Project "Development of innovative biostimulants for agriculture"

BG 06RDNP0001-16.001-0026, AD № RD50-62/26.02.2021

The project is funded by the EU under sub-measure 16.1 "Support for the formation and functioning of operational groups within the European Innovation Partnership" of the Rural Development Programme 2014-2020.

Activities during the third implementation period of the project cover the period from January to December 2023, including:

- The derivation of 3 precision field trials to study the effect of treating the vegetative mass of wheat, barley and hazelnut with biostimulants - samples developed by ICHT - Sofia.

In modern agriculture, the search for environmentally friendly ways to stimulate plant growth and increase crop productivity is paramount. Due to the global nutrient imbalance and increased demands for higher yields and quality of food and raw materials, the search for environmentally friendly and sustainable ways to produce bio-based fertilization reagents has become a major goal in agriculture. In this respect, the use of plant biostimulants is of growing scientific and production interest. Biostimulants are a group of substances of natural origin that contribute to increasing the yield and uptake of nutrients by plants while reducing dependence on chemical fertilisers. They are products that can influence metabolic and enzymatic processes in plants, improving crop productivity and quality. Biostimulants also help plants cope with abiotic stress, especially in the early stages of development. Over the years, different authors have identified several types of biostimulants based on starting material, mode of action and other parameters, which are categorized into 7 classes: humic and fulvic acids, protein hydrolysates, seaweed extracts, chitosan, inorganic compounds, beneficial fungi and bacteria (du Jardin, 2015). The results reported by different authors regarding the effectiveness of their application in laboratory and production conditions are sometimes contradictory in nature. This is a prerequisite for forming a sceptical attitude among farmers regarding the efficacy and benefits of their application.

The above is the basis for the concept and implementation tasks of this project. The project is aimed at testing the possibilities of greening agricultural production by limiting the negative impact on the soil of the application of phytostimulants of natural origin in semi-production trials with the direct participation of farmers. The project is based on the hypothesis that phytostimulants applied in the critical phases of vegetation, in an appropriate dose-minimum, contribute to increasing crop productivity.

The aim is to establish, with the joint participation of the scientific team and the producers, the degree of effective impact of foliar application of biostimulants, developed on the basis of natural organic sources, on the yield, technological and biological quality of production, when incorporating the stimulants in the technology of cultivation of 3 types of crops - barley, wheat and hazelnut. To achieve the final objective, the following tasks were formulated:

I. The creation of complex preparations-samples with growth-stimulating action and testing of their physiological activity by means of biotechnological experiments with seeds.

II. Setting up and conducting, together with farmers, field trials with wheat, barley and hazelnuts under semi-productive conditions under conventional cultivation technology, to report the effect of foliar application of biostimulants on growth and productivity indicators over 2 marketing years.

III. Evaluation of the effect of treatment with biostimulants on production quality.

Testing of three /3/ biostimulants, developed by the Institute of Cryobiology and Food Technologies - Sofia in wheat, barley and hazelnut, in production fields showed that these foliar biostimulants have a strong positive effect on the vegetative and generative development of the tested plants and their resistance to abiotic and biotic stress.

In the production fields of. Two field experimental trials for testing the effect of biostimulants with No. 1, 2, 3 and a control variant, respectively, in two winter cereal crops - wheat variety Annapurna and barley variety Kalypso - were set up for two consecutive agricultural years on an area of 720 m2. The trials were laid out using the Shanin block method in 3 replications per variant, with an experimental plot size of 30 m2. A control variant was maintained for the entire growing season of the study crops, which was treated with water in an amount equal to the amount of detergent with which the other variants were treated (Scheme 1).

Scheme of the experiment

1. Control - 30 l da-1 water;

2. I variant - 30 l da-1 working solution;

3. II variant - 30 l da-1 working solution;

4. III variant - 30 l da-1 working solution;

On the basis of accumulated experimental experience in the laboratory "Biologically Active Substances for Plant Production" at ICHT in the creation of complex preparations on an organic basis, 3 new bio-product samples have been developed that meet the maximum criteria for use in the technologies for greening production. In accordance with the current concept of circular economy for waste utilization, the samples were prepared after applying different chemo-technological treatment regimes to the initial organic substrates - compost and chitosan, which are by-products from waste organic sources. The formulations were formulated for foliar treatment.

The humic preparation was created after the development of the vermicompost extraction technology scheme. A cycle of extraction procedures (solid-liquid extraction) were applied while controlling the parameters temperature, extraction time, settling time (centrifugation). The procedure includes the following steps: homogenisation of the vermicompost; drying to an absolute dry state; weighing and addition of an aliquot of extractant (I portion); mixing, centrifugation, decanting (separation of volume 1 from the supernatant); addition to the precipitate of an aliquot volume of extractant (II portion), mixing, centrifuging, decanting (separation of volume 2 from the overlying liquid); filtration of volume1 and volume2; make-up of the resulting filtrate to final volume. Two extractants were used. The technological procedure was completed by mixing certain volumes of the two extracts and preparing a combined extract. The composition of the humic preparation was formulated by dilution of the initial extracts on the basis of the analytical data obtained on the concentration in the humic extracts of 10 macro- and trace elements essential for plants, determined by inductively coupled plasma mass spectrometry taking into account the physiological tolerance norms in plants to the elements.

The preparation based on glucosamine complex was prepared on the basis of a solution of the active component with a certain concentration in the implementation of the following technological procedures: dissolution of the organic substrate (chitosan), hydrolysis, neutralization and dilution.

The microbial preparation was created on the basis of fermentation of brewer's yeast.

The physiological activity of the developed biostimulant samples was tested by soaking seeds and monitoring the effect at different concentrations on germination dynamics, germination ability and initial sprout growth intensity. Five biotechnology experiments of duration were conducted. The experimental design included the following treatments: control, treated seeds with sample preparations applied at different concentrations. The experimental setup was arranged according to the standards of International Seed Testing Association (ISTA, 2009). Each design involved the plating of 4 parallel samples of 50 pre-treated seeds each. Seeds were placed in petri dishes on a substrate moistened filter paper in a climate incubator at the optimum temperature regime for the respective crop. The effect of the dose and composition of the active substances was established by controlling the parameters characterizing the seed properties: mean germination time, mean daily germination, mean germination rate, germination energy, germination rate, stem length, stem mass, root mass, stem dry matter, root dry matter, resistance index.

The analytical data obtained showed that the applied composition and dose of the stimulant-samples was within the physiological tolerance range for the plants - no reduction in laboratory germination parameters was detected (Figure 1)

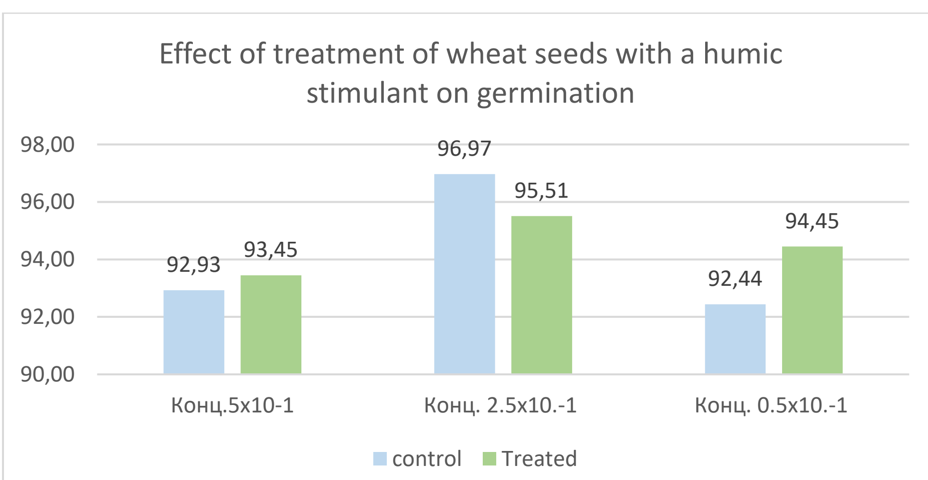


Figure 1.

At 5-fold dilution of the humic preparation, germination was 2% higher (Figure1).

