*horticulturae*

*Article*

# The Effect of Humic-Based Biostimulants on the Yield and Quality Parameters of Chili Peppers

**Ivana Mezeyová [1,*], Ivana Kollárová [1], Marcel Golian [1], Július Árvay [2], Ján Mezey [1], Miroslav Šlosár [1], Lucia Galovičová [1], Robert Rosa [3], Martin Bakalár [1] and Tereza Horečná [1]**

[1] Institute of Horticulture, Horticulture and Landscape Engineering Faculty, Slovak University of Agriculture in Nitra, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia; xkollarovai@uniag.sk (I.K.); marcel.golian@uniag.sk (M.G.); jan.mezey@uniag.sk (J.M.); miroslav.slosar@uniag.sk (M.Š.); lucia.galovicova@uniag.sk (L.G.); martin.bakalar97@gmail.com (M.B.); thorecna@gmail.com (T.H.)
[2] Institute of Food Sciences, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia; julius.arvay@uniag.sk
[3] Institute of Agriculture and Horticulture, Faculty of Agricultural Sciences, University of Siedlce, B. Prusa 14, 08-110 Siedlce, Poland; robert.rosa@uws.edu.pl
* Correspondence: ivana.mezeyova@uniag.sk; Tel.: +421376414243

**Abstract:** Chili peppers are globally cultivated for their rich bioactive compound profile. This study investigates the impact of two biostimulants, Humix® and Energen, on *Capsicum chinense* 'Habanero Orange' and *Capsicum annuum* 'Kristian', focusing on quantitative and qualitative parameters. Conducted over two years with three annual harvests, the research assesses the effects of biostimulant application on yield, fresh fruit number, fruit weight, drying ratio, capsaicin, dihydrocapsaicin, and ascorbic acid content (via HPLC-DAD analysis), as well as carotenoid levels (via spectrophotometric analysis). Biostimulant application significantly increased ($p \leq 0.05$) total yields and capsaicin levels. Harvest timing also influenced dihydrocapsaicin and capsaicin levels, with the third harvest showing the highest values ($p \leq 0.001$). The effects on ascorbic acid and carotenoids were variable and depended on genotype, harvest, and treatment. Thus, our study provides insights into the dynamic responses of *Capsicum* species to biostimulants under variable climatic conditions, contributing new knowledge to agricultural practices and the scientific understanding of biostimulant effects in *Capsicum* production.

**Keywords:** ascorbic acid; biostimulants; capsaicin; *Capsicum* spp.; carotenoids; yield



**Citation:** Mezeyová, I.; Kollárová, I.; Golian, M.; Árvay, J.; Mezey, J.; Šlosár, M.; Galovičová, L.; Rosa, R.; Bakalár, M.; Horečná, T. The Effect of Humic-Based Biostimulants on the Yield and Quality Parameters of Chili Peppers. *Horticulturae* **2024**, *10*, 998. https://doi.org/10.3390/horticulturae10090998

Academic Editors: Marjorie Reyes-Díaz and Jorge González-Villagra

Received: 15 August 2024
Revised: 10 September 2024
Accepted: 18 September 2024
Published: 20 September 2024



## 1. Introduction

Chili peppers (*Capsicum* spp.), belonging to the *Solanaceae* family, constitute an economically important group of crops consumed worldwide for their pungent and non-pungent fruits. These crops are a significant source of numerous dietary and nutritional components, such as capsaicinoids, vitamins, pigments, minerals [1], glucosides (sinapoyl and feruloyl), carotenoids, alkaloids, tannins, terpenoids, coumarins, flavonoids, and essential oils. These compounds have antimicrobial, antioxidant, antihyperglycemic, cardioprotective, and anticancer activities [2–4]. Among this diverse spectrum of bioactive compounds, two principal capsaicinoids, capsaicin and dihydrocapsaicin, are prevalent. The concentration of capsaicin in chili pepper plays an important role in defining the spiciness levels across various *Capsicum* species, evaluated by the Scoville scale [3]. In recent decades, numerous studies have been conducted to investigate the impact of capsaicin on human health. Notably, capsaicin has demonstrated therapeutic potential in the treatment of pain, inflammation, and rheumatoid arthritis. Furthermore, there is emerging evidence supporting its efficacy as an anti-cancer agent. Capsaicin exhibits considerable antioxidant activity and holds promise as an anti-obesity compound [5,6]. A multitude of beneficial effects of capsaicin in neurodegenerative diseases, epilepsy, stroke, and depression have been delineated through both animal and human studies. These findings indicate that dietary capsaicin has the potential to influence the structure and abundance of gut microbiota, thereby playing a significant role in the prevention of depression [7,8]. Dihydrocapsaicin also has a positive impact on the neurological system. Through antioxidant and anti-apoptotic pathways, which help combat harmful oxidative stress and are involved in preventing or reducing cell death (apoptosis) in the brain, dihydrocapsaicin has a potential effect on mitigating the pathological changes induced by cerebral ischemia [9].

Biostimulants are regularly used in today's cultivation practices. In the past, different techniques have been developed to reduce the usage of synthetic fertilizers and insecticides to protect the environment from hazardous chemicals and to increase plant yield. Among such environment-friendly approaches, one is the use of biostimulants, which is very crucial in the development of plants [10]. Natural biostimulants, foliar fertilizers, and plant growth regulators have been applied in horticultural production; however, their effect varies depending on the plant species treated, and those have been mainly cucumber, tomato, pepper, potato, and melon [11]. Plant biostimulants are characterized as any substances, seaweeds, humic and fulvic acids, different plant-growth-promoting bacteria, and extracts from algae applied to plants, seeds, or in the rhizosphere, with the aim to stimulate natural processes in plants and enhance nutrition efficiency and tolerance toward environmental stress [10,12,13]. These compounds have effects on metabolic and enzymatic processes in plants, and the main aim is increasing the yield and quality of the final product [14]. When utilized as a foliar spray, they are absorbed by the leaves, leading to heightened metabolic activity by activating enzymes in various biological processes. Furthermore, it amplifies the photosynthetic rate through an increased synthesis of photosynthetic pigments. This affects plant resilience against both biotic and abiotic stressors, prolongs the storage period of fruits, and consequently refines the growth characteristics and productivity of the plant [15].

Humic acid treatments helped mitigate environmental stresses, such as salinity, by promoting nutrient uptake and improving the chili pepper plant's ability to handle abiotic stress. This was achieved through improved osmotic and antioxidant defense systems, leading to better growth under challenging conditions [16]. The study of Karakurt et al. [17] demonstrated that both soil and foliar humic acid treatment might successfully be used to obtain higher fruit yield and can significantly enhance fruit quality in organically grown pepper. Application of humic acid improved the growth of pepper [18] and increased pepper plant dry matter production [19]. Three types of biostimulant application did not affect the marketable pepper yield, but enhanced the fruit quality, including fruit length, diameter, and green coloration [20]. Biostimulant application also increased total and individual capsaicinoids after 48 h in the chili placenta and pericarp [21]. Based on available scientific literature, the effects of humic-based biostimulants have been tested in few trials with peppers, including chili peppers. However, not much is understood about how biostimulants, including amino acids, impact peppers' enzymatic activity.

Objectives of this study are:

1. To examine the influence of commercial humic-based biostimulants, comprising humic substances, *Ascophyllum nodosum* seaweed extract, macro-elements, and microelements, on the growth parameters of two chili pepper varieties, *Capsicum chinense* 'Habanero Orange' and *Capsicum annuum* 'Kristian', focusing on yield and fruit weight.
2. To assess the effects of biostimulant application on critical qualitative traits, including capsaicinoid concentration, Scoville Heat Units (SHU), carotenoid levels, and ascorbic acid content, in the dried fruits of the aforementioned pepper varieties.

## 2. Materials and Methods

The experiment was conducted in the greenhouse of the Botanical Garden at the Slovak University of Agriculture in Nitra, as well as in the field of the Institute of Horticulture in Nitra, during the years 2022 and 2023.

### 2.1. Plant Material

For the study, two different species of chili peppers were selected. *Capsicum chinense* variety 'Habanero Orange' (Figure 1) is a branched lower plant, with lantern-shaped orange fruits that measure 4–7 cm. These chili peppers reach about 600,000 SHU. The second species is *Capsicum annuum* variety 'Kristian' (Figure 2). Fruits of this plant are thin, medium–long, and yellow in color. On the Scoville scale, these peppers reach approximately 50,000–60,000 SHU. The seeds of the pepper varieties were obtained from the company SEMO a.s. (Czech Republic) and the seedlings were grown in the greenhouses of the Botanical Garden at the Slovak University of Agriculture in Nitra.

**Figure 1.** Plant of Habanero Orange variety.

**Figure 2.** Plant of Kristian variety.

### 2.2. Biostimulants

Commercial biostimulants Energen and Humix were applied to the two selected varieties of chili peppers in this study. Energen Fulhum Plus (AV EKO-COLOR s.r.o., Ústí nad Labem, Czech Republic) is a modified aqueous solution of salts, which consists of 20% dry matter, at least 30% combustible substances in dry matter, and humic substances (HS) and their salts in a concentration of at least 8% with pH between 8 and 10. Energen Fulhum Plus is a modified and processed aqueous solution of salt compounds obtained by the original decomposition of the technical lignosulfonate. Additionally, an extract from the seaweed *Ascophyllum nodosum* is present in Energen. This biostimulant promotes the growth of fine root hairs, boosts nutrient and moisture uptake efficiency, and enhances photosynthetic efficiency. Plants will, therefore, become more resistant to abiotic stress. Additionally, it encourages metabolites to flow into fruits and seeds, which boosts crop growth [22].

