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Stimulation of Cellular Mechanisms of Potato Antivirus Resistance by the Action of a Preparation Based on *Bacillus subtilis* Bacteria

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Abstract—A comparative study of the antiviral activity of a new biopreparation based on bacteria *Bacillus subtilis* (strain 47) and commercial biopesticides (beta-protectin, phyto-protectin, frutin) in potato plants of Belarusian selection (Lileya and Scarb) was carried out in vivo and ex vitro. Pretreatment of plants with *B. subtilis* biopreparation and biopesticides prevents infection by potato X-viruses. The antiviral efficacy of *B. subtilis* does not depend on the conditions of plant growth and is more effective than biopesticides. Increased potato plant resistance to viral infection was accompanied by an effect leading to an increase in the mass of minitubers and the dry matter content in them. Treatment of potato plants with *B. subtilis* did not affect the molecular heterogeneity of peroxidase and superoxide dismutase, but it changed the relative activity of their isoforms. Treatment with a bacterial preparation increased the activity of superoxide dismutase when it was applied both to intact plants and those preinfected with virus. The results indicate that pretreatment of potato plants with the B. *subtilis* drug prevents virus infection, inducing the antiviral resistance of the potato, and is accompanied by a change in the activity of redox enzymes.

Keywords: potato, in vivo and ex vitro culture, *Bacillus subtilis*, potato X-virus, superoxide dismutase, peroxidase, antiviral drugs, plant immunity

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INTRODUCTION

At present, biologically active preparations—immunostimulators—are successfully applied to intensify plant protection reactions [1, 2]. The biological activity of such preparations is related to the presence of specific elicitor signal molecules that induce (or intensify) in the plant organism the pathogen penetration signal [3].

According to recent views, the mechanisms of the induction of plant immune reactions include enhancement of the expressive activity of genes that release a cascade of consecutive biochemical reactions, which leads to the synthesis of protective compounds (phytoalexines, PR proteins, active oxygen species), and activation of the hormonal and enzyme systems responsible for restructuring of the cell metabolism, which leads to modification of the physiological state of plants and, generally, to activation of their immune status [4, 5].

One of the first plant responses to a pathogen attack is the reaction of supersensitivity (SS), which is accompanied by oxidative stress and the formation of various oxygen active forms with the participation oxidoreductases (peroxidase, oxalate oxidase, superoxid dismutase, etc. [6].

At present, preparations based on bacteria *B. subtilis* are thus widely used for plant protection from phytophages, since they suppress pathogen growth and development and stimulate plant growth and resistance to unfavorable environmental factors [7]. The enhanced plant resistance to pathogens that results from the use of such preparations is attributed to the ability of bacteria *B. subtilis* to produce various metabolites having antiviral, antibacterial, and antifungal actions [8]. Lipopeptides produced by bacteria, as signal molecules, favor the development of systemic induced stability (**SIS**), which results from transduction of H_2O_2 generation and the lipoxigenase signal cascade [9]. Screening and isolation of new strains of *B. subtilis* possessing antiviral activity considerably widen the prospects of the development of new preparations. A purposeful search for and selection of *B. subtilis* strains having economically valuable char-

Table 1. Pattern of experiments

Experimental variant	Infestation	Expenditure dose of preparation	Mode of application
Control-1 (no viruses)	None	–	No preparation
Control-2 (infested with XVP and YVP)	XVP YVP	None	No preparation
Preparations for comparison: Betaprotectin, Phytoprotectin, Frutin	XVP YVP	10 mL in 1 L of medium MS per 300 plants	Under conditions in vitro at clonal microreproduction of potato; single introduction in medium MS
Bacillus subtilis	XVP YVP	10 mL in 1 L of medium MS per 300 plants	Under conditions in vitro at potato clonal microreproduction; single introduction in medium MS

acteristics requires knowledge of the mechanisms of plant resistance formation under their action.

