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Investigation of Antagonistic Properties of Bacteria Bacillus Subtilis against Carrot Phytopathogenes *in vitro* and *in vivo* Experiments

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Abstract

Pathogenic microorganisms that cause microbiological damage represent a potential threat to the production and provision of post-harvest storage of vegetables. This problem is relevant for the cultivation and storage of carrots in the southern regions of Russia due to the special climate conditions and physiological features of this root-crop.

Three strains of bacteria of the genus Bacillus have been studied for their potential as biological control agents for phytopathogens Sclerotinia sclerotiorum, Alternaria radicina and Erwinia carotovora, which cause carrot diseases during storage. Based on the evaluation of the antagonistic activity of strains of Bacillus subtilis against phytopathogenic microorganisms, the strain IMP 215 was chosen as the most promising. The potential of the strain for controlling carrot diseases was assessed in vitro and in vivo.

In comparison with the control there was found a significant decrease in the intensity of the damage caused by the investigated pathogens in all studies. The results allow to draw a conclusion about the expediency of using the strain Bacillus subtilis IMP 215 to control the development of carrot roots diseases during storage. Processing of carrots with strain Bacillus subtilis IMP 215 in combination with proper storage technologies can be a useful tool for preventing microbiological damage and reducing losses.

Keywords Bacillus subtilis, biocontrol, vegetables, carrots, microbiological damage, storage.

INTRODUCTION

The prevention of losses in crop production is increasing attention as a significant aspect of food security. Studies conducted by various international and national organizations have shown that more than half of all fruits and vegetables lose their marketable condition during their transportation and storage.

Endurance of storage of vegetables depends on their varietal characteristics, the protection system from vermin and diseases, storage time and harvesting methods, commodity processing and methods of preparation for storage. Physiological, biochemical and microbiological processes taking place in the vegetables during storage, usually lead to a decrease in their quality. The main causes of losses in the storage of vegetables are associated with the processes of respiration, moisture evaporation and microbiological damage [http://www.fao.org/3/a-i4068e.pdf].

Microbiological damage is the main cause of loss of crop production during storage. It creates a potential hazard to the consumer's health and leads to significant economic losses. The quantitative and qualitative composition of the epiphytic microflora variety depends on the type of plant production, on the agronomical techniques, on the features of the soil, on the location and climate conditions of cultivation.

The quantity and type of microorganisms on fresh vegetables always vary notably. The number of bacteria on vegetables after harvest depends on the type of product and it's growing conditions and usually is about 10^3-10^9 CFU/g [1].

Gram-negative bacteria predominate in the microflora of most vegetables. The microflora of vegetables is represented by Pseudomonas spp., Erwinia herbicola, Flavobacterium, Xanthomonas and Enterobacter agglomerans, as well as various forms of mold fungi of the genera Alternaria, Penicillium, Fusarium and Aspergillus. Also 50-90% of the microbial population on vegetables is represented by lactic acid bacteria, such as Leuconostoc mesenteroides, Lactobacillus spp. and Pseudomonas spp. [2].

Traditional ways to extend the storage time of vegetables are cooling, creating a modified atmosphere and chemical treatment. The use of protective cultures, bacteriophages and bacteriocins can become an alternative to chemical treatments, naturally inhibiting the growth of pathogenic microflora during storage [3].

Different types of bacteria of the genus Bacillus have many advantages over other bacteria. The most important of them is protection against phytopathogens due to the ability to produce a wide range of antibiotics such as atrimine, bacilipine, bacillizin, bacillomixin, bacillin, globicin, datemycin, debariocidin, uterine, iturin, xantelin, mycosubtilin, mycobainylline, neocidin, shtutin, petrin, polychlorosubtiline , rhizobacidin, subtilin, subtenolin, subtenolizin, subtilisin, subsporin, toximycin, trypanotoxin, fungistatin, fungocin, fluvomycin, endosubtilisin, eumycin, bacillomycin, etc. The ability of some strains of the genus Bacillus to inhibit the growth of Fusarium verticillioides and the accumulation of fumonisin B_1 in vitro has been established. An analysis of the ability of ten Bacillus strains to inhibit fungal growth and accumulate fumonisin B_1 in vitro has established that the best antagonist for F. verticillioides is B. subtilis CE1 [4].