Humix® Universal (AGROCULTUR BIO, Nitra, Slovakia) is a special liquid fertilizer that can be applied to the leaf or to the soil. It contains HS from Leonardite, macro-elements, and microelements intended for the nutrition of garden and field crops. HS was a minimum of 3.0% by weight, potassium ($K_2O$) a minimum of 2.5% by weight, and phosphorus ($P_2O_5$) a minimum of 1.0% by weight. Unknown quantities of the elements Cu, Zn, Fe, Co, B, Mn, and Mo are also included in the fertilizer. The pH ranges from 9 to 10. Microelements are bound in chelated form. According to the manufacturer, applying Humix® Universal to the soil reduces symptoms of microelement deficiencies (Cu, Zn, Fe, Co, B, Mn, and Mo), raises the level of the mentioned microelements in the soil, and enhances the content of both organic and inorganic substances. Foliar application intensifies plant nutrition and supports the growth of the root system and the entire plant, thanks to which higher and better-quality harvests are achieved. Watering causes an increase in the content of microelements and the formation of humus, enabling the creation of an optimal soil structure. The proliferation of soil bacteria and beneficial microorganisms is encouraged, leading to an overall enhancement in the soil structure, particularly in denser clay soils. Additionally, the conversion of phosphorus and nitrogen in the soil into plant-acceptable forms is facilitated. This results in an increased uptake of industrial fertilizers by plants, reducing their accumulation in the soil and minimizing leaching into groundwater [23].

### 2.3. Soil Nutrition, Fertilization, and Climatic Conditions

The experiment occurred over two years on medium–heavy soil with a high content of the clay fraction, especially in the subsoil (at a depth of 30–60 cm) fertilized with manure, coinciding with soil analyses conducted in the spring (Table 1). Based on the values, the soil was then fertilized according to the recommended standard for pepper growing [24], and the course of fertilization on the trial area is shown in Table 2. The nitrogen dose in the form of LAD 27% EC Fertilizer (27.0% N and 4.1% MgO; Duslo, a.s.) was applied two weeks before the planned planting (60% of the recommended standard), and the remaining nitrogen (40%) during vegetation six weeks later. Table 2 shows the scheme of the vegetation trial, in which three treatments were designed for the purpose of verifying the combination of tested preparations with commonly used nitrogen fertilizer.

**Table 1.** Soil analysis of the experimental area.

| | **pH** | **Nan** | **Nutrient Content (mg·kg$^{-1}$) (Mehl.III)** | | | | **Cox (Hummus)** |
|---|---|---|---|---|---|---|---|
| | | **mg·kg$^{-1}$** | ***p*** | **K** | **S** | **Mg** | **%** |
| 2022 | 6.90 | 191 | 148 | 480 | 27.5 | 1028 | 4.29 |
| 2023 | 7.45 | 8.9 | 232.5 | 600 | 85.0 | 729 | 4.38 |

**Table 2.** Field trial scheme.

| Treatment | Number of Repetitions | N Application | Application of Energen Fulhum Plus | Application of Energen Fruktus Plus | Application of Humix (New Composition) Universal |
|---|---|---|---|---|---|
| N (K) | 3 | 120 kg·ha$^{-1}$ | - | - | - |
| N + Energen Fulhum Plus + Energen Fruktus Plus<br>+ | 3 | 120 kg·ha$^{-1}$ | 0.5 L·ha$^{-1}$ (0.5%) | 0.5 L·ha$^{-1}$ (0.5%) | - |
| N + Humix (new composition) Univerzál | 3 | 120 kg·ha$^{-1}$ | - | - | 5 L·ha$^{-1}$ (1%) |

K—control treatment; N—nitrogen.

In Nitra, the climate is warm and temperate, where average annual temperatures range from 8.28 °C to 10.05 °C. The average annual rainfall in this district ranges from 529 mm to 895 mm [25]. Climatic data (Tables 3 and 4) from the years 2022 and 2023 were obtained from the meteorological station in the Botanical Garden, operated by the Institute of Landscape Engineering (ILE) of the Slovak University of Agriculture in Nitra. The climatic norm from the years 1991–2020, which is included in the tables, was also provided by the ILE, as they maintain all these data in their databases.

**Table 3.** Air temperature at the experimental site for years 2022 and 2023.

| Month | Normal 1991–2020 (°C) | t (°C) 2022 | Characteristic—2022 | t (°C) 2023 | Characteristic—2023 |
|---|---|---|---|---|---|
| April | 11.4 | 8.5 | very cold | 4.1 | extremely cold |
| May | 16.0 | 15.8 | normal | 9.9 | extremely cold |
| June | 19.6 | 20.7 | normal | 13.3 | extremely cold |
| July | 21.7 | 21.5 | normal | 17.4 | extremely cold |
| August | 21.1 | 21.9 | normal | 17.9 | extremely cold |
| September | 15.9 | 13.9 | cold | 16.9 | normal |
| October | 10.4 | 11.5 | normal | 12.5 | warm |

**Table 4.** Precipitation at the experimental site for years 2022 and 2023.

| Month | Normal 1991–2020 (mm) | Precipitation 2022 (mm) | Characteristic—2022 | Precipitation 2023 (mm) | Characteristic—2023 |
|---|---|---|---|---|---|
| April | 36 | 13 | extremely dry | 40 | normal |
| May | 59 | 13 | extremely dry | 111 | extremely wet |
| June | 59 | 88 | wet | 83 | wet |
| July | 65 | 60 | normal | 8 | extremely dry |
| August | 55 | 60 | normal | 46 | normal |
| September | 58 | 7 | extremely dry | 79 | wet |
| October | 46 | 28 | dry | 36 | normal |

### 2.4. The Course of the Experiment

A small plot experiment was established under field conditions using a randomized block design (RBD). This design was chosen to account for potential variability within the experimental field conditions, ensuring that differences in environmental factors, such as soil composition or microclimate, were minimized within each block. Each block contained all treatment combinations (variety and biostimulant), and the treatments were randomly assigned within each block. The experiment was conducted with three repetitions to ensure the reliability and statistical robustness of the results. The dates of individual cultivation measures are presented in Table 5. Planting of pre-grown transplants took place in the growth phase of 8–10 true leaves, while in each repetition, 5 plants were planted in a uniform growing space: 0.4 m × 0.5 m. The area of one experimental treatment was 3 $m^2$. Additional drip irrigation was applied as needed during the growing season. Soil loosening and weed control was carried out by hand hoeing. Throughout the growing season, the health status of the plants was regularly monitored, and appropriate plant protection measures were applied as required, using only approved phytosanitary preparations, in accordance with the established guidelines.

**Table 5.** Experiment course and application of Energen and Humix biostimulants.

| | 2022 | | 2023 | |
|---|---|---|---|---|
| | **Habanero Orange** | **Kristian** | **Habanero Orange** | **Kristian** |
| Sowing | 02/02 | 25/02 | 10/02 | 02/03 |
| Planting of plants with cotyledons | 11/03 | 11/03 | 14/03 | 20/03 |
| Planting in the field with root treatment of transplants with biostimulants | 19/05 | 18/05 | 23/05 | 23/05 |
| Spray on the leaf at the beginning of flowering (BF) | 20/06 | 20/06 | 13/06 | 13/06 |
| Spray on the leaf at the beginning of the first fruits' formation (FFF) | 30/06 | 30/06 | 19/06 | 19/09 |
| 1st harvest | 07/09 | 07/09 | 21/09 | 04/09 |
| 2nd harvest | 22/09 | 22/09 | 02/10 | 21/09 |
| 3rd harvest | 20/10 | - | 25/10 | 11/10 |

As part of the field experiment, Energen Fulhum Plus was applied once prior to transplanting by soaking the root ball of the young plants immediately before planting. The Energen Fruktus Plus preparation was applied twice during the growing season by foliar spraying (BF and FFF in Table 5). The Humix (new composition) Universal preparation was applied three times: initially by soaking the root ball of the transplants prior to planting, followed by two foliar applications during the growing season (BF and FFF in Table 5). Fruits at full botanical maturity were harvested progressively through selective sorting.

### 2.5. Evaluation of Quantitative Parameters

Each treatment was weighed individually after harvest using a Kern 440-53N scale (Kern and Sohn, Albstadt, Germany). The weight of fresh fruit per plant, the weight of fresh pepper fruit, and the number of pepper fruits per plant were evaluated. Fresh samples of chili peppers were taken and used immediately for analyses. The rest of the peppers were then dried in an air dryer (Memmert UF 110 Plus; Memmert, Schwabach, Germany) until a constant weight was achieved at a temperature of 60 °C. The yield of dry biomass from chili peppers and the drying ratio were assessed by weighing the dried fruits as well. The fruits of dried peppers were ground in a shear mill (Retsch SM 100; Retsch, Haan, Germany), and average samples (100 g) of both varieties and all treatments were prepared and stored in dark conditions until laboratory analyses took place. Based on the planting density and the number of plants, the yield for each variety in the individual treatments was calculated and expressed in tons per hectare ($t \cdot ha^{-1}$).

### *2.6. Evaluation of Qualitative Parameters*

#### 2.6.1. Determination of Capsaicin and Dihydrocapsaicin

Capsaicin and dihydrocapsaicin analyses were performed by HPLC-DAD (High-Performance Liquid Chromatography with Diode-Array Detection) according to [26].

#### 2.6.2. Determination of Vitamin C

Vitamin C was determined using an Agilent 1260 Infinity HPLC with DAD (Agilent Technologies GmbH, Wäldbronn, Germany) according to [26].

#### 2.6.3. Determination of SHU (Scoville Heat Units)

The conversion to Scoville Heat Units was performed by multiplying the capsaicin content in pepper dry weight by the coefficient corresponding to the heat value for pure capsaicin, according to [27].

