

***In vitro* Antifungal Activity of Plant Beneficial Microorganisms Against Phytopathogenic Fungi**

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Natural strains of plant beneficial fungi *Beauveria bassiana*, *Arthrobotrys oligospora* and *Duddingtonia flagrans* showed *in vitro* antifungal activity against the Siberian strains of *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizoctonia solani* phytopathogenic fungi. Adding entomopathogenic and nematophagous fungi to *Bacillus amyloliquefaciens* antagonistic bacterium increases its activity against plant pathogens. The results are promising for biocontrol of plant noxious organisms association *in vivo*.

Key words: Antagonistic bacteria, Antifungal activity, Double effect, Entomopathogenic fungi, Nematophagous fungi.

Plants, whose fruits can be eaten by human, are subject to attack by numerous competing organisms, such as phytophagous insects, plant pathogens, plant-parasitic nematodes and others. Throughout the world, the main method of protecting plants against pests and diseases is a chemical pest control. However, the use of chemical pesticides leads to contamination of soil and water, as well as accumulation of toxic residues in fruits of plants that ultimately negatively affects human health¹. Environmentally safe alternative to synthetic pesticides is the development and application of biological insecticides and fungicides, which are based on natural regulators of abundance of harmful organisms². Despite the increase in proportion of microbial biopesticides in plant protection in recent years, they hardly compete with the synthetic chemical pesticides. One reason is the narrow spectrum of activity of biopesticides

due to the specificity of their impact on the pest. In this respect, important are the worldwide enhanced studies to identify polyfunctional properties of natural regulators of the abundance of organisms that damage plants. It is revealed that entomopathogenic bacterium *Bacillus thuringiensis* Berliner has suppressive effect on plant pathogens *Rhizoctonia solani* and *Botrytis cinerea*^{3,4}, as well as antagonistic action of entomopathogenic fungi *Metarhizium* on pathogens of cotton⁵ and olive⁶. *Beauveria bassiana* fungi exhibit double effect of biological control. Being known entomopathogens, they suppressed in laboratory and field conditions the phytopathogenic fungi of *Pythium*, *Fusarium* and *Rhizoctonia*⁷ genera. Simultaneous manifestation of insecticidal and antagonistic properties of plant beneficial microorganisms greatly enhances the regulatory role of noted biological agents in natural ecosystems, as well as increases the effectiveness of biological pesticides, which are based on these microorganisms. The need to simultaneously protect the most important agricultural crops against pests and plant parasitic nematodes requires the development of biopesticides with

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polyfunctional properties. The basis for this is the initial *in vitro* evaluation of potential biological agents enabling control of harmful species abundance. This paper presents the results of *in vitro* manifestation of antifungal properties of entomopathogenic and nematophagous fungi, as well as their mixture with antagonistic bacteria of the *Bacillus* genus with regard to Siberian strains of plant phytopathogenic fungi of the *Fusarium*, *Botrytis* and *Rhizoctonia* genera.

MATERIALS AND METHODS

Microorganisms

Biocontrol strains of entomopathogenic fungus *Beauveria bassiana* IC-1480-25-1, nematophagous fungi *Arthrobotrys oligospora* IC-1482-26-1 and *Duddingtonia flagrans* IC-1481-24-1, bacteria *Bacillus amyloliquefaciens* of RNCIM (Russian National Collection of Industrial Microorganisms) were used as the plant beneficial microorganisms from collection of scientific and production company "Research Center" (Novosibirsk region), as well as their mixtures in concentrations of 10^3 , 10^4 , 10^5 , 10^6 CFU/ml. A mixture of biocontrol strains consisted of half of the volume of *B. amyloliquefaciens* suspension, and the other half of equal parts of *B. bassiana*, *A. oligospora* and *D. flagrans*. Antifungal activity was evaluated on the strains of plant pathogens from the collection of Biological Control and Biotechnology Laboratories of the Novosibirsk Agrarian University, isolated from infected plants in the Novosibirsk region, namely *Fusarium oxysporum*, *Botrytis cinerea*, and *Rhizoctonia solani*.

