



Article

The Use of a Plant-Based Biostimulant Improves Plant Performances and Fruit Quality in Tomato Plants Grown at Elevated Temperatures

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Abstract: Abiotic stresses can cause a substantial decline in fruit quality due to negative impacts on plant growth, physiology and reproduction. The objective of this study was to verify if the use of a biostimulant based on plant and yeast extracts, rich in amino acids and that contains microelements (boron, zinc and manganese) can ensure good crop yield and quality in tomato plants grown at elevated temperatures (up to 42 °C). We investigated physiological responses of four different tomato landraces that were cultivated under plastic tunnel and treated with the biostimulant CycoFlow. The application of the biostimulant stimulated growth (plants up to 48.5% taller) and number of fruits (up to 105.3%). In plants treated with the biostimulant, antioxidants contents were higher compared to non-treated plants, both in leaves and in fruits. In particular, the content of ascorbic acid increased after treatments with CycoFlow. For almost all the traits studied, the effect of the biostimulant depended on the genotype it was applied on. Altogether, the use of the biostimulant on tomato plants led to better plant performances at elevated temperatures, that could be attributed also to a stronger antioxidant defence system, and to a better fruit nutritional quality.

Keywords: antioxidants; biostimulant; tomato; fruit quality; abiotic stress

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables worldwide also owing to the development of products such as soups, juices, purees, and sauces [1]. Tomato is an essential component of the Mediterranean diet and of other traditional diets. However, heat can negatively affect vegetative and reproductive growth phases in tomato resulting in up to 70% harvest losses [2,3]. Indeed, in tomato, when temperatures exceed 35 °C different physiological functions result adversely affected including seed germination, seedling and vegetative growth, flowering and fruit set and ripening [3]. High temperature stress leads also to inhibition of chlorophyll biosynthesis and of photosystem II activity [4]. Indeed, photosynthesis is one of the processes most affected by elevated temperatures [5].

Considering the importance of this crop, the development of new management practices to enhance tolerance to abiotic stresses, including heat stress, could contribute to global food production. The use of biostimulants is proposed as an innovative solution to address the novel challenge to improve the sustainability of agricultural systems and reduce the use of chemical fertilizers [6,7]. The most accepted and complete definition of a biostimulant is the one from Du Jardin that defines

a plant biostimulant as “any substance or microorganism that applied to plants, regardless of its nutrients content, is able to enhance nutrition efficiency and also abiotic stress tolerance and quality traits” [8]. Du Jardin allocated the biostimulants into eight classes: humic substances, complex organic materials, beneficial chemical elements, inorganic salts, seaweed extracts, chitin and chitosan derivatives, anti-transpirant and free amino acids and considered other N-containing substances with microorganism a potential ninth category. The mechanisms activated in plants by the different biostimulants are still not known as they can act directly on plant metabolism and physiology or indirectly on soil conditions [9]. The effects of biostimulants compounds include stimulation of enzyme activities of glycolysis, Krebs cycle, nitrate assimilation, and of hormonal activities [10]. It has been also demonstrated that biostimulants application is able to enhance tolerance to different abiotic stresses, such as drought [11,12], salinity [7,13,14], and thermal stresses [15]. For example, it has been demonstrated that applications of algal extracts are able to promote tolerance to drought, salinity, and heat, while extracts rich in amino acids can help increasing tolerance to thermal stresses [16,17]. Lettuce plants (*Lactuca sativa*) treated with a mixture derived from enzymatic hydrolysis of proteins and subjected to cold showed higher fresh weights and better stomatal conductance compared to non-treated plants [18]. In another work, perennial ryegrass (*Lolium perenne* L.) treated with hydrolyzed amino acids had improved photosynthetic efficiency compared to non-treated plants at high temperatures (36 °C) [15]. In general, the application of amino acids was found to exert positive effects on plant growth due to their use for the biosynthesis of a large number of non-protein nitrogenous compounds (pigments, vitamins, coenzymes, purine, and pyrimidine bases). Therefore, amino acids applications could directly influence the physiological activity in plant growth and yield also under abiotic stress [19]. Protein hydrolysates can also improve soil respiration, microbial biomass and activity and impact on plant nutrition by forming complexes and chelates between amino acids and soil nutrients [20].

To improve the tolerance to high temperatures the use of biostimulants has been previously investigated, even if it is presently unclear to what extent these compounds are able to improve the physiological performances of tomato plants under elevated temperatures [7]. We hypothesize that the use of an amino acid-based biostimulant could stimulate natural processes to enhance plant performances also at elevated temperatures. Indeed, the use of protein hydrolysates could directly stimulate carbon and nitrogen metabolism and indirectly enhance nutrient availability, nutrient uptake and nutrient use-efficiency in plants [21]. To verify this hypothesis, we used a novel plant-based biostimulant named CycoFlow and we performed physiological and biochemical analyses on four different tomato landraces grown at elevated temperatures and treated or not with this biostimulant. We reasoned that treatments with CycoFlow could facilitate stress adaptation because of its putative cytokinin-like action and its high concentration of glycine betaine known to mitigate the effect of heat stress [7,22]. Considering climate changes and the expected rise of temperatures in the next few years, to understand the contribution of biostimulants to ensure good plant performances at high temperatures may become increasingly important.

