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Health—Promoting Properties of Highbush Blueberries Depending on Type of Fertilization

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Abstract: The purpose of the experiment was to demonstrate a comparison of fertilization with and without biostimulation. A study was carried out in an experimental blueberry field in central Poland (51°55′42.7″ N 20°59′28.7″ E) during the three growing seasons of 2019, 2020 and 2021, on 'Bluecrop' shrubs growing at a distance of 1 × 3 m. The plants were re-planted in the spring of each year and irrigated using drip irrigation. The experiment was conducted using a random block design (four fertilizer treatments × five replications × six bushes). The fruits were tested for antioxidant activity and amount of total polyphenols. Additionally, anthocyanin quantitative and qualitative analysis was performed. The results indicated a significant effect of fertilizer combinations on the values of the evaluated parameters. The positive effect of biostimulants on the content of antioxidant compounds in highbush blueberry fruit was significant. In most of the combinations in which additional biostimulants were used, higher values of the analyzed indicators (antioxidant activity and polyphenol content) were observed. The most noteworthy was the T4 fertilization program, where during treatment, soil and foliar fertilization were carried out with preparations that contained biostimulants.

Keywords: biostimulation; seaweed extract; blueberries; polyphenols; antioxidants; anthocyanins; fertilization

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1. Introduction

The ever-increasing consumer demand for blueberries means that the cultivation of this species in Poland, and around the world, rapidly rises each year. In addition to its health-promoting and flavorsome qualities, blueberry fruit is highly sought after due to the widespread marketing campaign promoting blueberries as superfoods [1–6]. Blueberries are considered a natural source of health-promoting substances, containing a diverse group of bioactive compounds that show positive effects on human health [7]. Beneficial elements such as fiber, minerals, vitamins, and phenolic compounds with antioxidant characteristics can be found in abundance in these fruits [8,9]. Blueberry fruits are widely consumed as a dessert fruit, but they are also commonly used in processing as a dried or frozen raw material, for juices, purees, jams, and even wine [10–12]. Berry fruits, distinguished by their antioxidant richness, also include blueberry (Vaccinium myrtillus), blackberry (Rubus fruticosus), chokeberry (Aronia melanocarpa), black currant (Ribes nigrum), cranberry (Vaccinium macrocarpon), raspberry (Rubus idaeus), grape (Vitis vinifera), and strawberry (Fragaria × ananassa) [13]. Antioxidants in the fruit are mainly represented by vitamin C and polyphenols such as anthocyanins, phenolic acids, flavanols and tannins [13]. Kęhkönen et al. [14] reported that berries are one of the richest sources of antioxidants in our diet. According to Prior et al. [15], in particular, the fruits of highbush blueberries deserve attention due to the high level of plant phenolic compounds which determines the high antioxidant activity.

Rashidinejad [7] described the beneficial effects of highbush blueberry fruit for the prevention of chronic diseases, including cancer, cardiovascular disorders, diabetes, and neurodegenerative diseases. Häkkinen and Törrönen [16] and Rimando et al. [17] reported on the antioxidant and anti-cancer effects of phenolic compounds contained in berries. Blueberry extracts reduce age-related decline in neurons and cognitive function, common in disorders such as Alzheimer's disease [18]. Bioactive substances with antioxidant properties have hydroxyl groups (-OH) on aromatic rings. These are components produced by secondary metabolism in plants. In addition to their health-promoting properties, they have an important physiological function in plants. They are known to protect plants from pests, pathogens and ultraviolet radiation, to regulate metabolic pathways and impart to color and flavor to the plant [19]. According to Piątkowska et al. [20] and Khoo et al. [21], anthocyanins are a group of phenolic compounds of very high health-promoting importance. A study by Lee et al. [22] indicated that the following anthocyanins are found in highbush blueberry fruit: cyanidins, delphinidins, malvinidins, peonidins, and petunidins. According to Khoo et al. [21], anthocyanins are used as an appetite-stimulating phytopharmaceutical, a cholagogue, and the highly bioavailable anthocyanin effectively reduces cellular lipid peroxidation. Tsuda et al. [23] indicated that anthocyanins extracted from beans (Phaseolus vulgaris) have antioxidant properties. The authors showed that a 'purple corn color' (PCC) diet rich in cyanidin 3-O-β-D-glucoside significantly inhibited the development of obesity and alleviated hyperglycemia in mice induced by a high fat (HF) diet. The PCC diet reduced mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis. The decrease in enzyme levels as a result of the PCC diet may contribute to a decrease in triacylglycerol accumulation in WAT (white adipose tissue). Tsuda et al. [23] also pointed to the use of anthocyanins as a functional food factor, which may have important implications in the prevention of obesity and diabetes. Numerous scientific studies have proved the anticancer effects of anthocyanins [24]. Anthocyanins and anthocyanin–pyruvic acids exhibit anticancer properties by inhibiting tumor cell proliferation and act as anti-cell invasion agents and chemo-inhibitors [25]. A study by Buena et al. [26] confirmed that the anthocyanin-rich fraction obtained from the Toro variety had the highest anthocyanin content and antioxidant activity, and inhibited melanoma cell proliferation in mice. The results of [26] indicated that anthocyanins from highbush blueberry fruit could be used as a chemopreventive or adjuvant agent in controlling the spread of cancer cells. Miyake et al. [27] confirmed that berries of the Vaccinium genus are rich in anthocyanins that have beneficial effects in the treatment of eye diseases. They showed that oral administration of blueberry extract to six-week-old mice prevented photoreceptor cell dysfunction during retinitis pigmentosa.

