Agricultural Sciences, 2015, 6, 1357-1366
Published Online November 2015 in SciRes. http://www.scirp.org/journal/as
http://dx.doi.org/10.4236/as.2015.611130

Antiviral Effect of Ribonuclease from *Bacillus pumilus* against Phytopathogenic Rna-Viruses

Margarita Sharipova[1], Annett Rockstroh[1], Nelly Balaban[1], Ayslu Mardanova[1], Anna Toymentseva[1], Anastasiya Tikhonova[1], Semen Vologin[2], Zenon Stashevsky[2]

[1]Kazan (Volga Region) Federal University, Kazan, Russia
[2]Tatar Research Institute of Agriculture, Kazan, Russia
Email: natalialrudakova@mail.ru

Received 10 July 2015; accepted 21 November 2015; published 24 November 2015

Copyright © 2015 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY).
http://creativecommons.org/licenses/by/4.0/
Open Access

Abstract

Background: Viruses can cause different diseases in plants. To prevent viral infections, plants are treated with chemical compounds and antiviral agents. Chemical antiviral agents usually have narrow specificity, which limits their wide application. Alternative antiviral strategy is associated with the use of microbial enzymes, which are less toxic and are readily decomposed without accumulation of harmful substances. The aim of this work is to study the effect of *Bacillus pumilus* ribonuclease on various phytopathogenic viruses with specific focus on the ability of enzyme to eliminate them from plant explants *in vitro*. Materials and methods: Extracellular ribonuclease of *B. pumilus* is tested as an antiviral agent. To study the antiviral effect of RNase, depending on concentration and the time of application several plant-virus model systems are used. Virus detection is conducted by serological testing and RT-PCR. Results: *Bacillus pumilus* ribonuclease possesses antiviral activity against plant Rna-viruses RCMV (red clover mottle virus), PVX (Potato Virus X) and AMV (Alfalfa Mosaic Virus). The maximum inhibitory effect against actively replicating viruses is observed when plants are treated with the enzyme in the concentration of 100 ug/ml prior to infection. In case of local necrosis ribonuclease in the concentration of 1 ug/ml completely inhibits the development of RCMV virus on bean plants. The enzyme is able to penetrate plants and inhibit the development of viral infection, inhibiting effect for untreated surfaces decreased on average for 20%. It is also found that *B. pumilus* ribonuclease protects apical explants of sprouts of potato tubers from PVM and PVS viruses. Conclusion: *B. pumilus* ribonuclease possesses antiviral activity against plant Rna-viruses and produces viruses-free plants in the apical meristem culture.

Keywords

***Bacillus Pumilus*, Ribonuclease, Phytopathogenic Rna-Viruses, Inhibition, Virus-Free Apical**

How to cite this paper: Sharipova, M., Rockstroh, A., Balaban, N., Mardanova, A., Toymentseva, A., Tikhonova, A., Vologin, S. and Stashevsky, Z. (2015) Antiviral Effect of Ribonuclease from *Bacillus pumilus* against Phytopathogenic Rna-Viruses. *Agricultural Sciences*, **6**, 1357-1366. http://dx.doi.org/10.4236/as.2015.611130

1. Introduction

Viruses, along with bacteria and fungi can cause different diseases in plants. Viral infections lead to plant damage, a significant drop in the crop yield and eventually affect the quality of the food products. The most significant economic losses due to viral infections are linked with potatoes, fruits and berries, as well as decorative flowers and perennial grasses.

Viral diseases in plants are difficult to treat during vegetative period of growth. The most effective antiviral strategy relies on using healthy planting material, obtaining resistant plant varieties, and employing the techniques of *in vitro* culturing in combination with chemo- and thermotherapy and implementation of phytosanitary norms [1].

Currently, cultures of apical meristem are most frequently used for the production of virus-free plants. The process of obtaining the healthy plants from meristems is laborious and time consuming. Because of the weak capacity of meristems for regeneration and low overall yield of healthy plants it is necessary to isolate multiple meristem explants.

Plants are treated with chemical compounds and antiviral agents such as ribavirin, interferon, benomyl, thiouracil, aziridines and others to prevent viral infections [2]-[4]. However, chemical antiviral agents usually have narrow specificity and can be toxic to plants, animals and humans, which limit their wide application.

The emerging alternative antiviral strategy is associated with the use of microbial enzymes. They are less toxic and are readily decomposed without accumulation of harmful substances. It is known that some enzymes that are capable to break down the nucleic acids have antiviral activity high resistance to pathogens which have been observed in transgenic plant expressed bacterial nuclease *Serratia marcescens* [5]. *Bacillus cereus* ZH14 is found to produce a new type of antiviral ribonuclease, which is secreted into medium and active against tobacco mosaic virus [6] [7]. Recent studies demonstrate the ability of artificial ribonucleases (a RNases, small organic RNA cleaving compounds) to inactivate Rna-viruses via the synergetic effect of viral RNA cleavage and disruption of viral envelope [8]. Taken together, these data allow us to explore the possibility of using target bacterial enzymes to inhibit plant pathogenic viruses.

The objective of this work is to study the effect of *B. pumilus* ribonuclease on various phytopathogenic viruses with specific focus on the ability of enzyme to eliminate them from plant explants *in vitro*.

