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A new protein hydrolysate-based biostimulant applied by fertigation promotes relief from drought stress in *Capsicum annuum* L.

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ABSTRACT

Recently, biostimulants have been used in sustainable agriculture as priming agents able to increase crop tolerance to abiotic stressors. Here, a soil application of GHI_16_VHL, a plant protein hydrolysate-based biostimulant, was tested for its capability to mitigate severe water stress effects on *Capsicum annuum* at flowering time. The biostimulant influence on plant physiological status was monitored upon stress and its relief, by measuring chlorophyll levels, stomatal density, stem water potential, leaf gas exchanges and plant growth. Moreover, leaf osmoregulation and oxidative stress levels were also evaluated by quantifying free proline, total non-structural carbohydrates (NSC), ROS-scavenging activity and H_2O_2 level.

Although biostimulant-primed plants showed a quicker decrease of stem water potential with respect to untreated plants upon drought imposition, they recovered faster probably due to the higher leaf osmolyte accumulation, namely NSC during drought. Moreover, leaf gas exchange recovery was prompted in biostimulant-treated plants, which showed an incremented stomatal density and the same chlorophyll level of well-watered plants at the end of the recovery phase. Hydrogen peroxide level was significantly lower during stress and early recovery in biostimulant primed plants, probably due to the higher catalase activity in treated plants before drought or to the higher level of non-enzymatic antioxidant scavengers in primed stressed plants. Finally, the biostimulant priming increased aboveground relative growth rate and final fruit yield of stressed plants. Taken together, our data suggest that the biostimulant priming treatment promotes a faster and more efficient plant recovery after drought.

1. Introduction

Nowadays, drought periods are getting more frequent and spread worldwide due to global climate change (Swann, 2018). Water stress interferes with plant physiological processes involved in nutrient and water relations, photosynthesis and assimilate partitioning, thus impairing plant health and growth, particularly affecting crop qualitative and quantitative productivity (Fathi and Tari, 2016). Thereby, one of the major challenges of modern agriculture is the improvement of crop productivity by promoting crop tolerance and resilience to drought stress (Fahad et al., 2017).

Drought effects on plants depend on its severity, duration, and timing along plant developmental stages (Farooq et al., 2009). One of the first drought-induced impairment is related to the overproduction of reactive oxygen species (ROS) in cell organelles, resulting in the induction of oxidative stress through the peroxidation of cellular membranes and the degradation of proteins and nucleic acids (Fathi and Tari, 2016). Plants can respond to drought by inducing tolerance mechanisms. These consist of physiological and biochemical processes whose activation at molecular, tissue, organ, and whole-plant levels is relatively fast (Yordanov et al., 2003). Responses to water stress include: minimizing water losses through regulation of stomatal closure and production of small leaves; improving water uptake by promoting the root system growth (and/or enhancing the root hydraulic conductivity) and accumulating osmoprotective substances (Osakabe et al., 2014). Moreover, scavenging of ROS by enzymatic and nonenzymatic mechanisms, cell

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osmoregulation, and expression of stress proteins are particularly involved in drought tolerance (Abass et al., 2017).

Recently, it has been shown that chemical priming can be an efficient practice to improve plant drought stress tolerance (Kerchev et al., 2020). It involves plant exposure to very low and not toxic concentrations of natural or synthetic priming agents before an up-coming stress. When priming occurs, plants can be 'prepared' to more successfully tolerate future stress conditions due to the fact that they enter the primed state (PS), in which activation of stress-related responses is faster or stronger when a stress pressure is faced (Kerchev et al., 2020). Plants can therefore acclimate to the drought events by modifications at morphological, metabolic, subcellular, proteomic and transcriptional levels (Wojtyla et al., 2020).

Plant biostimulants can be applied as priming agents (Kerchev et al., 2020) and due to their natural origin (especially waste-derived substances) they can be considered a potentially novel sustainable agriculture technology (Xu and Geelen, 2018). Since they exert positive effects on plant metabolism both in optimal and sub-optimal environmental conditions, they can stimulate plant growth, increase plant yield and mitigate stress-induced limitations (Bulgari et al., 2019; Drobek et al., 2019). However, their foliar or soil application can trigger different responses in plants (du Jardin, 2015). Foliar spray is generally applied to achieve a relatively short-term response, whereas soil application is used when a long-term effect is desired (Paul et al., 2019). Among the different biostimulant typologies, protein hydrolysate-based biostimulants (PHs) have been largely studied to counteract drought stress effects when applied at leaf level (Colla et al., 2017), but they seem to be more efficient in promoting plant growth under adverse conditions especially when applied by fertigation (Lucini et al., 2015; Sestili et al., 2018). Indeed, PHs can modulate leaf gas exchanges and water use efficiency (Van Oosten et al., 2017) and usually provide greater photosynthetic efficiency by increasing total chlorophyll index (Van Oosten et al., 2017). Moreover, they can promote osmolyte and osmo-protectant accumulation (e.g. proline), and they interfere with oxidative stress response by increasing the level of plant enzymatic and non-enzymatic antioxidant systems (Van Oosten et al., 2017). Despite several studies focused on PH effects during drought stress occurrence, few information is available on biochemical and physiological mechanisms promoted by this biostimulant class on plant recovery after drought exposure (Colla et al., 2015). Plant recovery consists of plant rehydration after a stress period, and it ends when the plant physiological status resembles that of well-watered plants. However, the recovered physiological status can be similar to that of not-primed plants or can be different, thus affecting the allocation of resources into growth or future faster responses to an abiotic stressor (Kollist et al., 2019). Consequently, the study of the recovery phase represents a critical window to determine biostimulant action as priming chemicals (Crisp et al., 2016).

Capsicum annuum L. is a crop of high economic importance especially in the Mediterranean area, but in the last three decades, sweet pepper cultivation has progressively become dependent from greenhouses due to its high susceptibility to water stress conditions (Ferrara et al., 2011). Drought stress adversely affects productivity of sweet pepper plants, especially when occurring during its flowering stage (Ferrara et al., 2011). Considering that pepper is one of the world's most important vegetable, the search for new agricultural strategies able to retain its yield despite drought events is one of the most important challenges for agricultural research (Sanusi and Ayinde, 2013). PHs have already been studied for their beneficial effects on pepper crop quality. In particular, they were shown to enhance carotenoid, flavonoid, and ascorbic acid content when applied at leaf level (Ertani et al., 2014). However, little is known about PH effects on pepper yield, drought tolerance and recovery after stress relief when applied at root level.

In the present study, we evaluated the effects derived from the soil application of a commercial PH on *Capsicum annuum* at flowering time and before the occurrence of severe water stress. The biostimulant was tested for its potential priming activity by evaluating its beneficial influence both on plant drought tolerance and on recovery efficiency after stress relief. In order to accomplish these purposes, biometric, anatomical (stomatal density), physiological (chlorophyll content, leaf gas exchanges and stem water potential), and biochemical (osmolyte accumulation and ROS scavenging system) parameters were monitored on both treated and untreated plants.

2. Materials and methods

2.1. Plant material and experimental design

Capsicum annuum L. plants ("Corno di Toro Giallo" variety) were grown in 4 L pots filled with a substrate composed of sand, expanded clay and peat (1:2:2 by weight) in the greenhouse under partially controlled conditions (25° ± 2 °C temperature and around 60% relative humidity). Fig. 1 graphically shows the adopted experimental design together with the sampling times.