2. Materials and Methods

2.1. Plant Growth, Experimental Design, and Treatments

One-month-old tomato seedlings (landraces E17, E36, E107, PDVIT, described in Table 1) were transplanted in May 2018 under walk-in plastic tunnel (22 × 8 m²) in Battipaglia in the Campania Region in Southern Italy (40°57'68"N 14°95'97"E). The tunnel was covered in polyethylene sheet and was open on both sides. Microclimatic conditions and temperatures were not regulated but were recorded during the growing season. All four genotypes have an indeterminate growth habit. The genotype E17 is characterized by large fruits (200–500 g), the genotype E107 is characterized by medium-sized fruits (70–100 g) and the E36 and the PDVIT genotypes are characterized by small cherry fruits (Table 1). Only the mature fruits of the E107 genotype are yellow while the fruits of the other genotypes are red. Tomato plants were grown following the standard cultural practices of the

area. The experimental design consisted of a completely randomized design with three replicates *per* treatment and ten plant *per* each biological replication. There were two different groups: one control, which did not receive any biostimulant, and one that was treated with the biostimulant. The biostimulant CycoFlow (Agriges, Benevento, Italy) was produced by mixing sugar cane molasses with yeast extract obtained by autolysis of previously grown *Saccharomyces cerevisiae* yeasts. It is rich in high free amino acids, peptides, nucleotides, B-vitamins, trace elements, and other growth factors. Its chemical composition contains total nitrogen of 4.5% and organic carbon of 19.5%. The aminogram of the Biostimulant Cyco Flow is reported in Supplementary Table S1. The product contains also Boron (0.2%), Manganese (1%) and Zinc (1.2%). The biostimulant has a pH of 5.0, a density of 1200 kg/m³ and an EC value of 15.0 dS/m. The Biostimulant, in liquid formulation, was initially applied directly to the soil (400 mL *per* plant) at the moment of transplanting, and thereafter every 15 days, until the end of the cultivation cycle for a total of four total applications. CycoFlow was applied by fertigation at a final concentration of 3 g/L. The control and the treatment groups received the same amount of water. No fertilizer has been applied. During the whole growing period climatic data (Figure S1) were recorded using the weather station VantagePro2 from Davis Instrument Corp. At the end of the cultivation cycle, plants were harvested and separated into leaves, stems, roots and fully ripe fruits. Plant height, numbers of leaves *per* plant, fresh weight of biomass, total number of fruits, weight of fruit and final yield were recorded. Dry biomass (in grams) was determined by drying plant tissues to constant weight in a forced-air-oven at 80 °C for 72 hours. Measurements were done on three randomly selected plants *per* each biological replication *per* genotypes for each treatment.

Table 1. Details of the tomato genotypes used in this study.

Genotype	Origin	Common Accession	Fruit Size	Fruit Color
E17	Italy	Pantano Romanesco	Big (200–250 g)	red
E36	Italy	Riccia San Vito	Small (25–30 g)	red
E107	Spain	E-L-19	Medium (70–100 g)	yellow
PDVIT	Italy	Caramella	Small (10–15 g)	red

2.2. Pollen Viability

Pollen viability was analyzed using five flowers *per* plant sampled from three different plants *per* replicate. In the laboratory, pollen grains were spread on microscope slides. One droplet of DAB solution (SIGMA) was added on each pollen sample; slides were gently warmed with a gas lighter and mounted with a cover slip [23]. Scoring was made using an LEITZ Laborlux12 microscope.

2.3. Ascorbic Acid Quantification

Reduced ascorbic acid (AsA) and total ascorbic acid (AsA + dehydroascorbate – DHA) measurements were carried out by using a colorimetric method [24] with modifications reported by Rigano et al. [25,26]. Briefly, 500 mg of frozen powder from tomato fruits or leaves were extracted with 300 µL of ice cold 6% trichloroacetic acid (TCA) and the mixture was then incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation, to 20 µL of supernatant were added 20 µL of 0.4 M phosphate buffer (pH 7.4), 10 µL of double distilled (dd) H₂O and 80 µL of color reagent solution. This solution was prepared by mixing solution A (31% (*w/v*) H₃PO₄, 4.6% (*w/v*) TCA and 0.6% (*w/v*) FeCl₃) with solution B (4% (*w/v*) 2,2'-Dipyridyl). For total AsA, to 20 µL of sample, 20 µL of 5 mM dithiotreitol in 0.4 M phosphate buffer (pH 7.4) were added and the mixture was incubated for 20 min at 37 °C. Ten microliters of N-ethyl maleimide (NEM; 0.5% (*w/v*) in water) were added and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both the final mixtures were incubated at 37 °C for 40 min and measured at 525 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated biological replicates for each sample and three technical assays for each biological repetition were measured. The concentration was expressed in mg/100 g of fresh weight (FW).

2.4. Total Carotenoids and Chlorophylls Content

The evaluation of total carotenoids and chlorophylls was carried out according to the method reported by Wellburn [27] and by Zouari et al. [28] as modified by Rigano et al. [2]. To obtain the lipophilic extract, 0.25 grams of sample were extracted with 24 mL of acetone/hexane (40/60, *v/v*). The mixture was centrifuged at 15,000 rpm for 5 min at 4 °C. Supernatants were collected and stored at −20 °C until analyses. For carotenoids and chlorophylls a and b levels determination, absorbance of lipophilic extracts was read at 470, 663, and 645 nm, respectively. For lycopene and β -carotene levels absorbance was read at 505 and 453 nm, respectively. Results were converted into mg/100 g FW. Three separated biological replicates for each sample and three technical assays for each biological repetition were measured.

