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Correlation between Influence of Polysaccharides on Hydrolase Activity and Their Antiviral Effect in Tobacco Leaves

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Abstract—The activities of hydrolases (acid phosphatase, RNase, and proteases) in healthy and tobacco mosaic virus-infected leaves of *Nicotiana tabacum* L. var. Samsun, both untreated and treated with polysaccharides (PS) (1,3;1,6-β-D-glucan, fucoidan, and κ/β-carrageenan), were determined. The PS lead to substantial increase in the hydrolase level. The percentage of viral particles undergoing destructive change also increases in leaves treated with PS 24 h before infection. We suppose that the PS-mediated hydrolase activation promotes intracellular destruction of the viral particles and, thus, comprises one of the PS-induced protective mechanisms limiting intracellular viral accumulation.

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Key words: 1,3;1,6-β-D-glucan, fucoidan, κ/β-carrageenan, acid phosphatase, RNase, protease, tobacco mosaic virus

Development of anti-pathogen methods [1, 2], particularly directed to viruses [1, 3], has an attractive possibility of plant defense based on stimulation of natural resistance using ecologically safe natural substances. Promising inducers of antiviral plant resistance include various polysaccharides (PS), such as 1,3;1,6-β-D-glucans from fungi [4] and brown algae [5, 6], 1,3;1,4-β-D-glucan from the lichen *Cetraria islandica* [7], chitosan [8], κ/β-carrageenan from the marine red alga *Tichocarpus crinitus* [9], and fucoidan from the alga *Fucus evanescens* [10]. Nevertheless, mechanisms of PS antiviral effect are yet not well understood.

Some data are indicative of an important role of hydrolases, such as chitinases and 1,3-β-glucanases [11], proteases [12, 13], and RNases [14] in plant protection against pathogens. Chitinases and glucanases degrade the cell wall of pathogenic fungi and bacteria, respectively [11]. Their antiviral action is likely associated with production of oligosaccharides that play a role as signaling molecules triggering various protective mechanisms in the cells [15]. P-69, an alkaline endoprotease induced by citrus exocortis viroid in tomato leaves, is one of major pathogenesis-related (PR) proteins of this host [16]. Vacuolar protease found in tobacco plants infected with tobacco mosaic virus (TMV) and carrying the resistance gene *N* possibly plays a crucial role in induction of virus-mediated hypersensitive response [17]. RNases may be involved in plant antiviral resistance caused by various agents (gibberellin, steroid glycosides, vaccination with viral weak strains, etc.) [18]. A PR protein isolated from hot pepper (*Capsicum annuum* L.) possesses both RNase and antiviral activities [19]. Antiviral proteins of some plants possess strong RNase activity [20, 21].

We conceptualize that a lytic compartment plays an important role in antiviral defense of cells by means of destruction of virions by hydrolases, such as proteases and RNases [3, 18]. It was shown [22] that 1,3;1,6-β-D-glucan activates the lytic compartment in leaves of tobacco (cultivar Samsun) and Jimson weed (*Datura stramonium* L.) infected with TMV and potato virus X (PVX), respectively, which manifests as increase in amount of smooth endoplasmic reticulum (ER) elements, dictyosomes, cytoplasmic vacuoles, multivesicular bodies, and cytosegresomes comprising [18] this compartment. Recently, similar data were obtained from microscopic observation of effects of fucoidan [23] and κ/β-carrageenan [24] on the development of PVX-induced infection in *D. stramonium* L. leaves.

Abbreviations: ER, endoplasmic reticulum; PS, polysaccharide; PTA, phosphotungstic acid; PVX, potato virus X; TMV, tobacco mosaic virus.

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One of the hallmarks of plant cells infected by viruses is smooth ER cistern swelling [3], which likely reflects abnormal changes of reticular membranes resulting in impairment of their barrier function. The swelling of ER cisterns may result in release of hydrolases from the ER lumen into the cytosol [3]. This view is supported by cytochemical data [25] suggesting localization of acid phosphatase in the lumen of swollen ER cisterns, on their membranes, and in adjacent cytoplasmic area in host cells infected with TMV and PVX. The hydrolases released from ER cisterns can cause lytic processes leading to destruction of both cell structures and viral particles. The destructive changes of viral particles manifest as appearance of subtle virions with low contrast in ultrathin sections [3, 25].

