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Studying the antagonistic properties of *Bacillus subtilis* bacteria to pathogens of fruits in in vitro and in vivo experiments

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Abstract

The antagonistic properties of bacterial strains Bacillus subtilis to apple phytopathogens Phytophtora cactorum, Botrytis cinerea, Penicillium glaucoma, Erwinia carotovora have been studied in *in vitro* and *in vivo* experiments. The antagonistic properties of bacteria strains Bacillus subtilis were assessed using the method of agar blocks based on measuring the zone of tested pathogens' growth inhibition. It has been established that strain Bacillus subtilis IPM 215 is more efficient against the studied pathogens. Dependence of the dynamics of the population of Bacillus subtilis strain IPM 215 on the temperature of the phytopathogen species, and the influence of Bacillus subtilis concentration and storage temperature on the incidence and the diameter of lesion caused by Phytophtora cactorum and Botrytis cinerea have been established. The research substantiates the use of strain Bacillus subtilis IPM 215 for obtaining preparations for biological monitoring of diseases caused by phytopathogenic microorganisms during apples' storage.

Keywords: biological monitoring, phytopathogens, fruit disease occurrence rate, storage.

INTRODUCTION

Damage by microbial organisms is the main reason for crops' loss in plant cultivation. Depending on the level of economy development, the loss of the yield from microbial damage during the cultivation and storage ranges between 25 and 50% [1]. The traditional method of preventing postharvesting diseases of fruits and vegetables is treatment with synthetic chemical fungicides [2].

However, pathogens that cause diseases during storage develop resistance to commonly used chemical fungicides, thus reducing their efficiency [3]. Besides, this increases the demand for products free from chemicals and pesticides, which makes it important to search for more environmentally friendly and sustainable alternatives [1, 2].

The use of microbial antagonists or biological reference agents is a promising alternative to chemical fungicides, since they are less risky to human health and to the environment [1].

The use of bacterial antagonists is most efficient after harvesting, during storage. It is known that mixed cultures of microorganisms ensure better monitoring of diseases that occur after infection, compared to individual cultures or strains. Also, efficiency of the antagonist may be increased if they are used in combination with small dosages of fungicides and salts, and physical effects, such as hot water fluctuations, irradiation with ultraviolet rays, etc. [4].

Bacterial strain Pseudomonas fluorescens is known, which is an antagonist of the Botrytis cinerea fungus, which causes fruits' grey mould. The use of Pseudomonas fluorescens significantly reduced the size of the lesion and the incidence of gray mold on apples during long-term cold storage [1, 5].

For efficient biological monitoring of the Penicillium expansum apple disease, a method was developed, where apples had been processed with strain Cryptococcus podzolicus reinforced with β-glucosane to combat blue rot of apples [6].

For preventive care and treatment of tropical and subtropical fruits and avocado fruit during storage, a strain of bacteria Bacillus atrophaeus B5 was developed. Efficiency of Bacillus Atrophaeus against bitter rot or anthracnose caused by fungal pathogen Colletotrichum gloeosporioides [7] had been proven.

The postharvesting diseases of fruits are mainly caused by fungus Penicillium expansum. The use of strains Candida membranfaciens and Rhodotorula mucilaginosa as antagonists to blue mold of apples is known. It had also been shown that these strains had the ability to suppress pathogens such as green mold – Penicillium digitatum – that affected oranges, and gray mold – Botrytis cinerea – that affected tomatoes. Strains Cryptococcus laurentii prevented spreading of pear fungal diseases. Combination of calcium chloride mixed with strains had significant synergistic effect on reducing fruit diseases [8, 9].

Postharvest processing of the crops with the Hansenias porauvarum strains is efficiently used for suppressing mold that affects strawberries. The study revealed that this strain not only prevented mold growth, but also preserved high quality of fruit [10].

Epiphytic bacterium Rahnella aquatilis had been isolated from the surface of apples and showed high efficiency in reducing rot caused by Botrytis cinerea and Penicillium expansum on apples during storage in a cold place. At 4^{o}C and 90 % relative humidity, Rahnella aquatilis significantly inhibited development of Botrytis cinerea and Penicillium expansum on Red delicious apple variety stored for 40 days, by 100 % and 60 %, respectively [11].