The experiment was conducted on 48 individuals and once pepper plants reached the flowering transition phase (3-month-old plants) they were divided into two groups. Twenty-four plants (group I, BIO) were treated with the biostimulant: two applications were provided respectively at 14 days and 7 days before the beginning of water stress treatment. The other 24 plants (group II, NO BIO) were not treated with the biostimulant. The biostimulant-treated and untreated pepper plants were further divided in two subgroups: control (CTR) and stressed (STRESSED) plants. Eight plants were kept as controls and watered every two days to pot capacity, while the remaining 16 plants were subjected to water stress by stopping irrigation until the stem water potential was below −2 MPa. When severe water stress level was reached, recovery phase started by re-watering plants to pot capacity in the morning at 10 a.m. Recovery dynamics were monitored for the following 6 days. Physiological parameters (stem water potential, Ψ_{stem}, and leaf gas exchanges, g_s and A_n) were monitored daily throughout the entire experiment (i.e., from the beginning of the stress treatment to the end of the recovery period) in stressed and control plants of both BIO and NO BIO groups.

Leaves from three plants were pooled (1 biological replicate) and three biological replicates were collected at the beginning and at the end of the stress period and during the recovery (4 h, 1 day, 4 days and 6 days after re-watering). The samples were immediately frozen in liquid nitrogen and then stored at −80 °C for further biochemical analyses. Relative plant growth was evaluated by measuring biometric parameters (leaf area, shoot height and diameter) before the pre-treatment phase, at the beginning and at the end of the stress phase and at the end of the recovery phase. Plant shoot and root dry weight was measured at the end of the stress and the recovery phases. In order to evaluate pepper final fruit yield, 5 plants for each group were monitored after the recovery ended.

2.2. Biostimulant and spectrophotometric characterization

The GHI_16_VHL biostimulant was provided by Green Has Italia S.p.a (Canale, CN, Italy) and applied by fertigation (1.5 ml L^{-1}). This product mainly derives from *Cruciferae* and *Leguminosae* protein hydrolysates, with a 7% (w/w) glutamic acid content. The label of the product claims to contain 5% (w/w) of organic nitrogen and 16% (w/w) of organic carbon. The product contains 20% (w/w) of total amino acids and 4% (w/w) of glycine betaine. The pH [in 1% (w/w) water solution] and Electrical Conductivity (in water solution 1 g L^{-1}) are respectively 6 ± 0.5 and 270 μS cm^{-1}. The quantification of bioactive compounds contained in GHI_16_VHL was spectrophotometrically determined by using 1 mL L^{-1} of GHI_16_VHL dissolved in water, as previously reported (Campobenedetto et al., 2020; Mannino and Campobenedetto, 2020). Precisely, Folin-Ciocalteu Assay was employed for the evaluation of the Total Polyphenol Content (TPC); pH differential method for the Total

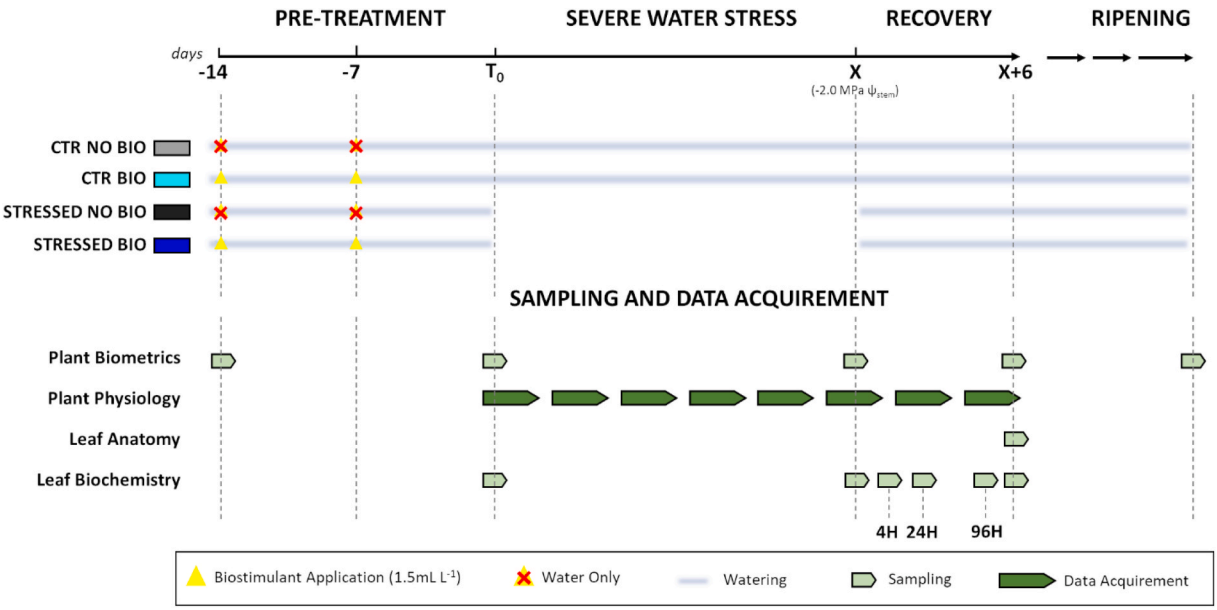

Fig. 1. Schematic representation of the adopted experimental design and timing of sampling and data acquirement.

Anthocyanin Content (TAC); Aluminium Chloride Assay was employed for the measurement of both Total Flavonol (TFlC) and Flavonoid (TFvC) content; and BL-DMAC assay for the measurement of the Total Flavan-3-ol Content (TF3C). Moreover, the potential antioxidant property of the biostimulant was also measured in terms of both radical scavenging (ABTS and DPPH assay) and reducing activity (FRAP assay).

2.3. Plant biometric and anatomical measurements

To evaluate the biostimulant influence on pepper vegetative and reproductive growth under control, stress and recovery conditions, destructive and non-destructive measurements were performed.

Plant height, stem diameter and leaf area were monitored throughout all the experiment and all data were expressed as relative growth rate (RGR) (Hoffmann and Poorter, 2002) related to the three different phases (pre-treatment, severe water stress and recovery). Leaf area relative growth rate was calculated on the same three selected young leaves on each plant.

Destructive samplings were performed to monitor plant biomass and final fruit yield. Plant aerial biomass and total root dry weight were measured on treated and untreated control and stressed plants sampled at the end of the stress and the recovery phases. Plant final fruit yield (expressed as g^{-1} per plant) and fruit weight distribution was measured on pepper plants, which were let grown until fruits reached their mature stage (switched to yellow colour).

Stomatal density was determined on three developed leaves of each selected plant at the end of the recovery phase. Clear nail polish was applied to three different areas of each leaf avoiding the midvein and allowed to dry. Clear packing tape was then used to peel off the nail polish from the abaxial epidermis, which was then placed onto a glass microscope slide. Images were captured with a digital camera connected to an optical microscope (Dialux 22, Leitz, Wetzlar, Germany) and stomata were counted using Image J software (https://imagej.nih.gov/ij/download.html).

2.4. Plant physiological measurements

Physiological parameters were monitored throughout the entire experiment. All data were collected from 10 a.m. to 1 p.m. except for those related to short recovery dynamics (2 h and 4 h after re-watering). Stem water potential (ψ_{stem}) was measured daily and throughout the entire experiment using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Mature leaves were inserted in a humidified plastic bag covered with aluminium foil to stop transpiration. After 30 min, leaves were cut and allowed to equilibrate in dark conditions before taking the measurements.

Stomatal conductance (g_s) and net photosynthesis (A_n) were measured on leaves using a portable infrared gas analyser (ADC-LCPro + system, the Analytical Development Company Ltd, Hoddesdon, UK). Measurements were performed using a 6.25 cm^2 leaf chamber equipped with artificial irradiation (1200 μmol photons m^{-2} s^{-1}), set with a chamber temperature of 25 °C. Measurements were taken on three fully expanded leaves per plant between 9 a.m. and 12 a.m. on each experimental day.