#### 2.6.4. Determination of Carotenoids

The carotenoid content was determined using a spectrophotometric method according to [28]. The calculation was performed according to [29].

#### 2.6.5. Data Analysis

The experiment utilized a multifactorial design, considering both the variety (*Capsicum chinense* 'Habanero Orange' and *Capsicum annuum* 'Kristian') and the biostimulant treatment (Humix® and Energen) as factors. A multifactor analysis of variance (ANOVA) was applied to assess the effects of these factors independently and their interaction on both quantitative and qualitative parameters. The factors analyzed included variety, treatment, and harvest term. This design allowed for the examination of how different biostimulant treatments impacted each variety, as well as how these effects varied across multiple harvests. Indeed, some results reflected data where multiple years and harvests were combined. In such cases, a one-way ANOVA was performed to analyze the overall effects of the treatments when summarized across the different variables. Our intention in doing so was to provide a holistic view of the data, capturing the combined influence of the treatments across the two years of experimentation. The mean values were tested using the least significant difference (LSD) test, performed at a significance level of 95% ($p \leq 0.05$). The statistical analysis was conducted using the Statgraphics Centurion XVII software (StatPoint Inc., The Plains, VA, USA).

## 3. Results and Discussion

### *3.1. Quantitative Parameters*

Biostimulant application significantly ($p \leq 0.05$) increased the fresh pepper total yield values for Kristian variety from 5.23 $t \cdot ha^{-1}$ (control) to 8.11 $t \cdot ha^{-1}$ (treatment with Humix application) and 8.14 $t \cdot ha^{-1}$ (treatment with Energen application; Table 6). Humix application significantly ($p \leq 0.05$) increased the total yields in the case of 'Habanero Orange' to 20.01 $t \cdot ha^{-1}$, compared to the control treatment (17.70 $t \cdot ha^{-1}$). The differences in chili pepper fruit yield, both in terms of fresh and dry matter, varied across each harvest and were specific to each variety (Table 7). Humic acids and seaweed in the biostimulants used can promote plant growth by speeding up cell division, encourage the synthesis of chlorophyll, sugar, and amino acids in plants, and improve nutrient uptake, which helps with photosynthesis. This may be the cause of a notable increase in total yields following biostimulant application [30,31].

**Table 6.** Assessment of different biostimulants on yield and quality parameters of chili (average for 2022–2023).

| Treatment | | Yield Parameters | | | | Qualitative Parameters | | |
|---|---|---|---|---|---|---|---|---|
| | | FM ($t{\cdot}ha^{-1}$) | DR | Average W1FF (g) | Total FFPP (pcs) | CAPS ($\mu g{\cdot}g^{-1}$) | DIH ($\mu g{\cdot}g^{-1}$) | SHU |
| Kristian | K | 5.23 a | 4:1 a | 2.93 a | 105.2 a | 1896.1 a | 2083.6 a | 66,051 a |
| | E | 8.14 b | 4:1 a | 3.23 ab | 111.6 a | 2063.8 a | 2242.4 a | 69,150 a |
| | H | 8.11 b | 4:1 a | 4.10 b | 90.0 a | 2110.8 a | 2397.6 a | 61,017 a |
| Habanero Orange | K | 17.70 c | 9:1 b | 5.86 c | 198.2 b | 8029.4 b | 4703.4 b | 200,277 b |
| | E | 17.60 c | 8:1 b | 5.45 c | 183.0 b | 8081.8 b | 4711.4 b | 199,748 b |
| | H | 20.01 d | 9:1 b | 5.68 c | 224.1 b | 8499.9 b | 4790.4 b | 206,537 b |

FM—fresh matter; DR—drying ratio from fresh to dry mass; Average W1FF—average weight of 1 fresh fruit (g)—average values from all harvests; Total FFPP (pcs)—number of fresh fruits per plant as the total sum from all harvests; CAPS—capsaicin content; DIH—dihydrocapsaicin content; SHU—Scoville Heat Units. Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

**Table 7.** The effect of the biostimulants Energen (E) and Humix (H) on the yield of fresh and dry chili pepper fruits.

| Treatment | | 1st Harvest | | 2nd Harvest | | 3rd Harvest | |
|---|---|---|---|---|---|---|---|
| | | $t.ha^{-1}$ (FM) | $t.ha^{-1}$ (DM) | $t.ha^{-1}$ (FM) | $t.ha^{-1}$ (DM) | $t.ha^{-1}$ (FM) | $t.ha^{-1}$ (DM) |
| 2022 | | | | | | | |
| Kristian | K | 2.48 ± 2.01 ab | 0.586 ± 0.474 ab | 1.15 ± 0.69 a | 0.251 ± 0.152 a | | |
| | E | 3.40 ± 0.26 ab | 0.780 ± 0.059 b | 1.09 ± 0.45 a | 0.229 ± 0.094 a | | |
| | H | 3.36 ± 1.68 ab | 0.943 ± 0.471 b | 1.90 ± 1.68 ab | 0.387 ± 0.341 a | | |
| Habanero Orange | K | 3.73 ± 1.05 b | 0.483 ± 0.136 ab | 3.31 ± 0.82 b | 0.381 ± 0.126 a | 7.75 ± 1 a | 1.201 ± 0.7 a |
| | E | 1.48 ± 0.71 a | 0.199 ± 0.096 a | 1.93 ± 0.79 ab | 0.215 ± 0.088 a | 11.30 ± 1 b | 1.275 ± 0.1 a |
| | H | 1.39 ± 0.34 a | 0.197 ± 0.048 a | 2.85 ± 0.52 b | 0.348 ± 0.064 a | 8.44 ± 1 a | 0.953 ± 0.1 a |
| 2023 | | | | | | | |
| Kristian | K | 2.22 ± 1.62 a | 0.490 ± 0.359 a | 4.62 ± 1.86 ab | 0.984 ± 0.397 a | 5.16 ± 3.60 a | 1.219 ± 0.850 a |
| | E | 2.07 ± 0.98 a | 0.498 ± 0.236 a | 3.43 ± 1.07 ab | 0.760 ± 0.237 a | 6.32 ± 1.84 a | 1.441 ± 0.419 a |
| | H | 0.99 ± 0.26 a | 0.244 ± 0.065 a | 2.61 ± 1.88 a | 0.574 ± 0.412 a | 7.35 ± 2.68 a | 1.653 ± 0.603 a |
| Habanero Orange | K | 3.51 ± 2.63 a | 0.425 ± 0.319 a | 6.82 ± 1.09 ab | 0.792 ± 0.127 a | 10.20 ± 0.85 ab | 1.211 ± 0.100 a |
| | E | 2.77 ± 1.58 a | 0.390 ± 0.222 a | 7.72 ± 1.39 ab | 0.843 ± 0.151 a | 10.05 ± 3.70 ab | 1.235 ± 0.454 a |
| | H | 3.04 ± 1.40 a | 0.368 ± 0.170 a | 8.72 ± 6.55 b | 0.885 ± 0.665 a | 15.57 ± 6.35 b | 1.720 ± 0.701 a |
| 2022–2023 | | | | | | | |
| Kristian | K | 2.35 ± 1.82 a | 0.538 ± 0.417 ab | 2.89 ± 1.28 ab | 0.618 ± 0.275 a | | |
| | E | 2.74 ± 0.62 a | 0.639 ± 0.148 b | 2.26 ± 0.76 a | 0.495 ± 0.166 a | | |
| | H | 2.18 ± 0.97 a | 0.594 ± 0.268 ab | 2.26 ± 1.78 a | 0.481 ± 0.377 a | | |
| Habanero Orange | K | 3.62 ± 1.84 a | 0.454 ± 0.228 ab | 5.07 ± 0.96 ab | 0.587 ± 0.127 a | 8.98 ± 0.92 a | 1.210 ± 0.383 a |
| | E | 2.13 ± 1.15 a | 0.295 ± 0.159 ab | 4.83 ± 1.09 ab | 0.529 ± 0.120 a | 10.67 ± 2.35 a | 1.255 ± 0.277 a |
| | H | 2.22 ± 0.87 a | 0.283 ± 0.109 a | 5.79 ± 3.54 b | 0.617 ± 0.365 a | 12.01 ± 3.67 a | 1.337 ± 0.401 a |

Mean ± SD (n = 3). FM—fresh matter; DM—dry matter; K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix. Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

Total yield was also significantly affected by chili varieties and humic acid levels, while the interaction of the treatments was found non-significant in [32], where they tested foliar application of humic acids on quantitative parameters of chili peppers. Singh et al. [33] found that the application of biostimulant treatments rendered a significant effect on almost all the growth and yield characteristics, as well as the quality of chili. According to [34], the Habanero chili pepper yields increased from 31 to 39 $t{\cdot}ha^{-1}$. Their higher results can be related to growing in a tunnel-type greenhouse during the spring–summer cycle in Mexico, which better corresponds to the thermophilic character of chili fruits compared to cultivation in Slovakia's climate. The marketable yield inside the greenhouse was 4.5-

and 4.8-fold higher, as compared to the open field in the case of the trial with *Capsicum annum* chili varieties [35]. The production of habanero chili peppers under greenhouse conditions increased the yield four times when compared with open-field production, with average values of 35 $t \cdot ha^{-1}$ and 8 $t \cdot ha^{-1}$, respectively [34]. Total yields in case of Habanero in Energen treatment (17.60 $t \cdot ha^{-1}$) were not significantly increased compared to the control treatment. The findings imply that enhanced nutrient uptake was not connected to the processes by which Energen impacted plant growth and development. The results indicated that the growth-promoting elements might be connected to the humic compounds' chemical structure, even though their precise identity is still unknown.

