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EFFECT OF *LACTOBACILLUS PLANTARUM* ON TUMOR FORMATION CAUSED BY *RHIZOBIUM RADIOBACTER*

*The **aim** of investigation was to study the effect of Lactobacillus plantarum bacteria and their metabolites on tumor formation in Kalanchoe daigremontiana Mill. plants experimentally inoculated with crown gall agents Rhizobium radiobacter. **Methods**. Leaves of test plants were inoculated with the cultures of bacteria R. radiobacter C58 and three Lactobacillus plantarum strains or their cell-free supernatants (CFS) with primary low pH (4.1–4.3) and with neutralized pH (6.5) in a ratio 1:1. After 45 days amount of samples with tumors and weight of tumor tissues were evaluated, and formation of necroses were checked out. **Results**. Lactobacilli and their supernatants effected tumor formation differently depending on the variant of treatment. Inhibiting activity in a case of all three investigated L. plantarum strains was exhibited by CFS with primary pH (4.1–4.3). Lactobacilli injected in plant tissues simultaneously with the pathogen decreased the amount of crown galled samples both in a case of low initial pH of cultural liquid (strain L. plantarum ONU 87 decreased in 86%, strain L. plantarum ONU 206 – in 95%), and in a case of neutralized pH of cultural liquid (strain L. plantarum ONU 87 decreased in 75%, strain L. plantarum ONU 206 – decreased in 94%). Bacteria of the strain L. plantarum ONU 991 didn't exhibit the inhibiting activity in cases of the treatments with cultures of lactobacilli and with the neutralized supernatant. At the same time, the overnight cultures of all strains showed clear antagonistic activity in vitro. Injecting the cultures of lactobacilli and their supernatants both with low and neutral pH resulted in wide zones of necrosis comparing to that obtained after the injection of tissues with sterile distillated water with the same pH. **Conclusion**. Lactic acid bacteria L. plantarum differ in the level of inhibition of tumor formation caused by R. radiobacter. Strain L. plantarum 206 was the best antagonist in these investigations: both the amount of infected samples and weight of formed tumors significantly decreased in all variants of the treatments. Inhibiting activity of metabolites from the cultural liquid of lactobacteria probably is explained not only with the action of organic acids, but also with the action of bacteriocins and other products of metabolism.*

K e y w o r d s : Rhizobium radiobacter, Lactobacillus plantarum, inhibition of tumor formation, crown gall, Kalanchoe daigremontiana.

Bacteria from *Lactobacillus* genus inhabit the rhizosphere and phyllosphere of plants [15], where they can comprise 0.01–1% from the total microbial population [4]. On the leaf surfaces the representatives of species *L. plantarum, L. paracasei, L. fermentum, L. brevis, L. buchneri* can be found [4; 13; 16]. Owing to their antagonistic properties, lactobacilli were studied as the potential agents of biological control of phytopathogens. The significant decrease in the symptoms on haricot beans infected with *Pseudomonas syringae* was detected when plants were treated with the strain *L. plantarum* L292 [18]. It is known that certain strains of lactobacilli and bacterial mixes containing lactobacilli can inhibit Ralstonia solanacearum [12], *Xanthomonas campestris* [5; 17], *Colletotrichum gloeosporioides* [7], *Fusarium* [8], *Aspergillus niger* and *Penicillium expansum* [2; 17].

In previous investigations there were revealed the antagonistic effect of *L. plantarum* in a mixture with autolysate of erwinias against crown gall agent *Agrobacterium tumefaciens* (*Rhizobium radiobacter* according to the new nomenclature proposed by Young et al., 2001 [19]), the agents of black foot and soft rot [11; 14]. The aim of the investigation was to study the effect of *Lactobacillus plantarum* bacteria and their metabolites on tumor formation in *Kalanchoe daigremontiana* Mill. plants experimentally inoculated with crown gall agents *Rhizobium radiobacter*.

