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Phenotyping to dissect the biostimulant action of a protein hydrolysate in tomato plants under combined abiotic stress

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ABSTRACT

Drought and heat stresses are the main constrains to agricultural crop production worldwide. Precise and efficient phenotyping is essential to understand the complexity of plant responses to abiotic stresses and to identify the best management strategies to increase plant tolerance. In the present study, two phenotyping platforms were used to investigate the effects of a protein hydrolysate-based biostimulant on the physiological response of two tomato genotypes ('E42' and 'LA3120') subjected to heat, drought, or combined stress. The free amino acids in the biostimulant, or other molecules, stimulated growth in treated plants subjected to combined stress, probably promoting endogenous phytohormonal biosynthesis. Moreover, biostimulant application increased the net photosynthetic rate and maximal efficiency of PSII photochemistry under drought, possibly related to the presence of glycine betaine and aspartic acid in the protein hydrolysate. Increased antioxidant content and a decreased accumulation of hydrogen peroxide, proline, and soluble sugars in treated plants under drought and combined stress further demonstrated that the biostimulant application mitigated the negative effects of abiotic stresses. Generally, the response to biostimulant in plants had a genotype-dependent effect, with 'E42' showing a stronger response to protein hydrolysate application than 'LA3120'. Altogether, in this study a fine and multi-level phenotyping revealed increased plant performances under water-limited conditions and elevated temperatures induced by a protein hydrolysate, thus highlighting the great potential biostimulants have in improving plant resilience to abiotic stresses.

1. Introduction

Plants are continuously subjected to a multitude of stresses during the whole life cycle. These stresses are commonly divided into two categories, biotic and abiotic, depending on the nature of the trigger factor. Among abiotic stresses, drought and heat stresses are the main constrains to crop production worldwide (Moore et al., 2021). Tomato (*Solanum lycopersicum* L.), widely grown optimally from 20 to 30 °C, is a crop sensitive to both heat stress and water shortage, although this sensitivity varies among different genotypes (Duc et al., 2018). Due to the global climate change, it is likely that crops will face in the next future a higher incidence of combined heat and drought stress, resulting in greater agricultural losses. Indeed, the Intergovernmental Panel on Climate Change (IPCC) predicted that in the next few years heat waves will occur more frequently and with increases in both duration and intensity, and that, at the same time, the mid-latitudes and drought-prone areas will experience a decrease in water supply (Hein et al., 2021). Despite this, a limited number of studies have focused on the response of crops to abiotic stresses occurring simultaneously during plant growth and on identifying strategies that could be applied to increase crop

Abbreviations: AsA, ascorbic acid; DHA, dehydroascorbate; IPCC, Intergovernmental Panel on Climate Change; EPPN, European Plant Phenotyping Network; PSII, photosystem II; P_N, Net photosynthetic rate; Ci, Intracellular CO_2 concentration; g_s, stomatal conductance; E, transpiration rate; Chl, chlorophyll; NBI, nitrogen balance index; FW, fresh weight; DW, dry weight; MDA, malondialdehyde; GSH, reduced glutathione; GSSG, glutathione disulfide; PCA, principal component analysis.

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resilience to combined water deficit and heat stress (Zhou et al., 2019a, 2019b). The occurrence of high temperature or soil water depletion can induce a wide range of morphological, anatomical, physiological, and biochemical changes in plants. Drought can directly induce alterations in existing physiological processes, or can indirectly promote alterations in the plant developmental pattern (Chaves et al., 2009). Moreover, abiotic stress caused by adverse environmental conditions, including drought, heat, heavy metal toxicity, affects the inhibition of photosystem II (PSII) damage repair (Gururani et al., 2015)Moore et al., 2021). Also, high temperatures can cause enzyme degradation that can hamper PSII function, decrease electron transport rates, inhibit Rubisco activase and decrease chlorophyll content (Moore et al., 2021).

Optimizing crop management and improving resource efficiency (*i.e.* fertilizers and water) can be a key to increase the ability of crops to maintain stable yield under stressful conditions (Colla et al., 2017). However, it is still unclear what best management practices could be used to alleviate plant abiotic stresses.

Application of algal extracts, protein hydrolysates, humic and fulvic acids, and other complex mixtures, are known to be able to improve plant performances, increase crop yield and quality and cut down abiotic stress effects (Van Oosten et al., 2017). The beneficial effect of the applications of protein hydrolysates on plant growth, development and final yield is reported in different papers, and could be due to the direct effect of bioactive compounds (signaling peptides and free amino acids) on plant metabolism and to the indirect effect resulting from the stimulation of plant microbiomes (Ertani et al., 2009; Paul et al., 2019; Van Oosten et al., 2017). Recent studies indicate that protein hydrolysates can positively affect carbon and nitrogen metabolism, interfere with hormonal activity, enhance nutrient availability of plant growth substrates, increase nutrient uptake and nutrient use efficiency (Colla et al., 2017; Paul et al., 2019). It has been previously reported that this class of biostimulants does not produce a fertilizer effect but a modulation of nitrogen uptake and assimilation, regulating enzymes involved in nitrogen assimilation and their structural genes and by acting on the signaling pathway of nitrogen acquisition (Du Jardin, 2015; Nardi et al., 2016). For instance, Ertani et al. (2009) observed that two protein hydrolysates increased nitrate reductase (NR) and glutamine synthetase (GS) activities in maize seedlings, thus suggesting a beneficial effect in inducing the conversion of nitrates to organic nitrogen (Ertani et al., 2009). Colla et al. (2014) demonstrated the beneficial effects of a plant-derived protein hydrolysate on the growth parameters of corn, pea, and tomato. In another study, a protein hydrolysate derived from alfalfa plants enhanced shoot biomass production, soluble sugar accumulation and nitrogen assimilation in hydroponically grown maize plants (Nardi et al., 2016). It has been demonstrated that the application of protein hydrolysates can also modulate leaf gas exchanges and water use efficiency, increase total chlorophyll index, promote the accumulation of osmolytes and osmo-protectants, interfere with the oxidative stress responses and, hence, improve plant tolerance to abiotic stresses including limited water availability, salinity and heavy metals (Agliassa et al., 2021; Ertani et al., 2013; Paul et al., 2019; Van Oosten et al., 2017). As an example, Ertani et al. (2013) tested the efficacy of these compounds to increase salinity tolerance in *Zea mays*. Recently, Agliassa et al. (2021) demonstrated that a protein hydrolysate-based biostimulant applied by fertigation was able to mitigate severe water stress effects on *Capsicum annum*. Also, Casadesús et al. (2019) demonstrated that in tomato the application of an animal-based protein hydrolysate (Pepton) under water stress increased the endogenous content of auxin, cytokinin and jasmonic acid in treated plants, helping plant growth under drought conditions (Casadesús et al., 2019).

In a previous study, we investigated the effects of a novel protein hydrolysate-based biostimulant (CycoFlow, Agriges) on the response of tomato plants grown in open fields under elevated temperatures, demonstrating that the biostimulant had a clearly positive effect on plant growth and final yield (Francesca et al., 2020). Altogether, these results indicated that the application of protein hydrolysates on crops induces a wide diversity of physiological plant responses. Therefore, their investigation requires the use of fine and multilevel phenotyping that may highlight the plant responses to this class of biostimulants, which could enhance tomato performances under abiotic stress. In this study, analyses were carried out by using two phenotyping platforms (the Dynapheno platform at the Aarhus University for plant growth and physiological analyses and the platform Bordeaux Metabolome, MetaboHUB, PHENOME-EMPHASIS for metabolic analyses), where two tomato genotypes ('E42' and 'LA3120'), with a different attitude to abiotic stress tolerance, were subjected to single and combined abiotic stresses under well-controlled conditions and treated or not with the biostimulant. The outcomes of this work will be important for the selection of the best management practices to be used to improve crop performance and productivity under water-limited conditions and elevated temperatures.