2.5. Antioxidant Activity Determination

Hydrophilic antioxidant activity (HAA) was evaluated in the water-soluble fraction, obtained by adding to 2 g of frozen powder 25 mL of 80% methanol, using the ferric reducing/antioxidant power (FRAP) method [29] with slight modifications. The FRAP assay was carried out by adding in a vial 2.5 mL of acetate buffer at pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (12 mM), and 150 μL of methanolic extract. The mixture was incubated for 30 min in the dark, and then readings of the colored products (ferrous tripyridyltriazine complex) were taken at 593 nm using a spectrophotometer. Results were expressed as micromoles of Trolox equivalents (TE) per 100 g FW. Lipophilic antioxidant activity (LAA) determination was carried out according to the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method, using the lipophilic extract obtained as described in the previous paragraph [30]. The ABTS assay was based on the reduction of the $\text{ABTS}^{\bullet+}$ radical action by the antioxidants present in the sample. A solution constituted by 7.4 mM $\text{ABTS}^{\bullet+}$ (5 mL) mixed with 140 mM $\text{K}_2\text{S}_2\text{O}_8$ (88 μL) was prepared and stabilized for 12 h. This mixture was then diluted by mixing $\text{ABTS}^{\bullet+}$ solution with ethanol (1:88) to obtain an absorbance of 0.70 ± 0.10 unit at 734 nm using a spectrophotometer. Methanolic extracts (100 μL) were allowed to react with 1 mL of diluted $\text{ABTS}^{\bullet+}$ solution for 2.5 min, and then the absorbance was taken at 734 nm using a spectrophotometer. All biological replicates of samples were analyzed in triplicate. Results were expressed as micromoles of TE per 100 g FW.

2.6. Fluorescence Emission Measurements

Fluorescence emission measurements were performed on five replicates *per* each treatment, coming from five different plants. A portable FluorPen FP100max fluorometer, equipped with a light sensor (Photon System Instruments, Brno, Czech) was used for measurements, following the procedure reported in Figlioli et al. [31]. The ground fluorescence signal, F_o , was induced on 40' dark adapted leaves, by a blue LED internal light of about $1\text{--}2 \mu\text{mol m}^{-2} \text{s}^{-1}$. The maximal fluorescence level in the dark, F_m , was induced by a 1s saturating light pulse of $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$. The maximum quantum efficiency of PSII photochemistry, F_v/F_m , was calculated as $(F_m - F_o)/F_m$, according to Kitajima and Butler [32].

2.7. Leaf Functional Traits Determination

Fully expanded leaves, without apparent damages, were collected to determine the functional leaf traits following Arena et al. [33]. Leaf area (LA) was measured by the program Image J 1.45 (Image Analysis Software) and expressed in *per* square centimeter, specific leaf area (SLA) was measured as the ratio of leaf area to leaf dry mass and expressed as square centimeter *per* gram dry weight (DW). For dry mass determination, leaves were dried at 70 °C for 48 h. Leaf dry matter content (LDMC) was measured as the oven-dry mass of a leaf divided by its water-saturated fresh mass and expressed as gram *per* gram of water saturated leaf mass (WSLM). Relative water content in leaves (RWC) was calculated by dividing the amount of water in the fresh leaf tissue by the water in the leaf tissue after rehydration multiplied by 100 [34].

2.8. Statistical Analysis

Data were subjected to analysis of variance using a two-way ANOVA. To separate means within each parameter, the Tukey-HSD's test was performed. Differences at $p < 0.05$ were considered to be significant. ANOVA was performed by using SPSS (Statistical Package for Social Sciences) Package 6, version 23.0. To explore the overall data, we used the R environment for statistical computing and graphics R Core Team (2018). We first selected variables of interest for each genotype, treatment and plant part ($4 \times 2 \times 2$) then calculated the arithmetic mean ($n = 3$), and finally used the scale function to center the data around the mean and scale it using the standard deviation. The transformed data were visualized using a heatmap (heatmap function). To aid interpretation of the data, we also performed an SVD-based Principal Component Analysis over the multivariate matrix (function prcomp in base R) after normalization.

3. Results

3.1. Phenotypic and Physiological Analyses

In this study four different tomato genotypes were transplanted under a plastic walk-in tunnel with a delay of one month compared to the usual transplanting period (tomato plants in the South of Italy are usually transplanted in April), thus imposing a high-temperature condition during flowering and fruit setting. Indeed, the maximum temperature of 32 °C during the day, which represents a critical threshold in the sensitive stages of reproductive development, was frequently exceeded in this trial [3] (Figure S1). The four different tomato landraces were treated with a plant-based biostimulant named CycoFlow. According to ANOVA analyses, the treatment with the biostimulant increased the height of genotypes E107 and PDVIT by 48.5% and 30.1%, respectively (Supplementary Table S2). Generally, the number of leaves was lower in the biostimulant treated group compared to the control, independently of the genotype it was applied to (no significant interaction G X T). For the fresh biomass parameter, in PDVIT the treatment with CycoFlow increased the above ground fresh biomass by 68.4% (Figure 1a). Genotypes E17 and E36 showed, instead, lower values in treated plants compared to non-treated ones (−53.8% and −21.1%, respectively). A slightly higher pollen viability was also observed in the genotypes treated with the biostimulant compared to the respective controls (Figure 1b). In particular, in the genotype E107 the treatment with the biostimulant increased pollen viability by 125%. Generally, the treatment with the biostimulant increased the number of fruits, independently of the genotype (no significant interaction G X T). In particular, the treatment with the biostimulant increased the number of fruits in the genotype PDVIT by 105.3% (Figure 1c). The medium fruit weight was significantly affected only by the factor genotype (Supplementary Table S2). Generally, the final yield (kg *per* plant) showed a tendency to be higher in all the samples from the treated genotypes, even though these differences were not significant (Figure 1d). Interestingly, the yield was significantly affected only by the factor treatment (Supplementary Table S2).