Materials and Methods

The effect of the three strains of *L. plantarum* from the Collection of the Chair of Microbiology, Virology and Biotechnology of Odesa National I.I. Mechnykov University *L. plantarum* ONU 87, *L. plantarum* ONU 206 and *L. plantarum* ONU 991 was studied. Lactobacilli were cultivated overnight in MRS broth at 37 ºC [6] and used in the experiments in concentration of 10^9 CFU (colony forming units)/ml typical for overnight cultures. To obtain the cell-free supernatant (CFS), overnight cultures in liquid medium were centrifuged at 10 000 g 10 min and filtrated through the bacterial filters with the diameters of pores 0.22 μm (Millipore Millex-GS, Merck Millipore). The effect of supernatants of lactobacilli with primary low and neutralized pH was studied *in vitro* on a lawn of *R. radiobacter* C58 by the well-diffusion method. In the same way the sterile distillated water (SDW) with the low and neutral pH was applied onto the lawn of phytopathogen. The presence of growth inhibition zones was observed after overnight cultivation at 28 ºC.

As test-plants *Kalanchoe daigremontiana* Mill. were used. Plants were infected with bacteria of *R. radiobacter* C58 strain kindly provided by Senior Research Associate of D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine Dr. F.I. Tovkach. Rhizobia were cultivated overnight in LB broth [1] at 28 ºC and used for further investigations in concentration of 10^8 CFU/ml.

Inoculations were carried out in three independent experiments by the injection method. In each of the independent experiments, 3–5 injections on a leaf of 25–30 plants were done. 10 μl of overnight rhizobial cultures and tested liquids (overnight cultures of lactobacilli and their supernatants with primary low and neutral pH) in a ratio 1:1 were injected in leaf tissues [9]. The positive control of tumor formation

was the mixture of overnight rhizobial culture and SDW with the same pH as in overnight cultures of lactobacilli. Also SDW was injected in leaves as a control for necroses formation.

After 45 days, amount of crown galled samples, weight of tumor tissues and character of necroses were checked out.

Significant differences between the mean values were estimated by Student's criteria on significance level not less than 95% ($p \leq 0,05$). The data were calculated by the Excel program.

Results of Investigation and Their Discussion

In vitro on Petry's dishes the tested strains of lactobacilli revealed the clear antagonistic activity of overnight cultures and CFS with low pH, which was exhibited as the zones of absence of phytopathogen growth with the diameter of 8–10 mm. The neutralized CFS did not inhibit the growth of *R. radiobacter* C58 that gave us the possibility to hypothesize that the key factor of phytopathogen inhibition was the low pH. To reveal the possible role of acidic medium in inhibition of tumor formation, further investigations *in vivo* were carried out both with the primary low pH 4.1–4.3 and with the neutral pH.

Further investigations *in vivo* have shown that the simultaneous injections of cultures of lactobacilli and their metabolites and inoculation of *K. daigremontiana* with the phytopathogen *R. radiobacter* C58 inhibit tumor formation in the majority of variants of the treatment. Inhibiting activity in cases of all three strains has been exhibited by the CFS with the primary low pH of overnight culture (pH 4.1–4.3). The treatment with the CFS of *L. plantarum* ONU 87 culture decreased the amount of crown galled samples in 71.7% (Fig. 1), *L. plantarum* ONU 206 – in 84,0% (Fig. 2), *L. plantarum* ONU 991 – in 68.1% (Fig. 3).

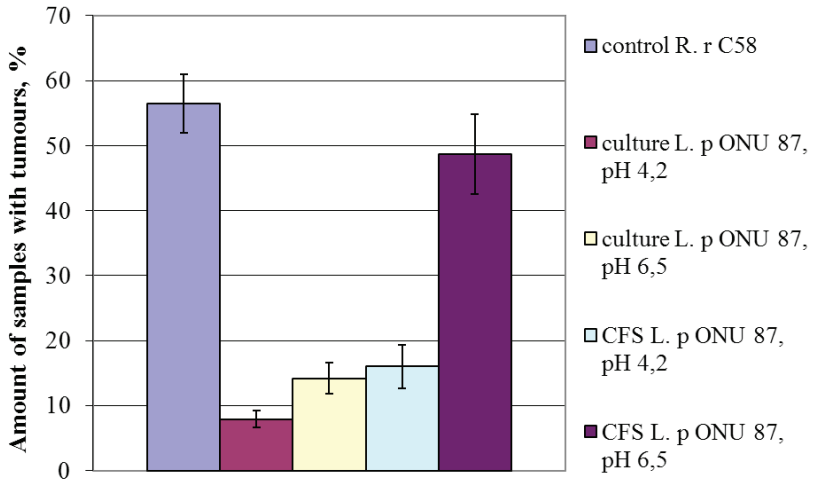

Fig. 1. The effect of bacterial culture and cell-free supernatant of *Lactobacillus plantarum* ONU 87 on tumor formation caused by *R. radiobacter* C58 in plants *K. daigremontiana*

Bacterial cultures injected in plant tissues simultaneously with the pathogen decreased the amount of crown galled samples both in case of low initial pH of cultural liquid (strain *L. plantarum* ONU 87 decreased in 86%, strain *L. plantarum* ONU 206 – in 95%) and in case of neutralized pH of cultural liquid (strain *L. plantarum* ONU 87 decreased in 75%, strain *L. plantarum* ONU 206 – decreased in 94%) (Fig. 1 and Fig. 2).