2. Materials and methods

2.1. Plant material, experimental design, and growth conditions

Two genotypes with a determinate growth habit were used in this study based on previous screening experiments (Francesca et al., 2021b; Olivieri et al., 2020): 'E42' generally cultivated in Southern Italy (Campania) available at the Department of Agricultural Sciences of the University of Naples Federico II and the heat-tolerant genotype 'LA3120' (Tomato Genetics Resource Center, TGRC, University of California, CA, USA) (Olivieri et al., 2021). Seeds were sown in plastic pots (11 cm diameter, 9 cm height) with commercial substrate (Pindstrup Færdigblanding 2, Pindstrup Mosebrug A/S, Ryomgaard, Denmark). The seedlings were grown in a greenhouse with 24 ± 3 °C air temperature during the day and 18 ± 3 °C during the night. The seedlings were irrigated by flooding the benches every morning for 10 min using the following full nutrient solution: Ca $(NO_3)_2{\cdot}4H_2O$, 13.56 g L^{-1}; $NH_4H_2PO_4$, 1.56 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 11.59 g L^{-1}; $MnSO_4{\cdot}4H_2O$, 0.20 g L^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.39 g L^{-1}; $CuSO_4{\cdot}5H_2O$, 0.02 g L^{-1}; KNO_3, 23.47 g L^{-1}; NH_4NO_3, 2.87 g L^{-1}; Mg $(NO_3)_2$, 5.17 g L^{-1}; K_2CO_3, 1.89 g L^{-1}; $Na_2B_4O_7{\cdot}5H_2O$, 0.05 g L^{-1}. The used biostimulant was CycoFlow, a protein hydrolysate produced by Agriges (Benevento, Italy) by mixing sugar cane molasses with yeast extract obtained by autolysis of previously grown *Saccharomyces cerevisiae* yeasts (Francesca et al., 2020). The product contains total nitrogen of 4.5% and organic carbon of 19.5%. It has a pH of 5.0, a density of 1200 kg m^{-3} and an electrical conductivity (EC) of 15.0 dS m^{-1}. The biostimulant contains glycine betaine (3.62 g 100 g^{-1}) and micronutrients (boron, 0.2 g 100 g^{-1}; manganese, 1.0 g 100 g^{-1}; zinc, 1.2 g 100 g^{-1}). The aminogram of CycoFlow is reported in Table S1. The 26 days-old seedlings were randomly divided into eight groups each consisting of ten plants per genotype. The experiments included 8 treatments: (1) Control: 25/20 °C day/night temperatures; (2) Control plus biostimulant; (3) Heat-stress: 31/30 °C day/night temperatures; (4) Heat-stress plus biostimulant; (5) Drought: 25/20 °C day/night temperatures, without irrigation; (6) Drought plus biostimulant; (7) Combined stress: 31/30 °C day/night temperatures, without irrigation; (8) Combined stress plus biostimulant. The treatments lasted for 3 day as the plants under single drought and combined stresses showed significant phenotypical changes. The biostimulant, in liquid formulation, was applied directly to the pots (50 mL per plant) at 20 DAS (days after sowing) and 26 DAS, which corresponds to the day before starting stress. The biostimulant was applied by a syringe at a final concentration of 3 g L^{-1}, according to a previous study (Francesca et al., 2020). The control treatment was composed by plants treated using the same experimental protocol but replacing the biostimulant with water. The experimental timeline is shown in Fig. S1.

2.2. Gas exchange and chlorophyll fluorescence measurements

Net photosynthetic rate (P_N), intracellular CO_2 concentration (C_i),

stomatal conductance (g_s) and transpiration rate (E) of the plants were measured using a portable photosynthesis system (CIRAS-2, PP Systems, Amesbury, USA). The temperature setting of the cuvette during the measurements corresponded to the respective growth conditions of plants at each treatment. The first fully expanded leaf was placed in the cuvette and the measurements were recorded when P_N and g_s reached a steady state, typically after 5–10 min. To maintain vapor pressure deficit (VPD) at 0.95–2.0 kPa, a moist cloth was placed on the water vapor equilibrator of the CIRAS-2 when the VPD was above 2.0 kPa during the measurements. Chlorophyll fluorescence (F_v/F_m) measurement was performed on the first fully expanded leaf on day 3 using a MINI-PAM (Walz, Effeltrich, Germany). Prior to measurement, leaves were dark adapted with a dark leaf clip for 30 min. All the measurements were determined on at least five plants per genotype per treatment. For each leaf, three random spots were measured.

2.3. Dualex measurements

The metabolite content was non-destructively monitored using a Dualex 4 Scientific (Dx 4) (FORCE-A, Orsay, France). All the measurements were determined on at least three plants per genotype per treatment. Three random spots from both adaxial and abaxial side of each leaf were monitored, and the six values were averaged. For each treatment, the measurements were performed on the same day as the gas exchange measurements. This allowed for measurement of the relative content of chlorophyll (Chl), anthocyanin and flavonol content, and nitrogen balance index (NBI).

2.4. Ascorbic acid content

Quantification of reduced ascorbic acid (AsA) and total ascorbic acid (AsA + DHA) measurements were carried out using a colorimetric method as reported in Rigano et al. (2014). Briefly, 500 mg of N_2-frozen powder from tomato leaves were extracted with 600 μL of ice cold 6% w/v trichloroacetic acid (TCA) and the mixture was then incubated for 15 min on ice and centrifuged at 14,000 rpm for 20 min. For reduced AsA evaluation, 20 μL of supernatant were added to 20 μL of 0.4 M phosphate buffer (pH 7.4), 10 μL of double distilled (dd) H2O and 80 μL of color reagent solution prepared by mixing 5.5 mL of solution A (31% w/v H3PO4, 4.6% w/v TCA and 0.6% w/v FeCl3) with 2 mL of solution B (4% w/v 2,20-Dipyridyl). For total AsA, to 20 μL of sample, 20 μL of 5 mM dithiotreitol in 0.4 M phosphate buffer (pH 7.4) were added and the mixture was incubated for 20 min at 37 °C. Ten microliters of N-ethylmaleimide (0.5% w/v) were added and left for 1 min at room temperature. Eighty microliters of color reagent were added as previously described for reduced AsA. Both final mixtures were incubated at 37 °C for 40 min and measured at 525 nm using a Nano Photometer TM (Implen, Munich, Germany). Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed. Concentrations were expressed in mg 100 g-1 of fresh weight (FW).

2.5. Glutathione content

Glutathione was quantified by cycling assay involving Glutathione reductase (GR) and 5,5′-dithio-bis-(acide 2-nitrobenzoïque) (DNTB, Ellman's reagent) as electron acceptor, which forms a yellow-colored product (2-Nitro-5-thiobenzoic-acid) absorbing at 412 nm. The method measures "total glutathione" that is reduced glutathione (GSH) plus its oxidized form glutathione disulfide (GSSG). Specific GSSG quantification can be achieved after 2-vinylpyridine (VPD) treatment (adapted from Queval and Noctor, 2007). Briefly, to 30 mg of frozen powder from tomato leaves 300 μL of HCl (0.1 M) were added in microplate wells and extracts were centrifuged at 12,000 g for 15 min at 4 °C. Supernatant (200 μL) was neutralized by adding 50 μL of Na phosphate buffer (0.2 M pH 5.6) and NaOH (0.1 M) until pH = 4.5–5. GR was freshly prepared to a final concentration of 20 U mL^{-1} in 0.2 M of NaH_2PO_4 (pH 7.5) and 10 mM EDTA. To measure total glutathione, aliquots in triplicate of 10 μL neutralized extract were added to microplate wells containing 170 μL of a mix of 10 mL HEPES (0.2 M)/EDTA (10 mM) (pH 7.5), 1 mL of freshly prepared DNTB (12 mM), 1 mL of NADPH (10 mM) and 6 mL of water. The reaction started with the addition of 10 μL GR followed by automatic mixing by shaking and monitoring of the increase in A_{412} for 30 min. GSH standards (0–100 μM) were run simultaneously in the same microplate in triplicate. Values were corrected for GSH-independent reduction of DTNB by subtraction of the blank values. GSSG was measured by the same principle after incubation of 150 μL neutralized extract with 4 μL of VPD for 30 min at room temperature to complex GSH. To remove excess VPD, the solution was centrifuged twice at 12,000 g for 15 min at 4 °C and triplicate of 20 μL aliquots of the supernatant were assayed as described above. The same VPD treatment was done concomitantly on GSSG standards (0–100 μM) in the microplate wells. Rates were calculated as for total glutathione and corrected by subtraction of the blank. Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed.

2.6. Hydrogen peroxide and malondialdehyde determination

Quantification of H_2O_2 content was carried out using a colorimetric method as reported in Francesca et al. (2021b). Briefly, 500 mg of frozen powder from tomato leaves were extracted with 5 mL of ice cold 0.1% w/v trichloroacetic acid (TCA) and the mixture was then incubated for 15 min on ice and centrifuged at 10,000 rpm for 15 min at 4 °C. To 500 μL of surnatant, 500 μL phosphate buffer 10 mM (pH 7.0) and 1 mL of potassium iodide (1 M) were added. The mixtures were then incubated in the dark for 20 min and measured at 390 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed. The concentration was expressed in mmol g^{-1} FW. The malondealdehyde (MDA) levels in leaf tissues indicate the levels of membrane lipid peroxidation. Briefly, 200 mg of frozen powder from leaf samples were extracted by adding 1 mL of ice cold 0.1% w/v trichloroacetic acid (TCA). The samples were incubated for 15 min on ice and centrifuged at 10,000 rpm for 10 min at 4 °C. Afterwards, 0.25 mL supernatant was mixed with 1250 mL reaction solution (TCA 20% w/v + TBA 0.5% w/v), incubated in a water bath for 30 min at 95 °C and measured at 532 nm and 600 nm by using a Nano Photometer TM (Implen, Munich, Germany). Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed. The concentration was expressed as quantity of MDA-TBA complex (Zhang and Kirkham, 1996).

2.7. Proline content measurement and soluble sugar determination

Proline content was determined according to the method of Claussen (2005). In details, 250 mg of frozen powder from tomato leaves were suspended in 3 mL of 3% w/v sulfosalicylic acid and filtered through a layer of glass-fiber filter (Macherey-Nagel, Ø 55 mm, Germany). One milliliter of glacial acetic acid and 1 mL ninhydrin reagent (2.5 g ninhydrin 100 mL^{-1} of a 6:3:1 solution of glacial acetic acid, distilled water and 85% v/v ortho-phosphoric acid, respectively) were added to 1 mL of the clear filtrate. The mixture was incubated for 1 h in a boiling water bath. The reaction was terminated at room temperature after 5 min. Readings were taken immediately at a wavelength of 546 nm. The proline concentration was determined by comparison with a standard curve. Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed. For soluble sugar determination, powder leaf material was extracted in 80% v/v ethanol three times at 80 °C for 60 min, 50 μL of ethanolic extract was added to 160 μl of reaction mixture containing HEPES Buffer, adenosine 5′-triphosphate (ATP), reduced

nicotinamide-adenine dinucleotide phosphate (NADP) and glucose 6-phosphate dehydrogenase (G6PDH). The absorption was recorded at 340 nm before adding 1 μL Hexokinase. When stabilised, the OD was measured and 1 μL of Phosphoglucose isomerase added, then 1 μL of invertase was added before OD reading. The reading from each sample was expressed as μmol glucose eq. g^{-1} FW. Three separated replicates from three plants per genotype per treatment were used. Three technical assays per replicate were analyzed.

