International Journal of Plant & Soil Science

22(6): 1-12, 2018; Article no.IJPSS.41148
ISSN: 2320-7035

Impact of *Bacillus subtilis* on Tomato Plants Growth and Some Biochemical Characteristics under Combined Application with Humic Fertilizer

V. N. Pishchik[1*], N. I. Vorobyev[2], Yu. V. Ostankova[3], A. V. Semenov[3], A. Totolian Areg[3], A. A. Popov[2], Y. V. Khomyakov[1], O. R. Udalova[1], D. V. Shibanov[1], V. E. Vertebny[1], V. I. Dubovitskaya[1], O. V. Sviridova[2], O. S. Walsh[4] and S. Shafian[4]

[1]Agrophysical Research Institute of Russian Agricultural Academy, St. Petersburg, Russia.
[2]All-Russian Research Institute for Agricultural Microbiology of Russian Academy of Agricultural Science, St. Petersburg, Russia.
[3]Research Institute Pasteur, Russia.
[4]Department of Plant Sciences, University of Idaho, Parma, ID, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author VNP designed the study, performed the statistical analysis, wrote the protocol and co-wrote the manuscript. Author NIV performed the statistical analysis and co-wrote the manuscript. Authors YVO and AVS determined protocol, conducted 16sRNA analysis, co-wrote manuscript. Author ATA analyzed the results of 16sRNA analysis. Authors AAP, ORU and DVS designed and conducted research trial. Authors YVK, VEV and VID managed plant biochemical analysis. Author OVS conducted statistical analysis. Authors OSW and SS conducted the literature review, co-analyzed data, assisted in statistical analysis and data interpretation, co-wrote the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2018/41148
Editor(s):
(1) Abigail Ogbonna, Department of Plant Science and Technology, Faculty of Natural Sciences, University of Jos, Nigeria.
Reviewers:
(1) V. Vasanthabharathi, Annamalai University, India.
(2) Haluk Caglar Kaymak, Atatürk University, Turkey.
(3) Vijendra Mishra, Banaras Hindu University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/24593

Original Research Article

Received 22nd February 2018
Accepted 9th May 2018
Published 12th May 2018

ABSTRACT

In this study we investigated the mechanisms of action and effect of plant growth promoting rhizobacteria (PGPR) *Bacillus subtilis* No.2 when utilized alone and in conjunction with a humic fertilizer (HF). Different mechanisms of action of *B. subtilis* No.2 and HF *Stimulife* on tomato plants

**Corresponding author: E-mail: veronika-bio@rambler.ru;*

were identified in pot experiments under controlled conditions. PGPR *B. subtilis* No.2 was identified by using the16s rRNA gene sequence method. We applied factor analyses to evaluate differences in the responses of plants to the individual effects of *B. subtilis* No.2 and HF when they were used together. Auxin-producing *B. subtilis* No.2 enhanced tomato yield by increased average number of fruits per plant. Humic fertilizer *Stimulife*, which also contains auxins, improved tomato yield by increasing average fruit weight. As shown by factor analysis, the impact of weight coefficients for plant responses (across tomato varieties) to *B. subtilis No.2* and *Stimulife* were 0.54 and 0.28, respectively, indicating a greater response to *B. subtilis No.2* than to HF *Stimulife*. Combined use of *Stimulife* and *B. subtilis No.2* had a positive impact on tomato yield, increasing fruit yield by 25-29%. Tomato fruit quality was improved by increasing the amounts of dry matter, carbohydrates, sugar-index acid, and ascorbic acid. Results suggest that HF *Stimulife* and *B. subtilis No.2* could be successfully used to enhance tomato plant growth and yield under controlled conditions. We hypothesize that, along with direct impact, HF may also indirectly affect plants by stimulating PGPR.

Keywords: PGPR Bacillus subtilis No.2; humic fertilizer; tomato physiological and biochemical characteristics.

1. INTRODUCTION

Application of optimal agricultural technologies results in improved efficiency of vegetable crop production. Various growth regulators, including humic and bacterial preparations, are used to increase crop production [1-3]. The active components of bacterial preparations are PGPR, which stimulate the plant growth through various mechanisms, such as synthesis of siderophores, plant hormones, organic acids, and nitrogen (N) fixation [4-8].

Humic substances (HS) are heterogeneous polydispersed N-containing compounds of phenolic nature. Their molecular composition includes an aromatic ring with substituents (generally, carboxyl and phenolic groups), N- and sulfur (S)-containing heterocycles, and aliphatic chains [9,10]. Previously, HS have been considered macromolecules [9]. More recently, HS have been described as supramolecular structures consisting of molecules linked together by covalent bonds [11].

The use of HS has been linked to improved soil structure [12] and increased water-holding capacity, creating beneficial conditions for the rhizosphere microorganisms' growth [13]. Humic substances have been also associated with stimulation of plant root growth [14,15]. The fact that plants can uptake HS has been known for quite some time [16]. Recently, a tritium-labeling technique has been used to demonstrate that humic acids are transferred among plant tissues [17]. The stimulatory effect of HS is associated with an increase in the uptake of iron (Fe) ions by plants [13]. Humic substances help to increase cell membrane permeability and, thus, enhance nutrient supply to plants [18,19]. Humic substances affect the ATPase activity of cell membranes [20,21], stimulate respiration in higher plants [14], and have a hormone-like effect on physiological activity [22,23].

Humic substances are transformed by indigenous microbial communities [24, 25], and may serve as a food source for soil microorganisms [26,27]. It has been shown that soil bacteria can absorb humic acids with Gram-positive bacteria having the highest sorption potential [28]. Application of HS was shown to increase the total number of rhizosphere microorganisms, particularly the N-fixers, amylolytic, and cellulolytic microorganisms [29]. At the same time, HS reduced the development of pathogenic fungi such as *Fusarium oxysporum* [29].