New information on the functioning of oxidative-reductive enzymes upon plant infestation with viruses and the manifestation of protective responses, including that induced by biopreparations based on bacteria *B. subtilis,* is interesting, both for fundamental science and applied science.

The goal of the study is to study the influence of preparations based on *B. subtilis* bacteria on potato antiviral resistance related to modification of the activity and molecular heterogeneity of oxidative-reductive enzymes.

METHODS

Objects and methods. The object of study was the potato (*Solanum tuberosum* L.) of middle-early cultvars of Belarus selection (Lilea and Scarb). For the experiments, sanitized culture of plants in vitro raised on medium Murasiga-Skuga (MS) was used; apical potato cuttings were rooted in vivo and grown to seedlings. The plants were grown under controlled conditions until a crop of minitubers was obtained.

The potato culture was grown in vivo on the artificial ion-exchange substratum Triona® [10]. The plants were placed on BTC-1 biotechnical complexes developed by the Institute of Experimental Botany, Belarus National Academy of Sciences, in vegetation conditions with a day/night photoperiod of 16/8 h and humidity of 60–70%. The light sources were DNaZ (produced in Saransk, RF) (λ_{max}= 594–600 nm). The plants were irrigated with distilled water (at the seedling stage) or with settled tap water (at the stage of tuber formation) with a pH of 6.4.

The spore-forming bacteria *Bacillus subtilis* strain 47 were used from the collection of the Institute of Microbiology, Belarus National Academy of Sciences. The submerged cultivation was carried out in flasks on a shaker over two days. The bacterial were then settled by centrifugation at 6000 *g* for 30 min. The obtained cells were suspended in a small quantity of supernatant (50 mL), processed in an RDN-1W (Russian Federation) ultrasound vertical laboratory disperser for 4 min, and then diluted with supernatant until a cell concentration of 3.4×10^9 CFU. The obtained suspension (preparation), diluted with distilled water 50 times, was used to spray and irrigate plants.

Treatment with commercial protective microbiological biopesticides (betaprotectin, phytoperotectin, frutin) was made according to the instructions (Institute of Microbiology, Belarus National Academy of Sciences).

The plant material was inoculated by a microinjection of 100 μL of cell sap with the virus into the apical part of 20-day old regenerants, with the simultaneous application of 250 μL of cell sap of XPV- and YPV-donor potato plants onto a damaged part of the leaf blade of the first upper leaf. The initial culture of potato infested with *XVP* virus taken from the collection of the Russian Federal Institute of Potato Growing (Korenevo, Moscow oblast, Russian Federation) is supported at the Institute of Experimental Botany, Belarus National Academy of Sciences. The general pattern of experiments is shown in Table 1.

The protein content was measured by the Bradford method, and the calibration curve was constructed with BSA (Sigma, USA).

Electrophoretic separation of proteins. For electrophoretic separation of proteins of the cytoplasmatic fraction, 0.5 g of leaves were homogenized in 2 mL of buffer containing 50 mM of tris-HCl pH 8.0; 0.3 M saccharose; 2 mM $MgCl_2$; 5 mM of mercaptoethanol; and 0.02% sodium ascorbate. The homogenate was centrifuged at 8500 *g* for 20 min in a Sigma 1-14K microcentrifuge (USA). The supernatant was washed by 0.5 mL and kept at –18°C. Thirty nanoliters of a sample containing 70–100 μg of protein as determined by the Bradford method was applied to an 8 × 10 cm plate with a 1.5 mm–thick apparatus for moni-electrophoresis SE-250 (Ameraham Bioscienses, Great Britain). Electriphoresis was carried out for 4 h.

Determination of peroxidase activity. For peroxidase extraction (EC 1.11.1.7), 0.5 g of leaf tissue was homogenized in 2 mL of 50 mM of tris-HCl buffer,

pH 6.8, containing 0.3 M saccharose, 0.02% sodium ascorbate, 5 mM mercaptoethanol, 2 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluorid (**PMSF**). The homogenate was filtered through a nylon filter (Ø 0.2 mm) and centrifuged at 8500 g for 20 min. The supernatant containing soluble proteins and lightweight membrane fractions was frozen at –18°C and was further used in analysis of the activity of soluble peroxidases.