It was hypothesized [22-24] that the observed activation of the lytic compartment by polysaccharides (1,3;1,6-β-D-glucan, fucoidan, and κ/β-carrageenan) in cells infected with TMV and PVX should cause appearance of thin, disintegrating viral particles, thus counteracting the intracellular accumulation of infectious virions.

In this work we studied effects of PS (1,3;1,6-β-D-glucan, fucoidan, and κ/β-carrageenan) on activity of hydrolases (acid phosphatase, RNase, and proteases) in healthy and TMV-infected tobacco leaves, as well as on the state of TM virions in the infected leaves.

MATERIALS AND METHODS

Preparation of 1,3;1,6-β-D-glucan Antivir. Laminarin was purified from the brown alga *Laminaria cichorioides* by the previously described method [26]. The 1,3;1,6-β-D-glucan was prepared by transformation of laminarin with endo-1,3-β-D-glucanase from the marine mollusk *Chlamys albidus* [27]. Thus obtained 1,3;1,6-β-D-glucan is of higher molecular weight and more branched than the initial laminarin.

Fucoidan was isolated from the brown alga *Fucus evanescens* [28]. It is a 1,3;1,4-α-L-fucan sulfated at C2-position of fucose residues [29].

κ/β-Carrageenan was isolated from the red marine alga *Tichocarpus crinitus* (Gmel.) Rupr. Dried alga biomass was disintegrated, mixed with 30 volumes of water, and incubated for 3 h at 90°C with continuous agitation. The extract was filtered and centrifuged at 4000 rpm for 20 min. Solid residues were subjected to two rounds of extraction under the same conditions. The extracts were pooled and fractionated into gelating and non-gelating PS according to a described method [30]. Using IR- and NMR-spectroscopy, the gelating PS were classified as κ/β-carrageenans [31].

Preparation of TMV. Tobacco mosaic virus (TMV), strain vulgare, was cultured on tobacco plants (*Nicotiana tabacum* L., cultivar Samsun) grown in a greenhouse. TM virions were purified as described in [32].

Determination of hydrolase activities in leaves. Four-week-old plants of *N. tabacum* L., cultivar Samsun, grown in a greenhouse were used. Young leaves (5-6 cm in length) were dissected along the costa and dusted with carborundum. The left halves of the leaves (experimental) were rubbed with PS (ether glucan or fucoidan or carrageenan) at concentration of 1 mg/ml and the right ones (control) with water. Following 10 min of incubation, the leaves were washed with tap water and placed into a humid chamber. After one day, the left and right halves of the leaves were dusted with carborundum again, and half was rubbed with TMV suspension (1 mg/ml) and another half with water. Then the leaves were washed and returned into the humid chamber. Enzymatic activities (see below) were determined in 50 disks (4 mm in diameter) that were cut from healthy and infected leaves four days after infection and expressed in terms of optical density. The experiments were repeated 10 times. The data were statistically processed using Student's *t*-test.

Acid phosphatase [33]. Leaf discs were pasted up in a chilled mortar with 2 ml of incubation mixture composed of 0.05 M Na-acetate buffer, pH 5, and 0.05 M β-glycerophosphate as a substrate. The same samples without substrate served as control. Thus prepared suspensions were incubated for 1 h at 37°C. Reaction was terminated by addition of 2 ml of 10% trichloroacetic acid (TCA). Samples were kept for 10 min in the cold and filtered. Filtrates (3 ml) were supplied with 1 ml of 2.5% ammonium molybdate solution in 1.5 M H_2SO_4 and 0.5 ml of 0.4% freshly prepared ascorbic acid solution in distilled water. The mixture volume was adjusted to 10 ml with ammonium molybdate solution and after 10 min the optical absorbance at 540 nm was measured using a photocolorimeter.