Changes in chlorophyll concentration during the treatments were measured using SPAD meter (SPAD 502 Plus Chlorophyll Meter, Spectrum, Plainfield, IL, USA) on three consecutive actively growing leaves. Measurements were repeated twice weekly throughout the entire experiment.

2.5. Plant biochemical measurements

2.5.1. Hydrogen peroxide (H_2O_2) quantification

The hydrogen peroxide level was assayed according to Velikova et al. (2000) with some modifications. Powdered leaves (0.1 g) were extracted with 1 mL of 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation at 12,000×*g* for 15 min, 0.5 mL of supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance was read at 390 nm and the H_2O_2 content, expressed as nmol g^{-1} leaf fresh weight (FW), was determined based on a H_2O_2 standard curve.

2.5.2. Antioxidant enzyme assays

Total proteins were extracted according to Campobenedetto et al. (2020). The obtained supernatant was used for enzymatic assays after evaluating its soluble protein content by the method of Bradford (1976).

SOD (EC 1.15.1.1) - Superoxide dismutase activity evaluation was based on the ability of this enzyme to inhibit the reduction of nitro blue tetrazolium (NBT), generated photochemically thanks to the superoxide anion (Krishnan et al., 2002). The reaction consisted in 1 mL final volume, containing 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and enzyme extract. The absorbance was detected at 560 nm after 15 min of light

exposure (4000 lux). One unit of total SOD activity was calculated as the amount of protein per milligram causing 50% inhibition of NBT reduction. Enzymatic activity results were expressed as U mg^{-1} proteins.

CAT (EC 1.11.1.6) - Catalase activity was detected spectrophotometrically by monitoring the decreased absorption of H_2O_2 (ϵ H_2O_2 = 39.4 mM^{-1} cm^{-1}) at 240 nm (Zhang and Kirkham, 1996). The reaction was prepared in 1 mL final volume, containing 50 mM sodium phosphate buffer (pH 7.0), 15 mM H_2O_2, and enzyme extract. The reaction started by H_2O_2 addition and lasted for 120 s. CAT activity was calculated as μmol of decomposed H_2O_2 per minute. Enzymatic activity results were expressed as nmol H_2O_2 min^{-1} mg^{-1} proteins.

2.5.3. Proline content

Before quantifying proline, ground frozen leaf material was extracted in 70% (v/v) EtOH overnight at 4 °C (1/20 w/v, 50 mg of leaf material in 1 ml). After centrifugation at 10000×*g* for 5 min, the supernatant was ready for nyhinidrin assay performed at 520 nm, according to Mannino and Nerva (2020). Results are expressed as μmol g^{-1} leaf FW, by using a proline standard curve in the linear absorbance range for quantification, from 0.5 to 0.04 mM.

2.5.4. Total soluble sugars content

Total soluble leaf sugars were extracted according to Pagliarani et al. (2019) with some modifications. Fifteen mg of ground frozen material were suspended in 0.5 mL of 80% (v/v) EtOH adding 1% w/v polyvinylpolypyrrolidone (PVPP) and heated at 80 °C for 30 min. After centrifugation at 10000×*g* for 3 min, the supernatant was separately collected, and the pellet was extracted again in 0.3 mL of 80% (v/v) EtOH at 80 °C for 30 min. The supernatant obtained after centrifugation at 10000×*g* for 3 min was added to that obtained in the first extraction and let dry in the oven at 50–56 °C overnight. The pellet was extracted again overnight at room temperature by adding 0.5 mL of water. After centrifugation at 10000×*g* for 3 min, the water supernatant was added to the sugars obtained from the ethanolic extraction, by heating and vortexing vigorously. The total extract was used to quantify total soluble sugars by using the anthrone assay (Leyva et al., 2008). Results were obtained by reading the absorbance at 620 nm and they were expressed as mg g^{-1} leaf FW using a glucose standard curve as reference.

2.6. Statistics

Statistical analyses and graph design were performed by using Sigma Plot 10.0 (Systat Software Inc., San Jose, CA, USA). Significant differences among treatments were analysed by applying a two-way or three-way analysis of variance (ANOVA) (see Tables S1-S21), followed by Tukey's *post hoc* test. Significant differences between pairwise comparisons were assessed by *t*-test. The significant threshold was imposed under 0.05.

3. Results

3.1. Bioactive compounds and antioxidant activity of the biostimulant formulation

The partial spectrophotometric characterization of the biostimulant GHI_16_VHL included the quantification of the total amount of polyphenols (TPC), anthocyanins (TAC), flavonols (TFlC), flavonoids (TFvC) and flavan-3-ols (TF3C). Results of the UV/Vis quantification are shown in Table 1. The tested biostimulant did not show a high content of bioactive compounds. Among the measured compounds, TPC had the highest value, followed by TFlC and TFvC. Moreover, the spectrophotometric determination showed that anthocyanins were not present in the biostimulant formulation (LOD: 3 μg mL^{-1}; LOQ: 10 μg mL^{-1}), and only 0.017% of the mixture was composed by flavan-3-ols.

Concerning the antioxidant properties, the biostimulant displayed antioxidant activity both in terms of radical scavenging and reducing activity, as measured by ABTS, DPPH and FRAP assay, respectively (Table 1).

Table 1
UV/Vis spectrophotometric determination of bioactive compounds and antioxidant properties of GHI_16_VHL. Values are expressed as a mean ± SD of three experiments carried out in triplicate.

BIOACTIVE COMPOUNDS	**TPC**	7.38 ± 0.32	mg GAE g^{-1} biostimulant
	TFlC	2.32 ± 0.23	mg QE g^{-1} biostimulant
	TFdC	2.56 ± 0.34	mg RE g^{-1} biostimulant
	TAC	n.d.	mg CE g^{-1} biostimulant
	TF3C	0.17 ± 0.01	mg PACE g^{-1} biostimulant
ANTIOXIDANT CAPACITY	**ABTS**	81.61 ± 2.21	μmol TE g^{-1} biostimulant
	DPPH	13.79 ± 0.11	μmol TE g^{-1} biostimulant
	FRAP	8.23 ± 0.11	μmol TE g^{-1} biostimulant

TPC = Total Polyphenol Content; TFlC = Total flavonol Content; TFdC = Total Flavonoid Content; TAC = Total Anthocyanin Content; TF3C = Total Flavan-3-ols; ABTS = radical scavenging activity measured via 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; DPPH = radical scavenging activity measured via 2,2-diphenyl-1-picryl-hydrazyl-hydrat; FRAP = Ferric Reducing Antioxidant Power; GAE = gallic acid equivalents; QE = quercetin equivalents; RE = rutin equivalents; PACE = A-type proanthocyanidin equivalent, CE = cyanidin-6-glucoside equivalents; TE trolox equivalents.

3.2. Effects of the biostimulant application on pepper biometric parameters

Table 2 shows the relative growth rate (RGR) of pepper plant stem diameter, stem height and leaf area during the three experimental phases. During the pre-treatment phase, pepper plant aerial part relative growth was not significantly influenced by the biostimulant application. However, despite not significantly, leaf area relative growth rate (RGR) was slightly higher in CTR BIO with respect to CTR NO BIO plants throughout the experiment and it reached significantly higher values in biostimulant-treated plants later on at recovery timing. Although drought imposition generally affected plant growth, the biostimulant fertigation mitigated these effects by promoting a higher leaf RGR and stem height in treated stressed plants compared to untreated stressed plants under adverse conditions. During recovery, stressed plant vegetative relative growth rate was generally higher than under stressed conditions. On the one hand, stem diameter and leaf area RGR of STRESSED BIO were higher with respect to that of STRESSED NO BIO plants, even if they did not reach control values. On the other hand, STRESSED BIO stem height was the same of well-watered controls and it was consistently higher than those of STRESSED NO BIO plants.

