Artículo de investigación

The Effect of Laser Irradiation on The Activity of The Bacteria Bacillus Subtilis And Pseudomonas Fluorescens

Влияние Лазерного Облучения на Активность Бактерий Bacillus Subtilis и Pseudomonas Fluorescens

El efecto de la irradiación con láser sobre la actividad de las bacterias Bacillus subtilis y Pseudomonas Fluorescens

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Abstract

The paper discusses the problem of increasing the activity of phytopathogen-antagonistic bacteria under the effect of laser irradiation. It has been shown that short-term treatment of cells of *Bacillus subtilis* and *Pseudomonas fluorescens* with coherent light can increase bacterial growth rate and improve their fungicidal activity. It ensures high efficiency of environmentally safe measures for biological monitoring of plant diseases, which is in line with the principles of organic farming.

Keywords: Laser irradiation *Bacillus subtilis*, *Pseudomonas fluorescens*, bacteria stimulation, biological monitoring of plant diseases.

Аннотация

Обсуждается проблема повышения активности бактерий-антагонистов фитопатогенов под действием лазерного облучения. Показано, что кратковременная обработка когерентным светом клеток Bacillus subtilis и Pseudomonas fluorescencs способна повысить скорость размножения бактерий и усилить их фунгицидную активность. Это обеспечивает высокую эффективность экологически безопасных мероприятий по биоконтролю болезней растений, что соответствует принципам органического земледелия.

Ключевые слова: Лазерное излучение, Bacillus subtilis, Pseudomonas fluorescens, стимуляция бактерий, биоконтроль болезней растений.

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Resumen

El artículo analiza el problema de aumentar la actividad de las bacterias antagonistas de fitopatógenos bajo el efecto de la irradiación con láser. Se ha demostrado que el tratamiento a corto plazo de las células de *Bacillus subtilis* y *Pseudomonas fluorescens* con luz coherente puede aumentar la tasa de crecimiento bacteriano y mejorar su actividad fungicida. Asegura una alta eficiencia de las medidas ambientalmente seguras para el monitoreo biológico de las enfermedades de las plantas, lo cual está en línea con los principios de la agricultura orgánica.

Palabras clave: Irradiación con láser *Bacillus subtilis*, *Pseudomonas fluorescens*, estimulación bacteriana, monitoreo biológico de enfermedades de las plantas.

Introduction

The main task of the world's agricultural production is obtaining high-quality food products. Successful accomplishment of this task is impossible without competently organized measures for plant protection. Various approaches are used to prevent, mitigate, and combat plant diseases; the leading role is still played by chemicals. Pesticides efficiently suppress the development of the pathogenic microbiota, however, their excessive and often unsubstantiated use results in environmental pollution. Chemical fungicides can accumulate in the soil, in the water, and in the food, with a negative effect on human health. Along with that, their use causes genetic variability of microorganisms, which results in the emergence of increasingly resistant and virulent races of pathogens. In this regard, the need arose for alternative ways of combating diseases in agricultural plants, which would be in line with the principles of organic farming (Pal, 2006; Rahmawati, 2016; Novikova, 2017).

Currently, there are many state and national standards for organic agriculture. The main documents that regulate the requirements to agricultural products are EU Regulation 2092/91 (EC 834/2007); Codex Alimentarius Guidelines for Organically produced food 1999/2001; IFOAM Basic Standard (IBS); norms EU 834/2007, EC 889/2008, National Organic Program (NOP); Japanese Agricultural Standard (JAS) (Nikolaeva, 2016; Morgera, 2012).

Development and introduction of the means for biological phytopathogens monitoring into the agricultural practice is a promising research area in combating agricultural plants diseases, which have been widely developed due to the necessity of implementing the concept of organic farming (Shternis, 2012; Petrovsky, 2017; Horuz, 2018). Cultures of bacteria of genera *Bacillus* and *Pseudomonas* are promising as the basis for polyfunctional biological preparations for plant breeding, since they show antagonistic activity against pathogens of plant diseases of bacterial and fungal etiology (Kiprushkina, 2017; Mohammadi, 2017).

