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A Phenomics and Metabolomics Investigation on the Modulation of Drought Stress by a Biostimulant Plant Extract in Tomato (*Solanum lycopersicum*)

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Abstract: Biostimulants are gaining increasing interest because of their ability to provide a green and effective strategy towards sustainable crop production. Nonetheless, their mode of action remains often unknown. The object of this work was to unravel the mechanisms through which 4-Vita, a biostimulant plant extract, can mitigate drought stress in tomato. To this aim, tomato plants were treated with two foliar applications of 4-Vita and drought stress imposed to both treated and control plants. Phenomics investigations were coupled to mass spectrometric untargeted metabolomics, and raw data were elaborated by multivariate statistics and pathway analysis. The biostimulant elicited a broad reprogramming of the tomato's secondary metabolism, including its phytohormones profile, corroborating an improved ability to cope with drought stress. A series of mechanisms could be identified in response to the biostimulant treatment under drought, pointing to the preservation of photosynthetic machinery functionality. The modulation of thylakoid membrane lipids, the increase in xanthins involved in ROS detoxification, and the modulation of chlorophylls synthesis could also be observed. Overall, a series of coordinated biochemical mechanisms were elicited by the biostimulant treatment, supporting the increased resilience to drought stress in tomato.

Keywords: *Solanum lycopersicum*; metabolomics; abiotic stress; chlorophyll biosynthesis; photosynthesis

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1. Introduction

Agricultural crops are constantly affected by biotic and abiotic stresses. Among abiotic stresses, drought is the most common and has a profound negative impact on the plants' morphological, physiological, and biochemical aspects [1]. In nature, plants have evolved to adapt to drought stress by modifying biochemical processes such as photosynthesis, sugar synthesis, hormone synthesis, and shaping a large variety of primary and secondary metabolites [2]. However, these modulations may not be sufficient to withstand drought stress, which can lead to severe impairment of cellular functions, reduction of yield, and plant death [3].

Many studies have shown that biostimulants can mitigate abiotic stresses by influencing metabolic processes and helping the plant strive during a period of moderate or intense stress [4–6]. Indeed, the adoption of plant biostimulants has grown in the agricultural industry in the last decade and is nowadays considered a key emerging strategy for enhancing productivity and resilience to changing climate [5–7]. A successful evaluation of biostimulant activity requires an accurate measurement of the morpho-physiological traits of plants over time. In addition, the so-called "omics sciences" such as metabolomics have been

proposed as essential tools for the characterization and understanding of the biological and agronomic phenomena triggered by biostimulants [8–10]. Indeed, metabolomics considers the entire complement of metabolites within and under a given set of conditions [4,8], allowing hypothesis-free untargeted approaches, including the study of the possible mechanisms involved in abiotic stresses response [11–13]. Metabolomic studies have previously shown that the use of biostimulants may impact phytohormones, reactive oxygen species (ROS) signaling, and osmotic [12–14] and antioxidant adjustments in plants [15]. For instance, some secondary metabolites, such as flavonoids, were found to play an important role as antioxidants by detoxifying and scavenging ROS [16]. Indeed, it has been proven that when applied through biostimulants, they impact plant growth crop yield and are supportive in abiotic stresses [17–19]. Notably, these processes can be related to priming, a prominent topic in the field of biostimulants [20], which allows highlighting the early changes in plant metabolic processes [20–22]. Previous studies showed that biostimulants applied on tomatoes could improve the drought stress tolerance. For instance, between 2014 and 2022, some teams of researchers investigated the use of different biostimulants on Solanum lycopersicum. Among others, Alpan® [17], a novel protein-hydrolysate-based biostimulant [18], and Megafol® [19] have been tested. All these products showed mitigation of the drought stress symptoms related to plant physiology, and yielded improvement compared to the untreated plants.

Even when the effects on plants are demonstrated, most biostimulants have a mechanism of action that remains unknown. Considering that biostimulants are complex multicomponent products, it is challenging to establish a specific biochemical target site and a mode of action. Yet, to enter into a regulatory process and implement effective agronomic and marketing strategies, it is often fundamental to suggest the mechanism of action of an agricultural product.

The present work aims at using a combined phenotypic, physiological, and metabolomic approach for unraveling the mode of action of "Plants for Plants 4-Vita", a plant biostimulant. This product is entirely plant-based and is composed of a standardized ratio of flavonoids and organic acids, developed in the framework of a 2-year European LIFE project during which open-field trials were performed in different EU countries (LIFE18 ENV/NL/000043).

For instance, in 2019, always in the frame of the LIFE project (LIFE18 ENV/NL/000043), an open-field trial was led on processing tomatoes (Quinto Vicentino, Veneto, Ital,). Water shortage had a significant effect on yield, in particular on the negative control (−30% irrigation) that showed a 34.1% reduction compared with well-watered control. However, the plants treated with 4-Vita showed a total yield statistically equal to the well-watered plants and even slightly anticipated fruits ripening. Moreover, 4-Vita increased the °Brix, indicating a steady maturation of the fruits (personal communication). Therefore, 4-Vita has shown striking evidence for improving resilience to abiotic stress, boosting water use efficiency (WUE), leading to up to 30% water savings in irrigated crops and a significant reduction of water deficiency symptoms, as well as an increased crop yield and quality in rain-fed crops in climatic zones with water deficiency [23]. Considering the lack of knowledge on the processes underlying such improved tolerance to drought following 4-Vita treatments, we aimed at unraveling the biochemical changes induced by this biostimulant, with reference to its mode of action, using the tomato as a model plant.

2. Materials and Methods

2.1. Plants Cultivation and Treatments

Money maker tomato seeds (from Sativa Biosaatgut GmbH, Jestetten, Germany) were grown in the nursery and were later transplanted into 180 pots (5 × 5 × 10 cm) filled with 80 g of peat moss (potting soil enriched in nutrients, Vigorplant—186 SER V14-20P). The plants were grown in a walk-in growth chamber where they remained for the whole duration of the trial. The growth chamber conditions were the following: temperatures

fluctuated between 18 °C (during the night) and 22.5 °C (during the day) as Figure 1, with a relative humidity of 60%.

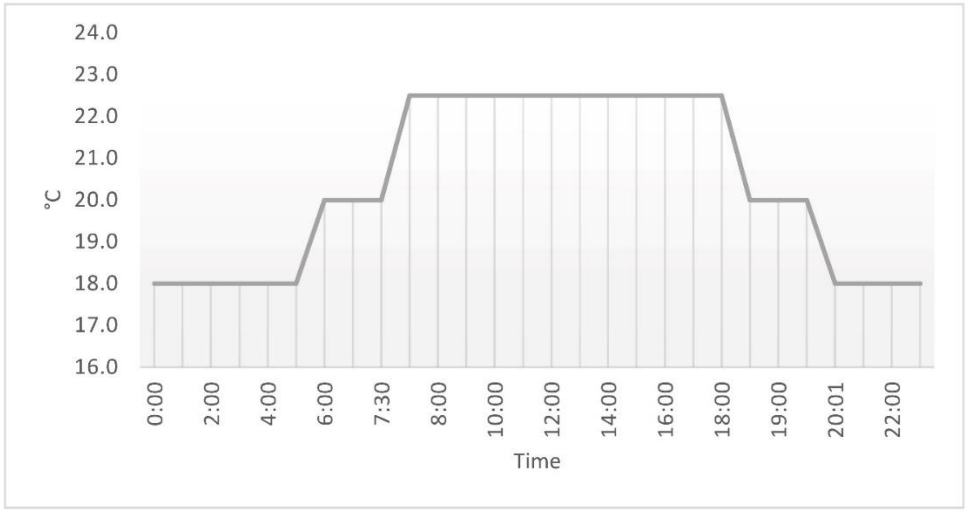

Figure 1. Temperatures cycle in the growing chamber over 24 h.