The wells of flat-bottomed plates for immunoenzyme analysis (Medpolimer, Russian Federation) were washed by 100 μL of plant extract preliminarily diluted 20 times with distilled water; 0.03 mL of chromogenous substrate ortho-phenyldiamine (**OPD**) (Reakhim, Russia) and 0.03 mL of 0.02% H_2O_2 were added. The reaction was by stopped by the addition of 0.05 mL of 4 n solution of H_2SO_4 to the incubated mixture. The plates were scanned in an apparatus for immunoezyme analysis (Vityaz M-430, Belarus) at $\lambda = 472$ nm. The activity unit of the enzyme was the quantity of oxidized substrate causing an increase in the unit of optic density ΔA in 1 min. For comparison, the activity was expressed in relative units/mg wet weight.

Determination of isoenzyme superoxide dismutase (SOD). To determine the superoxide dismutase (SOD) isoenzyme, leaf extracts were incubated in 100 mM tris-HCl buffer, pH 6.8 containing 10% glycerol, 0.5% mercaptoethanol, and 0.03% ascorbic acid. Separation (70 μg of protein) was carried out on vertical plates in 10% PAAG for 2–2.5 h at a current intensity of 20 mA and a voltage of 150 V.

For **SOD** development (EC 1.15.1.1), gel plates were submersed in incubation medium with the following composition: 100 mL of 100 mM tris-HCl mM, pH 8.3, containing 10 mg of riboflavin, 100 μL TEMED, and 40 mg nitroblue tetrazolium. They were incubated for 30 min at 37°C. The dying solution was poured off, and the gel plates were washed with water and exposed to artificial white light (60 W) for 30 min. Isoenzyme localization was revealed by uncolored bands on an intensively blue or violet background. The SOD activity was estimated by the total value of the color of digital image areas of gel corresponding to particular isoenzymes. The total value of the color of points was multiplied by 10^{-6} to obtain conventional units of enzymatic activity.

Peroxidase isoenzymes were determined in plant extracts of 30 μL volume (60 μg of protein) spread on plates with 10% PAAG 1.5 mm thick prepared with buffer containing 50 mM of tris-HCl, pH 6.8; 0.3 M saccharose; 0.02% sodium ascorbate; 2 mM $MgCl_2$; 1 mM PMSF.

Electrophoresis in PAAG was carried out for 4 h at 10 mA, 40 V until penetration of the separating gel and at 20 mA, 140 V in the separating gel in a apparatus for SE-250 mini-eletrophoresis (Amersham Bioscienses, Great Britain). The gel after eletrophoresis was exposed in 0.05% benzidin solution with 0.03% hydrogen peroxide until blue bands of peroxidase isoenzymes appeared.

Determination of potato phytoviruses by immunoenzyme analysis (IEA). Potato phytoviruses were identified by means of immunoenzyme analysis (IEA) with antibodies and conjugates according to standard methods [11]. Leaf material of potato plants in vivo and ex vitro was used. The leaves (weight 2–3 g) were crushed in a mortar and diluted with buffer for samples and conjugates at a ratio of 1 : 10 (wt/vol).

Antigens were determined by a "sandwich"-variant of IEA with standard immunoenzyme diagnostic kits to identify potato viruses (produced by the Russian Federal Institute of Potato Farming, Korenevo, Moscow oblast, Russian Federation), by adsorption on the surface of wells of polystyrol plates (0.1 mL of working solution of antibodies) and incubation overnight at +4°C or 2 h at +37°C in a thermostate. Unbound antibodies were removed from the plate. The tested material and immunosorbent (0.1 mL of the working solution of conjugate) were introduced to wells, and incubation was carried out at 37°C for 1 h. At the next stage, the conjugate of antibodies with horseradish peroxidase (by 0.1 mL of freshly prepared solution of the substrate) was introduced into wells and incubated to develop coloration at a room temperature for 20–30 min. The reaction was then stopped by the addition to each well of 0.05 mL of 3 M H_2SO_4. The quantity of the conjugate bound with antigene (proportional to the antigen content in the sample) was determined photometricallly.

