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EFFECT OF *Bacillus subtilis* BASED MICRROBIALS ON PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF SUGAR BEET (*Beta vulgaris* L.) PLANTS INFECTED WITH *Alternaria alternata*

O.V. LASTOCHKINA[1, 2], L.I. PUSENKOVA[1], R.A. YULDASHEV[1, 2], E.Yu. ILYASOVA[1], S. ALINIAEIFARD[3]

[1]*Bashkir Research Institute of Agriculture, Subdivision of the Ufa Federal Research Centre RAS,* Federal Agency for Scientific Organizations, 19, ul. Riharda Zorge, Ufa, 450059 Russia, e-mail oksanaibg@gmail.com (✉ corresponding author);
[2]*Institute of Biochemistry and Genetics, Subdivision of the Ufa Federal Research Centre RAS,* Federal Agency for Scientific Organizations, 71, prosp. Oktyabrya, Ufa, 450054 Russia, e-mail yuldashevra@gmail.com;
[3]*University of Tehran, Aburaihan campus,* PC 3391653775 Pakdasht, Tehran, Iran, e-mail aliniaeifard@ut.ac.ir
ORCID:
Lastochkina O.V. orcid.org/0000-0003-3398-1493
Pusenkova L.I. orcid.org/0000-0001-6341-0486
Yuldashev R.A. orcid.org/0000-0001-9033-6867
Ilyasova E.Y. orcid.org/0000-0001-5737-5469
Aliniaeifard S. orcid.org/0000-0001-9572-2839
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Abstract

Phytopathogenic *Alternaria* fungi are economically important causative agents of sugar beet (*Beta vulgaris* L.) leaf diseases which significantly reduce root yield and quality. Promising agents for plant disease biocontrol are *Bacillus subtilis* based biologicals due to the ability to stimulate plant growth and immunity to many biotic stressors. Starting our experiments, we could not find publications on *B. subtilis* effects towards physiological parameters of sugar beet plants affected by *Alternaria*. This paper is the first to report that *B. subtilis*-based biologicals including novel Bashkirian isolate *B. subtilis* 10-4 prevent a decrease in leaf photosynthetic activity in sugar beet plants affected by *A. alternata*, activate hydrolytic enzyme inhibitors, suppress proline production, and increase sugar content in roots. Our objective was to estimate effects of Fitosporin-M, Vitaplan, and endophytic strain *B. subtilis* 10-4 on leaf photosynthetic pigments (chlorophyll a, b and carotenoids), leaf area index, activity of hydrolases (proteases and amylases) and their inhibitors, as well as proline and sugar levels in leaves, root level of sucrose, and productivity in healthy plants as compared to those artificially infected with *A. alternata*. Our results show that Vitaplan, Fitosporin-M and strain *B. subtilis* 10-4 when used twice increase the concentrations of photosynthetic pigments (chlorophyll a, b and carotenoids) 1.2-1.9-fold in healthy plants whereas a decrease in photosynthetic activity in *A. alternata*-infected plants is 1.2-1.5 times lower, the leaf area is 30 % higher and leaf weight increases 1.8-2.9 times compared to the untreated plants. *A. alternata* infection increased the activity of hydrolases (proteinase, amylase) and suppressed their inhibitors, which indicates the intensive development of the pathogen and a decrease in plant resistance to enzymes produced by pathogen during plant tissue colonization. On contrary, biologicals suppress hydrolases and increase activity of their inhibitors both in infected and healthy leaves, which points out to the induction of protective reactions against *A. alternata* in plants. Interestingly, *B. subtilis* 10-4 and Fitosporin-M ensure the maximum activation of protective proteins. Furthermore, biologicals decrease stress-induced accumulation of proline and sugar, the markers of plant resistance to extremal factors in plants, which is in line with protective effect as well. Also, proline and sugar levels slightly elevated in healthy plants treated with the biologicals, which accentuate the role of these substances in induced resistance to *A. alternata*. Ultimately, larger roots with higher sucrose content confirm the positive effect of the used biologicals among which Fitosporin-M and strain *B. subtilis* 10-4 provide the maximum effect.