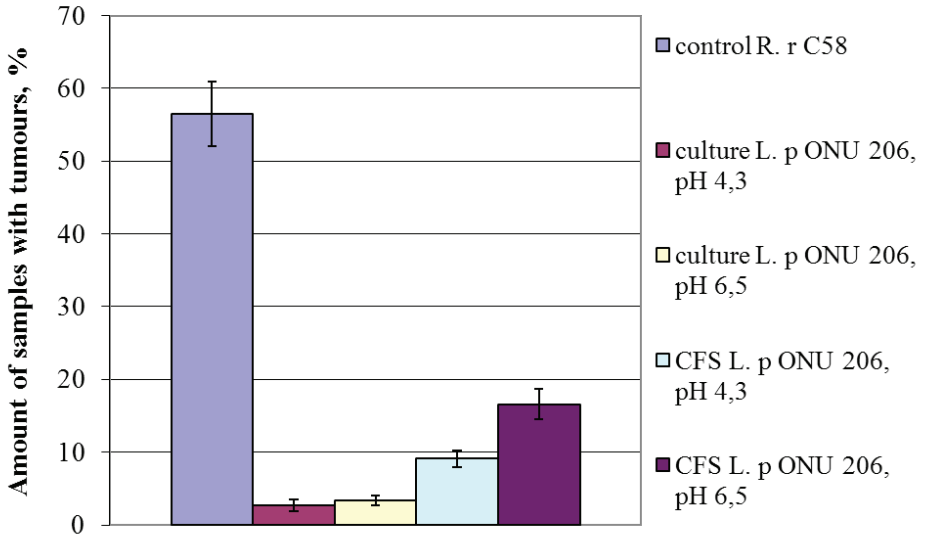

Fig. 2. The effect of bacterial culture and cell-free supernatant of *Lactobacillus plantarum* ONU 206 on tumor formation caused by *R. radiobacter* C58 in plants *K. daigremontiana*

The supernatant of the strain *L. plantarum* ONU 206 culture with pH 6.5 also had the inhibiting effect on tumor formation decreasing the amount of infected samples in 70.6%. The neutralized CFS of other strains – *L. plantarum* ONU 87 and *L. plantarum* ONU 991, did not decrease the manifestation of crown gall. Besides, the strain *L. plantarum* ONU 991 did not inhibit any tumorigenic activity of crown gall agent when overnight cultures both with primary pH (4.1) and neutralized pH (6.5) were applied. This fact does not coincide with the results of investigations *in vitro*, when CFS of the *L. plantarum* ONU 991 culture with a primary pH inhibited the growth of phytopathogen as the same effective as the CFS of other strains.

Although the strain *L. plantarum* ONU 991 exhibited antagonistic activity in the tests on phytopathogen lawns, in the investigations on plants its effect on tumor formation was not the same, and only the CFS of overnight culture with initial low pH inhibited the formation of tumors.

