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**EFFECT OF *LACTOBACILLUS PLANTARUM* ONU87
IN MIXTURE WITH AUTOLYSATE OF ERWINIAS
ON FORMATION OF TUMORS CAUSED
BY *RHIZOBIUM RADIOBACTER* C58**

The aim of the research was to investigate the effect of mixture of Lactobacillus plantarum ONU87 and autolysate of Erwinia carotovora ZM1 cells containing macromolecular bacteriocins and bacteriophages on the pathogenesis of the crown gall. Materials and Methods. As an infectious agent, a strain of Rhizobium radiobacter C58 characterized by its high virulence was chosen. As test-models, carrot roots (Daucuscarota subsp. sativus L.) and plants Kalanchoe daigremontiana Mill were used. Results. The effect of mixture of L. plantarum ONU87 and autolysate of E. carotovora ZM1 on survival of R. radiobacter C58 in vitro has been studied. Amount of viable cells of plant pathogenic bacteria decreased after 4 hours of culturing. Treatment with a mixture of lactobacilli and autolysate of erwinias results in complete inhibition of crown gall pathogenesis on Kalanchoe plants. Treatment of carrot roots with an experimental mixture results in decrease of the amount of infected explants in 14.3%. The mentioned results enabled us to conclude that depending on the sensitivity of the test-objects, treatment with the mixture of L. plantarum ONU87 and autolysate of E. carotovora ZM1 results in complete inhibition of crown gall pathogenesis or decrease of its symptoms.

Key words: Rhizobium radiobacter, crown gall, Lactobacillus plantarum, autolysate of Erwinia carotovora.

Revealing active antagonists, including lactic acid bacteria, in phyllosphere [9], provides a possibility of using them to protect plants against pathogenic microorganisms.

Inhibition of phytopathogens and food spoilage bacteria by lactobacilli were described for *Aspergillus flavus* [13], *Colletotrichum gloeosporioides* [4],



Aspergillus ochraceus [5], *Aspergillus niger* and *Penicillium expansum* [3], *Verticillium*, *Thielaviopsis*, *Fusarium* [6], *Monilinia laxa*, *Botrytis cinerea* and *Penicillium expansum* [10], *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae* [11]. Growth of *X. campestris* and *E. carotovora* was inhibited by representatives of *Enterococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Weissella* genera [10].

Our previous investigations showed an inhibitory effect of enterococci on *Ralstonia solanacearum* [7].

As to *Lactobacillus plantarum*, the significant antagonistic effect of *L. plantarum* ONU87 in the mixture with *Erwinia carotovora* ZM1 containing bacteriophages and macromolecular bacteriocins were demonstrated against soft rot and blackleg [1].

The aim of the research was to investigate the effect of *Lactobacillus plantarum* ONU87 in the mixture with autolysate of *Erwinia carotovora* ZM1 cells on crown gall pathogenesis.

Materials and methods

In this research, the phytopathogenic strain of *Rhizobium radiobacter* C58 characterized by its high virulence, *Erwinia carotovora* subsp. *carotovora* ZM1 (*Ecc* ZM1) strain – producer of bacteriophages and macromolecular bacteriocins (strains were kindly provided by Tovkach F.I. from Zabolotny Institute of Microbiology and Virology of NASU), and antagonistic strain *Lactobacillus plantarum* ONU87 were used. To model the infectious process of crown gall, bacteria *R. radiobacter* C58 were cultivated in LB-broth at 28 °C for 24 h and used for further investigations in concentration of 10⁸ CFU/ml. Lactic acid bacteria *L. plantarum* ONU87 were cultivated in MRS broth for 24 h at 37 °C and used for further experiments in concentration of 10¹⁰ CFU/ml. Autolysate of *Erwinia carotovora* ZM1 was prepared as previously described [1]. The experimental mixture of lactobacilli and autolysate of erwinias was prepared by mixing in ratio of 1:1.