The IEA results were estimated by means of an AFI M/430 immunoenzyme analyzer (Vityaz 430, Belarus) at $\lambda = 492$ nm.

The optical absorption of positive samples should exceed the mean absorption of negative samples by the value threefold standard deviation of negative samples:

$$P = X + 3E,$$

where P is the threshold of significance of positive results; X is the mean value at $\lambda = 492$ nm for the negative control; and 3E is the threefold value of the maximum positive deviation at $\lambda = 492$ nm from the mean in the negative control.

The samples infected with pathogen at $\lambda = 492$ nm had optical density (D_{492}) values exceeding 0.120 relative units.

Statistical treatment of the experimental data was done in Microsoft Excel 2003 and STATISTICA 6.0. In the selection of samples for the extraction of readily soluble proteins and for isoenezyme determination, no less than three plants per studied sample were taken.

Table 2. Antiviral activity of biopreparations on the potato cultivar Scarb under conditions in vivo and ex vitro by the results of immunoenzyme analysis (D_{492})

Experimental variant	Seedlings at BTC-1 (in vivo)	Plants in vessels (ex vitro)
Control 1, without treatment	0.117 ± 0.009	0.117 ± 0.010
Control 2, without treatment + XVP	0.318 ± 0.070	0.300 ± 0.087
Beta protectin + XVP	0.078 ± 0.016	0.104 ± 0.016
Phytoprotectin + XVP	0.126 ± 0.010	0.113 ± 0.009
Frutin + XVP	0.290 ± 0.023	0.386 ± 0.038
B. subtilis + XVP	0.096 ± 0.001	0.096 ± 0.002

RESULTS AND DISCUSSION

Influence of the *B. subtilis* strain 47 preparation on the antiviral resistance of potato leaves. Studies on the development of the presence of phytoviruses in potato leaf tissues by IEA demonstrated that plant pretreatment with the preparation fully protected plants from infestation. It is known that numerical values of the optical density (D_{492}) within 0.0–0.120 indicate the absence of viral infection (according to the methods of the test kit producer [11]). All numerical D_{492} values above 0.120 correspond to the presence of viruses in the incubational mixture; high D_{492} values indicate intensive tissue infestation.

The D_{492} value was 2.106 ± 0.069 for the incubation mixture reflecting the extent of viral infection of leaves of the cultivar Scarb and 2.232 ± 0.074 for that in leaves of the cultivar Lileya. After plant treatment with the *B. subtilis* preparation, the D_{492} values of these cultivars were 0.155 ± 0.098 and 0.102 ± 0.042, respectively.

The IEA results demonstrated the essential differences between viral infection of treated leaves both in the Scarb and Lileya cultivars in relation to nontreated leaves. The data show that pretreatment of potato plants with the bacterial preparation inhibited viral multiplication in tissues. In leaves of the plants treated with the *B. subtilis* drug, the manifestation of viral infection was minimal in the Scarb cultivar and absent in the leaves of the Lileya cultivar. According to modern views, antiviral activity is characteristic of many compounds and bacterial preparations used in in plant protection. This is primarily due to the nonspecific induction of immunity to a wide spectrum of pathogens, including viruses [1–4].

Comparative estimation of potato antiviral resistance under the action of treatment with various biopreparations. The antiviral efficiency of the *B. subtilis* preparation was estimated in comparison with the efficiency of commercial protective microbiological pesticides (betaprotectin, phytolectin, and frutin) widely used to protect the potato from fungal pathogens. The biological efficiency of the preparations was estimated by the decrease in the viral content in potato leaves after treatment with biopreparations. Thus, the used potato plants were grown in biotechnical complexes (BTCs) and in containers with the ion-exchange substratum Triona-M.