Strains have been identified and isolated that are characterized by complex action, have a positive effect on plant development, have antifungal action on a number of phytopathogens, and are characterized by phage resistance (Huang, 2012; Singh, 2016; Syamala, 2017; Kanjanamaneesathian, 2018; Yasmin, 2016; Suman, 2018).

Further development in this area will require deeper understanding of the complex interaction among the antagonist microbes, the plants, the environment, and the man.

Despite the fact that issues relating to human and environmental health and safety are fundamental in choosing a strategy for plant protection, the practical implementation of biological control of phytopathogens on a commercial scale is constrained by several factors: the high cost of biological preparations, their short shelf life, low efficiency and reliability compared to a number of chemicals, and limited temperature range.

The use of biological methods to combat diseases will become cost-effective if the total infection background or the target pathogen can be effectively suppressed by increasing the population of biological agents, which would contribute to successful colonization of the host plant with nonpathogenic strains of the microorganisms, as well as by increasing their antifungal activity. As research studies reveal various conditions that are required for successful implementation of biological control of plant diseases, the importance of this method in organizing measures for plant protection inevitably increases (Sindhu, 2016).

Based on the experiments on the effect of coherent light on microorganisms, its stimulating effect on growth processes and physiological activity of bacterial and fungal strains has been discovered. This fact is scientific substantiation for the efficiency of using laser irradiation for stimulating microbial antagonists to phytopathogens (Budagovsky, 2017).

This work was aimed at studying the effect of red laser irradiation on the activity of the bacteria that are antagonists to plant pathogens.

Materials and methods

The work was performed on the basis of the Biophotonics research problem laboratory at FSBEI Michurinsk State Agrarian University between 2014 and 2018.

In the experiments, aqueous suspensions of bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* with the bacterial biomass content of 0.001 % were used. Cultivation was performed on Saburo medium and on potato-glucose agar.

Saburo medium. Glucose – 40.0 g, peptone – 10.0 g, agar – 10 g, and distilled water – 1 l. The components were blended, and pH was brought to 6.5. The liquid was poured into glassware and sterilized at the pressure of 1 atm. for 15 min.
Potato-glucose agar. 300 g of peeled, washed, and sliced potatoes were put into 1 l of water and boiled for 40 min. Then the liquid was filtered, the volume restored to 1 l, and 10 g of glucose and 10 g of agar-agar were added. The liquid was boiled until dissolution. The liquid was poured in glassware and sterilized at the pressure of 1 atm. for 30 min.

To assess changes in the CFU number in bacterial suspensions after exposure, they were kept without illumination at 22...24ºC for two hours, and then irradiated by laser in transparent glassware with airtight lids. They were put on a rotating platform, which ensured uniformity of light action. Next, the suspension was seeded on the surface of the Saburo agarose nutrient medium. After the incubation for 24 hours in a thermostat at 37ºC, the number of the formed colonies was counted, and the number of CFU in 1 ml of the suspension was calculated.

The activity of *B. subtilis* and *P. fluorescens* was assessed by the volume of the formed colonies after one-day incubation at 37ºC. The bacterial biomass was seeded on dense potato-glucose medium in the center of a Petri dish.

The bacteria were irradiated with red helium-neon (He-Ne) and solid state (S/S) lasers (632.8 nm and 660 nm, respectively). In all experiments, power density was 2.5 W/m^2. To determine the optimal modes of micro-organisms light treatment, the time range from 15 to 960 sec. was used. The power and power density of quasi-monochromatic radiation were registered with high-precision measuring laser VEGA Ophir (Israel) and calorimetric meter IMO-2N (Etalon, Russia).

For treating green leaves of plants with antagonistic bacteria *B. subtilis* and *P. fluorescens*, suspensions with a concentration of 0.001 % were prepared. Irradiation was performed with a solid-state laser (wavelength of 660 nm, the power density of 2.5 W/m^2) for 60 sec; 120 sec; 240 sec; 480 sec; the reference was the variant without irradiation. These suspensions were used for treating the leaves of potted *Ormosia*, tomato, and pepper plants in the conditions of a film greenhouse. Two days later, washes were taken from the leaf surface and placed on a thermostatic shaker set to 37 ° C at 200 rpm for 40 minutes. 100 µl of each obtained washes were seeded on potato-glucose agar with penicillin and streptomycin (100 U/l each). On the 3rd day, the colonies of microorganisms were accounted for and identified, with subsequent recalculation for 1 cm^2 of the leaf surface.

