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Assessing the Biostimulant Effects of a Novel Plant-Based Formulation on Tomato Crop

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Received: 18 September 2020; Accepted: 7 October 2020; Published: 13 October 2020

Abstract: The aim of this research was to evaluate the biostimulant effects of an eco-product (EP) containing essential oils of rosemary and eucalyptus on tomato crop. Experiments were conducted to evaluate EP effects on plant growth and physiological parameters (e.g., chlorophyll content), total phenols and antioxidant activity, enzyme activities (e.g., catalase), plant macronutrient content and fresh tomato fruit quality. The application of an EP once (EP-1x) increased tomato plant height by 19%, stomatal conductance, and chlorophyll content compared to the control (sprayed with water). EP triplicate (EP-3x) application significantly increased yield (0.79 kg plant^{-1}) compared to the control (0.58 kg plant^{-1}). However, application of EP-3x was associated with a higher percentage of fruit cracking in comparison to the control. Total phenols and antioxidant activity were affected from both the use of EP and application frequency. EP application decreased by 27.3% the leaf damage index in comparison to the control. Nutrient content in leaves (N, Mg) was significantly decreased in the case of EP-3x treatment. Fruit firmness was decreased by 19.9% in the case of EP-1x application. Fruit marketability and tomato-like aroma, fresh weight and soluble solids did not differ among the treatments. Further research is required to examine the potential use of essential oils as biostimulants.

Keywords: essential oils; rosemary; eucalyptus; biostimulants; vegetable production; fruit quality

1. Introduction

A main issue for humanity is global food security, as demographic projections place the human population size at 9.5 billion by 2050. Securing and maximizing crop production under the threat of climatic change is a major challenge for the agricultural sector [1]. Research towards securing food production is focusing, in part, on the use of biostimulants to support agricultural production [2–4]. The term biostimulant refers to a broad range of substances and/or microorganisms used in agriculture to enhance soil fertility, plant tolerance to stress, plant growth, productivity and produce quality crops [2,5–7].

Biostimulants based on amino acids and marine algae have been applied in horticulture for many years [8]. Such materials are typically a rich source of phytohormones (e.g., auxins, cytokinins, gibberelins, abscisic acid) and polyphenol compounds [7,9]. More recently, substances such as humic and fulvic acids, protein hydrolysate and silicon have been tested with promising results to increase yield under normal conditions or maintain it under unfavorable conditions [1,10]. Moreover, the use of arbuscular mycorrhizal fungi (AMF), *Trichoderma* spp. and plant growth promoting rhizobacteria could support food production, especially in low input agriculture (e.g., reduce N and P fertilizers application) [5,11]. Low input agriculture supports climate change mitigation [12] and biostimulants application could support this goal [9,13].

Several research papers have demonstrated the positive effects of biostimulants use in vegetables and fruits. For instance, marine algae (*Ascophyllum nodosum*)-based products have been tested in lettuce and this resulted in increased yield [14] and alleviated the detrimental effects of potassium deficiency during plant growth and storage of processed products [15]. Biostimulants were also studied for their effects on water-stressed plants [9,16]. The results of Pereira et al. [16] highlighted the importance of using biostimulants in arid environments to increase plant yield and nutrients content. Under soil salinity conditions, root and leaf-root application of plant-derived protein hydrolysates increased fresh yield, dry biomass and root dry weight of lettuce [1]. Biostimulant application increased the phenolic antioxidants in broccoli heads and spinach [8,17]. In contrast, four biostimulant products (Megafol, Aminovert, Veramin, Twin Antistress) did not demonstrate any promising results concerning their ability to increase the content of phenolic antioxidants in two spinach varieties under water stress [16]. Soaking of bean seeds or foliar sprays with salicylic acid (SA) or *Moringa oleifera* leaf extract increased the nutrient content (N, P, K, Ca) of bean leaves [18]. In addition, concentrations of total chlorophylls, total carotenoids, total soluble sugars, free proline and ascorbic acid, were higher for treated bean plants compared to the tap water control. Biostimulants have been tested to evaluate their impact in enhancing tolerance to drought [19], enhancing yield and nutritional quality [20,21], preventing yield loss and oxidative stress [22]. Arbuscular mycorrhizae fungi and seaweed extract have been mainly tested in such experiments with tomato [23].

Even if there is currently a plethora of published papers on the use and effects of biostimulants, mainly on vegetables and fruits, the potential use of essential oils as biostimulants has received less attention. Rosemary (*Rosmarinus officinalis* L.) essential oil has been applied on tomato seedlings with beneficial effects on nutrient uptake and better plant growth [24]. Essential oils contain many organic substances that could possibly act as biostimulants, similar to what has already been observed for organic matter-related substances [25]. In addition, in contrast to the majority of papers related to biostimulant effects, which focus on the effects of biostimulants on plant growth and product yield/quality, less is known about their impact on enzymatic activities.

Essential oils from rosemary (*R. officinalis* L.) and eucalyptus (*Eucalyptus globulus* L.) are rich in 1,8-cineole (commonly known as eucalyptol) with diverse biocidal activities, such as insecticidal [26], antibacterial [27,28], herbicidal [29], antifungal [30], antiallergic, antitumoral and gastroprotective action as reviewed by Caldas et al. [31]. The aim of this research was to evaluate the biostimulant effects of an eco-product (containing essential oils of rosemary and eucalyptus) on tomato crop. More specifically its effects were evaluated on: (1) plant growth and physiological parameters (e.g., chlorophyll content), (2) total phenols and antioxidant activity, (3) enzyme activities (e.g., catalase), (4) plant nutrient content (macronutrients) and (5) fresh produce quantity and quality.

2. Materials and Methods

2.1. Plant Material and Experimental Set Up

The study took place at the experimental greenhouse of the Cyprus University of Technology, Limassol, Cyprus, during spring–summer of 2019. Tomato seedlings (*Solanum lycopersicum* cv. Brillande Grade A) were purchased from a commercial nursery. Young seedlings were transplanted in pots filled with soil (clay-loam texture, 1.41% organic matter; 24.28% total $CaCO_3$; pH 7.71; electrical conductivity (EC) 0.68 mS cm^{-1}; total nitrogen (N) 0.40 g kg^{-1}).

