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The use of organic biostimulants in hot pepper plants to help low input sustainable agriculture

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Abstract

Background: World demand for agricultural products is increasing. New insights are required in order to achieve sufficient and sustainable yields to meet global food request. Chemical fertilizers have been studied for almost 200 years, and it is unlikely that they could be improved. However, to produce food for a growing world population, various methods to increase the efficiency of chemical fertilizers are investigated. One approach to increasing crop productivity is the development of environment-friendly organic products named biostimulants which stimulate plant growth by enhancing the efficiency of chemical fertilizers. Most studies have tested these products in short-term experiments, but little information is available on their effect on plants at the maturity stage of growth. On this account, this paper focuses on the effects of two biostimulants, red grape skin extract (RG) and alfalfa hydrolyzate (AH), throughout the entire plant development.

Results: The findings obtained in the present investigation demonstrate the effectiveness of RG and AH in improving growth and the nutritional value of peppers. Specifically, the two biostimulants increased the phenol concentration, antioxidant activity, and ascorbic acid concentration in fruits, as well as the capsaicin concentration in plants. Differences in effectiveness between RG and AH were likely related to the characteristics of the starting matrixes as well as to the industrial processes used for their production. 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.

Conclusions: In the light of these results, the application of biostimulants could be considered as a good strategy for obtaining high yields of nutritionally valuable vegetables with lower environmental impact.

Keywords: Peppers; Biostimulants; Endogenous hormones; Phenols; Antioxidants

Background

The climate changes occurring in recent years have affected the farming, imposing constraints and objectives frequently ignored in the past. The environmental question and quality of the products affect farmers' choices in different ways, depending on the specific vulnerability of the environment where the crops are cultivated [1]. In this context, crop-derived food safety and nutritional value have become important issues worldwide [2]. For example, there is increasing interest in studying and quantifying the antioxidant and anti-inflammatory constituents of plants in terms of their potential health benefits [3,4].

To increase the content of nutritional constituents in plants, many approaches have been studied to increase plant nutrients capture and yield such as genetic selection, which has included allele selection, gene and genome duplication, and new genotypes creation. However, despite the advantages that these techniques offer, some of them may also pose potential problems for food safety and require special attention in order to ensure consumer health protection [5,6].

On this account, the use of biostimulants in agricultural practices is proposed as a safe tool to enhance the nutritional properties of food crops. Biostimulants are recognized as environment-friendly compounds with beneficial effects on plants [7,8]. In particular, they decrease the use of mineral fertilizers by increasing the amount of micro- and macro-nutrients taken up by plants, positively influencing root morphology and plant growth [9,10]. They also display hormone-

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like activity and influence plant metabolism by interacting with biochemical processes and physiological mechanisms, such as glycolysis and nitrogen assimilation [11,12-13].

The mechanisms behind the physiological and biochemical effects of biostimulants on plants are often unknown, because of the heterogeneous nature of the raw materials used for their production. Furthermore, these effects are often the result of many components that may work synergistically in different ways.

Recent studies suggest that active molecules contained in biostimulants can promote nitrogen assimilation through stimulation of the activity and transcription of N assimilation and Krebs' cycle enzymes [7-11]. Furthermore, the induction of the metabolic pathway associated with the synthesis of phenylpropanoids in plants treated with biostimulants may explain why these products can help plants to overcome stress situations [14,15]. In particular, a protein hydrolyzate-based fertilizer obtained from alfalfa hydrolyzate plants (AH) was proved to help maize plants to overcome salinity stress through stimulation of enzymes functioning in nitrogen metabolism, enhancement of phenylalanine ammonia-lyase (PAL) activity and transcription, and increase of flavonoid synthesis [16]. PAL is an important enzyme that catalyzes the first committed step in the biosynthesis of phenolics by converting phenylalanine to trans-cinnamic acid and tyrosine to p-coumaric acid, opening the way to the secondary metabolism. The biostimulant activity of AH was related to the presence of triacontanol (TRIA) and indole-3-acetic acid (IAA), two important regulators of plant metabolism [15].

In recent studies, the application of products with biostimulant action to pepper plants was found to exert positive effects on plant growth and yield without loss of fruit quality [13,17,18]. Pepper is an important agricultural crop due to the nutritional value of its fruits, which are an excellent source of a wide array of phytochemicals with well-known antioxidant properties. The main antioxidant compounds include carotenoids, capsaicinoids, and phenolic compounds, particularly flavonoids, quercetin, and luteolin [19,20].

Most of studies testing biostimulants analyze their effects on seed germination and plants growth in short-term experiments, but little information is available on their effects at flowering and maturity stages of plants. On this account, the purpose of this investigation was to study the effects of two biostimulants on plant growth, phenological development, and some metabolic parameters of pepper plants (total phenols, ascorbic acid, capsaicin, dihydrocapsaicin), throughout the development from seedling to fruit.