Procedure to evaluate the antifungal activity

Evaluation of antifungal activity of *B. bassiana*, *A. oligospora*, *D. flagrans*, bacteria of *Bacillus* genus, and microbe mixture *in vitro* was performed by a modified method of agar blocks and expressed in terms of the inhibitory activity. Test strains were grown on potato-dextrose agar (PDA) over 48 hours at 25°C in Petri dishes. At the center of the dishes, inoculated with test strains, a block with *F. oxysporum*, *B. cinerea*, or *R. solani* fungi (10 mm in diameter) was placed. Phytopathogenic fungi were grown preliminary on PDA. Dishes were incubated at 25°C during 7-14 days, at that, registering the diameter of fungal colonies. Each series included 5 replications. Petri

dishes without inoculation by test strains served as a control. Observations were carried out in 3, 5 and 7 days. The inhibitory activity (IA, %) was calculated by the formula:

$$IA = \frac{D_c - D_o}{D_c} \times 100$$

where

D_c – is the diameter of phytopathogenic fungus colonies in the control, cm;

D_o – is the diameter of phytopathogenic fungus colonies in the experiment, cm.

Statistical data processing was performed by standard methods using MS Excel and ANOVA program for Windows. Data were compared by calculating $LSD_{0.05}$.

RESULTS AND DISCUSSION

The effect of *B. bassiana* entomopathogenic fungi on the growth of phytopathogenic fungi *F. oxysporum*, *B. cinerea* and *R. solani* is shown in Tables 1-3. Antifungal activity of *B. bassiana* against *F. oxysporum* at all concentrations increased with time. The diameter of the colonies under the influence of *B. bassiana* decreased significantly (maximally, more than three times). The smallest inhibitory activity on the 7th day was observed when using a concentration of 10^5 CFU/ml, while the greatest inhibitory activity was revealed at a concentration of 10^6 CFU/ml (67.4%) (Table 1). Growth suppression of the *B. cinerea* fungus slightly depended on both the time of observation, and the concentration of the microbial suspension. The diameter of the *B. cinerea* colonies under the action of entomopathogenic fungi was reduced almost twice (Table 2).

As for the third type of plant pathogens, the lowest concentration of *B. bassiana*, equal to 10^4 CFU/ml, was ineffective in terms of its growth inhibition. When using suspension of entomopathogenic fungus in two higher concentrations, the maximum inhibitory activity (62.2-64.4%) was observed on the 5th day, whereas a slight decline in effect occurred on the 7th day (Table 3). In general, antifungal effect of entomopathogenic fungus *B. bassiana* was quite high for all the tested plant pathogens. However, each phytopathogenic fungus revealed some

Table 1. The effect of *Beauveria bassiana* entomopathogenic fungus on the growth of *Fusarium oxysporum* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		2.8	7.1	8.9			
<i>B. bassiana</i>	10 ⁴	1.9	3.0	3.9	32.1	57.7	55.2
<i>B. bassiana</i>	10 ⁵	2.5	4.3	4.5	10.7	39.4	48.3
<i>B. bassiana</i>	10 ⁶	2.5	3.0	2.9	10.7	57.7	67.4
LSD ₀₅ for concentration		0.3					
LSD ₀₅ for days		0.2					

Table 2. The effect of *Beauveria bassiana* entomopathogenic fungus on the growth of *Botrytis cinerea* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		6.5	8.0	8.9			
<i>B. bassiana</i>	10 ⁴	2.7	3.6	4.4	58.5	55.0	50.6
<i>B. bassiana</i>	10 ⁵	2.8	3.8	4.0	56.9	52.5	55.1
<i>B. bassiana</i>	10 ⁶	3.1	3.6	4.3	52.3	55.0	51.7
LSD ₀₅ for concentration		0.4					
LSD ₀₅ for days		0.3					