The PGPR are free-living bacteria found within the rhizosphere; these bacteria and beneficial for plant root growth. The PGPR include a wide variety of genera (*Azospirillium*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, and others). The PGPR have been found to have a direct effect on plant growth by 1) inducing the production of phytohormones, 2) making biologically fixed nitrogen available to plants, and 3) increasing uptake of phosphorus from the soil by solubilizing the inorganic phosphates. Additionally, the PGPR have many indirect positive effects on plant growth associated with minimizing the bacterial, fungal, viral, and nematode pathogens harmful to plants [30]. Although some equate PGPR with biological fertilizers (biofertilizers), it

is not accurate. Biofertilizers defined as a substance containing living microorganisms which, when applied to soil-plant system, colonizes the rhizosphere and enhances plant growth by increasing the supply and/or availability of primary nutrients. Based on this definition, not all PGPR can be considered as biofertilizers. For example, PGPR that improve plant growth by control of toxic organism are biopesticides, but not biofertilizers [31]. The major applications of PGPR for promoting plant growth include agriculture, horticulture, forestry and environmental restoration. In agriculture, benefits due to the application of PGPR include increased germination rate, root growth, increased shoot and root weights, increased leaf area, higher chlorophyll content, greater nitrogen content, higher protein content, enhanced tolerance to drought, delayed leaf senescence, and improved crop yield [32].

Despite the long-term interest by researchers in HS, their specific mechanisms of action on plants and on the rhizosphere microbial community have not been elucidated [33,34]. As evidenced by recent scientific reviews, the scientific community continues to be intrigued by the HS and their potential for improving plant yield [35,36]. Complex bacterial-humic fertilizers are used to improve the yield of crops [36,37]. However, the individual effects of components in these preparations have not been sufficiently studied.

We investigated the role of *B. subtilis* No.2 and HF *Stimulife* (Agrophysproduct, Saint Petersburg, Russia) [38] in increasing tomato yield and improving tomato fruit quality in pot experiments under controlled conditions.

2. MATERIALS AND METHODS

Two determinate tomato varieties *Licurich* and *Moldova Cup* (Vavilov Plant Industry Institute, Saint Petersburg, Russia,) were used in this study. Tomato cv. *Licurich* was released in Russian Federation and Ukraine and is characterized by early maturity (85-100 days) and medium-sized red, round fruit. Tomato cv. *Moldova Cup* was released in Ukraine and Moldova and has an average ripening period of 115-120 days and red, cylindrical medium-sized fruit.

The tomato plants were germinated under controlled illumination (DNaT-400 lamps, Photosynthetically Active Radiation (PAR) irradiance of 30 W/m^2 and 16-hour photoperiod). The greenhouse air temperature was maintained at 26(±2)ºC, and relative humidity was maintained at 65%. Tomato plants were grown in 5l pots filled with a growing medium composed of peat (97%) and Cambrian clay (3%). The peat was H1-H3 degree of decomposition, 95-99% organic matter content, 45-60% moisture content, 45-60% ash content, and pH of 7.0. The peat was spread out on racks to achieve a thickness layer of 25 cm and fertilized with basal solution at the rate of 10 l m^{-2}. The fertilizer solution contained 20 g of urea ($CO(NH_2)_2$), 60 g of triple superphosphate Ca $(H_2PO_4)_2$, 50 g of potassium nitrate (KNO_3), and 30 g of magnesium sulfate ($MgSO_4$). Tomato plants were fertigated daily with Knop solution ($CaNO_3$-1 g l^{-1}, KNO_3-0.25 g l^{-1}, KCl-0.12 g l^{-1}, KH_2PO_4-0.25 g l^{-1}, $MgSO_4$-0.25 g l^{-1}).

Humic fertilizer: Commercially available liquid humic fertilizer *Stimulife* was used in this study. *Stimulife* is produced from natural peat; it contains (on average) 12% total N, 47% carbon (C), 3.5% hydrogen (H), 25-27% oxygen (O), 0.3%, P_2O_5, 0.25% K_2O. The working concentration of *Stimulife* was 0.01% and it contained 0.04 µg ml^{-1} IAA.

Bacterial ribosomal RNA sequences by direct sequencing: Extraction of bacterial DNA was carried out in triplicate from three independently grown cultures. Extraction of DNA was carried out by the chloroform-saline standard method based on the lysis of cells and denaturation of cellular proteins with a solution that contained guanidine thiocyanate, followed by ethanol precipitation of nucleic acids [39]. Random combinations and ratios of DNA concentrations of the pure cultures - *Leptospira interrogans, Stenotrophomonas maltophilia, Escherichia coli, Bordetella pertussis* - were mixed and used as a control. The "blind" method was used.

For Polymerase Chain Reaction (PCR), two pairs of universal primers flanking fragment about 1500 bp were used (F1 5'-AGAGTTTGATCMTGGCTCAG - 3' and R1 5'-GGGGTATCTAATCCCGTTCG-3', F2 5'-AACTTCGTGCCAGCAGC -3' and R2 5'-GTCATCCCCACCTTCCTC -3' or R2' 5'-TACGGYTACCTTGTTACGACTT -3'). These were designed for the nucleotide sequencing of 16S rRNA. The PCR amplification was run in 25 mcl amplification mixture with the primers 15 pM each, plus 67 mM Tris HCl (pH 8.8), 16.6 mM ammonium sulphate, 6.7 mM $MgCl_2$, 6.7 mM EDTA, 10 mM mercaptoethanol, 170 mg BSA, 1.0 mM of each NTP, 1 U of Taq DNA-

polymerase (Fermentas). Denaturation (94°C for 5 min) was followed by 40 cycles of amplification: 94°C – for 30 sec, 55°C - for 30 sec, 72°C - for 1 min 20 sec, and the final elongation: 72°C – for 7 min.