Keywords: *Bacillus subtilis*, photosynthetic pigments, hydrolases, sugar, proline, sucrose, *Alternaria alternata*, resistance, productivity, *Beta vulgaris* L., sugar beet

Leaf diseases caused by pathogenic *Alternaria* fungi significantly reduce the productivity and quality of sugar beet plants (*Beta vulgaris* L.), an important sugar crops which serves as a source of raw materials for the sugar, food, confectionery, alcohol industries, bioethanol, fertilizers, animal feed manufacture [1]. Affected plants suffer from Alternaria spot which is characterized by spot for-

mation on the leaves surface [2]. Physiological functions are violated, anatomic-morphological indicators change, yield decreases, separate parts of the plant die, which leads to their complete destruction [3]. The premature loss of the assimilation area of the leaf apparatus, caused by Alternaria spot, leads to loss of plastic substances of the roots, spent on the formation of new leaves, the growth of the roots mass slows down, and the sucrose content reduces [2].

The advantages of the use of biological preparations for plant health improvement in comparison with chemical means of protection are the ecological safety and systemic immunomodulatory action [3, 4]. Promising agents for plant disease biocontrol are *Bacillus subtilis* based biologicals due to their antagonism to pathogens and positive effects on the productivity of crops [4-6]. Growth-stimulating and protective effects of these drugs are shown in many plant species [7-9] and in relation to various stress factors of biotic and abiotic nature [10-12]. It is considered that this action is due to the ability of *B. subtilis* to produce biologically active substances (insecticidal and antimicrobial components, phytohormones, siderophores, and chelators) [13-15], to reduce the content of ethylene in plants, to improve nitrogen fixation, absorption of macro- and microelements [16], to launch mechanisms of systemic plant resistance in response to stress [17] by activating salicylate- and jasmonate-dependent signaling pathways [18-20].

Hydrolytic enzymes (amylases, pectinases, and proteases) and their inhibitors [21-23] play an important role in the induction of plant resistance to pathogens. On the model potato and sugar beet plants, it is shown that the introduction of biologicals on the basis of *B. subtilis* promotes the activation of protease inhibitor synthesis and protects plants from the penetration and development of pathogenic microorganisms [6]. The development of protective reactions of plants to stresses of different nature can also be judged by the degree of accumulation of proline and sugars in them, which serve as markers of the resistance formation in extreme situations [24-26]. At the same time, despite the significant amount of experimental data, the sequence of protective mechanisms induced by *B. subtilis* is not completely clear. Starting the experiments, the authors could not find publications on *B. subtilis* effects towards the photosynthetic activity of sugar beet leaves as an integral characteristic of the physiological state of the whole plant and the nature of changes in the content of proline and sugars in the leaves in the conditions of infection with the pathogens of the Alternaria spots.

As a result of the research, the authors have revealed for the first time that the introduction of biological preparations based on *Bacillus subtilis* prevents the reduction of photosynthetic activity of the sugar beet leaf apparatus induced by the pathogen of the Alternaria spots, and initiates protective reactions, including the activation of inhibitors of hydrolytic enzymes, increasing the content of proline and sugars. It reduces the damaging effect of the pathogenic *A. alternata* fungus on sugar beet plants and promotes the formation of large root crops.

The work objective was to estimate the effects of Fitosporin-M, Vitaplan, and strain *Bacillus subtilis* 10-4 on the physiological and biochemical parameters and productivity of sugar beet infected with *Alternaria alternata*.

Techniques. The investigations were carried out on sugar beet (*Beta vulgaris* L.) plants, Kampai variety (OOO AgroSem-Invest, Krasnodar). In the experiments, the biological preparations Fitosporin-M (*B. subtilis* 26D, NVP Bashinkom, Ufa) (P, 30 g/10 l), Vitaplan (*B. subtilis* 2604D + *B. subtilis* 2605D, ZAO Agrobiotekhnologiya, Russia) (SP, 20 g/ha) and a new strain of *B. subtilis* 10-4 (Bashkir Research Institute of Agriculture; 10^5 CFU/ml) were used [8]. Plants were sprayed with suspensions of biological preparations 2 times, in the phase of 2-3 pairs and 4-6 pairs of real leaves, at a flow rate of 300 l/ha.