2.8. Stomatal anatomy and final harvest

Abaxial (lower) and adaxial (upper) surfaces of the mid-region of primary leaves were chosen to evaluate stomatal morphology. Leaf imprints were collected using impression material (elite HD+, Zhermack, Badia Polesine, Italy) in the morning. Three leaflets from three plants per genotype per treatment were sampled on the last day of stress treatment and three pictures were taken per leaflet using a magnification of 20 × (Leica DM R microscope equipped with a DFC 425 C camera, Leica Microsystems, Germany). Stomatal density was accessed on nine fields per treatment and stomatal characteristics (length, width) were determined. Stomatal density was expressed as the number of stomata per unit of leaf area. At harvest, the plant parameters were evaluated including plant height, leaf number (N° leaf), leaf area, fresh weight (FW) of the shoot. Plant material was put in a drying oven at 85 °C for 24 h and dry weight (DW) of shoot was measured. Relative water content was determined as follows: individual cut leaves from three plants per genotype per treatment were weighed (FW), saturated with distilled water for 4 h at room temperatures, blotted on filter paper and re-weighed (turgid weight - TW). The dry weight (DW) was measured drying the leaf at 80 °C for 24 h. The RWC was calculated as RWC (%) = [(FW – DW)/(TW – DW)] x 100 (Zhou et al., 2019a).

2.9. Statistical analysis

Data were subjected to analysis of variance using a three-way ANOVA. To separate means within each parameter, the Duncan's test was performed. Differences at $P < 0.05$ were considered to be significant. ANOVA and Principal component analysis (PCA) were performed by using SPSS (Statistical Package for Social Sciences) Package 6, version 23.0. A heatmap was generated by using the http://biit.cs.ut.ee/clustvis (accessed on October 10, 2021) program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage heatmap.

3. Results

3.1. Single and combined abiotic stresses and biostimulant treatment had effects on plant growth parameters and metabolite content

In this study two different tomato genotypes ('E42' and 'LA3120') were treated or not with a protein hydrolysate-based biostimulant and were subjected to single or combined drought and heat stresses. Drought and combined stress induced a significant reduction in plant height and leaf area in both genotypes (Table 1). For the shoot fresh weight, in 'E42' a decrease was evidenced under each single stress and was even more dramatic under combined stress, while in 'LA3120' a decrease was evidenced only under drought and combined stress. The treatment with the biostimulant increased plant height, leaf number and shoot fresh weight in 'E42' under combined stress and in non-stressed 'LA3120' plants (Table 1). In 'LA3120' biostimulant-treated plants an increase in leaf number under combined stress and of the leaf area under drought was also evidenced (Table 1). In both genotypes, plants under drought and combined stress showed wilting stems; however, the plants treated with the biostimulant under drought appeared less stressed than non-treated plants (Fig. 1a and b). On the contrary, plants under heat stress did not show clear differences compared to the control (Fig. 1a and b). The leaf RWC after drought and combined stress was significantly lower than in non-stressed plants in both genotypes (Fig. S2).

The chlorophyll content measured non-destructively increased by 12% in 'E42' subjected to combined stress and treated with the protein hydrolysate compared to non-treated plants (Table 2). In 'E42' a decrease in flavonol content under drought and combined stress and of anthocyanin content under combined stress was evidenced. Anthocyanin content decreased also in 'LA3120' under both drought and combined stress. An increase in NBI (nitrogen balanced index) was evidenced in 'E42' under drought and combined stress. Biostimulant application had no effects on the content of flavonol, anthocyanin and NBI (Table S2).

3.2. The effects of abiotic stresses and biostimulant treatment on leaf gas exchange and chlorophyll fluorescence were genotype dependent

Heat stress caused an increase of the transpiration rate in 'E42' and an increase of the stomatal conductance in both genotypes. The imposed drought and combined stress negatively affected transpiration rate (E), stomatal conductance (g_s) and net photosynthetic rate (P_N) in both genotypes. Compared to non-treated plants, higher E values were

Table 1
Plant height, leaf number, leaf area, shoot fresh weight (FW), shoot dry weight (DW) of two tomato genotypes ('E42' and 'LA3120') under control (CTRL), heat (H), drought (D), and combined (COMB) stress, without and with biostimulant (B). The data represent mean value ± SD (n = 3). Different letters indicate significant differences with Duncan's test ($P < 0.05$).

Parameters	Genotypes	Treatments							
		CTRL	CTRL_B	H	H_B	D	D_B	COMB	COMB_B
Plant height (cm)	**E42**	26 ± 2^{c}	27 ± 1^{c}	29.33 ± 0.58^{d}	27 ± 1^{c}	21.67 ± 0.58^{b}	22.67 ± 0.58^{b}	19.67 ± 1.53^{a}	22 ± 1^{b}
	LA3120	25.67 ± 2.08^{bc}	31 ± 1^{e}	27.33 ± 0.58^{cd}	28.67 ± 1.15^{de}	22.33 ± 1.53^{a}	23 ± 1^{a}	22.33 ± 0.58^{a}	24.67 ± 2.31^{ab}
Leaf number	**E42**	38.33 ± 2.08^{ab}	43 ± 4.36^{bc}	42.67 ± 3.79^{bc}	49.33 ± 4.93^{c}	36 ± 4.36^{ab}	40 ± 2.65^{b}	32.33 ± 2.52^{a}	42 ± 4^{b}
	LA3120	35 ± 3^{ab}	51.33 ± 2.52^{d}	44.67 ± 2.08^{cd}	47 ± 8.89^{cd}	40.33 ± 1.53^{bc}	42.67 ± 3.79^{c}	29 ± 1^{a}	40.67 ± 1.53^{bc}
Leaf area (cm^2)	**E42**	15.51 ± 1.15^{b}	15.45 ± 0.51^{b}	13.58 ± 1.25^{b}	15.51 ± 1.23^{b}	8.99 ± 2.54^{a}	5.59 ± 4.39^{a}	5.47 ± 0.29^{a}	7.14 ± 1.16^{a}
	LA3120	26.41 ± 3.55^{d}	25.19 ± 3.00^{d}	15.02 ± 2.94^{bc}	17.92 ± 1.16^{c}	9.86 ± 0.98^{a}	14.95 ± 3.51^{bc}	12.67 ± 0.55^{ab}	15.22 ± 2.02^{bc}
Shoot FW (g)	**E42**	27.89 ± 1.06^{e}	29.63 ± 1.62^{e}	22.62 ± 2.97^{d}	24.82 ± 1.87^{d}	16.03 ± 0.57^{c}	18.32 ± 0.37^{c}	9.85 ± 0.99^{a}	12.62 ± 0.83^{b}
	LA3120	25.62 ± 5.05^{b}	30.66 ± 0.56^{c}	26.03 ± 1.54^{b}	27.57 ± 3.75^{bc}	15.71 ± 0.88^{a}	18.66 ± 0.91^{a}	14.68 ± 1.47^{a}	16.15 ± 0.32^{a}
Shoot DW (g)	**E42**	1.97 ± 0.12^{ab}	2.09 ± 0.05^{ab}	1.86 ± 0.08^{ab}	3.22 ± 1.31^{b}	1.31 ± 0.49^{a}	2.51 ± 1.62^{ab}	1.17 ± 0.30^{a}	1.65 ± 0.43^{a}
	LA3120	1.93 ± 0.68^{ab}	2.58 ± 0.16^{b}	2.44 ± 0.69^{ab}	2.16 ± 0.42^{ab}	2.27 ± 0.78^{ab}	1.96 ± 0.45^{ab}	1.54 ± 0.19^{a}	1.49 ± 0.22^{a}

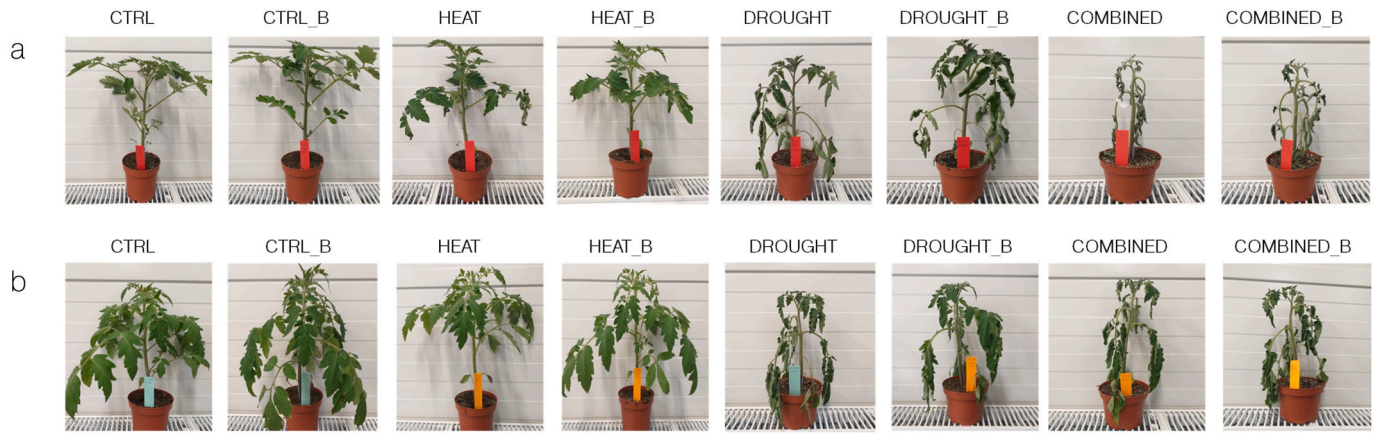

Fig. 1. The effect of heat stress, drought, and combined stress (heat stress + drought) on the phenotypes of the genotypes (a) 'E42' and (b) 'LA3120'.