The results of experiments demonstrated that pretreatment with biological preparations before infestation with *XVP* variously decreased plant infestation (Table 2). The preparation efficiency did not depend on plant growth conditions: the results were similar under conditions in vivo and ex vitro. The highest inhibiting activity toward the potato virus was manifested by betaprotectin and the *B. subtilis* strain 47 preparation. These preparations fully inhibited *XVP* development in the leaves of plants grown under BTC conditions and in containers on the ion-exchange substratum Triona-M. In all variants of the experiment with these preparations, the D_{492} values after IEA were below 0.120. The phytoprotectin antiviral efficiency was lower than that in the preparations based on *B. subtilis* and betaprotectin (D_{492} = 0.113 and 0.126, Table 2).

The preparation frutin under the conditions of our experiments did not show any inhibitory activity towards *XVP*. The results of IEA of potato leaves in variants with this preparation were at the level of infested control. It should be noted that the preparations based on *B. subtilis* and betaprotectin demonstrated a high antiviral activity also to *YVP,* the potato *Y* virus. In these experimental variants, the D_{492} values also did not exceed 0.100.

The antiviral activity of *B. subtilis* preparation was accompanied by stimulated growth, leading to an increase in the weight of minitubers and the dry matter content in them. Table 3 shows that treatment with the *B. subtilis* preparation was followed by a 22.5% increase in their relative dry matter as compared with untreated plants. Commercial preparations did not have a statistically significant influence on dry matter, which was at the level of the control (Table 3).

The economic efficiency of other preparations used in the experiment was also significantly lower.

The experimental data show that the *B. subtilis* preparation was not worse by its antiviral activity and economic efficiency under various potato growing conditions, and it surpassed commercial microbiological preparations in many cases.

It may be supposed that plant treatment with the *B. subtilis* preparaation stimulated biochemical reactions participating in the formation and manifestation of protective processes that occur during viral infesta-