To study the antagonistic effect of mixture of *L. plantarum* ONU87 and autolysate of *E. carotovora* ZM1 on growth of bacteria *R. radiobacter* C58 *in vitro*, the experimental suspension was mixed with the culture of phytopathogen and LB-broth in a ratio of 1:2:2 selected empirically in previous investigations. The control was the mixture of overnight culture of rhizobia and LB-broth in a ratio of 2:3 that simulated the phytopathogen concentration in the variants of experiment. The number of viable cells of rhizobia were measured by inoculating LB agar in certain time intervals, incubating overnight at 28 °C and counting the colonies. Three independent experiments were carried out in five replications each.

The effect of mixture of *Lactobacillus plantarum* ONU87 and autolysate of *Erwinia carotovora* ZM1 on crown gall pathogenesis was studied



in vitro on carrot roots (*Daucus carota* subsp. *sativus* L.) and *in vivo* on plants *Kalanchoe daigremontiana* Mill.

For test-object treatments, overnight culture of rhizobia in LB-broth and experimental mixture of lactobacilli and autolysate of erwinias were mixed in ratio of 1:1. Mixture of rhizobial overnight culture with sterile distilled water in ratio of 1:1 was used as positive control. The negative controls were sterile distilled water and the mixture of lactobacilli and autolysate of erwinias.

Carrot roots were thoroughly washed in chlorine-containing detergent, rinsed in running water, dipped in ethanol and flamed, peeled from the external tissues, and then cut into discs with the thickness of 0.5 cm [8]. The disks were placed in sterile Petri dishes with watered filter paper. On the surface of fresh cut discs (cambial ring) 100 µl of rhizobial culture mixed with lactobacilli and autolysate of erwinias, or the equal volume of positive or negative controls were applied. 56 carrot discs were treated in each control or experimental variant.

Disks were placed at 25 °C for 21 days, and after the results were evaluated by the following scale: “++++” – 100% cambial ring covered with tumors, “+++” – 75% of cambial ring have tumors, “++” – 50% cambial ring covered with tumors, “+” – less than 25% of cambial ring covered with tumors, “-” – no tumors.

The overall amount of infected explants was calculated as percentage of discs with crown gall symptoms from the total quantity of all inoculated discs.

Kalanchoe plants were inoculated by syringe injection of 50 µl of pathogen culture and mixture of lactobacilli and autolysate of erwinias (experimental variant) or culture of rhizobia and sterile water (positive control) or sterile distilled water, or the mixture of lactobacilli and autolysate of erwinias (negative controls) into the surface leaf layer. 30 leaves were treated in control and experimental variants in each of the three independent experiments. After 60 days tumors formed on injection sites were cut and weighed.

The experimental data were evaluated statistically using Microsoft Word Excel. The obtained results were expressed as means and their confidential intervals. Probability differences of the results were assessed at significance level of at least 95%.

Results and discussion

The investigation of *L. plantarum* ONU87 and autolysate of *E. carotovora* ZM1 effect on *R. radiobacter* C58 *in vitro* in a liquid medium has shown that the amount of viable cells of pathogenic bacteria decreased already after 4 hours of cultivation (fig. 1).