Table 2
Dualex measurements: chlorophyll content (Chl), flavonol content, anthocyanin content and nitrogen balanced index (NBI) in the leaves of two tomato genotypes ('E42' and 'LA3120') under control (CTRL), heat (H), drought (D), and combined (COMB) stress, without and with biostimulant (B). The data represent mean value ± SD (n = 3). Different letters indicate significant differences with Duncan's test ($P < 0.05$).

Parameters	Genotypes	Treatments							
		CTRL	CTRL_B	H	H_B	D	D_B	COMB	COMB_B
Chl (units)	**E42**	30.13 ± 1.04 abc	29.13 ± 5.00 cd	27.35 ± 0.94[a]	26.5 ± 2.88 ab	31.13 ± 2.56[c]	32.77 ± 0.91 cd	30.5 ± 1.2 bc	34.17 ± 2.47 d
	LA3120	23.38 ± 2.76 ab	25.1 ± 0.69 abc	22.75 ± 0.79 ab	21.77 ± 1.76[a]	27.73 ± 0.57 bc	28.17 ± 2.85 bc	26.67 ± 0.59 abc	30.43 ± 7.65 c
Flavonol (units)	**E42**	0.63 ± 0.09[d]	0.55 ± 0.11 bcd	0.40 ± 0.07 abcd	0.41 ± 0.05 cd	0.55 ± 0.03 [ab]	0.56 ± 0.12 abcd	0.37 ± 0.04 a	0.44 ± 0.03 abc
	LA3120	0.46 ± 0.05[a]	0.56 ± 0.12[a]	0.43 ± 0.07[a]	0.43 ± 0.08[a]	0.43 ± 0.03[a]	0.47 ± 0.03[a]	0.37 ± 0.04 a	0.41 ± 0.03 a
Anthocyanin (units)	**E42**	0.12 ± 0.01[b]	0.13 ± 0.01[b]	0.11 ± 0 [bc]	0.12 ± 0.01 bc	0.11 ± 0.02 [bc]	0.12 ± 0.01 [bc]	0.09 ± 0.01 a	0.1 ± 0.01 ab
	LA3120	0.14 ± 0.02[d]	0.13 ± 0.01 cd	0.13 ± 0.01 cd	0.14 ± 0.01 cd	0.12 ± 0.01 [bc]	0.11 ± 0.01 [ab]	0.10 ± 0.01 a	0.10 ± 0.02 a
NBI (units)	**E42**	48.88 ± 6.36[a]	53.48 ± 6.01 ab	49.78 ± 4.47[a]	47.8 ± 6.65[a]	68.77 ± 11.77 bc	60.73 ± 16.20 abc	70.53 ± 6.31 c	73.6 ± 7.04 c
	LA3120	61.98 ± 17.99 ab	62.23 ± 8.70 ab	61.9 ± 4.87 ab	49.93 ± 7.49[a]	66.37 ± 9.43 ab	66.77 ± 8.18 ab	72.2 ± 8.68 b	74.43 ± 15.17 b

evidenced in 'E42' treated non stressed plants and in 'LA3120' treated plants under heat stress. Biostimulant treatments increased the g_s in both genotypes subjected to heat stress (Fig. 2a and b). Moreover, under drought, 'E42' plants treated with the biostimulant, showed a greater net photosynthesis level compared to non-treated plants (Fig. 2c). Intracellular CO_2 concentration decreased under drought in 'E42' and under drought and combined stress in 'LA3120' (Fig. 2d), and biostimulant treatment had no effect on this trait (Table S2). The F_v/F_m value determined in 'E42' and 'LA3120' was significantly lower in plants subjected to drought stress compared to the control. Only in 'E42' biostimulant treatments significantly increased F_v/F_m levels under no stress condition and under drought (Fig. 3).

3.3. Treatment with the protein hydrolysates reduced H_2O_2 accumulation and MDA content and increased antioxidant content under stress

Total ascorbic acid (AsA) content increased under all stresses applied in 'E42' and under drought in 'LA3120' (Fig. 4a). The content of reduced AsA decreased under heat in 'E42' and, on the contrary, increased in 'LA3120' under all stresses (Fig. 4b). The use of biostimulant increased total AsA content in all treatments in 'E42' (Fig. 4a). Moreover, an increase in the content of reduced AsA in 'E42' under drought was evidenced. The H_2O_2 content of 'LA3120' and 'E42' significantly increased under drought and combined stress compared to control. The biostimulant application led to a decrease in the accumulation of H_2O_2 content in both genotypes under all stresses, except for 'E42' under heat stress (Fig. 4c). The MDA content in both genotypes significantly increased under heat and combined stress compared to the control and also under drought in 'LA3120' (Fig. 4d). Plants treated with the protein hydrolysate under drought in 'LA3120' and under no stress, heat, and combined stress in 'E42' showed a decrease in MDA content compared to non-treated plants. Total glutathione content decreased upon all stresses applied for both genotypes, except for 'E42' under drought (Fig. 4e). An increase in total glutathione content was observed with biostimulant treatment in 'E42' under heat stress and in 'LA3120' under combined stress (Fig. 4e). Glutathione disulfide (oxidized form) was non-detectable in all conditions, which indicated fully reduced glutathione (data not shown).

3.4. Protein hydrolysate treatment modulated proline and soluble sugars content during stress

Metabolic alterations induced by drought and combined stress may include leaf accumulation of proline and of soluble sugars. As expected, an increase in proline amount and soluble sugars concentration was evidenced under drought and combined stress in both genotypes (Fig. 5a–d). Notably, proline accumulation decreased in response to biostimulant application in 'E42' under both drought and combined stress (Fig. 5a). In the same genotype a decrease in glucose accumulation was also evidenced in biostimulant-treated plants subjected to drought.