The light system, made of LED, reproduced the photosynthetically active radiation (PAR) typically measured in the north of Italy during the month of May (max PAR: 177.67 µmol of photons/m^2/s (60% intensity) as reported in the Figure 2.

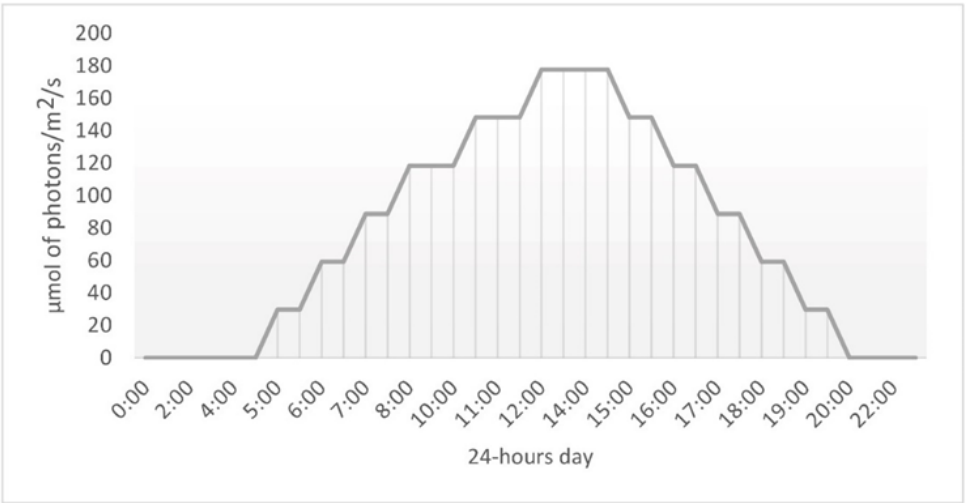

Figure 2. Photosynthetically active radiation in the growing chamber over 24 h (in µmol of photons/ m^2/s).

The plant nutrition was provided for all the growth cycle via fertigation, twice per week. The amount of NPK provided was 62.5, 19.5, and 97.7 mg/pot, respectively. The fertigation was performed using Universal Orange (16-5-25).

The first biostimulant (BST) application was performed once the plants reached the 4th leaf stage, using a high-performance sprayer with an application rate of 6.5 L ha^{-1} (as per the producer's instructions) and a carrier volume of 500 L ha^{-1}. The second application was performed a week later, following the same conditions of application.

The irrigation was provided on the basis of the plants request, in order to keep a field capacity around 0.85. A representative number of pots were weighed before planting, as reference, and were weighed before each irrigation. Prior to planting, the reference pots had been weighed dried and then saturated, to obtain the field capacity of the pots (saturated

− dry = maximum difference). Later, at each irrigation event, the water to restitute was calculated as follows:

$$((W_d + (W_s - W_d) \times 0.85) - W_i = W_r$$

W_d: weight dry pot.
W_s: weight saturated pot.
W_i: weight pot before irrigation.
W_r: amount of water to restitute per pot.

After completing the treatments cycle, a severe drought stress was imposed. The last irrigation was performed the day of the second BST application. No more irrigation was performed for 7 days. Only the positive control (well-watered) was regularly irrigated, always based on the plants request (field capacity = 0.85). After this period, a full irrigation was performed, until a complete recover of the plants. The plants were then grown two more weeks in comfort condition (both nutrients and water comfort).

2.2. Assessments

2.2.1. Visual Wilting Evaluation

A visual evaluation of the wilting of plant leaves was performed every day, starting from 4 days after treatment (DAT) until 7 DAT and at 4 days after recovery (DAR). The goal of this assessment was to monitor the development of stress symptoms. To describe this, a 0 to 5 scale was used and is shown below (Table 1).

Table 1. Visual assessment scale.

Wilting Value	Symptoms
5	Leaves in good health
4	Leaves starting to lose turgidity
3	Leaves starting to wilt
2	Leaves are strongly wilted
1	Leaves are completely wilted; plant close to death

2.2.2. Shoot Biomass

At 4 DAT and then 4 DAR, the shoot part was cut and weighed for 6 plants per entry when sampled for the metabolomics analysis. At 14 DAR, the shoots of the remaining plants were weighed, and the flowers/fruits were counted (18 plants per treatment).

2.2.3. Photosynthetic Activity

The photosynthetic activity was measured 4 DAT and 4 DAR, using a Fluorpen FP 100 (Photon Systems Instruments, Drásov, Czech Republic). The leaves were dark-adapted 20 min before the measurement. A dark-adapted pulse modulated fluorometry (PAM) fluorescence was performed at each assessment (chlorophyll fluorescence transient kinetics), which is one of the main methods for investigating PSII function and reactions under changing environmental and growth conditions. The Kautsky curve, also called OJIP curve, was computed at each assessment. This analysis allows the study of different parameters for the dark-adapted state of the photosynthetic systems. The following parameters were analyzed, according to the equations of the OJIP test: the maximum photochemical quantum yield of photosystem II (PSII) (Fv/Fm), the electron transfer efficiency caused by the captured excitons (Ψo), the quantum yield of electron transfer (φEo), the maximum quantum yield of nonphotochemical deexcitation (φDo), and the photosynthetic performance index (PI_Abs) were calculated.

2.2.4. Roots Surface via DIA

After removing the shoot part (14 DAR), the root part was separated from the pot together with the soil. For each of the four faces of the pot, a picture was taken. A box

built to put the object always at the same place and with constant light was used to obtain comparable pictures. The same parameters of the camera were used for all pictures (exposition). All the pictures collected were analyzed using WinCAM NDVI software (Regent Instruments Inc., Canada). This software tool allows a division by color, always maintaining the same set of colors and thus, it made it possible to calculate the roots surface.

2.2.5. Landlab Phenotyping Platform (LLPhP)

At 4 DAT, the plants were scanned through the Landlab Phenotyping Platform (LLPhP) in order to record the status of each plant at the sampling date. The LLPhP is based on a high-resolution 3D laser scanner (PlantEye F500, Phenospex B.V., Heerlen, The Netherlands) designed for plant phenotyping, hung on a high precision gantry, and moving above the plant in both directions (x and y). It is a high-throughput technology that allows one to scan plants and follow their modifications through time in different environments. The device can be used to assess morphological parameters such as digital biomass, maximum height, leaf angle, leaf area index (LAI), leaf inclination degree, and leaf area. The platform can also analyze physiological parameters such as the following: greenness (green leaf index), normalized difference vegetation index (NDVI), plant senescence reflectance index (PSRI), and normalized pigment chlorophyll ratio index (NPCI).

