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Research Article

Effect of Two Biological Formulations Based on *Bacillus subtilis* and *Pseudomonas fluorescens* on Control of *Didymella applanata*, the Causal Agent of Red Raspberry Cane Spur Blight

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In vitro and *in vivo* studies were conducted to estimate the efficacy of the two microbial formulations based on *Bacillus subtilis* Cohn. and *Pseudomonas fluorescens* Mig. on the fungus *Didymella applanata* (Niessl.) Sacc., the causal agent of red raspberry (*Rubus idaeus* L.) spur blight. *In vitro*, both bacteria reduced the growth of *D. applanata*. In inoculation experiments with raspberry canes in two cultivars with different susceptibility to *D. applanata*, these antagonistic bacteria suppressed fungal development by reducing the lesions area and the number of *D. applanata* fruiting bodies. Field trials of two biological formulations under natural conditions showed a significant suppression of the disease. *B. subtilis* and *P. fluorescens* included in the formulations revealed antagonistic activity towards *D. applanata* that depended on the red raspberry cultivar and weather conditions. In all cases, *B. subtilis* showed better results than *P. fluorescens* in biocontrol of the raspberry spur blight. This study demonstrated for the first time the ability of the biocontrol agents *B. subtilis* and *P. fluorescens* to suppress red raspberry cane spur blight, a serious worldwide disease.

1. Introduction

Red raspberry (*Rubus idaeus* L.) is one of the most common soft fruit growing in many countries. The general regions of different climatic conditions in which raspberries are widely grown include European countries, Russia, the USA, and Australia [1]. Some fungal diseases are harmful for red raspberry, and this fact requires plant protection. In Siberian region of Russia, raspberry suffers from a cane spur blight caused by the fungus *Didymella applanata* Niessl (Sacc.), as also revealed under other climatic conditions of different geographic locations. Chemical method of plant protection against this disease is well known [2, 3], while biological control of the fungus has been studied very little. However, biocontrol of plant diseases as an alternative to application of chemical fungicides is becoming more common all over the world [4, 5].

The main advantage of using native biological agents against plant disease causal agents including antagonistic bacteria compared with synthetic pesticides consists of environment pollution prevention and a reduction or full absence of chemical residues in fresh fruits [6] that are actual for fruit and berry production. Among the most common biological agents for plant disease control, bacteria of the *Bacillus* and *Pseudomonas* genera are well known [7–9]. As a rule, commercial biological formulations are based on *Bacillus subtilis* Cohn. and *Pseudomonas fluorescens* Mig. [10–12]. Antagonistic activity of *B. subtilis* [13, 14] and *P. fluorescens* [15, 16] against *Botrytis cinerea* was studied on strawberry, and *B. subtilis* was applied against grapevine diseases [17]. Both *B. subtilis* and *P. fluorescens* were used for apples gray mold control [18]. There are some more examples of successful biocontrol of several diseases by these beneficial bacteria on grapevine plants [19]. Beneficial rhizobacteria including *Bacillus* spp. and *Pseudomonas* spp. produce metabolites such as enzymes [20–22] and lipopeptides [23–26] that are considered to be responsible for antagonistic action against plant pathogens. For example, purified bacterial chitinase was

shown to inhibit development of some plant pathogenic fungi *in vitro* [27], and another bacterial chitinase reduced red raspberry spur blight under cane treatment [28].

This paper concerns the possibilities of the red raspberry spur blight biological control by commercial formulations based on *Bacillus subtilis* and *Pseudomonas fluorescens* both *in vitro* and through field experiments, in which raspberry canes were wound-inoculated and given prophylactic treatments with antagonists, and under natural conditions. The difference between two red raspberry cultivars in relation to the disease biocontrol was studied in two consecutive years.