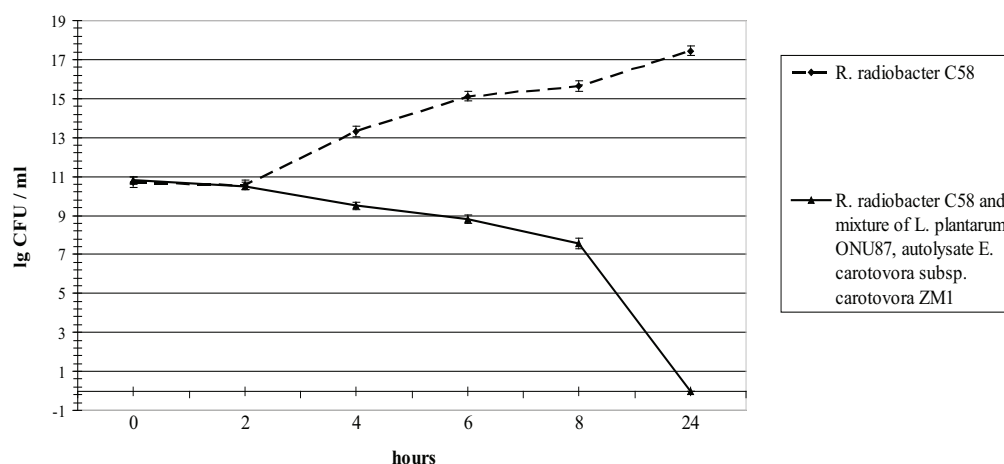


Fig. 1. Growth of phytopathogen *R. radiobacter* C58 in presence of *L. plantarum* ONU87 in mixture with autolysate of *E. carotovora* subsp. *carotovora* ZM1.

Probably, this effect was caused by antagonistic factors against phytopathogenic microorganisms produced by lactic acid bacteria such as hydrogen peroxide, bacteriocins and especially, organic acids [11]. After 24 h of incubation no viable cells of phytopathogens were recovered in contrast to positive control where *R. radiobacter* C58 continued to grow.

Investigations of *L. plantarum* ONU87 and autolysate of *E. carotovora* subsp. *carotovora* ZM1 effect on crown gall infection *in vivo* has shown the high efficiency of using this mixture.

Thus, comparing of kalanchoe leaves in positive controls inoculated only with crown gall agents with leaves treated with the mixture of pathogens, lactobacilli and autolysate of erwinias has shown that all the leaves of control plants infected with *R. radiobacter* C58 manifested the formation of tumors of various sizes (fig. 2).

The average weight of tumors was 40 ± 2 mg in three independent experiments. On the leaves of plants inoculated simultaneously with the mixture of lactobacilli and autolysate of erwinias and *R. radiobacter* C58 tumor formation was not observed. However, necroses of different levels were observed (fig. 2). On the leaves of kalanchoe in negative controls in which only lactobacilli and autolysate of erwinias were injected, necroses of tissues induced by defense reaction of the plants to damage the leaf blade and to interfere the introduction of foreign agent were also detected in some cases. Necrosis is a hypersensitivity reaction and an important part of plant immunity, thus presence of necrotic spots instead of tumors is the evidence of plant protection against the crown gall agent. In other case the plants may slowly die because of galls surrounding the trunks and interfering the normal water and nutrients supply [2].



Fig. 2. Leaves of kalanchoe inoculated with the phytopathogen *R. radiobacter* C58 and the mixture of lactobacilli and autolysate of erwinias (a) or with the culture of *R. radiobacter* C58 (b).

Opposite, necroses are restricted and don't cause damage of wide areas of the plant surfaces, and moreover, they localize the pathogen and don't allow it to disseminate in plant, whereas pathogenic rhizobia are able to rapid spread in xylema vessels causing systemic infection as a result of which tumors can be formed on any part of the plant.

Treatment of carrot discs with the mixture of lactobacilli and autolysate of erwinias simultaneously with their inoculation with crown gall agent resulted in 14.5% decrease in amount of infected explants. Absence of disease inhibition in 100% of cases as it was shown in investigations with kalanchoe, indicates the higher sensitivity of carrots to *R. radiobacter* C58 and allows to observe the effect of the studied mixture of lactobacilli and autolysate of erwinias on high susceptible plants (fig. 3).

Thus, in case of carrot explants, clear decrease of infection massiveness is observed, that increases in amount of explants with smaller manifestation of disease symptoms than in positive control (fig. 4). The amount of carrot discs with the crown gall symptom manifestation in «++++» decreases from 8.9% to 1.8%, that is – almost in 5 times, quantity of discs with manifestation in «+++» decreases from 19.6% to 3.6%, that is – in 5.4 times.