Plant total biomass was not different in CTR BIO with respect to CTR NO BIO plants along the stress and recovery phases (Table 3). At the end of the stress phase, total dry weight (DW) was higher in well-watered plants with respect to stressed plant groups whose total biomass values were the same. However, at the end of the recovery phase, only STRESSED BIO plants showed a total biomass accumulation significantly similar to CTR groups. Biomass data confirmed the observed higher rate of aerial part growth when pepper plants were treated with the biostimulant (Table 3). Indeed, plant biomass distribution was different in biostimulant-treated plants compared to untreated plants. The biostimulant treatment significantly enhanced shoot and leaf DW/total plant DW during the stress phase. Moreover, root/shoot DW was significantly higher in plants not treated with the biostimulant at the end of the stress phase (Table 3).

After water stress exposure, the biostimulant treatment succeeded in retaining the same final fruit yield of pepper groups that did not face stress, whereas fruit yield was severely affected in untreated plants (Table 3). Differently, biostimulant treatments did not affect the final fruit yield of well-watered plants. However, the biostimulant application enhanced the production of fruits of high-class weight in unstressed plants (data not shown), thus probably improving pepper marketable yield.

Table 2
Plant aerial part relative growth rate during pre-treatment, drought, and recovery phases. Relative growth rate (RGR) values are expressed as means ± SD.

	Experimental phase	Plant Group			
		CTR NO BIO	STRESSED NO BIO	CTR BIO	STRESSED BIO
STEM DIAMETER RGR	PRE-TREATMENT	0.0210 ± 0.0083^{a}	0.0204 ± 0.0061^{a}	0.0210 ± 0.0056^{a}	0.0223 ± 0.0118^{a}
	STRESS	0.0357 ± 0.0140^{a}	0.0035 ± 0.0003^{b}	0.0126 ± 0.0057^{a}	0.0047 ± 0.023^{bc}
	RECOVERY	0.0159 ± 0.0025^{a}	0.0036 ± 0.0005^{b}	0.0136 ± 0.0041^{a}	0.0059 ± 0.0003^{c}
STEM HEIGHT RGR	PRE-TREATMENT	0.0223 ± 0.0051^{a}	0.0210 ± 0.0041^{a}	0.0246 ± 0.0083^{a}	0.0247 ± 0.0053^{a}
	STRESS	0.0143 ± 0.0045^{a}	0.0063 ± 0.0110^{b}	0.0141 ± 0.0340^{a}	0.0102 ± 0.0110^{b}
	RECOVERY	0.0153 ± 0.0070^{a}	0.0131 ± 0.0006^{c}	0.0186 ± 0.0260^{a}	0.0209 ± 0.0007^{a}
LEAF AREA RGR	PRE-TREATMENT	0.0366 ± 0.0136^{a}	0.0372 ± 0.0170^{a}	0.0514 ± 0.0159^{a}	0.0516 ± 0.0118^{a}
	STRESS	0.0130 ± 0.0043^{b}	0.0021 ± 0.0006^{c}	0.0221 ± 0.0066^{be}	0.0039 ± 0.0008^{d}
	RECOVERY	0.0131 ± 0.0031^{b}	0.0028 ± 0.0002^{c}	0.0242 ± 0.0023^{e}	0.0107 ± 0.0035^{b}

Different letters correlate with statistically different data, as measured by three-way ANOVA (Tables S1-S3) followed by Tukey's test ($P < 0.05$). No significant interaction was found among the three independent variables for all the three analysed dependent variables.

Table 3
Plant dry weight at the end of stress and after 6 days from re-rewatering. Total plant dry weight (TOTAL DW), shoot and leaf dry weight with respect to TOTAL DW (SHOOT AND LEAF DW/TOTAL DW), root dry weight with respect to shoot dry weight (ROOT DW/SHOOT DW). Plant final yield at ripening time (FINAL FRUIT YIELD). Data are expressed as means ± SD.

	Experimental phase	Plant Group			
		CT NO BIO	STRESSED NO BIO	CTR BIO	STRESSED BIO
TOTAL DW (g)	STRESS END	17.910 ± 1.428^{a}	11.753 ± 1.572^{b}	18.997 ± 2.577^{a}	10.195 ± 0.658^{b}
	RECOVERY END	20.241 ± 1.868^{a}	14.215 ± 2.423^{b}	19.285 ± 1.746^{a}	14.680 ± 4.602^{ab}
SHOOT AND LEAF	STRESS END	0.543 ± 0.033^{a}	0.613 ± 0.030^{a}	0.682 ± 0.024^{b}	0.758 ± 0.041^{b}
DW/TOTAL DW	RECOVERY END	0.627 ± 0.152^{ab}	0.715 ± 0.029^{b}	0.676 ± 0.029^{b}	0.764 ± 0.092^{b}
ROOT DW/SHOOT DW	STRESS END	0.455 ± 0.007^{a}	0.615 ± 0.071^{a}	0.383 ± 0.055^{b}	0.301 ± 0.054^{b}
	RECOVERY END	0.360 ± 0.141^{ab}	0.342 ± 0.051^{b}	0.363 ± 0.040^{b}	0.278 ± 0.101^{b}
FINAL FRUIT YIELD (g plant^{-1})	RIPENING TIME	199.12 ± 21.14^{a}	136.95 ± 32.61^{b}	199.26 ± 27.04^{a}	225.485 ± 8.72^{a}

Different letters correlate with statistically different data, as measured by three-way ANOVA (for TOTAL DW, SHOOT AND LEAF DW/TOTAL DW and ROOT DW/SHOOT DW) (Tables S4-S6) or two-way ANOVA (FINAL FRUIT YIELD) (Table S7) followed by Tukey's test ($P < 0.05$). No significant interaction was found among the three independent variables for all the three analysed dependent variables.

3.3. *Effects of the biostimulant application on plant physiology and leaf stomatal density*

Under well-watered conditions, ψ_{stem} values were similar for both biostimulant-treated and untreated pepper plants (Fig. 2). Stem water potential progressively decreased during the stress imposition for all plants, but the water stress progression was significantly different between treated and untreated plants (Fig. 2a). STRESSED BIO plants started showing ψ_{stem} values lower than well-watered controls after 7 days of water stress imposition, and namely 2 days before STRESSED NO