The efficacy of the strain Bacillus subtilis V26 as an antagonist to Botrytis cinerea, the main cause of tomato disease in fruit rot, has been established. The antifungal activity of the strain of Bacillus subtilis V26 persisted under the influence of temperature, under UV treatment, also the strain was resistant to proteases. The treatment of tomatoes with Bacillus subtilis V26 strain reduces post-harvest diseases caused by B. cinerea by 79% [5].

A method for treating vegetable crops is known, which involves the use of Bacillus subtilis strain Ch-13 as a biocontrol preparation, which increases the effectiveness of protecting vegetables from phytopathogenic fungi [6].

Bacillus subtilis strain Ch-13 also forms the basis of the drug with the commercial name "Extrasol", effectively reducing the incidence of vegetables caused by phytopathogenic microorganisms Puccinia recondita, Erysiphe graminis and Fusarium culmorum [7].

It was found that Bacillus subtilis and Brevibacterium linens inhibited the infection of tomatoes caused by Alternaria solani and Botrytis cinerea. The combined use of bacteria has revealed synergistic effects. Bacillus subtilis produced antifungal agents from the group of surfactin lipopeptides. The most effective strains of Brevi bacterium (IC 10) and Bacillus subtilis have shown that the combined use of bacterial antagonists ($5x10^5$ or $5x10^6$ cells) with tomato pathogens causes inhibition of growth of B. cinerea up to 61% [8].

Also, the inhibitory effect of tomato treatment with Bacillus subtilis strain QST 713 on the development of diseases caused by Penicillium spp. and Rhizopus stolonifer was proved [9].

Rao and M. Kmamalnath in 2017 evaluated the strain of Bacillus subtilis IIHR BS-2 as a potential biocontrol agent. There was an inhibition of P. carotovorum growth (60.6%). The liquid composition of B. subtilis IIHR BS-2 (CFU-1 × 10^8 per ml) was tested in the field for the treatment of vegetable seeds (10 ml / kg

seed) comparing with the use of chemical treatment (carbofuran and streptocycline) and untreated control. Among all treatments, seed treatment along with soil application of enriched B. subtilis biomass (5 l/ha^{-1}) provided the maximum increase in carrot yield (28.8%) and a decrease in morbidity (70.2%) [10].

In the experiments in fighting against the cellular disease of apples there has been established the antifungal activity of the strain Bacillus subtilis 9407 against B. dothidea [11].

The efficacy of the Bacillus subtilis strain V26 as a biocontrol agent for potato diseases caused by Rhizoctonia solani has been established. The Bacillus subtilis strain V26 caused significant morphological deformations of fungal hyphae. Compared with the control, the incidence decreased by 81% [12].

The antagonistic activity of the Bacillus subtilis strain UK-9 against the phytopathogen Alternaria, which causes leaf disease of mustard, has been studied. Under the action of strain UK-9, spore germination was inhibited and morbidity decreased.

Biocontrol activity of the Bacillus subtilis G strain BO3, MBI600 towards Fusarium solani, which is a phytopathogen of legumes, has been established.

Despite a significant number of studies on the use of Bacillus subtilis for the biocontrol of plant products, there is still great interest in a study of the antagonistic activity of Bacillus subtilis strains against phytopathogens that cause root vegetable diseases during storage.

Carrot is one of the most difficult vegetables that are stored. Physiological features of this root crop require high humidity during storage. At the same time high humidity provokes intensive growth of pathogenic microflora. That's why researches is in the field of biological control of phytopathogens, which are agents of the most common carrot diseases, such as Sclerotinia sclerotiorum, which causes white rot, Alternaria radicina, which causes black rot, and Erwinia carotovora, which causes wet bacterial decay, are relevant.

The aim of our study was to evaluate the antagonistic efficacy of bacterial strains of Bacillus subtilis against carrot phytopathogens in in vitro and in vivo experiments.