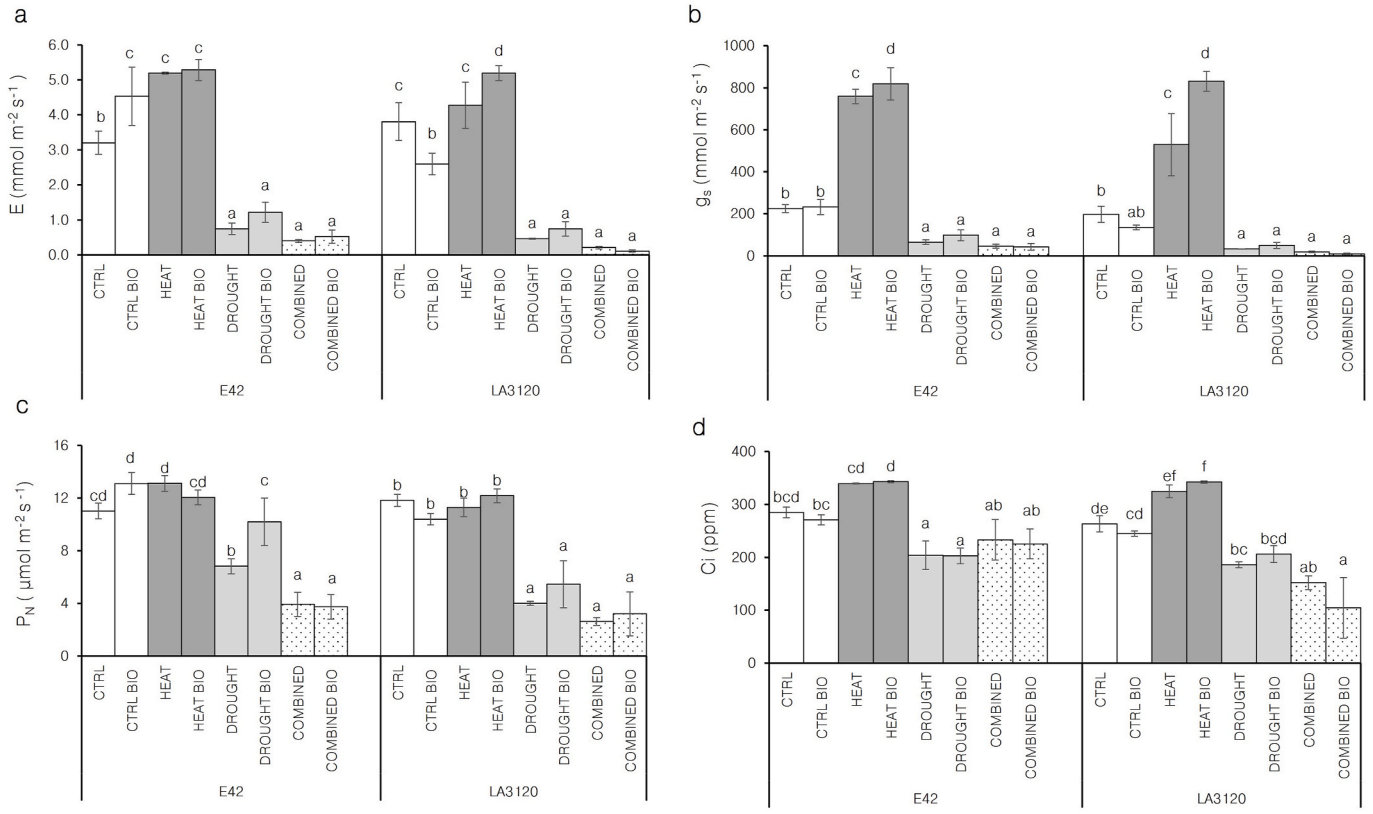

Fig. 2. Gas exchange measurements in the leaves of two tomato genotypes ('E42' and 'LA3120') under control, heat stress, drought, and combined stress, with and without biostimulant. Different sub-graphs represent: (a) transpiration rate (E), (b) stomatal conductance (g_s), (c) net photosynthetic rate (P_N) and (d) intracellular CO_2 concentration (C_i). The data represent mean value ± SE (n = 3). Different letters above the bars indicate significant differences ($P < 0.05$).

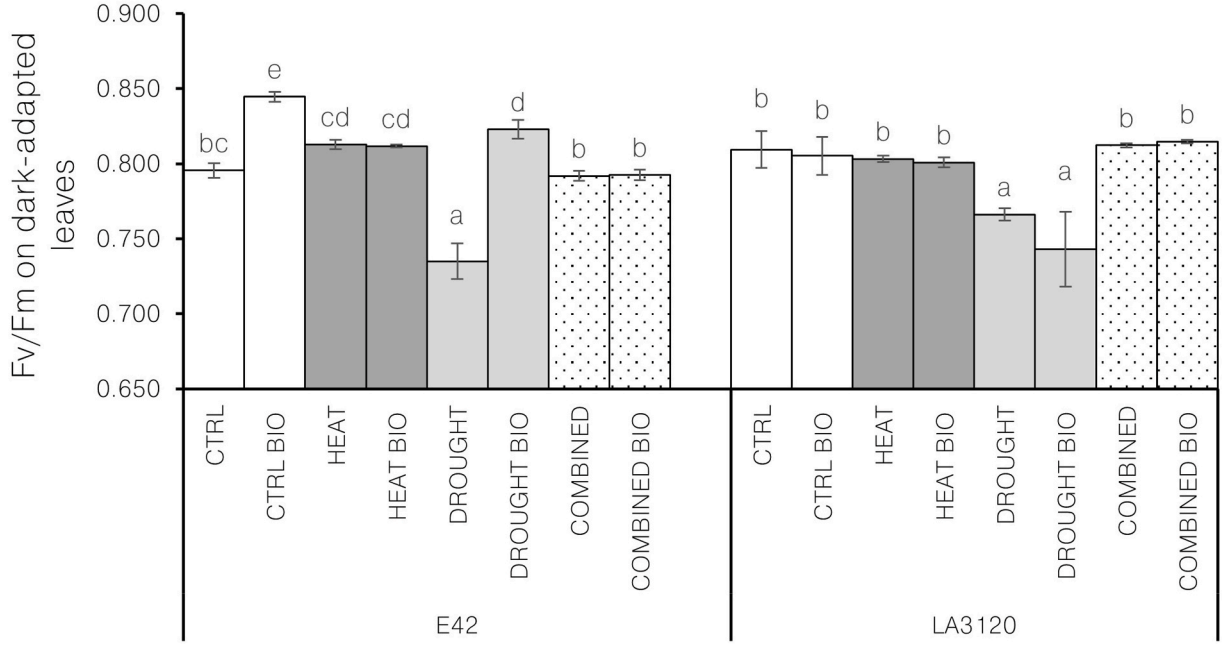

Fig. 3. F_v/F_m of two tomato genotypes ('E42' and 'LA3120') under control, heat stress, drought, and combined stress, with and without biostimulant. The data represent mean value ± SE (n = 3). Different letters above the bars indicate significant differences ($P < 0.05$).

In 'LA3120', under drought the content of sucrose and glucose were significantly reduced in protein hydrolysate-treated plants, as well as under combined stress a reduction in the content of fructose and glucose in treated plants was detected (Fig. 5b–d).

3.5. Stomatal anatomy changed in response to abiotic stresses and biostimulant treatment

The two genotypes showed a significant difference in stomatal

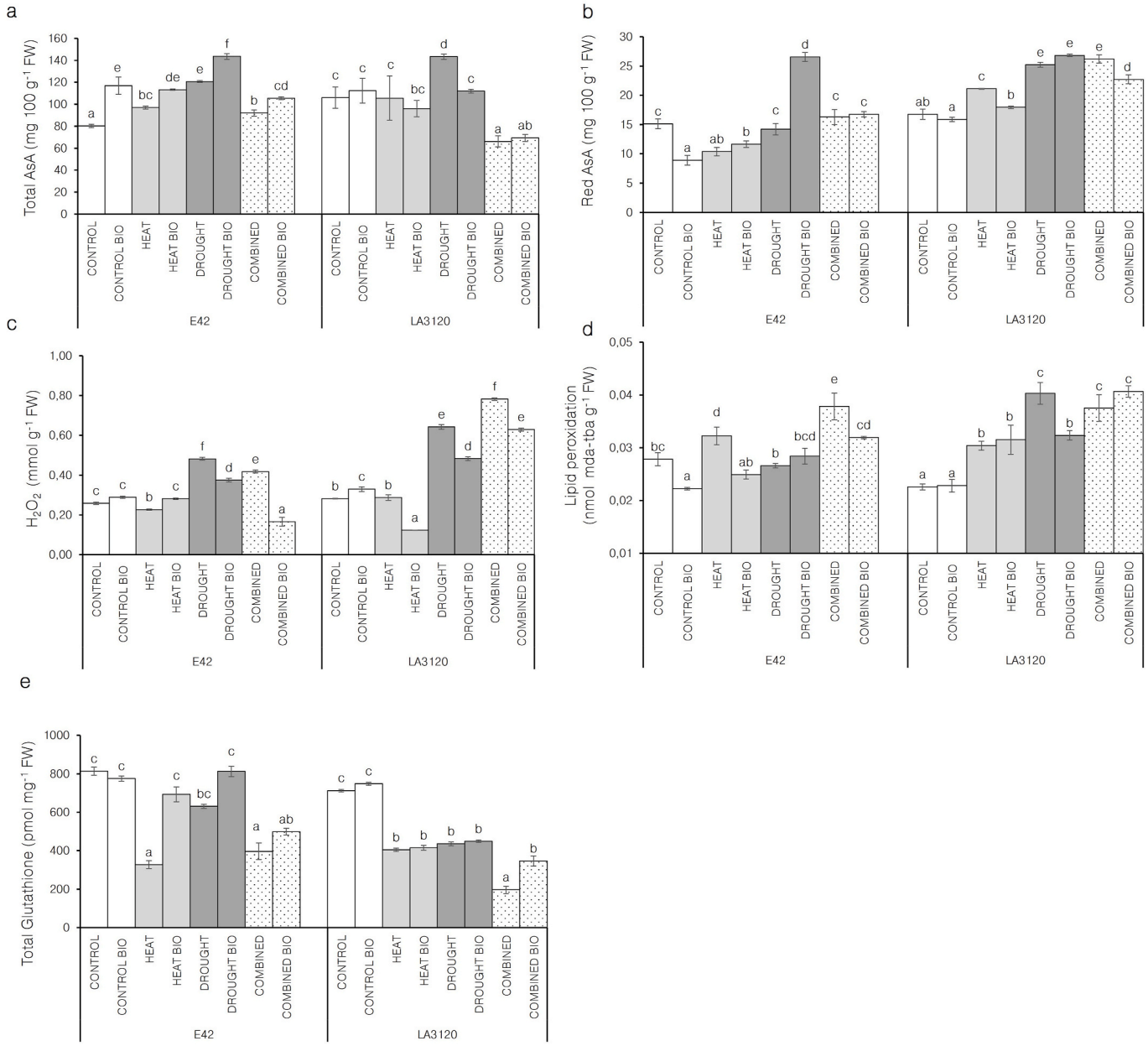

Fig. 4. Hydrophilic antioxidants and oxidative markers in the leaves of two tomato genotypes ('E42' and 'LA3120') under control, heat stress, drought, and combined stress, with and without biostimulant. Different sub-graphs represent (a) total ascorbic acid (AsA), (b) reduced ascorbic acid (AsA), (c) hydrogen peroxide (H_2O_2) concentration, (d) lipid peroxidation, measured as malondialdehyde (MDA-TBA) content and (e) total glutathione content. The data represent mean value ± SE (n = 3). Different letters above the bars indicate significant differences ($P < 0.05$).

anatomy under the different stresses applied (Fig. 6). The density of stomata in the plants grown at control temperature was 0.78 mm^{-2} for 'E42' and 1.46 mm^{-2} for 'LA3120', showing peculiar difference in leaves morphology. An increasing trend in stomatal density in 'E42' was observed in response to the different stresses applied and a very strong increase (+173%) was observed under heat stress (Fig. 6a). In the same genotype under all stresses applied a decrease in stomatal length and width was also observed (Fig. 6b and c). Under combined stress in 'LA3120' a significant increase (+39.72%) in stomatal density, that increased even more after biostimulant treatment, and in stomatal length was observed (Fig. 6a).