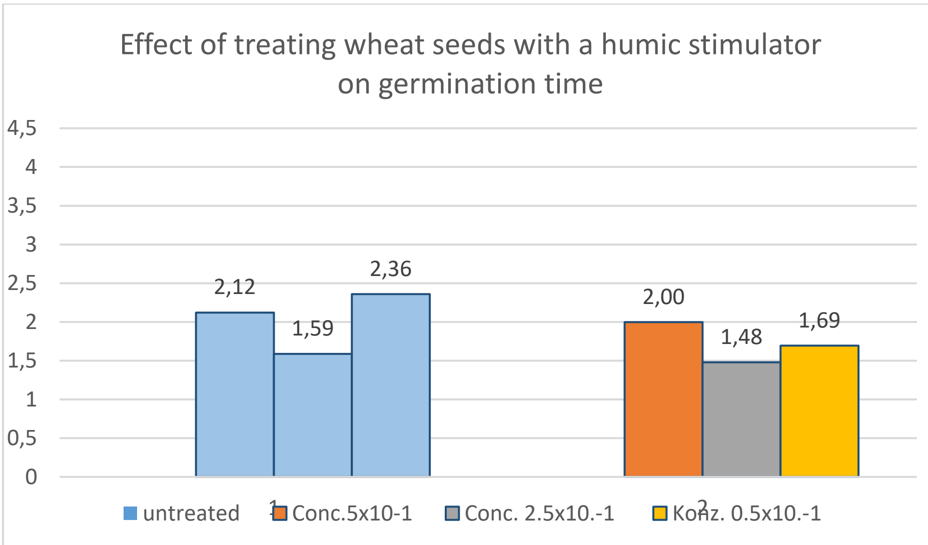


Figure 2.

The experimental results reported an effect of increase in the average daily germination rate when the seeds were soaked with humic stimulant, which accelerated the germination process and promoted the friendly seed germination (Figure 2). The most effective is the humic stimulant applied in 5-fold dilution.

Seed treatment with glucosamine applied in two concentrations did not affect the germination parameters: germination rate and mean germination time (Figure 3, 4).

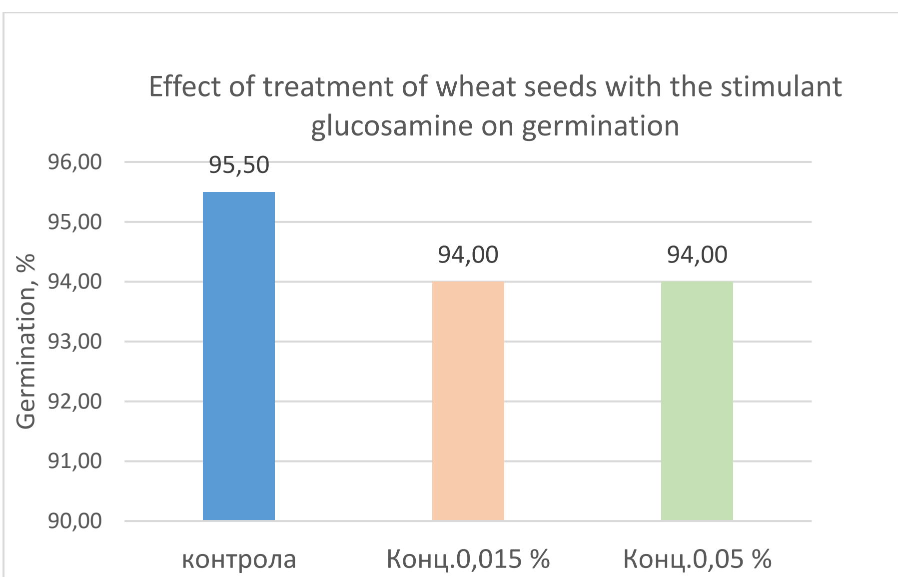


Figure 3.

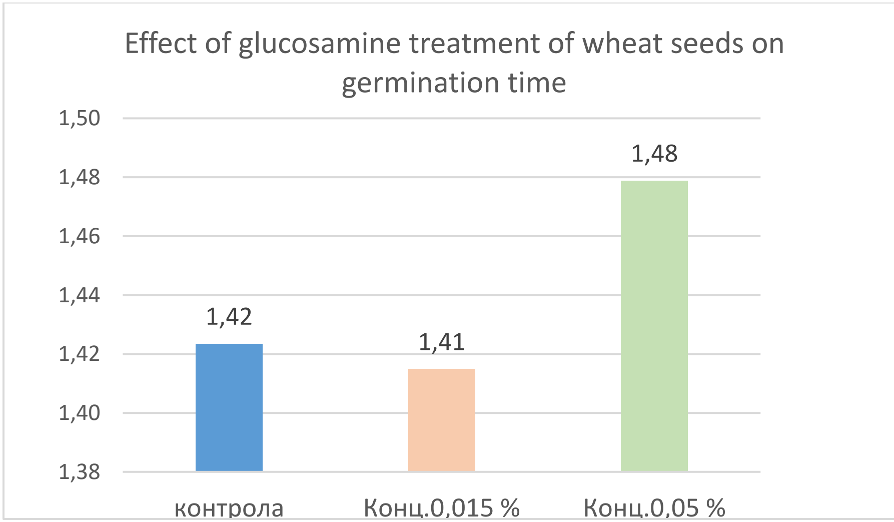
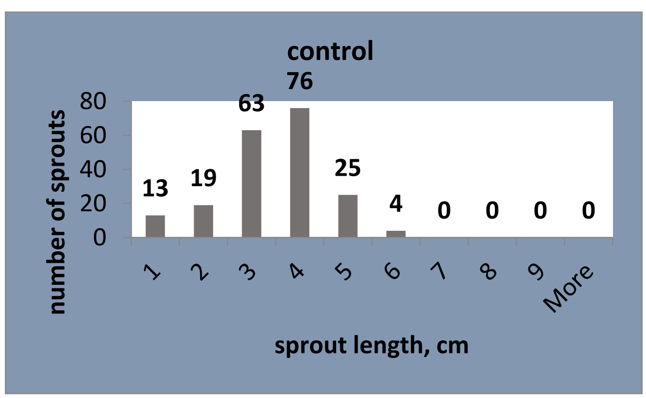
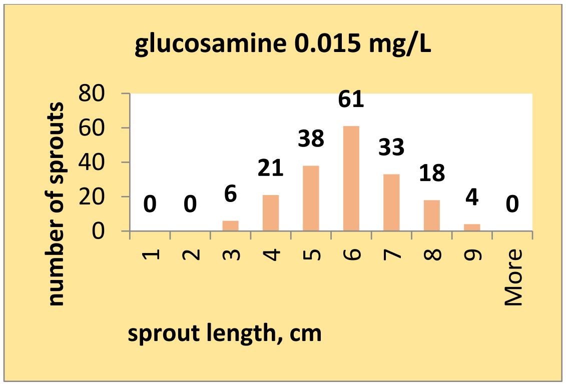


Figure 4.

The experimental results obtained showed that pre-soaking in biostimulant solutions positively affected the growth of the seedlings in the initial stages of development.

The attached histograms illustrate the positive effect of seed soaking on linear growth.