RNases [34]. Leaf disks were ground with 1.5 ml of distilled water, and the homogenates were centrifuged for 30 min at 4500 rpm. Supernatants (0.1 ml) were added to a reaction mixture composed of 0.5 ml of 0.1 M Na-acetate buffer, pH 5.6, and 0.4 ml of yeast RNA (3 mg/ml). The same samples without RNA served as control. The mixtures were incubated for 1 h at 37°C. The reaction was terminated by addition of 1 ml of 0.75% uranyl acetate solution in 25% perchloric acid. Following 15-min incubation at 2°C, the pellet was removed by centrifugation at 4500 rpm. Then 0.2 ml of supernatant was placed into 5 ml of 0.1 M Na-acetate buffer, pH 5.6, and absorbance at 260 nm was measured.

Proteases [35]. Disks from healthy and infected leaves were ground with 5 ml of 0.1 M Na-phosphate buffer, pH 5.7, and the homogenates were centrifuged for 15 min at 5500 rpm. Each sample was halved, and each half (2 ml) was placed into a separate tube; 1 ml of 1% casein was placed into one tube and 1 ml of distilled water into the other (control) tube. The mixtures were incubated for 18 h at 37°C. The reaction was terminated by addition of 2 ml of 10% TCA or $HClO_4$. Samples were

kept for 15 min in the cold, filtered, and absorbance at 280 nm was measured.

Transmission electron microscopy (TEM). Samples were negatively contrasted with phosphotungstic acid (PTA) as described in [36]. Hydrolase activities were preliminarily determined in the TMV-infected tobacco leaves. Suspension of succus from tobacco leaves four days after infection was applied onto formvar-coated TEM grids stabilized with evaporated carbon film, then a drop of 2% PTA, pH 7.0, was added. After 2 min the grids were carefully dried with a piece of filter paper and examined under a LIBRA-120 electron microscope (Carl Zeiss, Germany). In some experiments fragments of infected leaves were prefixed for 3 h with 6.5% glutaraldehyde dissolved in Na-phosphate buffer, pH 7.4. Diameter of virions negatively stained with PTA was measured in 20 random fields of the micrograph at overall 150,000× magnification. Four hundred virions were measured on each experimental and control preparation.

Immunoelectron microscopy of TMV particles in infected leaf succus was carried out using commercially available antisera prepared in the All-Russian Research and Development Institute of Potato Production, Moscow Region, Russia. Immunoelectron microscopic decoration was used [36]. The infected leaf fragments, either treated or untreated with PS, were shred with a blade in a drop of distilled water, the debris was removed, and a formvar-coated grid stabilized with evaporated carbon film was placed onto the surface of the drop. After 1 min the grid was dried with a piece of filter paper, placed onto a drop of TMV-specific antiserum, and incubated in a humid chamber for 15 min at 37°C. In some experiments either a PVX-specific antiserum or control serum were used. The prepared samples were thoroughly washed with distilled water, dried with filter paper, contrasted for 3-5 min with 2% PTA, pH 7.0, and examined under the electron microscope.

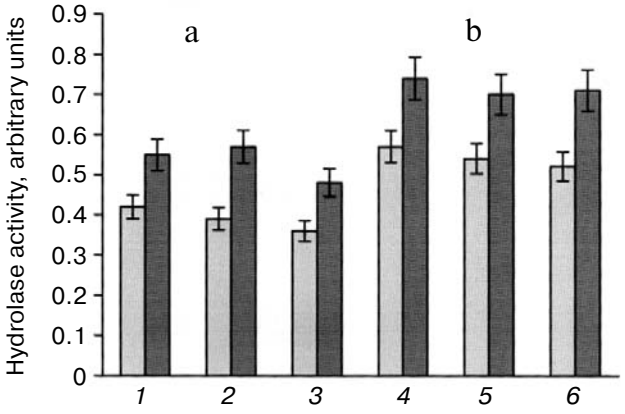

Fig. 1. Activities of acid phosphatase (*1*, *4*), RNase (*2*, *5*) and proteases (*3*, *6*) in healthy (a) and TMV-infected (b) tobacco leaves, either treated (dark-gray) or untreated (light-gray) with glucan. The difference was regarded as significant at $p < 0.05$.