3.6. *Principal component analysis clearly separated the different samples based on the stress applied and the treatment with the biostimulant*

A comprehensive overview of biostimulant application and abiotic stress effects on all parameters studied in the two tomato genotypes was obtained through a PCA. The first two PCs were associated with eigen values higher than one and explained cumulatively 64.46% of the total variance, with PC1 and PC2 accounting for 45.68% and 19.39%, respectively (Table S3). PC1 was highly and positively correlated with E, g_s, P_N, Ci and with biometric parameters, including height and shoot fresh weight (Table S3). PC2 was positively correlated with oxidative markers (proline, H_2O_2, lipid peroxidation, reduced AsA). The loading plot (Fig. 7a) revealed that variables clustered into four main groups, based on the type of the stress applied. Control samples with and without biostimulant treatment clustered on the lower right side of the

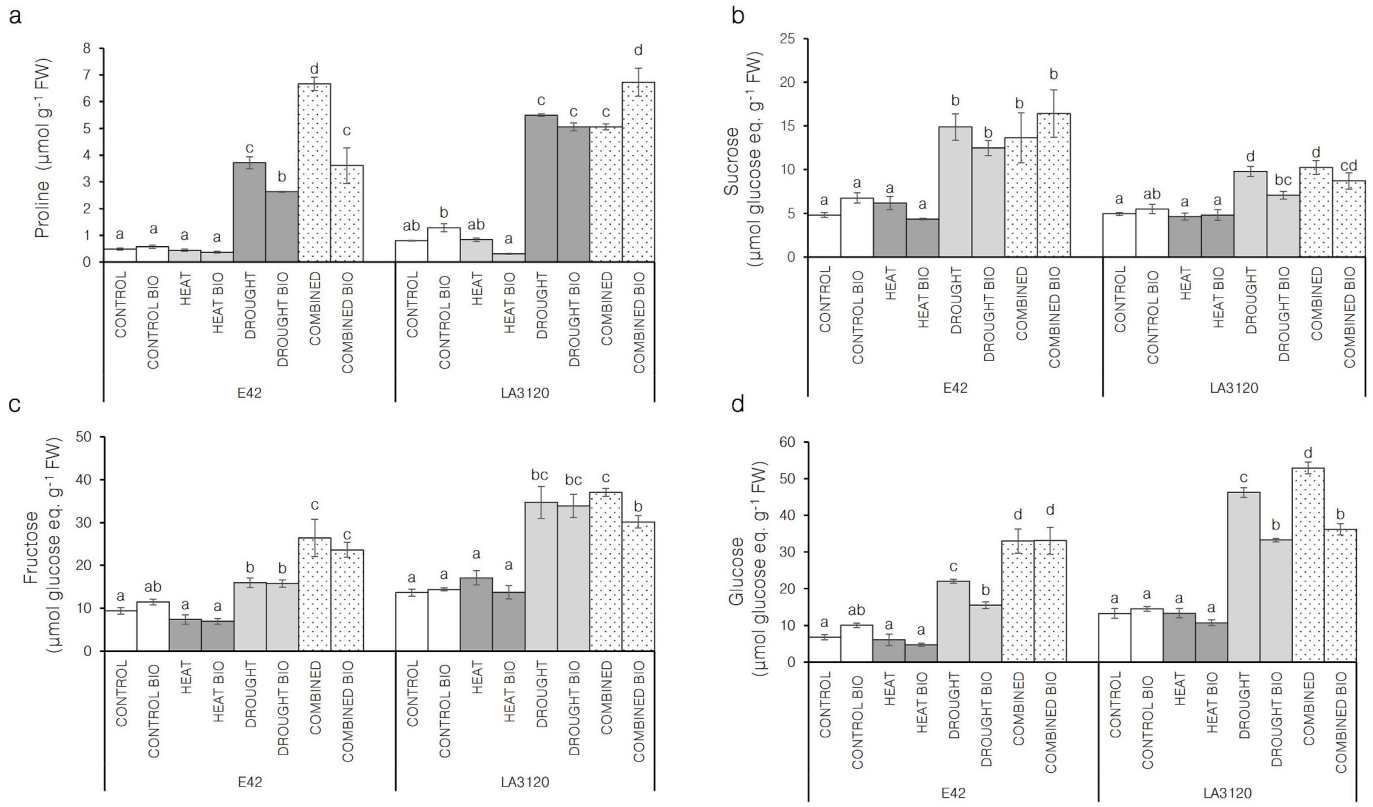

Fig. 5. Proline and soluble sugars content in the leaves of two tomato genotypes ('E42' and 'LA3120') under control, heat stress, drought, and combined stress, with and without biostimulant. Different sub-graphs represent the content of (a) proline, (b) sucrose, (c) fructose, and (d) glucose. The data represent mean value ± SE (n = 3). Different letters above the bars indicate significant differences ($P < 0.05$).

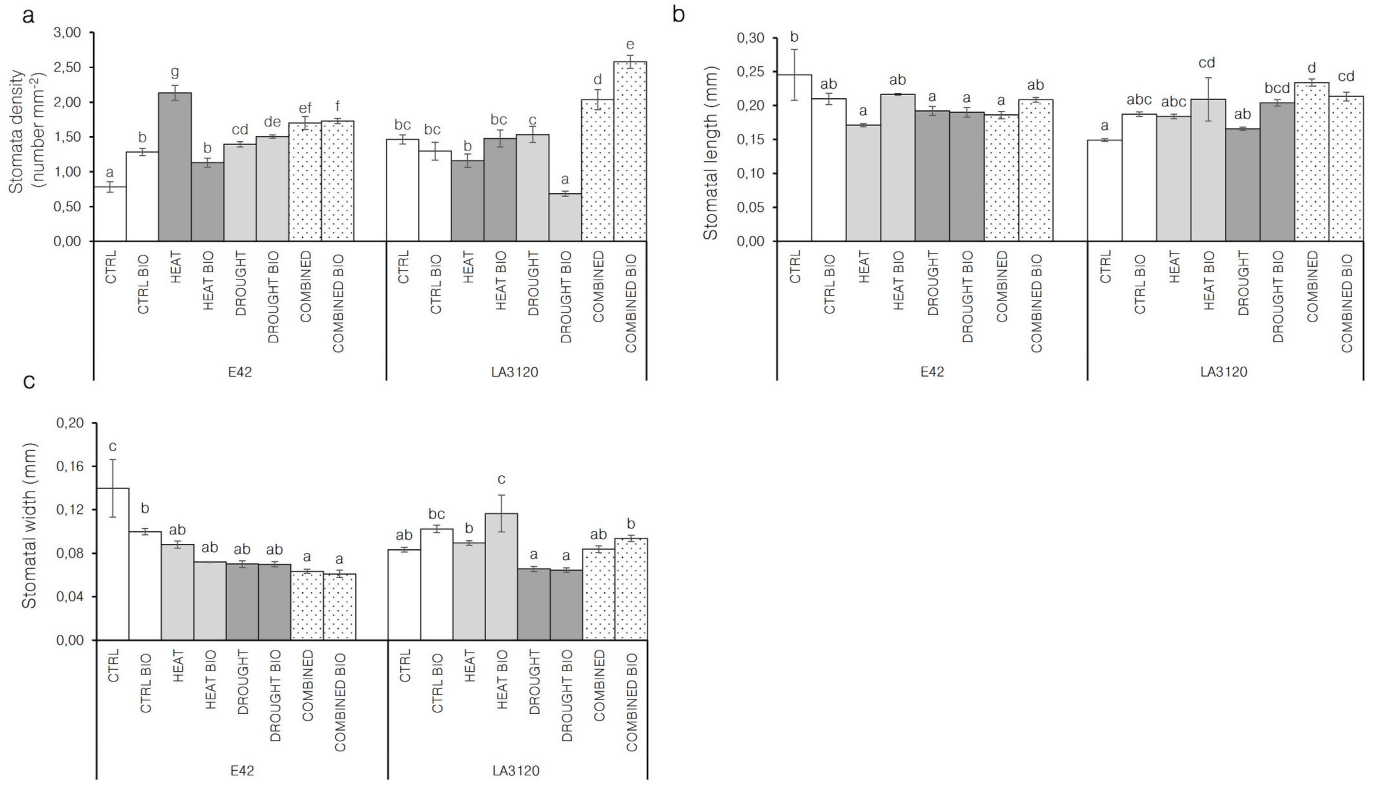

Fig. 6. Stomatal characterization of two tomato genotypes ('E42' and 'LA3120') under control, heat stress, drought, and combined stress, with and without biostimulant. The graphs represent (a) stomata density, (b) stomatal length and (c) stomatal width. The data represent mean value ± SE (n = 9). Different letters above the bars indicate significant differences ($P < 0.05$).