2. Materials and Methods

Extracellular ribonuclease of *B. pumilus* 7P/3 - 19 (RNase Bp, 12.3 kDa, CF 3.1.27.2) was tested as an antiviral agent. This endonuclease is thermostable with the specific activity 1.3×10^6 U/mg protein. The enzyme was produced and purified at the Riga Pharmaceutical Factory according to the previously described technique [9]. RNase activity was determined by RNA hydrolysis products soluble in 4% $HClO_4$ supplemented with 12% uranyl acetate [9]. One unit of activity was defined as the amount of enzyme necessary to increase the OD_{260} by 1 optical unit per 1 ml of enzyme solution for 1 h.

To study the antiviral effect of RNase Bp several plant-virus model systems were used (**Table 1**).

Plants were grown under constant controlled growth conditions at 20˚C ± 2˚C, 60% - 80% relative humidity with a daily photoperiodic cycle of 16 h light (9000^{lux} irradiation power) and 8h dark in pots with perlite, except

Table 1. Models of phytopathogenic viruses.

Phytopathogenic viruses	The hosts of viruses with systematic replication	Hypersensitive hosts
Red clover mottle virus (RCMV)	*Pisum sativum* L. convar. speciosum (Dierb) Alef Nadja	*Phaseolus vulgaris* L. Esto
Alfalfa mosaic virus (AMV)	*Nicotiana tabacum* L. Samsunnn	
Potato virus X (PVX)	*Nicotiana tabacum* L. Samsun	
Potato virus M (PVM)	Apical meristem of potato	
Potato virus S (PVS)	Apical meristem of potato	

tobacco plants, which were grown in soil.

40 - 45 days old tobacco plants cultivar Samsun were used for alfalfa mosaic virus (AMV) infection by sap of infected plants. AMV-infected leaves were disinte grated by French press in 0.025 M phosphate buffer pH 8.0 (100 g leaves per 50 ml buffer). The sap was applied to the leaves of healthy plants in the presence of an abrasive (silicon carbide). After 14 days two fully developed leaves of tobacco were collected and analyzed for the presence of virus.

10 days old pea plants were used for red clover mottle virus (RCMV) infection, which was performed with the sap of infected plants. After 10 days post-infection, the plants were collected and sap was serologically tested for the maintenance of viral antigen. 5 pots of peas with 5 - 6 plants in each were used for each experiment. In beans RCMV caused severe damage, leaf surface tissue was destroyed and areas of local necrosis were formed.

The sap of infected plants was prepared to infect the leaves of tobacco plants cultivar Samsun with potato virus X (PVX). Leaves were disintegrated by French Press; resulting sap was diluted with distilled water (1:1) and used for the infection of 40 - 45 days old tobacco plants.

Serological test was used to determine the RCMV as described in [10]. Determination of AMV was performed similarly to RCMV. Viral presence was determined on 4 - 5 day post infection. Leaves of the second growth were analyzed on 10 - 12 day after infection.

The determination of the PVX in tobacco leaves was determined by reaction of agglutination with rabbit antibodies to virus. Gamma-globulin fraction of rabbit serum was extracted twice by deposition of 20% aqueous solution of polyethylene glycol (M. W. 6 kDa) followed by dialysis against 0.01 MK-phosphate buffer (0.1 M NaCl) pH 7.4 to 7.8.The sap was incubated for 10 min at 42˚C and centrifuged for 20 min at 5000 rpm (centrifuge K-23 “Bekman”). The supernatant was serially two-fold diluted and the solution of antibodies to potato virus X in 0.025 M phosphate buffer, pH 8.0 was added. The agglutination reaction was monitored under the microscope. The average dilution at which the agglutination reaction took place was determined after five independent experiments.

We studied the dose-dependent effect of RNase Bp on the RCMV replication. On the 14th day of growth bean primary leaves were treated with RNase Bp (in the range of concentrations 1 - 100 ug/ml) followed by treatment with carborundum and then inoculated with virus (40 ug/ml) For each enzyme concentration tested at least 10 primary bean leaves were used. Untreated leaves, or the left side of the leaf when the right was treated with RNase were used as a control. After 4 - 5 days post-infection the number of local necrosis loci was determined and statistical analysis was performed.

To study the effect of RNase depending on the time of application, plants were treated with the enzyme (100 ug/ml) each day starting from 4 days prior to infection with 24-hour interval, at the time of infection, and 24 h after viral infection. In separate experiment seeds were treated with RNase by soaking in RNase solution (10 - 100 ug/ml) for 2 hours before planting.

Enzyme concentration was varied (1 ug/ml, 10 ug/ml, 100 ug/ml, 1000 ug/ml), pancreatic RNase (RNase A) was used for comparison. Enzyme concentration providing the same catalytic activity of both enzymes was used (RNase Bp100 ug/ml RNase A-300 ug/ml).

To study enzyme transport in the infected plants leaves of first, second and third tier of 10-days old pea plants (5 pots with 5 - 6 plants in each for every tier) were treated with RNase Bp (100 ug/ml) for 24 hours prior to infection. Leaves of the 2-ndtier only were infected with virus in all variants. After 10 days post- infection sap was collected and analyzed for the presence of RCMV.