Methods

Characterization of the products

The two products used in this study (red grape skin extract (RG) and AH) were manufactured by ILSA (Arzignano-Vicenza, Italy). AH was produced by a fully controlled enzymatic hydrolysis using vegetal material from alfalfa (*Medicago sativa* L.) plants. The elemental composition, organic matter content, and physical properties of AH were previously reported by Schiavon et al. [7]. Specifically, the water percentage in AH was 43.5% (*v*/*v*), and the amount of organic matter was low 37.6% (*w*/*v*). The content of ash and the electrical conductivity (ECw) were both high, with values of 18.9% (*w*/*v*) and 16 dS m-1, respectively. The percentage of inorganic nitrogen in the form of ammonia and nitrates was low (0.38% and 0.03 *w*/*w*, respectively), whereas the total amount of free amino acids was high up to 1.916% (*w*/*w*) and correlated with the free α-amino nitrogen (α-NH2-N). The hydrolytic process employed for EM production was effective in the weight-average molecular weight (MW) reduction, as confirmed by data on EM hydrolysis degree (DH) and MW.

RG was obtained from the skin of red grape wine via cool extraction according to the method of Machado [21]. RG aqueous extract was prepared by extracting 10 g of dried ground plant material with 50 mL of deionized water in a shaker for 2 h at room temperature. The extract was then filtered with cellulosic membrane filters at 0.8 μm (Membra-Fil® Whatman Brand, Whatman, Milan, Italy). The pH was determined in water (3:50 *w*/*v*). Total organic carbon (TOC) was measured using an element analyzer (varioMACRO CNS, Hanau, Germany).

Total phenols in RG extracts were determined according to Arnaldos et al. [22]. Specifically, RG (1 mL) was maintained in ice with pure methanol (1:1, *v*/*v*) for 30 min and centrifuged at 5,000*g* for 30 min at 4°C. One mL of 2% Na_2CO_3 and 75 μL of Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) were added to 100 μL of phenolic extract. After 15 min of incubation at 25°C in the dark, the absorbance at 725 nm was measured. Gallic acid was used as standard according to Meenakshi et al. [23].

For reducing sugars determination, a sample of RG product was dried for 48 h at 80°C, ground in liquid nitrogen, and then 100 mg were extracted with 2.5 mL 0.1 N H_2SO_4. Samples were incubated in a heating block for 40 min at 60°C and then centrifuged at 6,000*g* for 10 min at 4°C. After filtration (0.2 μm, Membra-Fil® Whatman Brand, Whatman, Milan, Italy), the supernatants were analyzed by HPLC (Perkin Elmer 410, Perkin Elmer, Waltham, MA, USA). The soluble sugars were separated through a Biorad Aminex 87 C column (300 × 7.8 mm; Bio-Rad Laboratories, Inc.,

Hercules, CA, USA) using H_2O as eluent at a flow rate of 0.6 mL min^{-1}.

Indole-3-acetic acid (IAA) in the RG was determined using an enzyme-linked immuno-sorbent assay (ELISA) standardized with methylated IAA (Phytodetek-IAA, Sigma, St. Louis, MO, USA). The ELISA test utilized a monoclonal antibody to IAA and was sensitive in the 0.05-to-100 pmol range. The tracer and standard solutions were prepared following the manufacturer's instructions, and absorbances were read at 405 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Isopentenyladenosine (IPA) in RG and AH was determined by ELISA, a Phytodetek-IPA with an anti-IPA monoclonal antibody was used (Sigma-Aldrich, St. Louis, MO, USA). The competitive antibody-binding method was adopted to measure the IPA concentration. IPA labeled with alkaline phosphatase (tracer) was added with the sample to antibody coated microwells. A competitive binding reaction was set up between a constant amount of the IPA tracer, a limited amount of the antibody and the unknown sample containing IPA (Sigma-Aldrich, St. Louis, MO, USA). One hundred μL of standard IPA concentration or serial dilutions of RG and AH and 100 μL diluted tracer were added to each well. For the standard curve, progressions of 100, 50, 20, 5, 1, 0.1, and 0.02 pmol IPA 100 μL^{-1} were used, whereas for RG and AH, the progressions were 20, 10, 7.5, 5.0, 3.5, and 2.5 μg C 100 μL^{-1}. After incubation at 4°C for 3 h, the wells were decanted and any unbound tracer was washed out by adding 200 μL of washing solution before adding 200 μL of substrate solution for colorimetric detection. After 60 min at 37°C, the optical density (OD) was read at 405 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Experimental design and growth conditions