MATERIALS AND METHODS

For our research we selected the root crops of Abako carrot, grown in the Dinskoy district of the Krasnodar territory. All carrots were 3-5 cm in diameter, without mechanical damage and signs of infection or physiological diseases.

Fungal pathogens Sclerotinia sclerotiorum and Alternaria radicina (causing white and black rot) were isolated earlier from affected carrot roots and cultivated on Saburo medium at (+ 27 ± 1) °C for 14 days.

Spores of pathogenic fungi were obtained by washing cultivated mold cultures with sterile distilled water containing 0.05% Tween-80. The suspensions were filtered through three layers of sterilized gauze and adjusted to a concentration of 1×10^7 spores/ml. Accounting was performed with the use of the Garyaev chamber.

The agent of wet bacterial rot, Erwinia carotovora, was isolated from the affected carrot and bred on standard nutrient media: DNA (dry nutrient agar) with glucose at a temperature of (+ 32 ± 1) °C for 48 hours.

Bacillus subtilis strains: Ch-13, IPM 215 and B-10 VIZR were used as antagonists.

The tested strains of Bacillus subtilis were cultivated on nutrient media: DNB (dry nutrient broth) (for culture accumulation) and DNA (dry nutrient agar) prepared from dry nutrient broth supplemented with 1% glucose (for agar blocks). The material was grown for 48 hours, the amount of Bacillus subtilis was adjusted to a concentration of 1×10^7 CFU/ml.

In vitro studies of the antagonistic properties of strains of bacteria of the genus Bacillus subtilis against phytopathogenic microorganisms were carried out by the method with agar blocks. A spore suspension of test cultures of phytopathogenic microorganisms was introduced into melted and cooled to +40 °C DNA (dry nutrient agar) and then this mixture was poured into Petri dishes. After agglomeration of the agar, agar blocks were curved with a sterile forstner bit from the lawn of the studied strain of Bacillus subtilis and placed on the agar surface. The lawn was grown previously using a DNA (dry nutrient agar). Agar blocks were placed with upward growth (lawn), tightly pressing to the agar plate.

The plates with agar blocks were incubated at + 25 °C. At the end of the 7th day monitoring of the areas of growth retardation of the test cultures was held.

Antagonistic properties of phytopathogenic microorganisms have been investigated in several strains of bacteria of the genus Bacillus subtilis. The strain that exhibited the most activity in vitro for a set of test cultures was selected.

The dynamics of the development of Bacillus subtilis strain IPM 215 (hereinafter Bacillus subtilis), Alternaria radicina and Erwinia carotovora were examined in root crop sections at +25 °C for 7 days and at +2 °C for 14 days.

Carrot roots were washed with flowing water, dried and treated with 70% ethyl alcohol.

With a sterile scalpel on the surface of the each root were made three snips, 3 by 3 mm in size, and each of the snips was pipetted with the amount of 10 µl of the suspension with one of the three studied microorganisms.

All root crops were placed in closed transparent plastic crispers. Each crisper contained a glass with liquid to ensure high humidity. The containers were stored at temperatures of +2 °C and +25 °C.

The number of microorganism cells in the snips on root crops was examined every 24 hours during 7 days at +25 °C and every 48 hours during 14 days at +2 °C.

To study the effect of Bacillus subtilis on the incidence and diameter of the lesion of diseases caused by Alternaria radicina and Erwinia carotovora depending on temperature, there were made punctures with a sterile needle on the carrot roots, and a suspension of Bacillus subtilis was added at a dose of 10 µl in an amount of 1×10^7 CFU/ml. Sterile distilled water was used for reference samples.

In the same puncture were added 10 µl of a spore suspensions of Alternaria radicina or Erwinia carotovora containing 1×10^7 spores/ml. All root crops were placed in closed transparent plastic containers and stored at temperatures of +2 °C and +25 °C. There was also placed a flask with liquid in all containers to provide high humidity.

Diagnosis of the incidence and size of lesions caused by Alternaria radicina and Erwinia carotovora was carried out after 7 days of storage at a temperature of +25 °C and after 7, 14 and 28 days of storage at a temperature of +2 °C. Each treatment was repeated three times. The experiment was carried out twice.