The initial diagnosis of potato viruses was performed by ELISA using kits Adgen (Neogen Europe, Scotland) according to the instructions of the manufacturer. The isolation of the apical explants was performed from the sprouts of potato tubers No # 3 - 23 - 2 (interbreeding 91.29/2 X Ausoniya) infected with potato virus M (PVM) and potato virus S (PVS). Dissection was done with needle under binocular microscope with 24 times magnification. Plant tissue containing the apical meristem, the cone and 4 primordial leaves was isolated. Samples were sterilized by 0.1% mercuric chloride solution and then washed three times with sterile distilled water. Explants were maintained on MS medium [11], supplemented with RNase Bp at different concentrations; the enzyme was introduced into the medium through the filter (Millipore filter). Plants were grown in vitro at 24˚C - 25˚C and 16-hour photoperiod (illuminance 2000 lux). Virus detection was conducted by reverse transcription PCR after three cycles of microclonal proliferation of regenerated plants.RNA was isolated from plants by “Ribo-sorb” kit (Inter Lab Service, Russia). For RT-PCR reagents from Sib Enzyme Ltd. (Russia) were used. The reaction was

performed in 25 ul containing 2.5 ul 20 mm solution of dNTP, 1.25 ul of 10 x RT buffer, 1.25 ul of 10 x PCR buffer, 0.2 ul of 5U M-MuLV reverse transcriptase, 0.2 ul of 5U Taq DNA-polymerase, 0.5 ul of 50 uM sense and antisense primers (for PVM or PVS), 2 ul of total RNA sample. For PVM detection the following oligonucleotide primers were used: forward 5'-gccacatcygaggacatgat-3', reverse 5'-gtgagctcsggaccattcat-3'; for PVS detection: forward 5'-gaggctatgctggagcagag-3', reverse 5'-aatctcagcgccaagcatcc-3' [12]. Reaction of amplification was performed on "Mastercycler Gradient" (Eppendorf, Germany) under following conditions: 1 cycle: 37˚C-60 min; 1 cycle: 94˚C-5 min; 42 cycles: 9˚C-1 min, 60˚C-1 min, 72˚C-1 min; 1 cycle: 72˚C-5 min; 4˚C-storage. RT-PCR productes were analyzed by electrophoresis in 2% agarose gels containing 1 x TBE buffer with ethidium bromide at the concentration of 0.5 ug/ml. The size of the amplified fragments (PVM-524 Bp, PVS-738 Bp) was estimated by mobility comparison with DNA molecular size markers (Sib Enzyme Ltd, Russia).

Statistical analysis was performed using the software package SPSS 12.0. Standard deviation (σ) was calculated and the results were considered significant when $\sigma \leq 10\%$.

3. Results and Discussion

3.1. The Effect of RNase Bp on Pea Plants Infected by Plant RNA-Virus RCMV

Plants were treated with the bacterial enzyme at 1 - 4 days prior to infection, at the time of infection (enzyme solution and virus were applied simultaneously) and one day post infection. Enzyme was used in the range of concentrations from 10 to 1000 ug/ml (**Figure 1**); in a separate experiment seeds were treated with RNase in the same concentrations (**Figure 2**).

It has been experimentally established that pretreatment of pea plants with the enzyme solution in different concentrations and at different times before the infection with RCMV significantly ($P \leq 0.05$) suppressed viral

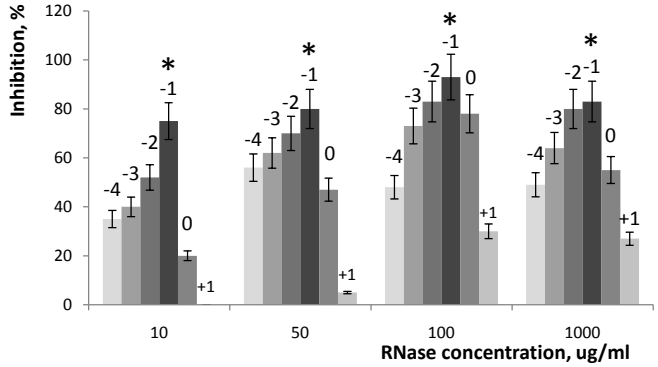

Figure 1. The effect of RNase Bp on RCMV spread in pea plants depending on the time of enzyme treatment. 0—viral infection and enzyme treatment was conducted simultaneously. −4, −3, −2, −1—correspond to the number of days pasted prior to infection; +1—indicates the number of days after infection. Viral spread was evaluated two days after infection. Viral spread in the control plants, infected with RCMV but not treated with the enzyme, was taken for 100%.

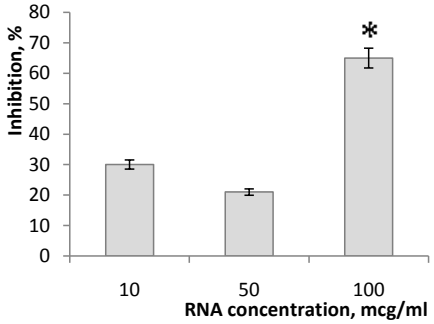

Figure 2. Resistance of pea plants germinated from the seeds, treated with RNase Bp in different concentrations to virus RCMV. The content of viruses in control plants, which were not treated with the enzyme, was taken for 100%. Asterisk indicates $P \leq 0.05\%$.

spread. Inhibition increased with the reduction of time of plant pre-treatment with the bacterial RNase before viral infection and reached the maximum value (75% - 93%) for all concentrations of the enzyme when RNase treatment was performed one-day prior the infection. When RNase was applied simultaneously with the virus (Time 0), the inhibition decreased to 20% - 78% depending on the concentration of the enzyme. It was found that treatment of plants with the enzyme after infection with viruses was less efficient (0% - 25% inhibition) compared to plants pre-treated with the enzyme.