The experimental trial was derived from the factorial combination of three types of treatment: no biostimulant, a cool extract from RG, and an AH. Two doses (50 and 100 mL L^{-1} for RG, and 25 and 50 mL L^{-1} for AH) of biostimulants were applied to two randomized blocks of pots, each consisting of 50 pots (ten pots per treatment to test biostimulants at flowering stage and ten pots per treatment to test biostimulants at maturity stage). The two doses for each biostimulant were in accordance with previous tests showing that doses in the 1-to-200 mL L^{-1} range were those that more interfered with pepper metabolism. Each pot was filled with 2 L perlite/vermiculite mixture. At the beginning of the trial, 7-day-old seedlings of chili pepper (*Capsicum chinense* L. *cv.* Fuoco della Prateria) were transplanted at a density of one plant per pot. Plants were grown until maturity in a tunnel maintained at 25°C/15°C day/night, receiving natural light. Plants were treated twice, i.e., at the second and fourth week after transplanting, by spraying each one with 4.5 mL of RG or AH on the leaves. All treatments were irrigated with a half-strength Hoagland's nutrient solution. Plants from 50 pots were sampled at flowering (4 weeks after transplanting), and plants from the remaining 50 pots at maturity (6 weeks after transplanting), harvesting leaves and fruits separately.

Analysis of sugars in plant material

Leaf and fruit samples (2 g) were homogenized in water (20 mL) with an Ultra Turrax T25 (IKA, Staufen, Germany) at 13,500 rpm until uniform consistency. Samples were filtered with filter paper (589 Schleicher) and further filtered through cellulose acetate syringe filters (0.45 μm). The analysis of the extracts was performed using an HPLC apparatus (Jasco X.LC system, Jasco Co., Tokyo, Japan) consisting of a model PU-2080 pump, a model RI-2031 refractive index detector, a model AS-2055 autosampler and a model CO-2060 column oven. ChromNAV Chromatography Data System was used as software. Sugars were separated on a Hyper-Rez XP Carbohydrate Ca^{++} analytical column (7.7 × 300 mm, ThermoScientific, Waltham, MA, USA) operating at 80°C. Isocratic elution was effected using water at a flow rate of 0.6 mL/min. The peaks were identified by comparing the retention time with those of standard compounds. To calculate the concentrations in the extract, a calibration curve was drawn for four solutions of known concentration in water.

Determination of total phenols in plant material

The concentration of total phenols was determined by the Folin-Ciocalteau (FC) assay with gallic acid as calibration standard, using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corp., Columbia, MD, USA). The FC assay was performed by pipetting 200 μL of plant extract (obtained as described above for sugars analysis) into a 10-mL PP tube. This operation was followed by addition of 1 mL of Folin-Ciocalteau's reagent. The mixture was vortexed for 20 to 30 s. Eight hundred microliters of sodium carbonate solution (20% *w*/*v*) was added to the mixture 5 min after the addition of the FC reagent. This was recorded as time zero; the mixture was then vortexed for 20 to 30 s after addition of sodium carbonate. After 2 h at room temperature, the absorbance of the colored reaction product was measured at 765 nm. The total phenols concentration in the extracts was calculated from a standard calibration curve obtained with different concentrations of gallic acid, ranging from 0 to 600 μg mL^{-1}. Results were expressed as milligrams of gallic acid equivalent per kilogram of fresh weight [24].

Determination of total antioxidant activity by ferric reducing antioxidant power

The assay was based on the methodology of Benzie and Strain [25]. Ten grams of plant material was homogenized in 20 mL of HPLC grade methanol using an Ultra-Turrax tissue homogenizer (Takmar, Cincinnati, OH, USA) at moderate speed (setting of 60) for 30 s. The ferric-reducing antioxidant power (FRAP) reagent was freshly prepared, containing 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate buffer at pH 3.6. One hundred microliters of the methanol extract was added to 1,900 μL of FRAP reagent and accurately mixed. After leaving the mixture at 20°C for 4 min, the absorbance was determined at 593 nm. Calibration was against a standard curve (0 to 1,200 μg mL^{-1} ferrous ion) obtained by the addition of freshly prepared ammonium ferrous sulfate. FRAP values were calculated as microgram per milliliter ferrous ion (ferric-reducing power) and are presented as milligram per kilogram of Fe^{2+E} (ferrous ion equivalent).

Extraction and determination of ascorbic acid

After harvest, the samples were immediately stored at −80°C before analysis. Five-gram samples were homogenized until uniform consistency in a meta-phosphoric acid and acetic acid solution. This solution was used for the ascorbic acid extraction after quantitative reduction of 2,6-dichlorophenolindophenol dyestuff by ascorbic acid and extraction of the excess dyestuff using xylene. The excess of ascorbic acid was measured at 500 nm in a Shimadzu UV 160A spectrophotometer (Shimadzu Corp., Columbia, MD, USA) and compared with a vitamin C reference standard (ISO/6557-2-1984 method).