RESULTS AND DISCUSSION

Table 1 presents data characterizing the antagonistic activity of the studied strains of Bacillus subtilis Ch-13, B-10 VIZR, IPM 215.

Table 1 – Antagonistic activity of strains of Bacillus subtilis in relation to phytopathogenic microorganisms.

Phytopathogen	The Bacillus subtilis strain / growth retardation zone, mm		
	Ch-13	B-10 VIZR	IPM 215
Sclerotiniasclerotiorum	2,1	1,3	2,5
Alternaria radicina	2,0	1,4	4,0
Erwinia carotovora	3,0	1,8	3,3

From the data presented in the table, it can be concluded that all investigated strains of Bacillus subtilis cause a delay in growth of the investigated phytopathogens. The highest activity against the test set of phytopathogens in *in vitro* experiments showed the strain of Bacillus subtilis IPM 215. Further studies were conducted with this strain.

At the next stage of the research was held a comparative analysis of the growth dynamics of the studied Bacillus subtilis strain and phytopathogens causing carrot diseases, depending on the storage temperature.

The dynamics of the population of the investigated microorganisms in the sections of carrot roots at +25 °C for 7 days of storage is shown in Figure 1.

The dynamics of the population of the investigated microorganisms in the sections of carrot roots at +2 °C for 14 days of storage is shown in Figure 2.

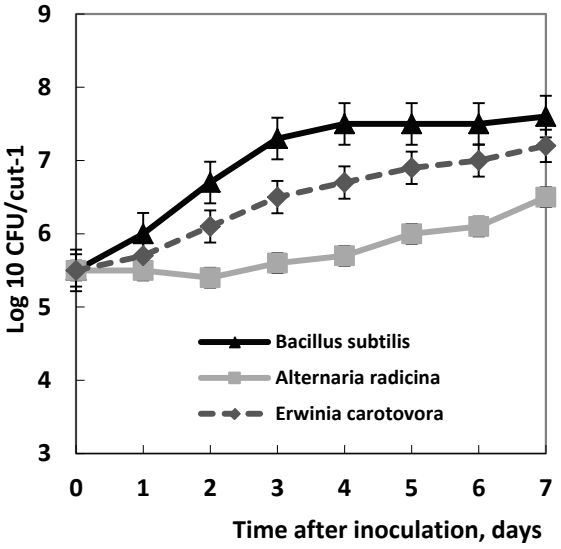

Figure 1 – Dynamics of the population of the investigated microorganisms in sections of carrot roots at +25 °C for 7 days of storage.

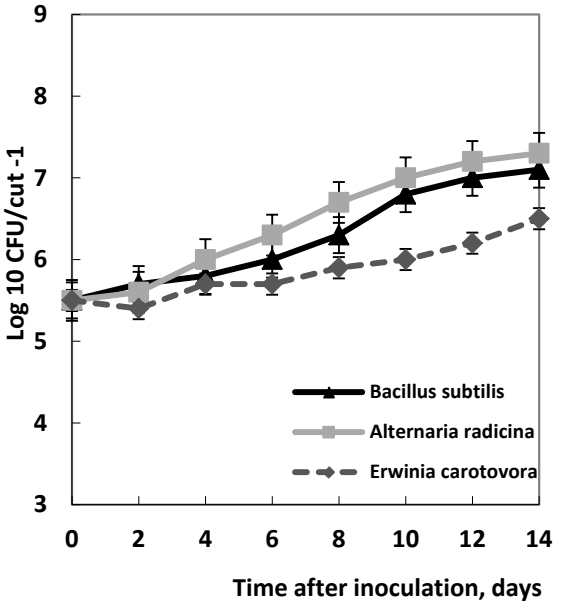

Figure 2 – Dynamics of the population of the investigated microorganisms in sections of carrot roots at +2 °C for 14 days of storage.