In positive control the percentage of explants with tumor formation characterized as «++» was 14.3%, and in experimental samples –10.7%;

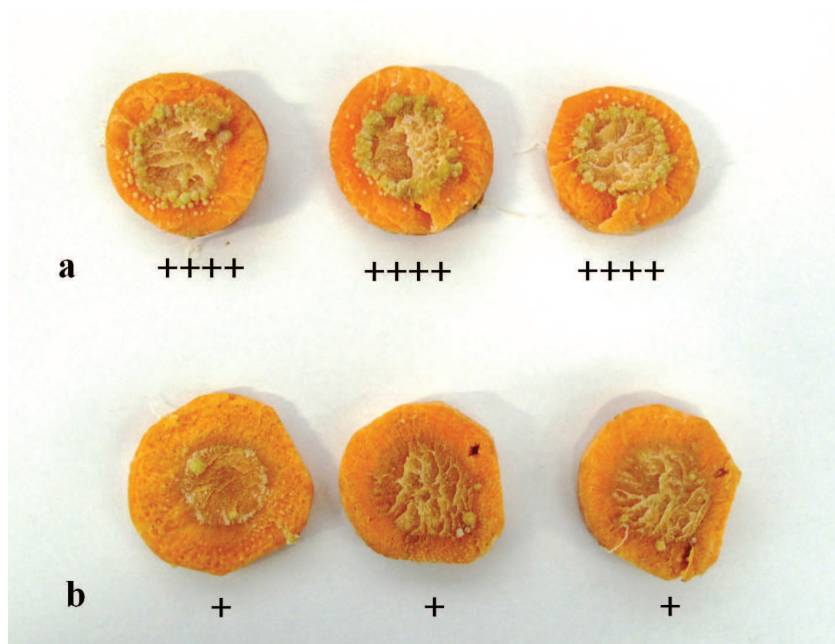


Fig. 3. Carrot discs with manifestation of crown gall symptoms: a – positive control – *R. radiobacter* C58; b – *R. radiobacter* C58 and the mixture of lactobacilli and autolysate of erwinias.

Thus, the shift of the levels of crown gall symptom manifestation to smaller tumor formation, and as a result, to smaller disease massiveness, is observed.

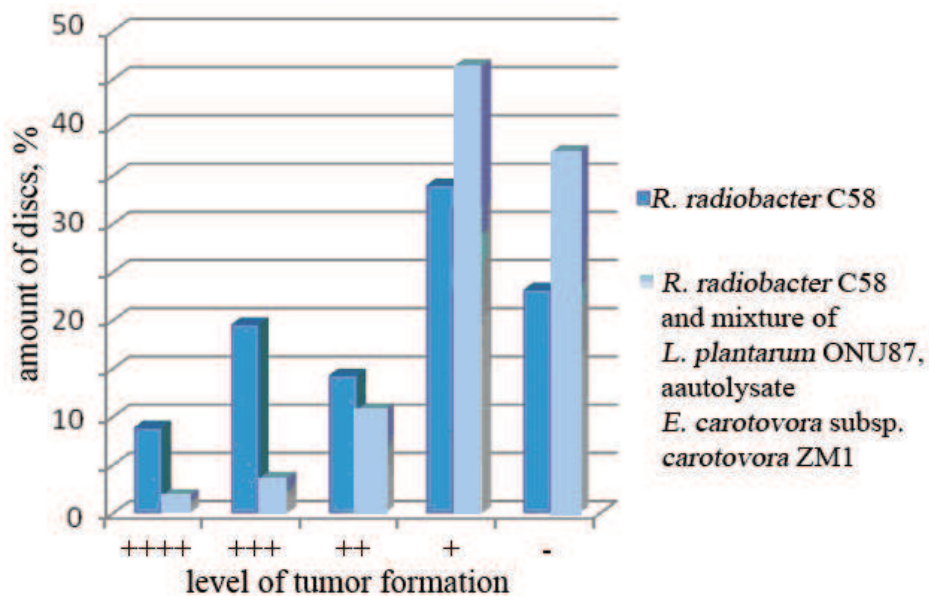


Fig. 4. Amount of carrot discs with different intensity of tumor formation.

the amount of discs with «+» manifestation in positive control was 34%, and in experiment with treatment with lactobacilli and autolysate of erwinias it increased to 46.4%. In negative controls no visible deformations in explant tissues were detected.