The purpose of this study was to evaluate the effect of different fertilizer and biostimulant combinations on the level of antioxidant activity, and polyphenol and anthocyanin content in highbush blueberry fruit.

2. Materials and Methods

2.1. Location and Layout of the Study

This work was completed as part of a Ministry of Science and Higher Education program "Doktorat Wdrożeniowy" no. um. 0060/DW/2018/02. The program's goal is to introduce, as part of doctoral studies, the possibility of developing cooperation between the scientific and socioeconomic communities by introducing the possibility of educating a doctoral student in collaboration with the entrepreneur (or other entity) employing them. The experiment was conducted at the Warsaw University of Life Sciences' Experimental Blueberry Field in Bonie, central Poland (51°55′42.7″ N 20°59′28.7″ E), over a three-year period from 2019 to 2021.

In the experimental field, the 'Bluecrop' shrub cultivar was spread out in a 1×3 m spacing, and the quarters were drip-irrigated. The highest average monthly air temperatures and total rainfall, throughout the three-year field experiment, were noted in 2019 and 2021, respectively (Table 1).

Table 1. Average air temperature and total rainfall recorded during the described experiment.

	2019		2020		2021	
Month	**Total Rainfall ($mm \cdot m^{-2}$)**	**Average Temp. (°C)**	**Total Rainfall ($mm \cdot m^{-2}$)**	**Average Temp. (°C)**	**Total Rainfall ($mm \cdot m^{-2}$)**	**Average Temp. (°C)**
March	25.1	6.8	13.2	5	18.3	4
April	2.2	10.7	7.4	9	55.3	6.5
May	77.8	14.1	65.2	11.9	62.3	12.4
June	16	22.7	140.9	19.6	69.2	19.7
July	34.8	19.5	45.9	19.1	118.8	21.7
August	34.4	21.1	83.1	20.7	140.1	17.2

A Davis Vantage Pro weather station was used at the experimental field to conduct the measurements. The substrate's pH during the experiment ranged from 4.5 to 4.8. In order to conduct the experiment, the soil was examined in a licensed laboratory of the Regional Chemical and Agricultural Station in Łódź (Table 2), and based on the findings, nutrients were added to the soil at the proper levels (Table 3).

Table 2. Soil testing report no. GO/502/18.

Salinity	**pH**	**Content in mg/L**						
g NaCl/L	**in H_2O**	**$N\text{-}NO_3$**	**$N\text{-}NH_4$**	**P**	**K**	**Ca**	**Mg**	**Cl**
0.08	4.9	<10.0 *	<10.0 *	<20.0 *	<20.0 *	245	20	<10.0 *
PB 02 ed.3 from 1 March 2018 **	PB 01 ed.2 from 1 March 2018 **	PB 06 ed.1 from 28 May 2004 **	PB 69 ed.1 from 3 April 2017 **	PB 03 ed.2 from 19 March 2007 **	PB 04 ed.1 from 21 May 2004 **	PB 04 ed.1 from 21 May 2004 **	PB 05 ed.1 from 28 May 2004 **	PB 07 ed.1 from 28 May 2004 **

*/—result below the lower range of the method. **/—Research standard.