2.3. Untargeted Metabolomics Analysis

The untargeted metabolomics analysis was carried out following extraction, centrifugation (8000× g for 15 min) and filtration (cellulose membrane, 0.22 mm) as previously described [24]. To this aim, an ultra-high-pressure liquid chromatograph was coupled to a quadrupole time-of-flight mass spectrometer (UHPLC/QTOF-MS) from Agilent Technologies (Santa Clara, CA, USA), according to previously defined operative conditions [25]. In brief, liquid chromatography was carried out on a Pursuit 3 pentafluorophenyl column (2.0 × 100 mm, 3 mm, Agilent Technologies, Santa Clara, CA, USA), and separation was achieved through a 33 min gradient elution (6–94% acetonitrile LC–MS grade, VWR, Milan, Italy) and a flow rate 200 µL min−1. For detection, a SCAN MS acquisition (100–1000 m/z, 30,000 FWHM, 1 Hz) was adopted. The spectra were next processed in Agilent Profinder B.07, using the isotopic spacing and ratio "find-by-formula" algorithm (5 ppm mass accuracy for monoisotopic mass, 0.05 min for retention time alignment) to putatively annotate compounds, as previously described [26]. The database PlantCyc 12.6 (Plant Metabolic Network; release: April 2018) was used for compounds annotations [27]; the approach used allowed a Level 2 of confidence, with reference to COSMOS Metabolomics Standards Initiative [28].

2.4. Statistical Analysis and Data Interpretation

Metabolomics data were aligned, normalized, and baselined in Mass Profiler Professional B.12.06 (Agilent Technologies, Santa Clara, CA, USA) as previously described [24]. Therein, a fold-change (FC) analysis and a volcano plot analysis (an ANOVA with Bonferroni's multiple testing correction was combined with FC). The differential metabolites identified by the volcano plot were finally interpreted in the Omics Viewer Pathway Tool of PlantCyc (Stanford, CA, USA) for pathway analysis [29], and a Venn analysis was performed to investigate the similarities and dissimilarities of the metabolomic response across the treatments.

All morphological and physiological data were subjected to an analysis of variance (ANOVA) appropriate to the experimental design to evaluate the effects of treatments. XLSTAT software—Addinsoft (2021), XLSTAT statistical and data analysis solution; Paris, France—was used to conduct the analysis of variance. Means were significantly different at $p < 0.05$. The comparison of treatment means was carried out using the Duncan test at a significant level of $p \leq 0.05$.

3. Results

3.1. Plant Morphological and Physiological Changes

During the phase of water withdrawal, the plants were scored daily considering their wilting level. The effect of the drought stress started to be visible from 5 DAT (days after last treatment), 1 day after the sampling for metabolomic analysis, when the leaves started to lose turgor. Although at the beginning (5 DAT and 6 DAT), the level of wilting was similar between the treated plants and the negative control plants (UTC, drought), on the last day before recovery the treated plants showed less wilting compared to the negative control, suggesting that 4-Vita application induced the activation of some tolerant mechanisms in the plants. In fact, no difference in wilting between 6 DAT and 7 DAT can be seen in the treated plants, whereas the stressed plants showed a continuous increase in wilting, and, therefore, in water loss. At 4 DAR (days after recovery) some signs of wilting were still visible in both untreated and treated drought stressed plants, with no statistical difference (Table 2).

Table 2. Visual assessment of the wilting (scale going from 1 to 5, with 1 showing a plant completely wilted and 5 a plant in good health). For each trait, at least one letter in common indicates no significant difference.

Treatment	4 DAT *		5 DAT *		6 DAT *		7 DAT *		4 DAR **	
UTC, drought	4.91	a	4.30	a	3.61	a	2.83	a	3.78	a
4-Vita, drought	4.91	a	4.17	a	3.48	a	3.48	b	4.04	a
UTC, well-watered	5.00	a	5.00	b	5.00	b	5.00	c	5.00	b

* DAT: days after last treatment; ** DAR: days after recovery.

At 4 DAT, six plants per treatment were sampled for metabolomic analysis and their fresh biomass was assessed. At 4 DAR, the fresh biomass was assessed to evaluate the effect of the drought stress in the early recovery phase. The trends were similar to what was observed in the visual evaluation: at 4 DAT, no difference between the treatments could be found, while at 4 DAR, the biomass was significantly affected by the drought stress with no statistically significant difference between treated and untreated drought-stressed plants (Table 3).

Table 3. Average of the fresh biomass (FB, g) of 6 plants per entry at 4 DAT and 4 DAR. For each trait, at least one letter in common indicates no significant difference.

Treatment	4 DAT *		4 DAR **	
UTC, drought	13.5	a	12.2	a
4-Vita, drought	13.9	a	13.0	a
UTC, well-watered	13.7	a	16.1	b

* DAT: days after last treatment; ** DAR: days after recovery.

At 14 DAR, the fresh biomass, as well as the number of flowers and fruits and the root growth, were evaluated for all the plants (18 plants per treatment). Drought stress had a significant impact on the final biomass of the plant compared to well-watered plants (−13% for UTC, drought). However, treatment with 4-Vita partially recovered plant growth compared to the negative control (+7.7%). Moreover, the number of fruits and flowers was affected by drought stress. As a trend, this parameter was more impacted when 4-Vita was not applied. Roots growth was instead impacted by drought stress in a similar way in both treated and untreated plants (Table 4).

Table 4. Assessments at 14 DAR of shoot biomass (FB, g), flowers and fruits, and on % of roots on the remaining plants. For each trait, at least one letter in common indicates no significant difference.

Treatment	FB (g)		No. Flowers + Fruits		% Roots	
UTC, drought	26.0	a	4.6	a	5.1	a
4-Vita, drought	28.0	b	5.4	ab	5.9	a
UTC, well-watered	29.9	c	6.2	b	7.9	b

3.2. Photosynthetic Activity

On the same dates, different photosynthetic parameters were also evaluated. At 4 DAT, the value of Fv/Fm was still optimal (>0.75). However, treatment with 4-Vita slightly increased this parameter. At 4 DAT, the differences between plants were very small; however, the start of drought stress seemed to have slightly increased the electron transfer efficiency (Ψo) and quantum yield of electron transfer (φEo) compared to the well-watered plants. Treated plants showed a statistically significant lower φDo compared to the untreated plants in both comfort and noncomfort conditions, as well as the highest performance index, consistent with all the other parameters. At 4 DAR, the differences between the photosynthetic parameters among the treatments were not statistically significant, except for Ψo that was slightly higher for plants treated with 4-Vita (Table 5).

Table 5. Different photosynthetic parameters 4 DAT and 4 DAR. For each trait, at least one letter in common indicates no significant difference.

Treatment	4 DAT *					4 DAR **				
	Fv/Fm	Ψo	φEo	φDo	PI_Abs	Fv/Fm	Ψo	φEo	φDo	PI_Abs
UTC, drought	0.76 b	0.50 a	0.38 a	0.24 a	0.90 b	0.78 a	0.45 ab	0.35 a	0.22 a	1.03 a
4-Vita, drought	0.79 a	0.48 a	0.38 a	0.21 b	1.30 a	0.76 a	0.49 a	0.38 a	0.24 a	0.99 a
UTC, well-watered	0.75 b	0.43 b	0.32 b	0.25 a	0.73 b	0.76 a	0.41 b	0.32 a	0.24 a	0.85 a

* DAT: days after last treatment; ** DAR: days after recovery.