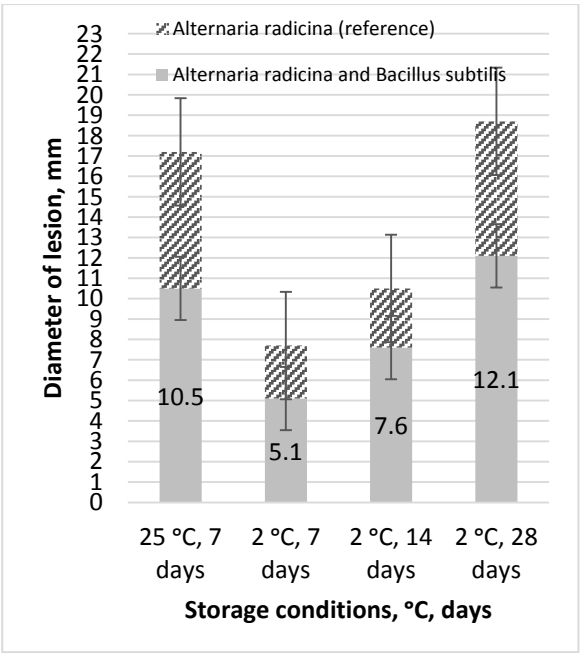

Figure 3 – The effect of Bacillus subtilis on the diameter of the lesion caused by Alternaria radicina depending on the temperature after 7, 14 and 28 days of storage.

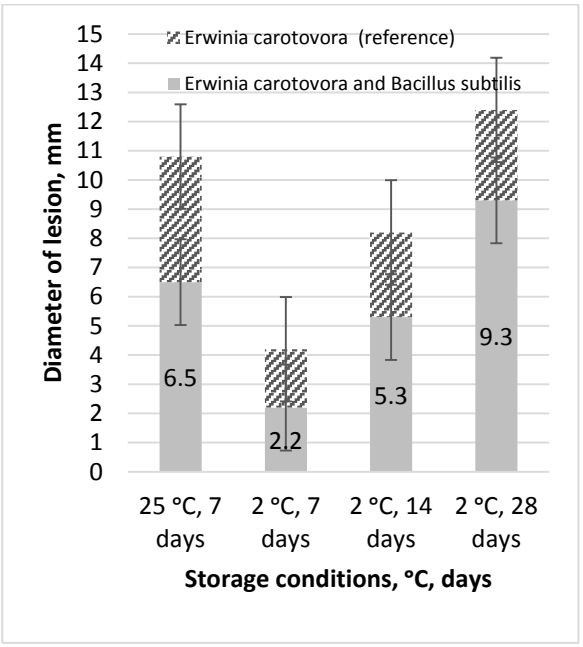

Figure 4 – The effect of Bacillus subtilis on the diameter of the lesion caused by Erwinia carotovora depending on temperature after 7, 14 and 28 days of storage.

In samples with a population of Bacillus subtilis bacteria introduced into the incision in the amount of 8.9×10^5 CFU/slice, their concentration increased after 7 days to 5.7×10^7 CFU/slice at +25 °C and up to 1.5×10^7 CFU/slice after 14 days at +2 °C. This corresponded to an increase of 64 and 19 times compared to the initial amount at +25 °C and +2 °C, respectively.

In samples with Alternaria radicina, introduced in an amount of 5.5×10^5 CFU/slice, their concentration increased after 7 days to 5.2×10^6 CFU/slice at +25 °C and up to 3.5×10^7 CFU/slice after 14 days at +2 °C. This corresponds to an increase of 9.4 and 63.6 times compared to the initial amount at +25 °C and +2°C respectively.

In samples infected with Erwinia carotovora, introduced in the amount of 5×10^5 CFU/slice, their concentration increased after 7 days to 3.1×10^7 CFU/slice at +25 °C and 5×10^6 CFU/slice after 14 days at +2 °C. This corresponded to an increase of 62 and 10 times compared to the initial amount at +25 °C and +2 °C, respectively.

The analysis of the obtained data leads to the conclusion that at a temperature of +25 °C, the growth rate of Bacillus subtilis population on carrot roots in the first stage of storage exceeds the growth rate of Alternaria radicina and Erwinia carotovora populations. After three days of storage, the growth dynamics of Bacillus subtilis decreases. This fact allows to assume expediency of processing of root crops by Bacillus subtilis strain which on the first stage of storage, owing to more intensive growth, creates competition in fight for survival. In addition, the waste products of Bacillus subtilis are effective inhibitors of pathogens [5].