Capsaicin and dihydrocapsaicin determination

Fresh leaves and green and red peppers (2 g) were extracted with 20 mL of acetone, followed by homogenization with an Ultra-Turrax T 25 (IKA, Staufen, Germany) for 30 s at 17,500 rpm. The extract was filtered with filter paper and then through regenerated cellulose syringe filters (0.45 μm). The analysis of the extracts was performed using an HPLC system (X-LC Jasco Co., Tokyo, Japan) equipped with a DAD detector (MD-2015, Jasco Co., Tokyo, Japan) and autosampler (AS-2055, Jasco Co., Tokyo, Japan). Samples (20 μL injection volume) were separated on a Tracer Extrasil ODS-2 (250 × 45 mm, 5 μm, Teknokroma, Barcelona, Spain) HPLC column. The mobile phase consisted of two solvents: water (A) and methanol (B) (50:50, *v*/*v*). Isocratic elution for 10 min was used, followed by gradient elution 50% to 90% B for 10 min. The flow rate was 1 mL/min and column temperature was 25°C. Detection was set at 278 nm. To calculate the concentrations in the extract, a calibration curve was drawn for four solutions of known concentration in acetone.

Data analysis

The data represent the means of measurements from five plants per treatment, each representing one biological replicate. Analysis of variance (ANOVA) was performed using the SPSS for Windows software, version 18.0 (SPSS, Chicago, IL, USA) and was followed by pairwise *post hoc* analyses (Student-Newman-Keuls test) to determine which means differed significantly at $p < 0.05$.

Results

Characterization of biostimulants and hormones quantification

The chemical characteristics of RG and AH are reported in Table 1. The values of pH were acid in both cases: 2.9 for RG and 5.9 for AH. Total carbon (TC) was 1.23% for RG and 18.8 for AH. The total sugars content was 5,700 mg/L for RG and 4,642 mg/L for AH. The level of total phenols in RG and AH was appreciable: 970 and 2,576 mg/L, respectively. Hormone determination via ELISA assay evidenced an IAA concentration of 2.92 nmol mg $carbon^{-1}$ and an IPA concentration of 0.073 nmol mg $carbon^{-1}$ in RG; 18.46 nmol mg $carbon^{-1}$ for IAA and 0.055 nmol mg $carbon^{-1}$ for IPA in AH.

Effects of RG and AH on growth parameters

To establish the stimulatory effects of RG and AH on growth, the fresh weight of leaves and peppers was measured (Figures 1A,B). At the first sampling time, RG and AH increased the leaf biomass at both tested rates. More specifically, RG at the lower and AH at the higher rate enhanced leaf weight (+68% and +165%) in comparison to untreated plants. The weight of green peppers showed a similar trend as the leaves, but both RG and AH rates, reduced the biomass of red peppers.

At the second sampling time (Figure 1B), the opposite trend of leaf and pepper biomass production in relation to the application of biostimulants was observed: RG and AH addition weakly stimulated the leaf biomass, decreased green pepper weight, and significantly increased the red pepper weight.

Table 1 Chemical properties and hormone content of red grape (RG) and alfalfa hydrolyzate (AH) products

Property	Unit	RG	AH
pH		2.9	5.9
Total carbon (TC)	%	1.23	18.8
Total sugars	mg L^{-1}	5,700	4,642
Total phenols	mg L^{-1}	970	2,576
Indole-3-acetic acid (IAA)	nmol mg $carbon^{-1}$	2.92	18.46
Isopentenyladenosine (IPA)	nmol mg $carbon^{-1}$	0.073	0.055

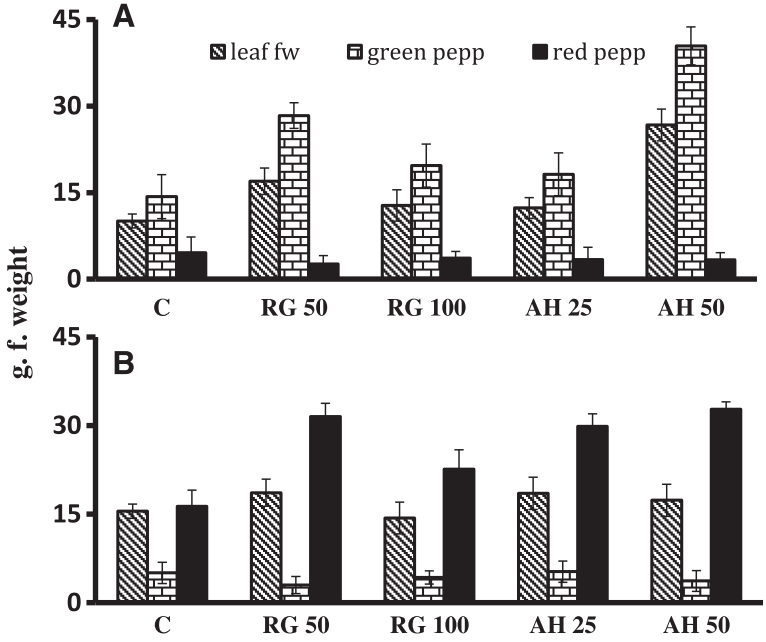

Figure 1 Effect of spray-solutions with red grape skin extract (RG) or alfalfa hydrolyzate (AH). On fresh weight of leaf and green and red fruits of hot pepper. **(A)** first harvest (flowering); **(B)** second harvest (maturity); error bars represent ± standard deviation (n = 5).