Table 3. Sum of nutrients used in the experiment in all assessed treatments (T1,T2,T3,T4).

N (kg/ha^{-1})	**P_2O_5 (kg/ha^{-1})**	**K_2O (kg/ha^{-1})**	**SO_3 (kg/ha^{-1})**	**$CaCO_3$ (kg/ha^{-1})**
100	30	92.5	142	64

All study/experimental protocols involving plant materials were carried out in accordance with institutional, national, and international rules and regulations, and with permission from the Warsaw University of Life Sciences.

The research subject was 'Bluecrop' highbush cultivar blueberries, using a random block mechanism to conduct the experiment. Five repetitions of each of the four fertilizer treatments were examined. Six plants were present in each replicate. The harvest was conducted in 2019 from 1 July to 10 August; in 2020 from 5 July to 5 August; and in 2021 from 10 July to 10 August, respectively. Harvested fruit samples were averaged-out. The experiment evaluated how biostimulation affected antioxidant activity, polyphenol content, and quantitative and qualitative anthocyanin analyses. The following treatment scenarios were used:

- 'Treatment T1'—consisted of standard fertilization and foliar sprinkling without the use of bioactive components (control treatment);
- 'Treatment T2'—included foliar fertilization and typical sprinkling in addition to a solution containing phytohormone precursors and biostimulants;

- ‘Treatment T3’—comprised traditional foliar fertilizing and sprinkling, with a new method of biostimulation based on extracts from various plants and sea algae, with bioactive qualities intended to improve the physiological processes in crops;
- ‘Treatment T4’—biostimulant-containing formulations were used to fertilize the soil and the leaves.

In their study, Lenart et al. [28] provided a thorough explanation of the combination of nutrients provided in each treatment option used in their study; the amount of nutrients (N, K, P, Mg, etc.) given to plants in each treatment of our experiment was equal or very close to it. Our study evaluated the impact of several fertilization methods on plants’ capacity to promote health and how the bioactive substances employed in biostimulant formulations affected the nutritive value of the plants.

2.2. Research Methods

All reagents used for HPLC were of HPLC grade and purchased at Sigma-Aldrich (Poznan, Poland) and Merck (Warsaw, Poland). Other chemicals were of analytical purity grade and purchased at Alchem (Warsaw, Poland).

The phenolics were isolated by solid-phase extraction as described by Latocha et al. [29]. The total phenols content (TPC) was determined by the spectrophotometric method described by Singleton et al. [30] by applying Folin & Ciocalteu’s reagent. The absorbance of the solution was measured using a Marcel 330S PRO spectrophotometer (Marcel, Zielonka, Poland) at the wavelength λ = 700 nm. The result was expressed in milligrams of gallic acid equivalent (GAE) per 100 g of fresh weight (FW). The antioxidant activity (AA) was determined according to Saint Criq de Gaulejac et al. [31] using DPPH free radical (1,1-diphenyl-2-picrylhydrazine). The AA was calculated on the basis of absorbance measurements for the sample (0.75 mL diluted fruit extract + 0.75 mL DPPH) performed after 10 min at λ = 517 nm in relation to the control sample (0.75 mL H_2O + 0.75 mL DPPH). The results were expressed in milligrams of ascorbic acid equivalent (AAE) per gram of FW. The identification and quantitative analysis of anthocyanins were conducted separately using the HPLC technique described by Szpadzik et al. [32], performed by means of a PerkinElmer series 200 HPLC with a diode array detector (Perkin Elmer, Krakow, Poland), using a LiChroCART® 125-3 (Merck KGaA, Darmstadt, Germany) column with a 1.0 mL/min flow rate, detected at 520 nm. The mobile phase was a mixture of water (A), 20% formic acid (B), and acetonitrile (C), with variable parameters of the gradient (A) and (C). The anthocyanin content was given as milligrams per 100 g of fresh weight of fruit as cyanidin-3-glucoside equivalent.