In a range of enzyme concentration tasted the maximum inhibition corresponded to the concentration of 100 ug/ml. Increase in enzyme concentration to 1000 ug/ml did not lead to increased inhibition of viral infection. Therefore, RNase concentration of 100 ug/ml was chosen for further experiments.

Pre-treatment of pea seeds with bacterial RNase resulted in 24% - 62% inhibition of viral spread; maximum inhibitory effect (65%) was observedwhen seeds were treated with RNase at 100 ug/ml (**Figure 2**).

Thus, data obtained for RCMV virus, indicate that the bacterial enzyme is effective when applied prior to viral infection and potentially can be used as a prophylactic antiviral agent. It should be emphasized that pre-treatment of seeds is simple and feasible in terms of agricultural practices-also contributes to the development of plant resistance to viral infection.

3.2. Mechanisms of Penetration and Transport of RNase *B. pumilus* in Plants

Next, the mechanisms of penetration and transport of RNase Bp in plants infected with RCMV were addressed. To study relationship between the enzyme application site and the distribution anti-viral effect in pea plants RNase Bp (100 ug/ml) was applied to the leaves of one of the three tiers of pea plants one-day prior to infection. One day later the leaves of the second tier only were infected. The content of viruses was determined in the sap of each variant (see **Table 2**).

Viral infection was suppressed in all three experimental groups: in the leaves of the second tier by 90%, in the leaves of the first and third tier-by 70% and 64%, respectively (**Table 2**), *i.e*. the maximum inhibition was observed for the tier where leaves were treated both with the enzyme and virus. The obtained data indicated that bacillary RNase is able to penetrate plant tissue and be transported both upwards and downwards. The difference in inhibition of RCMV spread after the treatment of pea with enzyme a day prior to the infection could be due to a different transport speed through phloem and xylem and dilution of the enzyme in plant tissue, as well as its partial inactivation in the course of several days by non-specific plant inhibitors or by plant proteases.

3.3. Inhibitory Effect and Duration of Enzyme Action, Depending on the Application Site of the Rnase on Various Parts of Plants

We studied the duration of RNase antiviral action after infection. Maximal concentration of RCMV virus in infected pea plants was observed on the 15th day post infection. The effect of the enzyme was studied on the 10th and 21st days after viral infection (**Table 3**). The data showed that RNase is active for a long time. On day 10 virus was serologically detected at the dilution of 1.2 (average value) compared to dilution of 3.5 for the control plants (without enzyme treatment). On day 21 post in fectionvirus was detected in RNase-treated plants at the dilution of 1.8 and in the control plants—at 7.0. It is possible that the difference between treated and control groups on day 21 post infection is due to continuous reinfection of plants with virus. These data indicate that RNase Bp has a pronounced effect on viral spread during the initial phase of infection, but also the inhibitory effect of enzyme was observed in secondary infected leaves (on the 21st day).

Table 2. Effect of RNase Bp (100 ug/ml) on the spread of RCMV virus in systemic infection of pea plants after the treatment of leaves of different tiers with the enzyme.

Tier, treated with the enzyme*	Inhibition effect of enzyme on viral infection %**, ($\sigma \leq 10\%$)
1 tier	70 ± 6
2 tier	90 ± 8
3 tier	64 ± 5

*RNase Bp (100 ug/ml) was applied to the leaves of one of the three tiers of pea plants one-day prior to infection, only the leaves of the second tier were infected. **The content of viruses in control plants, which were infected but not treated with the enzyme, was taken as 100%.

3.4. The Effect of Rnase *B. Pumilus* on Necrosis Formation in Bean Plants

The effect of RNase Bpon necrosis formation in bean plants (model for localized infection of plants with virus) depending on the concentration of enzyme, its site of application and duration of the plant treatment was studied. The right halves of fully developed primary leaves of beans were treated with RNase Bp in concentration of 1 - 1000 ug/ml on days four and two as well as1 hour, prior to infection, at the time of infection, and one hour and 1 day post infection. The left halves of the leaves were treated with distilled water. Both sides of these leaves and control leaves on the other plants were infected with RCMV virus. The number of developed necrotic lociwas determined after 5 days on the both sides of the leaves (**Figure 3**).

The right side of the leaf was treated with the enzyme in all experimental groups, and complete suppression of viral infectionwas observed when plants were treated with the enzyme prior to and at the time of infection (Time

Table 3. Duration of antiviral action of RNase Bp against the RCMV virus at system-infection of peas.

Conditions of the experiment	Inhibition, %* (after infection), $\sigma \leq 10\%$	
	Day 10	Day 21
Viral spread in plants treated with an enzyme (100 ug/ml)	79 ± 7	99 ± 9

*The content of viruses in control infected plants not treated with the enzyme, was taken for 100%.