An eco-product (EP; named as ''Agriculture Green-tech E", Meydan Solution Ltd, Larnaca, Cyprus) based on rosemary (*R. officinalis* L. synonym of *Salvia rosmarinus* L.) and eucalyptus (*E. globulus* L.) essential oils was used. The main constituents of rosemary oil were 1,8-cineole (32.94%), α-pinene (12.01%), and camphor (20.86%) [32] and eucalyptus oil were 1,8-cineole (74.3%) and α-terpineol (10.3%) [33]. This product was a mixture of these two essential oils (eucalyptus: rosemary in approximately 2:1 *v/v* ratio) and it also contained vinegar <5% *w/w* as well as emulsifier-treated water (<80%). A commercial product (Razormin Atlantica agricola SA; Alicante, Spain) based on amino acids

and biostimulants was used at 0.25% *v*/*v* as a positive control. Razormin contains free amino acids 7% *w*/*w*, iron (Fe) 0.4% *w*/*w*, manganese (Mn) 0.1% *w*/*w*, zinc (Zn) 0.085% *w*/*w*, copper (Cu) 0.02% *w*/*w*, boron (B) 0.1% *w*/*w* and molybdenum (Mo) 0.01% *w*/*w*, all water soluble.

2.2. Preliminary Test

A preliminary experimental set up was conducted to determine the possible phytotoxicity effects of the EP on tomato plants after application via spraying. Tomato seedlings were transplanted in soil in 5L pots. Seven concentrations of the EP were evaluated, from 0 to 3% (*v*/*v*) at 0.5% increments. Plants at a stage of 2–3 true leaves were transplanted and grown for 25 days, at the same environmental conditions as the main experiment (see Section 2.3 and Figure S1). Three replications were used for each concentration, resulting in a total of 21 tomato plants. Plants were monitored for phytotoxicity (marked spots) every second day for a period of 10 days. Based on the results of the preliminary experiment, the concertation of 2.0% (*v*/*v*) was selected for further evaluation, as this was the highest concentration at which no phytotoxicity effects were observed in tomato.

2.3. Main Experiment

A total of eighty-four young tomato seedlings were transplanted individually in 9-L pots filled with soil. The pots were placed in twin rows, with plants spaced 0.45 m from each other within the row, at 0.8 m within twin rows, and 1.2 m between rows. Tomato plants were trained on a string according to the single pruning scheme (the main stem grew vertically and all lateral shoots were removed) and were grown for 13 weeks.

Drip irrigation emitters (1 emitter/pot) were installed and irrigation occurred daily for 5 min at the early stage of plant development; after the 4th week, plants were watered every second day for 10 min, or according to crop needs, using a timer. Fertigation (EC: 2.5 mS cm^{-1}; 200 mL $plant^{-1}$) with commercial (i.e., 20-20-20) fertilizers was applied once, 10 days after transplanting (DAT) of seedlings in pots. Yellow sticky traps were placed to monitor insects, and pesticides were applied following common cultivation practices. In brief, 15 DAT foliar spraying for two-spotted spider mite (*Tetranychus urticae*) and whitefly (*Bemisia tabaci*) with Oberon® SC 240 (Bayer Hellas ABEE, Athens, Greece) at 0.06% *v*/*v* as well as 23 DAT foliar spraying for vegetable leaf miner (*Liriomyza* spp). with Tracer 48SC (Spinosad, Dow Agrosciences Export SAS, Montigny-le-Bretonneux, France) at 0.05% *v*/*v*. Average day and night temperature and relative humidity during the growing period were recorded with a meteorological station and are shown in Figure S1.

Plants were assigned into one of four treatments (Supplementary material Figure S2): (i) plants sprayed with water as a control (ii) foliar spray with the Razormin commercial product (CP) at 2.5 mL L^{-1} every 20 days beginning on day 31 after transplanting, for a total of three applications (iii) foliar spray with the EP at 2% once (EP-1x) and (iv) foliar spray with the EP at 2% every 20 days for a total of three applications (EP-3x). Multiple foliar sprays took place every 20 days. The first spray was at the 1st fruit setting. Each treatment was replicated in three plots and each plot had seven plants in a complete randomized design (see Figure S2).

2.4. Plant Growth and Physiological Parameters

Plant growth-related parameters were evaluated on site. Tomato plant height, leaf number, plant fresh and dry biomass were measured on six replicates per treatment. Plant yield as the total harvested fruit for each plant (21 plant/treatment) was measured through the whole harvesting period, and yield was expressed as kg of fruits per plant.

Leaf tissue (four replications per treatment; each replicate consisted of a pool of 2–3 different plant tissue samples; 0.1 g) was incubated in a heat bath at 65 °C for 30 min, in the dark, with 10 mL dimethyl sulfoxide (DMSO) for chlorophyll extraction. Photosynthetic pigments, i.e., chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (total Chl) contents were calculated as described by Chrysargyris et al. [34]. Maximum F_v/F_m photochemical quantum yields of photosystem II (PSII) were

measured with an OptiSci OS-30p Chlorophyll Fluorometer (Opti-Sciences). Leaves were incubated in the dark for 20 min prior to F_v/F_m measurements. Leaf stomatal conductance was measured on the 4th–5th leaf from the top of the plant (2 measurements per leaf in each plant), by using a ΔT-Porometer AP4 (Delta-T Devices-Cambridge, UK) according to the manufacturer's instructions [32]. All leaves were fully mature and sun-exposed in different individual plants (4 plants/treatment).

