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Endophytic *Bacillus* bacteria with RNase activity in the resistance of potato plants to viruses

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Viral diseases annually cause significant crop losses and significantly reduce the quality of products, including potatoes, some of the most important crops. Currently, viruses cannot be controlled with chemical pesticides, since known antiviral compounds are teratogenic and hazardous to people's health. Biocontrol agents based on endophytic microorganisms may be an alternative to them. Many strains of *Bacillus* produce ribonucleases (RNases). Our laboratory possesses a collection of bacteria that produce various metabolites and have RNase activity. The results showed that the inoculation of potato with *B. subtilis* 26D and *B. thuringiensis* increased the grain yield by 32–43 %. In addition, the treatment of potato plants with *Bacillus* spp. significantly reduced the infection of potato plants with virus M. The prevalence of the disease in potato plants was significantly reduced from 60 % in the control to 18 % (*B. subtillis* 26D) and 25–33 % (*B. thuringiensis*) in the inoculated plants. Similarly, the infection index decreased from 14 in the control to 1 in the inoculated plants. The further study of molecular mechanisms related to bacterial induction of plant defense reactions in response to viral infections will lead to a better understanding of stress resistance problems. The endophytic microorganisms studied in this report may become the basis for the creation of biological agents for plant protection.
Key words: ribonuclease; phytopathogenic RNA-viruses; *Solanum tuberosum*.

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Эндофитные бактерии *Bacillus* spp. с РНКазной активностью и устойчивость картофеля к вирусам

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Вирусные заболевания ежегодно вызывают существенные потери урожая и заметно снижают качество продукции, в том числе важнейшей сельскохозяйственной культуры – картофеля. Антивирусных препаратов для растениеводства, которые были бы безопасны для человека и животных, не существует, и в этих условиях перспективным методом защиты растений является использование биопрепаратов на основе эндофитных микроорганизмов, продуцирующих РНКазы. В работе проанализирована способность ряда эндофитных штаммов *Bacillus* spp. продуцировать РНКазы и влиять на пораженность растений вирусом М, широко распространенным в средней полосе России, и урожайность раннеспелого сорта картофеля Удача в полевых условиях. Обнаружено, что обработка штаммами бактерий *B. subtilis* 26Д и *B. thuringiensis* снижала степень инфицированности растений вирусом М с 60 % в контроле до 18–30 % на участках, обработанных микроорганизмами. Аналогичным образом снизился индекс развития вирусной инфекции: с 14 % в контроле до 1–7 % у инокулированных растений. Кроме того, бактерии с высокой РНКазной активностью вызывали прибавку урожая картофеля до 40 %. Предполагается, что изученные бактерии способны не только повышать рост и урожайность картофеля, но и, благодаря своей РНКазной активности, подавлять распространение вируса М. Дальнейшее изучение молекулярных механизмов, связанных с индукцией эндофитными бактериями защитных реакций растений в ответ на вирусные инфекции, приведет к лучшему пониманию фитоиммунитета растений. Более того, эндофитные штаммы *Bacillus* spp. с высокой РНКазной активностью могут стать основой для биопрепаратов комплексной защиты растений.
Ключевые слова: бактерии; РНКазы; вирусы; картофель.

Introduction

Viral diseases cause the significant loss (up to 30 %) of potato (*Solanum tuberosum*) yield and a marked deterioration of its quality annually. For instance, cultivated plants are affected by at least 450 different viruses, more than 40 of them infect potatoes, significantly reducing their productivity and deteriorating the quality of tubers which is known as the cultivar degeneration (Makarova et al., 2017). The main directions of

plant protection against viral infections are: the rehabilitation of seeds by isolating and cultivating the apical meristem *in vitro*, the generation of transgenic plants resistant to viral infections using genes of specific and nonspecific defense, use of plant protection preparations of a chemical and biological nature against viral vectors, the application of inductors of plant resistance, etc. (Nicaise, 2017). Genome-editing technologies, such as genetic transformation (Prins et al., 2008) and CRISPR/Cas9 system (Romay, Bragard, 2017), are more efficient to control viral diseases. For instance, overexpression of genes encoded antiviral proteins (interferon, ribonucleases), proteins toxic to insects or viral proteins (capsid protein) were shown to enhance resistance against viruses (Stasevski, Ilinskaya, 2009; Chung et al., 2013).