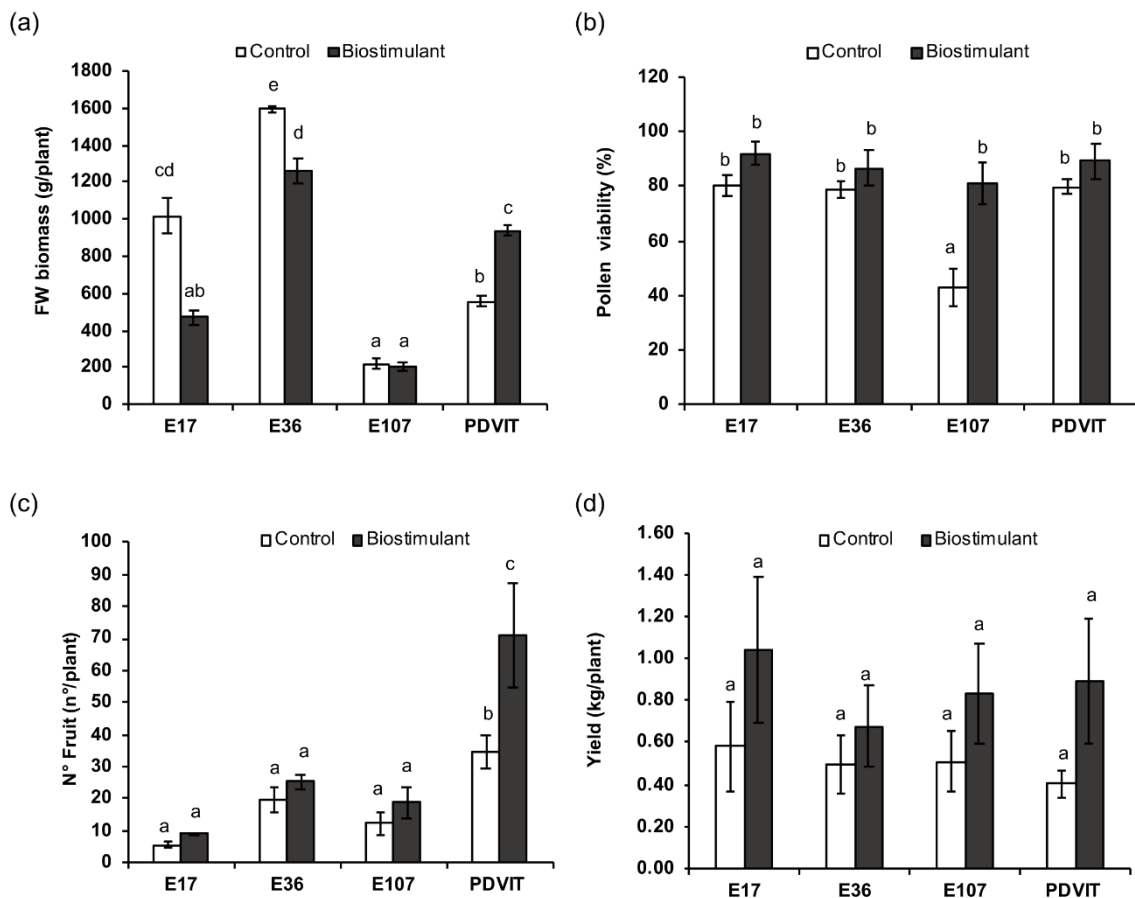


Figure 1. Effect of CycoFlow on: (a) Fresh weight (FW) biomass, (b) pollen viability, (c) fruit number, and (d) final yield in four tomato genotypes. Values are mean \pm SE. Different letters indicate significant differences based on Tukey-HSD test ($p \leq 0.05$).

The treatment with the biostimulant CycoFlow also increased the maximal PSII photochemical efficiency (F_v/F_m) in the E107 and PDVIT genotypes (Figure 2). The monitoring of leaf functional traits evidenced that biostimulant application did not affect these traits significantly (Supplementary Table S3).

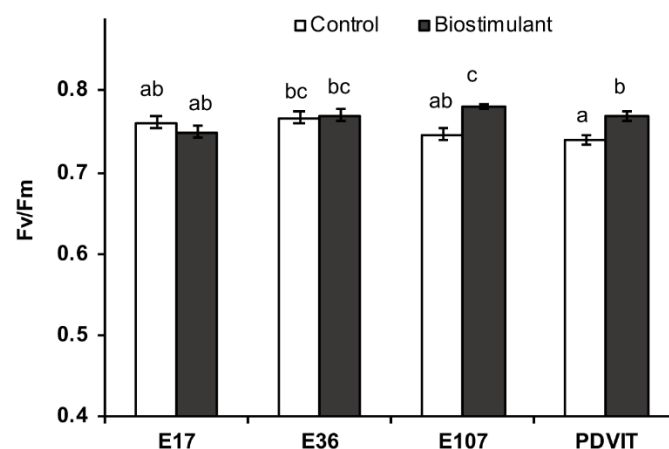


Figure 2. Maximal photochemical efficiency (F_v/F_m) in leaves of four tomato genotypes. Data are mean \pm SE ($n = 5$). Different letters indicate significant differences based on Tukey-HSD test ($p \leq 0.05$).