Field trials were carried out in the pre-Ural steppe zone of the Republic of Bashkortostan (OOO Chishmy Agroinvest) in 2013 on small plots (5 m^2). The soil is leached chernozem (pH 5.25), Hg 5.50 mg eq/100 g of soil, humus content 8.69%, potassium and phosphorus 29.0 and 23.0 mg/100 g, respectively. Sugar beet was planted according the terms generally accepted for the region; seedlings emerged on days 12-14. The shoots were artificially infected by applying 100 μl of the *A. alternata* fungus suspension (10^6 CFU/ml). The disease development was assessed visually during the growing season by a 5-point scale: 0 points no symptoms, 1 point lesion from 1 to 25% of the leaf area, 2 points lesion from 26 to 50%, 3 points lesion from 51 to 75%, and 4 points more than 75%.

During vegetation (phases of 4-6 pairs of leaves, closure of leaves in rows, technical maturity), control (untreated and healthy) and experimental (treated with biological preparations and infected by *A. alternata*) whole plants or detached roots and shoots were selected three times to assess physiological and biochemical parameters.

Chlorophyll a and b content was determined according to S.W. Jefferey et al. [27]. Leaf samples were weighed, ground, mashed in a mortar calcium carbonate and 90% acetone (at the rate of 0.05 g of leaves per 10 ml acetone); the obtained extract was filtered. The optical density of extracted pigments was measured at λ = 436 nm and λ = 680 nm (a UV-2401PC spectrophotometer, Shimadzu, Japan). The concentration of carotenoids in the total extract of the pigments was calculated by the P. Wettstein's formula [28].

The proline formation in leaves was evaluated by method of L.S. Bates et al. [29] modified by L.G. Kalinkina [30]. For this purpose, 2 g of the test material was taken and poured with 2.5 ml of boiling distilled water. The tubes were brought to a boil in a water bath and cooled. Then, tubes with 2 ml of the cold test, 2 ml of ninhydrin reagent, 2 ml of glacial acetic acid were placed in a water bath, boiled for 1 h and cooled. The color density of the proline complex with ninhydrin was determined (λ = 522 nm, a SF-26 spectrophotometer, LOMO, Russia). The proline content was determined by the calibration curve using standard solutions of chemically pure L-proline (Sigma Aldrich, USA).

The enzymatic activity of proteinases, amylases, and their inhibitors was determined by the spectrophotometric method [21, 31], accumulation of sugar in leaves – by the photometric method with the use of 2,4-dinitrophenol according to GOST R 51636-2000, the amount of sucrose in roots by the cold water digestion method with the use of the polarimeter P161-M (Russia) [32]. The leaf area was measured with a photoplanner, aerial parts of plants and roots were mass with the weighed [33].

All experiments were carried out in 3-4 biological and 4-5 analytical repeats. Statistical processing was performed with STATISTICA 6.0 software (StatSoft, Inc., USA.) The figures and tables show the mean values (*M*) and their standard deviations (±SD) at P = 0.95.

Results. Alternaria spot affects the leaf surface of plants, forming spots, and leads to a decrease in the photosynthetic surface of the leaves [2]. Photosynthesis is the main process in the formation of plant productivity; the total biological yield of crops depends on its intensity [34]. In turn, the content of the main photosynthetic pigments (chlorophylls a, b, and carotenoids) is one of the indirect indices of the photosynthetic activity and the most important biochemical indicator of the plant, which determines the intensity of photosynthesis [24, 34]. In the experiments, infection of sugar beet with *A. alternata* led to a decrease in the content of chlorophyll a (up to 1.5-fold) and b (up to 1.2-fold) in the leaves compared to healthy plants (Fig. 1), which indicates a violation of the photosynthesis process and a decrease in the photosynthetic activity of plants.