At a temperature of + 2 °C at the first stage of storage, the number of Bacillus subtilis cells varies slightly, but after 7 days of storage adaptation occurs, and the population begins to increase. By the end of 14 days storage, the size of population of Bacillus subtilis is greater than the size of the population of Erwinia carotovora and practically compared with the population of Alternaria radicina.

The obtained data allow to conclude that for the application of Bacillus subtilis for the purpose of inhibiting diseases caused by phytopathogens of carrots, it is advisable to process Bacillus subtilis at a temperature of + 25 °C, exposure for three days at a given temperature and subsequent cooling to a temperature of + 2 °C.

In the next phase of the research we tested the hypothesis of inhibition of diseases caused by phytopathogens Erwinia carotovora and Alternaria radicina with Bacillus subtilis, during processing of carrots.

The effect of Bacillus subtilis on the diameter of the lesion caused by Alternaria radicina depending on the temperature after 7, 14 and 28 days of storage is shown in Figure 3.

After storage at + 25 °C for 7 days, the average diameter of the lesion in reference (untreated Bacillus subtilis) samples infected with Alternaria radicina was 17.2 mm.

In samples treated with Bacillus subtilis and infected with Alternaria radicina, the average diameter of the lesion was 10.5 mm.

After storage at +2 °C for 7 days, the average diameter of the lesion in samples infected with Alternaria radicina was 7.7 mm.

In samples treated with Bacillus subtilis and infected with Alternaria radicina, the average diameter of the lesion was 5.1 mm.

After storage at +2 °C for 14 days, the average diameter of the lesion in samples infected with Alternaria radicina was 5.5 mm.

In samples treated with Bacillus subtilis and infected with Alternaria radicina, the average diameter of the lesion was 7.6 mm.

After storage at + 2 °C for 28 days, the average diameter of the lesion in samples infected with Alternaria radicina was 18.7 mm.

In samples treated with Bacillus subtilis and infected with Alternaria radicina, the average diameter of the lesion was 12.1 mm.

The effect of Bacillus subtilis on the degree of damage to wet bacterial rot caused by Erwinia carotovora in carrot roots, depending on the temperature after 7, 14 and 28 days of storage is shown in Figure 4.

After storage at +25 °C for 7 days, the average diameter of the lesion in samples infected with Erwinia carotovora was 7.2 mm.

In samples treated with Bacillus subtilis and infected with Erwinia carotovora, the average diameter of the lesion was 6.5 mm.

After storage at +2 °C for 7 days, the average diameter of the lesion in samples infected with Erwinia carotovora was 4.2 mm.

In samples treated with Bacillus subtilis and infected with Erwinia carotovora, the average diameter of the lesion was 2.2 mm.

After storage at +2 °C for 14 days, the average diameter of the affected samples infected with Erwinia carotovora was 8.2 mm.

In samples treated with Bacillus subtilis and infected with Erwinia carotovora, the average diameter of the lesion was 5.3 mm.

After storage at +2 °C for 28 days, the average diameter of the affected samples infected with Erwinia carotovora was 12.4 mm.

In samples treated with Bacillus subtilis and infected with Erwinia carotovora, the average diameter of the lesion was 9.3 mm.

CONCLUSIONS

On the basis of the conducted researches antagonistic properties of strains of bacteria Bacillus subtilis concerning phytopathogens of carrot Sclerotinia sclerotiorum, Alternaria radicina and Erwinia carotovora in experiments in vitro are established.

It was found that the strain Bacillus subtilis IPM 215 is the most effective in relation to the studied phytopathogens.

The treatment of Bacillus subtilis affects the degree of development of diseases caused by Alternaria radicina and Erwinia carotovora.

Conducted research justify the use of the strain Bacillus subtilis IPM 215 for the production of drugs in biological control of diseases caused by phytopathogenic microorganisms during storage of carrot roots.

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