2. Materials and Methods

2.1. Raspberry Cultivars, Phytopathogenic Fungus, and Biocontrol Agents. Red raspberry cultivar Kirzhach and cultivar Kolokolchik were used for experiments. The first cultivar, selected by Dr. Kichina at the All-Russian Institute for Breeding and Technology in Horticulture, Moscow, is highly susceptible to spur blight. The second cultivar, selected by Dr. Sokolova at the Lisavenko's Institute of Siberian Horticulture, Barnaul, is more resistant to this disease.

Didymella applanata, isolate Da-99, was obtained from diseased raspberry canes in Novosibirsk. This fungus was cultivated on the Czapek medium as described earlier [28].

Two biological formulations based on *B. subtilis* spores (2×10^9 CFU/mL) (Sibbiofarm, Russia) and *P. fluorescens* living cells (2.5×10^{10} CFU/mL) (Alsiko-Agroprom, Russia) were used. The formulations were approved in Russia for application against cereals and vegetables diseases only, and no data were obtained for raspberry disease control so far.

2.2. Antifungal Activity In Vitro. Evaluation of antifungal activity *in vitro* was performed by a modified method of agar blocks and expressed in terms of the inhibitory activity. *D. applanata* was grown on Czapek medium at 25°C in Petri dishes. At the center of the plates, inoculated with *D. applanata*, a block with the bacterium (10 mm in diameter) was placed. Plates were incubated at 25° (for 7–14 days), registering the diameter of fungal colonies. Each series included 5 replications. Petri dishes without inoculation by bacteria served as a control. Observations were carried out in 3, 5, 7, and 10 days.

The inhibitory activity (IA, %) was calculated by the following formula:

$$\mathrm{IA} = \frac{D_C - D_o}{D_C} \times 100, \quad (1)$$

where D_C is the diameter of pathogenic fungus colonies in the control, cm; D_o is the diameter of pathogenic fungus colonies in the experiment, cm.

2.3. Inoculation of Raspberry Canes. These experiments were carried out on the raspberry plantations at a producer farm under Novosibirsk (55°04′N latitude, 82°93′E longitude, and 164 m altitude). Agar plugs 10 mm in diameter were cut out of plates of 15-day-old *D. applanata*. The inoculum consisted of mycelium and native pycnidia. For inoculations, primocanes of 70 cm height were used. A zone 30 cm above the soil level and 15 cm wide was pruned to remove leaves (petioles of 3 cm were left after cutting). This zone was damaged with the aid of glass crude powder. The size of each wound was 2 cm^2. Immediately prior to application of the fungal inoculum, the canes were sprayed once with bacterial suspension applied at a volume application rate of 10 mL/cane using a hand-held (Quazar Corp. Warsow, Poland). The agar plugs were then positioned so that the fungus was in direct contact with the damaged epidermis and wrapped with moist cotton wool. Each inoculation was then individually covered with plastic film (12 × 12 cm) to provide adequate humidity and temperature conditions for fungal growth. The plants were incubated for 7 days. After this incubation period, the cotton with film was removed. Five replicates were used for each bacterial application treatment. The control canes were treated with fungal inoculum, but not with bacteria. A randomized design was applied in the experiment. To estimate the effect of bacteria on spur blight the lesion squares were measured after 7 and 30 days and at the end of the vegetation period. The number of fungal fruiting bodies was measured at the end of vegetation only. The area of lesions was quantified using cellophane film overlays of cane damage. To quantify the number of fruiting bodies of *D. applanata* per 1 cm^2 of patch, the light microscope was used with a calculated eyepiece reticle.

Table 1: Monthly rainfall and air temperatures for 2013 and 2014 growing seasons[a].

Month	Rainfall, mm		Temperature, °C	
	2013	2014	2013	2014
June	33	31	14	17
July	69	77	19	20
August	143	32	17	18
Total	255	140	58	55

[a]Data obtained from the hydrometeorological station.

2.4. Spur Blight Control by B. subtilis and P. fluorescens in a Field. The experiments under natural conditions were carried out in 2013-2014, when the independent spur blight started to develop on canes. Cumulative monthly rainfalls and medium air temperatures are presented in Table 1.