2.3. Statistical Analysis

The results were analyzed statistically in Statistica 13.3 (StatSoft Polska, Krakow, Poland), using the two-way analysis of variance. Tukey test was used for evaluation of the significance of differences between the means, accepting the significance level as 5%.

3. Results

The fertilizer combinations used in the experiment influenced the level of antioxidant activity (DPPH) in the fruits studied. Fruits from the control combination, where fertilizers with bioactive substances were not applied, showed significantly lower levels of antioxidant activity (Table 4). In the combinations in which additional biostimulants were applied, a higher value of the analyzed index was observed, and the T4 and T3 fertilization programs deserve special mention (Figure 1).

Table 4. Influence of fertilization technology on antioxidant activity—data averaged over the years of the study. * Data followed by the same letter are not significantly different.

Combination	Year	Antioxidant Activity [μM Trolox·100 g^{-1}]	3 Years Average Antioxidant Activity [μM Trolox·100 g^{-1}]
T1	2019 2020 2021	0.356 ab * 0.356 ab 0.414 bc	0.375 a
T2	2019 2020 2021	0.241 a 0.430 bc 0.472 c	0.381 a
T3	2019 2020 2021	0.481 c 0.413 bc 0.416 bc	0.437 ab
T4	2019 2020 2021	0.517 c 0.487 c 0.463 bc	0.489 b
p-value		<0.01	<0.01

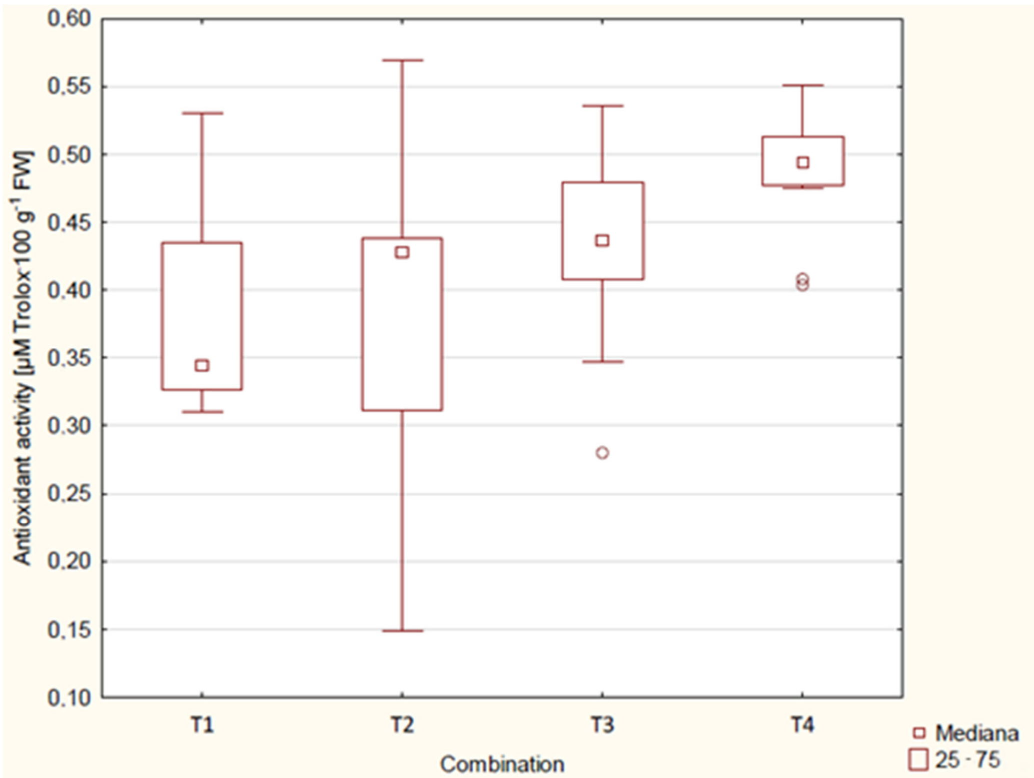

Figure 1. Influence of fertilization technology on antioxidant activity—average per harvest year, and three year average.