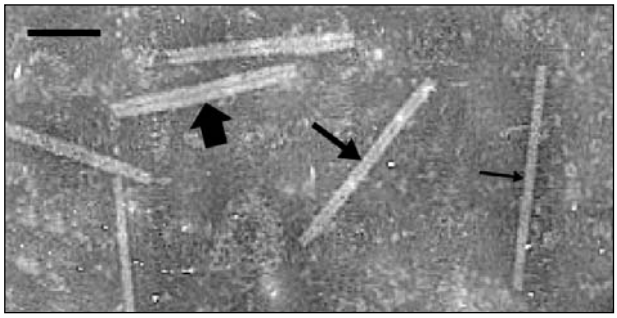

Fig. 2. PTA-stained TMV particles from glucan-treated tobacco leaves 4 days after infection. Medium, bold, and thin arrows indicate normal, swollen, and thin virions, respectively. Scale bar is 100 nm.

RESULTS AND DISCUSSION

Effect of PS on hydrolase activities in tobacco leaves. The treatment of tobacco leaves, both healthy and TMV-infected, with PS (1 mg/ml) results in considerable increase in activity of hydrolytic enzymes (acid phosphatase, RNase, and proteases). Activity of acid phosphatase was 1.31 times, RNase 1.46 times, and proteases 1.33 times higher in healthy leaves treated with glucan in comparison with control (Fig. 1). Similarly, the treatment with fucoidan or carrageenan resulted in increase of activities of acid phosphatase by 1.34 and 1.33 times, RNase by 1.5 and 1.37 times, and proteases by 1.36 and 1.3 times, respectively. Infection of PS-untreated tobacco leaves with TMV resulted in average elevation of activities of acid phosphatase by 1.36 times, RNase by 1.38 times, and proteases by 1.44 times in comparison with control (Fig. 1). Infection of PS-treated leaves with TMV also led to significant increase in activity of hydrolases. The treatment with glucan followed by the infection resulted in increase in activity of acid phosphatase by 1.3 times, RNase by 1.3 times, and proteases by 1.29 times in comparison with the untreated infected leaves (Fig. 1). The treatment of leaves with fucoidan or carrageenan and infection with TMV one day after the treatment was accompanied by elevation (in comparison with control) of acid phosphatase, RNase, and protease activities by 1.36 and 1.3 times, 1.3 and 1.29 times, and 1.49 and 1.32 times, respectively (data not shown).

Effect of PS on the state of TMV particles in infected tobacco leaves. Together with determining activities of hydrolytic enzymes in PS-treated and untreated leaves infected with TMV, we examined the state of viral particles in TEM preparations from these leaves negatively stained with PTA. Along with normal TMV particles (~18 nm in diameter), these preparations also contained