Table 2 reports the number of peppers at different maturity stages. Application of RG and AH at both tested doses increased the number of peppers in comparison to the untreated plants at flowering stage. Specifically, the amount of green peppers increased (+104% and +175%) when RG and AH were furnished at the lower and at the higher rate, respectively. The number of red peppers was slightly enhanced after biostimulants treatment. On the contrary, at maturity stage (Table 2), both RG dosages increased the number of green and red peppers with increments ranging from +19% to +78% in comparison to the control plants. The application of AH to plants induced the greatest effect on red peppers, regardless of the tested dose.

Table 2 Effect of RG and AH on green and red pepper number at flowering and maturity

Treatment	mL L^{-1}	Flowering	%	Maturity	%
Green pepper number					
Control	-	18.2 ± 1.2c	100	4.3 ± 0.3c	100
RG	50	37.1 ± 1.7b	204	5.1 ± 0.4b	119
RG	100	21.5 ± 1.1c	118	5.0 ± 0.1b	116
AH	25	20.2 ± 0.8c	111	6.0 ± 0.4a	140
AH	50	50.1 ± 6.1a	275	4.2 ± 0.6c	98
Red pepper number					
Control	-	4.3 ± 0.3a	100	18.3 ± 0.9c	100
RG	50	2.1 ± 0.1b	49	32.5 ± 1.6b	178
RG	100	4.3 ± 0.3a	100	30.1 ± 1.5b	164
AH	25	4.2 ± 0.5a	98	36.0 ± 1.8a	197
AH	50	4.1 ± 0.1a	95	37.0 ± 1.8a	202

Data represent the means of measurements using five plants per treatment. Different letters in the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

Effects of RG and AH on reducing sugars concentration

The effects of RG and AH on pepper plants were first investigated by determining the concentration of reducing sugars (Table 3). At the flowering phase, there was an increase in sugar concentration in leaves of pepper plants sprayed with RG, at both doses. More specifically, the increase was by about 111% at the lower and by about 75% at the higher RG dose. With respect to green peppers, the concentration of sugars was enhanced by the two treatments at the maturity stage. In particular, RG increased the sugars concentration by 71% and 354% at the lower and higher rates, respectively. At the same stage, the AH addition increased the reducing sugars with increments of 77% and +50% in red peppers of plants treated with the lower and higher rates, respectively.

Total phenols, FRAP, and ascorbic acid concentration

The samples were analyzed for total phenolic compounds, which are mainly responsible for the antioxidant activity of plant extracts. Different concentrations of total phenols were found in plants in response to RG and AH addition (Table 4). Their amount was significantly enhanced in leaves of plants treated with AH, at

Table 3 Effect of RG and AH on reducing sugar content in pepper plants at flowering

Treatment		Reducing sugars (glucose+fructose)			
		µg	(%)	µg	(%)
		Flowering		Maturity	
Leaf					
C	-	4.19 ± 1.12c	100	19.37 ± 1.05a	100
RG	50	8.83 ± 1.78b	211	16.61 ± 0.89b	86
RG	100	7.34 ± 0.54b	175	17.46 ± 1.13ab	90
AH	25	7.00 ± 1.82b	167	18.39 ± 1.23ab	95
AH	50	14.89 ± 2.45a	355	16.35 ± 0.97b	84
Green peppers					
C	-	17.56 ± 2.33b	100	2.64 ± 0.23c	100
RG	50	28.77 ± 2.59a	164	4.52 ± 0.36b	171
RG	100	14.28 ± 1.24b	81	11.99 ± 0.99a	454
AH	25	10.82 ± 0.78c	62	9.47 ± 0.85a	359
AH	50	32.89 ± 3.01c	187	5.66 ± 0.68b	214
Red peppers					
C	-	23.87 ± 3.48a	100	67.12 ± 4.66d	100
RG	50	13.84 ± 1.55c	58	123.80 ± 7.48a	184
RG	100	19.14 ± 2.95b	80	84.09 ± 5.51c	125
AH	25	17.68 ± 2.66b	74	118.86 ± 6.36a	177
AH	50	17.48 ± 2.59b	73	100.83 ± 5.88b	150

Data represent the means of measurements using five plants per treatment. Different letters in the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test. Data are expressed as µg reducing sugars per leaf or pepper dry weight.

both tested rates. The increments ranged from +5% in leaves of plants sprayed with RG at the lower rate to +58% in those supplied with AH at the higher rate, in comparison to the untreated plants. In both red and green peppers, the amount of total phenols increased after RG treatment at the higher dose and after application of AH at both tested doses. More specifically, the highest values were observed in red (+55%) and green peppers (+54%) of plants sprayed with RG at the higher dose.