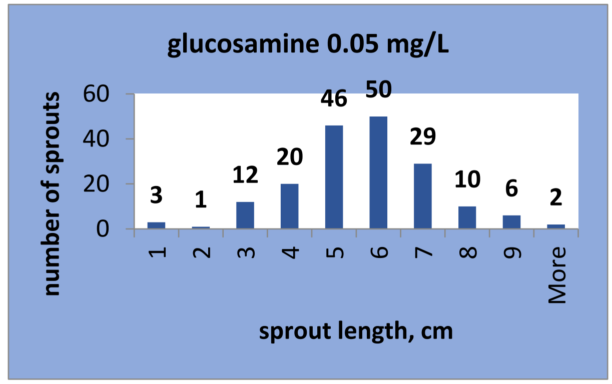
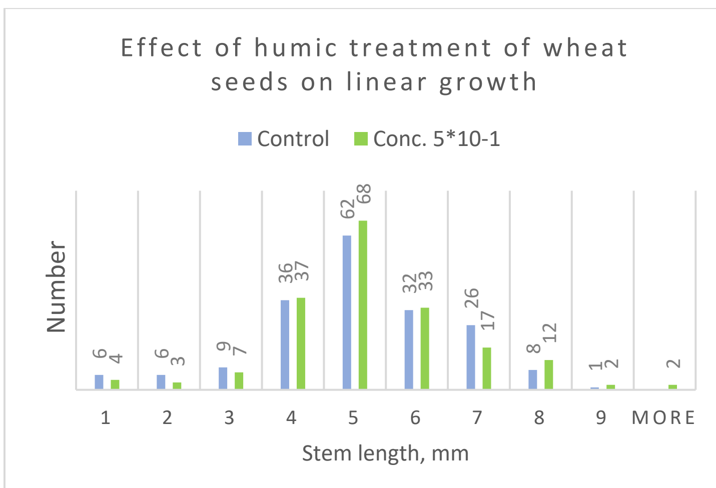
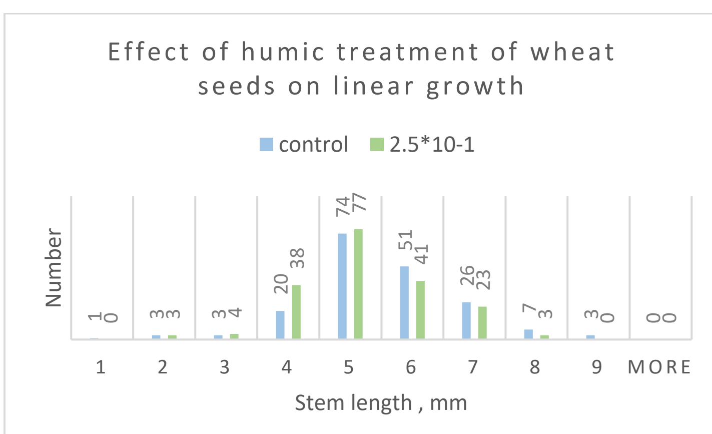


Figure 5 a, b, c

Sprout length measured on the seventh day of the laboratory experiment in untreated seeds was between 2 and 5 cm, with a predominant length of 3-4 cm. Sprout lengths in seeds pre-soaked in glucosamine solutions at a concentration of 0.015 mg/L were 4 to 8 cm and predominantly in the range 5-7 cm.





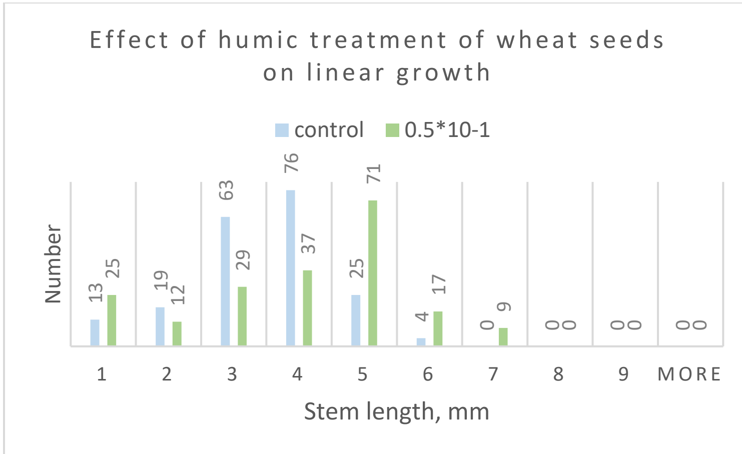


Figure 6 a, b, c

Pretreatment of seeds with humic stimulator accelerated linear stem growth, the effect being more pronounced in the treatment with the highest concentration - a 5-fold dilution of the preparation (Figure 6c). Compared to glucosamine, the applied humic stimulator at the tested concentrations had a less pronounced effect.

The stimulating effect of the tested preparations was confirmed by the results of increased biomass growth after seed pre-soaking (Table 1, 2).

Table 1. Effect of treatment of wheat seeds with humic stimulator on biomass growth

Biomass, g		Conc. 5x10 v/v^{-1}	Conc. 2.5x10^{-1} v/v	Konz. 0.5x10^{-1} v/v	Control
Roots	secondary	**0.727**	**0.941**	**0.702**	0.686
	SD	0.247	0.244	0.109	0.170
Stems	average	**1.489**	**1.828**	**1.4793**	1.461
	SD	0.171	0.098	0.3120	0.167

The determining factor is concentration. A 25-fold dilution of the humic preparation is effective. In this treatment regime, root biomass increased by 37% and stem biomass by 25% relative to the untreated control.

Table 2. Effect of glucosamine treatment of wheat seeds on biomass growth

Biomass, g		Konz. 0.015%	Konz. 0.05%	Control
Roots	average	**1.417**	**1.166**	1.257
	SD	0.237	0.143	0.107
Stems	average	**1.949**	**1.869**	1.828
	SD	0.133	0.119	0.260

Analytical data showed that glucosamine applied at a lower concentration stimulated root biomass growth by 12% relative to the control and had no effect on stem biomass. The effect of the high concentration was neutral.

A pronounced positive effect of treating seeds with a humic stimulator is an increase in dry matter content, which increases the resistance of the sprouts.

Table 3. Effect of treatment of wheat seeds with humic stimulator on dry matter content

Indicator		**Humic stimulator**		
Dry matter, %		$5x10^{-1}$	Conc. $2.5x10^{-1}$	Konz. $0.5x10^{-1}$
Roots	average	**32.69**	**24.39**	**30.57**
	SD	4.81	6.75	2.82
Control	average	**33.94**	**17.04**	**24.47**
Stems	secondary	**13.54**	**12.36**	13.60
	SD	**0.25**	**0.44**	0.50
Control	secondary	**13.824**	**16.360**	**13.23**

Soaking the seeds in humic preparation diluted 5 and 25 times contributes to an increase in dry matter by 25% and 43% in the roots. The dry matter content of the stem is not affected by seed treatment.

Table 4. Effect of treatment of wheat seeds with glucosamine stimulator on dry matter content

Indicator		**Glucosamine stimulator**		
Dry matter, %		Konz. 0,015%	Konz. 0,05%	Control
Roots	average	**17.175**	**17.83**	**17.99**
	SD	1.49	0.85	0.94
Stems	secondary	**11.62**	**11.29**	**11.71**
	SD	0.42	0.23	0.41

Soaking the seeds in glucosamine solutions did not affect the dry matter content of the root and stem (Table 4).

The analytical data obtained from the biotechnological experiments proved the biological activity of the developed stimulant samples. The composition is within the limits of physiological tolerance for plants - no decrease in laboratory germination parameters and depression in initial sprout growth were detected.

A major factor determining the effectiveness of seed treatment is the concentration of active substances. The results showed that soaking the seeds in the experimental solutions of humic stimulant had a positive effect by accelerating the germination process and promoting the friendly germination of seeds. Humic and glucosamine stimulants increased the intensity of initial growth: root biomass increased by 37% and 12%, respectively. With the humic preparation, stem biomass increased by 25%. The treatment with humic contributes to higher resistance during the most stress-sensitive periods of the biological cycle, germination and early development, which is manifested by an increase in root dry matter content of 25%/40% at different concentrations.

Results achieved

3 sample preparations were developed. Their harmlessness and phytostimulating activity in seed treatment has been proved. The possibilities of their application to improve seed properties by pre-sowing treatment are demonstrated. A methodology for foliar application of the stimulants during the growing season was developed and made available to farmers.

CONDUCTING FIELD TRIALS WITH FARMERS UNDER SEMI-PRODUCTIVE CONDITIONS IN WHEAT, BARLEY AND HAZELNUTS.

Field trials were conducted over 2 crop years to report the effect of foliar application of the developed biostimulants in the ICHT on growth and performance.

In the 2021/2022 and 2022/2023 marketing years, the production fields of the town of. Two field experimental trials to test the effect of biostimulants with No. 1 (glucosamine), No. 2 (humic complex), No. 3 (microbial extract) in two winter cereal crops - wheat variety Annapurna and barley variety Kalypso - were set up on an area of 720 m^2 . The experiments were laid out according to Shanin's block method, in 3 replications per variant, with an experimental plot size of 30 m^2 . A control variant was maintained for the entire growing season of the study crops, which was treated with water in an amount equal to the amount of detergent with which the other variants were treated (Scheme 1).