An additional method for the separation of the amplification products was used for accurate identification. A mixture of the amplification products was separated on denaturing polyacrylamide gels according to standard procedures with modifications [40]. Electrophoresis was carried out in 8% polyacrylamide gel with a gradient of 45 to 80% (100% gel contains 7 M urea solution and 40% deionized formamide solution). The gel was stained for 30 min in Tris-acetate - EDTA buffer containing ethidium bromide, washed with deionized water, and visualized under ultraviolet light. Fragments were excised from the gel with amplification products and homogenized in 1.5 ml tubes. Then, 25 mcl buffer was added to the DNA elute and incubated for 15 min at 37°C. The mixture was then frozen and thawed several times. Incubation was carried out overnight at 37 °C and the mixture was centrifuged at 13 k rpm for 10 min.

Purified fragments were used for sequencing reactions formulated using a set Genome Lab DTCS-Quick Start Kit (Beckman Coulter Inc., USA). To analyze the reaction product, the purified precipitate was sequenced and dissolved in SLS-buffer containing formamide and then placed in a genetic analyzer (GenomeLabGeXP).

The primary analysis of the obtained sequences was performed using the NCBI Blast program to compare to the sequences shown in the international GenBank database. In each case, the comparison was carried out on the identical sample of nucleotide sequences.

Comparative analysis of the complete sequence of the 16S rRNA allowed identification of the precise composition of the bacterial culture. In all three variants, independently grown bacterial cultures were identified as a Gram-positive, spore-forming aerobic bacteria *Bacillus subtilis*.

B. subtilis No.2 were cultured on Omelyanskiy agar medium and incubated for 48 h at 27°C. The cells were rinsed with sterilized water, suspended in sterilized water, and adjusted to 10^6 cells ml^{-1}. The bacterial titer was 4-6×10^6 cells ml^{-1}, total auxin-like hormones 7 $\mu g ml^{-1}$. Fifty ml of inoculum or fifty ml of humic fertilizer in working concentrations, or 50 ml of bacterial inoculum combined with 50 ml humic fertilizer were applied to each plant, according to treatment structure. The preparations were applied twice to each plant – at the beginning of budding and at flowering.

Biochemical analysis: At flowering, chlorophyll was extracted from three tomato leaves per plant via ethanol extraction. The total chlorophyll (a+b) content of the tomato leaves was determined by spectrophotometric analysis at wavelengths of 649 nm and 664 nm [41] with SF-46 spectrometer (LOMO, St. Petersburg, Russia). At full ripening, biochemical analysis of tomato fruit was carried out. Determination of ascorbic acid was carried out utilizing the titration with Tillmans dye method [42]; the total amount of sugars was determined by the Bertrand method [43]; the total content of malic acid and citric acid was quantified using organic Trilon B, as described by Ermakov [44].

Tomato yield: Tomato varieties *Licurich* and *Moldova Cup* were harvested 75 and 85 days after planting, respectively; tomato fruit yield was determined. All fruits per plant were harvested and weighed. The experiment was repeated twice, and the same trends were observed.

Multidimensional statistical analysis: Factor analysis, cluster analysis and correlation analysis [45], in combination with the Microsoft Office 2007-based Visual Basic for Applications (VBA), were carried out to clarify the effect of HF and PGPR *B. subtilis* No.2 on tomato growth, fruit yield, and quality.

3. RESULTS AND DISCUSSION

All treatments resulted in a significant increase in vegetative green biomass of the tomato plants. Tomato green biomass weight increased by up to 9% as a result of *Stimulife* applied alone. A combination of *Stimulife* and *B. subtilis* No.2 resulted in up to a 22% increase in vegetative biomass production. Comparable biomass was achieved with *B. subtilis* No.2 alone, and with combined application of *B. subtilis* No.2 with *Stimulife*. Similar to vegetative green biomass, total chlorophyll (a + b) concentration in the tomato leaves was significantly increased by the combined application of *B. subtilis* No.2 with *Stimulife*, indicating an increase in the photosynthetic activity of tomato plants. Chlorophyll content of *Licurich* and *Moldova Cup* tomato plants was increased by 0.62 mg $plant^{-1}$ and 0.56 mg $plant^{-1}$, respectively. An additive effect was observed

when both HS *Stimulife* and *B. subtilis* No.2 were applied.

Table 1 details results of the Duncan's Multiple Range test ($p<0.001$) for all measured parameters.

All treatments increased tomato yield (Table 1). Application of HS *Stimulife* increased yield of tomato cv. *Licurich* by 16% and cv. *Moldova Cup* – by 14%. The inoculation with *B. subtilis* No.2 increased tomato yield by 24 and 21% for *Licurich* and *Moldova Cup*, respectively. Combined application of HS *Stimulife* and *B. subtilis* No.2 resulted in substantially greater increase in tomato yield, with yields increased by 29 and 25% for *Licurich* and *Moldova Cup*, respectively.

Application of *B. subtilis* No.2 significantly increased the number of fruits per plant (Table 1). The biochemical analysis shows that *B. subtilis* No.2 also significantly increased the levels of total carbohydrates, ascorbic acid, and organic acids in tomato fruit of both cultivars (Table 1).

Factor analysis demonstrates the trends in the changes of the plant characteristics of interest, under different treatments (Table 2). As shown, the combined treatment resulted in higher amounts of total organic acids (B_{12} = 0.28; 0.22 > 0), but had a negative synergistic effect on ascorbic acid content (B_{12} = -0.02; -0.18 < 0), indicating the acceleration of fruit ripening. This result was supported by the observation of shorter ripening periods for tomato plants that had received the combined treatment; ripening periods for *Moldova Cup* and *Licurich* were shorter by 5 and 7 days, respectively. Factor analysis confirmed that the plant response to applied treatments was not straightforward. The average value of weight coefficients for plant responses to *B. subtilis* No.2 impact was 0.54, while for HF *Stimulife* impact, the value of this index was lower – 0.26-0.28 (Table 2). The average value of weight coefficients for some plant characteristics (No. 1 through 5, 8; No. 10 for *Likurich;* Table 2) had negative values, while other plant characteristics (No.6, 7, 9; No. 10 for *Moldova Cup*; Table 2) had positive values.