3.3. Parameters LLPhP at 4DAT

The following parameters were assessed with the LLPhP 4 DAT. The greenness index (Figure 3A) represents the relation between the reflectance in the green channel compared to the other two visible light channels (red and blue). The normalized differential vegetation index (NDVI) (Figure 3B) is an indicator of the plant greenness and of the global health state of the plants. NPCI (Figure 3C) is the normalized pigment chlorophyll ratio index and PSRI (Figure 3D) is the plant senescence reflectance index.

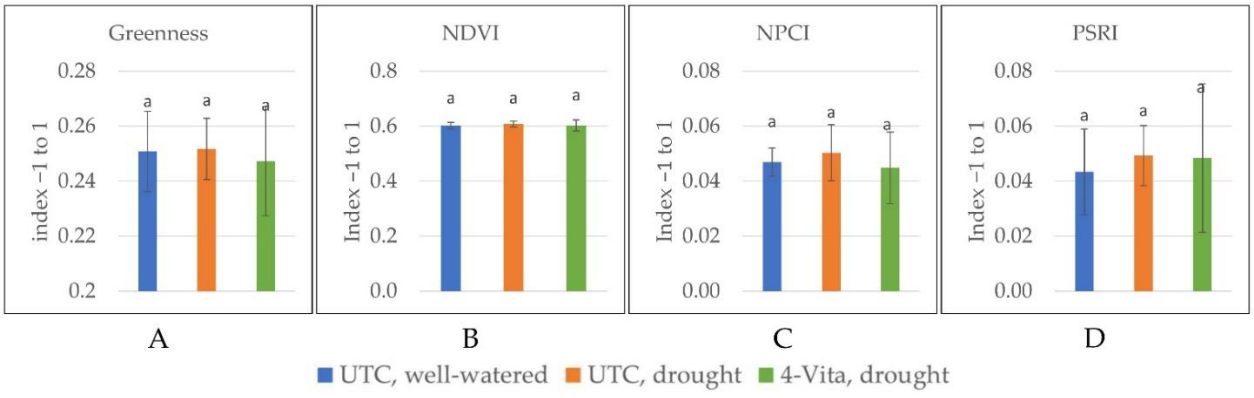

Figure 3. Physiological parameters observed 4 DAT by the LLPhP (scanning platform). (**A**) Greenness (green leaf index, GLI), (**B**) NDVI, (**C**) NPCI, (**D**) PSRI. For each trait, at least one letter in common indicates no significant difference.

The morphological parameters analyzed were digital biomass (Figure 4a), height (Figure 4B), leaf angle (Figure 4C), leaf area index (Figure 4D), leaf inclination (Figure 4E), and leaf area (Figure 4F). None of these morphological parameters showed a statistical difference between the different conditions.

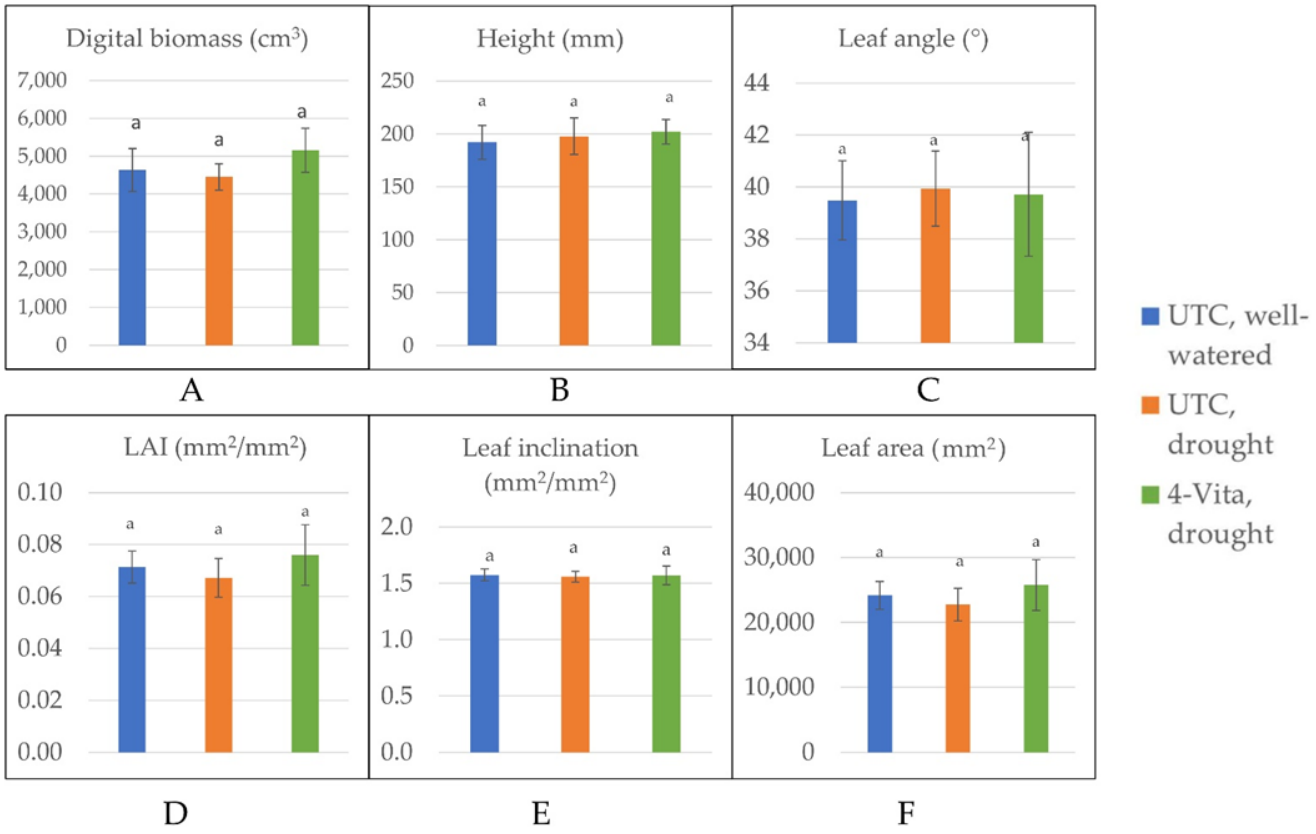

Figure 4. Morphological parameters observed 4 DAT by the LLPhP (scanning platform). (**A**) Digital biomass, (**B**) height, (**C**) leaf angle, (**D**) LAI (leaf area index), (**E**) leaf inclination, (**F**) leaf area. For each trait, at least one letter in common indicates no significant difference.

3.4. Effect of the Treatment on Metabolomic Profiles under Drought Stress Conditions

An untargeted metabolomics approach was performed using a hybrid quadrupole time-of-flight mass spectrometer coupled to a UHPLC chromatographic system (UHPLC-ESI/QTOF-MS) to investigate plants' metabolic responses induced by the 4-Vita treatment on tomato plants subjected to drought stress. The metabolomic analysis was performed on sampled tomato leaves after 3 days from the last irrigation or 4 days from the last treatment. Overall, the method allowed us to detect 572 metabolites, and a comprehensive list of compounds is provided in the Supplementary Materials, including ontology classification, picks abundances, and mass composite spectrum (Table S1). To investigate the mechanism of action of 4-Vita on tomato plants under drought stress, an online tool, Omics Dashboard from PlantCyc, was employed. Specifically, the entire metabolites in the dataset were investigated for their significance by using an ANOVA statistical analysis ($p < 0.05$) coupled with a fold-change analysis (FC) with a fold-change threshold of 2. A complete list of compounds passing the ANOVA and FC analysis is provided in the Supplementary Materials (Table S2).