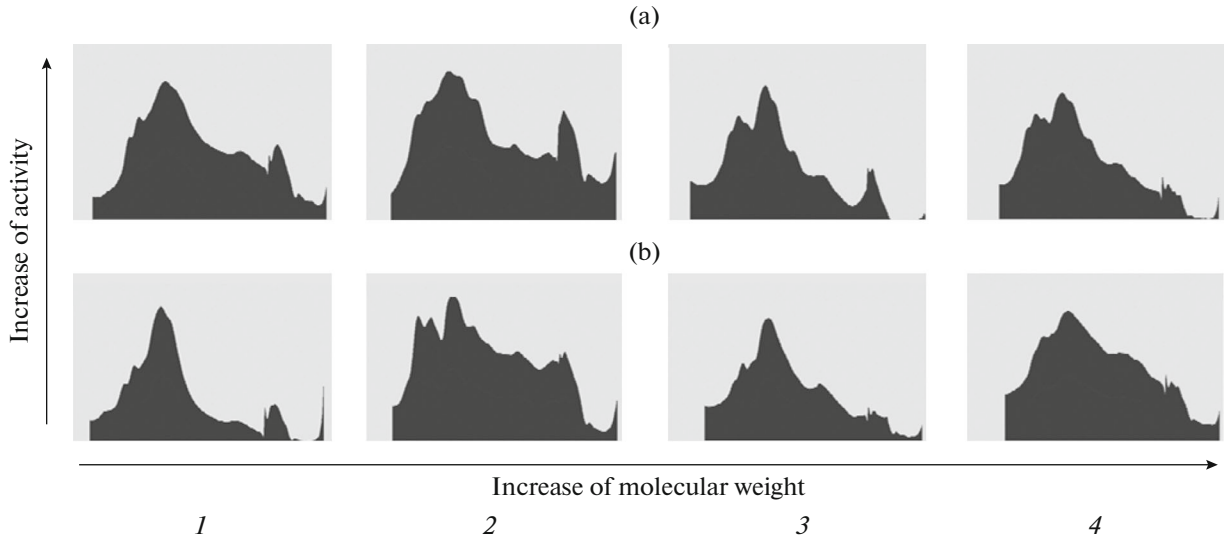

Fig. 1. Densitogram of peroxidase isoforms from leaves of poato plants of cultivars Scarb (a) and Lilea (b) pretreated with *B. subtilis* preparation and infested with XVP. *1*—Untreated noninfected plants; *2*—pretreatment with B. *subtilis*; *3*—pretreatment with B. *subtilis* + *XVP; 4*—*XVP*-infected plants without treatment.

tion. One of these reactions is the activation of oxidative-reducing enzymes, in particular, peroxidase. The latter is a major link in the signal system, the activity of which increases in pathogenesis [12, 13] and causes the expression of protective genes, HR genes, receptor-like kinases, proteinase inhibitors, APA metabolic enzymes, and PR proteins, which control the cellular protective response to infestation [14, 15].

The goal of the experiments was to study the activity and molecular heterogeneity of peroxidases in the process of potato viral infestation before and after treatments with the *B. subtilis* preparation.

Figure 1 shows the densitograms of peroxidase isoforms in the leaves of potato plants treated with the bacterial preparation and infested with *XVP*. Peroxidases were determined electrophoretically 14 days after treatment with the *B. subtilis* preparation and 7 days after plant infestation.

As seen in Fig. 1, in the soluble fraction of potato leaf proteins, there are up to six peroxidase isoforms differing in molecular weight. Plant treatment with *B. subtilis* bacteria and *XVP* infestation did not influence the molecular heterogeneity of these enzymes but modified the relative isoform activity (Fig. 1).

In general, treatment with the *B. subtilis* preparation enhanced the total peroxidase activity in the plant leaves of both cultivars. In potato leaves of Scarb and Lileya cultivars treated with *B. subtilis*, the total peroxidase activity was 1.2 times and 1.96 times higher than in untreated plant leaves of the corresponding cultivars (Fig. 2). In the treated Scarb plants, the total enzymatic activity increased due to the activation of high-molecular forms of enzymes; in the Lileya cultivar, it increased due to the activation of almost all isoenzymes. *XVP* infestation had differing effects on the peroxidase activity in tissues of the studied cultivars. This parameter

Table 3. Weight of tubers and dry weight content in minitubers of potato cultivar Scarb upon plant treatment with biopreparations

Experimental variant	Wet weight of tubers, g	Economic efficiency, % of control 1*	Dry weight of tubers, %	
			of wet weight	of control 1
Control 1 (non infested)	12.4 ± 1.6	–	19.5 ± 0.7	100
Control 2 (infested without treatment)	10.4 + 1.3	45.4	21 + 1.3	100
Betaprotectin + XVP	17.0 ± 2.9	37.1	17.0 ± 0.3	87.2
Phytoprotectin + XVP	14.3 ± 1.3	15.3	21.0 ± 0.3	107.7
Frutin + XVP	16.6 ± 3.0	33.9	18.8 ± 0.5	96.4
B. subtilis + XVP	18.6 ± 3.3	50.0	23.8 ± 0.1	122.5

*Economic efficiency of biological preparations was calculated by the formula $B = \frac{F - P_1}{P} \times 100$, where B is the economic efficiency (%) and P and P_1 are the disease development in the control and experiment, respectively (%).