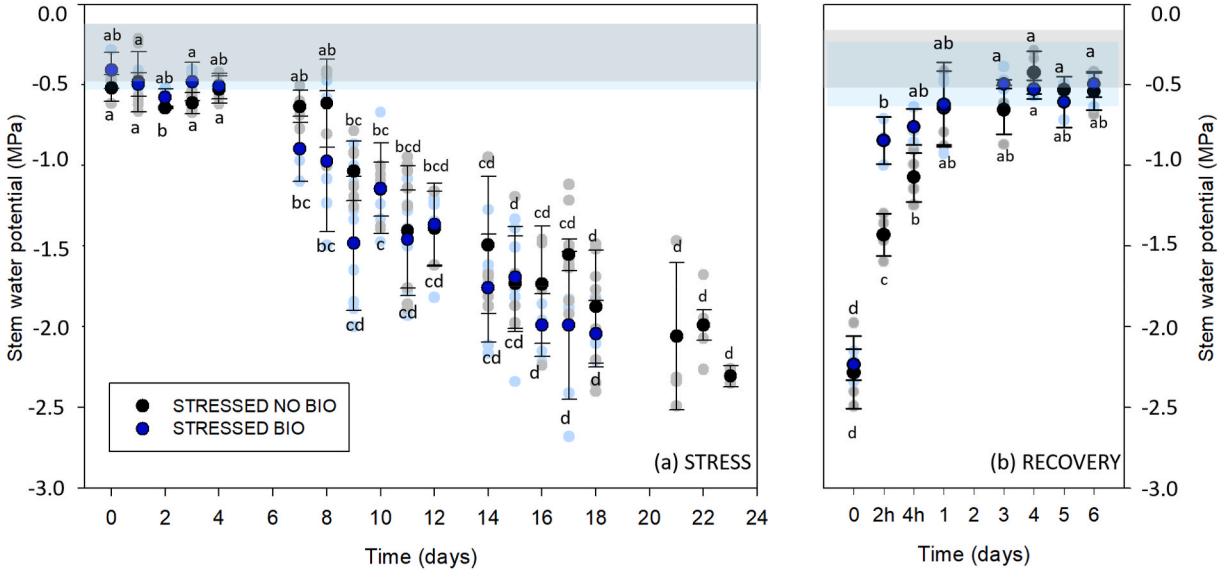

Fig. 2. Stem water potential trend along stress (a) and recovery (b) phases. Black and blue dots indicate average values for untreated stressed and treated stressed plants, whereas light grey and light blue dots refer to single plant measurements taken on stressed and recovered plants (NO BIO and BIO) during the experimental days. The light blue and light grey rectangles represent the average value of ψ_{stem} measured on well-irrigated treated and untreated plants, respectively. Data are expressed as means ± SD. Different letters correlate with statistically ($P < 0.05$) different data, as measured by three-way ANOVA (Tables S8,S9) followed by Tukey's test. Upper letters refer to untreated plants, lower letters to treated plants. Squares refer to CTR NO BIO and CTR NO BIO values, which are statistically ($P > 0.05$) equal, as measured by *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

BIO plants.

Interestingly, STRESSED BIO plants showed a faster decreasing of pot weight, especially at 7 days after drought starting (Fig. S1). Untreated plants exposed to drought reached the severe stress level of approximately −2 MPa in 23 days while in primed pepper plants it was achieved within 18 days. Following re-watering, biostimulant application promoted a faster ψ_{stem} recovery (Fig. 2b); stem water potential in treated plants recovered to the pre-stress values within 4 h, while the full ψ_{stem} recovery in untreated plants occurred after 1 day of irrigation.

Net photosynthesis and g_s showed a similar trend in response to stress in both stressed pepper groups and both treated and untreated plants revealed similar values of assimilation under well irrigated and stress conditions (Table 4).

Recovery of g_s and A_n was slower than that of stem water potential and differed between treated and untreated plants (Fig. 3). The STRESSED BIO plants showed a slight g_s recovery at 4 h after rewatering and a fully recovery occurred at 1 day after the return of irrigation. Differently, STRESSED NO BIO plants reached values of g_s similar to those of control plants three days after stress relief. Rewatering completely restored photosynthetic activity to pre-stress measurements after stomatal were fully open (within 1 and 3 days of irrigation for treated and untreated plants, respectively). Overall, g_s and A_n maintained slightly but not significantly higher values in STRESSED BIO plants compared to STRESSED NO BIO plants during the entire duration of recovery phase.

Stomatal density of the abaxial epidermis did not statistically change for leaves collected at the end of the recovery phase, except for STRESSED BIO plants; indeed, their stomatal density appeared to be significantly higher (Table 5).

Well-watered plants did not show chlorophyll accumulation differences when treated or not with the biostimulant (Table 5). No differences were found in chlorophyll content of treated leaves during the entire experiment (Table 5). However, STRESSED NO BIO plants showed a reduction in chlorophyll content both at the end of severe water stress and at 6 days after re-watering (Table 5).

3.4. Effects of the biostimulant application on leaf H_2O_2 level and antioxidant enzymatic machinery

Biostimulant application did not change H_2O_2 accumulation under well-watered control conditions (Fig. 4a). At the end of severe water stress, H_2O_2 content was sensibly higher than in well-watered plants in both pepper stressed groups and it recovered to H_2O_2 control concentration at 4 days after re-watering (Fig. 4a).

Table 4
Leaf gas exchanges along stress phase. Data are expressed as means ± SD. Data refer to BIO and NO BIO plants at the same stress level, whose range is expressed as means ± SD of ψ_{stem} values of both plant groups together.

	Stem Water Potential (MPa)	Plant Group	
		NO BIO	BIO
g_s, (mmol H_2O m^{-2} sec^{-1})	−0.44 ± 0.02	120.92 ± 29.59^{a}	118.07 ± 40.10^{a}
	−0.95 ± 0.12	46.67 ± 20.21^{b}	36.00 ± 13.87^{b}
	−1.43 ± 0.07	25.00 ± 7.07^{b}	23.75 ± 2.50^{b}
	−1.80 ± 0.06	15.00 ± 5.00^{b}	15.00 ± 7.07^{b}
	−2.19 ± 0.21	13.75 ± 4.79^{b}	13.33 ± 4.08^{b}
A_n (μmol CO_2 m^{-2} sec^{-1})	−0.44 ± 0.02	8.26 ± 0.94^{a}	8.05 ± 2.24^{a}
	−0.95 ± 0.12	3.94 ± 0.35^{b}	3.50 ± 1.96^{b}
	−1.43 ± 0.07	2.32 ± 0.09^{bc}	2.34 ± 0.07^{bc}
	−1.80 ± 0.06	1.30 ± 0.68^{c}	1.31 ± 0.43^{c}
	−2.19 ± 0.21	1.27 ± 0.33^{c}	1.14 ± 0.69^{c}

Different letters correlate with statistically different data, as measured by two-way ANOVA (Tables S10, S11) followed by Tukey's test. No significant interaction was found between the two independent variables for the analysed dependent variables ($P < 0.05$). .

However, STRESSED BIO plants showed a lower (about 20%) H_2O_2 leaf concentration with respect to STRESSED NO BIO pepper plants at the end of severe water stress and during the early recovery phases, namely at 4 h and 1 day after re-watering (Fig. 4a). As for the antioxidant enzymatic machinery, catalase activity (CAT) was two times higher in leaves of plants treated with the biostimulant under well-watered conditions (Fig. 4b). On the other hand, CAT activity was significantly downregulated when severe water stress level was reached and its recovery to control condition values was significantly affected by the biostimulant treatments (Fig. 4b). Indeed, CAT activity showed values similar to those of control plants at 4 h after re-watering when the biostimulant was not applied (Fig. 4b). Differently, CAT activity in STRESSED BIO plants did not recover to pre-stress values even at 6 days after re-watering (Fig. 4b). Finally, under the drought and recovery phases, SOD leaf activity was not different with respect to that of well-watered controls (Fig. 4c). Moreover, it was not influenced by the biostimulant application, either under control, drought stress or recovery conditions (Fig. 4c).

3.5. Effects of the biostimulant application on leaf osmolyte accumulation

Biostimulant treatments did not change proline leaf concentration under watered control conditions (Fig. 5a). Moreover, at the end of severe water stress, proline accumulation was 7 times higher than well-watered controls both in STRESSED BIO and STRESSED NO BIO plants. During the recovery phase, proline values gradually reached control values in STRESSED NO BIO plants. Differently, at 4 h after re-watering, proline leaf level was still at its highest level in STRESSED BIO plants, while it started to decrease in untreated pepper plants. Both plant treatments recovered to the proline level of control plants at 4 days after re-watering.

Total non-structural carbohydrates (NSC) concentration was slightly higher in CTR BIO with respect to CTR NO BIO plants (Fig. 5b). At the end of severe water stress leaf NSC generally increased, but their concentration was two times higher in STRESSED BIO plants with respect to STRESSED NO BIO plants. During the recovery phase, STRESSED NO BIO total soluble sugars followed a decreasing trend to gradually reach control values. Differently, after a drop at 4 h after recovery, NSC content raised again in STRESSED BIO leaves before went back to control values at 4 days after re-watering.