The resulting omics dashboard for the biosynthesis metabolism is reported in Figure 5 and the summary pathway in Table 6. In the table, the metabolites found to be differentially accumulated are expressed as Log fold change (Log FC) compared to the well-watered plants.

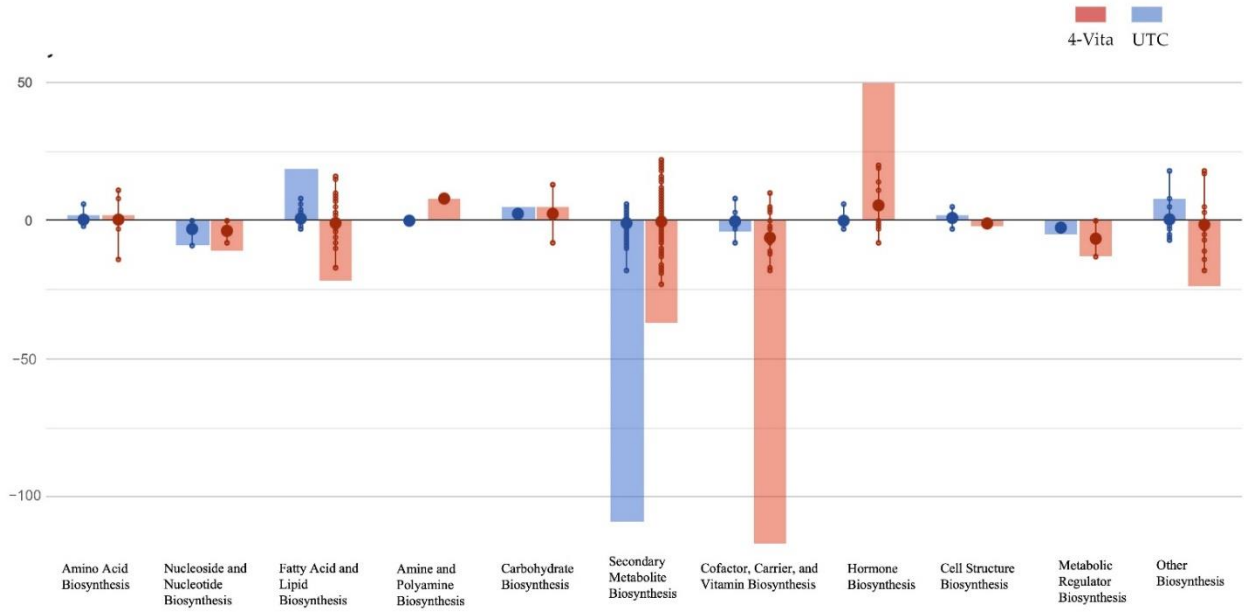

Figure 5. Metabolic biosynthetic processes modulated in tomato leaves following the treatments with 4-Vita under drought conditions, compared to untreated control (UTC). The *x*-axis represents each set of metabolic subcategories, while the *y*-axis corresponds to the accumulative Log fold change (Log FC). The large dots represent the average (mean) of all Log FCs in the class, whereas small dots represent individual Log FC values. Abbreviations: AA: amino acid, Nucleo: nucleotide, FA/Lip: fatty acid and lipid, Carbo: carbohydrate, Sec Metab: secondary metabolites, Cell struct: cell structure, Metab Reg: metabolic regulation, Syn: synthesis.

Table 6. Biosynthetic processes modulated by 4-Vita in tomato plants under drought stress, compared to untreated control (UTC).

Compounds	Avg Log FC		Sum Log FC		No. of Compounds Log FC > 2		No. of Compounds Log FC < −2		No. of Compounds Modulated	
	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought
Amino acid biosynthesis	0.28	0.49	1.40	2.43	1	2	2	2	3	4
Nucleoside and nucleotide biosynthesis	−3.11	−3.89	−9.32	−11.67	0	0	1	2	1	2
Fatty acid and lipid biosynthesis	0.82	−0.95	20.55	−23.73	7	11	4	10	11	21
Amine and polyamine biosynthesis	0.07	8.32	0.07	8.32	0	1			0	1
Carbohydrate biosynthesis	3.18	2.49	6.35	4.97	2	1	0	1	2	2
Secondary metabolite biosynthesis	−0.99	−0.43	−120.87	−52.31	21	45	37	50	58	95
Cofactor, carrier, and vitamin biosynthesis	−0.34	−6.40	−6.37	−121.62	2	4	4	12	6	16
Hormone biosynthesis	−0.04	5.61	−0.39	50.46	1	4	2	3	3	7
Cell structure biosynthesis	1.24	−1.33	2.48	−2.65	1	0	1	1	2	1
Metabolic regulator biosynthesis	−3.03	−6.75	−6.06	−13.49	0	0	2	1	2	1
Other biosynthesis	0.41	−1.53	6.99	−25.98	3	6	5	11	8	17

Data are expressed as cumulative Log fold-change (FC) values.

A general overview indicated that the drought stress modulated the metabolites profile of tomato plants, mainly in the biosynthetic pathways. Interestingly, this modulation was highly different in 4-Vita-treated plants. Based on our finding, the treatment with 4-Vita significantly and distinctively shaped the metabolomic profile of the plants compared to

droughts stressed. As reported in Table 6, 167 discriminant metabolites were reported modulated by the 4-Vita treatment under drought stress, compared to 96 metabolites in the nontreated ones, suggesting a fastest and higher rearrangement of the metabolic pathways. Of these metabolites, only 60 were commonly modulated, while 19 showed the opposite behavior, 88 were modulated only by 4-Vita-treated plants, and 17 were only modulated in negative control plants (Figure 6).

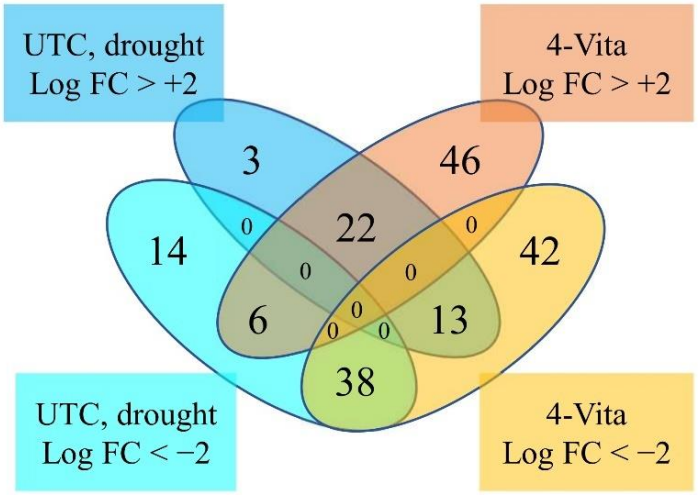

Figure 6. Venn diagram showing up- and downaccumulated metabolites under either well-watered or drought conditions, following application of 4-Vita to the tomato.