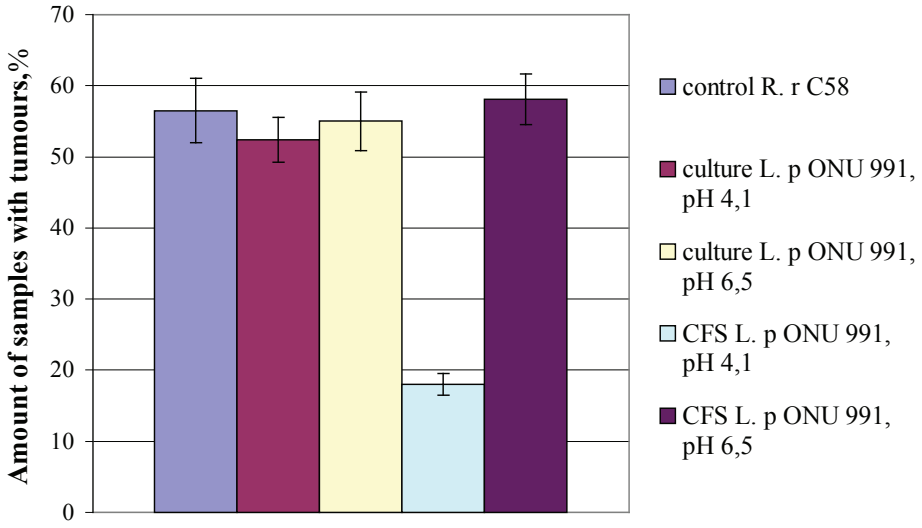

Fig. 3. The effect of bacterial culture and cell-free supernatant of *Lactobacillus plantarum* ONU 991 on tumor formation caused by *R. radiobacter* C58 in plants *K. daigremontiana*

The strain *L. plantarum* ONU 206 was the best antagonist in these investigations. Both the amount of infected samples and weight of formed tumors significantly decreased in all variants of the treatments (Fig. 2, Fig. 4, Fig. 5).

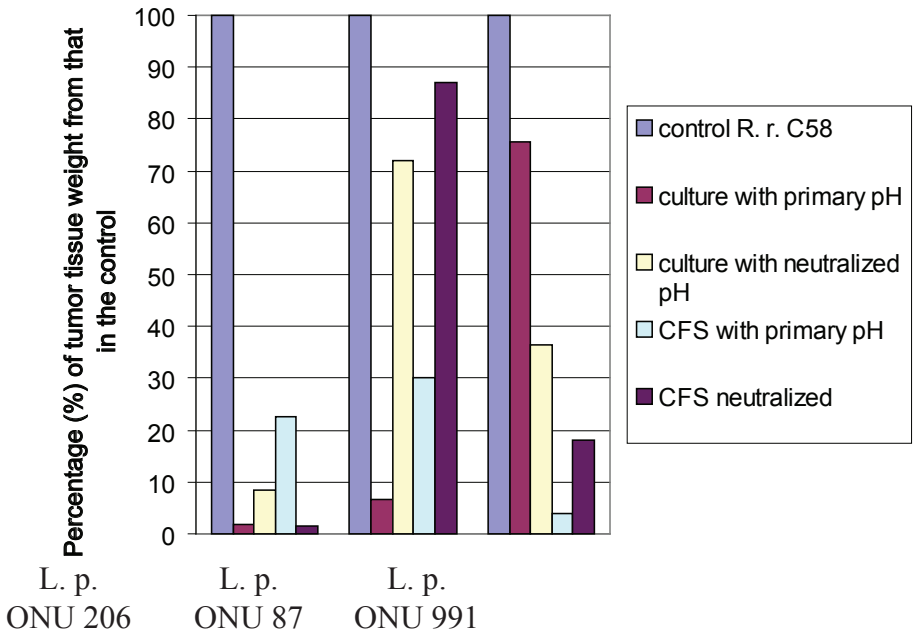

Fig. 4. The percentage of tumor tissue weight in the variants of treatment with the culture and supernatant of *Lactobacillus plantarum* ONU 206 comparing to the control

It is possible to hypothesize that the inhibiting activity of CFS with pH 6.5 is probably caused by the effect of bacteriocins or other antagonistic compounds which should be revealed during the further investigations.