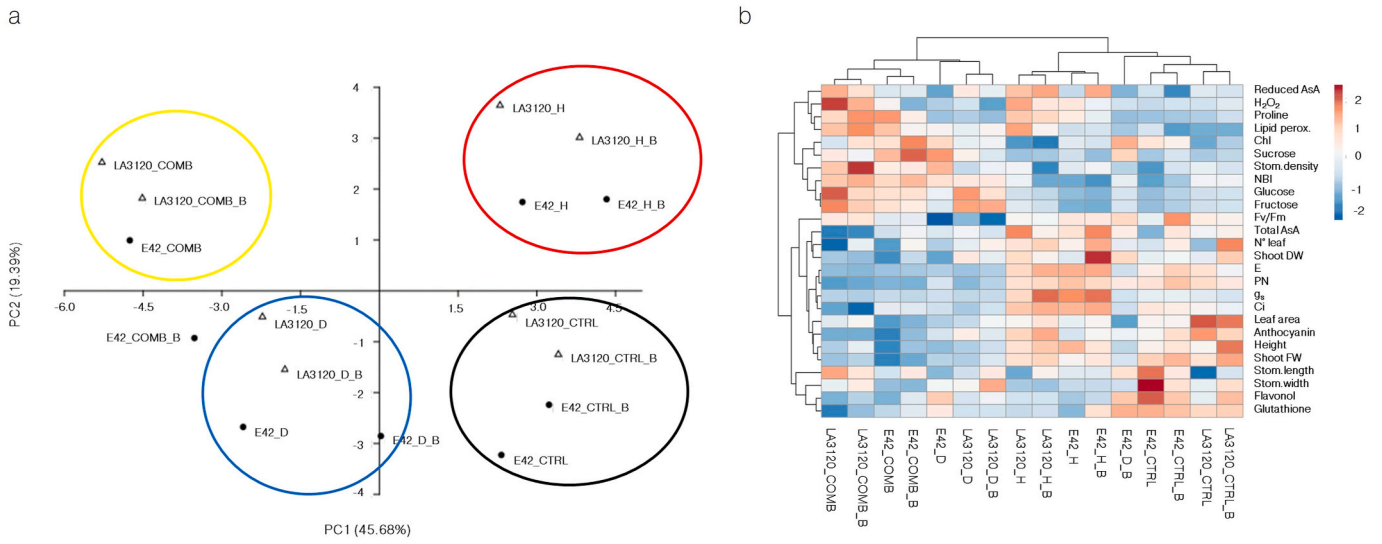

Fig. 7. Principal component loading plot and scores of principal component analysis (PCA) (a) and cluster heat map (b) of two tomato genotypes under control (CTRL), drought (D), heat (H) and combined (COMB) stress, without and with biostimulant (B).

PCA (black circle), with negative values for PC2 and positive values for PC1 (Fig. 7a). Samples under heat treatments clustered on the upper right side of the PCA (red circle), with positive values for both components. Samples under drought treatments clustered on the lower left side of the PCA (blue circle), with negative values for both components, except for the sample "E42 drought plus biostimulant". Most of the samples under combined stress (yellow circle) clustered on the negative PC1 axis and positive PC2. Only the sample "E42 combined plus biostimulant" had negative values for both components. A heat map providing the morphological, biochemical, and physiological changes in the two tomato genotypes in response to the different stress conditions is displayed in Fig. 7b. The heat map identified two main clusters, which distinguished the analyzed treatments. The first cluster separated the control and heat treatments from the other two treatments (drought and combined) and associated 'E42' and 'LA3120' control samples in a sub-group and 'E42' and 'LA3120' heat samples in another sub-group. This indicates that the presence/absence of water was the main clustering factor responsible for the different effects evidenced. Control and heat treatments were separated from drought and combined treatments mainly due to gas exchange and biometric parameters. The second cluster included the samples under combined stress in a sub-group and drought samples in another sub-group. In this last sub-group, the samples "E42 drought plus biostimulant" were further separated (Fig. 7b). Considering all the analyzed traits in the genotypes, both photosynthetic (P_N, C_i, g_s, E) and biometric traits (height, N° leaves, leaf area) were positively correlated with the biomass (Shoot FW) and were negatively correlated with the soluble sugar analyzed (Fig. S3).

4. Discussion

Previous studies on tomato plants grown in open fields suggest that amino acid-based biostimulants can enhance crop performances under elevated temperature or limited water availability (Francesca et al., 2021a). However, the potential of protein hydrolysates to increase crop resilience to combined abiotic stress, a frequent condition in nature and agricultural contexts, is underexplored (Francesca et al., 2021a; Zhou et al., 2017b). Herein, analyses were performed in two different tomato genotypes ('E42' and 'LA3120') grown under single and combined abiotic stress and treated or not with a protein hydrolysate (CycoFlow). 'LA3120' is a heat-tolerant genotype retrieved from the Tomato Genetic Resource Center that showed high yield in open field trials (Olivieri et al., 2021). The genotype 'E42' also demonstrated high yield when grown in open field and seems to have an efficient capacity to use limiting water resources under a prolonged (3 weeks) combined mild water and heat stress; however, its tolerance to abiotic stress remains unclear and needs further investigation (Francesca et al., 2021b; Olivieri et al., 2020). In this study we adopted a short-term harsh stress to better highlight the differences between the genotypes analyzed (Zhou et al., 2017b). Moreover, to define the physiological mechanisms activated by the combined action of multiple abiotic stresses and biostimulant application and to clarify its mode of action, two different phenotyping platforms were used and analyses were performed in a controlled environment to minimize genetic × environment effects on plant phenotype (Francesca et al., 2021a).

As expected, the different stresses had a clear effect in both genotypes, as evidenced by the PCA in Fig. 7. Notably, considering all the analyzed traits, drought had a predominant effect over heat stress in agreement with results by Zhou et al. (2017b).

In absence of stress, the treatment with the protein hydrolysate increased stomatal density, transpiration rate and maximal photochemical efficiency in 'E42' and had a growth-promoting effect in 'LA3120'. A stress protective effect of the biostimulant was instead mainly observed in 'E42' under drought and combined stress. These results are consistent with our previous work, which demonstrated that the treatment with this biostimulant of 'E42' grown in open field under water deficit (50% irrigation) induced better performances in terms of plant growth, final yield and yield components (Francesca et al., 2021a).

Under elevated temperature, a decrease in shoot fresh weight was recorded only in 'E42', as observed in the same genotypes subjected to long-term stress (Francesca et al., 2021b). Under heat stress, stomatal conductance (g_s) tends to rise to increase intracellular CO_2 concentration, reduce photorespiratory processes, facilitate enhanced transpiration and evaporative cooling. Heat stress can also induce alteration in stomatal density per unit leaf area to increase leaf cooling and/or stomatal size, which impact on g_s (Moore et al., 2021). Accordingly, heat stress increased stomatal conductance in both genotypes, maintaining stable Ci, and biostimulant application further contribute to increase this parameter. An increase in the transpiration rate (E) and in stomatal density, compensated by a decrease in stomatal length and width, was observed in 'E42' (Agliassa et al., 2021; Moore et al., 2021; Zhou et al., 2017a). In the heat-tolerant genotype 'LA3120' the transpiration rate and the stomatal anatomy did not change under heat stress and a decrease in leaf area to maintain higher leaf water content was evidenced. That said, heat stress had no effect on the net photosynthetic

rate and on F_v/F_m values, suggesting that in both genotypes a photoprotective mechanism was able to avoid photoinhibition events (Zhou et al., 2017b). These data, together with the fact that under elevated temperatures the content of H_2O_2 was lower in 'E42' and did not change in the tolerant genotype 'LA3120', suggest a partial heat tolerance of 'E42', as previously hypothesized (Olivieri et al., 2020).

Contrary to heat stress, drought had a clear detrimental effect on plant growth in both genotypes and the combination of drought and heat stress led to the most severe reduction in plant biomass, as previously seen (Francesca et al., 2021b). Under these stresses the effect of the biostimulant was more pronounced and a higher biomass and height was evidenced in 'E42' under combined stress treated with the protein hydrolysate. A possible explanation is that free amino acids, or other molecules/peptides, in the biostimulant may have promoted endogenous phytohormonal biosynthesis by stimulating growth in 'E42' treated plants, as suggested by Rouphael et al. (2017). A reason for this could be also a cytokinin-like activity promoting cell division triggered by the biostimulant (Matsuo et al., 2012).