An increase in FRAP was found in leaves of pepper plants sprayed with AH at both tested rates, with values ranging from +22% to +24% compared to the controls (Table 4). The stimulation of FRAP in green peppers by AH was much more pronounced (+61% at the lower and +33% at the higher rate). On the contrary, the effect of biostimulants on red peppers led to a weak increment of FRAP. The application of biostimulants caused a slight increase in the ascorbic acid concentration, with the highest values observed in leaves and green peppers of plants sprayed with AH at the lower dose (+18% and +20%, respectively).

Overall, the content of phenolics, ascorbic acid, and FRAP were more enhanced at the second sampling time corresponding to plant maturity (Table 5). The greatest increments of total phenols were observed in leaves (+45%) and green peppers (+140%) of plants treated with both AH doses. The FRAP in peppers was increased after AH treatment, with values ranging from +59% in red peppers at the higher rate to +27% in green peppers at the lower one. The ascorbic acid concentration was enhanced in leaves of plants supplied with RG at the lower (+17%) and higher (+24%) dose, as well as after treatment with the lower dose of AH (+37%).

With respect to red peppers, the greatest increase of ascorbic acid (+45%) was evident in plants supplied with RG at the higher dose, while in green pepper both AH and RG at all the tested doses increased ascorbic acid concentration. Specifically, the greater increments were observed in plants treated with RG at the higher rate (+106%) and with AH at the lower rate (+153%).

Capsaicin and dihydrocapsaicin determination

Capsaicin was not detected in leaves of pepper plants (Table 6). In red peppers, RG at the lower rate determined an enhancement of capsaicin content (+24%) in comparison to untreated plants, while AH increased the amount of this compound at both tested doses: +30% and +37% at the lower and higher rate, respectively. On the contrary, in green peppers, biostimulants did not stimulate the synthesis of capsaicin (Table 6). The dihydrocapsaicin concentration was enhanced in leaves of RG- and AH-treated plants, with increments ranging from +117% in plants supplied with RG at the lower rate to +98% in plants sprayed with AH at the same dose. With respect to the concentration of this metabolite in red and green peppers, there was only an increase of dihydrocapsaicin in the case of red peppers on plants with AH added at the higher dose (+46%). At the second sampling time, neither capsaicin nor dihydrocapsaicin were present in leaves, while their concentration was increased in red peppers by the two biostimulants at both rates, with increments ranging from +162% to +341%. RG and AH treatments increased these two metabolites to a lesser extent in green peppers.

Discussion

A number of researches have described the positive effects of biostimulants on plant growth and physiology. In particular, previous studies reported the improvement of pepper plant biomass and yield by either natural biostimulants [26] or humic substances from composted sludge [13]. Accordingly, RG and AH application to plants resulted in increased leaf biomass and weight of green peppers at the flowering stage, as well as in increased red pepper number and growth at maturity. The stimulation of plant growth by RG and AH was possibly due to their content in IAA and IPA, as both hormones

Table 4 Effect of biostimulants on total phenols, FRAP and ascorbic acid in pepper plants at flowering

Treat		Total phenols		FRAP		Ascorbic acid	
	mL L^{-1}	mg GAE kg^{-1} fw	(%)	mg $Fe^{2+}E$ kg^{-1} fw	(%)	mg kg^{-1} fw	(%)
Leaf							
C	-	1,102.1 ± 60.3c	100	3,042.5 ± 121.1b	100	1,857.1 ± 83.1ab	100
RG	50	1,161.4 ± 53.1c	105	2,907.3 ± 116.4b	96	1,709.2 ± 73.1b	92
RG	100	1,364.4 ± 65.3b	124	3,211.1 ± 130.1b	106	1,962.2 ± 38.4a	106
AH	25	1,686.1 ± 73.1a	153	3,697.1 ± 190.1a	122	2,190.4 ± 93.1a	118
AH	50	1,745.1 ± 80.2a	158	3,781.3 ± 173.3a	124	1,906.3 ± 87.3a	103
Red peppers							
C	-	1,081.1 ± 51.1c	100	3,090.1 ± 152.3b	100	1,740.2 ± 81.1a	100
RG	50	1,080.2 ± 38.1c	100	3,178.3 ± 120.3b	103	1,677.3 ± 63.2b	96
RG	100	1,678.1 ± 80.7a	155	4,510.5 ± 220.1a	146	1,754.3 ± 75.1ab	101
AH	25	1,317.3 ± 60.1b	122	3,135.2 ± 130.4b	101	1,905.0 ± 91.0a	110
AH	50	1,138.3 ± 60.7c	105	3,280.2 ± 133.0b	106	1,877.1 ± 83.1a	108
Green peppers							
C	-	3,621.4 ± 153.2b	100	1,078.0 ± 38.2c	100	1,502.6 ± 63.1b	100
RG	50	2,797.2 ± 110.2c	77	942.4 ± 40.2d	87	1,687.4 ± 80.2a	112
RG	100	5,590.2 ± 216.2a	154	1,584.1 ± 83.8a	147	1,639.4 ± 53.2a	109
AH	25	5,559.1 ± 230.8a	154	1,733.1 ± 73.1a	161	1,806.0 ± 63.2a	120
AH	50	5,439.4 ± 250.4a	150	1,432.3 ± 53.2b	133	1,697.0 ± 80.1a	112

Data represent the means of measurements using five plants per treatment. Different letters in the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

are known to play a pivotal role in plant development and initiation of fruit ripening. In support of this, previous work showed that physiological active concentrations of IAA contained in high molecular-weight humic substances [14] and in an alfalfa-based biostimulant EM [7] accounted for the enhancement of maize biomass production.