Despite of a huge amount of applied experimental works on the development of biocontrol agents on the basis of rhyzosphaeric and endophytic microorganisms, there are very few data about their antiviral activity, influence on distribution and severity of plant viruses. Biocidal activity of *Bacillus* strains resulted from the synthesis of specific insecticidal proteins (Cry and Vip of *B. thuringiensis*), bacteriocines and lipopeptides (Rodríguez et al., 2018). This perspective tends to favour the view that *Bacillus* can protect plants against viral diseases by affecting insect, bacterial and fungal phytopathogens, nematodes, which are vectors of viral particles. It's worth noting that currently the search of endophytic microorganisms which inhabit in the internal tissues of plants and less influenced by environmental factors and more integrated in plant metabolism than rhyzosphaeric and fillosphaeric microorganisms is of great interest.

A lot of *Bacillus* species can inhabit internal plant tissues (Burkhanova et al., 2017) and produce ribonucleases (Ulyanova et al., 2016; Ilinskaya et al., 2018). Thus, 73 % of *Bacillus* which were isolated from Cucurbitaceae produce nucleases (Khalaf, Raizada, 2018). Synthesis of secreted enzymes including RNases, which participate in mobilization of organic phosphates is one of the mechanisms of adaptation to changing environmental conditions. Low concentrations of RNases stimulate plant growth and resistance to a broad spectrum of stress factors, high levels of them show antiviral properties by destroying viral RNA. Microbial RNases are potential therapeutic agents which are suggested for the treatment of human viral diseases (Mahmud et al., 2017). In this regard, the identification of the biological properties of endophytic *Bacillus* spp. is relevant for the development of biological products with complex (antiviral, immunizing and growth stimulating) activity for the environmentally safe protection of potato plants from diseases and pests.

The aim of this work was to evaluate the RNase activity of a number of endophytic bacteria strains from the collection of the Laboratory of Biochemistry of Plant Immunity of the Institute of Biochemistry and Genetics UFRC (http://ibg.anrb.ru/wp-content/uploads/2019/04/Katalog-endofit.doc) and their effect on resistance of potato plants of early-ripening variety Udacha to phytopathogenic viruses (potato virus M) and potato productivity under the field conditions.

Materials and metods

Bacterial strains *B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* (B-5689) and *B. thuringiensis* var. *kurstaki* (B-5351) courtesy of the limited liability company "Bashincom". Isolates *B. subtilis* Stl-7, *B. subtilis* Stk-18, *B. subtilis* Stk-22 were obtained from surface-sterilized leaves tissues of potato varieties, which were cultivated on the territory of Iglinskiy district of Bashkortostan Republic. Identification of the all strains was carried out through DNA sequencing of 16S RNA gene fragments. Nucleotide sequence analysis was carried out by using international database GenBank. Bacterial culture was growth on Lysogeny broth basal medium (0.5 g/l NaCl) in TS 1/20 thermostat (SPU, Russia) at a temperature 28 °C. 16-hours old cultures were used for endophytic properties, antiviral activity and influence on potato productivity estimation.

Activity of extracellular RNases culture medium was estimated using the method reported by Hole et al. (2004). Strains were growth on LB agar with addition of yeast RNA (6 g/l) (Sigma, USA) at 30 °C. The plates were than incubated at required temperatures until growth was clearly visible (48 h). After incubation the plates were flooded with 3 ml of the precipitant (perchloric acid) and left to stand for 5 min. The plates were then visualized for transparent halos formed around the grown colonies, against an opaque background. RNase activity was shown as the distance between colony edge and halo edge. Quantitative estimation of extracellular RNase activity in liquid cultural medium was carried out using spectrophotometer UNICO 2800 (USA). The absorbance at 260 nm was measured according to (Margulis et al., 2012). Rate of reaction was evaluated as tangent of the slope of the rectilinear ascending part of the curve of light absorption versus reaction time, and expressed as an increase in absorption for 1 min per 1 mg of protein.