3.2. Leaf and Fruit Antioxidant Content

The main interaction effects of the biostimulant Cyco Flow on the content of antioxidants in leaves from treated and non-treated plants is reported in Table 2.

Table 2. Analyses of variance and mean comparison for reduced and total ascorbic acid (AsA), total phenols, carotenoids, chlorophylls a and b and total lipophilic and hydrophilic antioxidant activities (LAA and HAA, respectively) in leaves of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation four times. Means \pm SD within rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

		E17	E36	E107	PDVIT	SIGNIFICANCE	
Reduced AsA (mg/100 g FW)	control treated	6 \pm 0.43 a	7.95 \pm 1.33 a	10.83 \pm 1 ab	18.20 \pm 0.91 bc	G	**
		20.05 \pm 3.30 c	20.12 \pm 1.42 c	17.41 \pm 1.91 bc	19.34 \pm 1.33 c	T	***
						G X T	**
Total AsA (mg/100 g FW)	control treated	16.79 \pm 0.73 ab	14.45 \pm 0.51 a	24.52 \pm 2.03 bc	21.28 \pm 0.86 bc	G	***
		21.15 \pm 0.90 bc	24.40 \pm 2.55 cd	20.27 \pm 0.83 cd	26.85 \pm 0.69 d	T	***
						G X T	***
Phenols (mg/100 g FW)	control treated	43.38 \pm 0.98 e	26.91 \pm 1.19 a	35.14 \pm 0.48 c	35.30 \pm 0.56 c	G	***
		25.33 \pm 1.20 a	25.58 \pm 0.27 a	31.57 \pm 0.52 b	39.57 \pm 0.54 d	T	***
						G X T	***
Carotenoids (mg/100 g FW)	control treated	23.91 \pm 1.06 ab	26.06 \pm 0.53 abc	23.80 \pm 0.75 a	28.73 \pm 0.23 de	G	***
		23.78 \pm 0.48 a	30.17 \pm 0.24 e	28.10 \pm 0.47 cde	26.42 \pm 0.46 bcd	T	***
						G X T	***
Chl a (mg/100 g FW)	control treated	108.78 \pm 3.05 a	113.30 \pm 4.3 6 ab	128.22 \pm 5.34 bc	140.30 \pm 4.25 c	G	***
		110.13 \pm 1.37 a	137.08 \pm 2.07 c	138.61 \pm 3.32 c	130.20 \pm 2.80 bc	T	**
						G X T	***
Chl b (mg/100 g FW)	control treated	38.65 \pm 3.96 a	37.45 \pm 2.12 a	45.84 \pm 3.67 ab	55.75 \pm 3.74 b	G	***
		37.29 \pm 2.73 a	55.41 \pm 2.11 b	59.47 \pm 2.69 b	45.85 \pm 5.72 ab	T	**
						G X T	***
LAA (mg/100 g FW)	control treated	18.88 \pm 0.14 a	18.75 \pm 0.07 a	18.86 \pm 0.04 a	18.62 \pm 0.05 b	G	***
		18.98 \pm 0.04 a	19.07 \pm 0.21 a	19.90 \pm 0.08 b	19.75 \pm 0.10 a	T	***
						G X T	***
HAA (mg/100 g FW)	control treated	828.58 \pm 140.08 a	493.19 \pm 220.27 bc	599.85 \pm 118.33 ab	434.30 \pm 88.34 cd	G	***
		255.57 \pm 91.31 d	390.49 \pm 25.34 bc	510.33 \pm 53.59 ab	438.26 \pm 125.38 bc	T	***
						G X T	***

G = genotype; T = treatment; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

For the hydrophilic antioxidants, the treatment with the biostimulant increased the content of reduced AsA in the genotypes E17 and E36 and of total AsA in the leaves of the genotypes E36 and PDVIT. In particular, in the genotype E107 a 60.8% higher content of total AsA was registered in leaves treated with the biostimulant. As for the content of phenolic compounds, two genotypes (E17 and E107) showed lower contents of total phenols in the leaf after treatment with the biostimulant. In particular, in the E17 genotype a 41.6% decrease in the treated compared to the non-treated samples was demonstrated. Only in the PDVIT genotype the treatment with the biostimulant increased phenols content. It has been reported that phenolics compounds are the most important contributors to HAA [35]. Accordingly, in the leaves of the treated plants, HAA was lower in E17 compared to the respective non-treated control. For the lipophilic antioxidants, the treatment with the biostimulant increased the content of carotenoids in the genotypes E36 and E107 and the content of chlorophylls a and b only in the genotype E36. Particularly, the E36 genotype showed a 15.8% higher content of carotenoids in the treated leaves compared to the non-treated one, and 17.35% and 48% higher levels of chlorophyll a and b, respectively. The treatment with the biostimulant also increased total lipophilic antioxidant activities in E107 and surprisingly also in PDVIT, suggesting that other compounds outside of carotenoids contributed to this parameter.