The antioxidant capacity in each year of the study ranged from the lowest in the T1 combination in 2019 and 2020 at 0.356 μM Trolox·100 g^{-1} FW, to the highest at 0.517 μM Trolox·100 g^{-1} FW in the T4 combination in 2019 (Table 1).

The fertilizer combinations used in the experiment influenced the polyphenol content of the fruits studied (Table 5). Fruits from the control combination, where products with biostimulants were not used, had a significantly lower content of total polyphenols. In most of the combinations in which additional biostimulants were applied, a higher value of the analyzed index was observed, and the most noteworthy was the T4 fertilization program (Figure 2). Fertilization with the several biostimulants used in the T4 combination caused a significantly higher content of total polyphenols in the fruit. The application of biostimulation increased the content of total polyphenols by 13% in blueberry fruit, on average, for the year of the study.

Table 5. Influence of fertilization technology on amount of polyphenols—data averaged over the years of the study. * Data followed by the same letter are not significantly different.

Combination	Year	Amount of Polyphenols [mg 100 g^{-1} FW]	3-Year Average [mg 100 g^{-1} FW]
T1	2019 2020 2021	659 a * 766 ab 801 bc	742 a
T2	2019 2020 2021	759 ab 845 bc 802 bc	802 ab
T3	2019 2020 2021	859 bc 752 ab 848 bc	820 ab
T4	2019 2020 2021	759 ab 878 c 875 c	837 b
p-value		<0.01	<0.01

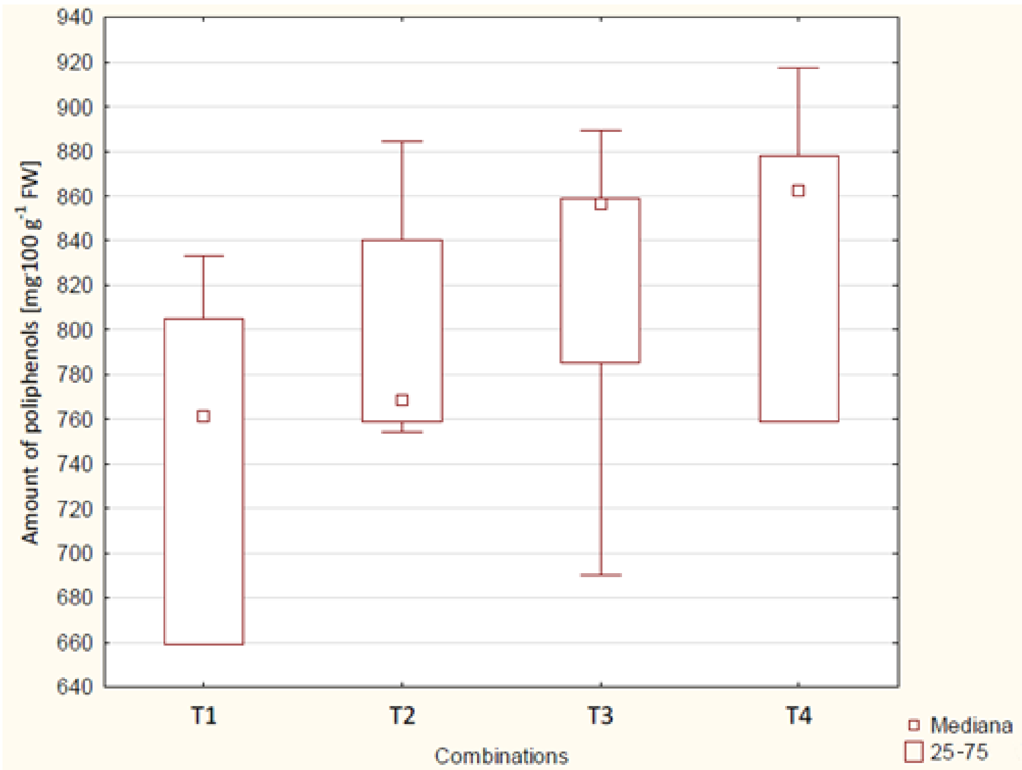

Figure 2. Influence of fertilization technology on amount of polyphenols, expressed as mg 100·g^{-1} FW; average per year and over three years of research.