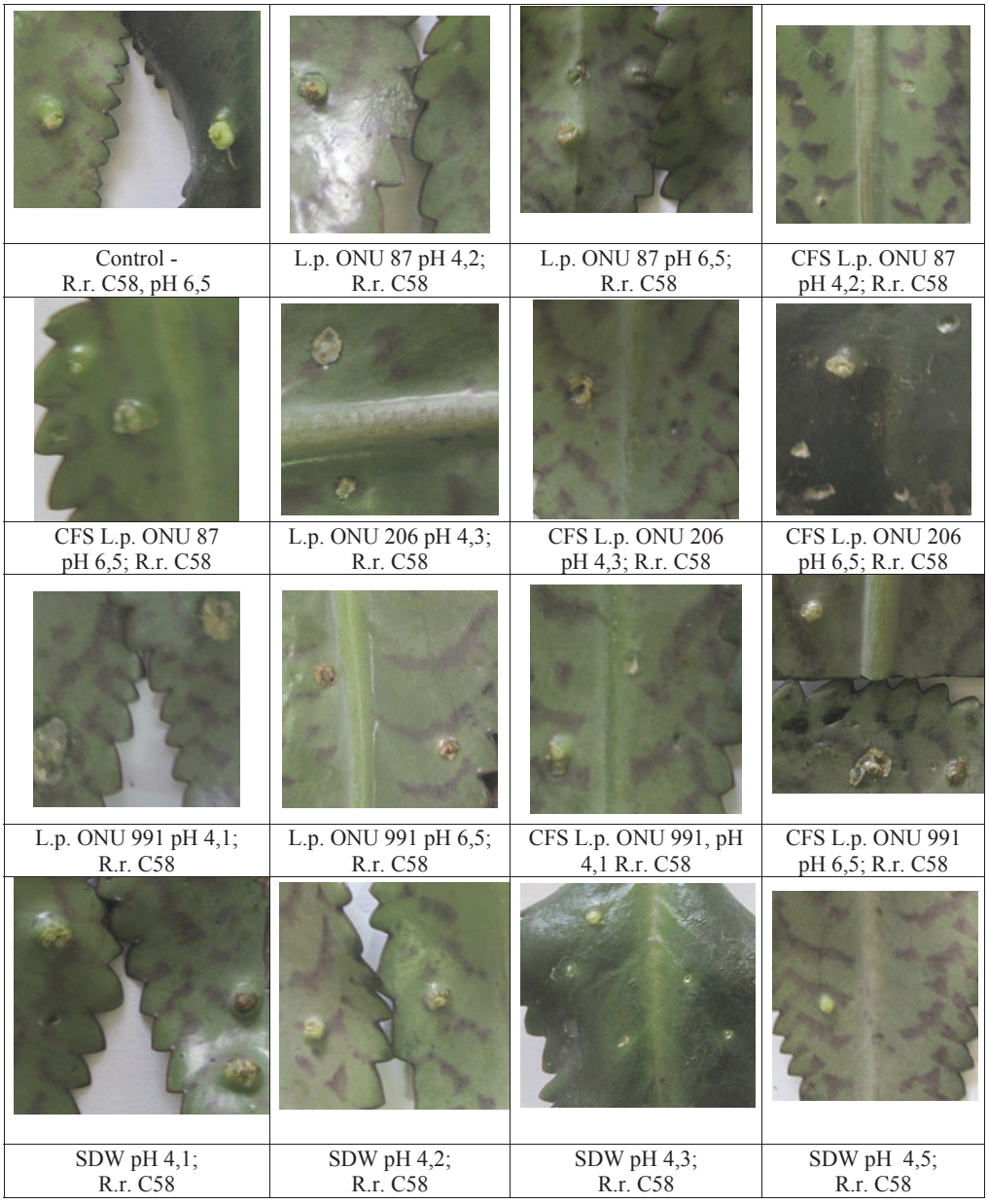

Fig. 5. Tumors formed on *K. daigremontiana* leaves after the inoculations with *R. radiobacter* C58 and different variants of the treatments with cultures of lactobacilli and their CFS

The obtained data show that the injections of bacteria of *L. plantarum* ONU 87 and ONU 206 strains both with pH 4.1–4.3 and pH 6.5 did not differ significantly by the effect of action (Fig. 1, Fig. 2). Thus, in the case of the tested strains the

antagonism is ensured by the presence of bacteria in plant tissues which actively inhibit the phytopathogen action not depending on the primary pH of the injected cultural liquid.

To check out if the low pH of the cultures of lactic acid bacteria and their CFS is the main factor of antagonism, the tests for the effect of SDW with the pH typical for the overnight cultures of *L. plantarum in vitro* and *in vivo* have been carried out. There were no zones of growth inhibition on the lawns of *R. radiobacter* C58. As it is known [10], rhizobia can grow in a wide range of pH including pH 4.0. Thus, only the low pH of the cultures of lactobacilli cannot explain their inhibition effect on tumor formation.

Necroses on kalanchoe leaves formed as the result of separated injections of lactobacilli, their metabolites and SDW with the same low pH were also different (Fig. 6).