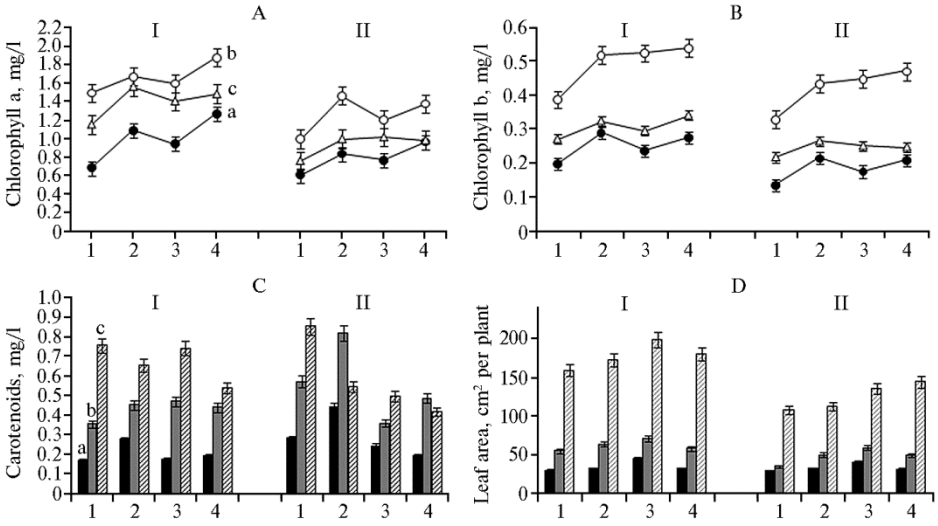

Fig. 1. The content of chlorophyll a (A)**, chlorophyll b** (B)**, carotenoids** (C) **and leaf area** (D) **in healthy** (I) **and infected with *Alternaria alternata*** (II) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** 1 C — control, 2 — Fitosporin-M, 3 — Vitaplan, 4 — *Bacillus subtilis* 10-4; a — the first treatment, b — the second treatment, c — harvesting (OOO Chishmy Agroinvest, the Republic of Bashkortostan, 2013).

Inoculation with *B. subtilis* 10-4 and treatment with Fitosporin-M and Vitaplan biologicals restored the photosynthetic activity of plants. For example, 2-fold treatment with biological preparations prevented stress-induced reduction of chlorophyll a and b in all variants of the experiment (see Fig. 1, A, B). The content of carotenoids in the leaves increased when infecting with *A. alternata* (see Fig. 1, B). Treatment with Vitaplan and *B. subtilis* 10-4 contributed to the decline of their number, whereas after two treatments with Fitosporin-M, a significant accumulation of carotenoids more than the control values was observed. However, the content of carotenoids decreased to harvesting and was comparable with that in the variants in which Vitaplan and the strain 10-4 were used (see Fig. 1, B). It is necessary to note that in uninfected plants, although 2-fold treatment with biological preparations led to a slight increase in the number of carotenoids, this figure was lower than in the control variant (see Fig. 1, B).

The introduction of biological preparations under normal growing conditions stimulated the photosynthetic activity of plants, probably due to an increase in the content of physiologically active chlorophyll a. Indeed, the results obtained in assessing the leaf surface area (see Fig. 1, D) correlated with the effect of the studied compounds on the chlorophyll a and b content. Plants treated with bioactive preparations in all variants during the whole vegetation period were characterized by a much larger leaf area, both without and with *A. alternata* infection (see Fig. 1, D).

It is obvious that the revealed increase in the content of photosynthetic pigments in case of the use of biologicals in the conditions of infection with *A. alternata* (see Fig. 1, A, B, C), in addition to their direct role in the process of photosynthesis and increasing leaf size (see Fig. 1, D), may contribute to the development of protective reactions of plants [34]. In particular, carotenoids perform photoprotective and antioxidant functions [35-38] by preventing damages caused by the formation of singlet oxygen and triplet chlorophyll [37]. In addition, they can take the excitation energy of triplet chlorophyll, and then dissipate it as heat or extinguish singlet oxygen molecules [38, 39]. However, it is necessary to note that despite the obvious role of carotenoids in the antioxidant protection

of plants, data on changes of their content under the influence of stress are very contradictory [22, 24, 36]. Such effects can be explained, on the one hand, by the induction of carotenoids formation under the influence of the stress factor, on the other hand, by its enhanced degradation under severe stress [22].