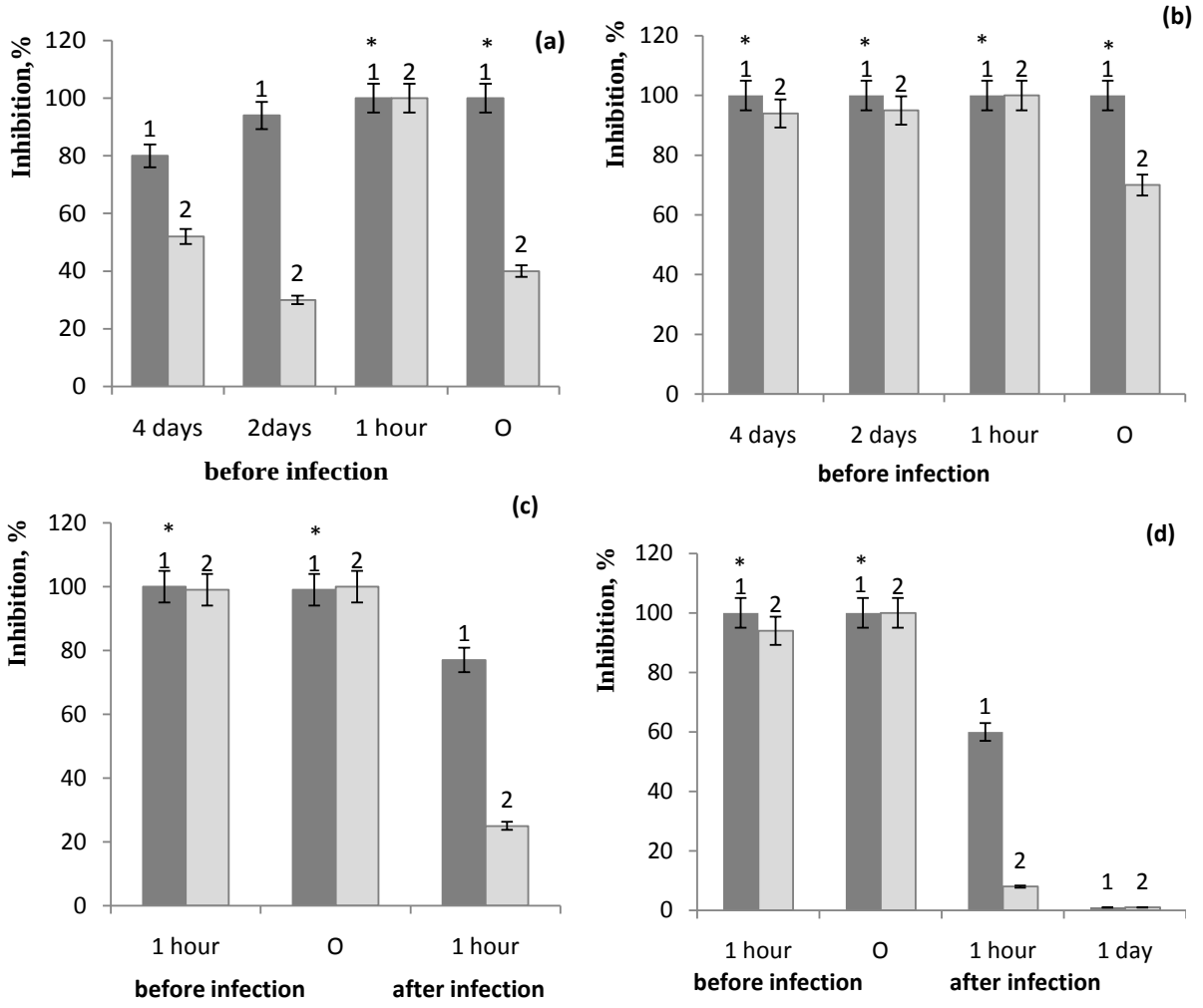

Figure 3. The inhibitory effect of RNase Bp on RCMV viral infection depending on the duration of treatment and the site of application (1—inhibition of virus on the right side of the leaf treated with RNase Bp; 2—inhibition of the virus on the left side of the leaf, treated with distilled water) and the concentration of enzyme (a: 1 ug/ml; b: 10 ug/ml; c: 100 ug/ml; d: 1000 ug/ml).

0). When leaves were treated with RNase Bp (1000 ug/ml) 1 hour post infection with RCMV, the number of necrotic loci was reduced by 57%. Nochange in the number of necrotic loci was detected after treatment with RNase Bp (1000 ug/ml) one day post infection. It was found, that RNase Bp in the concentration of 1 ug/ml completely prevented RCMV-mediated necrotic loci formation. RNase Bp treatment was most effective when enzyme was introduced 1 hour prior to infection or was used at the time of infection. Higher concentration of the enzyme (10 ug/ml) prevented necrotic loci formation after treatments performed four, two days and one hour prior to infection. Therefore, that RNase Bp protected leaves from necrotic loci formation most efficiently when treatment took place either prior to viral infection or during early stages of plant infection by phytopathogenic viruses. Necrotic loci formation was also suppressed on the left side of the leaf, infected with the virus, but not treated with the enzyme. Most likely, that could be a result of the RNase Bp transport inside the plant. The structural features of RNase Bp can contribute to the spread of the enzyme inside the plant, suggesting that RNase translocation occurs by spontaneous inversion through the membrane [13]. It does not exclude other mechanisms of plant defence, which are activated by the RNase.