Efficiency of the antagonist bacteria Metschnikowia fructicola strain AL27 against Penicillium expansum had been proven. The study was conducted on apple varieties "Golden delicious", "Fuji", "Red delicious" and "Granny Smith" at room temperature and at low temperatures [12].

For efficient monitoring of blue and gray mold (Penicillium expansum and Botrytis cinerea), scientists had developed a strain of antagonist bacteria Cryptococcus albidus extracted from peaches. With the concentration of 10^8 CFU/ml of rinsed suspension of cells, the strains completely inhibited decay of apples stored both at room temperature and in a cold place [13].

The biologically active strain Aureobasidium pullulans was also successfully used for preventing development of mold and bitter rot on apples [14].

For monitoring postharvesting diseases of citruses, a biopreparation was developed based on the Metschnikowia pulcherrima and Pichiaguilli ermondii bacteria, which efficiently inhibited the development of blue and green mold [15].

For the same purposes, scientists from China developed bacterial strain Rhodosporidium paludigenum that increased stability of citruses against blue mold during storage [16].

The inhibitory effect of pathogens Penicillium digitatum was also achieved during the use of strains Pseudomonas spp. and Trichoderma spp. [17].

Strain R. mucilaginosa that was created for monitoring and preventing development of apple diseases caused by phytopathogenic fungi of genera Penicillium expansum and Botrytis cinerea might be used for cold storage [18].

Microorganisms Pichiacaribbica were used to monitor Penicillium expansum on fruits. The method of using Pichiacaribbica and phytic acid for treating postharvesting fruit diseases is known [19, 20].

Strains of bacteria Candidas aitoana and Aureobasidium pullulans have biomonitoring activity to phytopathogenic fungi and induce biochemical defense reactions in fruit tissues [21, 22].

Efficiency of strain Pichiaguillie rmondii M8 against Botrytis cinerea on apples was assessed in terms of the long-term storage conditions. As a result of storage at 1° C for 120 days, the occurrence rate of fruit gray mold was reduced by more than 25 % [23].

It had been found that treatment with Pseudomonas syringae strain MA-4 was more efficient for monitoring blue and gray fruit mold, compared to treatment with chemical fungicides in various storage conditions [24].

Strains Rhodotorula glutinis perform biological monitoring of fruit diseases during storage. Inhibition of pathogens was most successfully achieved at Rhodotorula Glutinis concentration equal to 10^8 CFU/ml [25].

Successful application of antagonistic yeast Sporidiobolus pararoseus Y16 against black mold of table grapes caused by pathogen Aspergillus niger and strains Saccharomyces cerevisiae, Wickerhamomy cesanomalus that had antagonists to phytopathogen Botrytis cinerea [26, 27] had been studied.

Brown rot caused by Monilinia spp. is one of the most important postharvesting diseases resulting from storage, which usually affect stone fruit. It had been found that strains Pichia membranaefaciens and Kloeckera apiculata showed antifungal activity in postharvesting monitoring of Monilinia spp. [28]. Also, for monitoring brown rot on peach fruit, yeast Cryptococcus laurentii, Candida guilliermondii and Rhodotorula glutinis were successfully used [29].

Efficiency of Bacillus subtilis enhanced by the action of sodium bicarbonate had been proven for combating phytopathogenic bacteria Botryosphaeria berengeriana that caused fruit rot [30].

Thus, biological monitoring has become an important area of research in recent years, as it is a promising alternative to fungicides for fighting postharvesting fruit diseases.

In this regard, it was interesting to study the antagonistic properties of various strains of Bacillus Subtilis toward pathogens that most commonly affected apples zoned in the Krasnodar region, with the aim of breeding the strain that would be the most efficient against most pathogens, determining dependence of the antagonistic properties on the concentration of Bacillus subtilis suspension and the storage temperature.

METHODS

Fruits and preparations. Apples of the Ida Red variety were harvested in the phase of picking maturity in a garden in the town of Slavyansk-on-Kuban. 8-9 cm diameter fruit without physical damage, symptoms of infectious and physiological diseases were washed with running water, and their surface was disinfected with ethyl alcohol of 70% concentration.

Antagonists' strains were strains of Bacillus subtilis: VKM V-2604D, M-22 VIZR, IPM 215.