The more pronounced growth of pepper plants treated with RG and AH was consistent with the reduction in concentration of soluble sugars in leaves at maturity, which could be partially ascribed to the higher consumption of these compounds in the respiratory process. Indeed, biostimulants can induce the activity and gene expression of several enzymes involved in the tricarboxylic acid cycle (TCA), as previously observed in maize plants treated with an alfalfa based-hydrolizate [7]. Additionally, soluble sugars in leaves decreased because of their translocation into the fruits at plant maturity. Sugars provide energy for fruit development [27] and represent a major determinant of fruit quality. Therefore, changes in their composition and content lead to easily perceptible alterations in fruit flavor, which is an important, non-visual attribute for customers [28,29]. In this respect, the higher content of reducing sugars in peppers of plants treated with RG and AH compared to peppers of untreated plants indicated that both biostimulants can efficiently improve the fruit quality.

The increased accumulation of sugars in peppers appeared to be strongly associated to the level of ascorbic acid. Pepper is a good source of this antioxidant compound, which is notoriously involved in the photosynthetic process and in the synthesis of ethylene, gibberellins and anthocyanins, as well as in the control of cell growth and in the reduction of oxidative stress. Research performed on the ascorbate deficient mutant of *Arabidopsis thaliana* provided evidence that the ascorbic acid biosynthetic pathway starts from glucose, thus underlining a positive relationship between ascorbic acid and sugars concentrations in plants. In agreement with these findings, in the current investigation, the amount of ascorbic acid was enhanced in green peppers by RG and AH mainly at the maturity stage of plants, as in the case of soluble sugars. Increased levels of ascorbic acid were also observed in tomato plants treated with a seaweed-based biostimulant [30].

A recent study showed that agro-industrial residues and an alfalfa-based hydrolizate EM could function as biostimulants in agriculture as they increased maize plant yield and resistance to stress conditions through the enhancement of phenol production [15,16]. In particular, these biostimulants were able to induce the activity of the PAL enzyme, which is a notorious key regulator of phenol compound biosynthesis [16]. Possibly, RG and AH enhanced

Table 5 Effect of biostimulants on total phenols, FRAP and ascorbic acid in pepper plants at maturity

Treatment	mL L^{-1}	Total phenols		FRAP		Ascorbic acid	
		mg GAE kg^{-1} fw	(%)	mg $Fe^{2+}E$ kg^{-1} fw	(%)	mg kg^{-1} fw	(%)
Leaf							
C	-	1,318 ± 55.7c	100	3,620.4 ± 62.3c	100	528.3 ± 42.3c	100
RG	50	1,784 ± 80.3ab	135	3,740.2 ± 72.2b	103	618.4 ± 56.3d	117
RG	100	1,649 ± 60.2b	125	3,634.2 ± 48.4c	100	652.7 ± 25.2b	124
AH	25	1,906 ± 62.84a	145	3,890.0 ± 53.3a	107	723.3 ± 38.3a	137
AH	50	1,894 ± 82.2a	144	3,990.1 ± 25.8a	110	525.4 ± 52.2c	100
Red peppers							
C	-	1,196.4 ± 38.7c	100	4,218.1 ± 113.2c	100	900.3 ± 76.7c	100
RG	50	1,425.5 ± 82.5b	119	4,610.1 ± 216.1c	109	1,084.2 ± 112.1b	120
RG	100	1,684.4 ± 32.8a	141	4,748.3 ± 221.3c	112	1,307.5 ± 103.0a	145
AH	25	1,732.3 ± 83.1a	145	5,770.0 ± 286.3b	136	1,045.1 ± 128.0b	116
AH	50	1,667.1 ± 77.0a	139	6,721.0 ± 283.3a	159	1,185.1 ± 176.3ab	131
Green peppers							
C	-	2,364.4 ± 101.2d	100	12,010.7 ± 421.6c	100	498.5 ± 56.2d	100
RG	50	3,447.4 ± 128.3c	146	12,749.5 ± 526.3c	106	705.3 ± 123.0c	142
RG	100	4,320.5 ± 222.1b	183	13,645.8 ± 628.3bc	113	1,029.5 ± 101.81b	206
AH	25	5,670.0 ± 271.5a	240	15,260.1 ± 623.5a	127	1,263.2 ± 112.21a	253
AH	50	5,678.0 ± 215.4a	240	14,539.1 ± 618.6a	121	1,021.1 ± 116.07b	205

Data represent the means of measurements using five plants per treatment. Different letters in the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

the phenol content of peppers, especially at the maturity stage, by acting on PAL enzyme, too. This increase in phenolic compounds was likely responsible for the higher values of FRAP activity measured in peppers, as supported by a number of authors [31,32].