Table 3. The effect of *Beauveria bassiana* entomopathogenic fungus on the growth of *Rhizoctonia solani* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		3.9	9.0	9.0			
<i>B. bassiana</i>	10 ⁴	3.6	7.6	9.0	7.7	15.6	0.0
<i>B. bassiana</i>	10 ⁵	1.7	3.2	3.6	56.4	64.4	60.0
<i>B. bassiana</i>	10 ⁶	2.7	3.4	4.4	30.8	62.2	51.1
LSD ₀₅ for concentration		0.5					
LSD ₀₅ for days		0.3					

Table 4. The effect of *Arthrobotrys oligospora* and *Duddingtonia flagrans* nematophagous fungi on the growth of *Fusarium oxysporum* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		2.8	7.1	8.7			
<i>A. oligospora</i>	10 ⁴	1.7	2.2	3.7	39.3	69.0	57.5
<i>A. oligospora</i>	10 ⁵	2.5	3.4	3.0	10.7	52.1	65.5
<i>A. oligospora</i>	10 ⁶	2.4	3.2	3.1	14.3	54.9	64.4
<i>D. flagrans</i>	10 ⁴	1.8	3.0	4.0	35.7	57.7	54.0
<i>D. flagrans</i>	10 ⁵	1.8	2.3	3.1	35.7	67.6	64.4
<i>D. flagrans</i>	10 ⁶	1.6	2.1	3.2	42.9	70.4	63.2
LSD ₀₅ for concentration		0.3					
LSD ₀₅ for days		0.2					

specific features in terms of effect of beneficial organisms (Tables 1-3).

Data on the antifungal activity of nematophagous fungi *A. oligospora* and *D. flagrans* against phytopathogenic fungi are shown in Tables 4-6. For the first fungus, the increase of inhibitory activity with increasing the interaction time with the phytopathogenic fungus *F. oxysporum* was observed at all concentrations. At that, no pronounced antifungal activity was detected depending on the concentration of *A. oligospora* suspension. There was a maximum decrease in the diameter of the phytopathogenic fungal colonies more than three times (Table 4). Effect of concentration for *D. flagrans* was somewhat higher, though the maximum value of the inhibitory activity was almost equal to the corresponding value for *A. oligospora* (Table 4).

More differences were found for the grey mold causal agent *B.cinerea* in terms of the impact

of two types of nematophagous fungi (Table 5). At the least concentration used, the higher inhibitory activity was observed for *A. oligospora*. In addition, for the largest concentration of 10^6 CFU/ml, the antifungal effect decreased with increasing interaction time with nematophagous fungus and phytopathogenic fungus (Table 5). The opposite pattern was observed in the case of *D. flagrans*: at the lowest concentration the antifungal effect was the weakest, though for other concentrations the antifungal effect was also decreased with increasing interaction time with nematophagous fungus and phytopathogenic fungus (Table 5).

As to *R. solani*, nematophagous fungi showed the highest level of antifungal activity and the lowest dependence of biocontrol strains on concentration and the interaction time with nematophagous fungus and phytopathogenic fungus (Table 6).

It was of interest to compare the

Table 5. The effect of *Arthrobotrys oligospora* and *Duddingtonia flagrans* nematophagous fungi on the growth of *B. cinerea* phytopathogen

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		6.5	8.0	8.9			
<i>A. oligospora</i>	10^4	2.5	2.8	2.9	61.5	65.0	67.4
<i>A. oligospora</i>	10^5	2.7	3.8	4.9	58.5	52.5	44.9
<i>A. oligospora</i>	10^6	2.6	4.2	6.1	60.0	47.5	31.5
<i>D. flagrans</i>	10^4	5.5	6.9	7.8	15.4	13.8	12.4
<i>D. flagrans</i>	10^5	2.5	4.9	7.3	61.5	38.8	18.0
<i>D. flagrans</i>	10^6	2.6	4.7	7.3	60.0	41.3	18.0
LSD ₀₅ for concentration		0.4					
LSD ₀₅ for days		0.3					