The studied strains of Bacillus subtilis were bred on standard nutrient media: DNB (dry nutrient broth) and DNA (dry nutrient agar) with the addition of 1% of glucose.

The studied strains were cultivated in 750 ml Erlenmeyer flasks containing 100 ml of the medium on a rotary rocker (220 ± 10 rpm) at 27 °C for 48 hours. Then the titer of the obtained suspension was determined using the method of screening dilutions on solid nutrient media. The concentrations of the suspensions equal to 1×10^7 1×10^8 CFU/ml were achieved.

Fungal pathogens Botrytis cinerea, Penicillium glaucum, and Phytophtora cactorum were isolated from affected apples and bred on the potato - dextrose agar at 25 °C.

Spores of pathogenic fungi were obtained by flushing the cultures with sterile distilled water containing 0,05% of Tween 80. The suspensions were filtered through 3-4 layers of sterilized gauze and brought to the concentration of 1×10^5 spores/ml. Accounting was performed with the use of the Garyaev chamber.

The antagonistic properties of bacteria of species Bacillus subtilis against phytopathogenic fungi were studied in vitro using the method of agar blocks. A suspension of spores of the tested cultures of phytopathogenic fungi was introduced into molten agar and poured into Petri dishes. After agar solidification, agar blocks cut from the lawn of the examined strain Bacillus subtilis with a sterile forstner bit (6-8 mm in diameter) were placed on its surface. Agar blocks were placed lawn up, at equal distances from each other, by pressing them tightly to the agar plate. The lawn had been grown beforehand using a medium prepared from dried nutrient agar.

Cups with agar blocks were incubated at 27°C for 8 days, the zones of the tested fungi cultures' growth inhibition were checked on the 8th day.

In vivo studies.

To study the effect of Bacillus subtilis concentration and the temperature of storage on the incidence rate and diameter of lesions caused by Phytophtora cactorum and Botrytis cinerea, punctures were made with a sterile needle in apples, and 1 µl of suspension of Bacillus subtilis was introduced in the dosage of 1×10^7 and 1×10^8 CFU/ml. Sterile distilled water was used for reference samples.

After 5 hours, 1 µl of the suspension of spores Phytophtora cactorum or Botrytis cinerea containing 1×10^5 spores/ml was introduced into the same puncture. All fruits were placed in closed transparent plastic containers; a container with liquid was also placed there to ensure high humidity. The containers were stored at +2 °C and at +25 °C.

The disease occurrence rate and the characteristic lesions caused by Phytophtora cactorum and Botrytis cinerea were diagnosed after 5 days at + 25 °C and after 35 days at +2 °C. Each treatment was repeated three times. The experiment was made twice.

Studying the effect of the storage temperature and the type of phytopathogen on the dynamics of Bacillus subtilis population.

Using a sterile scalpel, 3 by 3 mm incisions were made in apples.

10 µl of the suspension of Bacillus subtilis strain IPM 215 in the dosage of 1×10^7 CFU/ml was uniformly introduced with a pipette.

After 5 hours, 10 µl of the suspension of spores Phytophtora cactorum or Botrytis cinerea containing 1×10^5 spores/ml was introduced into the same incision. All fruits were placed in closed transparent plastic containers; a container with liquid was also placed there to ensure high humidity. The containers were stored at +2 °C and at +25 °C.

The number of cells of Bacillus subtilis in the incisions in the apples was checked every 24 hours within 5 days at +25 °C and every 3 days within 15 days at +2 °C.

The experimental data were mathematically processed by the methods of descriptive statistics and ANOVA using the

Microsoft Excel software package. The difference at P≤0.05 was considered significant.

Results and discussion

The antagonistic activity to phytopathogenic fungi was studied for several strains of genus Bacillus subtilis bacteria: VKM V-2604D, M-22 VIZR, IPM 215. The results are shown in Table 1.

Table 1 - Antagonistic activity of Bacillus subtilis strains to phytopathogenic microorganisms

Phytopathogen	Bacillus subtilis strain/ zone of growth inhibition, mm		
	VKM V-2604D	M-22 VIZR	IPM 215
Phytophtora cactorum	3.5	4.0	5.2
Botrytis scinerea	4.2	2.5	4.5
Penicillium glaucum	1.4	3.0	2.9
Erwinia carotovora	1.3	1.8	2.2

The highest activity to the test set of pathogens in the in vitro experiments was shown by strain Bacillus subtilis IPM 215. The antagonistic activity of this strain was most evident to Phytophtora cactorum and Erwinia carotovora. In the next stage, the studies were performed with this strain.