Endophyticity of the tested strains was evaluated by counting the colony-forming units (CFU) of microorganisms in plant tissues 7 days after inoculation of sterile test-tube potato plants (Udacha variety) cultivated for 25 days at 16-h illumination (Osram L 36W/77 bulbs, Germany) in the KS200 climate chamber (Smolensk SKTB SPU, Russia) on the agarised Murashige–Skoog medium. For this purpose, 100-mg samples of experimental plants were superficially sterilized in the following order: 70 % ethanol (1 min) → 0.1 % Diacide-1 (3 min) → distilled water. The samples were homogenized in sterile mortars with 2 ml sterile water added. Two consecutive 10-fold dilutions of the resultant homogenate were then performed. Aliquots (100 μL) were spread over the surface of potato-glucose agar by a microbiological loop until complete drying. Petri dishes were then incubated at 28 °C in the TS-1/20 SPU thermostat (Smolensk SKTB SPU, Russia) for 24 h. CFU were counted in second and third dilutions, and their number was recalculated per 1 g of plant wet weight.

The study was carried out at the experimental fields of the Ufa Federal Research Center (Birsk experimental station, 55°24′27″ N, 55°36′39″ E). Fields were located on gray forest soils (northern forest-steppe). The plants of the original Udacha variety under study were planted in three repeats of 30 plants for each line. Three plots were used as replicates for each treatment (*B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* B-5689, *B. thuringiensis* var. *kurstakii* B-55351) as well as for the untreated control treatment (water-sprayed). 2-weeks seedlings were sprayed with different strains of *Bacillus* suspensions (10^6 cells/plant). Spraying was duplicated after flowering in the same man-

ner. Viral spreading was estimated using diagnostic sets for immune-chromatographic detection of viral particles of X, Y, S, M and PLRV according to the recommendations of manufacturer (LLC Agrodiagnostica, Russia). Phenotypic observations and diagnostics of viral infection were carried out according to methodic instructions (Methodology…, 1995) before flowering. Data on the productivity of potato was prepared according to Dospechov B.A. (1985). The experiments were performed three times in three replicates. Data presented are mean values with standard errors (±SE). Statistical analyses were performed with Microsoft Excel 2013 for Windows (Microsoft Corporation, 2013).

Results

Screening of bacterial strains in collection of the Laboratory of Biochemistry of Plant Immunity of the Institute of Biochemistry and Genetics UFRC RAS and isolates collected from the field population showed the presence of RNase activity in all *Bacillus* strains under investigation (Table 1, Figure). The maximal halo was observed on culture medium of the isolated from potato leaves *B. subtilis* Stl-7, minimal – in *B. thuringiensis* var. *kurstaki* B-5351 medium. Thus, isolated from Colorado potato beetle *Enterobacter* spp. which were earlier identified using specific primers, didn't express any RNase activity (see Table 1).

The significant RNase activity was observed in culture medium of *B. thuringiensis* var. *kurstaki* B-6066, *B. subtilis* Stl-7, *B. subtilis* 26D, *B. thuringiensis* var. *thuringiensis* B-5689 (see the Figure). These measures suggested the usability of the method of Hole et al. (2004) for screening, since the large of halo correlated with enzyme activity in liquid medium.

Bacteria *B. subtilis* 26D and *B. thuringiensis* var. *kurstaki* B-5351 were found in potato plant tissues in the amount of 10^5 CFU/g wet weight. The CFU number of *B. thuringiensis* var. *thuringiensis* B-5689 in potato plant tissues was diminished more than two orders of magnitude. Thus, it was shown that the strains *B. subtilis* 26D and *B. thuringiensis* var. *kurstaki* B-5351 have a greater ability to actively invade and colonize plant tissues as compared to *B. thuringiensis* var. *thuringiensis* B-5689.