4. Discussion

4.1. The biostimulant formulation contains bioactive compounds with antioxidant properties

Due to their different origin, biostimulants are commercial products displaying significant variations in their quantitative and qualitative chemical composition (Campobenedetto et al., 2021). Chemical characterization of biostimulants is a hard challenge. In particular, in addition to the complexity of profiling the starting raw matrices, the difficulty to characterize the compounds derived from hydrolysis and fermentation processes during their production also exists (Ugena et al., 2018). Consequently, most of the scientific reports related to biostimulant application simply describe the biological effects on plants without investigating their chemical composition. In this work, a preliminary spectrophotometric characterization of GHI_16_VHL biostimulant was carried out in order to provide a general profile that may be useful for elucidating which phytochemicals could be involved in the potential biological effects observed on plants treated with this product and grown under control or drought stress conditions. Our spectrophotometric analyses showed that the tested biostimulant contained a discrete amount of polyphenol compounds, especially flavonoids and flavonols, but a very low amount of flavan-3-ols and no amount of anthocyanin compounds was detected. Concerning the antioxidant properties, the biostimulant displayed both radical scavenging and

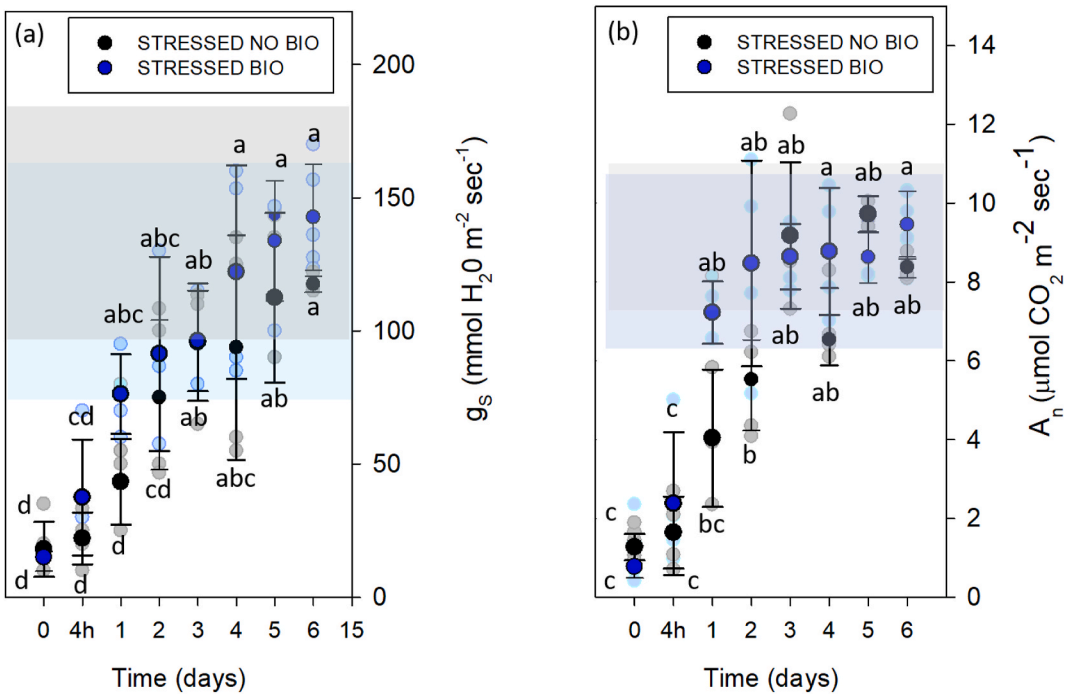

Fig. 3. Leaf gas exchange trend along recovery phase: (a) stomatal conductance (g_s); (b) net photosynthesis (A_n). Data are expressed as means ± SD. Different letters correlate with statistically ($P < 0.05$) different data, as measured by three-way ANOVA (Tables S12, S13) followed by Tukey's test. Upper letters refer to treated plants, lower letters to untreated plants. Squares refer to CTR NO BIO and CTR NO BIO values, which are statistically ($P > 0.05$) equal, as measured by *t*-test.

Table 5
Stomatal density at the end of the recovery phase, and Chlorophyll Content (SPAD values) at the beginning of the stress phase (T_0), at the end of drought and at the end of recovery. Data are expressed as means ± SD.

	Experimental phase	Plant Group			
		CTR NO BIO	STRESSED NO BIO	CTR BIO	STRESSED BIO
STOMATAL DENSITY (mm^{-2})	RECOVERY END	329.28 ± 12.47^{a}	335.46 ± 33.07^{a}	322.40 ± 43.46^{a}	505.63 ± 32.34^{b}
	T_0	44.56 ± 1.67^{a}	44.50 ± 1.86^{a}	47.72 ± 4.05^{a}	48.24 ± 2.42^{a}
CHLOROPHYLL LEVEL (SPAD)	STRESS END	43.90 ± 0.65^{a}	39.12 ± 0.65^{b}	44.56 ± 1.67^{a}	43.86 ± 3.14^{a}
	RECOVERY END	46.85 ± 5.42^{a}	35.43 ± 1.69^{b}	43.90 ± 0.65^{a}	41.1 ± 1.69^{a}

Different letters correlate with statistically different data, as measured by two-way ANOVA (STOMATAL DENSITY) (Table S14) or three-way ANOVA (CHLOROPHYLL LEVEL) (Table S15) followed by Tukey's test ($P < 0.05$). No significant interaction was found among the independent variables for both the measurements.

reducing activity. In particular, the biostimulant had highest ABTS and DPPH values with respect to FRAP value, suggesting the prevalence of polyphenolic compounds with para-oriented hydroxyl groups (Gu et al., 2019) (Table 1).

Generally, polyphenols are compounds known in literature to exert a strong antioxidant activity both in animals (Gessner et al., 2017) and plants (Ceccarini et al., 2019). Recently, Ceccarini and colleagues treated maize plants with two different extracts enriched in polyphenols under salt-stress conditions and observed an interesting correlation between the polyphenols and the attenuation of the generated oxidative stress (Ceccarini et al., 2019). Moreover, in a previous work, we investigated the chemical composition and the biological effects derived from the application of VIVEMA TWIN biostimulant on tomatoes grown under salt stress conditions, and we identified several compounds potentially involved in the plant stress attenuation (Campobenedetto et al., 2021).

4.2. Biostimulant application enhanced pepper growth and final fruit yield despite severe water stress

Drought is known to cause physiological permanent consequences by limiting sweet pepper growth and yield (Campos et al., 2014; López-Serrano et al., 2019). Our data confirmed that "Corno di Toro giallo" pepper variety yield and aboveground growth is sensibly reduced when water stress is applied at flowering stage (Tables 2 and 3), thus affecting pepper stress resilience.

Evaluation of recovery from drought is an important step in assessing drought resilience. It reveals the plant's ability to recover to its pre-stress conditions, thus reflecting the extent of the damage caused by severe drought (Dalal et al., 2019). The PH-based biostimulant used in this study appears to act as a resilience-promoting agent when applied at root level. Indeed, after severe water stress experience, treated plants showed a fruit yield which is not different from well-watered control peppers (Table 3). Similarly, Rouphael et al. (2017) reported that the combination of a microbial biostimulant product with PH derivatives induced a significant increase in crop productivity after salinity stress.