Scheme of the experiment (Scheme 1)

1. I variant - 30 L da^{-1} working solution;
2. II variant - 30 L da^{-1} working solution;
3. III variant - 30 L da^{-1} working solution;
4. Control variant - 30 L da^{-1} water;

The crops were sown at the optimum time for the area, mechanically, following a sunflower precursor. With the main tillage, wheat was fertilized with 25 kg da^{-1} of diammonium phosphate (DAP) once (first disking, mid-October) and barley with 12 kg da^{-1} of nitrogen-phosphorus fertilizer (NP) and 15 kg da^{-1} of triple superphosphate (TSP), with two subsequent pre-sowing diskings. The sowing of the cereal crops (wheat and barley) was carried out at the end of October.

The plot on which the study was carried out was located on black soil, on which the humus lies at a depth of 100 - 110 cm and the thickness of the humus horizon varies from 60 - 70 cm. The location of the experimental plot is in the eastern foothill region of the Balkan Mountains. Climatically, the area belongs to the temperate-continental climatic region with mild winters, wet springs, dry summers and variable autumns.

Plant treatments, in the experimental plots, were carried out at the tillering (BBCH 20-29) and flowering (BBCH 51-54) stages of wheat and barley. The treatments were applied with a backpack sprayer at a working solution rate of 30 L da^{-1} . Immediately prior to harvest of wheat and barley, when seeds reached the maturity stage (BBCH 92-99), 20 plants per replicate were sampled for biometric measurements. The experimental plots, per treatment, were harvested with a grain harvester and the biological yield of the crops was recorded. Seeds were collected from each variant for laboratory analyses.

Statistical analyses were conducted using the SPSS 17.0 software program, and differences in observed plant parameters among treatments were determined using one-way analysis of variance (ANOVA) followed by Tukey's test. All significance tests were two-sided and P≤0.05 was considered statistically significant.

Effect of biostimulants on growth and productivity in wheat

The tested biostimulants, in wheat, have a positive effect on the structural elements of yield. The highest values of the investigated biometric indicators were recorded for the humic stimulant (variant No. 2), except for the plant height indicator. This correlation was maintained both in the individual years of the field trial and on average over the period (Tables 5-7).

Table 5: Biometric parameters in wheat variety Annapurna, 2022**.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	80.10 a	1.35 b	1.79 b	39 c	1.10 b	43.10 b
2 - Khumin complex	71.40 b	3.70 a	4.12 a	75 a	1.62 a	45.10 a
3-Microbial Extract	72.25 b	1.80 b	1.91 b	45 bc	1.29 b	44.80 b
4 (Control)	72.45 b	1.90 b	2.14 b	53 b	1.22 b	42.30 b

Table 6: Biometric parameters in wheat variety Annapurna, 2023**.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	80.65 a	1.70 b	1.94 c	44 c	1.53 c	45.21 bc
2 - Khumin complex	72.50 c	4.00 a	4.62 a	60 a	2.47 a	47.15 a
3-Microbial Extract	74.90 bc	2.25 b	2.56 bc	53 bc	1.99 b	44.80 b
4 (Control)	77.10 b	2.35 b	2.81 b	56 b	1.88 bc	45.30 b

Table 7: Biometric indices in wheat variety Annapurna, **average for the period 2022-2023.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	80.37 a	1.52 c	1.86 c	41 c	1.31 c	44.15 bc

2 - Khumin complex	71.95 c	3.85 a	4.32 a	77 a	2.04 a	46.12 a
3-Microbial Extract	73.57 bc	2.02 bc	2.23 bc	49 bc	1.63 b	44.80 b
4 (Control)	74.77 b	2.12 b	2.47 b	55 b	1.55 bc	43.80 c

The highest plants were measured in the glucosamine complex (variant #1), whose values ranged from 80.10 cm to 80.65 cm, and the lowest in the humic stimulator (#2).

According to the results obtained, after the application of biostimulants, a significant difference was reported, which was statistically significant at $P \leq 0.05$.

The number of class-bearing stems of variant No. 2 exceeded the control by 1.8 pcs (2022), 1.65 pcs (2023) and 1.73 pcs (average over the period), respectively, and the realized positive differences were statistically significant at $P \leq 0.05$. For the other two options (#1 and #3), the values are close to the control and no statistically significant relationships were found.

The indicator of the mass of class-bearing stems is similar to their number, both for the years of the study and on average over the period. Positive differences were realized in favour of variant 2 and were proved at $P \leq 0.05$. The values of the indicator were not affected by the application of stimulants #1 and #3.

The applied humic biostimulant (No. 2) also had a significant effect on the number and mass of seeds per class, and contributed to their increase, by 22 pcs and 0.4 g (2022), 4 pcs and 0.59 g (2023) and 22 pcs and 0.49 g (mean), respectively, compared to the control. The reported positive differences in these two parameters were also statistically significant at $P \leq 0.05$.

In spite of the reported lower values of the biometric parameters characterizing the yield, the values reported for the mass per 1000 seeds for the three applied stimulants exceeded those of the control, both in the individual years and on average over the period. Statistically significant differences, at $P \leq 0.05$, were recorded only for the humic preparation. Despite the reported higher values of mass per 1000 grains relative to the control, no statistically significant relationships were found for the glucosamine and microbial extract treated variants.

The results of the present study showed that foliar application of biostimulants in wheat contributed to increased yields (Figures 7, 8, 9). In the control plot, where the crop was grown without biostimulant application, the yield averaged 497 kg da^{-1} , and remained relatively stable over the two years.