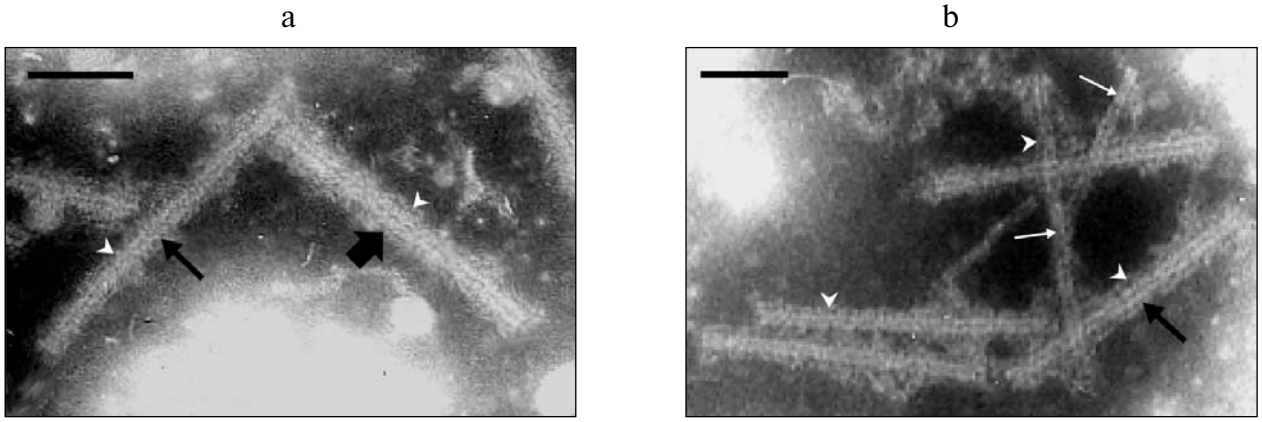

Fig. 3. TMV particles isolated from glucan-treated tobacco leaves 4 days after infection, treated with specific antiserum, and stained with PTA. Medium, bold, and thin arrows indicate normal, swollen, and thin virions, respectively. Arrowheads point to TMV-specific antiserum. Scale bar is 100 nm.

swollen and thin virions (Fig. 2). The PTA-stained preparations of infected leaves prefixed with glutaraldehyde contained about the same percentage of normal and abnormal (swollen and thin) virions as did the preparations from infected leaves prepared without prefixation.

Immunoelectron microscopy has shown that normal TMV particles (Fig. 3, a and b), as well as swollen virions (Fig. 3a) are completely coated with specific antiserum. Thin virions lose ability for decoration with antibodies; only some areas of them can interact with the antiserum (Fig. 3b). Control and anti-PVX sera do not bind with TMV particles.

Analysis of distribution of TMV particles by diameter showed that suspensions from PS-treated leaves contained significantly higher percentage of thin virions than those from untreated leaves. Preparations from the leaves treated with glucan contain 42% normal, 13% swollen, and 45% thin virions (Fig. 4). These values are 59, 11, and 30%, respectively, in preparations from infected leaves not treated with PS (Fig. 4).

Infected leaves treated with fucoidan or carrageenan one day before the infection contained 39 and 45% normal, 11.5 and 15.9% swollen, and 49.5 and 39.1% thin virions, respectively. Preparations from the infected leaves not treated with PS contained 58 and 56% normal, 13.6 and 14.5% swollen, and 28.4 and 29.5% thin virions, respectively.

Thus, the treatment of healthy and TMV-infected tobacco leaves with PS (1,3;1,6-β-D-glucan, fucoidan, and κ/β-carrageenan) is accompanied by increase in activity of hydrolases (acid phosphatase, RNase, and proteases). These data correlate with PS-mediated lytic compartment activation, which was observed earlier in host plant cells infected by viruses [22-24].

Electron microscopy of PTA-stained preparations of succus from TMV-infected tobacco leaves (untreated and treated with PS) has confirmed the earlier data [25] on destructive changes of virus particles manifested as appearance of swollen and thin virions. It is worth noting that prefixation with glutaraldehyde has no significant effect on the ratio between normal and abnormal virions, so the observed abnormal virus particles in TEM preparations are not an artifact.

We presume that formation of thin virions in the infected cells is a biphasic process. Alterations of certain physical and chemical parameters, in particular, pH, in cell compartments containing virus can lead to conformational modification (loosening and partial untwisting) of viral protein subunits. The loosening of protein subunits likely contributes to appearance of swollen virions, while the partial untwisting to limited proteolysis of exposed peptide bonds resulting in virion thinning. A similar explanation was proposed for destruction of adenovirus 2 proteins [37]. The fact that thin TMV particles, unlike normal virions, lose capability of binding with a