The average weight of one fresh fruit (Table 6) was significantly ($p \leq 0.05$) increased in case of the variety 'Kristian' under the influence of the Humix biostimulant, compared to the control (from 2.93 to 4.10 g/plant), while, on the other hand, the number of fresh fruits per plant decreased. Total yields were higher following the application of the biostimulants, resulting in increased fruit weight, although the number of fruits per plant decreased.

This aspect was also observed in the case of 'Habanero Orange', though the differences were statistically insignificant. Biostimulants can stimulate flowering and improve fruit set in hot peppers, leading to higher yields. This is particularly beneficial in hot climates, where high temperatures may negatively impact flower development and pollination. The weather throughout the examined years was normal or extremely cold during flowering. Arthur et al. [20] compared the quantitative characteristics of chili peppers under biostimulants' influence, and the weight of *Capsicum annum* varieties was 1.3–2.5 g/plant ('Chile de Arbol') and 2.4–4.3 g/plant ('Cayenne purple'). Biostimulant application did not affect the marketable yield either, but it enhanced the fruit quality, including fruit length, diameter, and green coloration. In a study conducted by Deori et al. [36], the liquid biostimulant Dhanzyme Gold, derived from seaweed containing cytokinins, hydrolyzed protein complexes, amino acids, and many other minerals, was used. After foliar application, this biostimulant influenced different parameters of chili peppers (*Capsicum annuum* L. cv. Kashi Anmol), including the average fruit weight, with the highest values at 3.66 g, compared to the control at 2.93 g. The results depended on the type of biostimulant and pepper species.

Similar to our results (Table 8), discussions about the biostimulant effect are variable. According to Ertani et al. [37], the weight of fresh leaves and fruits and the number of fruits were affected by treatment ($p \leq 0.001$), concentration ($p \leq 0.001$), and time from treatment ($p \leq 0.001$) in a study of two biostimulants' influence on *Capsicum chinense*. They used biostimulants derived from alfalfa plants (AH), and the second one was obtained from red grape (RG). In the study by Majkowska-Gadomska et al. [14], where the experimental materials comprised two hot ('Cyklon' and 'Palivec') cultivars of *C. annuum*, it was found that the combined application of environmentally friendly microbial-based biostimulants did not clearly improve the morphological traits of pepper fruit, the yield, or the concentrations of sugars and organic acids in the fruit; therefore, their use is not economically justified. Concerning humic biostimulants, according to Azcona et al. [19], in general, the treatments with HSS (humic substances derived from composted sludge) and HSL (derived from leonardite) did not markedly affect chlorophyll and nutrient concentrations in the leaves. At maturity, only small differences in total fruit yield, number of fruits per plant, and fruit size were observed between the amended and control plants. On the other hand, in a study performed by Noushad et al. [38], they used the biostimulant Dollar, containing 15% protein hydrolysate and 15% seaweed extract, essential amino acids, micronutrients, and growth factors, which stimulated plant yield and quality, as well as the number of fruits per plant. The maximum number of fruits per plant was recorded in T8 (Dollar 2.5 $mL \cdot L^{-1}$), with 73.66, followed by T9 (Dollar 3.0 $mL \cdot L^{-1}$) with 72.44, while the minimum fruits per plant was recorded in T0 (Control, water spray) with 58.00, respectively. Regarding the study by González-Cortés et al. [34], the NFP (number of fruits per plant) using T2 (240–200–180 + 25% of K from liquid earthworm humus) showed the highest number of fruits (207), resulting in a lower fruit weight, in contrast with V3 (240–200–120 +

50% of K from vermicompost), which showed the lowest number of fruits (n = 118) but with higher weights.

**Table 8.** The effect of the biostimulants Energen (E) and Humix (H) on the number of fresh fruits per plant and the weight of one fresh fruit of selected varieties of chili peppers.

| Treatment | | 1st Harvest | | 2nd Harvest | | 3rd Harvest | |
|---|---|---|---|---|---|---|---|
| | | FFPP (pcs) | W1FF (g) | FFPP (pcs) | W1FF (g) | FFPP (pcs) | W1FF (g) |
| | | | | 2022 | | | |
| Kristian | K | 21.67 ± 16.68 bc | 2.25 ± 0.10 a | 56.33 ± 35.53 a | 2.19 ± 0.56 a | | |
| | E | 25.20 ± 0.80 c | 2.70 ± 0.19 a | 56.33 ± 22.12 a | 1.94 ± 0.22 a | | |
| | H | 26.40 ± 3.33 c | 2.48 ± 1.05 a | 33.67 ± 11.93 a | 5.89 ± 5.95 ab | | |
| Habanero Orange | K | 10.73 ± 3.92 ab | 7.11 ± 0.79 b | 43.67 ± 4.62 a | 6.43 ± 0.60 b | 123.65 ± 10.12 a | 6.27 ± 0.99 a |
| | E | 4.45 ± 1.72 a | 6.58 ± 1.01 b | 33.67 ± 14.84 a | 4.98 ± 0.54 ab | 136.87 ± 13.78 a | 6.60 ± 0.32 a |
| | H | 4.30 ± 1.08 a | 6.54 ± 0.98 b | 43.00 ± 1.00 a | 5.82 ± 0.30 ab | 120.76 ± 10.27 a | 5.59 ± 0.09 a |
| | | | | 2023 | | | |
| Kristian | K | 12.64 ± 9.92 a | 3.61 ± 0.20 a | 27.16 ± 16.01 a | 3.68 ± 0.94 a | 92.67 ± 29.74 a | 2.90 ± 0.60 a |
| | E | 10.93 ± 5.59 a | 3.82 ± 0.18 a | 20.35 ± 12.27 a | 3.77 ± 1.11 a | 110.33 ± 5.51 a | 3.94 ± 0.25 a |
| | H | 5.25 ± 1.56 a | 3.81 ± 0.42 a | 12.44 ± 9.14 a | 4.36 ± 0.37 ab | 102.33 ± 52.35 a | 3.94 ± 0.75 a |
| Habanero Orange | K | 15.00 ± 10.47 a | 4.35 ± 0.71 a | 25.93 ± 4.27 a | 5.25 ± 0.24 b | 177.50 ± 55.15 ab | 6.01 ± 1.39 b |
| | E | 14.07 ± 5.25 a | 3.77 ± 0.71 a | 28.28 ± 5.66 a | 5.46 ± 0.11 b | 148.06 ± 23.99 ab | 6.08 ± 0.65 b |
| | H | 13.48 ± 5.14 a | 4.47 ± 0.81 a | 31.05 ± 21.13 a | 5.42 ± 0.40 b | 235.24 ± 103.08 b | 6.21 ± 0.49 b |
| | | | | 2022–2023 | | | |
| Kristian | K | 17.16 ± 13.30 a | 2.93 ± 0.15 a | 41.75 ± 25.77 a | 2.94 ± 0.75 a | | |
| | E | 18.07 ± 3.20 a | 3.26 ± 0.19 a | 38.34 ± 17.20 a | 2.86 ± 0.67 a | | |
| | H | 15.83 ± 2.45 a | 3.15 ± 0.74 a | 23.06 ± 10.54 a | 5.13 ± 3.16 b | | |
| Habanero Orange | K | 12.87 ± 7.20 a | 5.73 ± 0.75 b | 34.80 ± 4.45 a | 5.84 ± 0.42 b | 150.57 ± 32.57 a | 6.14 ± 1.19 a |
| | E | 9.26 ± 3.49 a | 5.18 ± 0.86 b | 30.98 ± 10.25 a | 5.22 ± 0.33 b | 142.47 ± 16.99 a | 6.34 ± 0.82 a |
| | H | 8.89 ± 3.11 a | 5.51 ± 0.90 b | 37.03 ± 11.07 a | 5.62 ± 0.35 b | 178.00 ± 56.54 a | 5.90 ± 0.75 a |

Mean ± SD (n = 3). FFPP—fresh fruits per plant (pieces); W1FF—weight of one fresh fruit (g); K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix. Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

The drying ratio did not show significant differences ($p \leq 0.05$) among the treatments evaluated in our study (Table 6). When seaweed biostimulants are applied to chili plants, they can stimulate root growth, leading to increased nutrient absorption and improved water uptake [38].

### 3.2. *Qualitative Parameters*

#### 3.2.1. Capsaicin, Dihydrocapsaicin, and SHU of Dried Chili Pepper Fruits

Capsaicin content values for both tested stimulants were higher compared to the control (Table 6), based on the average data from all harvests across both years, for both the 'Habanero Orange' and 'Kristian' varieties. The effect of the applied biostimulants on capsaicin and dihydrocapsaicin content varied across individual harvests when assessed separately (Table 9).

No significant response to nutrition and moisture levels was found in the study by Borges-Gómez et al. [39], where, on average, the contents of capsaicin and dihydrocapsaicin in Habanero peppers were 8.4 and 4.7 $g \cdot kg^{-1}$ fruit dry weight, respectively. Their results showed a significant positive relationship between plant age and capsaicin content, but not dihydrocapsaicin, which is also comparable to our data, where the term of harvest had a significant effect ($p \leq 0.001$) on the capsaicin and dihydrocapsaicin contents (Table 10). A detailed statistical analysis is shown in Table 11.