Other additional positive effects of AH and RG in pepper included the increased number of red fruits and stimulation of capsaicinoid production. In the first case, the higher number of fruits indicated an acceleration of the phenological development in plants supplied with the two biostimulants. This kind of 'priming' can be very important in countries with a temperate climate, where the cultivation period of peppers is relatively short and early cold temperatures in autumn can have a negative effect on flower and fruit development, as well as on fruit quality [33]. Priming may also result in economic and environmental benefits, since it can contribute to a reduction in the number of pesticide applications needed for the control of pepper pests and diseases [17].

The induction of capsaicinoid synthesis was particularly remarkable at the maturity stage of plants, probably because of the cumulative effects of the treatments. Capsaicin is one of the most important pepper metabolites as it is responsible for the fruit pungency. The increase of this compound in treated plants is of practical interest and has great relevance in medicine because capsaicin possesses analgesic and anti-inflammatory activities [34].

Although the RG and AH induced similar responses in pepper plants, the degree of each response could vary depending on the type of biostimulant. Differences in effectiveness between RG and AH could be related to the characteristics of the starting matrixes and/or to the industrial processes employed for their production. The cool-extraction method used to produce RG was had the advantage of preserving the organoleptic properties of the raw materials [21], while the hydrolytic process used for AH production could cause chemical changes in thermolabile bioactive compounds [35]. Moreover, the effectiveness of RG and AH could be in part due to their content in IAA and IPA and in part to the presence of compounds with known biological activity, such as phenols.

Conclusions

Several studies investigate the effects of biostimulants originated from different sources on the morphological and physiological parameters of plants. The novelty of this research is the attention paid to the effects of RG and AH on the nutritional features of fruits in consideration of the increased demand for functional foods by consumers. The obtained results demonstrated the effectiveness of RG and

Table 6 Effect of biostimulants on capsaicin and dihydrocapsaicin concentration in pepper plants at flowering and maturity

Treatment	mL L^{-1}	Capsaicin		Dihydrocapsaicin		Capsaicin		Dihydrocapsaicin	
		(μg g^{-1} dw)	(%)	(μg g^{-1} dw)	(%)	(μg g^{-1} dw)	(%)	(μg g^{-1} dw)	(%)
		Flowering				Maturity			
Leaf									
C	-	-	-	0.91 ± 0.02b	100	-	-	-	-
RG	50	-	-	1.98 ± 0.10a	217	-	-	-	-
RG	100	-	-	1.80 ± 0.08a	198	-	-	-	-
AH	25	-	-	1.80 ± 0.06a	198	-	-	-	-
AH	50	-	-	0.76 ± 0.021c	84	-	-	-	-
Red peppers									
C	-	155.4 ± 7.2c	100	42.8 ± 1.18b	100	48.3 ± 3.8d	100	17.2 ± 0.6c	100
RG	50	192.0 ± 8.3b	124	34.7 ± 1.53c	80	321.4 ± 12.1a	668	70.2 ± 12.5a	394
RG	100	124.6 ± 5.8d	81	26.3 ± 1.12d	61	261.0 ± 61.6b	544	50.3 ± 11.4b	281
AH	25	201.1 ± 6.2a	130	38.1 ± 1.76b	91	220.1 ± 52.5c	459	46.3 ± 12.0b	262
AH	50	211.2 ± 7.8a	137	62.7 ± 3.83a	146	335.0 ± 72.1a	696	78.2 ± 14.1a	441
Green peppers									
C	-	179.3 ± 18.3a	100	46.35 ± 11.2a	100	89.0 ± 13.2c	100	27.2 ± 1.9c	100
RG	50	183.1 ± 17.5a	102	40.14 ± 11.7b	87	162.1 ± 25.7a	182	28.3 ± 1.2c	106
RG	100	152.2 ± 15.4b	85	31.53 ± 11.8c	68	157.2 ± 25.3a	176	54.5 ± 12.8a	199
AH	25	153.3 ± 15.8b	86	38.74 ± 11.5b	84	98.2 ± 12.8c	111	25.4 ± 13.1c	93
AH	50	160.2 ± 13.2b	89	47.27 ± 12.16a	102	117.1 ± 16.2b	132	43.6 ± 15.5b	161

Data represent the means of measurements using five plants per treatment. Different letters in the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

AH in improving the nutritional value of peppers by increasing their content in phytochemicals with nutritional value.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AE carried out the laboratory studies, drafted the manuscript, and made the corrections. CN and SS carried out the laboratory studies. PS participated in the drafted the manuscript. MS helped to draft the manuscript. SN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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