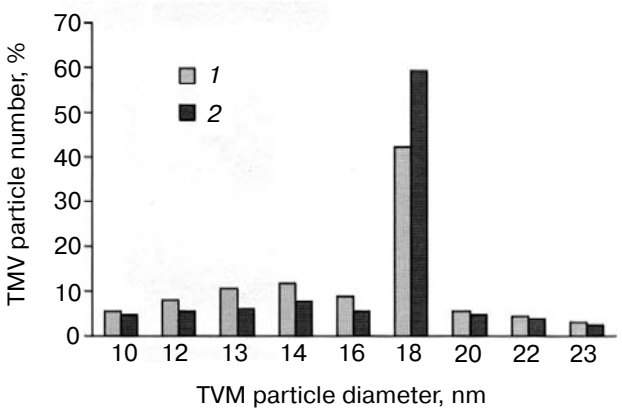

Fig. 4. TMV diameter distribution in preparations (suspensions) from glucan-treated (*1*) and untreated (*2*) tobacco leaves 4 days after infection.

specific antiserum seems to depend on limited proteolysis of the capsid protein accompanied by elimination of domains responsible for the binding with antibodies.

Increase in amount of thin virions in the infected leaves pretreated with PS suggests correlation between the PS-determined elevation of hydrolase (particularly protease) activities and the intracellular virion destruction. We anticipate that elevation of hydrolase activities favoring virion destruction is one of the chemical-induced protective mechanisms limiting intracellular viral accumulation.

It is important that cell treatment with PS also leads to elevation of hydrolase activities in healthy plant tissues. It is likely that the elevated level of lytic activity in the cells of PS-treated leaves is a factor facilitating destruction of potential viral pathogen and thus elevating virus resistance of plant tissues.