**Table 9.** The effect of the biostimulants Energen (E) and Humix (H) on the capsaicin (mg·kg$^{-1}$) and dihydrocapsaicin (mg·kg$^{-1}$) content in dried chili pepper fruits.

| Treatment | | 1st Harvest | | 2nd Harvest | | 3rd Harvest | |
|---|---|---|---|---|---|---|---|
| | | CAP | DHC | CAP | DHC | CAP | DHC |
| 2022 | | | | | | | |
| Kristian | K | 1710.6 ± 24.7 c | 1782.3 ± 91.5 b | 1843.5 ± 12.7 c | 1677.5 ± 115.1 a | | |
| | E | 1351.4 ± 15.5 a | 1608.3 ± 39.6 a | 1759.6 ± 28.8 b | 1693.2 ± 131.8 a | | |
| | H | 1474.8 ± 16.7 b | 1585.6 ± 195.3 a | 1619.4 ± 27.8 a | 1507.1 ± 154.6 a | | |
| Habanero Orange | K | 7964.4 ± 21.8 d | 4109.9 ± 23.8 c | 8215.9 ± 28.9 f | 5582.7 ± 28.2 c | 8080.7 ± 25.0 a | 4826.3 ± 19.4 c |
| | E | 8240.8 ± 57.4 e | 4579.8 ± 50.3 d | 7860.7 ± 49.8 d | 3747.9 ± 28.7 b | 8136.4 ± 33.3 a | 4143.6 ± 17.7 a |
| | H | 8539.1 ± 139.2 f | 4720.7 ± 24.5 e | 8164.3 ± 48.3 e | 3929.1 ± 243.2 b | 8256.3 ± 52.4 b | 4191.4 ± 30.0 b |
| 2023 | | | | | | | |
| Kristian | K | 2421.3 ± 8.0 b | 2471.1 ± 57.8 b | 1835.9 ± 28.7 a | 2262.1 ± 17.6 a | 2272.8 ± 10.9 c | 2877.5 ± 20.7 b |
| | E | 2850.7 ± 13.7 c | 2761.5 ± 68.1 c | 2504.3 ± 8.1 c | 3245.9 ± 222.3 b | 1826.0 ± 3.4 a | 2502.9 ± 12.2 a |
| | H | 2184.7 ± 7.0 a | 2283.6 ± 5.8 a | 1984.8 ± 10.3 b | 2300.6 ± 9.6 a | 1977.2 ± 8.2 b | 2596.7 ± 167.1 a |
| Habanero Orange | K | 7328.3 ± 116.8 e | 4200.1 ± 12.9 d | 7724.8 ± 115.9 e | 4587.1 ± 8.5 c | 8905.0 ± 81.3 d | 5643.3 ± 22.8 c |
| | E | 7086.0 ± 19.6 d | 4261.6 ± 25.1 e | 7592.1 ± 92.1 d | 4783.9 ± 17.4 d | 9528.9 ± 123.1 f | 6368.5 ± 42.4 d |
| | H | 7516.2 ± 119.1 f | 4227.7 ± 22.3 de | 8987.1 ± 121.4 f | 5094.2 ± 14.0 e | 9387.6 ± 8.2 e | 5735.5 ± 7.9 c |
| 2022–2023 | | | | | | | |
| Kristian | K | 2066.0 ± 16.3 a | 2126.7 ± 74.7 a | 1839.7 ± 20.7 a | 1969.8 ± 66.3 a | | |
| | E | 2101.0 ± 14.6 a | 2184.9 ± 53.9 a | 2132.0 ± 18.4 b | 2469.6 ± 177.0 a | | |
| | H | 1829.8 ± 11.8 a | 1934.6 ± 100.5 a | 1802.1 ± 19.0 a | 1903.9 ± 82.1 a | | |
| Habanero Orange | K | 7646.4 ± 69.3 b | 4155.0 ± 18.4 b | 7970.3 ± 72.4 c | 5084.9 ± 18.3 c | 8492.8 ± 53.2 a | 5234.8 ± 21.1 a |
| | E | 7663.4 ± 38.5 b | 4420.7 ± 37.7 b | 7726.4 ± 70.9 c | 4265.9 ± 23.1 b | 8832.6 ± 78.2 a | 5256.0 ± 30.0 a |
| | H | 8027.7 ± 129.1 b | 4474.2 ± 23.4 b | 8575.7 ± 84.9 d | 4511.7 ± 128.6 bc | 8822.0 ± 30.3 a | 4963.4 ± 18.9 a |

Mean ± SD (n = 3). CAP—capsaicin; DHC—dihydrocapsaicin; K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix. Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

**Table 10.** Statistical analysis of qualitative parameters related to chili pepper pungency (DM) according to average values from all tested chili varieties and years.

| | Capsaicin | Dihydrocapsaicin | SHU |
|---|---|---|---|
| Year | 0.0333 | 0.0000 *** | 0.0045 * |
| Harvest | 0.0000 *** | 0.0000 *** | 0.0000 *** |
| Treatment | 0.2885 | 0.3016 | 0.9236 |
| Variety | 0.0000 *** | 0.0000 *** | 0.0000 *** |

Statistically significant differences at $p \leq 0.05$ (*), and $p \leq 0.001$ (***).

**Table 11.** The effect of harvest term on the capsaicin content of dry pepper fruits in 2022–2023.

| Harvest | Capsaicin (mg·kg$^{-1}$) | | Dihydrocapsaicin (mg·kg$^{-1}$) | |
|---|---|---|---|---|
| | Habanero Orange | Kristian | Habanero Orange | Kristian |
| 1 | 7779.2 ± 111.2 a | 1951.9 ± 89.4 a | 4350.0 ± 113.7 a | 2082.1 ± 100.7 a |
| 2 | 8093.8 ± 119.1 a | 1998.3 ± 83.6 a | 4612.3 ± 121.2 a | 2184.2 ± 107.6 a |
| 3 | 8794.7 ± 130.3 b | 2025.4 ± 118.3 a | 5223.4 ± 142.5 b | 2659.1 ± 142.3 b |

Mean ± SD (n = 3). Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

Capsaicin and dihydrocapsaicin contents can vary depending on the planting density and the season of the year in which they are produced, according to Blum et al. [40] and Moirangthem et al. [41]. Sahid et al. [42] also found that capsaicin content was negatively correlated and varied with fruit weight, fruit diameter, fruit length, thick fruit flesh, total fruit per plant, and fruit weight per plant. According to Zamljen et al. [43], in terms of total phenolics, all three cultivars of *Capsicum* spp. were positively affected by amino acid treatment, but not in each fruit part. In terms of capsaicinoid content, the greatest effect of the two stimulants was on 'Somborka', which varied from 4 (pericarp, seed) to 16 (placenta) times compared to the control. Amino acid extract decreased 'Habanero Red Caribbean' capsaicinoid content in placenta by about 40%. Two biostimulants, based on red grape skin extract (RG) and alfalfa hydrolysate (AH), in the study by Ertani et al. [44], improved the phenol concentration, antioxidant activity, and ascorbic acid concentration in fruits, as well as the capsaicin concentration in plants. The efficiency of RG and AH in promoting plant growth and yield could also be due to their content in indole-3-acetic acid (IAA), isopentenyladenosine (IPA), phenols, and amino acids. Our experiment was established as part of a wider study on peppers, where several locations and species were measured with the given preparation. We focused on two species of hot peppers in the framework of individual harvests over two years. In the two-year experiment, it was shown that it is necessary to repeat the experiments in variable sub-climatic conditions of the given environment, as the complex results in these cases did not confirm some conclusions of the study with the tested preparations, according to Golian et al. [26].

The precise determination of pungency is critical for consumers and the industrial use of chili peppers. However, due to the long-lasting and fatigue-inducing nature of pungency, means to complement or partially replace sensory evaluation to determine pungency are needed [45]. The Scoville Heat Unit (SHU) has been proposed to represent the heat level or pungency of peppers, which is obtained by converting the concentrations of capsaicin and dihydrocapsaicin in samples [46] (Tables 6 and 11).

The results of the Scoville calculations showed that the two analyzed chili varieties had very different values of SHU. The average value for the Kristian variety in our study was 65,406 SHU, and Habanero Orange had, on average, 202,187 SHU. Therefore, according to the literature, Kristian falls under the highly pungent category of chili peppers, and Habanero Orange [47] is considered a very highly pungent pepper, although this can be a subjective metric. From the statistical analysis, in regard to experimental variants, there were not any significant differences between the biostimulant treatments and the control group. SHU values were statistically significantly affected by the harvest date, the variety of cultivated chili pepper, and the year of the experimental study (Table 11).

### 3.2.2. Ascorbic Acid

The average content of ascorbic acid in fresh fruits of tested peppers ranged from 2078 mg·kg$^{-1}$ ('Habanero Orange') to 2820 mg.kg$^{-1}$ ('Kristian'; Figure 3), and in the case of the Kristian variety, especially in the first harvest, both biostimulants proved to have an effect on ascorbic acid content compared to the control. The ascorbic acid content of 22 high-yielding chili pepper (*Capsicum annum*) landraces was tested by Orobiyi et al. [48]. Vitamin C content varied from 84.64 mg to 192.64 mg/100 g of fresh weight, with an average of 125.70 mg/100 g FM. The impact of the tested biostimulants was variable in each harvest, separately (Table 12).

When comparing all data together (Figure 3), although significance ($p \leq 0.05$) of the biostimulants' effect on ascorbic acid content was not found, the values of vitamin C in case of biostimulant treatments were generally increased. Humic acids can improve photosynthetic efficiency and carbon fixation in plants. Ascorbic acid is synthesized from sugars produced during photosynthesis. Therefore, by enhancing photosynthetic activity, humic acids can provide more substrates for the biosynthesis of ascorbic acid. Some authors stated that humic acids and sugars present in biostimulants improved the biosynthesis of low-molecular-weight antioxidative compounds, such as ascorbate and phenols [44]. In a study by Noushad et al. [38], they determined that foliar application of a seaweed extract and protein-based biostimulant (Dollar) at a concentration of 2.5 mL·L$^{-1}$ stimulated ascorbic acid production in chili peppers (118.52 mg/100 g), in comparison to the control (110.58 mg/100 g). The biostimulant-balanced application in liquid form improved seaweed availability, leading to increased ascorbic acid levels in chili fruits. This enhancement in ascorbic acid contributes to improved nutritional value and quality of chili crops. The application of biostimulants caused a slight increase in the ascorbic acid concentration in the study by Ertani et al. [44], with the highest values observed in leaves and green peppers of plants sprayed with AH (alfalfa hydrolysate), at the lower dose (+18% and +20%, respectively). There were differences in the biostimulant treatments assessed for vitamin C according to González-Cortés et al. [34], where the content of vitamin C was the same by supplementing with 50% of organic K from liquid earthworm humus or vermicompost as the results obtained by using 100% chemical fertilization. The other two biostimulants showed a lower content, with values of 149 mg/100 gand 164 mg/100 gof fresh weight.