The results of studying the effect of Bacillus subtilis concentration and storage temperature on the disease occurrence rate and diameter of lesions caused by Phytophtora cactorum and Botrytis cinerea are shown in Figures 1 through 8.

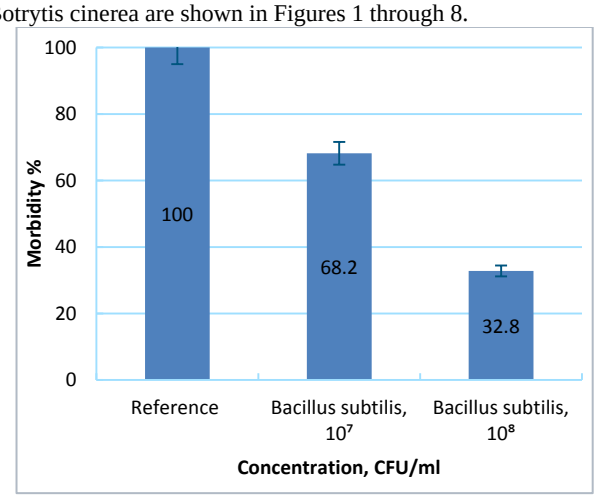

Figure 1 – The influence of Bacillus subtilis concentration on the occurrence rate of diseases caused by Phytophtora cactorum in apples stored at +25°C after 5 days

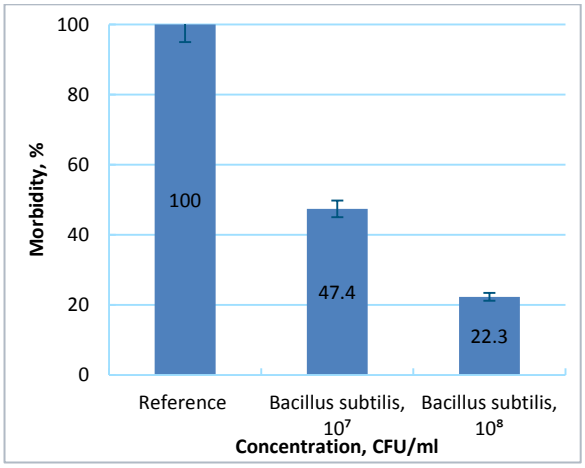

Figure 2 – The influence of Bacillus subtilis concentration on the occurrence rate of diseases caused by Botrytis cinerea in apples stored at +25°C after 5 days

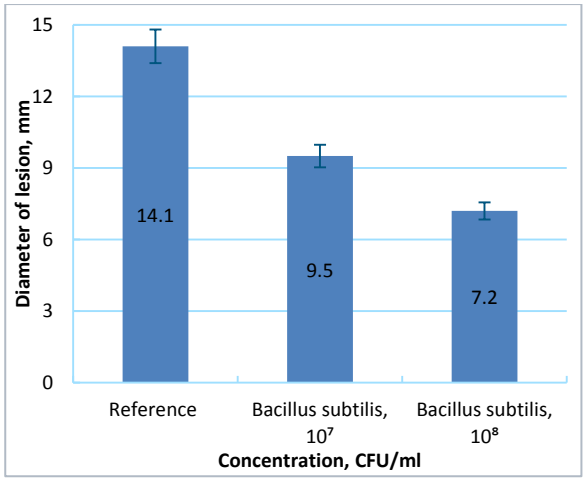

Figure 3 – The influence of Bacillus subtilis concentration on the diameter of lesions caused by Phytophtora cactorum in apples stored at +25°C after 5 days