As a result of qualitative studies, ten compounds from the anthocyanin group were determined in highbush blueberry fruit, including compounds from the delphinidin, petunidin, peonidin and malvinidin groups. The applied fertilization combinations had a significant effect on the content of individual compounds isolated during the study (Table 6). In the delphinidin group, delphinidin-3-glucoside (Dp-3-glu), delphinidin-3-galactoside (Dp-3-gal) and delphinidin-3-arabinoside (Dp-3-ara) were identified. Of the three delphinidins mentioned, only Dp-3-ara was not determined by the biostimulants used in the fertilization programs. Fruit from the T4 combination, in which fertilization with a range of different complementary biostimulants was applied, recorded higher contents of Dp-3-glu and Dp-3-gal than the other combinations. For the other combinations, the effect of the applied preparations was similar, and there were no significant differences between the evaluated fertilization programs on the content of anthocyanins of the group in question (Table 6).

Table 6. Content of anthocyanin group active compounds in relation to fertilizer combination (mg·100 g^{-1} FW). * Data followed by the same letter are not significantly different.

		Dp-3-glu	Dp-3-gal	Dp-3-ara	Pt-3-glu	Pt-3-gal	Pt-3-ara	Pn-3-glu	Mv-3-glu	Mv-3-gal	Mv-3-ara
Combinations	T1	19.83 ab *	10.58 a	18.53 a	10.76 a	7.36 a	6.42 a	1.88 b	26.5 a	26.08 a	19.00 a
	T2	18.88 ab	11.18 a	20.22 a	10.98 a	7.56 a	6.50 a	2.0 ab	36.73 b	26.40 a	19.09 a
	T3	17.19 a	10.38 a	21.10 a	11.36 a	7.90 a	7.51 a	2.36 b	28.62 a	27.52 a	22.79 a
	T4	21.78 b	12.96 b	22.88 a	12.74 a	10.31 a	8.04 a	1.38 a	26.16 a	28.84 a	23.43 a
p-value		<0.0001	<0.0001	0.0816	0.0816	0.0816	0.0816	<0.0001	<0.0001	0.0816	0.0816

The preparations and biostimulants used in the study had no significant effect on the content of anthocyanins from the petunidin group. The study isolated petunidin-3-glucoside (Pt-3-glu), petunidin-3-galactoside (Pt-3-gal) and petunidin-3-arabidoside (Pt-3-ara). Although no significant correlations were shown when evaluating petunidins, it was notable that, as in the case of delphinidins, the content of individual petunidins was higher in fruit from the T4 fertilization combination (in which the full range of products with biostimulation was applied), compared with T1, T2 or T3. The control combination in which no biostimulants were applied (T1) had a slightly lower petunidin content in the fruit. The study highlighted the significant effect of the tested fertilizer combinations on peonidin-3-glucoside (Pn-3-glu) content. Its content ranged from 1.38 mg cy-3-gl -100 g-1 FW in the T4 combination, to 1.88 mg cy-3-gl-100 g-1 FW in the T1 control combination, and significantly depended on the type of fertilization used. Unexpectedly, statistical evaluation proved a significantly higher content of Pn-3-glu in fruit from the control combination, where only conventional fertilization was applied, compared to T2, T3 or T4. In the fruits studied, malvinidins were the largest group and also the least active, and the fertilization combinations used slightly determined the content of these anthocyanins. Malvinidin-3-glucoside (Mv-3-glu), malvinidin-3-galactoside (Mv-3-gal) and malvinidin-3-arabidoside (Mv-3-ara) were identified; the Mv-3-glu content in blueberry fruits was highest using the T2 combination, while the other combinations showed no significant effect. The other two anthocyanins, Mv-3-gal and Mv-3-ara, also showed no significant effect of the treatment combinations.