Moreover, the biostimulant application reduced the negative effects induced by drought stress on plant stem growth and leaf area relative growth rate (Table 2). Furthermore, STRESSED BIO plants showed a higher ratio of aboveground biomass with respect to the total dry biomass at the end of the stress phase and their total dry weight biomass was not significantly different from well-watered plants at the end of the recovery phase (Table 3). Several studies testing the action of PH-based biostimulants on plants have demonstrated that plant relative growth rate and growth performance are significantly improved by PHs even when exposed to adverse abiotic stressors (Zhang et al., 2015). In particular, the inhibition of lettuce growth was higher in untreated

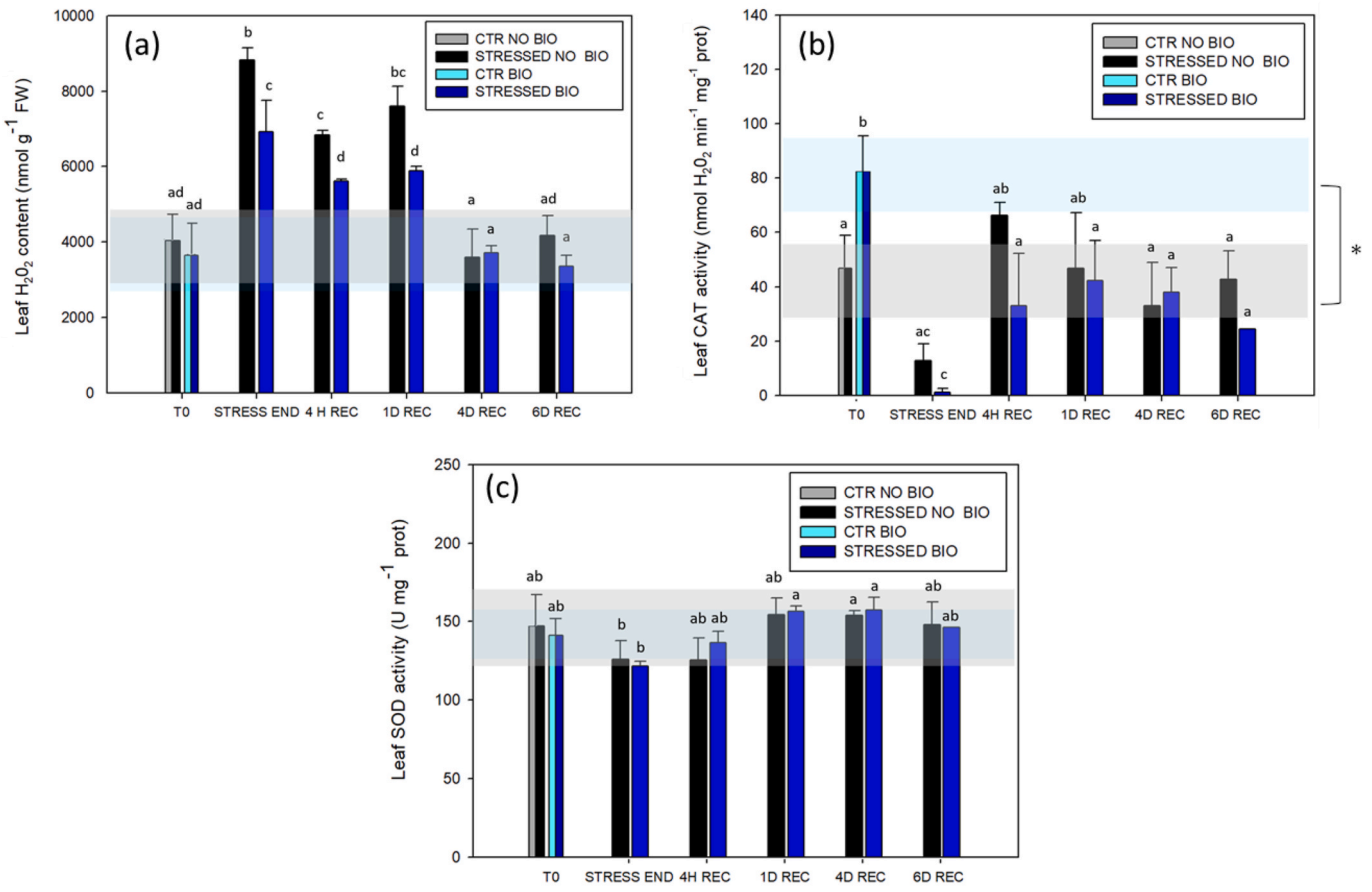

Fig. 4. H_2O_2 leaf content and enzymatic scavenging during severe water stress and its relief. a) leaf hydrogen peroxide content; (b) CAT leaf activity; (c) SOD leaf activity. Data are expressed as means ± SD. Different letters correlate with statistically ($P < 0.05$) different data along time with respect to their well-watered control, as measured by three-way ANOVA (Tables S16-S18) followed by Tukey's test. No interaction was found among the independent variables, with the exception of those related to CAT (irr * treat * tim, $F = 4.410$, $p = 0.002$). Squares refer to CTR NO BIO and CTR BIO values and *refers to significant statistical ($P < 0.05$) difference between the two controls, as measured by *t*-test.

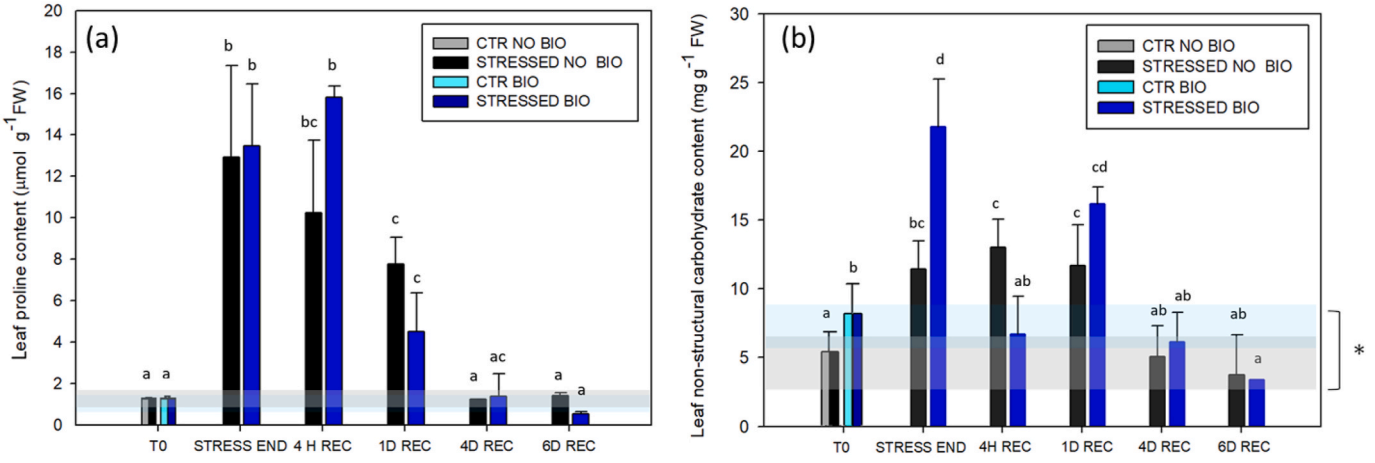

Fig. 5. Osmolyte leaf content dynamics during severe water stress and its relief. (a) Proline content, (b) NSC content. Data are expressed as means ± SD. Different letters correlate with statistically ($P < 0.05$) different data along the decreasing trend with respect to their well-watered control, as measured by three-way ANOVA (Tables S19,S20) followed by Tukey's test. No interaction was found among the independent variables, with the exception of those related to NSC (irr * treat * time, $F = 4.742$, $p = 0.001$). Squares refer to CTR NO BIO and CTR BIO values and *refers to significant statistical ($P < 0.05$) difference between the two controls, as measured by *t*-test.

plants than in plants treated with PH spray under salinity stress conditions (Lucini et al., 2015). Moreover, a PH biostimulant tested on grapevine roots before imposing water deprivation sustained the growth of the younger aboveground vegetative organs during the stress and the following recovery (Meggio et al., 2020).