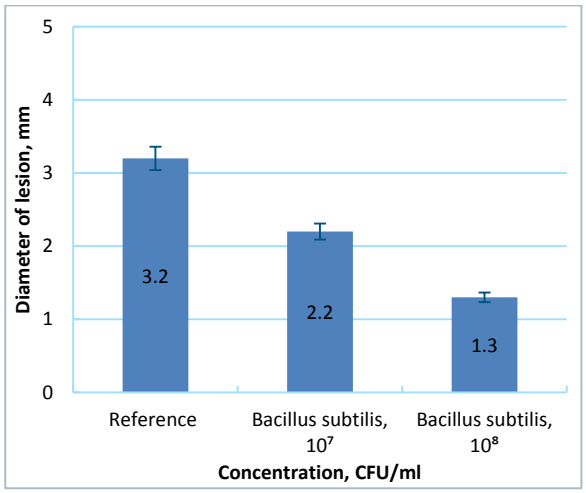

Figure 4 – The influence of Bacillus subtilis concentration on the diameter of lesions caused by Botrytis cinerea in apples stored at +25°C after 5 days

After storing at +25°C for 5 days, the disease occurrence rate in the reference fruits was 100% (with the diameter of lesion in case of infection with Phytophtora cactorum equal to 14.1 mm, in case of infection with Botrytis cinerea – to 3.2 mm).

The fruits processed with Bacillus subtilis with the concentration of inoculum equal to 1×10^7 and 1×10^8 CFU/ml and infected with Phytophtora cactorum were affected by 68.2% and 32.8%, respectively.

The diameter of the lesion caused by Phytophtora cactorum on the fruits processed with Bacillus subtilis in the concentration of 1×10^7 and 1×10^8 CFU/ml was 9.5 and 7.2 mm, respectively.

The fruits processed with Bacillus subtilis with the concentration of inoculum equal to 1×10^7 and 1×10^8 CFU/ml and infected with Botrytis cinerea were affected by 47.4% and 22.3%, respectively.

The diameter of the lesion caused by Botrytis cinerea on the fruits processed with Bacillus subtilis in the concentration of 1×10^7 and 1×10^8 CFU/ml was 2.2 and 1.3 mm, respectively.

The obtained data make it possible to make a conclusion that strain Bacillus subtilis IPM 215 inhibits development of the diseases caused by phytopathogenic microorganisms Phytophtora cactorum and Botrytis cinerea. The degree of inhibition depends on inoculum concentration.

The influence of Bacillus subtilis concentration on the development of apple diseases at +2 °C.

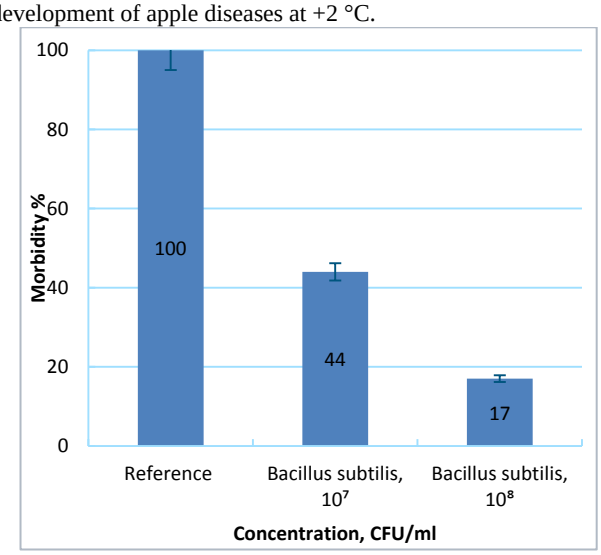

Figure 5 – The influence of Bacillus subtilis concentration on the occurrence rate of diseases caused by Phytophtora cactorum in apples stored at +2°C after 15 days

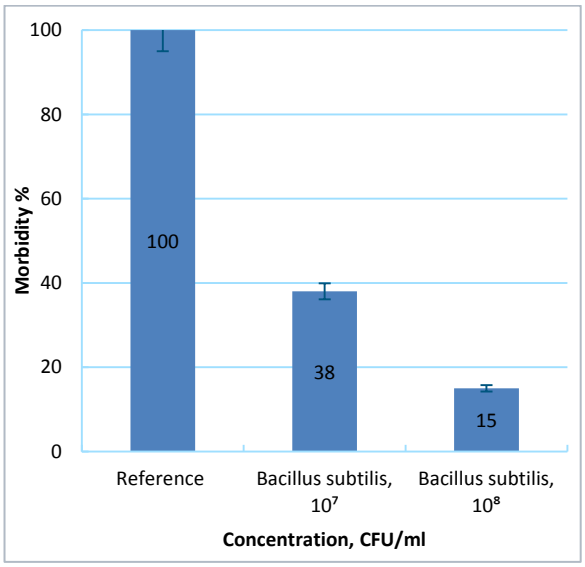

Figure 6 – The influence of Bacillus subtilis concentration on the occurrence rate of diseases caused by Botrytis cinerea in apples stored at +2°C after 15 days