The treatment of the right side of the leaf in the lower concentration (1 ug/ml) of the enzyme inhibited necrosis formation on the left side by 28% - 51% (treatment for 2 - 4 days prior to the infection). The application of the enzyme 1 h before the infection resulted in complete prevention of necrotic loci formation on the left side of the leaves. Importantly, RNase Bp treatment of the right half of the leaf with increasing concentration of enzyme-1, 100, 1000 ug/ml at the time of infection directly correlated with suppression of necrotic loci formation on the left side of the leaf (52%, 70% and 100%, respectively). Thus, RNase Bp is able to prevent necrotic loci formation on bean plant leaves even in the case of uneven enzyme distribution during application.

3.5. The Effect of RNase Bp on the PVX Viral Spread in Tobacco Plants

To study the effect of RNase Bp on the PVX virus in tobacco plants, plant leaves were treated with the enzyme (100 ug/ml) at 2 days and 30 min prior to infection (**Figure 4**).

The content of viruses was determined in the sap from plant leaves 5 days after infection. In both variants almost complete suppression of viral infection (93%) was observed. To compare antiviral effect of RNase Bp with other RNases we used pancreatic RNase in the concentration that provides the same catalytic activity as bacillary RNase. The inhibitory effect of RNase A was 92% - 94% (30 min and 2 days prior to the infection, respectively).

On day 13 we determined the virus content in secondary infected tobacco leaves, which were not infected at the time of infection and the presence of PVX was due viral spread. Viral spread in these leaves was inhibited by 88% after RNase Bp treatment and by 86% after treatment with RNase A. Thus, both enzymes were able to prevent PVX spread in primary and secondary infected tobacco leaves. Therefore, it confirms that RNases could be used to prevent spread of RNA-viruses.

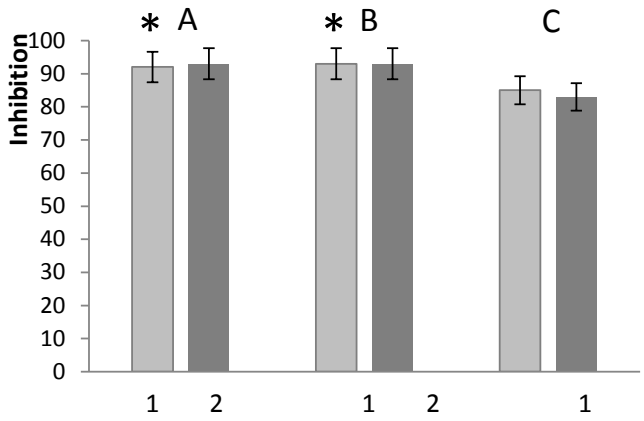

Figure 4. Antiviral effect of RNase Bp (1) (100 ug/ml) and pancreatic RNase (2) (300 ug/ml) on PVX virus spread in tobacco plants. A—enzyme treatment was performed 2 days prior to infection with PVX, virus content was determined 5 days after the infection; B—enzyme treatment was carried out 30 minutes before the infection with PVX, virus content was determined 5 days after the infection; C—enzyme treatment was carried out 30 minutes before the infection with PVX, virus content was determined 13 days after the infection.

3.6. The Effect of RNase BP on AMV Virus

To study the effect of RNase BP on AMV virus, tobacco leaves were treated with the enzyme 2 days prior to infection (enzyme was applied 3 times a day) (**Figure 5**). RNase treatment inhibited viral infection by 60% on day 6 after the infection, 41% on day 13 (at secondary infected leaves) and no inhibition was observed on day 21. Treatment of tobacco leaves with the higher concentration of enzyme (1000 ug/ml) did not increased efficiency of inhibition. Thus, the inhibitory effect of RNase Bp against AMV virus is weaker in comparison with other RNA-containing viruses. Therefore, the efficiency of inhibition depends on the virus type.

Therefore, the bacterial RNase showed antiviral activity against RCMV, PVX and AMV viruses. The maximal inhibitory effect against systematically replicating viruses was achieved when the enzyme was applied 1 - 2 days before the infection at the enzyme concentration of 100 ug/ml. Thus, RNase Bp treatment is most effective on the initial stages of viral plant infection. RCMV infection was suppressed by 91%, PVX and AMV infections were suppressed by 93% and 60%, respectively. It is possible that the differences in inhibitory effect of RNase Bp against various viruses are due to the peculiarities in viruses' structure. RNase Bp in the concentration of 1 ug/ml completely inhibited the necrotic loci formation hypersensitive RCMV-infected bean plants. The reason why systemic infection caused by RCMV virus was inhibited by RNase in the concentration of 100 ug/ml while a process of necrosis formation caused by the same virus in the concentration of 1 ug/ml, can be related to the nature of viral diseases. According to our data the enzyme in plants was transported both upwards and downwardsand prevented viral infection spread. Note that the maximum antiviral effect was observed when the infected pant surface was treated with an enzyme, the effect was less pronounced for the plant surfaces left untreated. It was determined that in RCMV-infected peas RNase BP had antiviral effect lasted for 3 weeks. It is important to mention, that guanyl-specific RNase Bp is a compact globular protein with the high content of hydrophobic amino acids, which ensures its high thermal stability in a wide pH range of 2.5 - 11.0 [14].