The biosynthetic pathways most affected by treatments were represented by fatty acid and lipid biosynthesis, secondary metabolism, hormones, and cofactor biosynthetic pathways (Figure 5). Particularly, the drought stress condition induced a downaccumulation in secondary metabolism compounds, specifically the nitrogen-containing metabolites—i.e., alkaloids and glucosinolates, as well as phenylpropanoids, phytoalexins, and terpenoids—and an upaccumulation in the fatty acid and lipids, which were mainly characterized by phospholipids. Regarding the treated plants with the 4-Vita product, a relevant modulation in phytohormone, secondary metabolites, and the cofactor, carrier and vitamin biosynthesis was observed, as well as other biosynthesis such as porphyrin and chlorophyll metabolism.

3.4.1. Fatty Acid and Lipid Biosynthesis

The fatty acid and lipid biosynthesis pathways were highly modulated by drought stress. Specifically, a different trend has been reported among plants subjected to drought stress (upmodulation) and drought stress with 4-Vita treatment (downmodulation). The drought stress caused an up-accumulation of phosphatidylglycerol (PG; sum Log FC 11.30). However, the treatment with 4-Vita improved this quantity by threefold (sum Log FC 36.33). Regarding the class of galactosyldiacylglycerol lipids, the monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were the most affected lipids by the treatment with 4-Vita, showing values of sum log FC equal to −52.99 and −10.47, respectively (Table 7). Moreover, the treatment with 4-Vita produced an increase in the 18:3 polyunsaturated MGDG (Log FC 7.06). The treatment with 4-Vita also produced a relevant increase in steroid lipids accumulation (sum Log FC 25.9) compared to the nontreated one (sum Log FC 3.6).

Table 7. Fatty acid and lipid biosynthesis processes modulated by 4-Vita in tomato plants under drought stress conditions, compared to untreated control (UTC, well-watered).

Compounds	Avg Log FC		Sum Log FC		No. of Compounds Log FC > 2		No. of Compounds Log FC < −2		No. of Compounds Modulated	
	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought
MGDG	0.08	−7.57	0.54	−52.99	0	1	0	4	0	5
DGDG	0.08	−10.47	0.08	−10.47			0	1	0	1
PG	2.26	7.27	11.30	36.33	3	5	1	0	4	5

Data are expressed as Log fold-change (FC) values. Abbreviation: MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; PG: phosphatidylglycerol.

3.4.2. Secondary Metabolite Biosynthesis

The secondary metabolites biosynthesis was detected to be the most affected by plants subjected to drought stress and after 4-Vita application. Overall, 58 metabolites were included in response to drought stress, while the application of 4-Vita treatment stimulated 95 metabolites, suggesting a different mechanism of action. Indeed, a general downmodulation of the secondary metabolism was observed in response to stress; however, the 4-Vita-treated plants showed a lower decrease (Figure 4 and Table 6).

In detail, the drought stress induced a downaccumulation in nitrogen-containing metabolites and terpenoids, followed by a downmodulation in phenylpropanoids and phytoalexins compounds (Table 8). Notably, we observed a variation in the quantity of alkaloids (mainly in magnoflorine and quinidinone), L-dehydroascorbate, and glucosinolates (glucoiberverin).

Table 8. N-containing compounds, phytoalexins, phenylpropanoids, and terpenoids modulated by 4-Vita in tomato plants under drought stress conditions, compared to untreated control (UTC).

Compounds	Avg Log FC		Sum Log FC		No. of Compounds Log FC > 2		No. of Compounds Log FC < −2		No. of Compounds Modulated	
	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought
N-containing compounds	−1.43	1.45	−51.45	52.21	4	13	14	13	18	26
Phenylpropanoid derivative biosynthesis	−0.08	−1.78	−2.20	−46.15	10	8	7	12	17	20
Phytoalexin biosynthesis	−1.30	3.65	−7.78	21.87	1	3	3	2	4	5
Terpenoid biosynthesis	−1.12	−1.11	−71.61	−70.88	6	24	17	29	23	53

Data are expressed as Log fold-change (FC) values.

Regarding 4-Vita-treated plants, the nitrogen-containing and phytoalexins accumulated while phenylpropanoids and terpenoids downaccumulated. Moreover, in this case, the alkaloids were the class of compounds most influenced with a strong upaccumulation of 2-descarboxy-cyclo-dopa and vanillylamine. Among alkaloids, the reduction of L-dehydroascorbate observed could be implied in lower oxidative stress. Among others, flavonoids, mainly anthocyanins, and terpenoids were negatively affected by the 4-Vita treatment, similar to what was observed in drought-stressed samples. However, among the terpenoids class of compounds, adonixanthin and enochinone were reported highly downmodulated (Log FC −19.9 and −18.6, respectively), with a consequently positive modulation of canthaxanthin (Log FC +3.8), suggesting the activation of the xanthophyll pathways activity (Table 9). The phytoalexins compounds oryzalide, camalexin, and kauralexin were upmodulated in 4-Vita-treated plants.

Table 9. Terpenoids compound: alkaloids, xanthin and anthocyanins modulated by 4-Vita in tomato plants under drought stress conditions, compared to untreated control (UTC).

Compounds	Avg Log FC		Sum Log FC		No. of Compounds Log FC > 2		No. of Compounds Log FC < −2		No. of Compounds modulated	
	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought	UTC, Drought	4-Vita, Drought
Alkaloids	−0.36	2.13	−6.88	40.4	4	9	5	6	9	15
Xanthin	−3.04	−3.90	−51.64	−66.24	0	6	8	9	8	15
Anthocyanins	−0.43	−7.65	−2.17	−38.24	0	0	2	4	2	4

Data are expressed as Log fold-change (FC) values.

3.4.3. Phytohormone Biosynthesis, Cofactor, and Other Biosynthesis Pathways

The application of 4-Vita produced a more extensive modulation of phytohormones compared to drought stress alone. Specifically, the treatment with 4 Vita increased the levels of brassinosteroids, gibberellins, and jasmonate-associated compounds, although the number of involved compounds was much lower than the other categories, as reported in Figure 7. However, a downaccumulation was observed for compounds involved in the ethylene, cytokinin (antagonists of auxins in root development), and abscisic acid synthesis (inhibitor of root elongation).