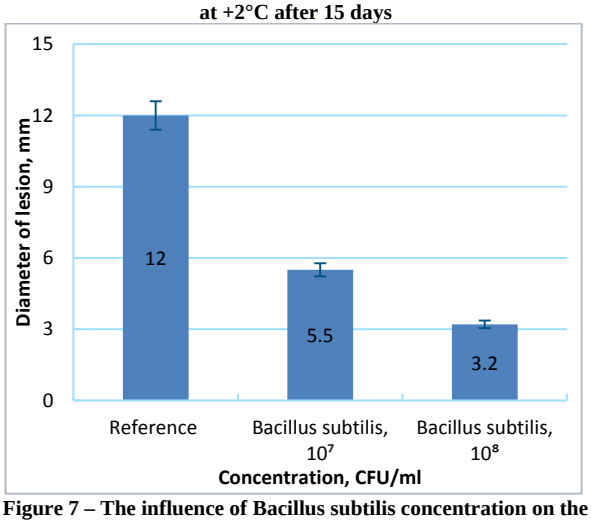

Figure 7 – The influence of Bacillus subtilis concentration on the diameter of lesions caused by Phytophtora cactorum in apples stored at +2°C after 15 days

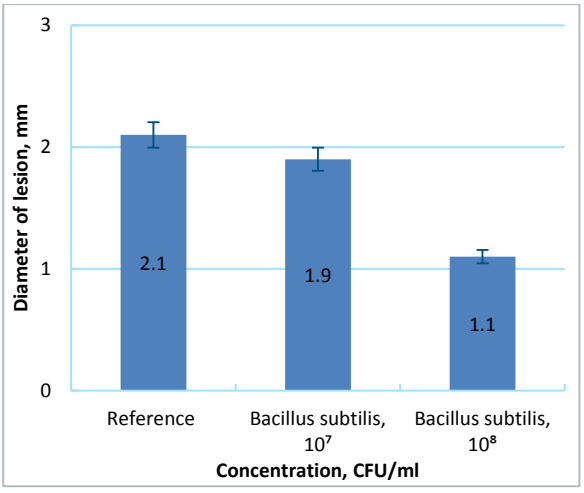

Figure 8 – The influence of Bacillus subtilis concentration on the diameter of lesions caused by Botrytis cinerea in apples stored at +2°C after 15 days

After storing at +25°C for 5 days, the disease occurrence rate in the reference fruits was 100% (with the diameter of lesion in case of infection with Phytophtora cactorum equal to 12.0 mm, in case of infection with Botrytis cinerea – to 2.1 mm).

The fruits processed with Bacillus subtilis with the concentration of inoculum equal to 1×10^7 and 1×10^8 CFU/ml and infected with Phytophtora cactorum were affected by 44.0% and 17.0%, respectively.

The diameter of the lesion caused by Phytophtora cactorum on the fruits processed with Bacillus subtilis was 5.5 and 3.2 mm.

The fruits processed with Bacillus subtilis IPM 215 with the concentration of inoculum equal to 1×10^7 and 1×10^8 CFU/ml and infected with Botrytis cinerea were affected by 38.0% and 15.0%, respectively.

The diameter of the lesion caused by Botrytis cinerea was 1.9 and 1.1 mm.

Based on these data, one can conclude that with increasing concentration of Bacillus subtilis, the diameter of the lesion caused by phytopathogenic microorganisms is reduced both at +25 °C and at +2 °C, which is an indicator of Bacillus subtilis cells' adaptation to the temperature parameters.

The dynamics of Bacillus subtilis population were studied with and without introducing fungal pathogens in slices of apples at + 25 °C within 5 days at +2 °C within 15 days. The results are shown in Figures 9 and 10.

