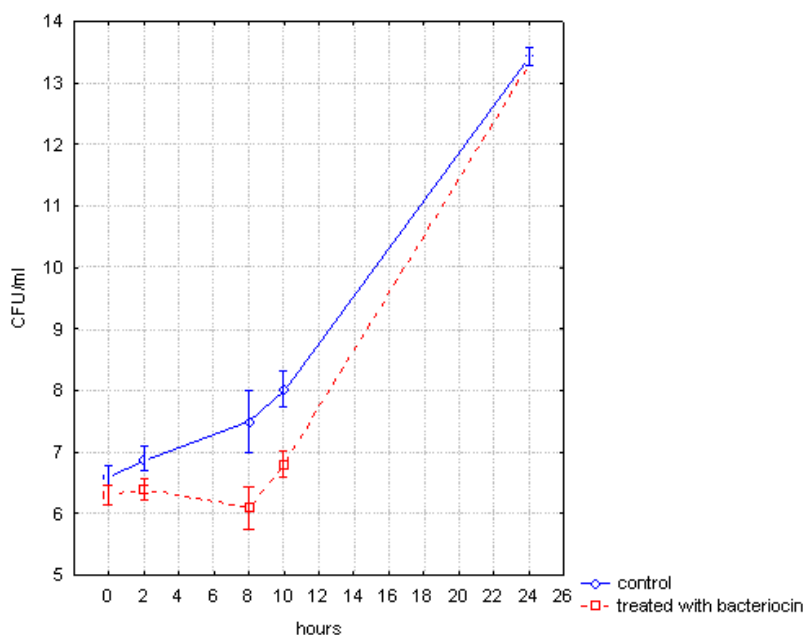


Fig. 3. Growth of *R. solanacearum* 7859 in LB broth in presence of *E. durans* A5-11 bacteriocin (concentration 0.03 mg/ml); bars show standard deviation.

The next step undertaken was to check antagonistic possibilities of LAB on test-plants.



Lactic acid bacteria antagonistic activity *in vivo*

Dipping plant roots into suspensions with the pathogens and bacteriocin of *E. durans* A5-11 resulted in decreasing numbers of wilted tomatoes in case of the strain *R. solanacearum* 6189 (Fig. 4).

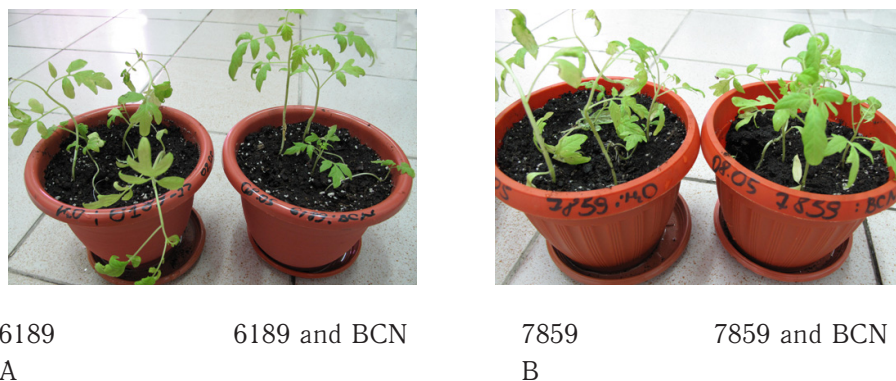


Fig. 4. Tomatoes treated with pathogen suspension and the mixture of pathogen and bacteriocin

A – *R. solanacearum* 6189 and enterococcal bacteriocin (BCN),
 B – *R. solanacearum* 7859 and BCN.

Thus, after 15 days of experiment, severity of wilt on 6189-inoculated plants was one unit lower as measured using Kelman and Person scale [16] (Table).

Table

Estimation of wilt symptoms on treated tomatoes

Assessment of wilt symptoms	<i>Ralstonia solanacearum</i> 6189	<i>Ralstonia solanacearum</i> 6189 and <i>E. durans</i> bacteriocin	<i>Ralstonia solanacearum</i> 7859	<i>Ralstonia solanacearum</i> 7859 and <i>E. durans</i> bacteriocin
Kelman and Person scale	4	3	4	4
Number of diseased plants (%)	63.6%	45.0%	72.7%	75.0%

The inhibition of certain *Ralstonia* strains by LAB and by their secondary metabolites is a promising method of biological control meriting further study. However, several problems should be solved before. The laborious way of bacteriocin purification forces to search for the LAB strains releasing antagonistic compounds against Gram-negative bacteria during their growth on nutritional media.

This will eliminate the stage of bacteriocin extraction and purification from cell suspension. A lot of LAB strains originate from soil and plant surfaces [29; 33] hence they should survive well on plant surfaces.

Further investigations should be carried out including screening for even more effective antagonistic LAB and laboratory and field trials should confirm usefulness of their application on plants.

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ВПЛИВ БАКТЕРІОЦИНУ *ENTEROCOCCUS DURANS* НА ЗБУДНИКА БАКТЕРІАЛЬНОГО ВІЛТУ

Реферат

Досліджено вплив бактеріоцину *Enterococcus durans* А5-11 на ріст бактерій *Ralstonia solanacearum* та здатність їх викликати вілт у томатів. Нанесення бактеріоцину на газони штамів *Ralstonia solanacearum* показало, що 33% тестованих штамів фітопатогенів були чутливими до інгібуючої дії бактеріоцину. Мінімальні інгібуючі концентрації бактеріоцину та розміри зон лізису або інгібування росту бактерій варіювали залежно від штаму. Такі ж відмінності були виявлені щодо впливу бактеріоцину на бактерії штамів *Ralstonia solanacearum* у рідкому середовищі. Обробка коренів рослин томатів *Lycopersicon esculentum* Mill бактеріоцином *E. durans* А5-11 одночасно із зараженням бактеріями *R. solanacearum* 6189 призвела до зменшення кількості рослин томатів із симптомами вілту.

Ключові слова: бактеріоцин, *Enterococcus durans*, вілт томатів, *Ralstonia solanacearum*.

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ВЛИЯНИЕ БАКТЕРИОЦИНА *ENTEROCOCCUS DURANS* НА ВОЗБУДИТЕЛЯ БАКТЕРИАЛЬНОГО ВИЛТА

Реферат

Исследовано влияние бактериоцина *Enterococcus durans* А5-11 на рост бактерий *Ralstonia solanacearum* и способность их вызывать вилт у томатов. Нанесение бактериоцина на газоны штаммов *Ralstonia*



solanacearum показало, что 33% тестированных штаммов фитопатогенов были чувствительными к ингибирующему действию бактериоцина. Минимальные ингибирующие концентрации бактериоцина и размеры зон лизиса или ингибирования роста бактерий варьировали в зависимости от штамма. Такие же различия были установлены для влияния бактериоцина на бактерии штаммов *Ralstonia solanacearum* в жидкой среде. Обработка корней растений бактериоцином *E. durans* А5-11 одновременно с заражением бактериями штамма *R. solanacearum* 6189 привела к уменьшению количества растений томатов с симптомами вилта.

Ключевые слова: *Enterococcus durans*, бактериоцин, вилт томатов, *Ralstonia solanacearum*.