3.7. The Effect of Rnase Bp Treatment on PVM and PVS Elimination during the *in Vitro* Regeneration of Potato Plants from Apical Explants of Tuber Sprouts

To study were used potato tubers, already infected with potato virus S (PVS) and potato virus M (PVM). Using ELISA-test it previously was found that all the experimental potato tubers are mix-infected with potato virus S (PVS) and potato virus M (PVM). From tuber sprouts explants were isolated apical meristem, from which a nutrient medium containing RNase Bp 6 - 8 months raised plants. Pathogens were tested by RT-PCR.

The effect of RNase Bp treatment on virus elimination during the *in vitro* regeneration of potato plants from apical explants of tuber sprouts. The results are shown in **Table 4**.

Supplementation of culture medium with RNase Bp promoted elimination of viruses. According to our data PVS-free plants were obtained with all concentrations of the enzyme. The number of PVS-free plants was 1% - 37% higher compared to the control group. Yield of PVM-free plants was for 6% - 9% compared to the control

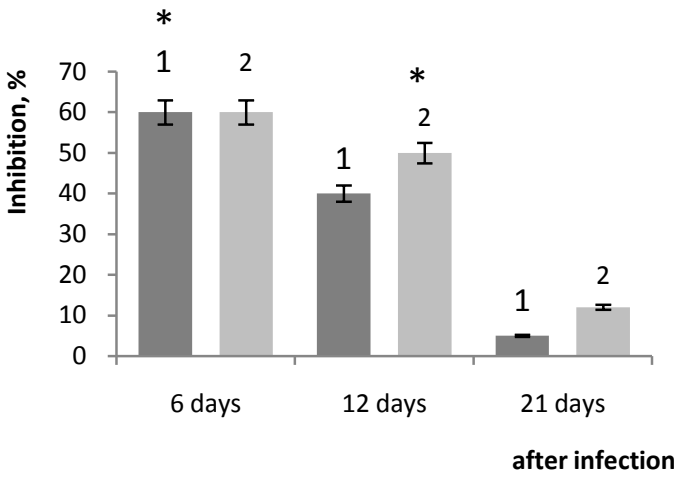

Figure 5. Inhibitory effect of RNase Bp on AMV virus, replicating systemically in tobacco plants: 1—the plants treated with the enzyme in the concentration of 100 ug/ml; 2—the plants treated with the enzyme in the concentration of 1000 ug/ml.

Table 4. The effect of RNase Bp on the process of potato viruses' elimination.

Group	RNase Bp, ug/ml	Number of explants	Number of virus-free plants			
			PVM		PVS	
			number	%*	number	%*
Control	0	47	2	4	6	13
	1	20	2	10	6	30
	10	18	0	0	4	22
RNase Bp	50	12	0	0	2	17
	100	16	2	13	1	6
	500	4	0	0	2	50

*100%—Total number of explants.

(completely infected explants). Inverse relationship between the content of RNase Bp in a nutrient medium and the number of received uninfected samples was established. The maximum yield of PVS-free plants was observed at the enzyme concentration of 1 ug/ml. Yield of PVM-free plants was for 6% - 9% compared to the control. Liberation of potato plants PVM occurred when the content of RNase Bp in the nutrient medium at concentrations of 1 and 100 ug/ml. Only at a concentration of 1 ug/ml we obtained virus-free plants, completely free of PVS and PVM. At a concentration of fermenta 100 ug/ml, this result is not achieved. Thus, we concluded that the most optimal for the removal of viral pathogens in the apical meristem culture of potatoes is to apply Bp RNase at a concentration of 1 ug/ml.

Both, PVS and PVM viruses, belong to the genus Carlaviridae, which are known to be difficult to treat. The relatively low efficiency of PVM elimination may be associated with deeper presence of viral particles in the apical meristem tissue. According to the electron microscopy data PVM is localized in the zone of 70 - 80 microns and PVS-90 - 95 microns [15]. Such localization PVM difficult to produce virus-free forms. We found effective antiviral effect at low enzyme concentrations the conditions for penetration and transport of RNase Bp in plant tissue are optimal.

In work published by others the stimulating effect of several bacterial enzymes on the development of potato plants *in vitro* is described. For example, application of endonuclease of *Serratia marsescens* intensifies regeneration of potato plants from the apical explants and inhibits proliferation of DNA-and RNA-containing phytoviruses [5]. Antiviral action of endogenous plant ribonucleases, localized on the surface of plant cells in the apoplastis reported [16]. It is known that virus inactivation occurs through depolymerisation of nucleic acids by nucleases when they nascent viral DNA or RNA are released from the protein coat. According to our data RNase Bp showed antiviral activity on the initial stages of plant infection by viruses. One of the ways to penetrate the eukaryotic cells for viruses is pinocytosis [17]; the mechanism of antiviral action of RNases may include the destruction of viral RNA with ribonucleases during this process, resulting in inactivation of immature, but infectious virions. It is known that, the inhibitory effect is associated with the formation of virus-ribonuclease complex followed by cleavage of viral RNA by enzyme [18]. We hypothesize that RNase Bp has a similar mechanism of action.

4. Conclusion

In conclusion, data obtained for different plant RNA-viruses indicate that the bacterial enzyme is effective when is applied prior to viral infection and potentially can be used as a prophylactic antiviral agent. It is important to note that pre-treatment of seeds is simple for agricultural practices and it can be used to protect plants against viruses. Furthermore, *B. pumilus* ribonuclease intensifies regeneration of potato plants from the apical explants to inhibit proliferation RNA-containing phytoviruses. Therefore, it confirms that RNases can be used to prevent the spread of RNA-viruses.