The combined treatment of HF *Stimulife* and *B. subtilis* No.2 showed a clear additive effect on ascorbic acid of tomato plant cv. *Likurich.* (B_{12}=0). Despite the individual treatments of *B. subtilis* No.2 and *Stimulife* alone resulting in increased values for biochemical characteristics of plants, the combine treatment had a negative synergistic effect on most biochemical characteristics of plants. This indicates that the ratio of preparations should be changed when they are used together.

Application of both *Stimulife* and *B. subtilis* No. 2 resulted in an increase in tomato fruit yield increase of 29% and 25% for *Likurich* and *Moldova Cup*, respectively. These increases were less than those obtained with the individual application of these treatments. Specifically, when applied alone, *Stimulife* resulted in a 40% yield increase, while *B. subtilis* No.2 resulted in a 35% yield increase.

In cluster analysis, the data were grouped into two clusters for plants of both tomato varieties (Figs 1a and 1b). The first cluster included an untreated variant and a variant with addition of *Stimulife* alone, and the second cluster included all variants with inoculation with *B. subtilis* No.2. Cluster analysis confirmed that *B. subtilis* No.2 had a greater impact on tomato plant yield than *Stimulife*.

The correlation analysis (Figs 2 and 3) demonstrated that *B. subtilis* No.2 had a positive impact on most of the tomato plant characteristics of interest (values of correlation coefficients were between 0.71 and 0.96), with the exception of the average weight of the fruit.

The correlation coefficients values were lower when *Stimulife* was applied alone. The highest correlation coefficients were noted for *Stimulife* application and the average weight of tomato fruit (r =0.89-0.90) for both cultivars. For *Moldova Cup*, the treatment with HS *Stimulife* correlated with the concentrations of ascorbic acid (r = 0.6) and organic acids (r = 0.5) in fruits.

The increases in tomato green biomass and chlorophyll content are consistent with results obtained by other researchers [46,47]. As an example, the number of leaves of indeterminate Dutch tomato hybrids *Gayana* F1 and *Raisa* F1 increased by 12-32% when HS and biological preparation Agate 25-K (based on *Pseudomonas sp.* bacteria) were applied in a greenhouse experiment [47]. Previous work has demonstrated that the key growth parameters of tomato plants, such as plant height and number of branches per plant, significantly increased with a combined inoculation of PGPR and humic acid, compared to individual humic acid effect [46].

The increase in green biomass shows that auxin-producing bacteria *B. subtilis* No.2 has potential to improve tomato yield. Our results for tomato yield agree with results previously obtained by other authors [48]. For example, the yield of the *Likurich* tomato grown under controlled conditions reached 15-20 kg m^2 in 75-80 days of vegetation [48]. Our study demonstrated that

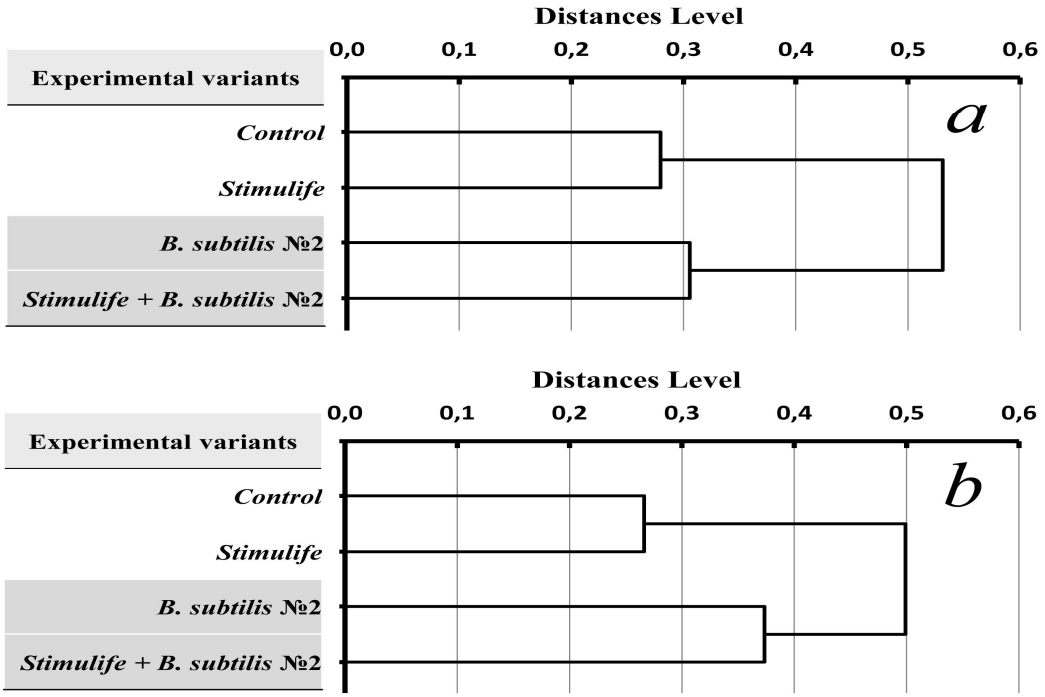

Fig. 1. Dendrogram of similarity level between experimental variants. A-for tomato cv. *Licurich*, b-for tomato cv. *Moldova Cup* Dendrogram is constructed by average-linkage method

Experimental variants: 1 – Control (without treatment); 2 – Treatment with Stimulife; 3 – Treatment with Bacillus subtilis No.2; 4 – Combined treatment with Stimulife and Bacillus subtilis No.2.