Experimental plots were 10 m^2. A randomized complete block design was used to assign treatments to four replicates. Plants were located in rows 40 cm wide at a spacing of 2.5 m between rows. Twenty-five plants were in each 1 m and the size of the buffer zone between plots was 2 m. Scale for assessment of the independent spur blight was described earlier [29]. Topaz (Syngenta) as a chemical standard was used. These were applied at a volume application rate of 0.1 L/m^2 using a hand-held Orion-6 sprayer. The concentration of both bacterial suspensions was 10^7 CFU/mL. The control plots were left untreated.

2.5. Data Analysis. Data of laboratory experiments were analyzed by paired t-test. Data of inoculation experiments and field trials were subjected to two-way ANOVA. Treatment

Table 2: The effect of the biocontrol agents on *D. applanata* growth *in vitro*.

Treatments	Diameter of colony (cm), days				Inhibitory activity, %			
	3	5	7	10	3	5	7	10
Control	4.1	6.1	6.9	8.6				
P. fluorescens	2.4[s]	4.3[s]	5.0[s]	5.4[s]	41.5	29.5	27.5	37.2
B. subtilis	2.4[s]	2.7[s]	3.1[s]	3.3[s]	41.5	55.7	55.1	61.6
LSD_{05}		0,4						

[s]Significant at $p = 0.05$; $F = 48.12$; df = 11.48.

Table 3: Influence of the biocontrol agents on cane lesion due to spur blight development in inoculation experiment with *D. applanata* in cultivar Kirzhach.

Treatments	Area of lesion, cm^2, days after treatment			The number of fruiting bodies/cm^2
	7	30	90 (end of vegetation)	
P. fluorescens	2.5	2.9	12.2[s]	5.6
B. subtilis	1.0[s]	1.2[s]	7.0[s]	2.5[s]
Control[a]	3.9	4.1	19.9	8.1
LSD_{05}		2.6[b]		4.7[c]

[a]Control includes inoculation by the fungus lacking sprayed bacteria.
[s]Significant at $p = 0.05$; [b]$F = 4.51$; df = 23,22; $p = 0.05$; [c]$F = 2.58$; df = 3,0; $p = 0.05$.

Table 4: Influence of the biocontrol agents on cane lesion due to spur blight development in cultivar Kolokolchik.

Treatments	Area of lesion, cm^2, days after treatment			The number of fruiting bodies/cm^2
	7	30	90 (end of vegetation)	
P. fluorescens	0.8	0.7	8.6[s]	6.4
B. subtilis	0.8	0.9	4.0[s]	3.6
[a]Control	1.6	2.1	11.2	7.8
LSD_{05}		2.6[b]		4.7[c]

[a]Control includes inoculation by the fungus lacking sprayed bacteria.
[s]Significant at $p = 0.05$; [b]$F = 4.51$; df = 23.22; $p = 0.05$; [c]$F = 2.58$; df = 3.0; $p = 0.05$.

means were compared with the least significant difference (LSD). The significant level used was 0.05.

3. Results

3.1. Effect of B. subtilis and P. fluorescens on D. applanata Growth. The results of laboratory experiments are summarized in Table 2. In three days, both biocontrol agents significantly reduced the growth of *D. applanata* (1.7 times) compared with control ($p < 0.05$). However, further observations showed that *B. subtilis* was more effective than *P. fluorescens* (1.3–1.6 times) and the differences between two bacteria and between those bacteria and control were significant ($p < 0.05$).

3.2. Inoculation of Canes with D. applanata. Data on the influence of *B. subtilis* and *P. fluorescens* on spur blight lesion development under the treatment of two raspberry cultivars are presented in Tables 3 and 4. Areas of lesions were measured at 7 and 30 days after inoculation and at the end of vegetation.