Acknowledgements

We thank Professor Z. Kluge (Germany) for the initiation of research and precious advice. This work was supported by the subsidy allocated to Kazan Federal University for the state assignment in the sphere of scientific

activities (Project No. 14-83 0211/02.11.10083.001) accordance with the Russian Government Program of Competitive Growth of Kazan Federal University.

References

[1] Halterman, D., Charkowski, A. and Verchot, J. (2012) Potato, Viruses, and Seed Certification in the USA to Provide Healthy Propagated Tubers. *Pest Technology*, **6**, 1-14.

[2] Conrad, P.L. (1991) Potato Virus S-Free Plants Obtained Using Antiviral Compounds and Nodal Segment Culture of Potato. *American Journal of Potato Research*, 68, 507-511.

[3] Brown, F. (2001) Inactivation of Viruses by Aziridines. *Vaccine*, **20**, 322-327.

[4] Nascimento, L.C., Pio-Ribeiro, G., Willadino, L. and Andrade, G.P. (2003) Stock Indexing and Potato Virus Y Elimination from Potato Plants Cultivated *in Vitro*. *Scientia Agricola*, **60**, 525-530. http://dx.doi.org/10.1590/S0103-90162003000300017

[5] Trifonova, E.A., Komarova, M.L., Leonova, N.S., Shcherban, A.B., Kochetov, A.V., Malinovskii, V.I. and Shumnyi, V.K. (2004) Transgenic Potato (*Solanum tuberosum* L.) Plants Expressing the Gene of Secretory Nuclease from *Serratia marcescens*. *Doklady Biochemistry and Biophysics*, **394**, 39-41.

[6] Zhou, W.W. and Niu, T.G. (2009) Purification and Some Properties of an Extracellular Ribonuclease with Antiviral Activity against Tobacco Mosaic Virus from *Bacillus cereus*. *Biotechnology Letters*, **31**, 101-105.

[7] Zhou, W.W., He, Y.L., Niu, T.G. and Zhong. J.J. (2010) Optimization of Fermentation Conditions for Production of Anti-TMV Extracellular Ribonuclease by *Bacillus cereus* Using Response Surface Methodology. *Bioprocess and Biosystems Engineering*, **33**, 657-663.

[8] Fedorova, A.A., Azzami, K., Ryabchikova, E.I., Spitsyna, Y.E., Silnikov, V.N., Ritter, W., Gross, H.J., Tautz, J., Vlassov, V.V., Beier, H. and Zenkova, M.A. (2011) Inactivation of a Non-Enveloped RNA Virus by Artificial Ribonucleases: Honey Bees and Acute Bee Paralysis Virus as a New Experimental Model for *in Vivo* Antiviral Activity Assessment. *Antiviral Research*, **91**, 267-277. http://dx.doi.org/10.1016/j.antiviral.2011.06.011

[9] Leshchinskaia, I.B., Kleĭner, G.I., Volkova, T.I., Balaban, N.P. and Sharipova, M.R. (1981) Isolation and Purification of Alkaline Ribonuclease from *Bacillus* Intermedius. *PriklBiokhimMikrobiol*, **17**, 241-246.

[10] Kluge, S. and Marcinka, K. (1979) The Effects of Polyacrylic Acid and Virazole on the Replication and Component Formation of Red Clover Mottle Virus. *Acta Virologica*, **23**, 148-152.

[11] Murashige, T.A. (1962) Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Plant Physiology*, **15**, 473-497.

[12] Crosslin, J.M. and Hamlin, L.L. (2011) Standardized RT-PCR Conditions for Detection and Identification of Eleven Viruses of Potato and Potato Spindle Tuber Viroid. *American Journal of Potato Research*, **88**, 333-338.

[13] Sharipova, M.R. (2002) Late Stages of Protein Secretion in *Bacilli*. *Biochemistry*, **67**, 1207-1216. http://dx.doi.org/10.1023/A:1021301502160

[14] Grishina, I.B., Makarov, A.A., Kuznetsov, D.V., Vasil'ev, D.G., Palovskiĭ, A.G., Aĭzenmenger, F. and Esipova, N.G. (1993) Ionic Pairs in *Bacillus* Intermedius 7P Ribonuclease. *Biofizika*, **38**, 22-30.

[15] Demchuk, I.V., Zaritsky, N.M. and Volkova, I.V. (2011) Efficiency of Biotechnology Disease Eradication System for the Potato Varieties. *POTATO-GROWING*: *Proceedings*, Vol. 19, RUE: Research and Practical Center of NAS of Belarus for Potato, Fruit and Vegetable Growing, Minsk, 221-230.

[16] Malinovsky, V.I. (2010) Resistance Mechanisms of Plants to Viruses. Dalnauka, Vladivostok, 191-192.

[17] Mercer, J.L., Schelhaas, M. and Helenius, A. (2010) Virus Entry by Endocytosis. *Annual Review of Biochemistry*, **79**, 803-833.

[18] Fartais, L. (1998) The Regeneration Capacity of Potato Explains Stored on Media with Growth Inhibitors. Vol. 44, Analele Stiintificeaie Universitatii Al. I. Cuza din lasi (Romania) Sec.2a, 69-73.