2.5. Total Phenols Content and Antioxidant Activity

Total phenols content in leaves (four replications per treatment; each replicate consisted of a pool of 2-3 different plant leaf tissue samples) was determined with the Folin–Ciocalteu method at 755 nm according to Chondraki et al. [35], and results were expressed as equivalents of gallic acid per gram of fresh weight (μmol of GAE g^{-1} fresh weight). The antioxidant capacity was measured using the ferric reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods as previously described [36]. The results for antioxidant activities were expressed as trolox equivalents (mg of trolox g^{-1} fresh weight).

2.6. Hydrogen Peroxide, Lipid Peroxidation and Antioxidative Enzyme Activities

Hydrogen peroxide (H_2O_2) content in leaves was determined as described previously [36]. Leaf tissue (four replicates/treatment; 0.2 g) was homogenized in ice-cold 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000× *g* for 15 min, and an aliquot of the supernatant was used for the reaction mixture. The H_2O_2 concentration was evaluated using standards prepared from dilutions of H_2O_2. The absorbance was measured at 390 nm, and results were expressed as μmol H_2O_2 g^{-1} fresh weight. Lipid peroxidation was assessed and measured in terms of the malondialdehyde content (MDA) [36]. Absorbance of the reaction mixture was measured at 532 nm and corrected at 600 nm. The amount of MDA was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}. Results were expressed as nmol of MDA g^{-1} fresh weight.

For antioxidative enzyme activities, fresh leaf tissue (four replicates/treatment) was homogenized using an ice-cold extraction buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (*w/v*) polyvinylpyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% Triton X-100 in 50 mM potassium-phosphate buffer (pH 7.0). Protein content was determined using bovine serum albumin (BSA) as a standard [36]. Catalase activity (CAT) (EC 1.11.1.6), superoxide dismutase activity (SOD) (EC 1.15.1.1) and peroxidase activity (POD) (EC 1.11.1.6) were assayed following the methods described previously [36]. In brief, catalase activity was assayed in a reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2 and plant extract. The decomposition of H_2O_2 was measured at 240 nm. The results were expressed as CAT units mg^{-1} of protein (1 unit = 1 mM of H_2O_2 reduction min^{-1}). SOD was assayed using a photochemical method; a reaction mixture (1.5 mL) containing 50 mM K-phosphate buffer (pH 7.5), 13 mM methionine, 75 μM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 2 μM riboflavin and plant extract was used. The reaction began by exposing the mixture to a light source of two 15-W fluorescent lamps for 15 min, and terminated by placing the tubes in the dark. Absorbance was determined at 560 nm, and activity was expressed as units mg^{-1} of protein. Peroxidase activity (POD) (EC 1.11.1.6) was determined following the increase in absorbance at 430 nm. Calculations were performed using the coefficient of extinction of 2.47 mM cm^{-1}. One POD unit was defined as the amount of enzyme to decompose 1 μmol of H_2O_2 per minute. Results were expressed as units of peroxidase per milligram of protein.

2.7. Plant Nutrient Content

The nutrient content in leaves (four replications per treatment; each replicate consisted of a pool of 2-3 different plant tissue samples) was determined. Leaves were collected, dried at 65 °C for 4 d, weighed, and ground in a Wiley mill to pass through 40 mesh screens as described in Chrysargyris et al. [32]. Nitrogen (N) content was determined by the Kjeldahl method (BUCHI, Digest automat K-439 and Distillation Kjelflex K-360, Flawil, Switzerland) [37]. Potassium (K) and sodium (Na)

were determined photometrically (Flame photometer, Lasany Model 1832, Lasany International, Panchkula, India), phosphorus (P) was determined spectrophotometrically (Multiskan GO, Thermo Fischer Scientific, Waltham, MA, USA), and magnesium (Mg) and calcium (Ca) by an atomic absorption spectrophotometer (PG Instruments AA500FG, Leicestershire, UK) following Chrysargyris et al. [37]. Data were expressed in g kg^{-1} of dry weight.

2.8. Fresh Produce Quality

Tomatoes freshly harvested from the 2nd truss were evaluated for their quality attributes. At least six biological replicates (pool of 2–3 fruit subsamples or sub-measurements) were used in each treatment. Number of fruits, mean fresh weight and yield were recorded.

Fruit firmness was assessed at two points on each tomato's shoulder using a texture-meter FT 011 (TR Scientific Instruments, Forli, Italy) with an 8-mm plunger. The amount of force (in Newtons; N) needed to break through a tomato's radial pericarp (i.e., surface) in eight replicates was measured at room temperature [38].

Color was measured using the Hunter Lab System and a Minolta colorimeter model CR400 (Konica Minolta, Osaka, Japan). Following the recording at 2 points of each tomato for the individual L^*, a^*, and b^* parameters, chroma value (C) was calculated by the following equations $C = (a^{*2} + b^{*2})^{1/2}$ as described previously [38].

Tomato juice was obtained from 2-3 pooled fruit for each replication (with six replicates per treatment), and total soluble solids (TSS, expressed in percentage) measured with a temperature-compensated digital refractometer (model Atago PR-101, Atago Co. Ltd., Tokyo, Japan) at 20 °C. Titratable acidity (TA) was measured via potentiometric titration (Mettler Toledo DL22, Columbus, OH, USA) of 5 mL supernatant diluted to 50 mL with distilled water using 0.1 N NaOH up to pH 8.1. Results were expressed in citric acid percentage [39]. The fruit sweetness/ripening index was calculated using TSS/TA ratio. Fruit juice pH and EC were also measured.

Ascorbic acid (AA) was determined by the 2,6-Dichloroindophenol titrimetric method as described previously [38]. An aliquot of 5 mL of pooled tomato juice was diluted with 5 mL of water and was titrated by the dye solution until the color changed. Data were expressed as mg of AA per gram of fresh weight.

Total phenolic content was measured on blended fruit tissue (5 g) extracts following repeated (4-fold) addition of 2.5 mL of 50% (*v*/*v*) methanol as reported previously [35]. Results were expressed as gallic acid equivalents (GAE) per gram of fresh weight.