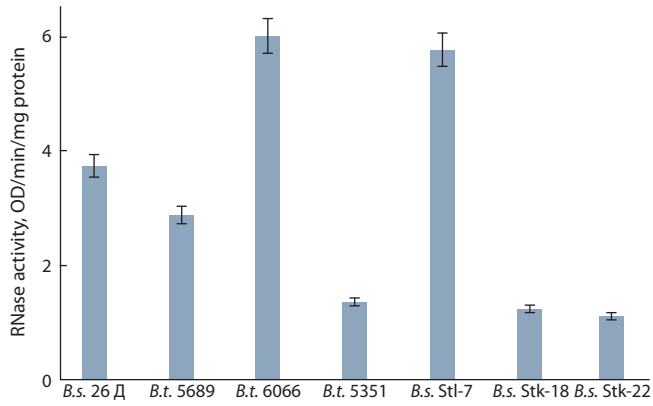
It's worth noting that application with strains, which have high RNase activity and ability to colonize internal plant tissues can promote plant resistance to viral diseases. Thus, the influence of endophytic strains *B. subtilis* 26D, *B. thuringiensis* var. *kurstaki* B-5351 and *B. thuringiensis* var. *thuringiensis* B-5689 on potato plants (Udacha variety) were investigated at the field conditions.

The presence of X, Y, S, M viruses and PLRV was preliminary estimated using immune-chromatographic sets manufactured by LLC Agrodiagnostica (Russia). High incidence of M virus was observed (Table 2). 60 % of plants growing on non-treated plots were infected with M virus. About 14 % on average leaves on each plant showed visible manifestation of disease symptoms.

The lowest number of plants with simptoms of viral desease was detected on the plots which were treated with *B. subtilis* 26D. Treatment with *B. thuringiensis* var. *thuringiensis* B-5689 (low endophytic rate, but high RNase activity) decreased at twice the rate of plants with virose symptoms. Disease severity was no more than 1 % of leaves on each plant.

Table 1. RNase activity *in vitro* of strains from collection

Strains	Halo, mm
B. subtilis 26D	4.5
B. subtilis Stl-7	6.5
B. subtilis Stk-18	3.0
B. subtilis Stk-22	4.5
B. thuringiensis var. *kurstaki* B-6066	4.0
B. thuringiensis var. *thuringiensis* B-5689	5.0
B. thuringiensis var. *kurstaki* B-5351	2.0
Enterobacter sp. BeP	0
Enterobacter sp. m10	0
Enterobacter sp. m9	0



RNase activity of cell filtrate strains of *Bacillus* spp.

B. thuringiensis var. *kurstaki* B-5351 (low RNase activity, but high endophytic rate) prevented the spread of the virus under the same conditions. However, the rate of disease symptoms manifestation in this case was higher than in plots which were treated with strains which displayed high RNase activity *in vitro*. Probably, spreading of infection was limited by through insecticidal properties of *B. thuringiensis* var. *kurstaki* B-5351 (Sorokan et al., 2018).

It's important that *B. subtilis* 26D and *B. thuringiensis* var. *thuringiensis* B-5689 treatment significantly increased potato productivity. It could be attributable to their direct antiviral activity and plant growth-stimulating properties.

Discussion

It is known that many bacteria, especially from the genus *Bacillus*, have a wide range of enzymes with RNase activity, as well as nucleases and other proteins responsible for RNA interference (Aguiar-Pulido et al., 2016). For example, bacteria *B. amyloliquefaciens*, *B. intermedius*, and *B. licheniformis* can produce extracellular ribonucleases called baRNases, binases,

Table 2. Content of CFU of *Bacillus* spp. in internal tissues of potato plants Udacha cv, their virus M infection rates and yield