ЕВРОПЕЙСКИ СЪЮЗ

ПРОГРАМА ЗА РАЗВИТИЕ НА СЕЛСКИТЕ РАЙОНИ

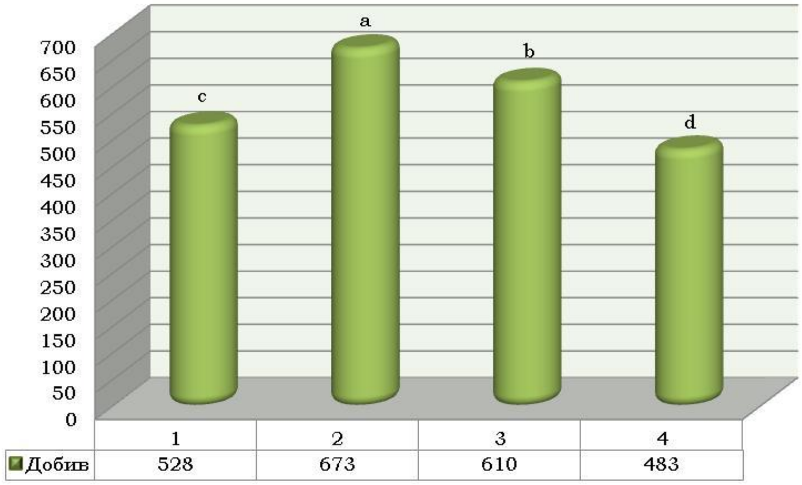


Figure 7: Seed yield in wheat variety Annapurna (kg da^{-1}), in 2022**.**

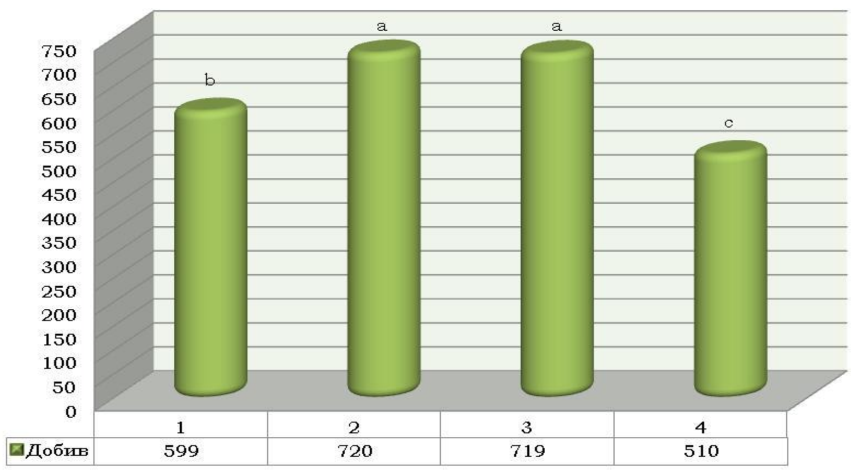


Figure 8: Seed yield in wheat variety Annapurna (kg da^{-1}), in **2023.**

ЕВРОПЕЙСКИ СЪЮЗ

ПРОГРАМА ЗА РАЗВИТИЕ НА СЕЛСКИТЕ РАЙОНИ

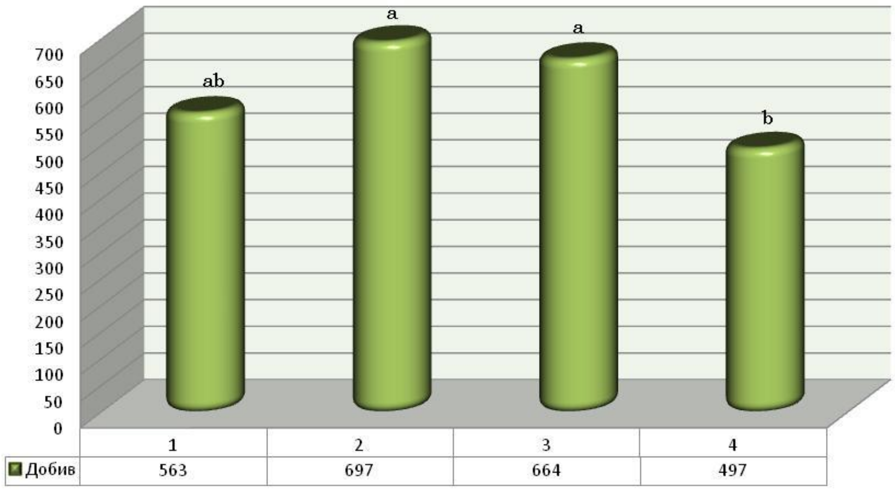


Figure 9. Seed yield of wheat variety Annapurna (kg da^{-1}), **average for the period 2022-2023.**

1-glucosamine ; 2-humic complex; 3-microbial extract; 4-controls

Effect of biostimulants on growth and productivity in barley

The values obtained from the biometric measurements in barley are similar to those in wheat (Tables 8-10).

Table 8: Biometric parameters in barley variety Calypso, 2022**.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	52.80 bc	1.40 a	1.63 ab	25.20 b	0.75 a	44.90 a
2 - Khumin complex	58.70 a	1.70 a	2.01 a	26.30 a	0.85 a	45.70 a
3-Microbial Extract	56.35 ab	1.60 a	1.91 ab	25.10 ab	0.80 a	45.10 a
4 (Control)	50.70 c	1.35 a	1.55 b	24.10 b	0.73 a	44.20 a

Table 9. Biometric indicators in barley variety Calypso, 2023**.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	54.05 bc	1.70 a	1.95 a	24.70 c	0.96 ab	45.90 ab
2 - Khumin complex	60.70 a	2.00 a	2.26 a	26.95 a	1.05 a	46.70 a
3-Microbial Extract	57.90 ab	1.95 a	2.16 a	26.10 ab	1.15 a	46.10 a
4 (Control)	53.20 c	1.70 a	1.93 a	25.00 bc	0.73 b	45.20 b

Table 10. Biometric indicators in barley variety Calypso, **average for the period 2022-2023.**

Option	Plant height cm.	Number of class-bearing stems	Weight of class-bearing stems, g	Number of seeds in 1 class	Seed weight in class 1, g	Weight per 1000 seeds, g
1 - Glucosamine	53.42 b	1.55 b	1.79 b	24.40 b	0.85 ab	45.40 a
2 - Khumin complex	59.70 a	1.85 a	2.13 a	26.62 a	0.95 a	45.70 a
3-Microbial Extract	57.12 a	1.77 ab	2.03 ab	25.77 a	0.97 a	45.60 a
4 (Control)	51.95 b	1.52 b	1.74 b	24.50 b	0.73 b	44.70 a

With the best biometric indicators characterizing the yield was the humic complex (preparation No. 2). The observed indices, in this variant, were higher than the control and were statistically significant at the level of evidence P≤0.05. The reported positive differences were maintained both during the years of the study and on average.

In contrast to wheat, in barley, a more pronounced positive influence of the glucosamine complex (No. 1) and microbial extract (No. 3) on the structural yield elements: number of ear-bearing stems, mass of ear-bearing stems, number of seeds in 1 class, mass of seeds in 1 class, mass per 1000 seeds was observed. The values of the studied parameters are slightly higher than the control. In the case of glucosamine complex they were not statistically confirmed. In the case of microbial extract, the indicators number of seeds in 1 class and mass of seeds in 1 class were statistically higher than the control variant.

The results shown in Figures 10-12 reflect the effect of foliar application of the tested biostimulants on the magnitude of grain yield obtained in barley cv. Calypso by year and on average over the period.

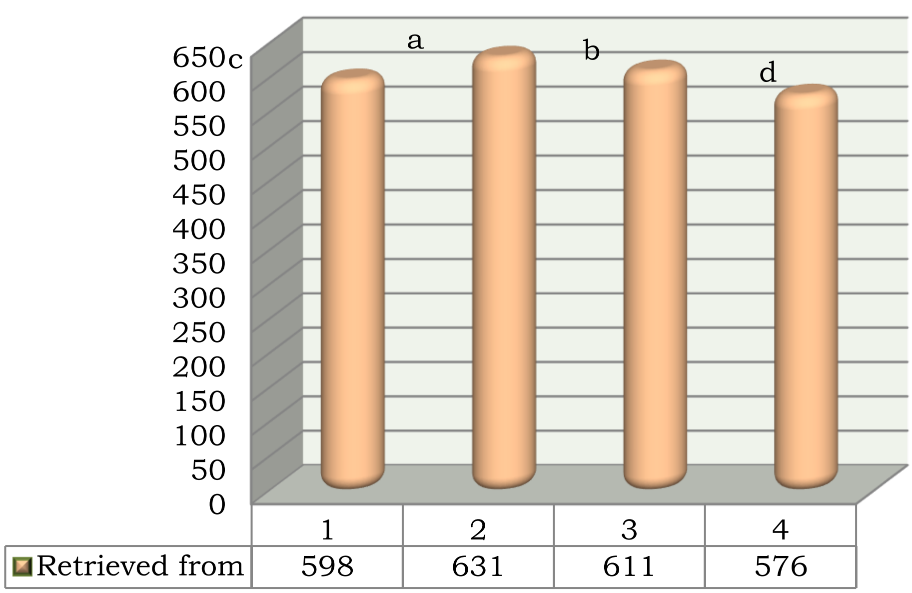


Figure 10. Seed yield of barley variety Calypso (kg da^{-1}), in 2022.