For treating greenhouse soil with suspensions of *B. subtilis* and *P. fluorescens* at the concentration of 0.001 %, they were preliminarily irradiated with solid-state laser (wavelength of 660 nm, power density of 2.5 W/m^2) for 60 sec; 120 sec; 240 sec; 480 sec; the reference was the variant without irradiation. After that, these suspensions were used for watering the roots of the plants. After two days, suspensions were prepared from 1 cm^3 of the soil in the reference variant and in the variants treated with laser; suspensions were prepared in 30 ml of sterile water, then seeded by 100 µl on potato-glucose agar with an antibiotic (penicillin and streptomycin at the concentration of 100 U/l).

On day 3, the colonies of microorganisms were accounted for and identified, with subsequent recalculation for 1 cm^3 of soil.

The experiments were repeated at least five times. For statistical data processing, analytical tools of Microsoft Office Excel 2007 was used.

Results and discussion

The experiment for studying the effects of laser irradiation on the viability of bacterial cells allowed revealing the stimulating effect of coherent light on *P. fluorescens* and *B. subtilis*. The number of CFU after irradiation increased the most in the suspension with bacteria *P. fluorescens* – on average by 64.1 %. In the

irradiated suspension with these bacteria (in the experiment), the number of living cells, depending on the duration of exposure, varied between $7.5x10^5$ and $16.1x10^5$, and was $7.3x10^5$ in the reference variant.

Laser stimulation of bacterial cells viability was detected upon irradiation of the suspension with *B. subtilis*. The number of CFU after irradiation increased on average by 46.0 %, compared to the reference. With that, the number of living structures after irradiation was $9.8x10^5$ – $13.3x10^5$, without irradiation – $7.9x10^5$ (Fig. 1). The effect of laser irradiation on the growth of bacterial colonies *B. subtilis* and *P. fluorescens* was assessed. It has been found that the greatest colony-growing stimulation effect, compared to the non-irradiated variants, was observed with the *P. fluorescens* bacteria (15.3 %), which formed colonies with moderate rate (biomass volume without irradiation was on average 480.6 mm^3).

While bacteria *B. subtilis* formed larger colonies on solid nutrient media (biomass volume without irradiation was on average 5,742.1 mm^3), the growth rate increase after laser treatment was insignificant (4.9 %) (Fig. 2).

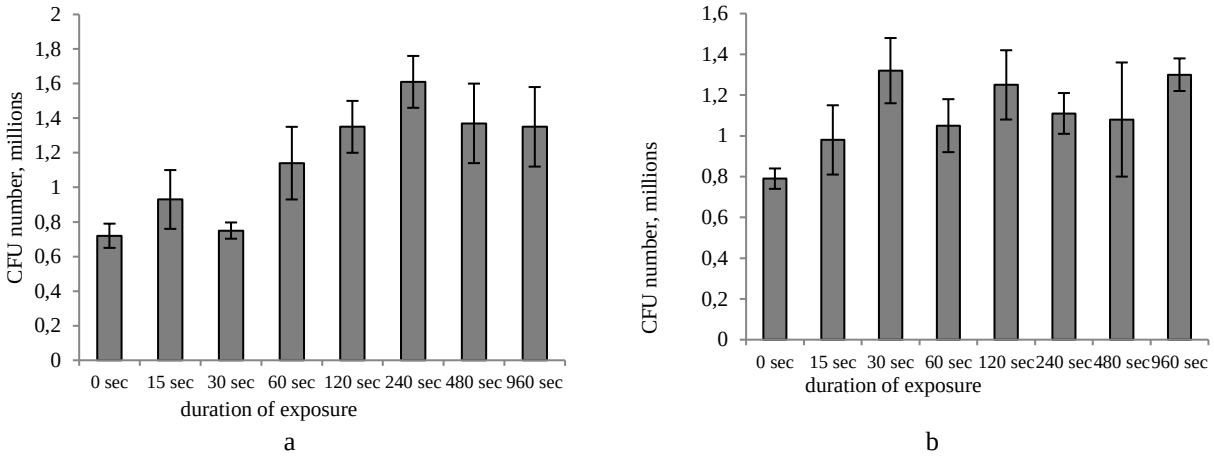

Figure 1. The effect of laser irradiation on changes in the CFU number of *P. fluorescens* (a) and *B. subtilis* (b) in the suspension.