Fresh produce marketability, aroma and appearance were recorded by using a 1-10 scale (1: not marketable quality (i.e., malformation, wounds, infection); 3: low marketable with malformation; 5: marketable with few defects i.e., small size, decolorization (medium quality); 8: marketable (good quality); 10: marketable with no defects (extra quality)) and results were expressed in percentage [38].

Additionally, symptoms of blossom end rot (BER), cracking, insect attack and russeting were recorded and results expressed in percentages.

2.9. Statistical Methods

Statistical analysis was performed using IBM SPSS version 22 comparing data means (± SE) with one way-ANOVA, and Duncan's multiple range tests were calculated at $p < 0.05$. Measurements were performed in four-to-six biological replications/treatment (each replication consisted of a pool of two to three individual measurements/sample).

3. Results

Foliar application with CP or EP affected plant growth-related attributes (Table 1). The application of an eco-product once (EP-1x) increased (by 19%) plant height compared to the control. Tomato yield was increased with the foliar applications but differed significantly only in the case of EP-3x application compared to the control treatment. The highest plant dry weight was found in the CP application

and significantly differed from the control and EP-3x treatment. Plant biomass, leaf and fruit number did not differ among the different foliar applications. Blossom-end-rot (BER) symptoms were absent in EP-1x-treated plants while BER levels were at 4.13%, 6.59% and 4.20% for the control, CP and EP-3x, respectively. Application of EP-3x was associated with a higher percentage of fruit cracking in comparison to control and CP applications. Symptoms of insect injury (average 1.22%) and russeting (average 1.29%) were similar to all treatments (data not presented).

Table 1. Yield and plant growth characteristics of tomato plants in relation to biostimulants foliar application.

Plant Growth	Control	CP	EP-1x	EP-3x
Height (m)	1.47 ± 0.03b	1.59 ± 0.05ab	1.75 ± 0.05a	1.63 ± 0.09ab
Leaf number	28.33 ± 0.42a	30.00 ± 1.29a	32.17 ± 1.25a	29.00 ± 1.65a
Plant biomass (g)	433.90 ± 30.39a	510.75 ± 41.27a	524.48 ± 70.38a	421.33 ± 42.99a
Plant dry weight (g)	83.88 ± 3.98b	107.38 ± 6.41a	93.43 ± 8.15ab	82.93 ± 5.86b
Yield (kg plant^{-1})	0.58 ± 0.06b	0.74 ± 0.05ab	0.71 ± 0.06ab	0.79 ± 0.06a
Fruit number	9.58 ± 0.89a	9.46 ± 0.63a	9.10 ± 0.79a	10.00 ± 0.41a
Blossom end rot (BER) (%)	4.13 ± 2.80a	6.59 ± 2.16a	0.00 ± 0.00b	4.20 ± 2.03a
Fruit cracking (%)	8.23 ± 2.06b	7.97 ± 2.68b	12.77 ± 3.05ab	18.43 ± 2.688a

Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT (p = 0.05).

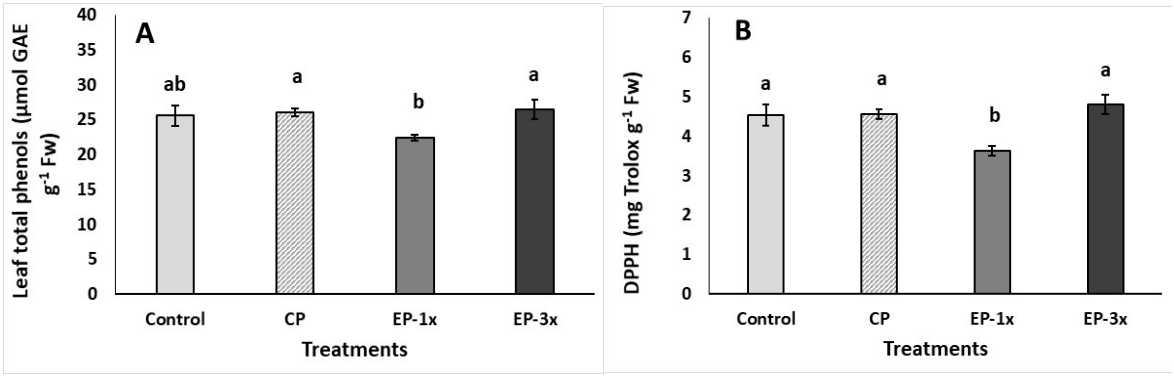
Plants subjected to EP-1x showed higher stomatal conductance compared with the plants sprayed with water (control) (Table 2). Interestingly, EP-1x-treated plants had increased content of chlorophylls (chlorophyll a, chlorophyll b and total chlorophylls) compared to control, CP or EP-3x treatments. However, leaf chlorophyll fluorescence was similar in all treatments and averaged at 0.825 of the Fv/Fm value.

Table 2. Leaf stomatal conductance, chlorophyll fluorescence and content of chlorophyll a, chlorophyll b and total chlorophylls of tomato plants in relation to biostimulants foliar application.

Plant Physiology	Control	CP	EP-1x	EP-3x
Stomatal conductance (s cm^{-1})	1.18 ± 0.16b	1.56 ± 0.13ab	1.81 ± 0.21a	1.59 ± 0.21ab
Chlorophyll fluorescence (Fv/Fm)	0.82 ± 0.01a	0.83 ± 0.00a	0.82 ± 0.01a	0.83 ± 0.00a
Chlorophyll a (mg g^{-1} Fw)	0.31 ± 0.07b	0.29 ± 0.03b	0.65 ± 0.06a	0.25 ± 0.03b
Chlorophyll b (mg g^{-1} Fw)	0.08 ± 0.02b	0.07 ± 0.01b	0.17 ± 0.02a	0.06 ± 0.01b
Total Chlorophylls (mg g^{-1} Fw)	0.39 ± 0.09b	0.37 ± 0.03b	0.82 ± 0.08a	0.31 ± 0.04b

Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT (p = 0.05).