In Table 3 is reported the content of hydrophilic antioxidants determined in red ripe fruit from genotypes treated or non-treated with the biostimulant CycoFlow. In general, the content of hydrophilic

antioxidants in the fruits was higher in almost all the genotypes treated with biostimulants compared to the non-treated ones. The treatment with the biostimulant increased the content of reduced AsA independently of the genotype it was applied on (not significant interaction G X T). The content of reduced AsA was 28.7%–58.7% higher in fruits from treated genotypes compared to non-treated genotypes. Moreover, a content 112.8% higher of total AsA was registered in fruits from PDVIT treated with the biostimulant compared to the respective non-treated control. Contrary to what seen in the leaf, the content of total phenols in berries of treated E17 and E36 genotypes was higher compared to the non-treated control. In particular, in the E17 genotype 72.8% higher values were registered. Moreover, a significantly higher antioxidant activity HAA was demonstrated in fruits from E36 plants treated with CycoFlow, according to ANOVA analyses. Assessing the content of lipophilic antioxidants, the treatment with the biostimulant had no effects on the content of carotenoids and chlorophylls but only on the total lipophilic antioxidant activity, as reported in Supplementary Table S4. In particular, LAA was higher in fruits from the treated genotypes E17, E36, and E107.

Table 3. Analyses of variance and mean comparison for reduced and total ascorbic acid (AsA), total phenols, hydrophilic antioxidant activities (HAA) in fruits of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation four times. Means \pm SD within rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$).

		E17	E36	E107	PDVIT	SIGNIFICANCE	
Reduced AsA (mg/100 g FW)	control	33.31 \pm 2.99 a	39.56 \pm 2.30 ab	47.14 \pm 1.66 bc	50.36 \pm 1.84 bc	G	***
	treated	47.36 \pm 1.60 bc	59.87 \pm 4.34 cd	74.79 \pm 3.25 e	64.83 \pm 2.34 de	T	***
						G X T	ns
Total AsA (mg/100 g FW)	control	61.97 \pm 0.57 ab	78.03 \pm 3.29 bc	85.40 \pm 3.75 c	52.87 \pm 4.24 a	G	**
	treated	79.34 \pm 4.44 bc	87.15 \pm 2.35 c	93.36 \pm 6.19 cd	112.53 \pm 4.08 d	T	***
						G X T	***
Phenols (mg/100 g FW)	control	9.62 \pm 0.46 a	13.70 \pm 0.68 b	16.17 \pm 0.58 c	22.35 \pm 0.37 e	G	***
	treated	16.62 \pm 0.46 c	18.92 \pm 0.76 d	16.88 \pm 0.44 c	22.55 \pm 0.19 e	T	***
						G X T	***
HAA (mg/100 g FW)	control	129.28 \pm 33.95 a	189.22 \pm 49.66 b	179.38 \pm 20.62 bc	309.06 \pm 39.51 d	G	***
	treated	151.57 \pm 8.71 c	304.38 \pm 30.92 c	212.47 \pm 7.08 c	333.03 \pm 46.91 d	T	***
						G X T	***

G = genotype; T = treatment; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; ns = not significant.

3.3. Heat Map Analysis

A heat map providing the morphological, biochemical, and physiological changes in leaves and fruits of four different tomato genotypes in response to the addition of one biostimulant is displayed in Figure 3. With regard to leaves, the heat-map identified two main clusters which divided the analyzed samples differently (Figure 3, panel a). The first cluster separated the control genotypes E107 and E17 from the other genotypes and respective treated samples, the second cluster associated the treated genotypes E107, E17, and PDVIT in a sub-group and control PDVIT and E36 genotypes in another sub-group (Figure 3). Our data indicate that biostimulant application was the main clustering factor for E107, E17, and PDVIT genotypes, on the basis of differences in some leaf traits, F_v/F_m , phenols, yield and HAA, suggesting that the biostimulant utilization produces significant effect on many metabolites. The heat map built on tomato fruits clearly separated the treated PDVIT genotype from all others, in particular for number of fruits and reduced AsA (Figure 3, panel b), indicating this genotype as the most responsive to biostimulant application for fruit characteristics. A remarkable separation was also evident for control E107 and E36 compared to treated genotypes, grouped in two sub-clusters on the basis of pigments (chlorophylls and carotenoids) and LAA. A PCA analyses was also performed (Supplementary Figure S2). The PCA output further showed an evident separation between the treated and the non- treated genotypes.