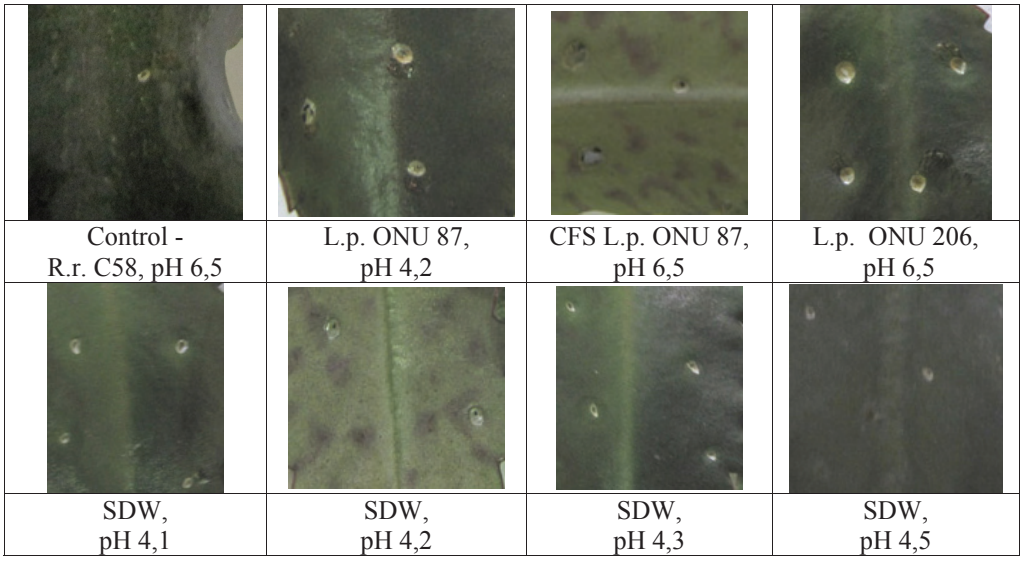

Fig. 6. Necroses formed as the results of injections of bacteria, their metabolites and sterile distillated water with low pH into the kalanchoe leaves

SDW with the low pH caused only the slight necroses spots. Opposite, the injections of the cultures of lactobacilli and their CFS both with low and neutral pH caused the vast zones of necroses. This makes possible to hypothesize that the injection of lactobacilli or their metabolites results in a hypersensitivity reaction which probably leads to inhibition of tumor formation. The fermentative activity of lactobacilli towards wounded plant tissues [3] with the same probability could result in some damages.

Thus, the studied lactic acid bacteria of *L. plantarum* strains differ in the level of inhibition of tumor formation caused by *R. radiobacter* in the experiments *in vivo* on *K. daigremontiana* plants. Strain *L. plantarum* 206 was the best antagonist. Bacteria of this strain significantly decreased both the amount of infected samples

and weight of formed tumors. It was hypothesized that the inhibiting activity of metabolites from the cultural liquid of lactobacteria is explained not only with the action of organic acids, but also with the action of bacteriocins or other products of metabolism which will be the subject of further investigation.

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ВПЛИВ *LACTOBACILLUS PLANTARUM* НА УТВОРЕННЯ ПУХЛИН, СПРИЧИНЕНИХ *RHIZOBIUM RADIOBACTER*