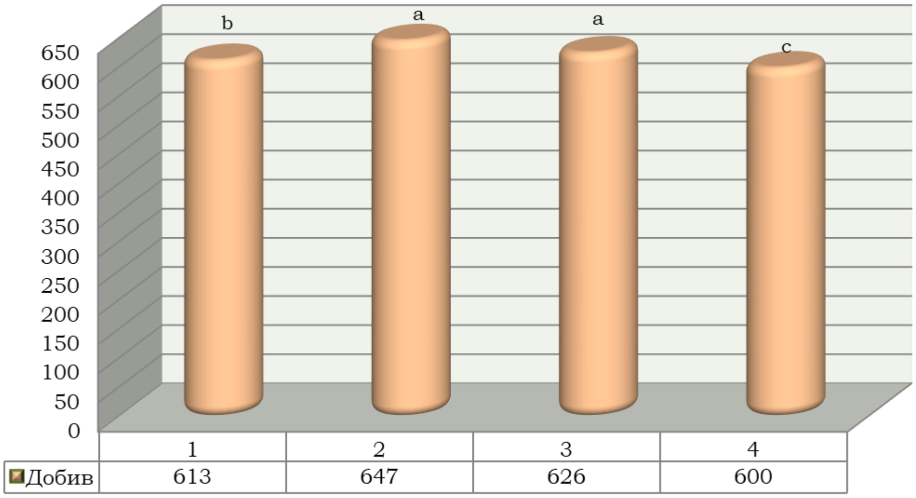


Figure 11. Seed yield of barley variety Calypso (kg da^{-1}), in **2023.**

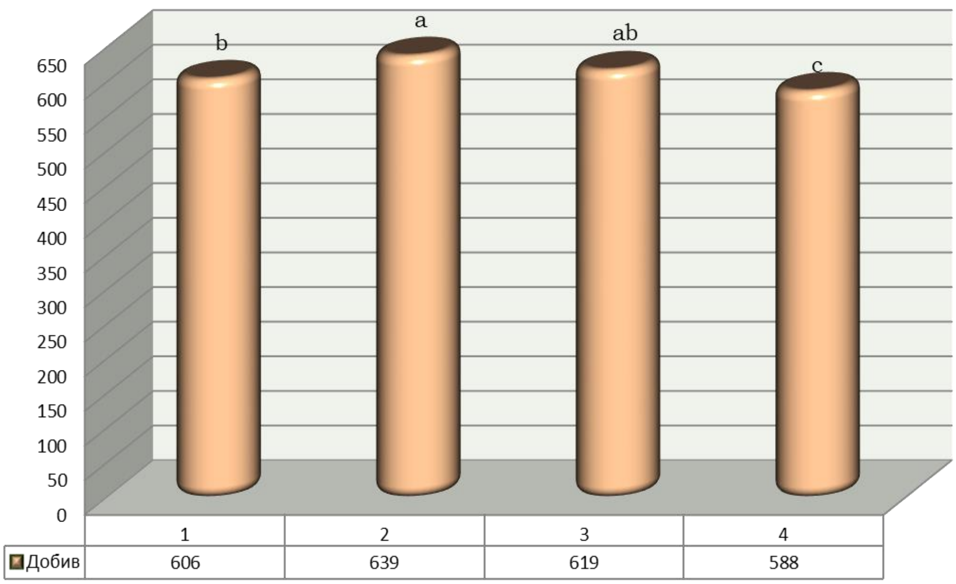


Figure 12. Seed yield of barley variety Calypso (kg da^{-1}), **average for the period 2022-2023.**

1-glucosamine ; 2-humic complex; 3-microbial extract; 4-controls

Over the two marketing years, the average yield reported for the control was 588 kg da^{-1} . In 2022, from barley, the highest yield of 631 kg da^{-1} was harvested from variant #2 with humic stimulator, followed by variant #3-microbial extract (611 kg da^{-1}) and variant #1-glucosamine (598 kg da^{-1}) The data obtained, from barley, were statistically proven at P≤0.05, only in the humic stimulator (#2). In the other variants the increase in values, was not statistically proven. In 2023 the results are confirmed. The highest yield of 647 kg da^{-1} was obtained from the variant with applied humic biostimulator, followed by the microbial extract (626 kg da^{-1}) and glucosamine (613 kg da^{-1}).

Wheat and barley are crops that are responsive to fertilization. Excessive application of synthetic soil fertilisers leads to soil fertility contamination and an increase in plant height, which may later become lodged. Nowadays, an alternative to synthetic soil fertilisers is considered to be biostimulants, which are predominantly based on natural organic products. The results of precise field trials confirm this possibility.

Results achieved

The results obtained from the field trials conducted with wheat and barley demonstrate to farmers in real production conditions that the application of the developed organic-based stimulants at low concentration and dose by foliar spraying at appropriate stages of crop development can contribute to increased yields. Of the tested preparations, the influence of the humic stimulator was statistically proven, with yields increasing on average over 2 marketing years by 40% in wheat and 12% in barley as a result of better ear ripening and higher grain mass compared to the control. The other treatments also had a positive effect, but it was not mathematically proven. This may be due to variation in composition, climatic factors, inappropriate application rate in the particular conditions of the field trials.

Polish experience with hazelnuts

In the spring of 2022 and 2023, on an area of 4 da, in the hazelnut gardens of the village of Mlada gvardiya, municipality of Vetrino, Varna district, a spring hand pruning of hazelnut bushes was carried out. A field trial was built, with three organic fertilizers and a control, in four /4/ replications, with 20 shrubs per variant and 40 shrubs per control, and randomized placement of the variants. The hazelnut orchard was located on black soils with very good soil structure. The location of the experimental plot is in the eastern foothill region of the Balkan Mountains. Climatically, the area belongs to the temperate-continental climatic region with relatively mild winters characterised by snowfalls, wet springs and cool summers. The hazelnut orchard is cultivated organically, without the use of chemical fertilisers or plant protection products. In April of 2022 and 2023, marking of the hazel bushes was carried out, according to a previously prepared scheme, with signs marking the exact location of the variants and their repetitions. Between 10 and 25 May 2022 and 2023, biologically active biostimulants, developed by ICHT - Sofia, were introduced to increase the productivity and quality of hazel bushes. The biostimulants were applied twice (leafing phase and 30 days after the first application of the biostimulants), in the morning, in dry weather and at temperatures up to 22 ºC, according to the following schedule.

Scheme of the experiment

1. Control variant - 50 L da^{-1} water;
2. I variant- glucosamine - 50 L da^{-1} working solution;
3. II variant- humic complex - 50 L da^{-1} working solution;
4. III option - microbial extract - 50 L da^{-1} working solution.

At harvest, no effect of foliar spraying with biostimulants on yield was reported. This may be due to suboptimal dose of foliar treatment applied, also to different species-specific requirements of the crop.

EVALUATION OF THE EFFECT OF BIOSTIMULANT TREATMENT ON PRODUCTION QUALITY.

Introduction

Wheat (Triticum aestivum L.) is the most important crop used for human and animal nutrition worldwide as cereals are an important source of protein and energy and due to its high demand, varieties have been developed that are adapted to different environmental conditions (Ghasemi-Mobtaker et al.

2020). Nitrogen (N) is often a limiting factor for increasing both yield and quality (Zhu et al. 2016). Nitrogen fertilizer is commonly used in large quantities to improve crop production worldwide (Bakhtiari et al. 2020). However, application of nitrogen fertilizer beyond crop demand causes adverse effects on the environment. Excessive nitrogen application pollutes soil, water, and air locally; accelerates global climate change; and affects human health (Sainju et al. 2019). Humic acid, as an organic fertilizer, is a naturally occurring ring-shaped polymeric heterocyclic organic compound that contains carboxylic (COOH-), phenolic (OH-), alcoholic, and carbonyl fractions (Khan et al. 2018). Humic acids have been reported to increase the transport and availability of nutrients (Olk et al. 2018; Yildiztekin et al. 2018). Humic complexes, due to their chemically active groups, can alter biochemical processes in plants, resulting in improved photosynthesis, respiration rates, and increased hormone and protein production (Olk et al. 2018). Overall, the positive role of humic acid on plant physiology has been described in terms of enhancing root growth and nutrient uptake (Dinçsoy and Sönmez 2019). Recently, some farmers have turned to organic fertilizers (composts, microbial cultures, algal extracts) as a substitute for inorganic fertilizers to improve productivity. Integrated use of organic and inorganic fertilizers can play an important role in sustainable soil fertility and crop productivity (Bakhtiari et al. 2020). Hamad et al. (2020) observed improvement in following soil fertility and protein content of wheat grains - application of organic fertilizers. However, they also reported that synthetic fertilizer resulted in the highest grain yield. Improved plant productivity due to the combination of chemical and organic fertilizers were reported in rice (Mi et al. 2018; Moe et al. 2019), millet (Abebe and Deressa 2017), wheat (Arif et al. 2017), and maize (Azeem and Ula 2016).