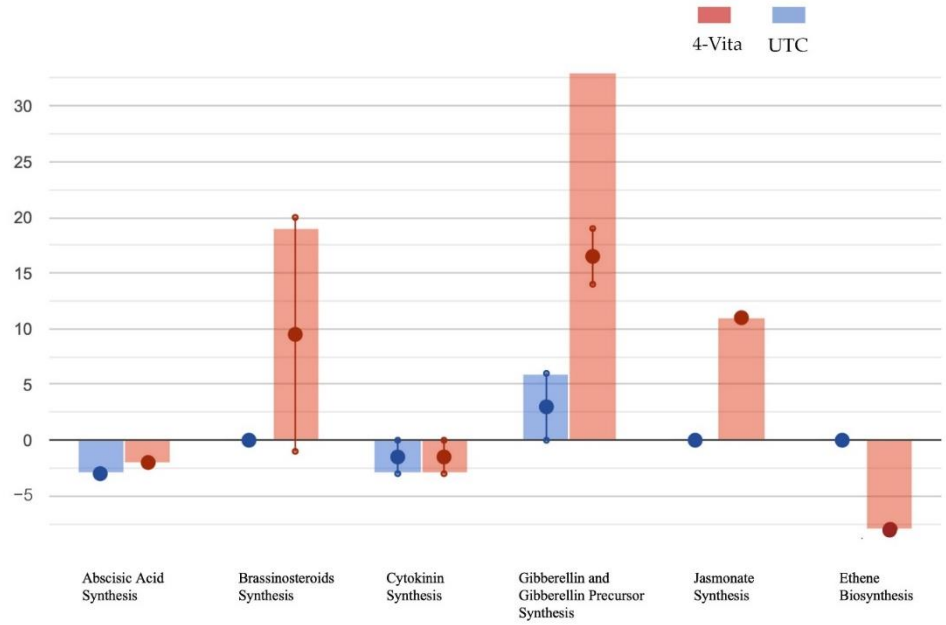

Figure 7. Phytohormones being modulated in tomato leaves following the treatments with 4-Vita conditions, compared to untreated control (UTC). The *x*-axis represents each set of metabolic subcategories, while the *y*-axis corresponds to the accumulative Log fold change (Log FC). The large dots represent the average (mean) of all Log FCs in the class, whereas small dots represent individual Log FC values. Abbreviations: Syn: synthesis.

The cofactor category showed a dramatic decrease in 4-Vita-treated plants, with two-thirds of the compounds involved downmodulated. The change in these compounds was less extreme in the UTC, drought (Table 6). The 4-Vita-treated plants showed a decrease in cofactors involved mainly in the ABA biosynthesis while they showed an increase in cofactors involved in the NAD+ biosynthesis/salvage pathway (increase β-nicotinate D-ribonucleotide (NaMN), no decrease nicotinamide). The untreated plants showed a downaccumulation of nicotinamide.

Moreover, 4-Vita increased the levels of S-adenosyl 3-(methylsulfanyl)propylamine (dAdoMet), which serves as an aminopropyl donor for the synthesis of polyamines that are

known to protect plant cells from the damages of drought by enhancing the antioxidant defense system, restricting ethylene synthesis, maintaining cell pH and ion homeostasis, and preventing chlorophyll loss [30,31].

The metabolites involved in the porphyrin metabolism were differentially modulated as well [32]. In higher plants, Chl a and b serve as the major and minor antenna pigments in the light harvesting complexes (LHCs), while only Chl a are bound to the core antenna complexes [33]. UTC, drought plants showed a loss of chlorophyll a (Chl a, Log FC −5.6), while chlorophyll b (Chl b) was not affected, whereas 4-Vita-treated plants showed a loss of both Chl a (Log FC −5.4) and b (Log FC −3.1).

4. Discussion

Climate change is a major threat to global food security and food availability worldwide [34]. The main reason is that environmental stresses affect morphophysiological traits, biochemical signaling, and molecular changes, consequently reducing plant growth and productivity [35]. Two different types of stresses threaten global food security, i.e., biotic and abiotic stresses. Concerning abiotic stresses, a drought triggers the activation of downstream pathways, mainly through phytohormones homeostasis and their signaling networks, which further involves the phytochemical accumulation and secondary metabolites production. Stress-tolerance mechanisms are activated at the molecular level, downstream producing physiological and morphological changes.

The lack of statistical differences in the physiological and morphological parameters at 4 DAT showed that at the early beginning of drought stress, there was still no visible differences between the plants. This was also confirmed by the visual evaluation of the wilting, and by the fresh biomass of the plants taken at this time point. However, signals of drought stress were already present, as shown by the metabolomic analysis.

Accordingly, in this study, tomato plants subjected to drought showed a downmodulation of secondary metabolites, cofactors, carriers, and vitamins biosynthesis, and an up modulation of fatty acids and lipid biosynthesis, well-known to play an important role in drought resistance [36]. Drought stress, as well as other stresses, induces the stimulation of the calcium signaling as an early response to stress in plants, followed by the generation and accumulation of reactive oxygen species (ROS) and nitrogen–oxygen species (NOS) [37]. Membrane potential changes and accumulation of signal molecules have the purpose of activating the downstream drought-specific phytohormones, osmolytes, secondary metabolites, antioxidants, and polyamines pathways, and this phase was referred to as active responses [38]. Indeed, drought stress conditions significantly altered the metabolism of hormones biosynthesis of tomato, compared to well-watered plants. However, the treatment with 4-Vita reported an upmodulation of the main phytohormones involved in the drought stress (Figure 7). The brassinosteroids, gibberellins, abscisic acid, jasmonates, and their precursors were found highly modulated by the treatment with 4-Vita under drought stress. As known, brassinosteroids are required for plant growth and development and gibberellin in the stimulation of cell elongation and division [39]. They are involved in the signaling pathways of plants adaptation and survival under environmental stresses [40,41]. The critical role of endogenous brassinosteroids in drought tolerance was assessed in maize, where differences in morphology, water content, photosynthesis activity, and protection against cell damage were reported in drought-tolerant cultivars, compared to more sensitive cultivars. The drought-stress-tolerant cultivar reported a high accumulation of typhasterol, an intermediate involved in the biosynthesis of brassinolide [42]. Accordingly, the metabolite 3-dehydro-6-hydroxyteasterone, involved in the same biosynthetic pathway of brassinolide, was found upaccumulated in plants treated with 4-Vita, suggesting a relevant improvement of drought stress by the action of the 4-Vita treatment. Indeed, a reduced level of brassinosteroids leads to a lower improvement of growth parameters, osmolytes production, antioxidants capacity, and photosynthesis activities, as noted in the plants nontreated with 4-Vita (Tables 4 and 5).

Different works reported the effect of abscisic acid treatment under drought in the synthesis of chlorophyll [43]. Abscisic acid is a terpene synthesized from carotenoids (C30) through the methylerythritol phosphate pathway, and it was found downmodulated under drought stress and treatment with 4-Vita. Upon drought stress, abscisic acid signaling is able to reduce water loss due to transpiration by modulating stomatal closure and increasing root cells elongation, allowing plants to capture more water [44]. This study pointed out the relationship between abscisic acid and NAD+ homeostasis, modulation of stomatal development, and thus the final responses to drought. The correlation was observed using a nadk2-null A. thaliana line, depredated by the gene coding for NAD kinase 2. The mutant nadk2-null showed a compromised abscisic acid-mediated generation of messengers promoting stomatal closure, suggesting the important function of NAD+ homeostasis on upstream and downstream regulation of abscisic acid. The upstream regulation involves the NAD+ as a cofactor of abscisic acid biosynthesis, while the downstream regulation involves abscisic acid content in regulating target genes related to the biosynthesis of NAD+ [45]. In accordance with this observation, the treatment with 4-Vita on tomato plants under drought stress reported a relevant upaccumulation of β-nicotinate D-ribonucleotide, the precursor of NAD+ biosynthesis, as well as the modulation of abscisic acid biosynthesis, confirming the activation of mitigating mechanisms through the regulation of phytohormones.