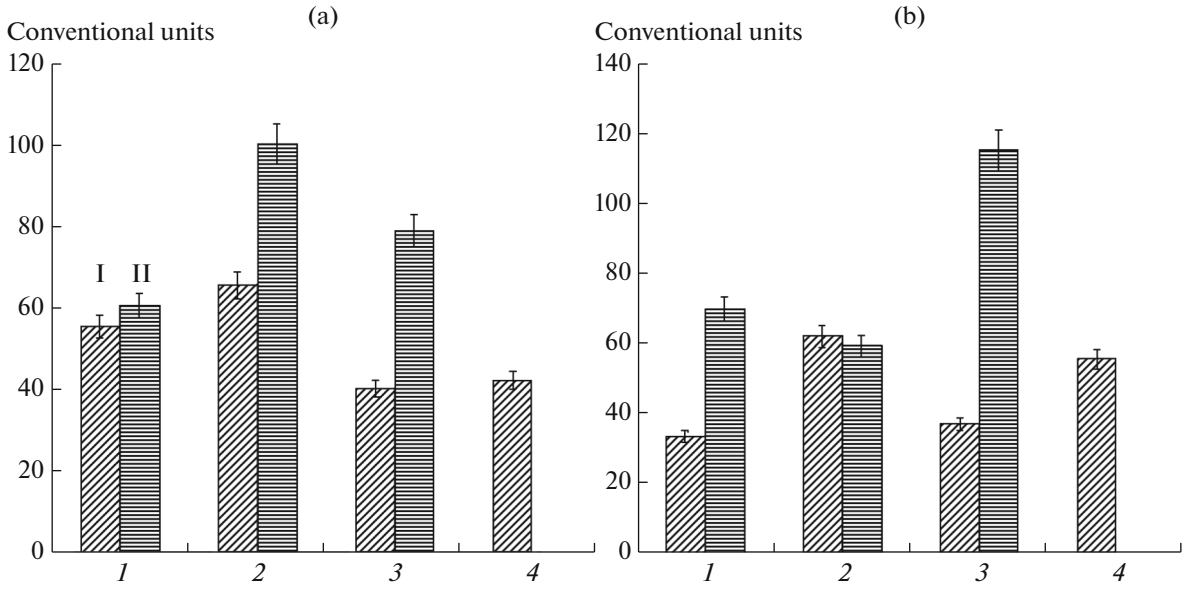

Fig. 2. Total peroxidase and SOD activity in potato leaves of Scarb (a) and Lileya (b) cultivars in the case of treatment with the *B. subtilis* preparation and *XVP* infection. I—Peroxidase activity, conventional units, II—SOD activity, conventional units. *1*—Untreated nonintected plants, *2*—treatment with B. *subtilis*, *3*—treatment with B. *subtilis* + *XVP, 4*—*XVP* infestation.

decreased in infested Scarb leaves. Infestation of the Lileya cultivar led to a significant increase in peroxidase activity (Figs. 1, 2). *XVP* infestation of plants of both cultivars pretreated with *B. subtilis* decreased the total peroxidase activity in comparison with infested plants that were not pretreated.

It should be noted that drug treatment of infested plants also led to a 1.3- to 1.4-fold decrease in the total peroxidase activity in leaves of both cultivars (Fig. 2a). In this case the total peroxidase activity decreased mainly due to the lower relative activity of low molecular isoforms (Fig. 1). Thus, the relative activity of the form with the lowest molecular weight in Lileya leaves infested with *XVP* after their treatment with the *B. subtilis* preparation decreased by 3.6 times.

Modification of the activity of oxireductases, the molecular basis of the signal system, is an important criterion of the metabolic responses that form under the action of stress factors [6].

Several signal systems triggering and regulating the organism's response reactions to the impact of biological factors have been discovered in plants. One of these is the superoxide synthase signal system [16]. The oxidation of membrane NADP-H with molecular oxygen leads to the formation of superoxide-anions. The latter are transformed to hydrogen peroxide in the reaction catalyzed by **SOD** [17, 18]. Abrupt increase in concentration of AFO, including hydrogen peroxide (so-called- oxidative explosion) in the cell becomes the initial stage of biochemical response aimed at suppression of the stress reaction and the process of infestation. As is known, hydrogen peroxide is a secondary intermediary causing the activation of factors regulating transcription and the expression of protective genes [6, 17].