Total phenols content in tomato leaves decreased with the EP-1x application compared with the EP-3x and CP, but remained at similar levels to the control treatment (Figure 1A). Antioxidant activity, as assayed by DPPH, decreased with the EP-1x application compared to control and/or CP or EP-3x applications (Figure 1B). No differences were found in FRAP antioxidant activity among treatments (Figure 1C).



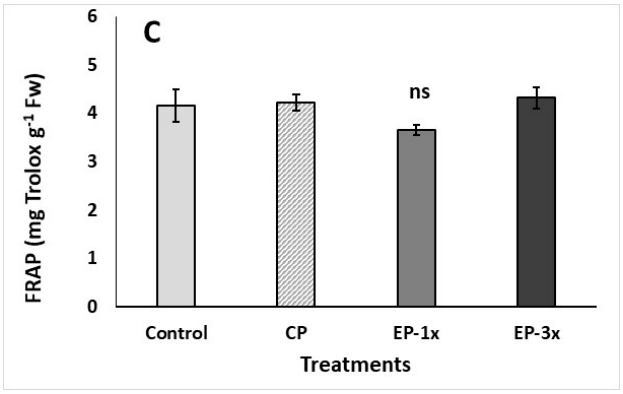
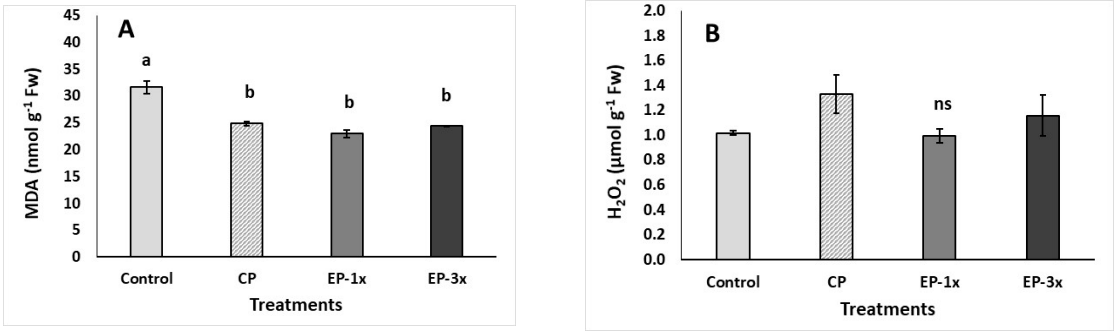


Figure 1. Total phenols content and antioxidant activity of tomato plants in relation to biostimulants foliar application (Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications). (**A**) Total Phenols (**B**) DPPH and (**C**) FRAP. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT ($p = 0.05$); ns indicates non-significant.

Foliar application with CP or EP decreased (up to 27.3%) leaf damage index indicated by the decreased lipid peroxidation (MDA) compared with the control (Figure 2A). Hydrogen peroxide production and CAT activity were similar among the treatments (Figure 2B,D). SOD activity increased with the application of EP-3x compared to the control, CP and EP-1x applications (Figure 2C). POD activity decreased with the CP and EP-1x application compared to control and EP-3x treatment (Figure 2E).



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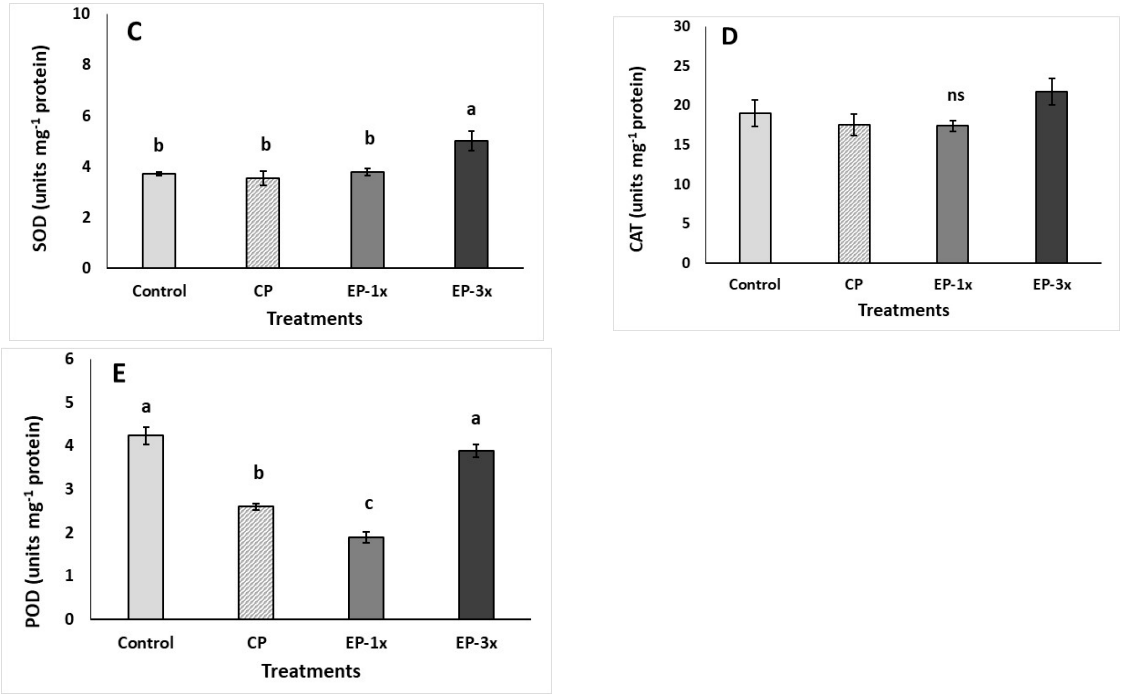

Figure 2. Damage index and antioxidant enzymes activities in tomato plants in relation to biostimulants foliar application (Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications). (**A**) lipid peroxidation (malondialdehyde)- MDA, (**B**) hydrogen peroxide—H_2O_2, (**C**) superoxide dismutase—SOD, (**D**) catalase—CAT, and (**E**) peroxidase—POD. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT ($p = 0.05$); ns indicates non-significant.