While a response to heat stress is stomata opening to increase leaf cooling by enhancing transpiration, water deficit induces stomata closure preventing water loss. Herein, in both genotypes drought led to a strong decrease in transpiration parameters (E and g_s), a reduction in intracellular CO_2 concentration, and a strong decrease in net photosynthetic rate due to stomatal limitation. Under combined stress a strong decrease in P_N was observed as a consequence of stomatal limitation in 'LA3120', where a decreased g_s was accompanied by a decreased Ci, and of non-stomatal factors in 'E42', where no significant differences in Ci were observed (Zhou et al., 2017b). In both genotypes under drought and combined stress a decrease in leaf area was also demonstrated to minimize water loss. Water stress generally induces an increase in the stomata density to rise the supply of CO_2 to leaves and a reduction in the size of the stomata to limit water loss (Conti et al., 2021). Accordingly, it was observed an increase in stomata density under drought and combined stress in 'E42' that was again compensated by a reduction in stomata size (Agliassa et al., 2021; Moore et al., 2021; Zhou et al., 2017b). A different morphological behaviour of stomata was instead seen in 'LA3120' and under drought no differences in stomatal anatomy was observed, while under combined stress an increase in stomatal density was seen that was accompanied by an increase in stomatal length. During drought a strong decrease of maximal efficiency of PSII photochemistry was evidenced in both genotypes and these values, together with P_N values, increased in 'E42' upon biostimulant treatment. One hypothesis is that this higher photosynthetic activity in treated plants is related to the presence of glycine betaine and aspartic acid in the biostimulant. In particular, it is known that glycine betaine has a crucial role in repairing photodamaged PSII and maintaining the activity of Rubisco (Sorwong and Sakhonwasee, 2015). Different studies have reported the positive effect of these molecules under stressful conditions. For example, glycine betaine accumulation enhanced the P_N and quantum yield of photosynthesis under salt stress in tobacco (Zhang et al., 2008), and foliar application of aspartic acid increased gas exchange attributes in rice (Rizwan et al., 2017). The hypothesis that this class of biostimulant may help to protect the photosynthetic apparatus under stress was reinforced by the increased chlorophyll accumulation observed in treated 'E42' under combined stress caused by the down-regulation of chlorophyll breakdown and/or acceleration of its biosynthesis (Kravic et al., 2021). Under combined stress both genotypes, treated or not with the biostimulant, were able to activate photoprotective mechanisms of PSII and no decrease in F_v/F_m values was evidenced.

Oxidative damage is a subsequent stage of abiotic stresses in plants that causes an overproduction of reactive oxygen species (ROS) mainly targeting membrane lipids. Hydrogen peroxide is considered to be the main compound involved in membrane lipid peroxidation (Miller et al., 2010). Accordingly, an increase in H_2O_2 content and lipid peroxidation under drought and combined stress was here observed in stressed plants. These changes were accompanied by an increased total and/or reduced AsA content and a reduced total glutathione content, knowing that these non-enzymatic antioxidant are interconnected through the Ascorbate – Glutathione pathway (Hasanuzzaman et al., 2019).

The application of the biostimulant generally increased antioxidant content, as previously shown (Francesca et al., 2020), and decreased hydrogen peroxide and MDA levels under drought and combined stress. This aspect could be related with specific metabolites that are at high concentrations in the biostimulant: the glutamine, which is part of the glutathione structure and could explain the higher levels of total glutathione measured after biostimulant application (Hasanuzzaman et al., 2019). Different studies have established a interlink between glutamine and oxidative stress responses (Ji et al., 2019). For instance, Ji et al. (2019) revealed that this amino acid is involved in the regulation of cellular redox state under abiotic stress in *Arabidopsis thaliana* mutants. Drought generally also affects the accumulation of flavonol and anthocyanin depending on the plant species, the genotype, and the developmental stage (Rodríguez-Calzada et al., 2019). Herein, a general decrease of these compounds was observed under drought and combined stress, as previously seen in maize under water deficit (Kravic et al., 2021).

Osmotic adjustment by increasing the concentration of total soluble sugars and proline is a further physiological adaptation of plants associated with water stress tolerance to keep proteins functional and protect other cellular macromolecules from damages induced by drought (Agliassa et al., 2021). Leaf soluble sugars accumulation during stress events have been also associated in tomato source and sink organs with a complex modulation of the carbon metabolism enzymes and with an increase in sucrose-synthesizing enzyme activity (Osorio et al., 2014). The current study observed higher levels of proline content and of soluble sugars in tomato leaves under drought and combined stresses. Interestingly, in stressed 'E42' plants proline content decreased in the biostimulant treated plants, demonstrating the improved drought tolerance of this genotype after treatment with the protein hydrolysate. Also, a decrease in soluble sugars accumulation after biostimulant applications was demonstrated in both genotypes under stress. This decrease in proline and sugar accumulation further demonstrated that the application of this class of biostimulants can mitigate the effect of abiotic stress likely due to the presence of glycine betaine and proline. Indeed, it has been previously demonstrated that the exogenous application of these compounds increased drought tolerance in plants grown under hyper-osmotic conditions (Ashraf and Foolad, 2007).

Altogether, in this paper we used two phenotyping platforms to perform multilevel plant growth, physiological and metabolic analyses that allowed us to demonstrate that the treatment with a novel plant-based protein hydrolysate induced physiological and biochemical changes in treated tomato plants (as shown in Fig. 8), thus increasing resilience to single and combined drought and heat stress. The genotype-specificity remains decisive in the response to the biostimulant treatment, as previously seen, and 'E42' showed a stronger response to protein hydrolysate application than 'LA3120', as evidenced by the photosynthetic parameters, the antioxidant content, and the final biomass (Francesca et al., 2020). One hypothesis, that should be further verified, is that this different effect is linked to the distinguishing sensitivity of the different genotypes to stress condition and to the higher tolerance of 'LA3120' to abiotic stress (Francesca et al., 2020; Zhou et al., 2020). Our findings indicate that the application of this class of biostimulants can be recommended as a sustainable crop enhancement technology to increase plant tolerance under abiotic stresses. In the future, additional studies will be necessary to fully understand how much and to what extent the single molecules in the formulation affect plant physiological mechanisms and to comprehend the full potential of plant-based protein hydrolysate in agriculture.

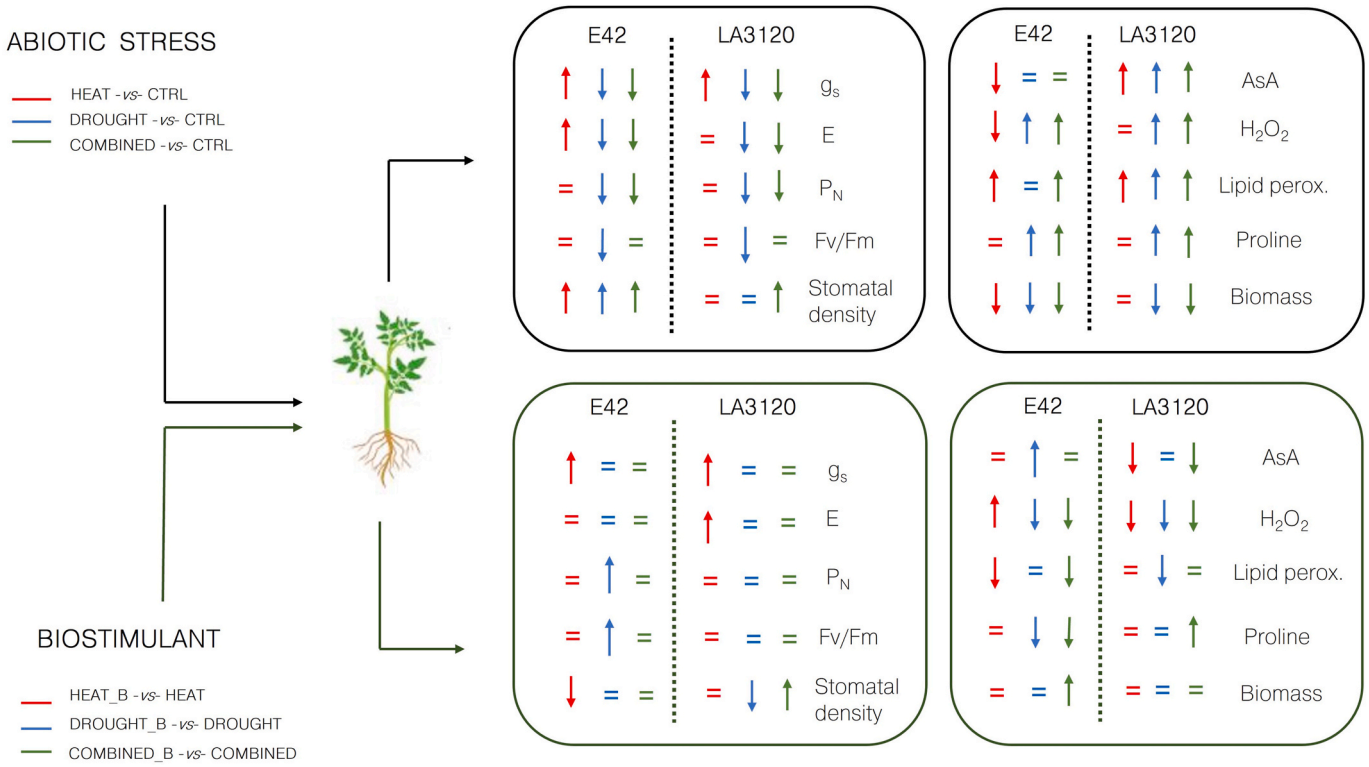

Fig. 8. The overall effect of heat stress, drought, and combined stress and of protein hydrolysate application in tomato plants.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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Author contributions

SF, SN, CC, GD: Investigation; SF, SN, RZ, FD, MMR conceptualization and Formal Analysis; RZ, FD, CO, AB, MMR: Supervision; SF, MMR, AB funding acquisition; SF, SN, RZ, FD, MMR Writing – Original Draft Preparation; CO, AB Writing – Review & Editing. All authors agree with this statement and individual contributions. The paper was revised and approved by all the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.03.012.

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