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REFERENCES

1. Lyon, G. D., Reglinski, T., and Newton, A. C. (1995) *Plant Pathol.*, **44**, 407-427.
2. Vasyukova, N. I., Chalenko, G. I., Gerasimova, N. G., Valueva, T. A., and Ozeretskovskaya, O. L. (2008) *Appl. Biochem. Microbiol.*, **44**, 213-217.
3. Reunov, A. V. (1999) *Viral Pathogenesis and Protective Mechanisms of Plants* [in Russian], Dal'nauka, Vladivostok.
4. Kopp, M., Rouster, J., Fritig, B., Darvill, A., and Albersheim, P. (1989) *Plant Physiol.*, **90**, 208-216.
5. Reunov, A. V., Lapshina, L. A., Nagorskaya, V. P., and Elyakova, L. A. (1996) *J. Phytopathol.*, **144**, 247-249.
6. Elyakova, L. A., Isakov, V. V., Lapshina, L. A., Nagorskaya, V. P., Likhatskaya, G. N., Zvyagintseva, T. N., and Reunov, A. V. (2007) *Biochemistry (Moscow)*, **72**, 29-36.
7. Stubler, D., and Buchenauer, H. J. (1996) *J. Phytopathol.*, **144**, 37-43.
8. Chirkov, S. N. (2002) *Appl. Biochem. Microbiol.*, **38**, 1-8.
9. Reunov, A., Nagorskaya, V., Lapshina, L., Yermak, I., and Barabanova, A. (2004) *J. Plant Dis. Protect.*, **111**, 165-172.
10. Lapshina, L. A., Reunov, A. V., Nagorskaya, V. P., Zvyagintseva, T. N., and Shevchenko, N. M. (2006) *Rus. J. Plant Physiol.*, **53**, 246-251.
11. Van Loon, L. C., Rep, M., and Pieterse, C. M. (2006) *Annu. Rev. Phytopathol.*, **44**, 135-162.
12. Van der Hoorn, R. A. L. (2008) *Annu. Rev. Plant Biol.*, **59**, 191-223.
13. Shindo, T., and van der Hoorn, R. A. L. (2008) *Mol. Plant Pathol.*, **9**, 119-125.
14. Trifonova, E. A., Kochetov, A. V., and Shumnyi, V. K. (2007) *Uspekhi Sovrem. Biol.*, **127**, 13-24.
15. Ryan, C. A. (1988) *Biochemistry*, **27**, 8879-8883.
16. Vera, P., and Conejero, V. (1988) *Plant Physiol.*, **87**, 58-63.
17. Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2004) *Science*, **305**, 855-858.
18. Reunov, A. V., and Reunov, A. A. (2008) *Lytic Function of the Cell* [in Russian], Nauka, Moscow.
19. Park, C. J., Kim, K. J., Shin, R., Park, J. M., Shin, Y. C., and Paek, K. H. (2004) *Plant J.*, **37**, 186-198.
20. Begam, M., Narwal, S., Roy, S., Kumar, S., Lodha, M. L., and Kapoor, H. C. (2006) *Biochemistry (Moscow)*, **71**, Suppl. 1, S44-S48.
21. Choudhary, N. L., Yadav, O. P., and Lodha, M. L. (2008) *Biochemistry (Moscow)*, **73**, 273-277.
22. Nagorskaya, V. P. (2000) *Ultrastructural Aspects of Viral Pathogenesis and Plant Resistance Induced with 1,3;1,6-β-D-Glucan*: Author's abstract of Candidate dissertation [in Russian], PIBC FEB RAS, Vladivistok.
23. Reunov, A. V., Lapshina, L. A., Nagorskaya, V. P., Zvyagintseva, T. N., and Shevchenko, N. M. (2009) *J. Plant Dis. Protect.*, **116**, 49-54.
24. Nagorskaya, V. P., Reunov, A. V., Lapshina, L. A., Ermak, I. M., and Barabanova, A. O. (2010) *Biol. Bull.*, **37**, 653-658.
25. Reunov, A. V. (1989) *Cytopathology of Plant Cell Infected with Viruses (TMV and PVX) and the Problem of Plant Resistance*: Author's abstract of doctoral dissertation [in Russian], Institute of Biology and Virology, Kiev.
26. Zvyagintseva, T. N., Shevchenko, N. M., Popivnich, I. B., Isakov, V. V., Scobun, A. S., Sundukova, E. V., and Elyakova, L. A. (1999) *Carbohydr. Res.*, **322**, 32-39.
27. Zvyagintseva, T. N., Elyakova, L. A., and Isakov, V. V. (1995) *Bioorg. Khim.*, **21**, 218-225.
28. Zvyagintseva, T. N., Shevchenko, N. M., Chizhov, A. O., Krupnova, T. N., Sundukova, E. V., and Isakov, V. V. (2003) *J. Exp. Marine Biol. Ecol.*, **294**, 1-13.
29. Kusaikin, M. I., Chizhov, A. O., Alekseeva, S. A., Bakunina, I. Yu., Nedashkovskaya, O. I., Sova, V. V., and Zvyagintseva, T. N. (2004) *Dokl. Ros. Akad. Nauk*, **396**, 691-693.
30. Yermak, I. M., Kim, Y. H., and Titlyanov, E. A. (1999) *J. Appl. Phycol.*, **11**, 41-48.
31. Barabanova, A. O., Yermak, I. M., Glazunov, V. P., Isakov, V. V., Titlyanov, E. A., and Solov'eva, T. F. (2005) *Biochemistry (Moscow)*, **70**, 350-356.
32. Otsuki, Y., Takebe, I., Onho, T., Fukuda, M., and Okada, Y. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1913-1917.
33. Rai, J. N., Srivastava, S. K., and Dhawan, S. (1979) *Microbios*, **25**, 107-110.
34. Tatarskaya, R. I., Abrosimova-Amelyanchik, N. M., Aksel'rod, A. I., Korenyako, A. A., Niedro, N. Ya., and Baev, A. A. (1966) *Biokhimiya*, **31**, 1017-1026.
35. Alekseenko, L. P. (1968) *Methods for Determination of Proteolytic Enzymes*, in *Modern Methods in Biochemistry* [in Russian], Meditsina, Moscow, pp. 115-130.
36. Korolev, M. B. (1980) *Electron Microscopic Methods for Virus Identification*, in *Electron Microscopy of Viruses and Viral Infections. Advances in Science and Technology, Series Virusology* [in Russian], VINITI, Moscow, pp. 114-157.
37. Everitt, E., Persson, M. J., and Wohlfart, C. (1988) *FEMS Microbiol. Lett.*, **49**, 229-233.