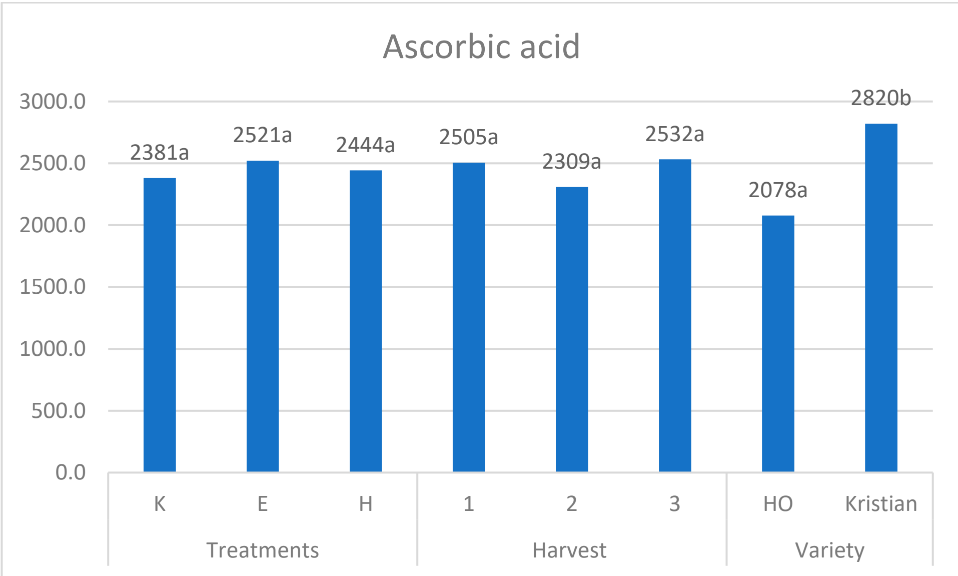


**Figure 3.** Influence of biostimulants, harvest, and variety on ascorbic acid content in chili peppers (mg·kg$^{-1}$ FM). AA—ascorbic acid; FM—fresh matter; K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix; HO—'Habanero Orange'. Different letters demonstrate statistically significant differences at $p \leq 0.05$.

**Table 12.** Qualitative parameters of chili fruits (mg.kg$^{-1}$ FM).

| Treatment | | 1st Harvest | | 2nd Harvest | | 3rd Harvest | |
|---|---|---|---|---|---|---|---|
| | | Vit. C | Carotenoids | Vit. C | Carotenoids | Vit. C | Carotenoids |
| Kristian | K | 2480.68 ± 80.78 a | 39.08 ± 7.90 b | 2723.88 ± 60.21 a | 36.65 ± 2.63 b | 2967.08 ± 166.83 ab | 38.41 ± 0.98 b |
| | E | 2690.78 ± 45.76 b | 27.34 ± 0.61 a | 2766.08 ± 47.55 a | 26.12 ± 4.66 a | 2841.39 ± 94.30 a | 27.24 ± 0.14 a |
| | H | 2802.59 ± 92.56 b | 21.87 ± 0.81 a | 2969.40 ± 50.00 b | 46.88 ± 1.92 c | 3136.21 ± 160.24 b | 41.88 ± 1.23 b |
| Habanero Orange | K | 2411.84 ± 169.12 b | 20.96 ± 1.52 a | 1659.07 ± 31.10 a | 34.22 ± 0.61 b | 2045.46 ± 42.52 a | 46.17 ± 4.86 a |
| | E | 2641.49 ± 99.26 b | 28.55 ± 3.24 b | 1971.52 ± 53.26 b | 29.97 ± 2.83 a | 2216.50 ± 71.12 b | 46.17 ± 2.83 a |
| | H | 2003.04 ± 51.61 a | 27.24 ± 0.10 b | 1763.52 ± 87.56 a | 43.54 ± 1.01 c | 1987.22 ± 30.41 a | 52.65 ± 0.40 a |

Mean ± SD (n = 3). FM—fresh matter; K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix. Different letters in columns demonstrate statistically significant differences at $p \leq 0.05$.

### 3.2.3. Carotenoids

The total carotenoid content for each variety across all three harvests is presented in Table 12. The effect of the biostimulants on carotenoid content in fresh fruits of the tested chili pepper varieties varied depending on both the specific variety and the harvest. Considering the average values from all data (Figure 4), a decrease was found in the case of the Energen biostimulant, while the control treatment and Humix did not differ significantly ($p \leq 0.05$).

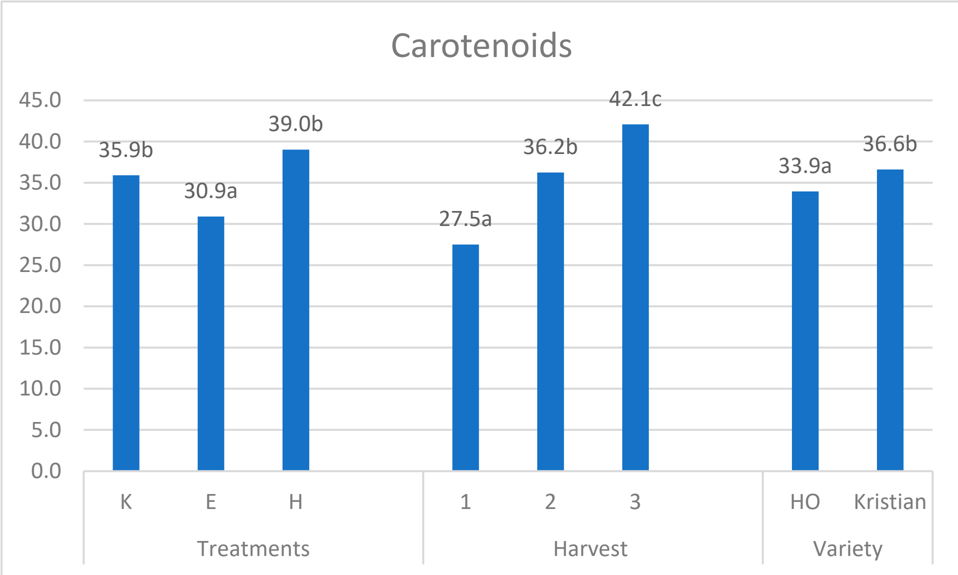


**Figure 4.** Influence of biostimulants, harvest, and variety on carotenoid content in chili peppers (mg·kg$^{-1}$ FM). FM—fresh matter; K—control treatment; E—treatment with application of the biostimulant Energen; H—treatment with application of the biostimulant Humix; HO—'Habanero Orange'. Different letters demonstrate statistically significant differences at $p \leq 0.05$.

Carotenoid biosynthesis is a complex process involving multiple enzymatic reactions and regulatory mechanisms. Biostimulants based on humic acids can influence various metabolic pathways in plants, and their specific effects on the enzymes and regulatory factors involved in carotenoid biosynthesis may be limited. Humic acids primarily enhance the availability and uptake of certain nutrients by chelating minerals and making them more accessible to plants. While some nutrients are essential for carotenoid biosynthesis (such as nitrogen, phosphorus, and magnesium), the relationship between nutrient availability and carotenoid accumulation can be complex and may vary depending on the specific nutrient and plant species. The treatment of *Capsicum* fruits with Actium® increased carotenoids, concomitant with an increase in some digalactosyl diacylglycerols, which are part of the chromoplasts lipid machinery of enzymes involved in the synthesis of carotenoids, according to Barrajón-Catalán et al. [49]. The discrepancies in our findings can be attributed to differences in the content of the biostimulant, where Actium® is a commercial biostimulant containing a lipo-complex formulation comprised mainly of polysaccharides, polypeptides, vitamins (40%), amino acids (2%), and potassium oxide (5%). Carotenoids have been determined to have a significant rise in content within the harvests, and the content grew progressively over each of the harvests, peaking in the third harvest (Figure 4). We assumed a relationship with pepper fruits' natural ripening. The carotenoid biosynthesis in pepper is an observable process of gradual changes in color during fruit ripening, from green to yellow, orange, and finally red, depending on the cultivars [50]. A significant difference was also observed in the case of variety, with ′Orange Habanero′ presenting a higher concentration of carotenoids in comparison to the yellow 'Kristian'. Orange landraces showed the highest variability in total carotenoid content, while the yellow ones showed the lowest amounts, in the study by Morales-Soriano et al. [51]. Carotenoid content varied from 1.54 mg (P116, *C. chinense*) to 54.11 mg β-carotene/100 gfresh weight in Acunha et al.'s study [52], where 72 accessions of *Capsicum annuum* L., *C. baccatum* L., *C. chinense* Jacq., and *C. frutescens* L. were evaluated. Besides the maturity stage at harvest, the variation in composition and relative content of carotenoids of the *Capsicum* species is influenced mainly by the differences in genotypes, agroclimatic conditions, post-harvest handling, processing, and preparation [53].