Regular consumption of nuts is recommended worldwide due to the beneficial effects of the chemical composition on health. The prevention of cardiovascular disease through the consumption of nuts, in particular hazelnuts, has been extensively studied and has shown positive effects, mostly based on improved blood lipid profiles. Hazelnuts contain significant amounts of macronutrients such as fat, protein and fiber, but also micronutrients such as minerals and vitamins. Hazelnuts are high in monounsaturated fatty acids (MUFA) and contain relatively small amounts of saturated fatty acids (SFA). This information has determined the interest in studies on variations of major components in the chemical profile of hazelnuts.

Based on available scientific information, it was assumed that crops have different responses in their yield and physicochemical characteristics to different organic and synthetic fertilizer treatments.

In this regard, the present study was justified on the assumption that conducting field trials to investigate the effect of organic-based biostimulants on physicochemical and biochemical parameters in wheat, barley and hazelnut crops would be of applied importance to establish the expected positive effect on the chemical composition of target components.

The aim of the study was to evaluate the effect of biostimulant dose loading on grain chemistry and performance by foliar spraying during the growing season for 2 crop years.

Material and methods

Crops studied: wheat variety Annapurna, barley variety Calypso, hazelnuts.

Location of the study: wheat and barley - Vetrino municipality - Varna region, hazelnuts - Dalgopol municipality - Varna region

Experimental design: The field experiment with wheat and barley was conducted in 5 replications using 20 m plots^2 (4 m × 5 m). A mean sample of biological material was collected from each plot for analysis.

For hazelnuts, the experiment was carried out in 5 replicates with each group containing 8 trees. The dose loading for these was 500 ml for all 40 trees.

Agro-remediation measures: spraying twice - at the tillering and flowering stages in wheat and barley with biostimulants; spraying twice at the flowering stage in hazelnuts with biostimulants.

Biostimulants used:

1. Glucosamine - dosage load - twice 500 ml/dka;
2. Humic complex - dosage load - twice 500 ml/dka;
3. Microbial extract - dose load - twice 500 ml/dka

Indicators studied:

Wheat - wet gluten, crude protein; total fat; fatty acid composition
Barley - wet gluten, crude protein, total fat, fatty acid composition;
Hazelnuts - crude protein, total fat, fatty acid composition.

Methods used:

- Wet gluten - BDS 754-79. The gluten content of wheat grain is determined by washing it from dough kneaded on a sample of grain, crushed to a specified size, weighing 25 g, with the addition of 14 ml of water. After kneading, the dough is allowed to rest (settle) for 20 minutes to swell the proteins of the gluten complex, after which the gluten is washed from it in water at 18 + 2 °C. The cereal hulls, water-soluble matter and starch are completely removed from the dough, leaving only the gluten proteins (glutenin and gliadin), forming a highly elastic jelly (gel). The washed gluten is called raw as it contains up to 70% water. After partial drying in the hands (to sticking point) and removal of excess water, the gluten is weighed on a laboratory balance to the nearest 0,1 g and its content is recalculated as a % of the sample weight.

- Crude protein - Protein content shall be determined by the Kjeldehl method as specified in AACC Method 46-10.

- Total Fat - Total fat was analyzed by Soxhlet extraction, immersing samples in a boiling solvent (petroleum ether from 30-60° C of the boiling range) that dissolves fats, oils, pigments, and other solutes, collectively referred to as "crude fat." The continuous flow of condensed soluble extractives, dissolved extractables and the resulting crude fat residue is determined gravimetrically after drying [Thiex et al.]

- Fatty acid composition-Fatty acid analysis of nuts was performed using gas chromatography (GC-17 V3; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and autosampler (AOC-5000) as described [Dawczynski C.]. Fatty acid concentrations were expressed as percentage of the total area of all fatty acid methyl esters (% of total fatty acid methyl esters, FAME) using GC solution software version 2.3 (Shimadzu). The results of the measurements of some major fatty acids that determine the fatty acid composition of the studied crops (palmitic C16:0; stearic C18:0; oleic C18:1; linoleic C18:2; linolenic - C18:3), as well as the ω6/ω3 ratio of the fatty acid composition of hazelnuts are shown.

- Statistical data processing - Statistical analysis of the data was performed using the ANOVA procedure of the JMP v.7 software package [JMP]. The effects of biostimulants were evaluated.

In case of significant effects, the difference between means was evaluated by Tukey HSD at P<0.05.

Results and discussion

Tables 11 -16 show the results of the analyses of wet gluten, crude protein and total fat of the crops studied. The amounts of some essential fatty acids that determine the fatty acid composition (palmitic C16:0; stearic C18:0 ; oleic C18:1; linoleic C18:2; linolenic - C18:3) and the ω6/ω3 ratio of the fatty acid composition of hazelnuts were also determined.

Table 11. Physicochemical and biochemical parameters in Wheat -variety Annapurna - 2022.

Biostimulant	Wet gluten (%)	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)				
				C 16:0	C 18:0	C 18:1	C 18:2	C 18:3
Control	31.46	12.15^{b}	2.13	16.95	6.19	19.33	47.05	5.52
Glucosamine	31.64	**12,62^{a}**	2.24	16.47	6.33	19.54	49.03	5,18
Microbial Extract	31.72	12.26^{ab}	2.18	17.12	6.13	19.82	46.54	5.54
Khumin complex	31.44	12.26^{ab}	2.11	16.85	6.37	19.23	48.24	5.35
SEM	0.75	0.22	0.10	0.85	0.21	0,50	1.35	0.33
(P)	0.912	0.020	0.214	0.342	0.251	0.584	0.124	0.296

Zab. *Values associated with different letter designations differ significantly, P<0.05*

In the first year, we found a reliable increase in crude protein content in wheat areas treated with glucosamine complex. The effect of the other biostimulants was in the same direction, but with very small differences compared to the control group. The wet gluten content, total lipids and essential fatty acids studied were not affected by the influence of the biostimulants used.

Table 12. Physicochemical and biochemical indices in Wheat variety Annapurna -2023.

Biostimulant	Wet gluten (%)	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)				
				C 16:0	C 18:0	C 18:1	C 18:2	C 18:3
Control	32.13	12,23^{b}	2.06	16.62	6.38	19.56	48.03	5.35

Glucosamine	31.95	12.84^{a}	2.15	16,45	6.40	19.83	47.44	5.60
Microbial Extract	31.54	12.79^{a}	2.12	16.28	6.56	19.15	48.45	5.42
Khumin complex	32.56	13.31^{a}	1.98	16.81	6.28	20.21	46.89	5.51
SEM	0.75	0.25	0.12	0.74	0.24	0.65	1.72	0.28
(P)	0.883	0.006	0.549	0.856	0.765	0.145	0.354	0.903

Zab. *Values associated with different letter meanings differ significantly, $P<0.05$*

In the second year of study, the influence of all biostimulants used resulted in a reliable increase in crude protein content in the samples studied. The highest values were obtained after the application of humic complex, where the values of the indicator increased by 8.8% compared to the control group. As in the previous year, the influence of biostimulants on the indicators wet gluten, total lipids and fatty acid composition was insignificant.

Table 13. Physicochemical and biochemical parameters in Barley - Calypso variety - 2022.

Biostimulant	Wet gluten (%)	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)				
				C 16:0	C 18:0	C 18:1	C 18:2	C 18:3
Control	19.15	13.27^{b}	2.27	18.13	4.56^{b}	21.24	45.02	6.03
Glucosamine	18.89	14.96^{a}	2.13	18.32	4.61^{b}	21.12	45.56	5.89
Microbial extract	18.93	13.74^{b}	2.32	17.86	5.02^{a}	20.98	44.97	6.12
Khumin complex	19.14	15.45^{a}	2.19	17.97	4.48^{b}	21.36	45.21	6.14
SEM	0.34	0.68	0.15	0.38	0.22	0.56	1.42	0.23
(P)	0.954	0.0012	0.458	0.650	0.047	0.766	0.934	0.365

Zab. *Values associated with different letter meanings differ significantly, $P<0.05$*

The results of the studies done for the crude protein indicator in 2022 of the study showed that the application of glucosamine and humic complex in barley led to the reliable increase in grain content - by 12.7% and 16.4%, respectively, compared to the untreated control. To a lesser extent, the same can be said for the microbial extract used as a biostimulant, but the difference with respect to the control is unreliable. The wet gluten and total lipid contents fluctuated around the values of the control. Of the essential fatty acids, we found a reliable increase in stearic acid (C 18:0) in the areas treated with microbial extract. For the other essential fatty acids, we found no significant effect of the biostimulants used.