Реферат

Метою *дослідження було вивчення впливу бактерій Lactobacillus plantarum та їх метаболітів на утворення пухлин у рослин Kalanchoe daigremontiana Mill. за інокуляції збудниками бактеріального раку Rhizobium radiobacter.* **Методи**. *Листя тест-рослин інокулювали культурами бактерій R. radiobacter C58 та трьох штамів Lactobacillus plantarum або їх надосадовими рідинами (НОР) з вихідним низьким значенням pH (4,1–4,3) та з нейтралізованим pH (6,5) у співвідношенні 1:1. Через 45 днів враховували кількість зразків, у яких утворилися пухлини, масу пухлинних тканин і відмічали утворення некрозів.* **Результати**. *Показано, що на утворення пухлин лактобацили та їх надосадові рідини впливали по-різному в залежності від варіанта обробки. Інгібувальну активність у випадку усіх трьох досліджених штамів L. plantarum проявила НОР з вихідним pH (4,1–4,3). Лактобацили, введені у рослинні тканини одночасно із патогеном, зменшували кількість зразків із симптомами бактеріального раку як у випадку низького вихідного pH культуральної рідини (штам L. plantarum ОНУ 87 зменшував на 86%, штам L. plantarum ОНУ 206 – на 95%), так і у випадку нейтралізованого pH культуральної рідини (штам L. plantarum ОНУ 87 зменшував на 75%, штам L. plantarum ОНУ 206 – на 94%). Бактерії штаму L. plantarum ОНУ 991 не проявляли інгібувальної активності у випадку обробки культурами лактобацил, а також нейтралізованою надосадовою рідиною. В той же час добові культури усіх штамів in vitro виявили чітку антагоністичну активність. Введення культур лактобацил та їх надосадових рідин як з низьким, так із нейтральним pH, призводило до поширених зон некрозу у порівнянні з такими за ін'єкцій тканин стерильною дистильованою водою з такими самими значеннями pH.* **Висновок**. *Молочнокислі бактерії штамів L. plantarum відрізняються за рівнем пригнічення пухлиноутворення, спричиненого R. radiobacter. Штам L. plantarum ОНУ 206 виявився найкращим антагоністом у даних випробуваннях: за усіма варіантами обробок істотно зменшувались як кількість інфікованих зразків, так і маса утворених пухлин. Інгібувальна активність продуктів метаболізму, що містяться в культуральній рідині лактобактерій, ймовірно, обумовлена дією не лише органічних кислот, а також бактеріоцинів або інших продуктів метаболізму.*

Ключові слова: Rhizobium radiobacter, Lactobacillus plantarum, пригнічення пухлиноутворення, бактеріальний рак, Kalanchoe daigremontiana.

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ВЛИЯНИЕ *LACTOBACILLUS PLANTARUM* НА ОБРАЗОВАНИЕ ОПУХОЛЕЙ, ВЫЗВАННЫХ *RHIZOBIUM RADIOBACTER*

Реферат

Целью *исследования было изучение влияния бактерий Lactobacillus plantarum и их метаболитов на образование опухолей у растений Kalanchoe daigremontiana Mill. при инокуляции возбудителями бактериального рака Rhizobium radiobacter.* **Методы**. *Листья тест-растений инокулировали культурами R. radiobacter C58 и трех штаммов Lactobacillus plantarum или их надосадочными жидкостями (НОЖ) как с первичными низкими значениями pH (4,1–4,3), так и с нейтрализованными pH (6,5) в соотношении 1:1. Через 45 дней учитывали количество образцов с опухолями, массу опухолевых тканей и отмечали образование некрозов.* **Результаты**. *Показано, что на образование опухолей лактобациллы и их надосадочные жидкости влияли по-разному в зависимости от варианта обработки. Ингибирующую активность в случае всех трех исследованных штаммов L. plantarum проявила НОЖ с первичным pH (4,1–4,3). Лактобациллы, введенные в растительные ткани одновременно с патогеном, уменьшали количество образцов с симптомами бактериального рака как в случае низкого первичного pH культуральной жидкости (штамм L. plantarum ОНУ 87 уменьшал на 86%, штамм L. plantarum ОНУ 206 – на 95%), так и в случае нейтрализованного pH культуральной жидкости (штамм L. plantarum ОНУ 87 уменьшал на 75%, штамм L. plantarum ОНУ 206 – на 94%). Бактерии штамма L. plantarum ОНУ 991 не проявляли ингибирующей активности в случае обработки культурами лактобацилл, а также нейтрализованной надосадочной жидкостью. В то же самое время суточные культуры всех штаммов in vitro выявили четкую антагонистическую активность. Введение культур лактобацилл и их надосадочных жидкостей как с низким, так и с нейтральным pH, приводило к обширным зонам некроза по сравнению с такими в случае инъекций тканей стерильной дистиллированной водой с такими же значениями pH.* **Вывод.** *Молочнокислые бактерии штаммов L. plantarum отличаются по уровню угнетения опухолеобразования, вызванного R. radiobacter. Штамм L. plantarum ОНУ 206 выявился лучшим антагонистом в данных испытаниях: во всех вариантах обработок существенно уменьшались как количество инфицированных образцов, так и масса образованных опухолей. Ингибирующая активность продуктов метаболизма, содержащихся в культуральной жидкости лактобактерий, вероятно, обусловлена действием не только органических кислот, а также бактериоцинов или других продуктов метаболизма.*

Ключевые слова: Rhizobium radiobacter, Lactobacillus plantarum, угнетение опухолеобразования, бактериальный рак, Kalanchoe daigremontiana.

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