## 4. Conclusions

This study investigated the effects of two commercial biostimulants, Humix® and Energen, on the yield and qualitative traits of two chili pepper genotypes, *Capsicum chinense* ('Habanero Orange') and *Capsicum annuum* ('Kristian'). Both biostimulants positively influenced various parameters, but their effects varied depending on the genotype and the specific quality trait measured. Humix® consistently increased the total fruit yield, especially in 'Kristian', which exhibited an increase from 5.23 $t \cdot ha^{-1}$ to 8.11 $t \cdot ha^{-1}$, while 'Habanero Orange' saw an improvement from 17.70 $t \cdot ha^{-1}$ to 20.01 $t \cdot ha^{-1}$. Energen showed similar yield improvements in both varieties.

Regarding capsaicinoid content, both biostimulants increased the capsaicin and dihydrocapsaicin levels in the peppers, though the exact response was variable depending on the harvest and variety. Notably, 'Habanero Orange' had significantly higher capsaicin levels across all harvests compared to 'Kristian'. The application of biostimulants influenced the accumulation of secondary metabolites, with Humix® leading to higher capsaicin concentrations than Energen, particularly in the later harvests. The term of harvest played a crucial role in determining these levels, with the third harvest showing the highest capsaicin content.

The effect on ascorbic acid and carotenoid content was more variable, with the genotype, biostimulant type, and harvest term influencing the outcomes. Humix® generally improved ascorbic acid content in both varieties, while Energen showed inconsistent effects on carotenoid content. 'Habanero Orange' consistently demonstrated higher carotenoid levels than 'Kristian', suggesting genotype-specific responses to the biostimulants.

Since the two monitored chili pepper varieties showed an increase in economically significant yield and capsaicin levels, using the tested biostimulants could be a strategic approach to producing nutrient-rich vegetables with no adverse environmental effects. Overall, both Humix® and Energen positively impacted yield and bioactive compound accumulation, but the responses were highly dependent on the chili variety and harvest timing. This study highlighted the potential for using biostimulants to enhance chili pepper production, while also suggesting that genotype-specific strategies may be necessary to optimize their benefits.

**Author Contributions:** Conceptualization, I.M.; methodology, I.M., M.G., J.Á. and L.G.; software, I.M. and I.K.; formal analysis, I.M.; investigation, I.M.; growing plant material, I.M., M.B. and T.H.; laboratory analyses, J.Á., L.G., M.B. and T.H.; writing—original draft preparation, I.M., I.K. and J.M.; writing—review and editing, I.M., I.K. and J.M.; supervision, R.R.; validation, J.M. and R.R.; funding acquisition, I.M., M.G. and M.Š. All authors have read and agreed to the published version of the manuscript. We insist that no author be omitted.

**Funding:** This research was funded by KEGA, grant number KEGA 004SPU-4/2022, "Interactive Classroom for Horticulture Study Program in the Context of Innovation of the Current Student's Teaching Process", and by APVV, grant number APVV-21-0168, "From Cell to Organism—From Protectivity to Toxicity".

**Data Availability Statement:** The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Kalita, R.; Bhorali, P.; Gogoi, M.B.; Gogoi, B. Advances in Chilli Pepper (*Capsicum* spp.) Improvement Using Modern Genetic Tools. In *Genetic Engineering of Crop Plants for Food and Health Security*; Springer Nature: Singapore, 2023; pp. 151–168.
2. Jeeatid, N.; Techawongstien, S.; Suriharn, B.; Bosland, P.W.; Techawongstien, S. Light Intensity Affects Capsaicinoid Accumulation in Hot Pepper (*Capsicum chinense* Jacq.) Cultivars. *Hortic. Environ. Biotechnol.* **2017**, *58*, 103–110. [CrossRef]
3. Batiha, G.E.-S.; Alqahtani, A.; Ojo, O.A.; Shaheen, H.M.; Wasef, L.; Elzeiny, M.; Ismail, M.; Shalaby, M.; Murata, T.; Zaragoza-Bastida, A.; et al. Biological Properties, Bioactive Constituents, and Pharmacokinetics of Some *Capsicum* spp. and Capsaicinoids. *Int. J. Mol. Sci.* **2020**, *21*, 5179. [CrossRef] [PubMed]

4. Ao, Z.; Huang, Z.; Liu, H. Spicy Food and Chili Peppers and Multiple Health Outcomes: Umbrella Review. *Mol. Nutr. Food Res.* **2022**, *66*, 2200167. [CrossRef] [PubMed]
5. Zheng, J.; Zheng, S.; Feng, Q.; Zhang, Q.; Xiao, X. Dietary Capsaicin and Its Anti-Obesity Potency: From Mechanism to Clinical Implications. *Biosci. Rep.* **2017**, *37*, BSR20170286. [CrossRef] [PubMed]
6. Xiang, Q.; Tang, X.; Cui, S.; Zhang, Q.; Liu, X.; Zhao, J.; Zhang, H.; Mao, B.; Chen, W. Capsaicin, the Spicy Ingredient of Chili Peppers: Effects on Gastrointestinal Tract and Composition of Gut Microbiota at Various Dosages. *Foods* **2022**, *11*, 686. [CrossRef]
7. Xia, J.; Gu, L.; Guo, Y.; Feng, H.; Chen, S.; Jurat, J.; Fu, W.; Zhang, D. Gut Microbiota Mediates the Preventive Effects of Dietary Capsaicin Against Depression-Like Behavior Induced by Lipopolysaccharide in Mice. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 627608. [CrossRef]
8. Pasierski, M.; Szulczyk, B. Beneficial Effects of Capsaicin in Disorders of the Central Nervous System. *Molecules* **2022**, *27*, 2484. [CrossRef]
9. Jittiwat, J.; Suksamrarn, A.; Tocharus, C.; Tocharus, J. Dihydrocapsaicin Effectively Mitigates Cerebral Ischemia-Induced Pathological Changes in Vivo, Partly via Antioxidant and Anti-Apoptotic Pathways. *Life Sci.* **2021**, *283*, 119842. [CrossRef]
10. Naqve, M.; Mukhtiar, A.; Arshad, T.; Zia, M.A.; Mahmood, A.; Javaid, M.M.; Aziz, A. Biostimulants in Sustainable Agriculture. In *Climate-Resilient Agriculture*; Springer International Publishing: Cham, Switzerland, 2023; Volume 1, pp. 535–548.
11. Gajc-Wolska, J.; Mazur, K.; Niedzińska, M.; Kowalczyk, K.; Żołnierczyk, P. The Influence of Foliar Fertilizers on the Quality and Yield of Sweet Pepper (*Capsicum annuum* L.). *Folia Hortic.* **2018**, *30*, 183–190. [CrossRef]
12. Rouphael, Y.; Colla, G. Editorial: Biostimulants in Agriculture. *Front. Plant Sci.* **2020**, *11*, 40. [CrossRef]
13. Drobek, M.; Frac, M.; Cybulska, J. Plant Biostimulants: Importance of the Quality and Yield of Horticultural Crops and the Improvement of Plant Tolerance to Abiotic Stress—A Review. *Agronomy* **2019**, *9*, 335. [CrossRef]
14. Majkowska-Gadomska, J.; Dobrowolski, A.; Jadwisieńczak, K.K.; Kaliniewicz, Z.; Francke, A. Effect of Biostimulants on the Growth, Yield and Nutritional Value of *Capsicum Annuum* Grown in an Unheated Plastic Tunnel. *Sci. Rep.* **2021**, *11*, 22335. [CrossRef]
15. Arumugam, R.; Rabert, G.A. Plant Biostimulants: Overview of Categories and Effects. In *Biostimulants: Exploring Sources and Applications*; Springer: Berlin/Heidelberg, Germany, 2022; pp. 1–29.
16. Abou-Sreea, A.I.B.; Azzam, C.R.; Al-Taweel, S.K.; Abdel-Aziz, R.M.; Belal, H.E.E.; Rady, M.M.; Abdel-Kader, A.A.S.; Majrashi, A.; Khaled, K.A.M. Natural Biostimulant Attenuates Salinity Stress Effects in Chili Pepper by Remodeling Antioxidant, Ion, and Phytohormone Balances, and Augments Gene Expression. *Plants* **2021**, *10*, 2316. [CrossRef] [PubMed]
17. Karakurt, Y.; Unlu, H.; Unlu, H.; Padem, H. The Influence of Foliar and Soil Fertilization of Humic Acid on Yield and Quality of Pepper. *Acta Agric. Scand. Sect. B Plant Soil Sci.* **2009**, *59*, 233–237. [CrossRef]
18. Canellas, L.P.; Olivares, F.L.; Aguiar, N.O.; Jones, D.L.; Nebbioso, A.; Mazzei, P.; Piccolo, A. Humic and Fulvic Acids as Biostimulants in Horticulture. *Sci. Hortic.* **2015**, *196*, 15–27. [CrossRef]
19. Azcona, I.; Pascual, I.; Aguirreolea, J.; Fuentes, M.; García-Mina, J.M.; Sánchez-Díaz, M. Growth and Development of Pepper Are Affected by Humic Substances Derived from Composted Sludge. *J. Plant Nutr. Soil Sci.* **2011**, *174*, 916–924. [CrossRef]
20. Arthur, J.D.; Li, T.; Bi, G. Plant Growth, Yield, and Quality of Containerized Heirloom Chile Pepper Cultivars Affected by Three Types of Biostimulants. *Horticulturae* **2022**, *9*, 12. [CrossRef]
21. Zamljen, T.; Medic, A.; Hudina, M.; Veberic, R.; Slatnar, A. Biostimulatory Effects of Amino Acids on Phenylalanine Ammonia Lyase, Capsaicin Synthase, and Peroxidase Activities in *Capsicum baccatum* L. *Biology* **2022**, *11*, 674. [CrossRef]
22. Energen. 2024. Available online: https://www.energen.info/cs/dokumenty-ke-stazeni/ (accessed on 9 September 2024).
23. Humix Univerzál®. 2024. Available online: https://www.agrocultur.sk/certifikaty/ (accessed on 9 September 2024).
24. Fecenko, J.; Ložek, O. *Výživa a hnojenie pol'ných plodín*; Slovenská polnohospodárska univerzita v Nitre: Nitre, Slovakia, 2000; 442p, ISBN 80-7137-777-5.
25. Kočický, D. Regionálny Územný Systém Ekologickej Stability Okresu Nitra. 2019. Available online: https://www.minv.sk/?regionalny-uzemny-system-ekologickej-stability-okresu-nove-zamky (accessed on 12 January 2024).
26. Golian, M.; Mezeyová, I.; Andrejiová, A.; Hegedűsová, A.; Adamec, S.; Štefániková, J.; Árvay, J. Effects of Selected Biostimulants on Qualitative and Quantitative Parameters of Nine Cultivars of the Genus *Capsicum* spp. *Open Agric* **2024**, *9*, 20220266. [CrossRef]
27. Al Othman, Z.A.; Ahmed, Y.B.H.; Habila, M.A.; Ghafar, A.A. Determination of Capsaicin and Dihydrocapsaicin in *Capsicum* Fruit Samples Using High Performance Liquid Chromatography. *Molecules* **2011**, *16*, 8919–8929. [CrossRef]
28. Hegedusova, A.; Slosar, M.; Mezeyova, I.; Hegedus, O.; Andrejiova, A.; Szarka, K. *Methods for Estimation of Selected Biologically Active Substances*; Slovak University of Agriculture in Nitra: Nitra, Slovakia, 2018; ISBN 978-80-552-1928-8.
29. Biehler, E.; Mayer, F.; Hoffmann, L.; Krause, E.; Bohn, T. Comparison of 3 Spectrophotometric Methods for Carotenoid Determination in Frequently Consumed Fruits and Vegetables. *J. Food Sci.* **2010**, *75*, C55–C61. [CrossRef] [PubMed]
30. Baltazar, M.; Correia, S.; Guinan, K.J.; Sujeeth, N.; Bragança, R.; Gonçalves, B. Recent Advances in the Molecular Effects of Biostimulants in Plants: An Overview. *Biomolecules* **2021**, *11*, 1096. [CrossRef]
31. Ali, O.; Ramsubhag, A.; Jayaraman, J. Biostimulant Properties of Seaweed Extracts in Plants: Implications towards Sustainable Crop Production. *Plants* **2021**, *10*, 531. [CrossRef]
32. Jan, J.A.; Nabi, G.; Khan, M.; Ahmad, S.; Shah, P.S.; Hussain, S.; Sehrish, S. Foliar Application of Humic Acid Improves Growth and Yield of Chilli (*Capsicum annum* L.) Varieties. *Pak. J. Agric. Res.* **2020**, *33*, 461. [CrossRef]