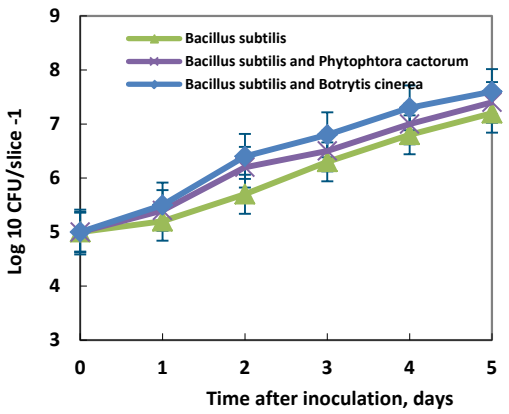

Figure 9 – The dynamics of Bacillus subtilis population were studied with and without introducing fungal pathogens in slices of apples at + 25 °C within 5 days

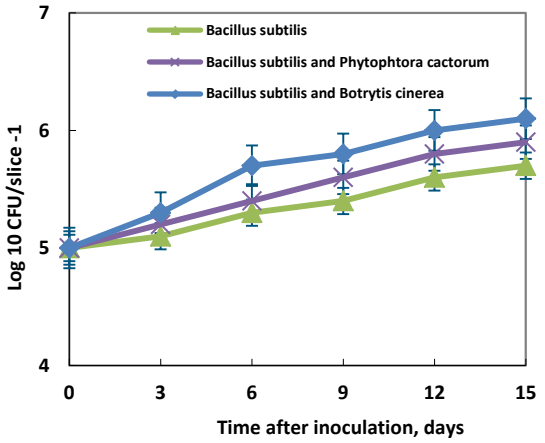

Figure 10 – The dynamics of Bacillus subtilis population were studied with and without introducing fungal pathogens in slices of apples at + 2 °C within 15 days

In the reference samples, the population of Bacillus subtilis bacteria introduced into the incision had been 6.9×10^5 CFU/slice, and increased after 5 days to 1.6×10^7 CFU at +25 °C, and to 7.7×10^5 CFU after 15 days at +2 °C. This corresponded to the growth equal to 23.1 and 1.1 times, compared to the initial numbers at +25 °C and +2 °C, respectively.

In the samples infected by Phytophtora cactorum, the population of bacteria after 5 days increased to 1.1×10^7 CFU at +25 °C, and to 8.2×10^5 CFU after 15 days at +2 °C. This corresponded to the growth equal to 15.9 and 1.26 times, compared to the initial numbers at +25 °C and +2 °C, respectively.

In the samples infected by Botrytis cinerea, the population of bacteria after 15 days increased to 1.2×10^7 CFU at +25 °C, and to 9.7×10^5 CFU after 15 days at +2 °C. This corresponded to the growth equal to 16.3 and 1.4 times, compared to the initial numbers at +25 °C and +2 °C, respectively.

The obtained data allow to draw the conclusion that after contact with the surface of fruits, strain Bacillus subtilis IPM 215 remains viable at +25 °C and +2 °C, and the population increases during the initial period of storage.

In the presence of the Botrytis cinerea and Phytophtora cactorum pathogenic fungi, the growth of Bacillus subtilis strain IPM 215 population is activated to some extent. The presence of Phytophtora cactorum has virtually no effect on the number of Bacillus subtilis.

Conclusion

Based on the study, antagonistic properties of bacterial strains Bacillus subtilis to apple phytopathogens Phytophtora cactorum, Botrytis cinerea, Penicillium glaucoma, Erwinia carotovora have been established in *in vitro* experiments.

It has been established that strain Bacillus subtilis IPM 215 is the most efficient against studied pathogens.

The concentration of Bacillus subtilis affects the disease occurrence rate and the diameter of lesions caused by Phytophtora cactorum and Botrytis cinerea.

It has been established that the population of strain Bacillus subtilis IPM 215 depends on the temperature and the species of the phytopathogen, and retains its viability at +25 °C and +2 °C. During the initial period of storage, the population grows.

In the presence of the Botrytis cinerea and Phytophtora cactorum pathogenic fungi, the growth of Bacillus subtilis strain IPM 215 population is activated to some extent. The presence of Botrytis cinerea affects the number of Bacillus subtilis to a greater extent.

The research substantiates the use of strain Bacillus subtilis IPM 215 for obtaining preparations for biological monitoring of diseases caused by phytopathogenic microorganisms during apples' storage.

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