Table 6. The effect of *Arthrobotrys oligospora* and *Duddingtonia flagrans* nematophagous fungi on the growth of *Rhizoctonia solani* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		3.9	9.0	9.0			
<i>A. oligospora</i>	10^4	1.3	1.2	1.8	66.7	86.7	80.0
<i>A. oligospora</i>	10^5	1.4	1.0	1.0	64.1	88.9	88.9
<i>A. oligospora</i>	10^6	1.3	1.0	1.0	66.7	88.9	88.9
<i>D. flagrans</i>	10^4	1.4	1.3	1.7	64.1	85.6	81.1
<i>D. flagrans</i>	10^5	1.3	1.3	2.0	66.7	85.6	77.8
<i>D. flagrans</i>	10^6	1.3	1.3	1.6	66.7	85.6	82.2
LSD ₀₅ for concentration		0.2					
LSD ₀₅ for days		0.1					

Table 7. The effect of *Bacillus amyloliquefaciens* on the growth of *Fusarium oxysporum*, *Botrytis cinerea* and *Rhizoctonia solani*

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
<i>Fusarium oxysporum</i>							
Control		2.2	5.1	9			
<i>B. amyloliquefaciens</i>	10 ⁴	1.4	2.5	3.2	27	50.9	64.4
<i>B. amyloliquefaciens</i>	10 ⁵	1.4	2.9	3.9	27	43.1	56.7
<i>B. amyloliquefaciens</i>	10 ⁶	1.3	1.7	1.8	40.1	66.7	80.0
LSD ₀₅ for concentration		0.2					
LSD ₀₅ for days		0.1					
<i>Botrytis cinerea</i>							
Control		2.8	9.0	9.0			
<i>B. amyloliquefaciens</i>	10 ⁴	1.8	4.1	2.9	35.7	54.0	67.3
<i>B. amyloliquefaciens</i>	10 ⁵	1.6	3.4	2.5	42.9	62.7	72.7
<i>B. amyloliquefaciens</i>	10 ⁶	1.7	3.6	2.5	39.1	59.5	72.7
LSD ₀₅ for concentration		0.1					
LSD ₀₅ for days		0.1					
<i>Rhizoctonia solani</i>							
Control		2.6	4.6	9.0			
<i>B. amyloliquefaciens</i>	10 ⁴	1.7	3.0	5.3	33.8	34.8	41.3
<i>B. amyloliquefaciens</i>	10 ⁵	1.2	2.0	3.1	52.3	56.5	65.6
<i>B. amyloliquefaciens</i>	10 ⁶	1.2	2.0	3.1	52.3	56.5	65.6
LSD ₀₅ for concentration		0.2					
LSD ₀₅ for days		0.1					

Table 8. The effect of strains mixture of different nature on the growth of *Fusarium oxysporum* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		2.8	7.1	8.9			
Mixture	10 ⁴	1.9	3.0	3.4	32.1	57.7	60.9
Mixture	10 ⁵	1.7	2.8	2.8	39.3	60.6	67.8
Mixture	10 ⁶	2.0	2.8	3.3	28.6	60.6	67.8
LSD ₀₅ for concentration		0.3					
LSD ₀₅ for days		0.2					

Table 9. The effect of strains mixture of different nature on the growth of *Botrytis cinerea* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		6.5	8.0	8.9			
Mixture	10 ⁴	3.0	3.9	5.2	53.8	51.3	41.6
Mixture	10 ⁵	2.6	3.4	3.6	60.0	57.5	59.6
Mixture	10 ⁶	2.3	3.0	2.8	64.6	62.5	68.5
LSD ₀₅ for concentration		0.4					
LSD ₀₅ for days		0.3					

antifungal activity of entomopathogenic and nematophagous fungi with the same parameter for the well-known antagonist of plant pathogens. It has been shown previously that *B. amyloliquefaciens* bacterium exhibits rather high antifungal activity against the tested phytopathogenic fungi⁸. Table 7 shows the results of the effect of *B. amyloliquefaciens* on the *in vitro* growth of phytopathogenic fungi under the same conditions as for the beneficial fungi. In some cases, antifungal activity was comparable to biocontrol strains of fungi and *B. amyloliquefaciens* (Table. 7). However, in the latter case, the growth of inhibitory activity with the increase of interaction time of antagonist with phytopathogenic fungi was observed in all cases.