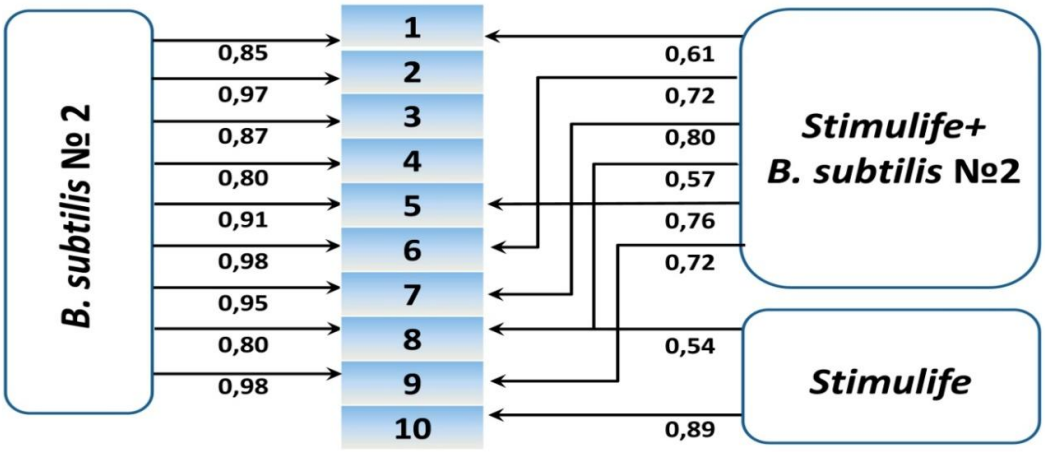

Fig. 2. Coefficients of multiple correlations between different treatments and «growth and yield» plant characters of tomato cv. *Likurich*. The numbers of characters are the same as in tables 1 and 2

1-Fruit mass, g/plant, 2-Fruit numbers per plant, 3- Plant height, cm, 4- Green mass, g/plant, 5-Ascorbic acid, mg/100 g fresh fruit mass, 6- Sum of carbohydrates, % 7 - Fruit dry matter, %, 8-Chlorophyll, FW, mg chl/g, 9 - Sum of organic acids, %, 10- Average fruit mass, g

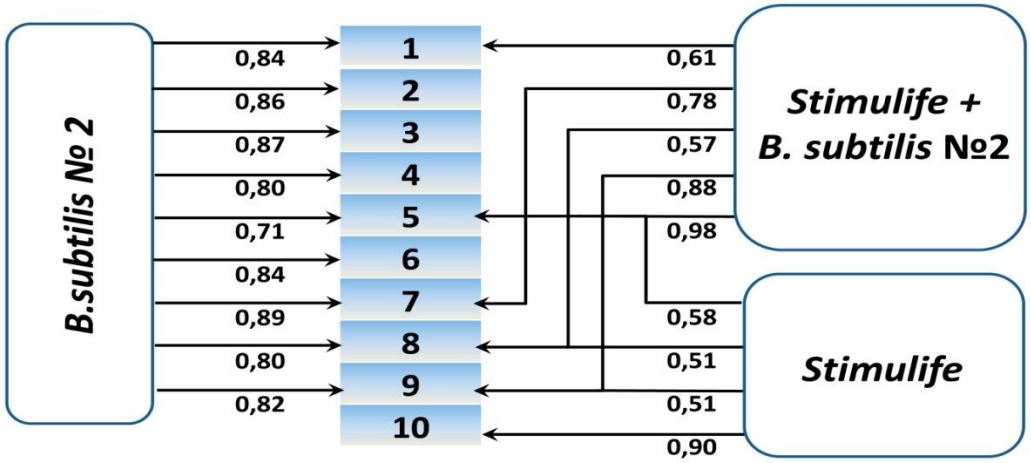

Fig. 3. Coefficients of multiple correlations between different treatments and «growth and yield» plant characters of tomato cv. *Moldova Cup*. The numbers of characters are the same as in tables 1 and 2

1-Fruit mass, g/plant, 2-Fruit numbers per plant, 3- Plant height, cm, 4- Green mass, g/plant, 5-Ascorbic acid, mg/100 g fresh fruit mass, 6- Sum of carbohydrates, % 7 - Fruit dry matter, %, 8-Chlorophyll, FW, mg chl/g, 9 - Sum of organic acids, %, 10- Average fruit mass, g

PGPR bacteria *B. subtilis* No.2 increased the number of fruit per branch and fruit per plant. Similar results were obtained by others [49]. The individual effect of bacterial inoculation resulted in overall increased tomato yield due to greater number of fruit per plant.

On the other hand, HS *Stimulife* increased tomato yield by increasing the average fruit weight, while the number of fruit per plant did not increase. This finding supports the theory of increase in ATP activity, leading to an increase in the electrochemical proton (H^+) gradient, which - in turn - increases ion transport through cell membranes under the action of HS [50]. Permeability of plasma membranes and supply of nutrients to the plant cells resulting from application of HS has been previously reported [18, 47].

Stimulife and *B. subtilis* No.2 both resulted in greater tomato yield, but via different mechanisms of action. Combined application of *Stimulife* and *B. subtilis* No.2 positively affected several plant characteristics, which were also affected in a similar way by *B. subtilis* No.2 alone. When used together, *B. subtilis* No.2 and HS *Stimulife* had a positive additive effect on the amounts of organic acids and carbohydrates in tomato fruit. At the same time, combined application of *B. subtilis* No.2 and *Stimulife* resulted in a decrease in several plant characteristics (plant height, green biomass, and numbers of fruit per plant) that were increased with application of *B. subtilis* No.2 alone. This antagonistic effect could be explained by the initial competition for nutrient uptake by plant root cells and by the activation of H-pumps; this, in turn, enhanced the nutrient transport into the roots and then - into the tomato plant shoots [37].