4. Discussion

Growing consumer demand for highbush blueberry fruit causes producers to look for modern agrotechnical solutions to ensure high yields and high quality of the obtained fruits [33,34]. Information campaigns promoting the health-promoting qualities of berries determine not only an increased awareness of consumers on this topic, but also the decisions of producers to cultivate in a sustainable manner. Seaweed-based products are of particular interest to growers [28,35]. Seaweed extracts often form the basis of fertilization programs with biostimulation [36,37]. According to Du Jardin [38], a biostimulant can be defined in the following manner: "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content. By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms." Numerous scientific studies have reported positive effects of seaweed extracts on plants, including improved yield and yield quality, and increased resistance to biotic and abiotic stresses [39]. As reported by Mooney and Van Staden [40], formulations based on seaweed extracts have beneficial effects on plant metabolism, promote growth, resistance to pathogens and increase antioxidant activity. Additionally, the studies conducted in this paper indicated the positive effect of active compounds extracted from marine algae. It should be noted that the high antioxidant activity of highbush blueberry fruit confirmed in the T4 combination may be due to the use of several active compounds obtained from marine algae and tropical and desert plants in the fertilization program. Interesting research results were presented by Grazani et al. [41], where the authors stressed the importance of using biostimulants in the cultivation of Olea europaea to mitigate the effects of abiotic stresses (high temperature and drought). The effect of preparations with seaweed extracts was confirmed by higher values in leaf number growth and leaf area, as well as the same treatments showing positively significant values for antioxidant activity and polyphenol content. Similar results for 'Gala Must' apple fruit were obtained by Nagy et al. [42] indicating that a biostimulant fertilizer formulation containing seaweed extracts significantly improved antioxidant properties in the studied variety of apples. Ambroszczyk et al. [43] proved that tomato fruits treated with biostimulation preparations had significantly higher antioxidant activity compared with fruits not treated with preparations containing marine algae extracts. In our own experiment, we found a higher content of total polyphenols in highbush blueberry fruits after application of preparations containing biostimulants (T4). A similar relationship was confirmed by Mikos-Bielak [44]. The author showed that a biostimulant obtained from seaweed extracts, when applied to raspberry crops, resulted in a 30% increase in the polyphenol content of fruits, compared with the control group. As reported by DeBoer [19], polyphenols in plants not only have a health-promoting effect on the human body but also perform important defined functions in plant organisms. Many authors have reported that the use of programs based on seaweed extracts increases the biological performance of plants, has an anti-stress effect on plants, and strengthens the defined functions of plant organisms [28,45,46]. In a research paper by Sylvia et al. [47], the authors evaluated the effects of seaweed extracts on the antioxidant activity and polyphenol content of fruit, and found a program containing a combination of several biostimulants to be the most effective. Similar observations were also obtained in the present study. Mukherjee [48] reported that seaweed is a source of elicitors due to the presence of several different polysaccharide compounds they take a direct part in the activation of plant secondary metabolism pathways and the mobilization of signaling molecules to trigger a defined response in the plant to a stress factor (biotic or abiotic). Higher concentrations of health-promoting components and nutrients in agri-horticultural crops following use of products containing seaweed extracts were found in maize [49], broccoli [50], Arabidopsis [51], strawberry [52], grape [53], and spinach [54]. Fan et al. [54] reported that the total content of phenols, flavonoids and antioxidant compounds in spinach was significantly higher after treatment with a marine algae extract, as a result of increased activity of, among other things, chalcone isomerase, which is a key enzyme in the biosynthesis of flavanone precursors and triggers activation of plant defined compounds. The effect of biostimulant extracts on the antioxidant properties of highbush blueberry fruit, proven in the present study, was also confirmed by Bi et al. [55] and Vera et al. [56]. The authors indicated that the use of brown algae extracts in agricultural crops influenced better assimilation and concentration of nutrients, increased plant metabolism, the rate of cell division and the photosynthetic index, which ultimately increased the physiological performance of plants.

5. Conclusions

The experiment showed that fertilizers with biostimulation have a significant effect on increasing the antioxidant activity of highbush blueberry fruit and the content of total polyphenols, and also have a significant effect on the content of anthocyanins. A notable result of the study was the fertilizer program in which various bioactive substances derived from marine algae and desert plants were used. Therefore, it can be concluded that the synergistic effect of the interaction of several biostimulants more effectively affects the mechanisms regulating plant metabolism, supports physiological functions and, in particular, activates defined mechanisms. Based on the present study, it can be concluded that the use of biostimulated products in horticulture has a significant positive impact on the health-promoting properties of fruits. The mechanisms affecting the concentration of antioxidants in plant fruits treated with seaweed extracts or bioactive substances still require further research.

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