33. Suhaini, N.; Singh, D.; Wesley, C.J. Effect of Different Biostimulants on Growth, Yield and Quality of Chilli (*Capsicum annuum* L.) under Prayagraj Agro Climatic Conditions. *Int. J. Environ. Clim. Change* **2023**, *13*, 191–197. [CrossRef]
34. González-Cortés, A.; Robledo-Torres, V.; Luna-García, L.R.; Mendoza-Villarreal, R.; Pérez-Rodríguez, M.Á. Yield and Antioxidant Quality of Habanero Chili Pepper by Supplementing Potassium with Organic Products. *Horticulturae* **2023**, *9*, 797. [CrossRef]
35. Angmo, P.; Dolma, T.; Phuntsog, N.; Chaurasia, O.P.; Stobdan, T. Effect of Shading and High Temperature Amplitude on Yield and Phenolic Contents of Greenhouse *Capsicum* (*Capsicum annuum* L.). *Open Access Res. J. Biol. Pharm.* **2022**, *4*, 030–039. [CrossRef]
36. Deori, M.; Singh, A.K.; Kumari, S. Effect of Foliar Application of Biostimulants on Growth and Yield of Chilli (*Capsicum annuum* L.). *Pharma Innov. J.* **2023**, *870*, 870–874.
37. Ertani, A.; Pizzeghello, D.; Francioso, O.; Sambo, P.; Sanchez-Cortes, S.; Nardi, S. *Capsicum chinensis* L. Growth and Nutraceutical Properties Are Enhanced by Biostimulants in a Long-Term Period: Chemical and Metabolomic Approaches. *Front. Plant Sci.* **2014**, *5*, 375. [CrossRef]
38. Noushad, R.; Singh, D.; Wesley, C.J.; Sneha, M.S. Assessment of Bioefficacy by Using Biostimulants on Growth, Quality, and Yield of Chilli (*Capsicum annum* L.). *Int. J. Environ. Clim. Change* **2023**, *13*, 2152–2162. [CrossRef]
39. Borges-Gómez, L.; Cervantes Cárdenas, L.; Ruiz Novelo, J.; Soria Fregoso, M.; Reyes Oregel, V.; Villanueva Couoh, E. Capsaicinoids in habanero pepper (*Capsicum chinense* Jacq.) under various humidity and nutritional conditions. *Terra Latinoam.* **2010**, *28*, 35–41.
40. Blum, E.; Mazourek, M.; O'Connell, M.; Curry, J.; Thorup, T.; Liu, K.; Jahn, M.; Paran, I. Molecular Mapping of Capsaicinoid Biosynthesis Genes and Quantitative Trait Loci Analysis for Capsaicinoid Content in *Capsicum*. *Theor. Appl. Genet.* **2003**, *108*, 79–86. [CrossRef] [PubMed]
41. Moirangthem, S.S.; Gogoi, S.; Thongbam, P.D.; Ramya, K.T.; Fiyaz, R.A.; Pandey, D.S. Effect of Sowing Time and Crop Geometry on the Capsaicinoid Content in Bhoot Jolokia (*Capsicum chinense* Jacq.). *J. Food Sci. Technol.* **2014**, *51*, 1974–1981. [CrossRef] [PubMed]
42. Sahid, Z.D.; Syukur, M.; Maharijaya, A. Diversity of Capsaicin Content, Quantitative, and Yield Components in Chili (*Capsicum annuum*) Genotypes and Their F1 Hybrid. *Biodiversitas* **2020**, *21*, 2251. [CrossRef]
43. Zamljen, T.; Hudina, M.; Veberič, R.; Slatnar, A. Biostimulative Effect of Amino Acids and Green Algae Extract on Capsaicinoid and Other Metabolite Contents in Fruits of *Capsicum* spp. *Chem. Biol. Technol. Agric.* **2021**, *8*, 63. [CrossRef]
44. Ertani, A.; Sambo, P.; Nicoletto, C.; Santagata, S.; Schiavon, M.; Nardi, S. The Use of Organic Biostimulants in Hot Pepper Plants to Help Low Input Sustainable Agriculture. *Chem. Biol. Technol. Agric.* **2015**, *2*, 11. [CrossRef]
45. Zhu, Y.; Li, X.; Jiang, S.; Zhang, Y.; Zhang, L.; Liu, Y. Multi-Dimensional Pungency and Sensory Profiles of Powder and Oil of Seven Chili Peppers Based on Descriptive Analysis and Scoville Heat Units. *Food Chem.* **2023**, *411*, 135488. [CrossRef]
46. Scoville, W.L. Note on *Capsicums*. *J. Am. Pharm. Assoc.* **1912**, *1*, 453–454. [CrossRef]
47. Moreb, N.; O'Dwyer, C.; Jaiswal, S.; Jaiswal, A.K. Pepper. In *Nutritional Composition and Antioxidant Properties of Fruits and Vegetables*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 223–238.
48. Orobiyi, A.; Ahissou, H.; Gbaguidi, F.; Sanoussi, F.; Houngbèmè, A.; Dansi, A.; Sanni, A. Capsaicin and Ascorbic Acid Content in the High Yielding Chili Pepper (*Capsicum annuum* L.) Landraces of Northern Benin. *Int. J. Curr. Microbiol. Appl. Sci.* **2015**, *4*, 394–403.
49. Barrajón-Catalán, E.; Álvarez-Martínez, F.J.; Borrás, F.; Pérez, D.; Herrero, N.; Ruiz, J.J.; Micol, V. Metabolomic Analysis of the Effects of a Commercial Complex Biostimulant on Pepper Crops. *Food Chem.* **2020**, *310*, 125818. [CrossRef]
50. Mohd Hassan, N.; Yusof, N.A.; Yahaya, A.F.; Mohd Rozali, N.N.; Othman, R. Carotenoids of *Capsicum* Fruits: Pigment Profile and Health-Promoting Functional Attributes. *Antioxidants* **2019**, *8*, 469. [CrossRef] [PubMed]
51. Morales-Soriano, E.; Panozzo, A.; Ugás, R.; Grauwet, T.; Van Loey, A.; Hendrickx, M. Carotenoid Profile and Basic Structural Indicators of Native Peruvian Chili Peppers. *Eur. Food Res. Technol.* **2019**, *245*, 717–732. [CrossRef]
52. Acunha, T.d.S.; Crizel, R.L.; Tavares, I.B.; Barbieri, R.L.; Pereira de Pereira, C.M.; Rombaldi, C.V.; Chaves, F.C. Bioactive Compound Variability in a Brazilian *Capsicum* Pepper Collection. *Crop Sci.* **2017**, *57*, 1611–1623. [CrossRef]
53. Pugliese, A.; O'Callaghan, Y.; Tundis, R.; Galvin, K.; Menichini, F.; O'Brien, N.; Loizzo, M.R. In Vitro Investigation of the Bioaccessibility of Carotenoids from Raw, Frozen and Boiled Red Chili Peppers (*Capsicum annuum*). *Eur. J. Nutr.* **2014**, *53*, 501–510. [CrossRef] [PubMed]