The average value of weight coefficients for plant responses to *B. subtilis* No.2 impact was 0.54 and for HF *Stimulife* was 0.28, indicating that *B. subtilis* No.2 was the primary influence on the characteristics of interest, regardless of tomato variety. Suppressive effects were observed when HF *Stimulife* and bacteria *B. subtilis* No.2 were used together (Table 2). These results agree with previous findings and suggest that the working concentration of HF *Stimulife* should be reduced in combined application with *B. subtilis* No.2 [51].

Previous studies indicate that the auxin-producing bacteria *B. subtilis* No.2 were able to not only stimulate plant growth, but also change the composition of the rhizosphere microbial community. The number and the activity of N-fixing bacteria of the wheat rhizosphere increased and affected N accumulation by wheat plants inoculated with *B. subtilis* No.2 [52]. The same effect was observed in the application of biological preparation based on a combination of microorganisms (non-N fixers), where application of preparation increased N uptake in rice by 52% [53].

Table 1. Effect of different preparations on tomato yield, biometric plant characteristics and biochemical fruit characteristics

Number	Measured parameters	Control	*Stimulife*	*B. subtilis* №2	*Stimulife* + *B. subtilis* № 2
	Tomato cv. Likurich				
1	Fruit mass, gram/plant	711 d	825 c	884 b	918 a
2	Fruit numbers per plant	17 c	18 c	22 a	21 b
3	Plant height, cm	45 c	45 c	52 a	48 b
4	Green mass/plant	460 d	503 c	550 a	520 b
5	Ascorbic acid, mg/100 g fresh fruit mass	20 d	28,6 c	38,2 b	46,2 a
6	Sum of carbohydrates, %	3.6 c	3.8 c	4.4 b	4.6 a
7	Fruit dry matter	5.7c	5.8 c	6.4 b	6.8 a
8	Chlorophyll, FW, mg chl/g	1.42 d	1.80 c	1.92 b	2.04 a
9	Sum of organic acids, %	0.60 a	0.60 a	0.64 a	0.66 a
10	Average fruit mass, gram	41.8 b	44.8a	40.2 b	43.7 a
	Tomato cv. Moldova cup				
1	Fruit mass, gram/plant	824 d	939 c	995 b	1030 a
2	Fruit numbers per plant	14 c	14 c	19 a	16 b
3	Plant height, cm	49.0 c	49.7 c	68.3 a	57.3 b
4	Green mass/plant	475 d	520 c	578 a	534 b
5	Ascorbic acid, mg/100 g fresh fruit mass	24.0 d	27.2 c	29.6 b	48.2 a
6	Sum of carbohydrates, %	3.7 b	3.8 b	4.4 a	4.0 b
7	Fruit dry matter	5.8 b	6.0 b	6.2 b	6.4 a
8	Chlorophyll, FW, mg chl/g	1.50 d	1.86 c	1.98 b	2.06 a
9	Sum of organic acids, %	0.58 b	0.60 b	0.62 b	0.66 a
10	Average fruit mass, gram	58.9 c	67.0 a	52.3 d	64.4 b

Note: Means within each lines followed by the same letter are not significantly different at p<0.001 as determined by Duncan's multiple range test.

Table 2. Factor loadings under effect of *Stimulife* and bacteria *B. subtilis* № 2 on the plants characters for tomato cv. *Likurich* and *Moldova Cup*

Number	Factor	Preparations					
		***Stimulife*, A_1**		***B. subtilis* № 2, A_2**		***Stimulife* +*B. subtilis* № 2 B_{12}**	
		Likurich	***Moldova Cup***	***Likurich***	***Moldova Cup***	***Likurich***	***Moldova Cup***
1	Fruit mass, gram/plant	0.35±0.04	0.40±0.03	0.58±0.03	0.59±0.03	-0.20±0.07	-0.28±0.06
2	Fruit numbers	0.15±0.04	0.00±0.04	0.77±0.02	0.93±0.01	-0.31±0.06	-0.56±0.05
3	Plant height, cm	0.00±0.04	0.03±0.03	0.79±0.04	0.92±0.04	-0.45±0.06	-0.56±0.06
4	Green mass g/plant	0.37±0.04	0.35±0.04	0.77±0.03	0.81±0.03	-0.63±0.08	-0.70±0.06
5	Ascorbic acid, mg/100 g fresh fruit mass	0.26±0.01	0.27±0.01	0.55±0.01	0.64±0.01	-0.02±0.02	-0.18±0.02
6	Sum of carbohydrates, %	0.09±0.06	0.13±0.04	0.61±0.03	0.52±0.02	0.09±0.09	0.20±0.06
7	Fruit dry matter %	0.27±0.09	0.08±0.06	0.54±0.07	0.54±0.04	0.00±0.16	0.23±0.10
8	Chlorophyll, FW, mg chl/g	0.43±0.02	0.44±0.02	0.57±0.02	0.59±0.02	-0.30±0.09	-0.34±0.04
9	Sum of organic acids%	0.00±0.12	0.22±0.08	0.56±0.07	0.44±0.06	0.28±0.19	0.22±0.14
10	Average fruit mass, gram	0.85±0.05	0.69±0.05	-0.34±0.13	-0.56±0.08	-0.11±0.17	0.33±0.11
	Average data	0.28	0.26	0.54	0.54	-0.17	-0.16

Note: when $B_{12}>0$ – is a positive additive effect

Plants, in turn, may control the composition and abundance of the rhizosphere microbial community. In the presence of the water-insoluble fraction of HS, maize plants increased the quantity of carbon production in root secretions, thereby increasing the number of HS-destructors in the microbial community of plant rhizosphere. When biologically available HS fractions were added, this effect was not observed. The authors suggested that the plants had stimulated rhizosphere microorganisms capable of degrading HS [31].