Figure 3 shows that eight molecular forms of SOD with different relative activities are found in potato leaves. According to published data, up to 20 different SOD isoenzymes are present in the leaves of various potato cultivars [18]. The experiments demonstrated that viral infestation and potato plant treatment with the *B. subtilis* preparation did not influence the molecular heterogeneity of SOD but had an essential influence on the activity of particular isoforms and, accordingly, on the level of total enzyme activity (Fig. 3).

In most cases treatment with the *B. subtilis* preparation increased the total SOD activity when applied both to noninfested plants and plants preinfested with XVP (Fig. 2b). The Scarb cultivar is more responsive to treatment with this preparation: the total SOD activity on infested plants of this cultivar increased up to 2.3 times in comparison with the noninfested plants. In Lileya plants, the analogous parameter was almost twice lower, 1.2. The biggest contribution to enhancement of the total enzyme activity in the case of the impact of the preparation and virus was made by isoforms of medium molecular weight. Thus, in infested and *B. subtilis*–treated Scarb leaves, the relative activity of these isoforms increased by 1.4–1.6 times.

CONCLUSIONS

Thus, the results demonstrated that potato pretreatment with the *B. subtilis* preparation prior to viral infestation completely suppressed plant infestation. The antiviral activity of the *B. subtilis* strain 47 preparation did not depend on the conditions of plant cultivation and surpassed the level of successful commercial biopesticides. Treatment with the preparation

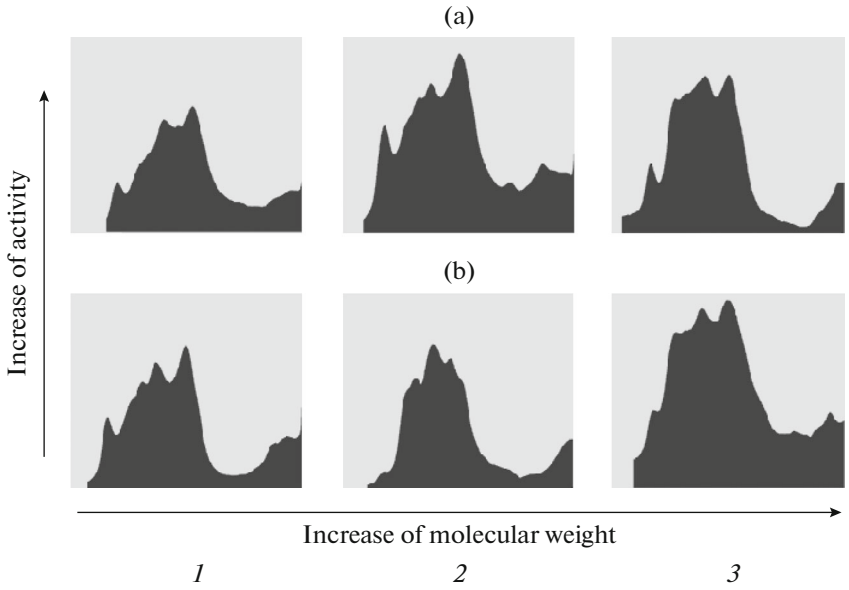

Fig. 3. Densitogram of SOD isoenzymes in potato leaves of Scarb (A) and Lileya (B) cultivars pretreated with the *B. subtilis* preparation and *XVP* infection. *1*—Noninfested plants; *2*—pretreatment with *B. subtilis*; *3*—*B. subtilis* + infestation.

enhanced the activity of peroxidase and superoxide dismutase in plant leaves der to modification of the relative activity of their isoforms. The *B. subtilis* preparation enhanced superdismutase activity in the case of its application to both infested and noninfested plants. It is supposed that modification of the activity of particular isoforms of oxidative-reducing enzymes controlling the level of AFO in treatment with bacterial preparations is one mechanism of plant preadaptation to viral infestation [20].

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