Foliar applications affected the tomato leaf nutrient content as presented in Figure 3. The application of EP-3x decreased N content in leaves compared to the control (Figure 3A). CP application decreased but EP-3x increased K content in leaves compared to the control treatment (Figure 3B). Phosphorus content was decreased with the application of CP and EP-1x but was increased with the EP-3x compared to the control (Figure 3C). Application of CP or EP increased Na but decreased Mg content in leaves (Figure 3D,F). Calcium content was decreased in CP and EP-1x treatment compared to the control treatment (Figure 3E).

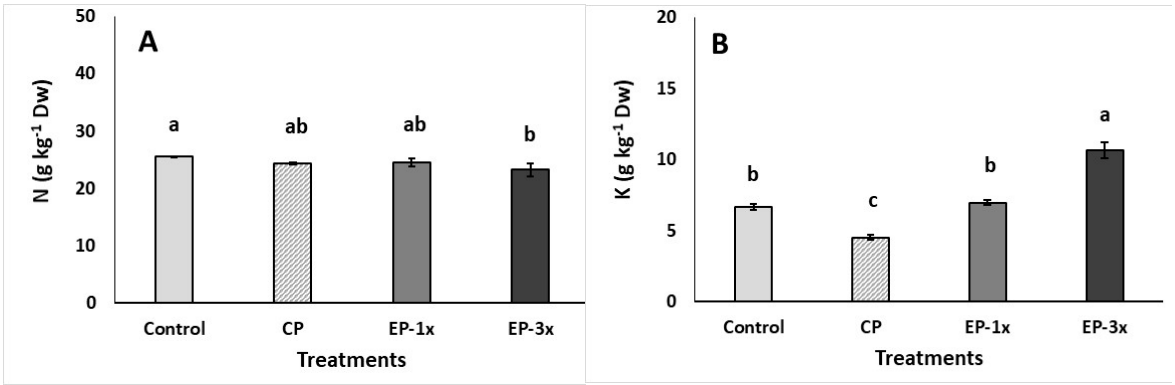

Figure 3. *Cont.*

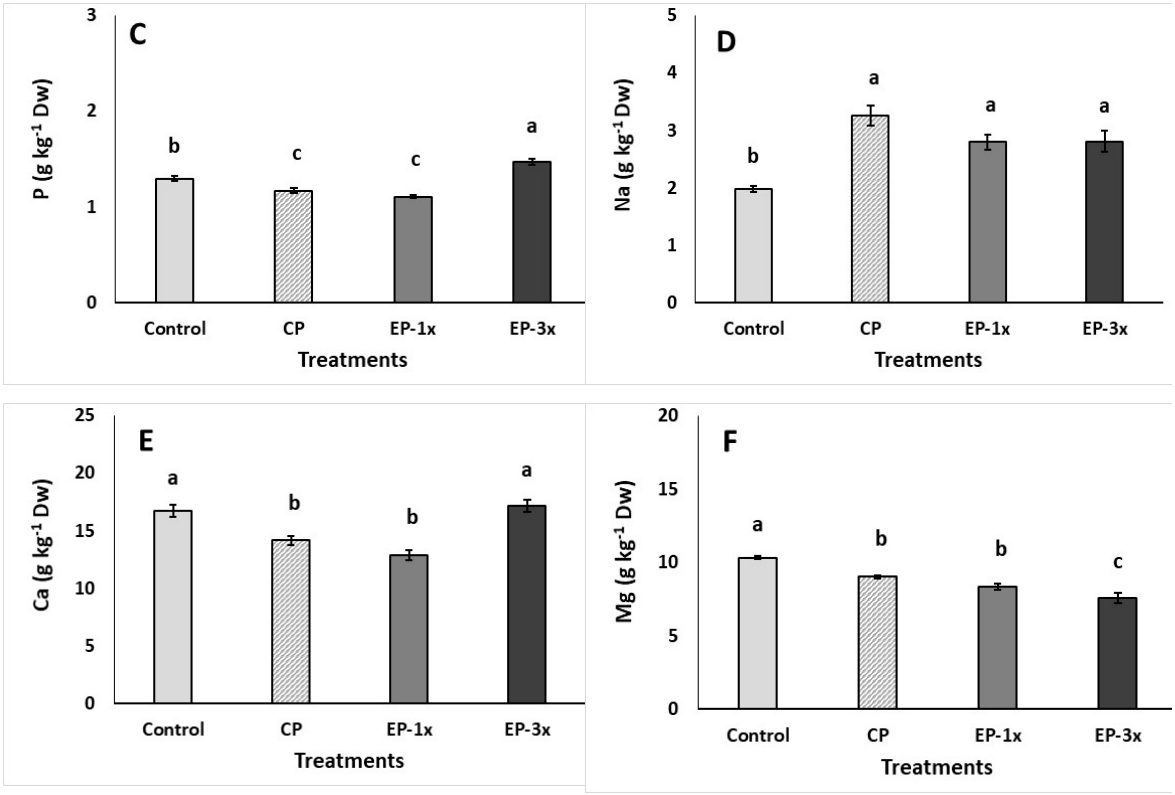

Figure 3. Nutrient content of tomato leaves in relation to biostimulants foliar application (Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications). (**A**) nitrogen-N, (**B**) potassium-K, (**C**) phosphorus-P, (**D**) sodium-Na, (**E**) calcium-Ca, and (**F**) magnesium-Mg. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT (p = 0.05).