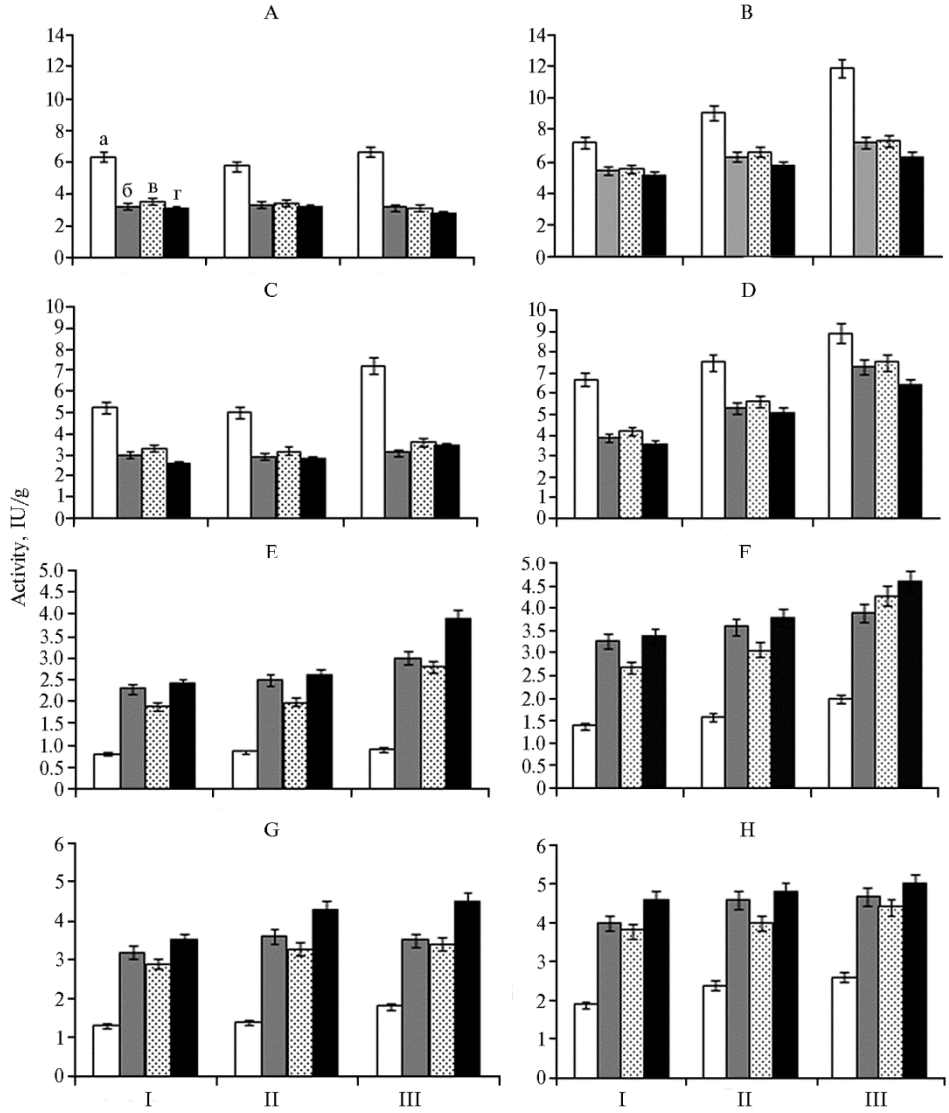

Fig. 2. Activity of proteinases (A, B)**, amylases** (C, D)**, proteinase inhibitors** (E, F) **and amylase inhibitors** (G, H) **in leaves of healthy** (A, C, E, G) **and infected with *Alternaria alternata*** (B, D, F, H) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** a — control, b — Fitosporin-M, c — Vitaplan, d — *Bacillus subtilis* 10-4; I — the first treatment, II — the second treatment, III — harvesting (OOO Chishmy Agroinvest, the Republic of Bashkortostan, 2013).

Hydrolytic enzymes (proteases, amylases) and their inhibitors play an important role in the formation of plant protective reactions to pathogens [21]. In infection with *A. alternata*, the increase in the activity of proteinases and amylases (Fig. 2, B, D) in the leaves of sugar beet occurred which indicates the intensive development of the pathogen in plant tissues. Probably, this process was caused by changes in the metabolism of the host plant under the influence of the pathogen, as well as by the secretion of hydrolytic enzymes by the pathogen itself capable of macerating tissues and destroying the components of the

cell wall, which allows the pathogen to overcome the natural resistance of the host plant. Plants treated with the strain of *B. subtilis* 10-4, Fitosporin-M and Vitaplan were characterized by a decrease in the activity of hydrolases in the infected and healthy leaves (Fig. 2, A-D), and the greatest decrease in hydrolytic activity was observed in variants with the use of Fitosporin-M and the strain of *B. subtilis* 10-4.