In 7 days after inoculation, the size of lesion caused by spur blight in cultivar Kirzhach susceptible to *D. applanata* was 3.9 times lower under the influence of *B. subtilis* compared with the control ($p < 0.05$). The difference between control and *P. fluorescens* was not significant ($p > 0.05$) (Table 3). This trend continued in the following days. At the end of vegetation, there were significant differences between the control and both *B. subtilis* and *P. fluorescens*; however, they were revealed more strongly in the case of the first bacteria (area of lesions was reduced by 2.8 times). The amount of fruiting bodies was also decreased after the treatment by *B. subtilis* by 3.2 times ($p < 0.05$) and to a lesser extent by the treatment by *P. fluorescens* with no significant differences ($p > 0.05$). In the case of more resistant cultivar Kolokolchik significant differences between the control and both biocontrol agents were observed at the end of vegetation only (Table 4). The size of lesion was reduced under the influence of *B. subtilis* by 2.8 times and, in another case, by 1.3 times ($p < 0.05$). Both bacteria reduced the number of fruiting bodies in experiments on inoculation of canes by *D. applanata* but the difference between the control and the treatments was not significant ($p > 0.05$).

3.3. Spur Blight Control by Bacteria in a Field. Field experiments on estimation of spur blight severity in two raspberry

Table 5: Effect of the biocontrol agents on raspberry spur blight severity[a], 2013.

Treatments	Cultivars					
	Kirzhach			Kolokolchik		
	Spur blight severity (means of four replications), %					
	Days after treatment					
	30	40	50	30	40	50
Control	22.5	26.3	28.1	10.0	12.5	13.1
P. fluorescens	11.9[s]	12.5[s]	15.0[s]	5.6[s]	6.7[s]	7.5[s]
B. subtilis	10.6[s]	11.3[s]	13.1[s]	5.0[s]	6.1[s]	6.9[s]
Topaz	7.5[s]	8.8[s]	10.0[s]	3.8[s]	5.6[s]	6.3[s]
LSD_{05} for agents			0.7			
LSD_{05} for cultivars			0.5			

[a]Experiments on spraying the raspberry canes under natural conditions.
[s]Significant at $p = 0.05$; $F = 39.59$; df = 31.96.

Table 6: Effect of the biocontrol agents on raspberry spur blight severity[a], 2014.

Treatments	Cultivars					
	Kirzhach			Kolokolchik		
	Spur blight severity (means of four replications), %					
	Days after treatment					
	30	40	50	30	40	50
Control	13.8	15.9	22.5	7.5	8.3	9.4
P. fluorescens	7.5[s]	8.9[s]	12.5[s]	4.4[s]	4.8[s]	5.6[s]
B. subtilis	6.3[s]	7.4[s]	10.6[s]	3.8[s]	4.2[s]	5.0[s]
Topaz	4.4[s]	5.4[s]	8.1[s]	2.5[s]	3.5[s]	4.4[s]
LSD_{05} for agents			0.7			
LSD_{05} for cultivars			0.5			

[a]Experiments on spraying the raspberry canes under natural conditions.
[s]Significant at $p = 0.05$; $F = 39.59$; df = 31.96.

cultivars, depending on spraying by two bacterial agents, were carried out in 2013-2014 (Tables 5 and 6). In 2013, disease severity in control was significantly more (not less than 2-fold) in susceptible cultivar Kirzhach ($p < 0.05$) compared with the second cultivar. The bacterial agents reduced disease severity by about 2 times compared with untreated canes; at that, efficacy was increased from *B. subtilis* to *P. fluorescens* ($p < 0.05$). Chemical fungicide topaz was some more effective. As to cultivar Kolokolchik, more resistant to the disease, the relationship between *B. subtilis* and *P. fluorescens* action was retained; however, there were no significant differences between their values ($p > 0.05$). In addition, in 40 and 50 days after treatment no significant differences between the action of the biological agent *B. subtilis* and the chemical fungicide topaz were observed (Table 5).

In 2014, principle differences between disease severity in two cultivars and the differences between the biocontrol agents influence were almost the same, in spite of less degree of disease severity in control (Table 6).