Fruit quality-related attributes by the plants subjected to different foliar applications were presented in Table 3. Fruit firmness decreased by 19.5% and 19.9% in CP and EP-1x treated plants, respectively, compared to the plants sprayed with water (control). Total phenolics and titratable acidity decreased with the application of CP or EP compared to the control. Foliar applications of CP and EP affected fruit sweetness as indicated by ripening index of TSS/TA ratio, and revealed the highest values with the EP-1x treatment. The higher ascorbic acid content was observed in tomatoes harvested by plants subjected to the EP-1x treatment, as AA was almost doubled in EP-1x compared to control. Fruit marketability and tomato-like aroma did not differ among the treatments; however, tomato appearance had the highest score in EP-1x compared to control and CP applications. Fruit fresh weight (averaged in 88.01 g) and total soluble solids (averaged in 5.14 °Brix) were similar among the treatments.

Table 3. Fruit quality related attributes harvested from tomato plants in relation to biostimulants foliar application.

Quality Attributes	Control	CP	EP-1x	EP-3x
Fruit fresh weight-Fw (g)	80.93 ± 3.4a	94.4 ± 5.36a	95.22 ± 6.65a	81.49 ± 4.69a
Firmness (Newton)	12.75 ± 0.97a	10.27 ± 0.32b	10.21 ± 0.54b	10.74 ± 0.75ab
Total soluble solids-TSS (°Brix)	5.30 ± 0.26a	5.10 ± 0.18a	5.08 ± 0.14a	5.08 ± 0.22a
Titratable acidity-TA (citric acid g L^{-1})	5.26 ± 0.24a	4.14 ± 0.11b	3.58 ± 0.12c	3.95 ± 0.16bc
TSS/TA	1.01 ± 0.04c	1.23 ± 0.02b	1.42 ± 0.03a	1.28 ± 0.03b
Ascorbic acid-AA (mg 100 g^{-1} Fw)	73.80 ± 3.71b	79.87 ± 8.11b	147.02 ± 4.66a	85.21 ± 3.15b
Phenols (μmol GAE g^{-1} Fw)	3.10 ± 0.20a	2.59 ± 0.20b	2.51 ± 0.04b	2.51 ± 0.11b
Marketability (1–10)	7.83 ± 0.49a	8.58 ± 0.40a	8.67 ± 0.17a	7.75 ± 0.31a
Aroma (1–10)	5.50 ± 0.53a	7.00 ± 0.41a	6.75 ± 0.28a	6.33 ± 0.69a
Appearance (1–10)	7.25 ± 0.54bc	8.25 ± 0.40ab	8.50 ± 0.26a	7.00 ± 0.26c

Control: foliar application of water; CP: commercial product; EP: eco-product; 1x: indicates application once; 3x: indicates three applications. Means ± SE in the same row followed by different letters are significantly different according to Duncan's MRT ($p = 0.05$).

4. Discussion

The application of biostimulants affects plant metabolism and quality of end-products [40]. Przygocka-Cyna and Grzebisz [41] associated the use of biostimulants with an improvement in plant nutrient uptake and therefore with better nutritional value of the end products. In the present study, plant growth was affected by the application of the EP as well as the CP. The EP increased the height and the yield of tomato plants. However, in previous studies, high rosemary essential oil application on tomato seedlings decreased plant height and that could be related to stress signaling molecules (i.e., ethylene) [24]. The applied EP, based on an essential oils mixture, seems to have affected plant growth characteristics by acting as a biostimulant, similar to what has been shown for other organic compounds such as humic acids, amino acids, salicylic acid and vitamins [42–44]. Similar observations to our results were found after the use of products based on the marine alga *Ascophyllum nodosum* and foliar fertilizers with many micronutrients (Megagreen) which enhanced plant growth and production in lettuce [14]. In another study, under salinity conditions, the use of plant-derived protein hydrolysates increased fresh yield, dry biomass and root dry weight of lettuce [1]. For tomato, biostimulant treatments (tropical plant extract (PE); legume-derived protein hydrolysate (PH)) resulted in higher plant biomass, even in higher leaf area index (PH), compared to non-treated control [20]. In addition, foliar applications of protein hydrolysate, plant and seaweed extracts increased yield in tomato [21]. However, our study showed that fruit cracking, a non-desirable phenomenon for tomato fruits mainly related to differences in temperature around the fruit, irrigation water availability and water content in the plant/fruit tissue, was increased in the case of EP application. An optimized irrigation schedule could possibly prevent fruit cracking when EP is sprayed, but this needs to be further researched. Despite the fact that our study showed fruit cracking in tomato, biostimulants can be considered as a useful mean to alleviate the severe effects of water stress and enhance quality of bean (*Phaseolus vulgaris* L.) pods and seeds [45].

In addition, the application of EP increased stomatal conductance and it also demonstrated an effect on chlorophyll content (at EP-1x). This was in agreement with findings from a study on rosemary oil foliar application on tomato young seedlings, as chlorophyll levels (assayed by leaf SPAD) were increased at 0.1% oil foliar treatment compared to the control (water) or 0.05% soil application [24]. Stomatal conductance was related to plant water relations and photosynthesis [46]. In this case, EP application might increase water uptake in tomato plants. However, as mentioned previously, irrigation schedule (water was applied at the same time intervals) probably determined the water uptake in plants and fruit quality (i.e., cracking). It should be noted that the application of CP also increased stomatal conductance. Chlorophyll content (as well as carotenoids) was also increased in lettuce in another study, after the use of a foliar fertilizer containing macronutrients [14] as well as after the use of fish-derived protein hydrolysates [47]. Other products, such as chitosan (deacetylated

form of chitin) enhanced lettuce photosynthetic activity, posing biostimulant effects on the examined crop [48].

On the other hand, in the case that the EP was applied only once, it decreased total phenols content in tomato leaves compared to CP and EP-3x treatment, but not compared to the control. Antioxidant activity (assayed with the DPPH method) decreased with the EP-1x application while no differences were found for FRAP antioxidant activity among the treatments. The above decreases might be related to the lesser stress conditions, as MDA remained at low levels and/or due to the possible biostimulant role that the EP could have on the plants. In contrast to our results, the application of amino acid-based biostimulants and amino acids in combination with *A. nodosum* filtrate significantly increased total phenols content in broccoli [8]. Additionally, the use of brown seaweed (*A. nodosum*) enhanced phenolic content in spinach [17].