A significant contribution to the regulation of the hydrolytic enzymes activity is made by protein inhibitors of plants that suppress the activity of their own and foreign enzymes, in particular, pathogenic fungi and bacteria [23, 40]. The activity of hydrolase inhibitors decreased in the leaves of sugar beet in response to infection with *A. alternata* (see Fig. 2, E, H), as a result of which, probably, the resistance of plants to the action of enzymes of the pathogen and its spread in tissues decreased. Treatment with the studied drugs, on the contrary, contributed to an increase in the activity of hydrolase inhibitors (see Fig. 2, E, H), indicating that the under their influence plants induct protective reactions against *A. alternata*. It is necessary to note that the maximum activation of protective proteins was caused by the use of the strain of *B. subtilis* 10-4 and Fitosporin-M.

Accumulation of sugar and proline in plant mass can be important biochemical markers of resistance formation [31]. Healthy plants of *B. vulgaris* gradually accumulated sugar in the leaves throughout the growing season (Fig. 3, A), which was quite typical and consistent with the available data in the literature [41]. The highest rate of sugar accumulation was observed in the initial stages of growth, when the plant formed leaves and roots vigorously, and slowed to the end of the formation of the third pair of real leaves. Apparently, it was due to the fact that in the phase of the closure of leaves in the rows, leaves growth slowed, the intense thickening and formation of root crops, accompanied by the continuation of sugars accumulation in them, was observed.

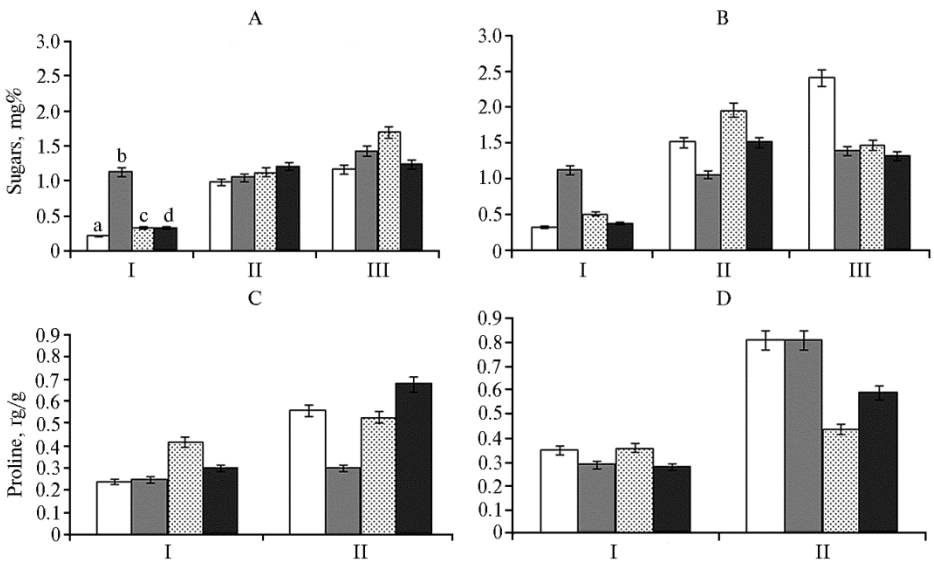

Fig. 3. The content of sugar (A, B) **and proline** (C, D) **in leaves of healthy** (A, C) **and infected with *Alternaria alternata*** (B, D) **sugar beet (*Beta vulgaris* L.) plants of variety Kampai treated with biological preparations:** a — control, b — Fitosporin-M, c — Vitaplan, d — the strain of *Bacillus subtilis* 10-4; I — the first treatment, II — the second treatment, III — harvesting (OOO "Chishmy Agroinvest", the Republic of Bashkortostan, 2013).

Infection with *A. alternata* led to a sharp increase in sugar content in the leaves compared to the control parameters of healthy plants, which apparently

performs a protective role and allows the plants to continue to grow under stress conditions (see Fig. 3, B). The properties of monosaccharides associated with an increase in the stability of biomembranes, anti-denaturation effects on proteins and antioxidant effect may contribute to this process [42]. In addition, accumulating carbohydrates help maintain the osmotic status of cells [33].

One of the most multifunctional plant stress metabolites is amino acid proline which plays the role of not only an osmolyte and antioxidant [24, 43] but also a low-molecular chaperone [40] involved in maintaining the native structure of enzymes [24]. Many investigations have reported an increase in the proline content in plants in response to the stress of different nature and its importance as a factor for plant survival in extreme situations [24-26]. However, we did not find any available data on changes of proline content in sugar beet plants in *A. alternata* infection and the use of *B. subtilis*-based preparations.