Humic substances can be utilized by microorganisms as effective electron acceptors for the oxidative degradation of organic carbon in an anaerobic environment. Alternatively, HS in its reduced forms can be utilized by microorganisms as effective electron donors for the assimilation of organic carbon.

The results of our experiments suggest that the HS may have an indirect hormonal effect on plants. We believe that HS primarily affects rhizosphere microorganisms, which, in turn, stimulate the plant, exhibiting a hormone-like activity of HS, as described by other authors [22, 23]

4. CONCLUSION

Our results showed a positive impact of *Stimulife* and bacteria *B. subtilis* No. 2 on tomato, increasing fruit production by 11-25%. Tomato fruit quality was improved by increasing the amounts of dry matter, carbohydrates, sugar-index acid, and ascorbic acid. Therefore, *Stimulife* and *B. subtilis* No.2 could be successfully used to enhance fruit production and fruit quality of tomato plants grown under controlled conditions.

When *Stimulife* and *B. subtilis* No.2 were applied together, results indicate that *B. subtilis* No.2 had the predominant influence on the biochemical characteristics of tomato plants. This allows us to suggest a newly proposed mechanism of HS action on the plants. It is likely, that the indirect physiological effect of HS on plants is due to increased PGPR activity. Further research is required to make definite conclusions about HS mechanisms of action.

ACKNOWLEDGEMENT

This work was partially supported by the Russian National Fund grant 14-26-00094.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Zahir ZA, Arshad M, Frankenberger WT. Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. Advances in Agronomy. 2004; 1(81):98-169.
2. Bogoslovskiy VN, Levinskiy BV, Sychev VT. Agrotechnologies in future. Moscow, RIF, “Antikva”, Book 1st. in Russian. 2004; 136.
3. Ruzzi M, Aroca R. Plant growth-promoting rhizobacteria act as biostimulants in horticulture. Scientia Horticulturae. 2015; 196:124-34.
4. Kloepper JW, Leong J, Teintze M, Schroth MN. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature. 1980; 286(5776):885.
5. Pishchik VN, Chernyaeva II, Kozhemaykov AP, Vorobyov NI, Lazarev AM, Kozlov LP. Effect of inoculation with nitrogen-fixing *Klebsiella* on potato yield. In Nitrogen Fixation with Non-Legumes. Springer, Dordrecht. 1998;223-235.
6. Kloepper JW, Ryu CM, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 2004;94(11):1259-66.
7. Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University-Science. 2014; 26(1):1-20.
8. Gond SK, Bergen MS, Torres MS, White Jr JF. Endophytic *Bacillus* spp. Produce antifungal lipopeptides and induce host defence gene expression in maize. Microbiological Research. 2015;172:79-87.
9. Orlov DS. Gumusovye kisloty pochv (Humic Acids of Soils), Moscow: Mosk. Gos. Univ; 1974.
10. Stevenson FJ. Humus chemistry: Genesis, composition, reactions. John Wiley & Sons. 1994;12.
11. Piccolo A. The supramolecular structure of humic substances. Soil Science. 2001; 166(11):810-32.
12. Piccolo A, Mbagwu JS. Effects of different organic waste amendments on soil microaggregates stability and molecular

sizes of humic substances. Plant and Soil. 1990;123(1):27-37.

13. Chen Y, Aviad T. Effects of humic substances on plant growth 1. Humic substances in soil and crop sciences: Selected readings. Humic Substances. 1990;161-86.
14. Vaughan D, Malcolm RE. Influence of humic substances on growth and physiological processes. In soil organic matter and biological activity. Springer, Dordrecht. 1985;37-75.
15. Arancon NQ, Edwards CA, Lee S, Byrne R. Effects of humic acids from vermicomposts on plant growth. European Journal of Soil Biology. 2006;(42):S65-9.
16. Vaughan D, Ord BG. Uptake and incorporation of 14C-labelled soil organic matter by roots of *Pisum sativum* L. Journal of Experimental Botany. 1981; 32(4):679-87.
17. Kulikova NA, Abroskin DP, Badun GA, Chernysheva MG, Korobkov VI, Beer AS, Tsvetkova EA, Senik SV, Klein OI, Perminova IV. Label distribution in tissues of wheat seedlings cultivated with tritium-labeled leonardite humic acid. Scientific Reports. 2016;6:28869.
18. Visser SA. Effects of humic substances on plant growth. Humic Substances Effect on Soil and Plants. 1986;89-135.
19. Varanini Z, Pinton R. Direct versus indirect effects of soil humic substances on plant growth and nutrition. The Rhizosphere. Marcel Dekker, New York. 2000;141-57.
20. Varanini Z, Pinton R, De Biasi MG, Astolfi S, Maggioni A. Low molecular weight humic substances stimulate H+-ATPase activity of plasma membrane vesicles isolated from oat (*Avena sativa* L.) roots. Plant and Soil. 1993;153(1):61-9.
21. Nardi S, Pizzeghello D, Muscolo A, Vianello A. Physiological effects of humic substances on higher plants. Soil Biology and Biochemistry. 2002;34(11):1527-36.
22. Müller-Wegener U. Interaction of humic substances with biota. Humic substances and their role in the environment. 1988;79-92.
23. Nardi S, Concheri G, Dell'Agnola G. Biological activity of humus. In Humic Substances in Terrestrial Ecosystems. 1996;361-406.
24. Filip Z, Kubát J. Microbial utilization and transformation of humic substances extracted from soils of long-term field experiments. European Journal of Soil Biology. 2001;37(3):167-74.
25. Sviridova OV, Vorobyov NI, Provorov NA, Orlova OV, Rusakova IV, Andronov EE, Pishchik VN, Popov AA, Kruglov YV. The alignment of soil's conditions for plant's development during microbial destruction of plant's residues by microbial preparations. Biology Agricultura; 664.
26. Tepper EZ. Microorganisms of the Nocardia Genus and the Humus Destruction.
27. Tikhonov VV, Yakushev AV, Zavgorodnyaya YA, Byzov BA, Demin VV. Effects of humic acids on the growth of bacteria. Eurasian Soil Science. 2010; 43(3):305-13.
28. Tikhonov VV, Orlov DS, Lisovitskaya OV, Zavgorodnyaya YA, Byzov BA, Demin VV. Sorption of humic acids by bacteria. Microbiology. 2013;82(6):707-12.
29. Costacurta A, Vanderleyden J. Synthesis of phytohormones by plant-associated bacteria. Critical Reviews in Microbiology. 1995;21:1-18.
30. Kaymak HC. Potential of PGPR in Agricultural Innovations. Plant growth and health promoting bacteria, Microbiology Monographs. 2011;18/2011:45-79.
DOI: 10.1007/978-3-642-13612-2_3
31. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil. 2003;255:571-586.
32. Lucy M, Reed E, Glick BR. Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology. 2004;86:1-25.
33. Sviridova OV, Vorobyov NI, Pishchik VN, Mikhaleva LV. Eeffect of humic preparation on plant-microbial associations. In New Approaches and Techniques in Breeding Sustainable Fodder Crops and Amenity Grasses. 2000;171-173.
34. Yakimenko OS, Terekhova VA. Humic preparations and the assessment of their biological activity for certification purposes. Eurasian Soil Science. 2011;44(11):1222.
35. Puglisi E, Trevisan M. Combining Rhizobox, reporter gene systems, and molecular analyses to assess the effects of humic substances on plant–microbes interactions in soil rhizosphere. Molecular Microbial Ecology of the Rhizosphere: 2013;1-2:933-42.
36. Trevisan S, Francioso O, Quaggiotti S, Nardi S. Humic substances biological activity at the plant-soil interface: From