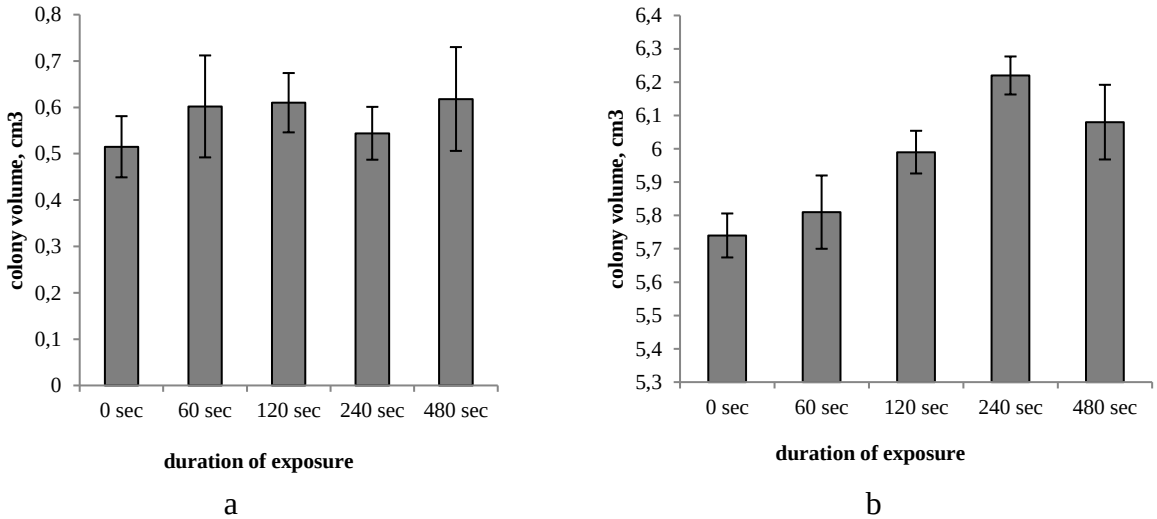

Figure 2. The effect of laser irradiation on the growth of colonies of *P. fluorescens* (a) and *B. subtilis* (b).

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P. fluorescens and *B. subtilis* are used for biological monitoring of plant diseases. The efficiency of these antagonistic bacteria irradiated with laser for combating fungal pathogens was assessed in the conditions of a film greenhouse. After the introduction of irradiated suspension with *B. subtilis* with the concentration of 0.001 %, the number of CFU of the bacteria per 1 cm^3 of soil in the root zone of plants ranged, depending on the duration of exposure, from 6.9×10^4 to 9.1×10^4, and on average amounted to 8.1×10^4, which was by 47.5 % higher than in the reference variant.

Along with the increased number of cells of the bioagent, in the variants with laser irradiation, improved antifungal properties were observed in variants with *Bacillus subtilis*. In this regard, a decreased number of cells of the *Penicillium* and *Alternaria* fungi by 44.0 % and 56.3 %, respectively, was observed in the soil.

Depending on the duration of irradiating the suspension with *Bacillus subtilis*, the number of plant penicilliosis pathogen CFU in 1 cm^3 of soil ranged from 120 to 186, while this value in the reference variant was 276. The number of plant alternarioses pathogen CFU in the test samples ranged from 0 to 120, on average, it was 52.5, and in the reference variant, 120 CFU were found per 1 cm^3 of soil.

The use of irradiated suspension of *Pseudomonas fluorescens* for the introduction to the roots of the studied plants allowed increasing the content of fungicidal biological agents in the soil, compared to the variant without irradiation.
As a result of using irradiated suspension, the number of bacterial cells in the soil was on average 0.88×10^4, while this indicator in the reference was equal to 0.58×10^4. Thus, the use of irradiated suspension of *P. fluorescens* allowed increasing the number of CFU of bacteria in greenhouse soil by 51.5 %, compared to the variant where no irradiation had been used.