Table 14. Physicochemical and biochemical parameters of Barley - Calypso variety - 2023.

Biostimulant	Wet gluten (%)	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)				
				C 16:0	C 18:0	C 18:1	C 18:2	C 18:3
Control	19.31	13.05^{b}	2.34	18.34	4.61	21.15	44.94	6.11
Glucosamine	19.12	14.63^{a}	2,26	18.61	4.49	21.45	45.14	6.05
Microbial Extract	19.17	14.42^{a}	2.18	17.93	4.71	20.87	43.87	6.21
Khumin complex	19.47	15.38^{a}	2.15	17.75	4.43	20.98	46.45	6.14
SEM	0.32	0.58	0.17	0.56	0.19	0.67	2.14	0.27
(P)	0.867	0.0023	0.656	0.293	0.123	0.713	0.592	0.964

Zab. Values associated with different letter meanings differ significantly, P<0.05

Similar trends were found in the second year of the barberry study. For the crude protein parameter, a significant increase was found after the use of all three biostimulants with values between 10.5% and 17.9% compared to the untreated control. The wet gluten content of barley cultivar 'Calypso' varied within a small range. While total lipid content decreased unconfidently in all biostimulant treated groups compared to the control. Fatty acid composition was also not affected by the biostimulant treatments used.

Table 15. Biochemical indicators in hazelnuts - 2022.

Biostimulant	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)					
			C 16:0	C 18:0	C18:1	C18:2	C 18:3	ω6/ω3
Control	18.43^{b}	50.62	5.32	3.61	81.39	8.74	0.31	29.10
Glucosamine	20.24^{a}	51.14	5.26	3.67	80.45	7.96	0.36	28.45

Microbial extract	18.86^{b}	50.29	5.24	3.48	81.38	8.64	0.35	25.96
Khumin complex	20.65^{a}	48.72	5.21	3.36	79.36	8.33	0.42	29.14
SEM	1.15	3.35	0.15	0.14	2.69	0.64	0.08	3.23
(P)	0.0057	0.817	0.912	0.523	0.903	0.545	0.341	0.186

Zab. *Values associated with different letter meanings differ significantly, P<0.05*

In 2022, the experimental results showed that the use of biostimulants led to a reliable increase in crude protein in hazelnuts of the order of about 10%, with the highest values recorded in the treatment with humic complex. On the other hand, a slight decrease in total lipid content of about 1 to 2% was found.

Table 16. Biochemical indicators in hazelnuts- 2023.

Biostimulant	Crude protein (%)	Common lipids (%)	Essential fatty acids (%)					
			C 16:0	C 18:0	C18:1	C18:2	C18:3	ω6/ω3
Control	18.09^{b}	53.32	5.52	3.64	81.19	8.55	0.37	26.43^{b}
Glucosamine	20.12^{a}	52.56	5.73	3.38	80.34	8.82	0.41	27.93^{b}
Microbial extract	19.85^{a}	53.11	5.94	3.43	78.61	8.93	0.40	29.45^{a}
Khumin complex	20.43^{a}	52.38	5.51	3.80	81.68	8.21	0.42	26.81^{b}
SEM	0.84	2.14	0.42	0.27	2.45	0.54	0.07	1.48
(P)	0.0001	0.947	0.734	0.228	0.550	0.681	0.903	0.038

Zab. *Values associated with different letter meanings differ significantly, P<0.05*

In 2023, similar to the 2022 results, a higher grain protein content was found in the treatments. In the second year, we also found significantly higher ω6/ω3 ratio values in the hazelnut group treated with yeast extract biostimulant. The essential fatty acids changed insignificantly in the treatments with the biostimulants used.

The total lipid content determined in hazelnut samples was within 48.72% to 51.14% for the first year and 52.38% to 53.32% for the second year. In the fatty acid profile, oleic acid (C18:1n-9) had the highest proportion, its content forming about 80% of the fatty acid composition. Although in much smaller amounts, but above 1% are linoleic (C18:2), palmitic (C16:0) and stearic (C18:0). Hazelnuts are characterized by a low content of saturated fatty acids (9.17%), the very low percentage of myristic acid (C14:0) being noteworthy. The latter, together with C16:0, is considered cholesterol-raising and its high content in foods is not desirable in order to reduce the risk of cardiovascular disease. Hazelnuts are characterised by a low α-linolenic acid content (C18:3) of 0.3-0.4 %, which determines the values of the n-6/n-3 fatty acid ratio, namely between 25 and 30 %. Considering the recommendations for values of this ratio <4 (Simopoulos, 2001), the values obtained for hazelnut composition are quite high, and by this indicator it could be considered that the fatty acid composition of hazelnuts is somewhat unbalanced. On the other hand, the ratio between poly- and saturated fatty acids for the two years varied between 0.95 and 0.98, which is above the recommended minimum of 0.4. The unfavourable values of the ratio between n-6 and n-3 fatty acids is compensated by the high percentage of oleic acid (C 18:2). A number of scientific studies (Karacor and Cam, 2015) have highlighted the positive effect of this fatty acid on human health It is also the basis of the Mediterranean diet, whose benefits for human health have been demonstrated. The results we obtained for the individual fatty acid content are in agreement with the studies of Granata et al. (2017) in hazelnuts grown in Italy and Tüfekci and Karataş (2018) in varieties grown in Turkey, and unlike us, the latter determined linolenic acid contents in the range 0.075%-0.096%.

References

Thiex, N.J.; Anderson, S.; Gildemeister, B. Crude fat, diethyl ether extraction, in feed, cereal grain, and forage (Randall/Soxtec/submersion method): Collaborative study. J. of. AOAC Int. 2003, 86, 888-898.

Karacor, K. ., & Cam, M. . (2015). Effects of oleic acid. Medical Science and Discovery, 2(1), 125-132. https://doi.org/10.36472/msd.v2i1.53

Granata, M. U.; Bracco, F.; Gratani, L.; Catoni, R.; Corana, F.; Mannucci, B.; Sartori, F.; Martino, E. (2016). Fatty acid content profile and main constituents of Corylus avellana kernel in wild type and cultivars growing in Italy. Natural Product Research, (), 1-6.

Tüfekci F, Karataş Ş. Determination of geographical origin Turkish hazelnuts according to fatty acid composition. Food Sci Nutr. 2018 Feb 2;6(3):557-562.

Simopoulos, A. P. n-3 fatty acids and human health: Defining strategies for public policy. Lipids 2001, 36(S1), S83-S89.

Dawczynski C., Schubert R., Jahreis G. Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chem.* 2007;103:891-899. doi: 10.1016/j.foodchem.2006.09.041.

JMP, Version 7 SAS Institute Inc., Cary, NC, 1989-2023

Ros, E. Nuts and CVD. Br. J. Nutr. 2015, 113, S111-S120. [CrossRef] [PubMed]

Perna, S.; Giacosa, A.; Bonitta, G.; Bologna, C.; Isu, A.; Guido, D.; Rondanelli, M. Effects of Hazelnut Consumption on Blood Lipids and Body Weight: A Systematic Review and Bayesian Meta-Analysis. Nutrients 2016, 8, 747 [CrossRef] [PubMed].

ЕВРОПЕЙСКИ СЪЮЗ

ПРОГРАМА ЗА
РАЗВИТИЕ НА
СЕЛСКИТЕ РАЙОНИ

Results achieved

In the two-year field trials conducted under production conditions with the crops - wheat - variety Lavandu, barley - variety Calypso and hazelnuts it was found that under the influence of the applied 3 biostimulants - polyglucosamine complex, microbial extract and humic complex the crude protein content increased reliably in all three crops grown. The highest values of the indicator were found after treatment with glucosamine in wheat and with humic complex in barley and hazelnut. The other examined parameters - wet gluten, total fat and fatty acid composition varied within small limits compared to the control group, with differences within the statistical error.