environmental aspects to molecular factors. Plant Signaling & Behavior. 2010; 5(6):635-43.
37. Canellas LP, Olivares FL. Physiological responses to humic substances as plant growth promoter. Chemical and Biological Technologies in Agriculture. 2014;1(1):3.
38. Agrophysprodukt. Stimulife, liquid humic fertilizer from peat; 2018. Available:http://www.agrophys.com/Agrophys_files/Stimullife/stimullife_eng.html
39. Pishchik VN, Ktitorova IN, Skobeleva OV, Mirskaya GV, Vorobyov NI. Method of instrumental assessment of plant-bacterial interaction in agricultural system in forecasting the yield. In EIITA/WCCA. Proc. 8th European Federation of Information Technology in Agriculture, Food and the Environment/World Congress on Computers in Agriculture, Prague, Czech Republic. 2011;11-14.
40. Graham DE. The isolation of high molecular weight DNA from whole organisms or large tissue masses. Analytical Biochemistry. 1978;85(2):609-13.
41. Cremonesi LS, Firpo S, Ferrari M, Righetti PG, Gelfi C. Double-gradient DGGE for optimized detection of DNA point mutations. Biotechniques. 1997;22(2):326-31.
42. Lichtenthaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In Methods in Enzymology Academic Press. 1987;148:350-382.
43. Tillmans J, Hirsch P, Hirsch W. Das Reduktionsvermögen pflanzlicher Lebensmittel und seine Beziehung zum Vitamin C. Zeitschrift für Untersuchung der Lebensmittel. 1932;63(1):1-21.
44. Badger PC. Ethanol from cellulose: A general review. Trends in New Crops and New Uses. 2002;1:17-21.
45. Ermakov AI, Arasimovich VV, Smirnova-Ikonnikova MI, Yarosh NP, Lukovnikova GA. Methods for the biochemical analysis of plants. Methods for the Biochemical Analysis of Plants (Ed. 2); 1972.
46. Kachigan SK. Multivariate statistical analysis: A conceptual introduction. Radius Press; 1991.
47. García JA, Probanza A, Ramos B, Palomino M, Mañero FJ. Effect of inoculation of *Bacillus licheniformis* on tomato and pepper. Agronomie. 2004; 24(4):169-76.
48. Rassokha NF, Naumova GV, Zhmakova NA. On the efficiency of growth regulators application to cultivate tomatoes on mineral substratum in winter hot houses. Prirodopolzovaniye. 2011;20:100-105.
49. Udalova OR, Sudakov VL, Anikina LM, Vilichko AK. Lighting technology for extreme natural conditions. Potato and Vegetables. 2013;8:12-15.
50. Abdel-Monaim MF, Abdel-Gaid MA, Armanious HA. Effect of chemical inducers on root rot and wilt diseases, yield and quality of tomato. International Journal of Agricultural Science. 2012;7:211-20.
51. Morsomme P, Boutry M. The plant plasma membrane H+-ATPase: Structure, function and regulation. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2000;1465(1):1-6.
52. Pishchik VN, Vorobyov NI, Walsh OS, Surin VG, Khomyakov YV. Estimation of synergistic effect of humic fertilizer and *Bacillus subtilis* on lettuce plants by reflectance measurements. Journal of Plant Nutrition. 2016;39(8):1074-86.
53. Pishchik VN, Vorobyev NI, Moiseev KG, Sviridova OV, Surin VG. Influence of *Bacillus subtilis* on the physiological state of wheat and the microbial community of the soil under different rates of nitrogen fertilizers. Eurasian Soil Science. 2015; 48(1):77-84.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/24593