Active development of nonpathogenic bacterial microbiota in the soil contributed to reducing the level of fungal infection accumulation. The number of CFU of fungi *Penicillium*, *Alternaria*, and *Fusarium* in the soil after the treatment with a non-irradiated suspension of *P. fluorescens* was 160, 60, and 90, respectively. In the variants with the use of laser irradiation, the number of viable cells of fungus *Penicillium* in the samples of the soil decreased on average to 45. *Alternaria* in the variants with irradiated suspension with the duration of exposure of 60 sec., 120 sec., and 240 sec. was totally absent; with laser irradiation for 480 s weak growth of this pathogen was observed (the number of CFU was 75). Development of fungus *Fusarium* was completely suppressed in all samples of the soil where irradiated suspension of *P. fluorescens* had been used.

In order to assess the efficiency of laser irradiation for increasing the activity of bacterial antagonists to phytopathogens, plants were treated with 0.001 % suspension, followed by seeding swabs from the surface of the leaves on a nutrient medium, which allowed to determine the degree of contamination by the bacteria and the fungi.

In treating the studied plants with a suspension with *B. subtilis*, the number of CFU of bacteria per 1 cm^2 of the leaf surface was on average 4.4×10^4 without the use of irradiation. In the experiment where the bacterial suspension was exposed to coherent light, the number of cells of the bioagent was 5.2×10^4. Thus, the use of laser irradiation resulted in a 17.7 % increase in the number of bacterial cells on the leaf surface, compared to non-irradiated variant.

With that, the degree of the leaves contamination by the fungi appropriately changed. The number of cells of *Penicillium* and *Alternaria* in 1 cm^2 of the leaf surface treated with the suspension with *B. subtilis* was 53.0 and 45.6, respectively. In the variants of the experiment where the irradiated bacterial suspension had been used, this value ranged from 8.3 to 39.0 for *Penicillium*, and from 22.1 to 35.3 for *Alternaria*, depending on the parameters of irradiation, and on average amounted to 20.9 and 28.6, respectively. The use of laser irradiation allowed increasing the antifungal activity of bacteria against fungal pathogens *Penicillium* and *Alternaria* by 60.5 % and 22.6 %, respectively.

As a result of processing the leaves of the studied plants with the suspension of *P. fluorescens*, it was found that the bioagent had been laser-stimulated. This resulted in an increased contamination of the leaves with non-pathogenic bacterial microbiota in the variants where the irradiated suspension had been used, compared to the samples without the use of laser irradiation.

In 1 cm^2 of the leaf surface treated with the suspension of *P. fluorescens*, 6.1×10^4 bacterial CFU were found. Irradiation helped increase the number of microbial antagonists of pathogenic fungi on average to 6.7×10^4 CFU (by 10.5 % compared to the non-irradiated variant). In the most efficient exposures (60 sec. and 240 sec.), this value was 8.3×10^4 and 7.1×10^4 cells.

It is important to note that with a slight excess of the number of bacterial cells in the experiment where irradiation was used, a significant increase

in their antifungal activity was noted. Microbiological analysis of the swabs from the surface of the leaves of agricultural plants after the treatment with the suspension with *P. fluorescens* showed that the number of cells of fungi *Penicillium* and *Alternaria* in 1 cm^2 of the leaf surface was 50.1 and 33.4, respectively, while in the variants with the use of laser irradiation, these values were on average 13.9 and 19.5, i.e., they decreased by 72.3 % and 51.7 %, respectively. Pathogenic fungus *Fusarium* in the samples without the use of radiation was found in the small amount of 5.6 cells per 1 cm^2 of the leaf surface, and not found when the irradiated suspension was used.

Conclusion

The performed experiments have shown that the short-term laser treatment of bacteria *Pseudomonas fluorescens* and *Bacillus subtilis* can increase their activity. The stimulation action is based on the photoregulatory effect of the quasi-monochromatic red light. Under its action, microbial cells start multiplying faster; and their fungicidal activity increases. The technologies based on the determined effect can be used in organic farming for improving the efficiency of plants biological protection from diseases.

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