The final research step was studying the effect of antifungal mixed suspension, consisting of *B. amyloliquefaciens*, *B. bassiana* A. *oligospora* and *D. flagrans*, to identify possible synergistic effect of all biocontrol strains under the joint action on phytopathogenic fungi. The results obtained have shown that the nature of the variations in inhibitory activity depending on number of days has predominantly the same tendency, as the impact of antagonistic bacteria. However, given the smaller proportion of biological agents in the mixture, there is an additive or a synergistic effect in interaction of all biocontrol strains (Tables 8-10).

Antifungal effect of all studied plant beneficial strains and their mixtures are also illustrated in Figures 1-3.

B. bassiana is a well-known biological agent to control the abundance of phytophagous insects as a plant beneficial soil-borne^{9,10} or endophytic⁷ fungus. Mostly, it is useful as abundance regulator of pests, which damage crops

grown in greenhouses, where one can create the stable humidity conditions, providing the maximum display of its pathogenic activity¹⁰⁻¹³. At the same time, high humidity promotes the development of fungal diseases of plants, and therefore the simultaneous manifestation of both insecticidal and antifungal properties by *B. bassiana* fungus is of great importance. In this regard, the *in vitro* antifungal activity, revealed in this study, is potentially important for the application of this biocontrol agent in greenhouses of Asian part of Russia, including Siberia, where the climatic conditions are severe and require the use of greenhouses for growing vegetables. The conventional phytopathogenic fungi, damaging plants in greenhouses, include fungi of the *Fusarium*, *Botrytis* and *Rhizoctonia* genera, which are sensitive to *in vitro* detected antifungal activity. Some authors have demonstrated the possibility of suppression of plant diseases by strains of *B. bassiana* fungus of various origins^{7,14}. It is believed that secondary metabolites of the entomopathogenic fungus are responsible for antifungal effects^{15,16}.

In the greenhouses of the Siberian region, phytoparasitic nematodes of *Meloidogina* genus are often reproduced. Their natural enemies are predatory nematophagous fungi *A. oligospora*^{7,18} and *D. flagrans*^{19,20}. Rather high antifungal *in vitro* activity of these fungi, revealed in the present study, suggests the possibility of their double effect in greenhouses. Likely, the impact of nematophagous fungi on plant pathogens may be caused due to their secretion of the protease or chitinase^{21,22}.

The most known antagonistic bacteria, used in the plant pathogens biocontrol, belong to

Table 10. The effect of strains mixture of different nature on the growth of *Rhizoctonia solani* phytopathogenic fungus

Treatment	Concentration, CFU/ml	Diameter of the colonies, cm.			Inhibitory activity, %		
		3 days	5 days	7 days	3 days	5 days	7 days
Control		3.9	9.0	9.0			
Mixture	10 ⁴	1.7	1.3	1.7	56.4	85.6	81.1
Mixture	10 ⁵	1.3	1.6	1.7	66.7	82.2	81.1
Mixture	10 ⁶	1.3	1.0	1.0	66.7	88.9	88.9
LSD ₀₅ for concentration		0.5					
LSD ₀₅ for days		0.3					

the *Bacillus* genus, in particular, *B. amyloliquefaciens*^{23,24}. Our data have shown that the admixture of entomopathogenic and nematophagous fungi enhances antifungal activity of antagonistic bacteria (the ability to use lower doses of bacteria to achieve higher effect). These data are consistent with the results on the use of a mixture of *B. bassiana* with antagonistic bacteria of the *Bacillus* genus to effectively reduce *in vivo* both the pest insects and plant pathogens on

tomato²⁵.