A. alternata infection led to a significant increase in the content of proline in sugar beet plants (see Fig. 3, D). At the same time, treatment with Fitosporin-M, Vitaplan and *B. subtilis* 10-4 contributed to the prevention of its accumulation, induced by stress (see Fig. 3, D). It is necessary to note that under the influence of biological preparations in healthy plants, a slight increase in the amount of proline, which further indicates the important role of this agent in the formation of induced resistance to the causative agent of Alternaria spots, was observed (see Fig. 3, B).

The combined indicator of the nature of physiological and biochemical processes for the entire period of vegetation can be the data on the external state of plants and the productivity of root crops. In the experiment, the artificial infection of plants with *A. alternata* led to a gradual increase in the affected leaf area. For example, to harvesting it reached 75% or more (4 points), while in plants treated with *B. subtilis* 10-4, Fitosporin-M, Vitaplan, less than 35% (2 points). The best effect was observed in use of *B. subtilis* 10-4 and Fitosporin-M. In these cases, the disease development did not exceed 25 and 30% respectively. At the same time, 2-fold treatment with biologicals led to a significant increase in the average weight of aerial parts of healthy plants 1.8-2.7-fold and roots crops 1.6-2.3-fold depending on the variant of the experiment (Table). Treatment with biological preparations preventes the stress-induced decline in the productivity of root crops in infection with *A. alternata* and contributes to stable growth of leaves and roots comparable to that in healthy plants.

Leaf and root weight in sugar beet (*Beta vulgaris* L.) plants of variety Kampai, healthy and infected with *Alternaria alternata*, as influenced by *Bacillus subtilis*-based microbial preparations

Variant	Aaerial part, g			Roots, g		
	I treatment	II treatment	harvesting	I treatment	II treatment	harvesting
Healthy plants						
Control	4.45±0.19	14.67±0.77	165.20±2.65	0.52±0.09	4.33±0.30	550.80±10.41
Fitosporin-M	6.11±0.30	27.96±0.82	304.80±2.32	0.92±0.19	10.42±0.48	971.00±11.89
Vitaplan	7.75±0.51	14.25±0.46	425.40±3.01	1.17±0.28	4.94±0.12	970.60±13.03
Bacillus subtilis 10-4	11.00±0.42	36.70±0.91	336.40±4.69	1.80±0.12	14.12±0.22	1142.40±12.62
Infected plants						
A. alternata	6.86±0.22	14.14±1.13	71.20±1.78	1.32±0.09	3.37±0.21	276.30±4.11
Fitosporin-M	8.84±0.14	15.61±0.91	182.00±1.99	1.81±0.12	7.11±0.29	463.00±5.33
Vitaplan	4.68±0.49	11.84±0.70	127.40±2.06	0.64±0.11	3.98±0.15	502.20±5.09
Bacillus subtilis 10-4	11.21±0.30	25.70±1.42	209.40±2.57	2.72±0.23	11.66±0.23	607.20±4.95

In addition to the positive impact on the intensity of growth processes and biomass accumulation, 2-fold application of biological preparations provided a higher content of sucrose in yield as compared to control of both healthy and infected plants. So, at harvesting in the control variant, the root crops contained 16.1% of sucrose, in tests from 17.9 to 19.0%. The maximum amount of sucrose

was in 2-fold treatment with Fitosporin-M and *B. subtilis* 10-4. In infection with *A. alternata*, the roots of all treated plants were characterized by an increased content of sucrose compared to untreated ones.

Thus, the preparations Fitosporin-M, Vitaplan, and *Bacillus subtilis* 10-4 contribute to increased synthesis of photosynthetic pigments (chlorophyll a, b, and carotenoids), increase activity of hydrolase inhibitors in leaves and reduce stress-induced accumulation of proline and sugars, providing a protective effect in infection of sugar beet plants with *Alternaria alternata*. When treated with *B. subtilis*-based microbial preparations, both healthy and infected plants show an increase in sucrose accumulation. The most effective variants were 2-fold use of Fitosporin-M and *B. subtilis* 10-4, in which the adverse impacts of *A. alternata* is smoothed to the maximum and root crops with the biggest weight and the highest sucrose content are obtained.

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