The phytohormones crosstalk led to the activation of different biosynthetic pathways involved in the stress-responsive action to mitigate drought stress [46–48]. As a product of the phytohormones-signaling cascade, a relevant modulation in the fatty acid and lipid and secondary metabolites biosynthesis was observed in our experiments. The lipid profile was highly modulated in terms of fatty acids, glycolipids, phospholipids, and sterols biosynthesis (Table 6). Particularly, an intensive modulation was observed for monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and phosphatidylglycerols (PG) (Table 7). These lipids are the main component of the thylakoid membrane and have a highly conserved composition, together with negatively charged lipids like sulfoquinovosyl diacylglycerol [49]. Thylakoid lipids are the site of photosystems I and II allocation and play important roles in the photodriven photochemical reactions and electron transfer in plants. It is well known that plants species respond to drought stress by altering the thylakoid membrane composition, particularly in the content of membrane polar lipids such as phosphatidylglycerol [50]. Accordingly, tomato leaves treated with 4-Vita dramatically increased phosphatidylglycerol levels, while they remained stable in the drought-stressed plants. The importance of phosphatidylglycerol was also confirmed by Kobayashi et al., (2015) [51], suggesting their essential function in the stabilization of both photosystems (I and II) due to their charged polarity [50]. Another feature that supports the tolerance to drought stress of plants treated with 4-Vita is an increased DGDG:MGDG ratio [52]. In fact, although both galactosyldiacylglycerol lipids were decreased in both stressed plants, the decrease in MGDG was more significant than in DGDG, thus producing a higher DGDG:MGDG ratio in 4-Vita treated plants, implying better tolerance to drought stress.

The biosynthesis of secondary metabolites under drought stress involves different routes such as shikimic acid, malonic acid, mevalonic acid, and methylerythritol phosphate pathway for providing protection and adaptation to stress. Accumulation of secondary metabolites such as isoprenoids, polyphenols, alkaloids, and flavonoids resulted in an enhancement of plant stress tolerance [53] Accordingly, the drought stress negatively modulated the biosynthesis of nitrogen-containing compounds, phenylpropanoids and derivatives, phytoalexins, and terpenoids. However, the treatment with 4-Vita could mitigate that effect by eliciting defense-related secondary metabolites such as nitrogen-containing and phytoalexin compounds (Table 8). Several studies have reported an enhancement of phenylpropanoids compounds in response to drought stress. For example, anthocyanins, rutin, quercetin, and betulinic acid accumulate in drought-tolerant plants [54]. However, in our results, these compounds were reported highly downmodulated in plants treated with 4-Vita. This suggests that the 4-Vita mechanism of action involves different pathways

than that of phenylpropanoids. Indeed, the treatment stimulated the biosynthesis of other secondary metabolites such as phytoalexins, alkaloids, and their derivatives. The latter are reported to be involved in drought stress response in stressed Catharanthus roseus plants by the action of vincristine and cephaeline [55,56]. Interestingly, phytoalexins are highly involved and accumulated in response to biotic stress; nonetheless, different papers have shown their accumulation in abiotic stress [57–59].

In particular, the accumulation of phytoalexins in maize roots was found to be associated with drought tolerance (mutant an2 plants deficient in kauralexin production are more sensitive to drought [58]). Therefore, this may be a signal of activation of tolerance mechanisms. The downaccumulation of terpenoids in our experiment is related to compounds involved in the xanthophyll biosynthetic pathways. Indeed, adonixanthin and enochinone metabolites were reported strongly downaccumulated in 4-Vita-treated plants with a consequently upaccumulation of canthaxanthin. Carotenoids play a pivotal role in protecting the photosystems from oxidative stress, and this is consistent with the aforementioned modulation of lipids biosynthesis. Together with canthaxanthin, anteraxanthin was found upaccumulated and thus involved in the protection of the photosystem [60]. Unlike mammals, the chloroplast is the main source of ROS in photosynthetically active cells, being connected with multiple redox reactions and electron transport chains localized in thylakoid membranes. Singlet oxygen (1O2) is a unique ROS species produced constitutively in plant leaves in light. Chlorophylls pigments in the antenna system and the reaction center of photosystem II are primary sources of 1O2 in plants, formed in a reaction between the molecular oxygen and chlorophyll molecules in the triplet state. The loss of only chl a and not chl b in the UTC, drought plants suggests that the core is disrupted, and not the LHCs. In plants treated with 4-Vita, the loss of both chl a and b suggests the possibility of a loss of core + LHC, or only LHC. Interestingly the decrease in amount of these metabolites keep a ratio close to the ratio of Chla/Chlb in LHCs, suggesting a reorganization of the photosynthetic apparatus in order to overcome the environmental condition. A lower amount of LHC, in fact, reduces the amount of harvested light and excitation of the photosystem leading to a lower amount of water needed for the photosynthetic reaction and less production of ROS.

Our biostimulant treatment preserved the functionality of the photosynthetic machinery through a coordinated action of thylakoid lipids remodeling, ABA-dependent NAD+ availability, and carotenoid content. In fact, a well-maintained photosynthesis during drought should be a key requirement for drought tolerance, and as seen in this study, slight differences in photosynthesis efficiency were observed (Table 5). Many studies used the analysis of the fluorescence properties of chlorophyll a (Chl a) in photosystem II (PSII) to investigate the physiological aspects of photosynthesis in plants under drought stress [33,61]. One of the main methods for investigating PSII function and reactions under changing environmental and growth conditions is the chlorophyll fluorescence induction curve (OJIP) [62–65]. In this study, the application of 4-Vita seemed to improve the activity of the PSII in the early drought phase (Table 5), which prepared the plants to the drought stress. Indeed, one studied parameter of the OJIP curve, the Fv/Fm, that measures the proportion of light absorbed by chlorophyll associated with PSII [66], was improved with the application of 4-Vita. This ratio is frequently used as an indicator of the photoinhibition or other kind of injury to the photosystem II caused by the growth conditions; it tends to decrease for plants under drought stress and it is the most widely used parameter to assess drought stress. Other parameters were seen to be improved with the application of the biostimulant in early drought stress; the maximum quantum yield of nonphotochemical deexcitation (φDo), which indicates the energy dissipation as thermal energy leading to downregulation of the PSII activity, was lower in plants treated with 4-Vita; thus, a lower amount of energy was dissipated. Hence, a higher amount of energy was used by photochemistry, and was therefore favorable for plants growth [33]. As a further proof, the photosynthetic performance index (PI_Abs) was higher in 4-Vita-treated plants.

5. Conclusions

The biostimulant tested allowed a broad reprogramming of the tomato's secondary metabolism that ultimately resulted in increased resilience to the detrimental effects of drought. Metabolomics highlighted a series of mechanisms in response to the biostimulant, such as the modulation of membrane lipids, the increase in xanthin compounds involved in ROS detoxification, and the modulation of chlorophylls synthesis. Considering that photosynthesis is the main source of oxidative stress in plants, its preservation decreases ROS production, thus reducing the need to activate the antioxidant system and supporting plant growth. Consistently, photosynthetic performance at the level of PSII was promoted by the treatment, in particular at earlier stages of drought stress. The present work contributes to the current scientific background on the mode of action of biostimulants, and paves the way towards future studies and applications in the biostimulant industry. Future research should aim at better identifying and understanding the processes resulting from the various metabolic and biochemical processes in plant-stress–biostimulant interactions. The comprehension of such mechanisms will allow the definition of optimized strategies towards a more sustainable low-input agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040764/s1, Table S1: Dataset; Table S2: Volcano compounds.

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