Thus, in the present study *in vitro* antifungal activity of *B. bassiana* entomopathogenic fungus and two nematophagous fungi *A. oligospora* and *D. flagrans* towards Siberian strains of phytopathogenic fungi *F. oxysporum*, *B. cinerea* and *R. solani* was revealed. Addition of plant beneficial fungi to *B. amyloliquefaciens* bacteria can lead to enhancing antagonistic effect against

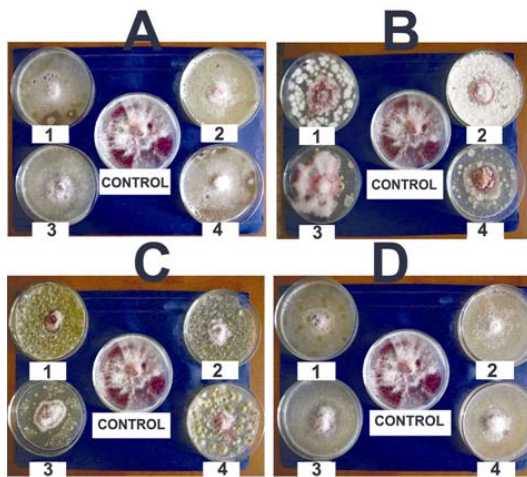


Fig. 1. Antifungal activity of beneficial microorganisms against *F. oxysporum*. A - *B. bassiana*; B - *A. oligospora*; C - *D. flagrans*; D - Mixture of microorganisms; Suspension concentration, CFU/ml: 1 - 10^3 , 2 - 10^4 , 3 - 10^5 , 4 - 10^6

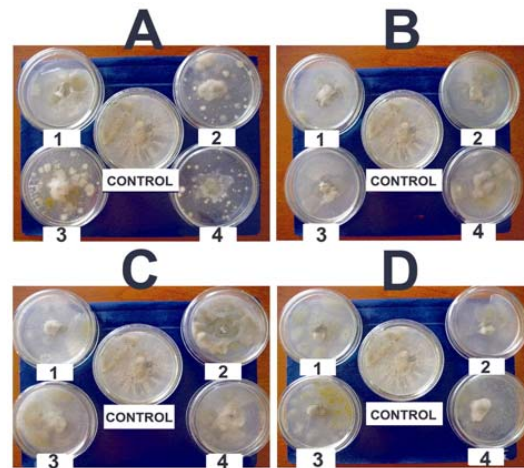


Fig. 2. Antifungal activity of beneficial microorganisms against *B. cinerea*. A - *B. bassiana*; B - *A. oligospora*; C - *D. flagrans*; D - Mixture of microorganisms; Suspension concentration, CFU/ml: 1 - 10^3 , 2 - 10^4 , 3 - 10^5 , 4 - 10^6

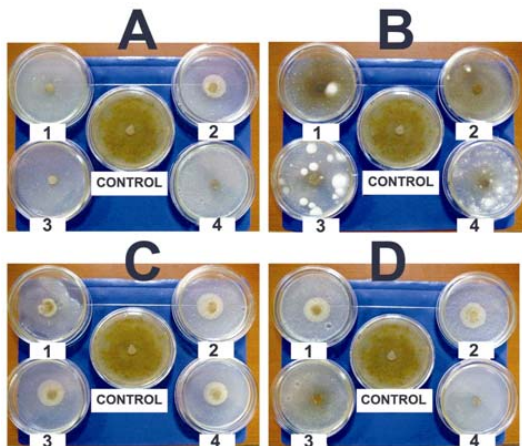


Fig. 3. Antifungal activity of beneficial microorganisms against *R. solani*. A - *B. bassiana*; B - *A. oligospora*; C - *D. flagrans*; D - Mixture of microorganisms; Suspension concentration, CFU/ml: 1 - 10^3 , 2 - 10^4 , 3 - 10^5 , 4 - 10^6 .

phytopathogenic fungi. The results obtained are promising in terms of using studied plant beneficial microorganisms in the biocontrol of plant pests.

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