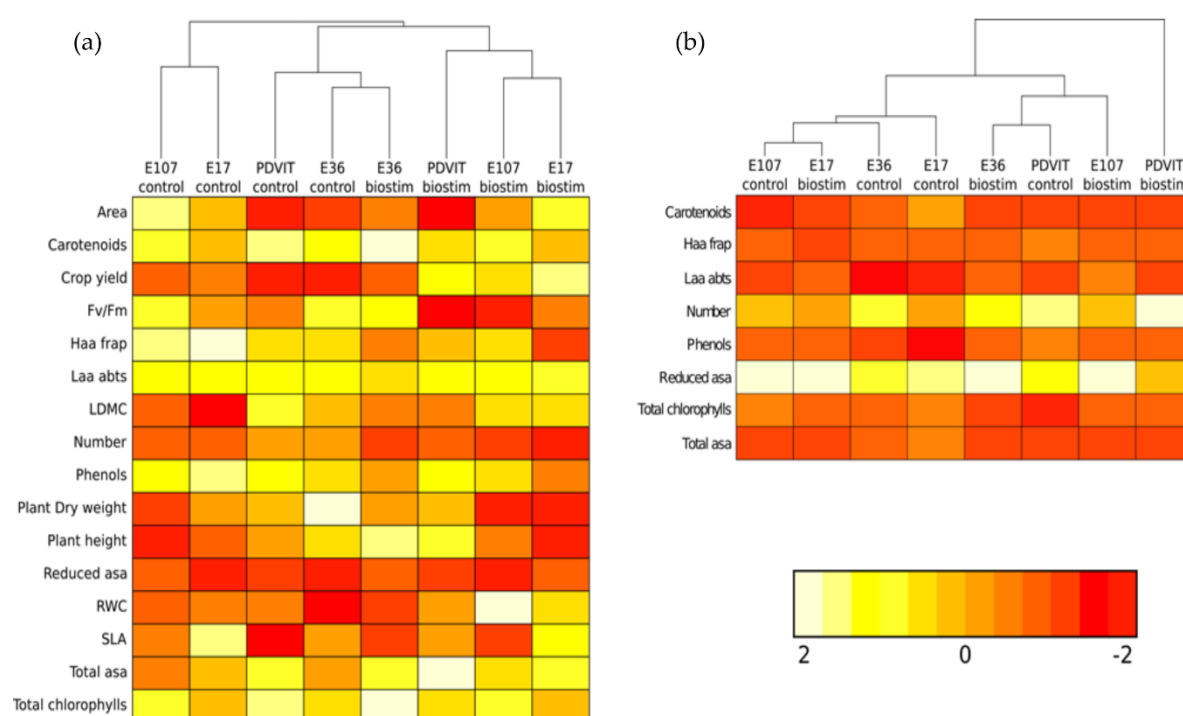


Figure 3. Cluster heat map analysis summarizing the behavior of the different tomato genotypes E36, E17, E107, PDVIT treated or non-treated with the biostimulant CycoFlow in leaf (panel a) and in fruit (panel b). The heat map was generated using the R environment for statistical computing and graphics (<https://www.R-project.org/online>) program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage.

4. Discussion

In this paper four different tomato landraces were grown at elevated temperatures under a plastic walk-in tunnel and were treated or not with a plant-based biostimulant named CycoFlow. The higher height demonstrated in the majority of the tomato plants treated with CycoFlow compared to non-treated plants is in agreement with previous studies on different plant species and biostimulants [36–40]. Probably, the presence of signaling molecules in the biostimulant, such as free amino acids, promoted endogenous phytohormonal biosynthesis thus stimulating growth and also fruit setting [41]. Indeed, several authors demonstrated that the application of plant-based biostimulants exhibited cytokinin-like activity promoting cell division [42]. Moreover, cytokinins mitigate stresses induced by free radicals by direct scavenging and also by preventing ROS formation inhibiting xanthine oxidation [39]. Also, the treatment with CycoFlow overall increased the number of fruits, as previously demonstrated also in tomatoes treated with other biostimulants [10,36,39–41]. For example, Rouphael et al. [41] demonstrated that application of a protein hydrolysate in tomato increased in one cultivar the fruit mean weight and in another cultivar the number of fruits. In this study, in the genotype E107, the higher number of fruits observed was also linked to a higher pollen vitality observed after CycoFlow treatment. This result could be due to a combination of multiple effects. While the cytokinin-like activity could have favored cell division, the high level of proline present in the biostimulant, an amino acid whose natural content in the flower organs is ten times higher than that in the leaves, may have played an important role [31]. Indeed, it is known that also the amino acid proline promotes the translocation of nutrients towards developing flowers (sink) [43]. The positive effects of biostimulants based on amino acid on growth and yield is also due to the fact that the amino acids present in plant-based biostimulants stimulate plant defenses, participate in the synthesis of organic compounds (such as amines, purines, pyrimidines, vitamins) and affect the uptake of macro and micronutrients [37]. The CycoFlow effects observed in this

study on yield and yield components are even more remarkable considering the elevated temperatures (up to 43 °C) reached under the plastic walk-in tunnel in Battipaglia. Indeed, this temperature normally impairs fertilization and reduces pollen viability [10]. It can be hypothesized that the presence of glycine betaine in the CycoFlow may have enhanced the tolerance of tomato plants to elevated temperatures. Indeed, it has been previously demonstrated that during tomato germination glycine betaine applied exogenously improved tolerance to high temperatures and enhanced the expression of heat shock genes [44]. At elevated temperatures, the glycine betaine compound may have also a crucial role in the repair of photodamaged PSII, in maintaining the activity of Rubisco and in alleviating the inhibition of gas exchanges [22]. Accordingly, a higher maximal photochemical efficiency was observed in the genotypes E107 and PDVIT treated with the biostimulant. These results are consistent with other papers, which demonstrated that applications of plant- and animal-based biostimulants are able to enhance photosynthetic rates and ensure a higher carbon assimilation efficiency [45,46]. For example, under drought stress conditions, *Arabidopsis* plants treated with an *Ascophyllum nodosum*-extract maintained a better photosynthetic performance compared to non-treated plants during the dehydration period, showing a higher capacity to dissipate thermally the excess of energy in the PSII reaction centers [47]. These results were linked to the fact that pre-treatments with the *Ascophyllum*-extracts induced partial stomatal closures and also modifications of the expression levels of genes involved in ABA-responsive and antioxidant system pathways [47]. Accordingly, our data indicate that biostimulant treatment induced the activation of the antioxidant defense system, as demonstrated by the higher content of reduced and total AsA in treated leaves. Although the precise reasons for these increases are not explained, it is known that biostimulants components, including glycine betaine, can promote the activity of specific enzymes involved in antioxidant homeostasis [22,41,48]. The ability to maintain an optimal chlorophyll content during heat stress is another key heat tolerance trait in tomato [49]. Interestingly, herein we observed higher contents of carotenoids and chlorophylls in two genotypes (E36 and E107) treated with the biostimulant compared to the non-treated samples. The higher chlorophylls content detected in these genotypes could be linked to limited chlorophyll degradation and leaf senescence [9]. In particular, this could be the case for the genotype E107 that demonstrated a higher maximal photochemical efficiency after treatment with the biostimulant.