The obtained data can lead to the conclusion that depending on plant sensitivity, treatment with lactobacilli and autolysate of erwinias results in complete inhibition of crown gall pathogenesis or in decrease of symptom manifestations.

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ВПЛИВ *LACTOBACILLUS PLANTARUM* ONU 87 У СУМІШІ З АВТОЛІЗАТОМ ЕРВІНІЙ НА УТВОРЕННЯ ПУХЛИН, СПРИЧИНЕНЕ *RHIZOBIUM RADIOBACTER* C58

Реферат

Метою дослідження було вивчення впливу *Lactobacillus plantarum* ONU87 у суміші з автолізатом клітин *Erwinia carotovora* ZM1, що містить бактеріофаги та макромолекулярні бактеріоцини, на розвиток інфекції бактеріального раку. **Методи.** Інфекційним агентом був штам *Rhizobium radiobacter* C58, який характеризується високою вірулентністю. Як тест-моделі використовували коренеплоди моркви (*Daucus carota* subsp. *sativus* L.) і рослини каланхое (*Kalanchoe daigremontiana* Mill.). **Результати.** Вивчено вплив суспензії лактобацил і автолізату ервіній на виживання бактерій штаму *R. radiobacter* C58 *in vitro*. Показано, що кількість життєздатних клітин фітопатогенних бактерій зменшується вже через чотири години. Обробка сумішшю лактобацил і автолізату ервіній призводить до повного пригнічення патогенезу бактеріального раку на рослинах каланхое. На коренеплодах моркви обробка експериментальною сумішшю веде до зменшення кількості уражених експлантів на 14,3%. Зроблено **висновок**, що залежно від сприйнятливості рослини обробка сумішшю лактобацил і автолізату ервіній призводить або до повного пригнічення патогенезу бактеріального раку, або до зменшення проявів його симптомів.

Ключові слова: *Rhizobium radiobacter*, бактеріальний рак рослин, *Lactobacillus plantarum*, автолізат *Erwinia carotovora*.



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ВЛИЯНИЕ *LACTOBACILLUS PLANTARUM* ОНУ 87 В СМЕСИ С АВТОЛИЗАТОМ ЭРВИНИЙ НА ОБРАЗОВАНИЕ ОПУХОЛЕЙ, ВЫЗВАННОЕ *RHIZOBIUM RADIOBACTER* С58

Реферат

Целью исследования было изучение влияния *Lactobacillus plantarum* ОНУ 87 в смеси с автолизатом клеток *Erwinia carotovora* ZM1, содержащим бактериофаги и макромолекулярные бактериоцины, на развитие инфекции бактериального рака. **Методы.** Инфекционным агентом был штамм *Rhizobium radiobacter* С58, характеризующийся высокой вирулентностью. В качестве тест-моделей использовали корнеплоды моркови (*Daucus carota* subsp. *sativus* L.) и растения каланхоэ (*Kalanchoe daigremontiana* Mill.). **Результаты.** Изучено влияние суспензии лактобацилл и автолизата эрвиний на выживание бактерий штамма *R. radiobacter* С58 *in vitro*. Показано, что количество жизнеспособных клеток фитопатогенных бактерий уменьшается уже через четыре часа. Обработка смесью лактобацилл и автолизата эрвиний приводит к полному подавлению патогенеза бактериального рака на растениях каланхоэ. На корнеплодах моркови обработка экспериментальной смесью приводит к уменьшению количества пораженных эксплантов на 14,3%. Сделан **вывод** о том, что в зависимости от восприимчивости растения, обработка смесью лактобацилл и автолизата эрвиний ведет к полному подавлению патогенеза бактериального рака или к уменьшению проявления его симптомов.

Ключевые слова: *Rhizobium radiobacter*, бактериальный рак растений, *Lactobacillus plantarum*, автолизат *Erwinia carotovora*.

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