4. Discussion

This study demonstrated that bacterial antagonists *B. subtilis* and *P. fluorescens* are able to suppress the raspberry cane spur blight caused by the fungus *D. applanata*. We have made the first attempt to estimate the effect of *B. subtilis* and *P. fluorescens* on the damage of two red raspberry cultivars differed by their susceptibility to *D. applanata* under artificial inoculation and natural conditions. First of all, the experiments *in vitro* revealed the higher inhibitory activity of *B. subtilis* spores compared with *P. fluorescens* living cells. These results were confirmed by the experiments on red raspberry cane inoculation by *D. applanata* where cultivar Kirzhach, susceptible to the disease, and relatively resistant cultivar Kolokolchik were used. It is well known, that susceptibility to raspberry cane spur blight is depended on a plant cultivar [30]; therefore, it was interesting to evaluate the influence of bacterial antagonists on *D. applanata* using the different raspberry varieties. Under the inoculation of susceptible cultivar Kirzhach with *D. applanata* the treatment by *B. subtilis* led to more considerable reduction of cane lesion area (about 2-fold) than the treatment by *P. fluorescens*. As to cultivar Kolokolchik, significant differences between the control and the treatments, on the one hand, and between two bacteria, on the other hand, were revealed at the end of vegetation only. However, the higher influence of *B. subtilis* compared with *P. fluorescens* was observed in both cultivars and this fact

was also confirmed by the number of fruiting bodies of *D. applanata* per cm^2.

Field trials of two biocontrol agents were carried out in 2013 and 2014 in order to confirm the results obtained *in vitro* and in the inoculation experiments and to show a possible role of weather conditions. To compare with bacterial biological agents, chemical fungicide topaz was used. The values of the disease severity in the control in both cultivars that were observed in 30 days after treatment in the first year were achieved later in the second year (in 50 days after treatment) (Tables 5 and 6). It could be explained by more favorable weather condition for *D. applanata* development in the first year. In 2013, 2-fold more precipitations and the higher average air temperature were observed compared with 2014. However, in both years, the damage of cultivar Kolokolchik by the disease was about 2-fold lower as compared with cultivar Kirzhach damage. In a field, formulations based on both antagonistic bacteria suppressed the raspberry cane spur blight, though in a less degree than the chemical fungicide. It should be noted that *B. subtilis* showed the higher antagonistic activity than *P. fluorescens* confirming the data obtained *in vitro* and under the inoculation condition. Other authors demonstrated similar results both *in vitro* [31] and in a field where these antagonists were used against blueberry disease [32].

Comparison of the results of the action of two bacterial antagonists in two cultivars showed that, in 2013, activity of *B. subtilis* was significantly higher than the activity of *P. fluorescens* in cultivar Kirzhach ($p < 0.05$). This difference was not significant in cultivar Kolokolchik; however, *B. subtilis* showed the same activity as the chemical fungicide (topaz) in this cultivar. This fact could be explained with the higher degree of surviving and multiplication of *Bacillus* spores compared with *Pseudomonas* cells [33]. In 2014, when weather conditions were less favorable for *D. applanata*, the difference in the influence of two biocontrol agents on plant pathogen in cultivar Kirzhach, more susceptible to the disease, was more evident. For more resistant raspberry cultivar, the similar situation was observed, as compared to the previous year.

5. Conclusion

The results obtained here provide some evidence that it is possible to reduce raspberry cane spur blight by application of the bacterial biocontrol agents. *B. subtilis* and *P. fluorescens* revealed antagonistic activity towards *D. applanata* that depended on the red raspberry cultivar and weather conditions. In all cases, *B. subtilis* showed better results than *P. fluorescens* in biocontrol of raspberry cane spur blight. This study demonstrated that biological formulations based on antagonistic bacteria *B. subtilis* and *P. fluorescens* could be ecologically safe alternative to chemical fungicides for the raspberry disease control. However, it may be stated that the antagonistic effect of the bacteria on raspberry cane spur blight depends on the concrete geographical location and weather conditions that should be taken into account.

Competing Interests

The authors declare that there is no conflict of interests regarding the publications of this paper.

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