4.3. Biostimulant application accelerated severe water stress reachment but also its physiological recovery in pepper

Plant yield and growth are directly linked to plant physiological trends during drought and stress relief. Indeed, drought stress strongly affected stomatal conductance and assimilation, which are directly linked to crop yield (Delfine et al., 2000). Among horticultural crops, pepper is one of the most susceptible to water stress because of its wide transpiring leaf surface and its elevated stomatal opening (Parkash and Singh, 2020). During severe water stress, *Capsicum annuum* "Corno di Toro giallo" variety showed a stomatal conductance reduction trend similar to that of other sweet pepper varieties (Campos et al., 2014).

The biostimulant treatment did not affect leaf gas exchange reduction trend during the progressive increase of stress level (Table 4). However, bio-treated plants were characterized by a faster stress progression, by reaching severe water stress 3 days in advance with respect to untreated pepper plants (Fig. 2). This could be related to the higher leaf relative growth rate in STRESSED BIO plants under stress conditions, which could explain the faster plant soil water absorption during the same experimental phase (Fig. S1). Similarly, under moderate water stress, tomato plants drenched with PH obtained a more favorable balance between carbon gain and water loss (Paul et al., 2019). Plants treated with PH-based biostimulants have already been shown to be able to recover more quickly when they had access to water (Van Oosten et al., 2017). Biostimulant treatment promoted a faster stem water potential and leaf gas exchange recovery, since STRESSED BIO plants reached leaf stomatal conductance values similar to those of control plants before STRESSED NO BIO plants.(Fig. 3). Similarly, lettuce plants subjected to cold stress in a controlled environment, when treated with sprayed PH, exhibited a higher stomatal conductance than untreated plants, thus implying productive improvements (Botta, 2013). At the end of the recovery phase, the youngest leaves of stress-experienced peppers exhibited a higher stomatal density when previously treated with the biostimulant (Table 5).

Concerning pigment levels, although some *Capsicum* species are able to maintain a high chlorophyll level during drought stress (Okunlola et al., 2017), our plants showed a reduction in SPAD index at the end of the drought exposure and at 6 days after the re-watering (Table 5). However, the chlorophyll level of biostimulant-treated pepper plants was the same of well-watered plants at recovery end. Similarly, after drought stress exposure, an increased total chlorophyll content was observed in some broccoli varieties when amino acid treatments occurred (Kałuzewicz et al., 2017). Moreover, similar results were also obtained by Petrozza et al. (2014) on tomato plants in response to treatments with a commercial biostimulant containing vitamins, amino acids, proteins, and betaines from plant and algal extracts.

4.4. Biostimulant application reduced pepper H_2O_2 leaf accumulation under drought and its relief by modulating catalase activity under well-watered condition

Plant photosynthetic apparatus could be functionally impaired because of the oxidation derived from the imposed stress (Foyer et al., 2014). Accordingly, in our experiment, drought enhances oxidative stress such as confirmed by the high leaf H_2O_2 levels observed at the end of the imposed stress (Fig. 4a). Drought is known to increase the antioxidant enzymatic activity in two *Capsicum annuum* cultivars under drought stress (Hu et al., 2010). On the one hand, this variety did not show any SOD activity regulation after drought exposure. On the other hand, CAT activity severely decreased at the end of the severe stress, but it went back to control values at 4 h after re-watering (Fig. 4c). Under our experimental conditions, stress-experienced plants were positively influenced by the biostimulant treatment, since they accumulated a lower H_2O_2 level in leaves, such as observed for pepper plants treated with garlic extract by soil application (Hayat et al., 2018). In some experiments, SOD and ascorbate peroxidase (APX) activity levels were increased by application of PH biostimulant in maize subjected to stress (Vasconcelos et al., 2009). The biostimulant we used did not change SOD activity during stress, while CAT activity was reduced in stressed plants with respect to their control, such as observed in Hayat et al. (2018). Finally, CAT activity was significantly higher in CTR BIO plants with respect to CTR NO BIO plants. Similarly, other biostimulant matrices were able to modify the enzymatic antioxidant system after their application even before stress occurrence on other pepper varieties and on eggplant (Hayat et al., 2018).

4.5. Biostimulant application differently modulate leaf proline and NSC accumulation during drought stress and its relief

During drought, osmotic adjustment occurred by increasing the concentration of total soluble sugars and proline thus maintaining membrane stability and keeping proteins functional (Zulfiqar et al., 2020). Other sweet pepper varieties subjected to severe water stress at flowering stage similarly showed a significantly increased accumulation of leaf total soluble sugars and proline with respect to well-irrigated controls and plants exposed to moderate water stress regimes (Okunlola et al., 2016).

In our experimental conditions, even if proline content did not change between CTR BIO and CTR NO BIO plants (Fig. 5a), NSC accumulation was significantly higher (Fig. 5b) in the treated control group. On the other hand, STRESSED BIO plants showed a higher accumulation of osmolytes compared to STRESS NO BIO group (Fig. 5), particularly total soluble sugars at the end of the stress and at 1 day after recovery (Fig. 5b). A protein hydrolysate derived from plants has already been shown to increase soluble sugar accumulation of hydroponically grown maize plants when applied by leaf spray (Schiavon et al., 2008). Moreover, drought relief promoted a high accumulation of proline at 4 h after recovery in STRESSED BIO plants, whereas proline accumulation reached its highest level at the end of stress in NO BIO plants (Fig. 5a). Differently, under saline stress conditions, biostimulant-treated maize plants exhibited higher proline concentrations than untreated plants during the stress phase (Ertani et al., 2013). In our experiments, we observed the highest proline level in biostimulant-treated plants at the early recovery phase, thus suggesting its involvement in promoting a faster recovery and inducing ROS non-enzymatic scavenging (Liang et al., 2013) (Fig. 5a). Proline high concentration could be correlated to the high total soluble sugar quantity at the end of the stress, since carbohydrates could be converted in proline and vice versa (Mohammadkhani and Heidari, 2008).

5. Conclusion

Despite the extensive literature suggesting that PH-based biostimulants mitigate the effects of drought stress, information regarding their mechanisms of action is limited. Our experimental set-up guaranteed us to monitor plant pre-treatment, drought, and recovery period dynamics with the aim to evaluate the possibility that the biostimulant treatments could not only promote plant growth, but also mitigate the effects of drought and prime the recovery after stress relief. Taken all together, our data point out the biological activity exerted by the PH-based biostimulant used in this study as resilience promotor and priming inducer when applied by fertigation on pepper. Indeed, its root application as priming chemical could represent an efficient agronomic technique to counteract severe water stress negative effects on growth and final fruit yield. The biostimulant seems to induce a new physiological homeostasis in stressed-experienced plants, such as suggested by the stomatal density values higher than those of untreated plants at the end of the recovery phase. Moreover, the biostimulant primed pepper plant drought tolerance not only by promoting a higher osmolyte and non-enzymatic scavenger accumulation in stressed plants during stress and its relief, but also by enhancing the antioxidant machinery in well-watered plants, preventively. Future works will aim to validate the

biostimulant efficiency as resilience-promotor by analyzing fruit quality and pepper marketable yield. Finally, the biostimulant action as priming chemical will be also evaluated on other crops.

Author contributions

Conceptualization, C.A., V.C., C.M.B., and F.S.; Data curation, C.A., F.S., D.M., and G.M.; Formal analysis, C.A, D.M., S.C., F.S., and G.M.; Investigation, C.A. and D.M.; Methodology, F.S., C.M.B., C.A., G.M.; Project administration, F.S. and C.M.B.; Supervision, F.S. and C.B; Validation, C.A., F.S., G.M. and C.M.B.; Writing – original draft, C.A. and D.M.; Writing – review & editing, G.M., C.A., C.M.B., V.C., S.C, D.M., and F.S. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Valeria Contartese by Green Has Italia S.p.A provided the biostimulant used for this experimentation free of charge. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.07.015.

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