Parameter	Control (H_2O)	*B. subtilis* 26D	*B. thuringiensis*	
			var. *thuringiensis* B-5689	var. *kurstaki* B-5351
CFU/g (fresh weight)	0	$5 \cdot 10^5$	$5 \cdot 10^3$	$1 \cdot 10^5$
Plants affected by virus M, %	60	18	33	25
Development of the virus M, % of affected leaves/plant	14	1	1	7
Yield, 100 kg/ha	182.45 ± 12.38	241.91 ± 22.67	261.03 ± 23.59	205.58 ± 15.60
Increase, % of control	–	32	43	12

and balifases, respectively (Ulyanova et al., 2011, 2016). Recent studies demonstrated that *B. subtilis* and *B. thuringiensis* contain bacterial RNases effectively inactivate RNA-containing viruses – baRNase and binase (Ulyanova et al., 2011). It has been established that *B. cereus* ZH14 produces a new type of extracellular ribonuclease which are active against tobacco mosaic virus (Zhou, Niu, 2009). Genetic transformation using bacterial RNase gene may be a promising approach for the engineering of plants with resistance to viral infection (Zhang, 2001; Cao et al., 2013). Soil treatment with *Pseudomonas putida* A3 prior to sowing reduced TMV infection in tobacco plants in comparison with the soil treatment with this PGPR after sowing (Guo et al., 2011). PGPR *P. putida* A3 was shown to destroy virus particles in the juice from tobacco leaves infected with TMV (Yang et al., 2012). Thus, in these works it was demonstrated not only the ability of PGPR of the genera *Pseudomonas* to suppress viral infection indirectly by stimulating the nonspecific plant defense mechanisms but also their direct viricidal activity (Guo et al., 2011; Yang et al., 2012). According to our data, strains with high *in vitro* RNase activity significantly reduced the intensity of the development of M virus symptoms on Udacha potato plants in the field compared to strains with low RNase activity.

It was shown that enhanced expression of PR-8 and NPR-1 defense genes contributes significantly to *B. amylolequifaciens*-induced multiple reduction in rhisomania infection caused by the BNYVV virus (Desoignies et al., 2013). The application of *B. subtilis* BS3A25 strain have been found to reduce cucumber mosaic virus (CMV) infection by inhibiting the development of its vector *Aphis gossipi* (Sudhakar et al., 2011).

It is of interest to develop antiviral biocontrol agents based on PGPR isolates having high RNase activity or the preparation of RNase itself to protect plants from viral infection, taking into account that the majority of phytoviruses are RNA viruses (Sharipova et al., 2015) as well as the generation of genetically modified plants expressing RNase genes (Trifonova et al., 2004). It has been found that potato plants expressing *Serratia marcescens* nuclease display enhanced resistance to pathogens (Trifonova et al., 2018).

Strains under investigation also displayed insecticidal effect against Colorado potato beetle (Sorokan et al., 2018) and wheat aphid (Veselova et al., 2019), representing about 20 % from all aphids species which damaged potato plants (Ekaterinskaja et al., 2016) and act as carriers for viral particles. In addition to developing increased resistance against viral infection, bacterial barnases can participate in plant protection against other diseases, for example, tobacco plants from the late blight desease, as evidenced by high resistance of transgenic plants producing barnase (Natsoulis, Boeke, 1991). Earlier we demonstrated that *B. subtilis* 26D effectively decreased disease severity of the late blight (Maksimov et al., 2015), which could contribute to the limitation of virus spreading in potato crops. It is important to note that effective suppression of viral diseases requires the constant presence of antiviral compounds in plant tissues. It makes endophytic microorganisms producing RNases promising viral biocontrol agents. These data can be a basis of approach to protection of plants from viral infection by using the "RNase enhanced" endophytic bacteria *Bacillus* spp., as an antiviral agent.

Conclusion

According to the data obtained, we can say that the use of biocontrol agents based on bacteria of the genus *Bacillus* reduced the natural viral infectious background, which depended on the endophytic properties of strains under investigation and the ability of bacteria to produce extracellular RNases. Thus, we suggest the development of biocontrol agents with complex (antiviral, insecticidal, fungicidal, bactericidal and growth stimulating) activity for environmentally safe system of plant protection from diseases and pests.

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