Foliar application of CP and EP resulted in decreased damage to tomato leaves (Figure 2). Enzymatic activity was also affected, as determined by SOD and POD. The decreased MDA was possibly related to the primary activation of antioxidant enzymes metabolism (initial increase in SOD and then POD, causing detoxification of reactive oxygen species (ROS) and therefore enzymes activities were decreased), or other non-enzymatic processes may be involved. Wozniak et al. [49] reviewed that with over 50 biostimulants (from seaweed extracts, humic compounds, hydrolyzed proteins, live microbial inoculums to synthetic compounds) when evaluated on over 30 agronomic and horticultural crops, one of the main impacts of biostimulants was related to the control of ROS overproduction and cell membranes protection. The protective mechanisms were reflected in the increased antioxidant enzymatic activity and secondary metabolites of the plants. Nevertheless, further studies are necessary to address this concern in more detail.

The application of EP affected the nutrient content in tomato leaves. Therefore, N leaf content decreased when EP was applied three times (EP-3x) while K as well as P content increased. Fanasca et al. [50] reported the positive effect of K in fruits on lycopene content, as K can be involved by its action on carbohydrate metabolism, and therefore involved in the carotenoid biosynthesis process [51]. For P, EP-1x treatment decreased the nutrient content in tomato leaves. Moreover, Na content increased and Mg decreased in leaves in the case of EP and CP application, indicating antagonistic effects among cations that could possibly reach deficient levels for key macronutrients on plant physiology processes (i.e., Mg deficiency is affecting the plant photosynthetic rates). Similar results were presented after the application of plant and seaweed extracts in tomato plants for the nutrient content of tomato fruit [21]. Additionally, the application of salicylic acid or *Moringa oleifera* leaf extract improved nutrients content in bean plants [18]. Souri and Bakhtiarizade [24] reported the increased N, K, Mg, Fe and Zn leaf content in tomato seedlings when plants were sprayed with 0.1% rosemary essential oil compared to the control and/or lower oil concentrations, indicating the beneficial effects of the essential oils to be depended on the selected rosemary oil levels. It is important to point here that biostimulants cannot be defined as fertilizers, since they do not provide nutrients directly to the plants, but indirectly could facilitate the acquisition of nutrients by supporting metabolic processes in plants and soil [4].

Fruit quality attributes are essential for the commercial success of vegetables and fruits. This study showed that fruit firmness was decreased in the case of EP or CP application, as a consequence, fruit were more subjected to the ripening process and senescence with decreased storage life [52]. Additionally, the application of CP and EP decreased total phenolics (antioxidants) and titratable acidity in tomato juice, reflecting increased ripening metabolism and fruit senescence, as was also indicated with higher TSS/TA values. Consequently, tomatoes produced under EP applications might have a shorter shelf life and additional postharvest handling might be required (i.e., ozone, 1-MCP) [52,53]. On the other hand, ascorbic acid was doubled in the case of EP-1x in comparison to the control, providing increased antioxidant status and better quality, characteristics that are well appreciated by consumers. In contrast, when tomato plants were subjected to foliar applications of protein hydrolysate, plant and seaweed extracts, the content of fruit total phenols and ascorbic acid did not differ compared

to the control treatment [21]. Quality attributes such as fruit marketability and aroma were not affected by the application of EP. The same was observed for fruit fresh weight and total soluble solids. However, EP-1x led to the highest scores related to fruit appearance. These parameters are important for assessing fruit ripening as well as the post-harvest treatment (e.g., handling and transportation) of tomato. In another study, where plant extracts and protein hydrolysates were applied in tomato, fruit brightness and redness as well as the target organic acids malate, oxalate, citrate and isocitrate were significantly higher in treated relative to untreated plants [20].

5. Conclusions

In this work, the potential use of an EP containing eucalyptus and rosemary essential oils as a biostimulant was tested in tomato plants. Plant yield, quality, nutritional, physiological and enzyme activity parameters were evaluated after its use. Plant growth was affected as EP increased height and yield in tomato. The EP affected stomatal conductance as well as chlorophyll content and it decreased phenolic content in leaves. Enzymatic activity was also affected as well as nutrient content in plant leaves. In tomato juice, total phenolics and titratable acidity were decreased while ascorbic acid was increased. Fruit marketability and aroma were not affected by the EP application. This research showed that essential oils could have potential application as biostimulants, as EP-1x application revealed superior fruit quality attributes compared to EP-3x and CP, considering the cost of the farmer for triple applications compared to once. The experiment is suggested to do 5x-EP to see weather show the similar patterns as shown in 3x-EP, and this is possible applicable in longer tomato crop cycle. Further research is required to test them in other crops as well as to identify the ingredients that are responsible for the biostimulant properties of essential oils. Considering the well-known role of 1,8-cineole, with a considerable content in rosemary and eucalyptus oils, other plant species with relevant considerable 1,8-cineole content could be tested for biostimulation, such as laurel, artemisia, lavender, to name a few.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/12/20/8432/s1, Figure S1. Average day and night temperature and relative humidity during growing period, Figure S2. Experimental design for the tomato greenhouse study.

Author Contributions: Conceptualization, A.C., V.L., N.T. and M.S.; methodology, A.C., S.C., P.X. and N.T.; data curation, A.C. and N.T.; Investigation, A.C., S.C., P.X., V.L. and N.T.; writing—original draft preparation, A.C., V.L., P.X. and N.T.; writing—review and editing, A.C., V.L., N.T. and M.S.; supervision, N.T.; project administration, M.S. and N.T.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Cyprus Research and Innovation Foundation programmes for research, technological development and innovation "RESTART 2016–2020", grant number ENTERPRISES/0916/0025; project PlantSafe. The project is co-funded by the European Regional Development Fund and the Republic of Cyprus.

Conflicts of Interest: The authors declare no conflict of interest.

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