The biostimulant-mediated effects on photosynthesis and secondary metabolism could also enhance fruit quality [10]. Indeed, one interesting finding of this study is the positive effect of the biostimulant CycoFlow on the quality of the tomato fruits. In general, the content of hydrophilic antioxidants in the fruits, including AsA, was higher in almost all the genotypes treated with biostimulants compared to the non-treated ones. Higher content of reduced AsA was observed in all the genotypes and of total AsA in the genotypes E17 and PDVIT. This result confirms data previously obtained in other studies that demonstrated an increase in AsA content in tomato, in kiwi fruits and in peppers after the application of plant-based biostimulants [36,41]. Contrary to what seen in the leaf, the content of total phenols in berries of treated E17 and E36 genotypes was higher compared to the non-treated control. Moreover, a significantly higher antioxidant activity HAA was demonstrated in fruits from E36 plants treated with CycoFlow. These results are in agreement with results previously obtained in other crops (soybean seeds, common bean, tomato, corn), even if the reported effects depended on the type of biostimulants, their concentrations and the number of applications [37]. Assessing the content of lipophilic antioxidants, the treatment with the biostimulant had no effects on the content of carotenoids and chlorophylls but only on the total lipophilic antioxidant activity. Similar results were obtained by Chehade et al. [36] in tomato. On the contrary, Roupheal et al. [41] demonstrated that in tomato foliar applications of a legume-derived protein hydrolysate had an effect also on lycopene content. Also, Colla et al. [10] demonstrated that foliar applications of protein hydrolysate, plant and seaweed extract affected lycopene content in greenhouse tomato. In the future, foliar application of CycoFlow will be also tested in order to verify if the results obtained in this study are also linked to the used application regimen.

Altogether, the genotypic factors remain decisive in the response obtained in the different tomato lines to the biostimulant. Indeed, for almost all the traits considered the effect of the biostimulant depended on the cultivar it was applied to, as seen by the interaction between the effect of the biostimulant and cultivars in most of the studied parameters. These variations can be explained by the differences in the genetic background between the different cultivars that were used in this study [33]. Indeed, the four genotypes here tested differed in terms of fruit shape and size and also in terms of fruit color (e.g., fruit of E107 is yellow). The geographical origin is also different with the E107 genotype coming from Spain and the other coming from Italy. These further highlight the fact that one biostimulant should be tested on a certain number of cultivars in order to assess its mechanisms of action.

5. Conclusions

In this paper we investigated the effects of the application of one plant-based biostimulant named CycoFlow on the nutritional quality and yield of tomatoes grown in walk-in tunnel under elevated temperatures. The application of the CycoFlow biostimulant had a clear effect on plant growth and final crop quality. Indeed, CycoFlow application had a significant effect on the content of hydrophilic antioxidants in both tomato leaves and fruits. In particular, the content of AsA increased after treatments with CycoFlow. Herein, the biostimulant application improved plant performances and fruit quality mostly in the genotypes E107 and PDVIT. In particular, in the genotype PDVIT application with CycoFlow determined a higher plant height, a higher number of fruits, a higher pollen vitality, a higher photochemical efficiency, a higher accumulation of AsA and a higher antioxidant activity. Additional studies are now planned in order to investigate if different applications regimen, such as foliar application, can also influence the observed effects.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/3/363/s1>, Table S1: Amino acid composition expressed in g/100 g of the biostimulant CycoFlow, Table S2: Analyses of variance and mean comparison for height, number of leaves, fresh weight (FW) and dry weight (DW) biomass, number of fruits, medium fruit weight, yields, and pollen viability (%) per plants of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation four times. Means \pm SD within rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$). Table S3: Analyses of variance and mean comparison for maximal PSII photochemical efficiency (F_v/F_m), leaf area (LA), specific leaf area (SLA), leaf dry matter content (LDMC) and relative water content (RWC) per plants of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation four times. Means \pm SD within rows and columns followed by the different letter are significantly different based on based on Tukey-HSD test ($p \leq 0.05$). Table S4: Analyses of variance and mean comparison for total lipophilic antioxidant activities (LAA), carotenoids, chlorophylls a and b (Chl A and Chl B, respectively) content in fruit of different tomato cultivars treated with the biostimulant CycoFlow applied by fertirrigation four times. Means \pm SD within rows and columns followed by the different letter are significantly different based on Tukey-HSD test ($p \leq 0.05$). Figure S1: Maximum temperatures recorded in the experimental field located in Battipaglia during the day from May to August 2018. Figure S2: Principal component analysis (PCA) of phenotypic and physiological traits in tomato plants treated or not with the biostimulant CycoFlow. The treated genotypes are indicated by the letter T after the name.

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